# CHARACTERIZATION OF STRUCTURAL AND FUNCTIONAL PROPERTIES OF ANTI-VEGF-IgG MOLECULES UNDER CERTAIN ENVIRONMENTAL STRESS CONDITIONS

by

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Submitted to the Graduate School of Engineering and Natural Sciences
in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

SABANCI UNIVERSITY
DECEMBER 2021

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APPROVED BY:

DATE OF APPROVAL: 10/12/2021

# ABSTRACT


INVESTIGATION OF STRUCTURAL AND FUNCTIONAL PROPERTIES OF ANTI-VEGF-IgG MOLECULES UNDER CERTAIN ENVIRONMENTAL STRESS CONDITIONS

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Ph.D. Thesis, December 2021

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Keywords: Monoclonal Antibodies, Biosimilar drugs, Forced degradation studies

Monoclonal antibodies (mAbs) are protein-structured molecules that bind to the target molecule with high specificity and have practical biotherapeutic effects. They are produced for therapeutic purposes in the pharmaceutical industry due to their essential functions, such as antigen-dependent cell cytotoxicity (ADCC), complement-dependent cell cytotoxicity (CDC), and target molecule neutralization. However, due to the protein nature of mAbs, they can be exposed to many different environmental stress factors during and after production. In the presence of these stress factors, the structures of antibodies and, accordingly, their biological functions can be negatively affected.

The mAbs are exposed to stress factors such as thermal, changing pH conditions, and oxidative environment during production and post-production processes such as shipping and storage. In the presence of these stress factors, in addition to the development of processes such as aggregation and fragmentation in the structures of antibodies, they can

undergo post-translational modifications (PTMs). These modifications and degradation processes can seriously affect the therapeutic activity of antibodies. Therefore, essential authorities in drug approval, such as the Food and Drug Administration (FDA), have made it mandatory to characterize the changes in the structure of mAbs and the changes in their biological activities in the presence of these stress factors.

For this purpose, a large field such as forced degradation studies has been created. Based on these studies, the antibody's characterization and biological activities can be determined quickly by imitating the stress factors that mAbs may encounter under normal environmental conditions in the laboratory environment. Within the scope of this study, both structural and functional analyzes of the antibody were performed by forming the most common stress factors such as thermal, pH change of the environment, and oxidative stress of the anti-VEGF IgG molecule *in vitro*. Therefore, by characterizing the PTMs and aggregation profiles that can occur, many preliminary studies will be carried out for the therapeutic use of this antibody in the pharmaceutical industry, and it will lead to studies for many industrial and therapeutic purposes.

# ÖZET


BELİRLİ ÇEVRESEL STRES FAKTÖRLERİNE MARUZ KALAN ANTİ-VEGF IgG MOLEKÜLLERİNİN YAPISAL VE FONKSİYONEL ÖZELLİKLERİNİN ARAŞTIRILMASI

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Doktora Tezi, Aralık 2021

Tez Danışmanı: Meral Yüce

Anahtar Kelimeler: Monoklonal Antikorlar, Biyobenzerler, Hızlandırılmış Degradasyon Çalışmaları

Monoklonal antikorlar (mAb'lar), hedef moleküle yüksek özgüllükle bağlanan ve etkili biyoterapötik etkilere sahip protein yapılı moleküllerdir. Antijene bağımlı hücre sitotoksisitesi (ADCC), kompleman bağımlı hücre sitotoksisitesi (CDC) ve hedef molekül nötralizasyonu gibi temel işlevleri nedeniyle ilaç endüstrisinde tedavi amaçlı üretilirler. Ancak mAb'lerin protein yapısından dolayı üretim sırasında ve sonrasında birçok farklı çevresel stres faktörüne maruz kalabilmektedirler. Bu stres faktörlerinin varlığında antikorların yapıları ve buna bağlı olarak biyolojik fonksiyonları olumsuz etkilenebilmektedir.

mAb'lar, üretim sırasında, nakliye ve depolama gibi üretim sonrası süreçlerde termal, değişen pH koşulları ve oksidatif ortam gibi stres faktörlerine maruz kalırlar. Bu stres faktörlerinin varlığında antikorların yapılarında agregasyon ve degradasyon gibi süreçlerin gelişmesine ek olarak post-translasyonel modifikasyonlara (PTM'ler) da uğrayabilmektedirler. Bu modifikasyonlar ve degradasyon süreçleri, antikorların terapötik aktivitesini ciddi şekilde etkileyebilmektedir. Bu nedenle, Gıda ve İlaç Dairesi (FDA) gibi ilaç onayındaki temel otoriteler, bu stres faktörlerinin varlığında mAB'lerin

yapısındaki değişiklikleri ve biyolojik aktivitelerindeki değişiklikleri karakterize etmeyi zorunlu hale getirmiştir.

Bu amaçla hızlandırılmış degradasyon çalışmaları gibi geniş bir alan oluşturulmuştur. Bu çalışmalarla, mAb'lerin laboratuvar ortamında normal çevre koşullarında karşılaşabileceği stres faktörleri taklit edilerek antikorun karakterizasyonu ve biyolojik aktiviteleri hızlı bir şekilde belirlenebilmektedir. Bu çalışma kapsamında anti-VEGF IgG molekülünün termal, ortamın pH değişimi ve oksidatif stres gibi en yaygın stres faktörleri oluşturularak antikorun hem yapısal hem de fonksiyonel analizleri *in vitro* olarak yapılmıştır. Bu nedenle oluşabilecek PTM'ler ve agregasyon profilleri karakterize edilerek bu antikorun ilaç endüstrisinde terapötik kullanımı için birçok ön çalışma yapılacak ve birçok endüstriyel ve terapötik amaçlı çalışmalara ön ayak olabilecektir.

*To my mom…*

## ACKNOWLEDGEMENTS

I would like to thank my advisor, Associate Prof, Doctor Meral Yüce, who always supported me during my doctoral education and gave me all the necessary dedication to become both a good scientist and a moral person. She made me a member of her lab and helped me do good works. She also instilled good work discipline and a sense of responsibility in all her students. In addition, she has consistently increased our motivation by showing very analytical behavior towards the problems we encounter both in our private life and the laboratory. In addition, she has been an exemplary person for us with her hard work, researcher identity, and sense of responsibility. I feel lucky to have met and worked with her.

I would also like to thank Hasan Kurt, assistant professor, who provided all kinds of scientific information and support during my doctoral studies. Together with Meral Yüce, he has always encouraged us to do good studies by creating a great team. I would also like to thank him very much for the family atmosphere he provided, especially by feeding us bagels every morning of the week.

I want to express my kindly appreciation to jury members Prof dr. Selim Çetiner, Asst. Prof Mehmet Kocatürk and Asst. Prof. Stuart James Lucas for accepting to participate in my thesis jury and their valuable feedback with great interest.

I would also like to thank my fellow lab mates, Dr. Büşra Gürel and Eda, who have given me tremendous and unmatched support during my thesis work.

No achievement can be made alone and always be accomplished as a group. Therefore, I would like to thank my lab mates Fatma Sert, Milad Torapham, Cemre Irmak Kayalan,

Deniz Yılmaz, Zeki Semih Pehlivan, Beyza Nur Günaydın, Parsa Pirshva, and Monireh Esmaeilli Rad for their unwavering support and motivation throughout my studies.

Also, I want to thank my other friends at Sabancı University. Although we belong to different groups, they always showed their friendship and supported me scientifically and psychologically during my thesis process. They never left me alone.

My deepest gratitude belongs to my family—their unconditional love and endless support encouraged me to complete my achievements. Hopefully, I will make them proud of me with their inspiration. I thank you for always believing in me.

Finally, I want to mention the most valuable person for me in the world. My mother is the main factor showing me that I can do everything. She is the source of my life. I love you so much, mother. Thank you so much. I am so glad I have you. Thinking about you and knowing of your presence encourages me. İnşallah uzun bir süre beraber yaşayacağız. Seni canımdan çok seviyorum. Benim güzel meleğim, anneciğim.

## TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| α | Alpha |
| β | Beta |
| γ | Gamma |
| ϵ | Epsilon |
| ζ | Zeta |
| δ | Delta |
| γ | Gamma |
| κ | Kappa |
| λ | Lamda |
| μ | Mu |
| μg | microgram |
| μl | Microliter |
| μM | Micromolar |
| 2,2’-Azobis | 2-amidinopropene |
| ADCC | Antibody-dependent cellular cytotoxicity |
| ADPC | Antibody-dependent Cellular Phagocytosis |
| Alpha screen | Amplified Luminescent proximity homogeneous assay |
| AMBIC | Ammonium bicarbonate |
| Asn | Asparagine |
| Asp | Aspartic acid |
| BLI | Bilayer interferometry |
| C1q | Complement componrnt 1q |
| CAN | Acetonitrile |
| CDC | Complement dependent cell cytotoxicity |
| CDR | Complementary determining region |
| CEX | Cation exchange Chromatography |
| CHO | Chinese Hamster Ovary |
| cIEF | Capillary isoelectric focusing |
| $CO_2$ | Carbon dioxide |
| CQA | Critical Quality Attribute |

| | |
|---|---|
| Cys | Cysteine |
| DAMPs | Damage-associated Molecular Patterns |
| $ddH_2O$ | Distilled water |
| DIA | Data-independent acquisition mode |
| DLS | Dynamic Light Scattering |
| DPBS | Dulbecco’s phosphate-buffered saline |
| DSC | Differential scanning calorimetry |
| DTT | Dithiothreitol |
| ECBS | Expert Committee on Biological Standardization |
| EDC | 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide |
| EDTA | Ethylenediaminetetraacetic acid |
| EGF | Endothelial Growth Factor |
| ELISA | Enzyme-linked ımmunosorbent assay |
| EMA | Europen Medicines Agency |
| ESI | Electrospray ionization |
| FA | Formic acid |
| Fab | Fragment antigen-binding |
| Fc | Fragment crystallizable |
| FcRn | Neonatal Fc Receptor |
| FcRs | Fc receptors |
| FcγR | Fc receptor gamma |
| FFF | Field flow Fractionation |
| FGF | Fibroblast Growth Factor |
| FRET | Fluorescence Resonance Energy Transfer |
| GlcNAc | N-acetylglucosamine |
| Gln | Glutamine |
| Glu | Glutamate |
| $H_2O_2$ | Hydrogen peroxide |
| His | Histidine |
| HRP | Horseradish peroxidase |
| HUVEC | Human umbilical vein endothelial cell |
| IAA | Indole-3-acetic acid |
| ICH | The International Council for Harmonization |
| Ig | Immunoglobulin |
| IgG | Immunoglobulin G |
| Iso-Asp | Iso aspartic acid |
| ITAM | Immunooreceptor tyrosine-based activation motif |
| ITIM | Immunooreceptor tyrosine-based inhibitory motif |
| $K_a$ | Association rate constant |
| $K_D$ | Equilibrium dissociation constant |
| $K_d$ | Dissociation rate constant |

| | |
|---|---|
| kDa | kilodalton |
| Lys | Lysine |
| mAb | Monoclonal antibody |
| MAC | Membrane attack complex |
| Met | Methionine |
| ml | Milliliter |
| mM | Milimolar |
| MS | Mass spectrometry |
| MS/MS | Tandem Mass Spectrometry |
| MTS | 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium |
| MWCO | Molecular weight cut off |
| NaICs | Sodium cesium iodide |
| Neu3Ac | N-acetylneuraminic acid |
| NHS | N-hydroxysuccinimide |
| NK | Natural Killer |
| NTA | Nanoparticle Track analysis |
| PAMP | Pathogen-associated molecular pattern |
| Pro | Proline |
| PRRs | Pattern Recognition Receptors |
| PTMs | Post Tranlational Modifications |
| PyroE | Pyro glutamate |
| Pyro-Glu | Pyro-glutamate |
| RT | Room temperature |
| RU | Response Unit |
| SBPs | Similar biotherapeutic products |
| SDS | Sodium dodecyl sulfate |
| SEC | Size exclusion high-performance liquid chromatography |
| Ser | Serine |
| SPR | Surface Plasmon Resonance |
| TEM | Transmission electron microscopy |
| Thr | Threonine |
| TIC | Total ion chromatogram |
| Trp | Tryptophan |
| US-FDA | The United States Food and Drug Administration |
| VEGF | Vascular Endothelial Growth factor |
| WHO | World Health Organization |

# 1. INTRODUCTION

## 1.1. The Immune System

The immune system consists of different cell types and soluble molecules responsible for protecting the organism against internal and external threats. These threats constitute a wide range from pathogenic microorganisms to tumor cells. In general, the immune system is divided into two main groups named innate immunity and adaptive immunity (**Figure 1**).

Innate immunity constitutes the first protective barrier in defense against microbial attack and tissue damage (damaged self). Innate immunity consists of many soluble factors (Cytokine, chemokine, complement system, and acute-phase proteins) and cells with different functions (Dendritic cells, Neutrophils, macrophages, mast cells, granulocyte, and natural killer cells), which have a very remarkable and rapid response to pathogenic invaders. Innate immunity can recognize microorganisms and ensure their destruction concisely (Janeway and Medzhitov 2002). Furthermore, it helps activate other parts of the immune system by generating an inflammatory response in the body. As a result of innate immunity working in this way, necessary time is provided for better elimination of internal and external threats and a better targeted immune response by the adaptive immune system.

Innate immunity has limited receptors recognizing common microbial components carried by pathogens. These receptors used in innate immunity do not have any genetic recombination and are genetically predetermined (germline-encoded). These receptors specifically and quickly recognize structures common among pathogens, generating an immune response. Therefore, innate immunity can recognize Pathogen Associated Molecular Patterns (PAMPs) that are common in pathogens (Janeway and Medzhitov 2002) and Damage-associated Molecular Patterns (DAMPs), which are stress factors occurring during inflammation and infection (Bianchi 2007). DAMPs are biological stress factors released during tissue damage and cell lysis. PAMPs and DAMPs are recognized by germline-encoded Pattern Recognition Receptors (PRRs) in innate immunity cells and trigger an effective immune response to eliminate pathogens (Kawai and Akira 2010; Takeuchi and Akira 2010).

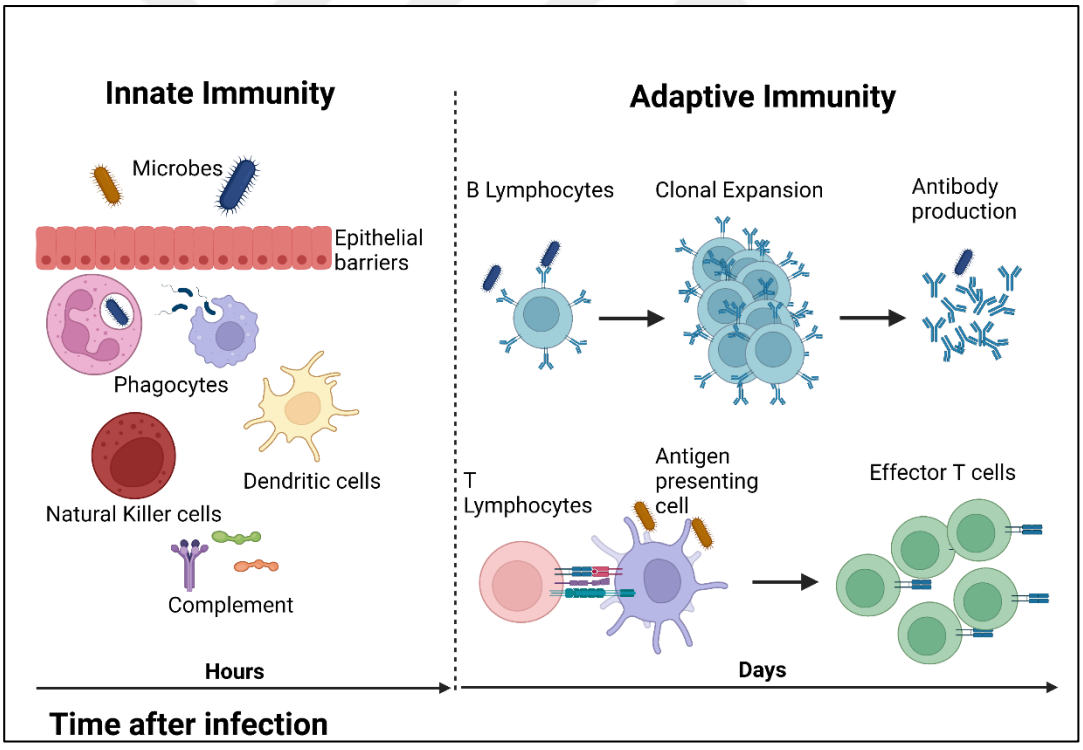



**Figure 1**: The Overview of Immune System

Although innate immunity recognizes many pathogens and creates a rapid and effective response, this effect is quite limited. An intense variation of target molecules that may

pose a threat to the organism cannot be fully recognized by the innate immune system and has driven the evolution of the adaptive immune system (Cooper and Alder 2006). Unlike innate immunity, receptors that can recognize a wide variety of targets in the adaptive immune system are generated from somatic recombination of the responsible gene segments.

Adaptive immunity can specifically recognize and distinguish self-molecules and non-self-molecules. Another defining feature of adaptive immunity is that it develops a memory system. As a result of the first entry of the pathogen, the adaptive immune system creates a memory against the antigenic molecules of this pathogen. It provides a rapid and effective defense by recognizing this pathogen in a specific way in a second encounter. Thus, damage to organisms is eliminated in a short time without losing much time in the face of threats. Lymphocytes are the cells that perform these tasks and play a central role in the adaptive immune system, and lymphocytes are functionally divided into two main cell groups: T and B cells. Both cell groups can form receptors with different structures due to somatic recombination and recognize various antigenic molecules (**Figure 1**). T lymphocytes are essential cells in generating the immune response by working with B cells and phagocytes.

On the other hand, B cells produce immunoglobulins (antibodies) and secrete them into the body. This thesis mainly covers the structural and functional characterization of antibodies. Therefore, the following sections will give information about antibodies' structure, importance, and functions.

## 1.2. The Immunoglobulins

### 1.2.1. The Classes of Immunoglobulins

The typical antibody molecules, also called immunoglobulins (Ig), are molecules produced by B cells against dangerous antigens such as bacteria, viruses, and tumor proteins. These molecules can bind to targets with high specificity, preventing antigen's

harmful activities. Antibodies are composed of five different isotypes (IgM, IgD, IgG, IgA, and IgE) (**Figure 2**) that create different functional roles due to different variants of their tail regions (Fc parts).

IgM exists as a receptor on B cells and is secreted in the form of a pentamer linked by disulfide bonds. Its pentameric structure increases its avidity, as it provides multi-site binding (Onoue et al. 1968; Mitchell, Edwards, and Collins 2001). IgM has five N-linked glycosylation sites. The high avidity of IgM and its ability to bind to the members of the complement system allow the destruction of pathogens early in infection during the formation of humoral immunity.

Like IgM, IgD also occurs as a B cell receptor, and it can be secreted then. The IgD molecule has a large hinge region, which increases its flexibility. The T-shaped IgD immunoglobulin can bind to surfaces with low epitope density. It binds to the surface of basophil and mast cells in the respiratory mucosa (Lutz et al. 1998; Choi et al. 2017), producing antimicrobial peptides and inflammatory cytokines (K. Chen et al. 2009). The IgD contains three N-linked and four O-linked glycan sites.

IgG constitutes 10-20 % of the total protein in the serum. There are four subclasses of human IgG, which are IgG1, IgG2, IgG3, and IgG4, in order of their abundance. They all carry different hinge structures in terms of disulfide bonds (Vidarsson, Dekkers, and Rispens 2014). IgG1 and IgG3 have a more extended and flexible hinge region than IgG2 and IgG4. This flexibility increases binding to antigen and Fc receptors for IgG1 and IgG3, increasing effector function (Dall'Acqua et al. 2006; Redpath et al. 1998). IgG molecules carry a single N-glycosylation site asparagine 297 (Asn 297) to regulate antibody function. But IgG3 has an additional N-linked glycosylation site at the Asn 392 (Stavenhagen, Plomp, and Wuhrer 2015) and three O-linked sites in the hinge region (Plomp et al. 2015).

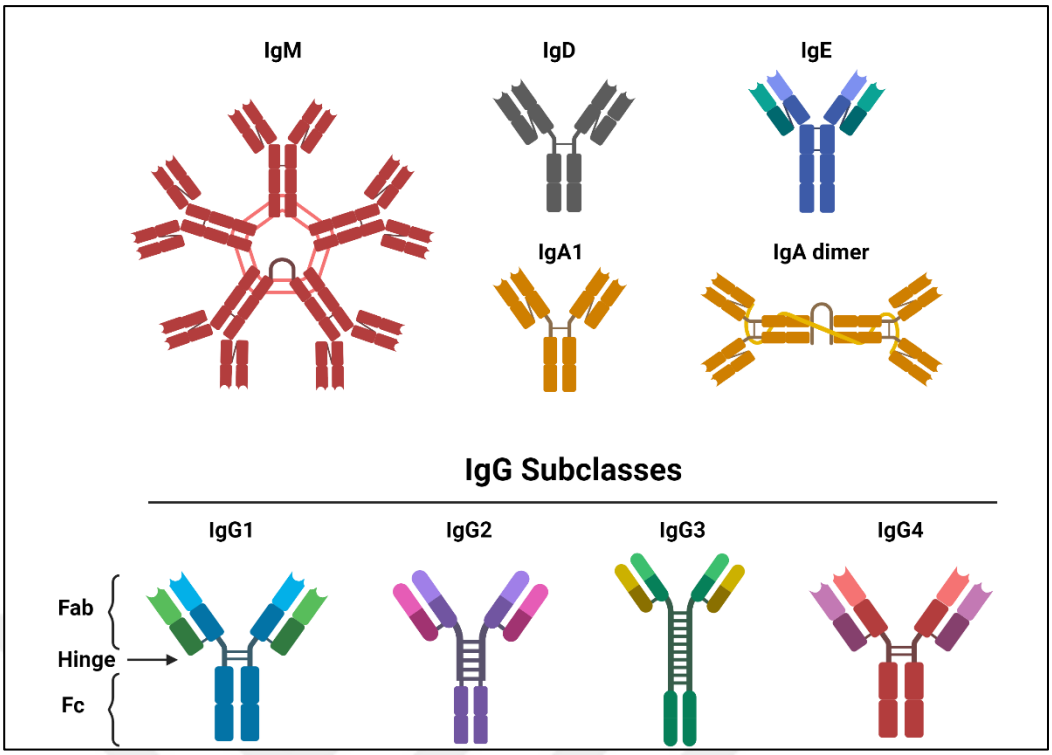



**Figure 2**: The Classes and Subclasses of Immunoglobulins

IgGs are molecules used for monoclonal antibody therapeutics due to the high abundance in the serum and long half-life. Also, since IgG molecules are the simple-structured immunoglobulin group, it is easier to study structurally and functionally for protein engineering.

The IgA molecule consists of two subclasses, which are IgA1 and IgA2. IgA2 is more rigid than IgA1. The IgA1 molecule is more flexible and predominantly has O-glycosylation in the hinge region (Boehm et al. 1999). Monomeric IgA is found in serum, while dimeric IgA bound by disulfide bridges is secreted into the mucosa (Macpherson et al., 2008). IgA1 contains two N-linked and four O-linked glycan sites, while IgA2 contains five N-linked glycan sites.

IgE is the class of antibody that is the lowest abundant in serum and has the shortest half-life among the isotypes. IgE has a high affinity for the Fc epsilon (ε) R1 receptor on mast cells and, once bound, can remain bound for weeks or even months (Kubo et al., 2003). The IgE molecule contains seven N-linked glycan sites, including position Asn 395 (Shade et al. 2015).

### 1.2.2. The Anatomy of IgG

Antibodies are flexible Y-shaped structures consisting of four polypeptides, two identical light (L), and two identical heavy (H) chains (Figure 3) (Rajpal et al., 2013). The light chain is approximately 22 kDa, while the heavy chain is approximately 50 kDa. Furthermore, there are five different types of light chain (α, δ, ε, γ, and μ) and two different types of the heavy chain (λ and κ) in mammals. These chains are linked by disulfide bonds formed between cysteine (Cys) amino acids. The N-terminal part of each polypeptide chain is varied to be specific to different antigen targets and forms the variable region (V). V domains have huge variability due to the gene rearrangements and somatic hypermutations. In addition, each V domain has three regions of high sequence variability called complementarity determining regions (CDRs). These three CDR regions in the H and L chains are paired to form an antigen-binding site.

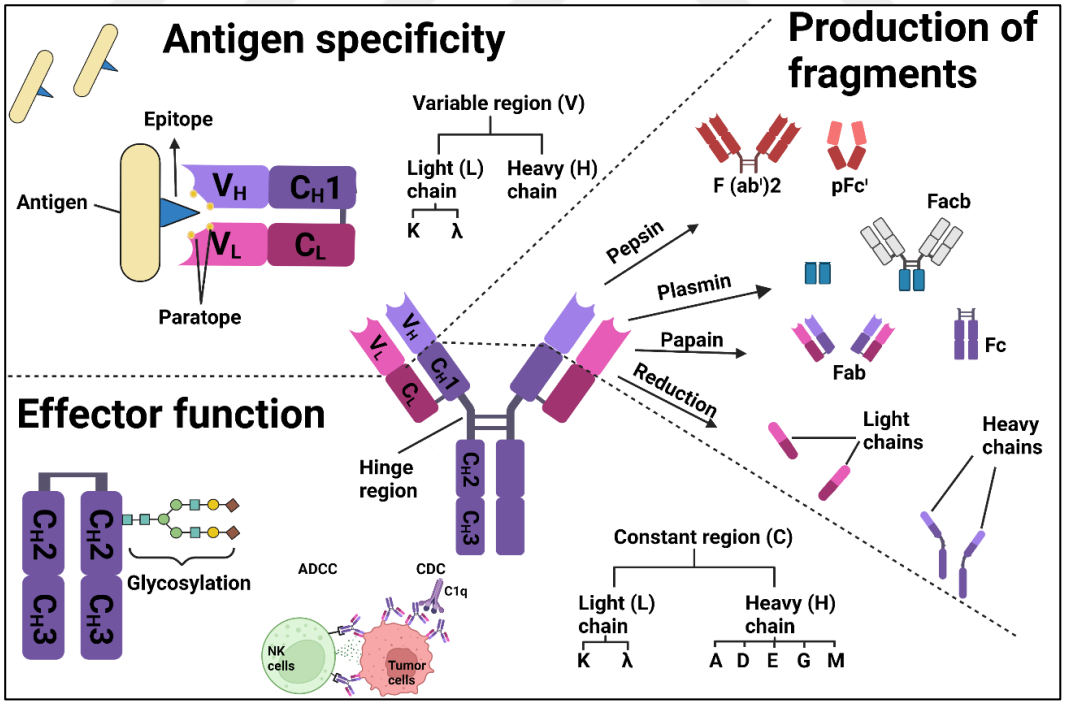



**Figure 3**: The structure of typical IgG molecule

Other parts of the antibody, which form the constant region, do not undergo much change. This region generally consists of CH1, CH2, and CH3 domains. These domains are

responsible for maintaining the stability of the constant region and the effector function of the antibody. Antibodies can be broken enzymatically into different fragments with critical biological activities. As a result of examining these fragments, the antibody's chemical structure and biological functions are elucidated. The disulfide bonds are broken during the reduction reactions, and the heavy and light chains are separated. When antibodies are treated with papain enzyme, the antibody is fragmented into two structures, antigen-binding (Fab fragment) and fragment crystallizable region (Fc region) (Figure 3).

The Fab fragment is the region responsible for specific binding to the antigen and contains the one variable and one constant region in each light and heavy chain. In the Fab fragment, the hypervariation regions in the CDR regions in both chains come together to form an antigen-binding site (paratope), which recognizes epitopes on the antigens (Chiu et al. 2019). In addition, a double Fab fragment (F(ab)' 2) is formed by digesting with the pepsin enzyme.

The Fc part of the antibody, the tail region, interacts with Fc receptors on the surface of many immune system cells and complement system proteins such as the complement component 1q (C1q). Due to this feature, antibodies can activate the immune system with their Fc parts and have an influential role in body defense. Besides, the Fc regions of the antibodies have a highly conserved N-glycosylation site. Last but not least, the Fc regions of IgG trigger antigen-dependent cell cytotoxicity (ADCC) by interacting with Fc receptors on the surface of immune system cells and by interacting with the complement system, triggering complement-dependent cell cytotoxicity (CDC). (Natsume 2008)

## 1.3. The Functions of Antibody

### 1.3.1. Fc gamma Receptors

Fc receptors (FcRs) are a cluster of cell surface proteins that specifically recognize the Fc regions of antibodies. Fc receptors are essential for performing the functional activities of immunoglobulins and regulating the immune response (Nimmerjahn and Ravetch 2008a). These receptors are found on specific immune cells, such as mast cells, neutrophils, macrophages, natural killer cells, and B lymphocytes. FcRs bind to Fc regions, recognize antigen-antibody complexes, and transmit the signal necessary to fulfill effector functions of immune system cells.

Although there are many classes of Fc receptors, the Fc gamma receptor (FcγR) class, which interacts with the IgG molecule, is the main class of receptors with essential functions. Three FcγR subclasses were described in humans (Geissmann et al. 2001). These are FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16). There are also different isoforms of FcγR such as A, B, and C (**Figure 4**). The binding affinities of FcγR to the IgG molecule differ. FcγRI can bind more strongly to the IgG molecule than FcγRII and FcγRIII. While there are two immunoglobulin-like domains in the extracellular part of other receptors, FcγRI has three domains. Except for the FcγRIIb, all FcγRs carry the ITAM (immunoreceptor tyrosine-based activation motif) in their cytoplasmic domains, which provides effector function activation. In contrast, FcγRIIb carries the ITIM (immunoreceptor tyrosine-based inhibitory motif) motif, which initiates signaling inhibiting effector activation (R. Liu et al. 2020).

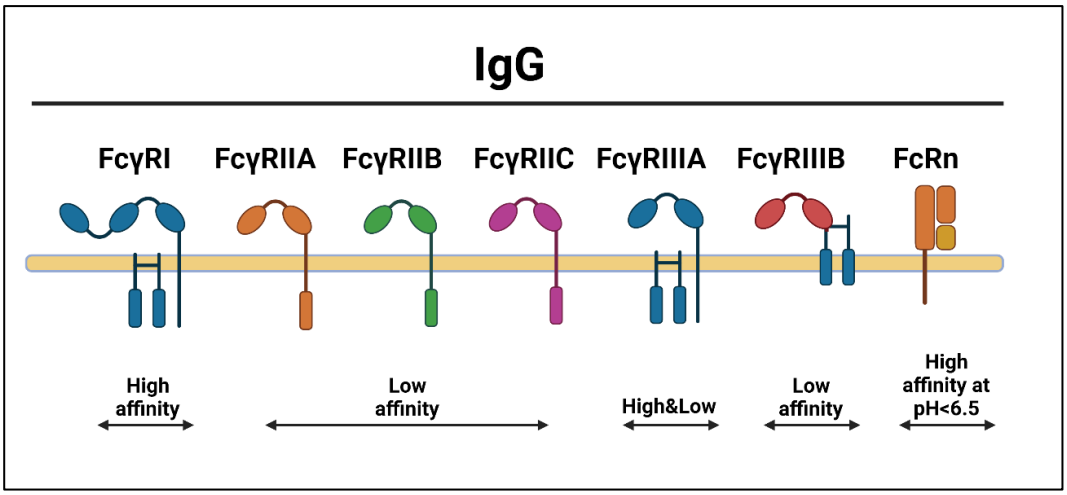



**Figure 4**: Fc gamma receptor (FcγR) classes interact with the IgG

Another FcγR, the neonatal Fc Receptor (FcRn), was initially discovered to be involved in the transcytosis of maternal IgG to the neonate (Ober, Martinez, Lai, et al. 2004). Later, it was understood that it has a vital role in regulating the serum IgG level. IgG binds to the FcRn receptor in acidic conditions (<6.5) within the endosome and protects it from lysosomal degradation (Ober, Martinez, Vaccaro, et al. 2004). Then, sending it back to the surface ensures that IgG molecules are released into the blood at neutral pH. Thus, IgG destruction is prevented (**Figure 5**).

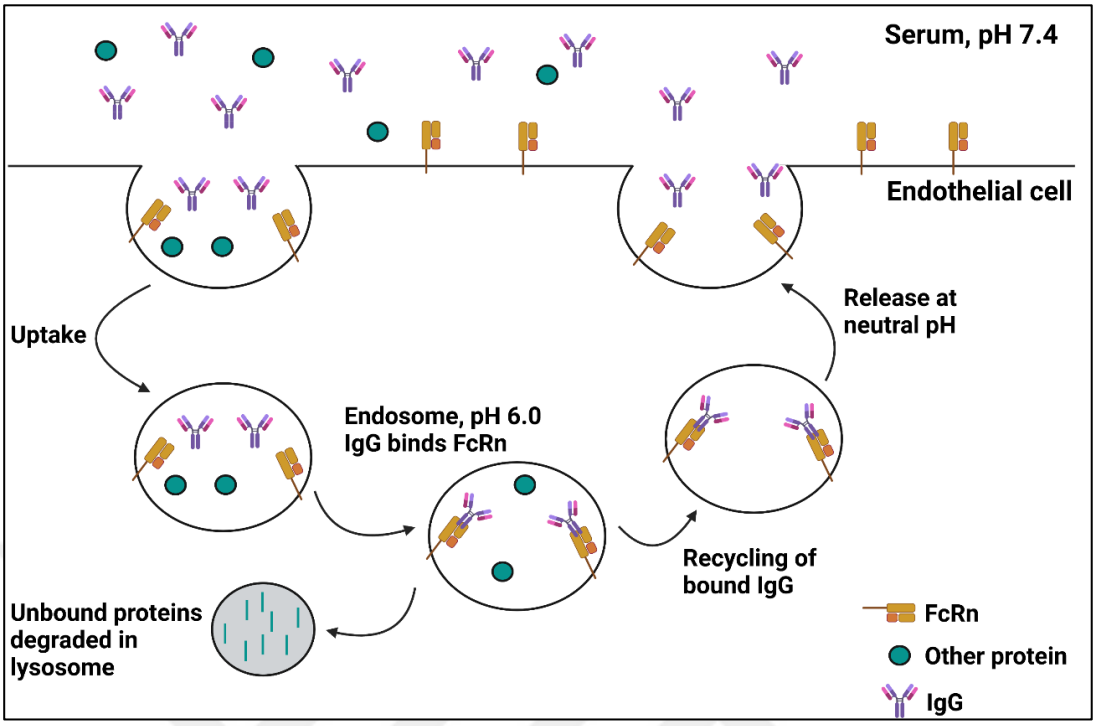



**Figure 5**: The mechanism of antibody recycling

### 1.3.2. Antibody-mediated neutralization

Neutralization is the most basic task of antibodies and is a mechanism to limit the effects of antigenic molecules by binding directly to the pathogen or pathogen-related toxins (**Figure 6**). The Fab domain interacts directly with microbial targets, preventing pathogens from binding to receptors on the cell surface of the organism, inhibiting the formation of infection or disease.

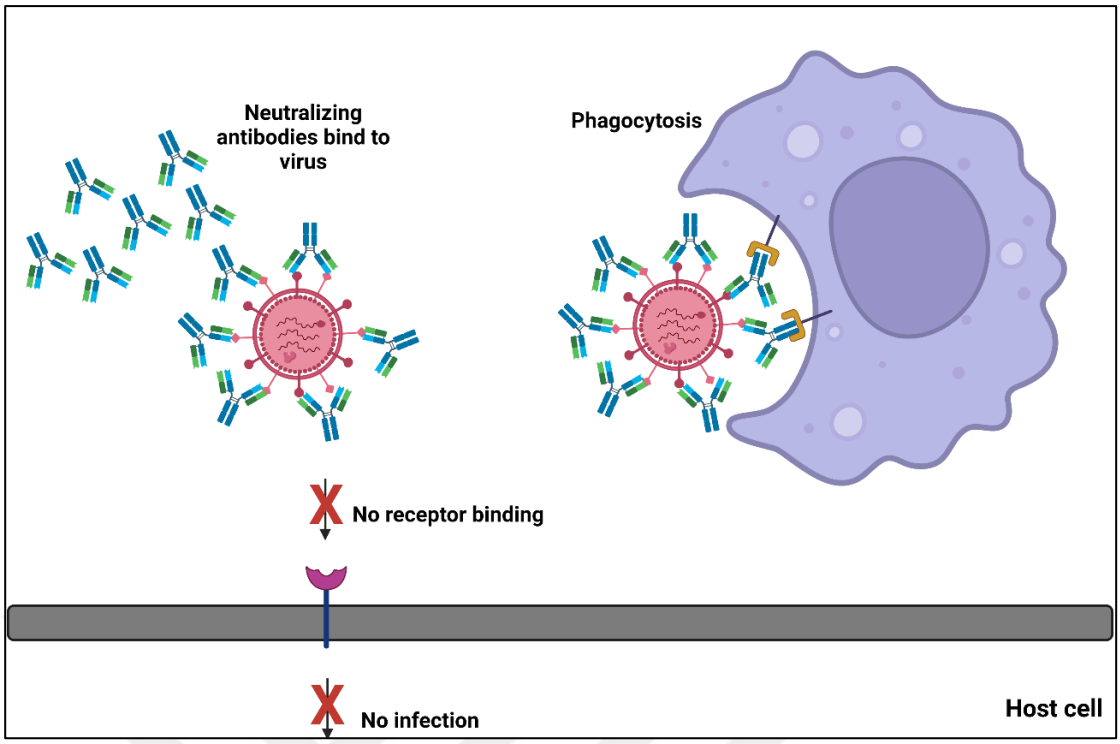



**Figure 6**: Antibody-mediated Neutralization

### 1.3.3. Antibody-mediated complement activation

Both IgM and IgG molecules can interact with the complement system found in the blood and tissues of mammalian organisms (Merle, Noe, et al., 2015; Merle, Church, et al., 2015). The complement system is composed of approximately 30 different proteins and is a system that works in communication with both adaptive and innate immunity for the destruction of pathogens (Dunkelberger and Song 2010; Sarma and Ward 2011). The complement system is activated in three different ways based on the diverse initiator molecules of the complement cascade (**Figure 7**).

The first is the classical pathway through which C1q proteins initiate the complement cascade. The second is the lectin pathway, where the opsonization of pathogens is carried out by binding to mannose groups on the surface of pathogens. The third is the alternative pathway, where the first two mechanisms are not used (Dunkelberger and Song 2010; Sarma and Ward 2011). The complement system first produces inflammatory proteins that recruit phagocytes and other responsible cells, whichever pathway is used. Then, it

performs opsonization with complement receptors on the surface of innate immune system cells. Finally, forming the membrane attack complex (MAC) ensures the destruction of the target cell. The MAC is the mechanism of opening transmembrane channels by gathering the complement system on the target cell (Bacteria or an infected host cell) following antibodies recognizing the antigens. As a result, the cell membrane loses its stability, causing cell death. And this is a crucial function that enables the use of antibodies for therapeutic purposes. The complement system is efficiently triggered by human IgG1, IgG3, and IgM antibodies, weakly by the IgG2 subclass, and IgG4 does not activate it (Schroeder and Cavacini 2010).

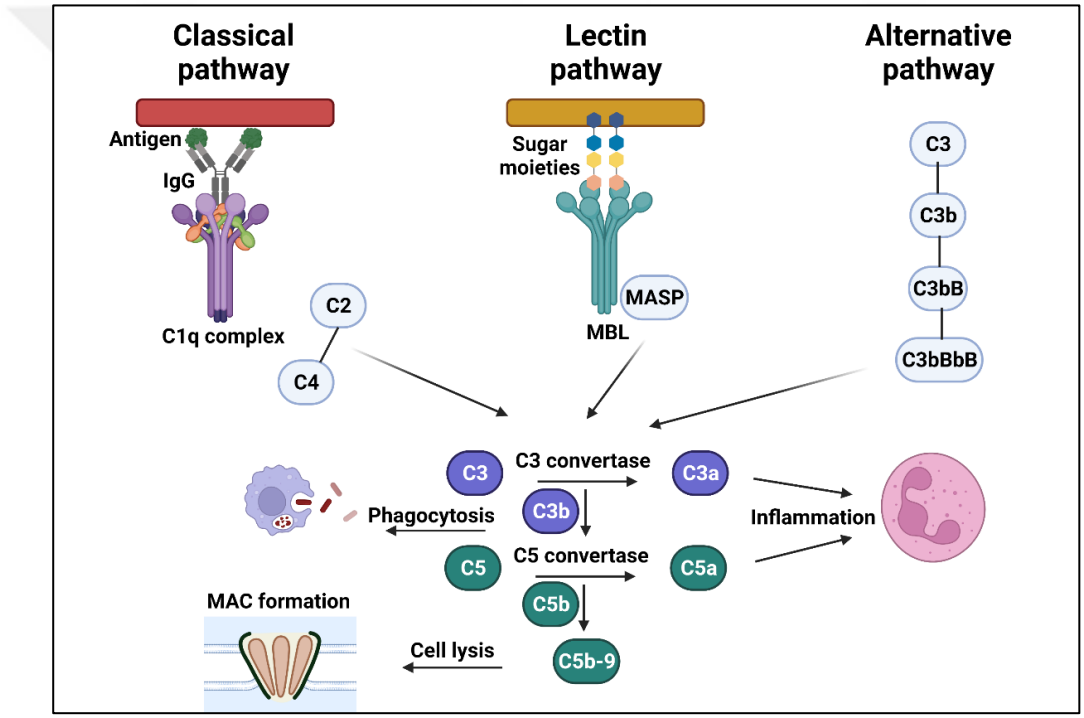



**Figure 7**: Antibody-Mediated Complement activation

As a result of the binding of C1q protein in the classical complement pathway, proteases become active, and C2 and C4 proteins are cleaved (**Figure 7**). With cleavage products, C3 convertase cleaves C3 to C3a and C3b. Highly reactive C3b binds to the surface of pathogens and infected cells to activate immune cells (Merle, Church, et al. 2015; Merle, Noe, et al. 2015). It finally creates a membrane attack complex and allows cells to lysis.

### 1.3.4. Antibody-dependent cellular cytotoxicity (ADCC)

Antibodies directly kill target cells by the mechanism of antibody-dependent cell cytotoxicity. Due to the existence of this mechanism, therapeutic monoclonal antibodies (mAbs) are used to destroy tumor cells (Weiner 2015). In the ADCC mechanism, the Fc part of the antibody bound to the target surface interacts with the FcγRIIIa on the innate immune system's natural killer cells (NK cells) (**Figure 8**). NK Cells secrete perforin and granzyme b onto the target tumor cell, which triggers lysis of the tumor cell. Therefore, the antibody helps to destroy the target by bringing the target and the effector cells together. Therefore, further activation studies were carried out in the therapeutic field by increasing NK-mediated cell cytotoxicity by modifying Fc parts (Jefferis 2012).

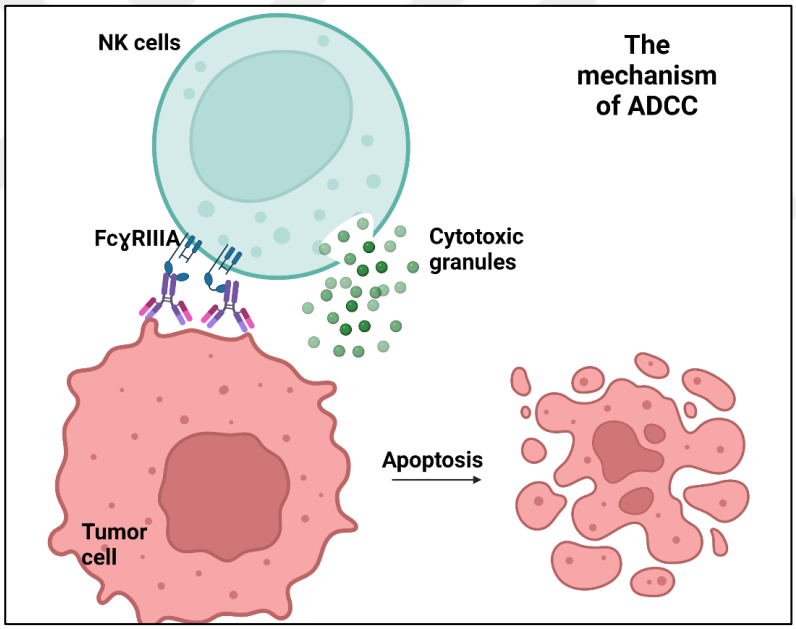



**Figure 8**: The Mechanism of ADCC

Moreover, studies on improving ADCC activity by changing the glycan profiles in the Fc part have also been carried out. For example, cleavage of fucose from the IgG glycan increased its affinity for the Fcgamma RIIIa receptor and was shown to increase ADCC (Shields et al. 2002; Zeitlin et al. 2011a). The addition of a bisecting N-acetylglucosamine

increases ADCC activity, preventing the addition of fucose (Umaña et al. 1999). Although NK cell activation is desired to be optimized in monoclonal therapeutic studies, the other innate immune system cells expressing FcγRIIIa (macrophages and dendritic cells) (Weiskopf and Weissman 2015; Biburger, Lux, and Nimmerjahn 2014; Bournazos, Wang, and Ravetch 2016), and FcγRIIIb (as CD16b, neutrophils) are also conducted on tumor clearance (Peipp et al. 2008; Nimmerjahn and Ravetch 2008b).

### 1.3.5. Antibody-dependent cellular phagocytosis (ADPC)

Opsonaphagocytosis, or elimination of pathogens marked by antibodies, is a killing mechanism by mononuclear phagocytes (monocytes, macrophages, and dendritic cells) and granulocytes (neutrophils, basophils, and eosinophils). In this mechanism (**Figure 9**), antibodies interact with complement receptors and FcRs (Reichert 2014). Apart from eliminating the pathogen, opsonophagocytosis also performs essential functions such as secretion of antimicrobial peptides, metalloproteases, cytokine production, and antigen presentation. Therefore, ADCP is of great importance in microbial clearance and immune memory formation.

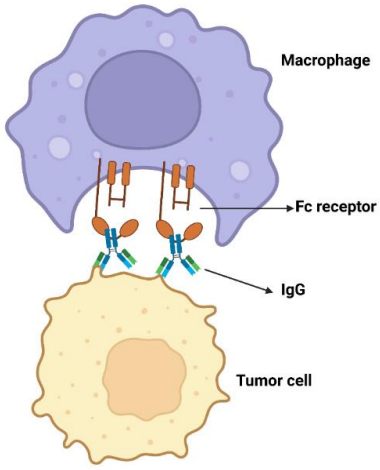



**Figure 9**: The Mechanism of ADPC

Antibody-coated pathogens are recognized by Fc receptors, allowing the pathogen recruitment in endocytic vesicles. Then the pathogen is degraded in different endosomal regions inside cells and is presented to lymphocytes for the further immune response (Reichert 2014).

## 1.4. Monoclonal Antibodies

### 1.4.1. Importance of Monoclonal antibodies

With the development of recombinant DNA technology in the 80s, the production and use of biopharmaceuticals began to spread rapidly (Fekete, Veuthey, and Guillarme 2012). In particular, mAbs, which have the largest share in this drug group, were used in many diseases, from cardiovascular diseases to cancer, from infectious diseases to asthma. The mAbs are immunoglobulins that can recognize an antigen with high specificity and are synthesized from a single B cell clone. After the development of hybridoma technology by Köhler and Millstein in 1975, mAb production was carried out in large-scale and high-purity. (Köhler and Milstein 1975). Thus, with the large-scale production of therapeutic mAbs, scientific research and potential clinical applications are increased. Currently, the most 570 mAbs are in clinical research worldwide. Approximately 80 mAbs (Kaplon and Reichert 2019) was approved by the United States Food and Drug Administration (US-FDA) and used effectively in the clinic. Thirty of these are used only for the treatment of cancer. The production of mAbs has gained significant importance with the increase in their clinical effectiveness. Therefore, production and development studies have increased significantly, and 48 mAbs were brought into the literature in 2008. As of 2017, the number of these global mAbs has increased to 61. (**Figure 10**).

Muromonab-CD3 (Orthoclone OKT3) is the first therapeutic mAb approved by the FDA in 1986 and was developed against CD3 in T cells for use in the treatment of acute transplant rejection (Ecker, Jones, and Levine 2015).

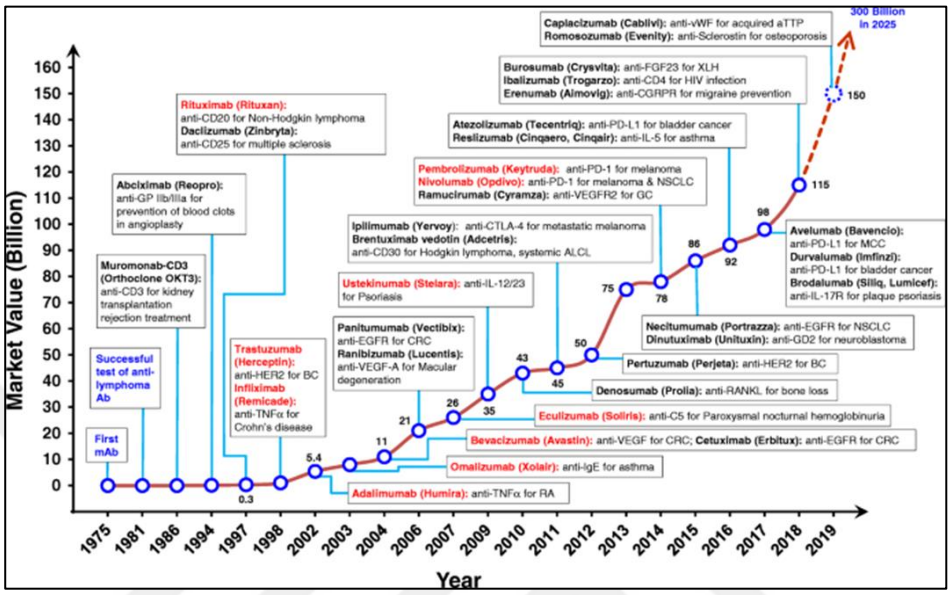



**Figure 10**: The history of Monoclonal Antibodies (R. M. Lu et al. 2020)

Briefly, in hybridoma technology, the relevant antigen is injected into a mouse or any mammal, thereby creating an immune response against the antigen. B cells that produce antibodies that specifically bind to this antigen are then isolated from the mouse. These isolated B cells are fused with myeloma, the immortal cancer cells, to form a hybridoma cell. Thus, the ability of the B cell to produce specific antibodies and the longevity and high production capability of myeloma are combined. Thus, high purity and large-scale mAbs are produced against a specific antigen. Each antibody produced here is the same as the other.

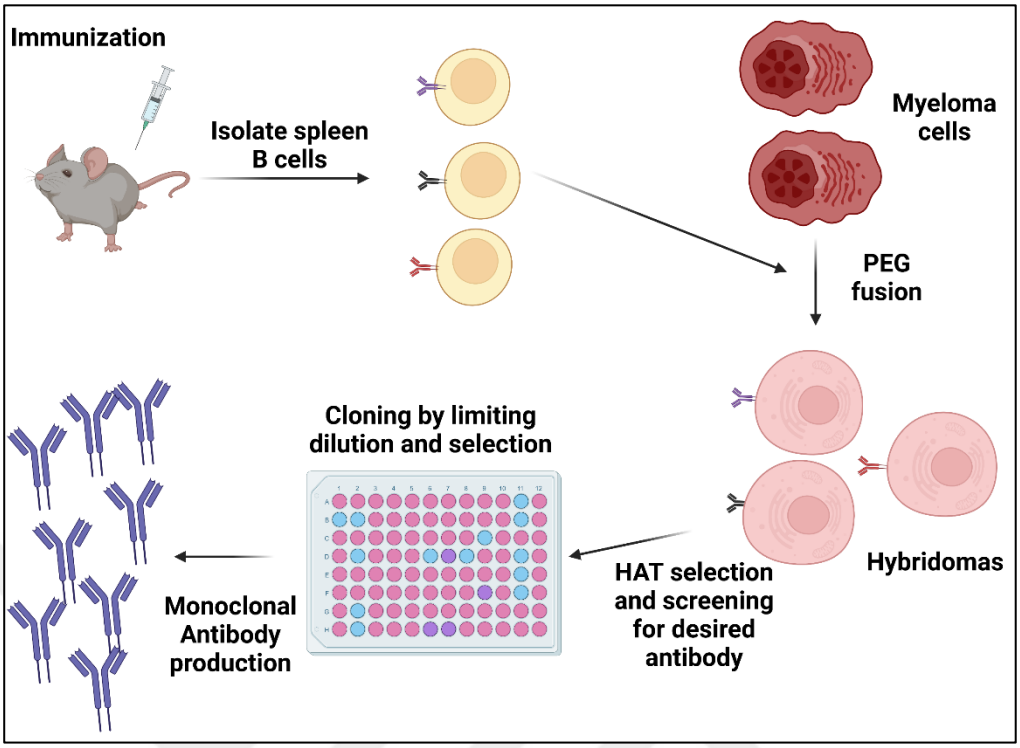



**Figure 11**: the mAb production by using the hybridoma technology

### 1.4.2. Monoclonal Antibody vs. Polyclonal Antibody

As mentioned above, mAbs are produced by a single B cell clone, and all antibodies produced are the same. The mAbs produced here are explicitly produced for only one antigen epitope (**Figure 12**).

On the other hand, polyclonal antibodies are heterogeneous mixtures of antibodies obtained from different B cell lines. These antibodies are a mixture of antibodies that can recognize different epitopes of a single antigen. In producing polyclonal antibodies, all antibodies in the serum of antigen-treated mice are collected. Polyclonal antibodies are more advantageous than mAbs because they are quicker and cheaper to produce. However, it is much more advantageous to produce mAbs because they are more specific to the target, and the production amount is unlimited because hybridoma cells are used.

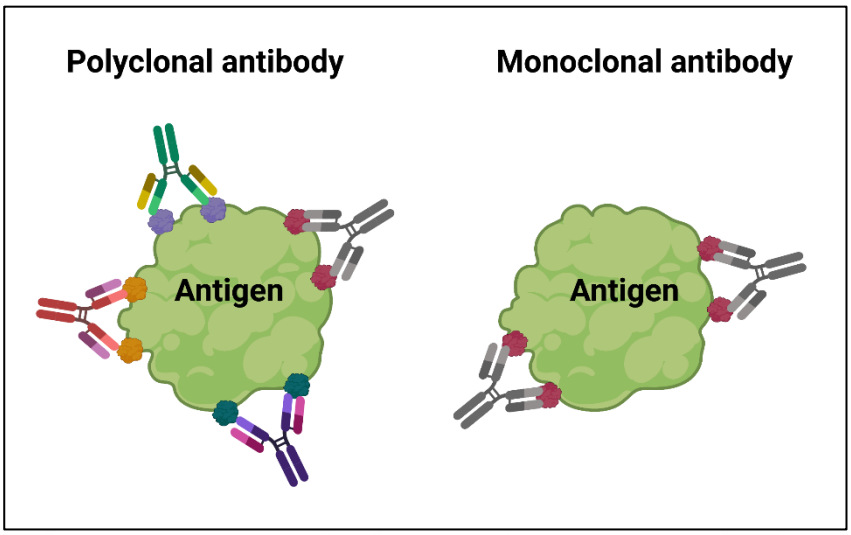



**Figure 12**: Monoclonal Antibody vs. Polyclonal Antibody

### 1.4.3. Biosimilars

The development of copies of pharmaceutical products such as generic drugs or biosimilars has started to develop as a significant trend in pharmaceutical markets. Unlike generic drugs that can be synthesized chemically, biosimilars are complex molecules with many post-translational modifications since mammalian cells are used to produce biosimilars. In addition, biosimilar products also have immunogenic potential. Therefore, during the production of biosimilar drugs, its biosimilarity to the reference molecule has to be proven by comparative studies (Camacho et al., 2014; Niederwieser and Schmitz 2011; Declerck et al. 2017). Biosimilars must be highly similar to licensed reference drugs with proven quality, efficacy, and safety. However, although the biosimilar concept is interpreted with slight differences by different regulatory agencies FDA and European Medicines Agency (EMA)), the basic requirements for biosimilars are the same (J. Wang and Chow 2012; Chugh and Roy 2014). For harmonization in the evaluation and regulation of biosimilars, a guideline for the evaluation of similar biotherapeutic products (SBPs) was determined by the WHO Expert Committee on Biological Standardization (ECBS) (World Health Organization 2013).

Since mABs can be used effectively in the clinic and have a significant share in the world market, they are produced under patent protection. The production and development of biosimilars of mAbs that have expired or are about to expire have also gained significant importance. Therefore, biosimilar mAb biotherapeutic products, also called biosimilar mAbs, must be highly similar in structure and clinical efficacy to the reference antibody. With the introduction of these products to the market, the increase in health expenditures has slowed down a bit, and the availability of drugs has increased. Since biosimilar mAbs are glycoprotein structured, products with significant heterogeneity can be formed because they undergo post-translational modifications (PTMs) and glycosylation profiles. Therefore, during the production of biosimilars, all proving studies must be completed so that all structural properties of the final product are similar to the reference product.

In 2016, ECBS added evaluation criteria for biosimilar mAb products to the WHO guideline (World Health Organization 2016). This document determines all the essential parameters required for the quality and characterization of the product for biosimilar mAb producers. There are no specific analytical methods identified so far, and manufacturers can apply their analytical methods to prove similarity with the reference molecule. These analytical methods must be scientifically validated.

The authorities have classified the quality attribute of the produced biosimilars. These are listed as tier 1, tier 2, and tier3 in order of risk. For example, the binding of the biosimilar to the target molecule and the demonstration of its biological activity are indicated as tier 1, which is the most important class. Demonstration of charge heterogeneity, analysis of binding with Fc receptors, determination of aggregation profile indicated as tier 2 and high order structures such as secondary and tertiary, and demonstration of properties such as ADCC and CDC constitute the category of tier 3. These risk criteria may vary according to the antibodies produced. Therefore, necessary controls should be provided by taking these categories into account in the production of biosimilars.

The manufacturing process can affect the quality attributes of biosimilar mAb products. In general, the amino acid sequence of biosimilar mAbs should be the same as the reference molecule. However, its structural properties, which will not change its effectiveness much, may differ in properties such as contents of impurities. These parameters can be approved as long as they do not change the drug's effectiveness. For the approval of the biosimilar by regulatory authorities, comparative studies must be done in terms of the structural properties and functional characterization of the biosimilar.

However, comparative clinical studies of biosimilar are also required. Since the production process of biosimilars is very complex, slight differences during production can have significant effects on the quality and effectiveness of the product.

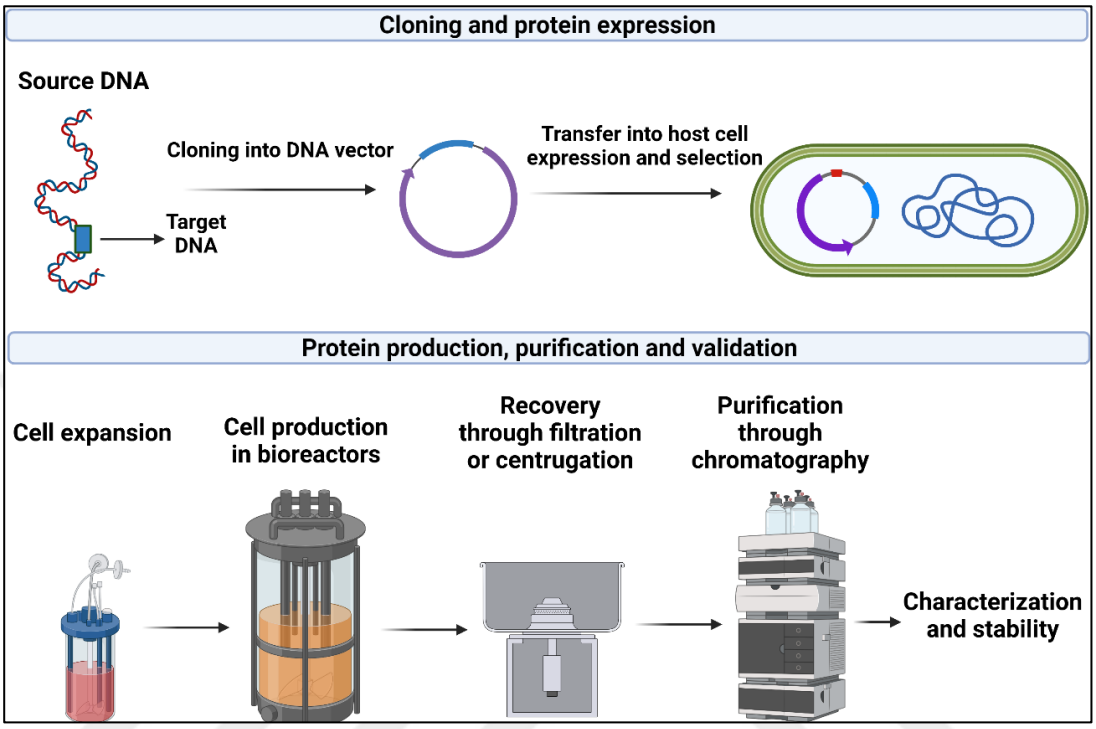



**Figure 13**: The production stages of biosimilar drugs

Production of biosimilars begins with cloning the corresponding antibody gene into the vector (**Figure 13**). The protein is then expressed by transferring this vector into the cell. Then, the cell line that produces the desired antibody in high yield is selected. The cell is grown in the appropriate medium and under appropriate environmental conditions using fermentation technology (Mellstedt, Niederwieser, and Ludwig 2008; Reichert, Beck, and Iyer 2009). Antibodies are then obtained with a complex settling time. Validated analytical methods are used to determine the structural and functional properties of the obtained biosimilars. After all the quality control parameters are completed, the high-scale fermentation process is continued (Mellstedt, Niederwieser, and Ludwig 2008; Reichert, Beck, and Iyer 2009). The final product is completely purified, and necessary

further characterization studies and clinical studies are completed. Then, the approval process is initiated by the regulatory agents.

### 1.5. Post-translational Modifications in Antibodies

Post-translational modifications (PTMs) alter the polypeptide chain processed enzymatically or nonenzymatically after translation. These modifications are essential in protein functions, signal transduction, cellular regulation, degradation, protein targeting, and localization (L. Chen, Keppler, and Schölz 2018). The most common PTMs in therapeutic antibodies are glycosylation, disulfide bond formation, and proteolytic cleavage in the protein backbone. In addition, in downstream processing, formulation, and storage states, mAbs can undergo many non-enzymatic chemical modifications: oxidation, glycation, pyro-glutamate formation (pyro-Glu), and deamidation. Since these modifications seriously affect mAbs' biological activity, immunogenicity, and stability, their characterization is essential in therapeutic antibody production. Although more than 500 PTMs were discovered, some are observed in mAbs, including glycosylation, N terminal pyro-Glu formation, C terminal Lysine (Lys) clipping deamidation, oxidation, glycation, cysteinylation, trisulfide bonding, hydroxylation, non-enzymatic fragmentation (clipping), and isomerization (**Figure 14**). But among these, the most common modifications are glycosylation, C-term Lys clipping, N terminal pyro-Glu formation, oxidation, and deamidation, especially in commercial biosimilar drugs.

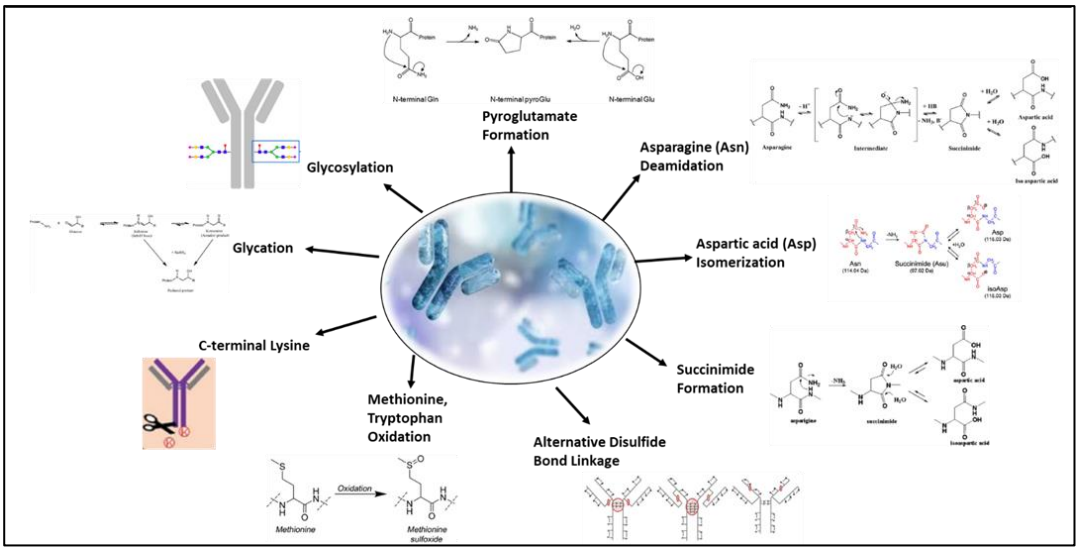



**Figure 14**: The most common modifications in mAbs

Others are present in very low abundance and do not severely affect biological activity. Most recombinant mAb therapeutics are produced using mammalian cells such as the Chinese hamster ovary (CHO) or murine NS0. These productions can generally be carried out as high fidelity and without much variation. However, many PTMs not generally found in the human body can occur in antibodies produced in non-human cells. However, these modifications can also occur during drug production. These modifications can reduce the effectiveness of antibodies and produce unexpected effects. Therefore, there are parameters to be regularly monitored while producing mAbs. It should be determined which modifications occur. These unnatural modifications create undesirable conditions, such as increased immunogenicity and activity loss (**Table 1**).

| Modifications | Biological effects | Reference |
|---|---|---|
| Removal of fucose residues | ADCC activity ↑ | Mimur et al. 2018 |
| Bisecting GlcNAc | ADCC activity ↑ | Tobia et al. 2017 |
| Terminal Galactose content | CDC activity ↑ | Hodoniczky, Zheng, James 2005 |
| High mannose | ADCC activity ↑ | Zhou et al. 2008 |
| High mannose | CDC activity ↓ | Kanda et al. 2007 |
| The isomerization of Asp 102 residue in the CDR region | The potency of drug ↓ | Harris et al. 2001 |
| Asn deamidation in CDR | The potency of drug ↓ | Bults et al. 2016 |
| Methionine oxidation in Fc | CDC activity ↓ | Jingije Mo, Qingrong 2016 |
| Methionine oxidation in Fc | Half-life(FcRn Binding) ↓ | Xuan Gao, Junyan A ji 2014 |

**Table 1**: The effects of PTMs on mAbs biological activities

### 1.5.1. Glycosylation

Glycosylation is a significant PTM, especially in the biology of cell surface and secreted proteins (Reily et al. 2019; Spiro 2002). In particular, glycosylation has severe effects on secretion, solubility, stability, packing, binding, conformation, biological activity, and antigenicity of proteins (Spiro 2002). Thus, this modification is performed in a highly controlled and conservative manner, and it does not occur spontaneously in normal physiological conditions (Mahan et al., 2016; Jennewein and Alter, 2017).

Because immunoglobulins are glycoproteins, they carry many glycosylation sites. Glycosylation is of great importance for immunoglobulin molecules and is essential for regulating the functions of antibodies (Wuhrer et al., 2007; Plomp et al., 2017).

The glycans are bound to immunoglobulins in two ways: either through Asn residues (N-glycans) or serine/threonine residues (O-glycans) (Spiro 2002). While most proteins carry these two different glycan-binding profiles, the glycans of IgG molecules are bound to the molecule from specific N- residues (Jennewein and Alter 2017). Importantly, all IgG subclasses are glycosylated in the Fc portion of the antibody through the Asn residue at amino acid 297 position (Asn297) (**Figure 15**). The sequence in which N-linked glycosylation occurs is Asn-Xaa-Serine/Threonine (Asn-Xaa-Ser/Thr). Here, Xaa can be any amino acid other than proline (Pro) (Sox and Hood 1970). This modification seriously affects the stability and effector function of the antibody (Jefferis 2009; 2012). In addition, approximately 20% of the Fab region contains the N-glycan site, but the glycosylation here is much more limited than in the Fc region (van de Bovenkamp et al., 2016).

Glycosylation of IgGs is an enzyme-directed chemical reaction in the cell's endoplasmic reticulum and Golgi apparatus. Since therapeutic antibodies are based on IgG, glycosylation is essential for the efficacy of mAbs. The glycosylation profile is essential for understanding the effector functions, pharmacokinetic properties, and stability of antibodies. The glycan structure affects the function due to conformational changes in the Fc part of the IgG molecule (Borrok et al., 2012). Deglycosylation causes mAbs to be less stable thermally. Thus, they are more prone to unfolding and degradation (Higel et al. 2016). While glycosylation enables the IgG molecule to interact with FcγRs by providing the "open" conformation, and deglycosylation prevents this interaction by converting it to the "closed" conformation (Krapp et al. 2003).

Glycosylation increases the stability and solubility of antibodies and prevents their tendency to form aggregates (Kayser et al., 2011). The heterogeneity of antibody glycoforms presents a significant challenge in biopharmaceuticals (Jefferis 2009). Knowing the sequence of the polypeptide structure alone is not enough; the whole structure must be accurately analyzed since carbohydrate residues are also involved in the polypeptide structure (Krapp et al., 2003).

The antibody glycan is composed of a structure with a biantennary heptasaccharide core. When this core structure is examined, two N-acetylglucosamine (GlcNAc) and mannose are attached to the end (**Figure 15**). One additional mannose was added by branching 1,3

and 1,6, and one additional GlcNAc residue was added to the ends of these mannoses (Kaur 2021). Then extra fucose, a bisecting GlcNAc, two galactoses, and two sialic acids can be attached to this sugar core to provide the glycan diversity of the antibody, which 30 different structures identified in human plasma (Rudd 2001).

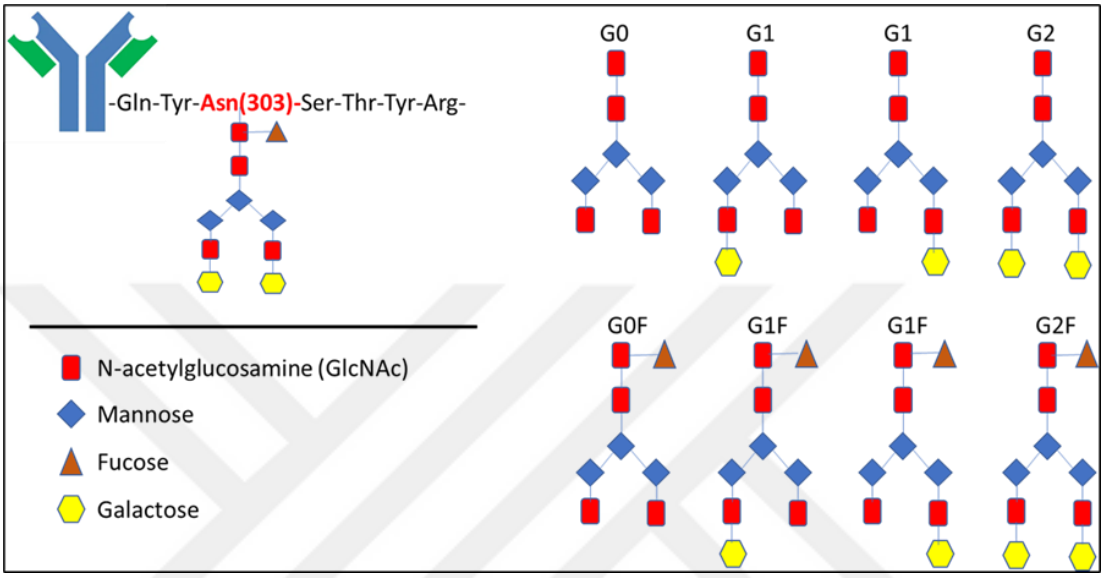



**Figure 15**: The Glycan Structure of IgGs

Specifically, heterogeneous glycans can be classified according to the number of galactose contained. The galactose-free glycans are shown as G0, G1 with a galactose residue, and G2 with two galactoses (Flynn et al. 2010; T. Shantha Raju and Jordan 2012). The alteration of galactosylation can have severe effects on the functions of antibodies and can cause changes in the immune response in many diseases (Schwab and Nimmerjahn 2013; Ren et al. 2016; Jennewein and Alter 2017; Tanaka et al. 2017; Trbojević Akmačić et al. 2015; Kanda et al. 2007). While terminal Galactose content has a vital role in CDC activity, it does not affect ADCC activity. Studies have shown that binding to the C1q complement member and CDC activity increases when the galactose ratio in the glycan structure increases (Hodoniczky, Zheng, and James 2005; T. Shantha Raju 2008). And it was shown in studies that CDC activity decreases due to removing galactose with glycosidase (P. N. Boyd, Lines, and Patel 1995). Moreover, terminal

galactose with afucosylation core makes a conformational change in the CH2 domain, allowing it to bind to Fcgamma receptors better, increasing ADCC activity (Houde et al. 2010).

The most common glycoform structures are fucosylated modifications with zero, one, or two beta-1,4-galactose. This is referred to as G0F, G1F, and G2F, respectively. Beta-1,4 galactose stabilizes the open conformational structure of the Fc region, allowing Fc receptors to interact with the antibody (Krapp et al., 2003; Houde et al., 2010). The removing fucose residues improve ADCC activity (T. Shantha Raju 2008; Mimura et al. 2018) since there is a conformational difference between fucosylated and non-fucosylated IgG (Matsumiya et al., 2007). ADCC activity is increased due to better binding to the FcγRIIIa in afucosylated IgG molecules (Jez et al. 2012; Houde et al. 2010; Ferrara et al. 2006). Because fucosylation sterically prevents this interaction (Ferrara et al. 2011). Fucosylation also does not significantly affect C1q binding and thus CDC activity (Kanda et al., 2007; Shields et al., 2002). But it does affect FcRn binding and thus antibody clearance (Kanda et al. 2007). In another study comparing the fucosylated and afucosylated forms of anti-Her2, the afucosylated form showed 100 times more ADCC activity (Shields et al. 2002).

In human serum, afucosylated glycosylation is common and therefore not immunogenic. As afucosylation is related to ADCC activity, this critical quality attribute (CQA) is a feature that has attracted attention by regulators in the field of therapeutic antibodies, especially in oncological drugs. Therefore, it is aimed to produce afucosyl groups in the production of antibodies for therapeutic purposes and should be controlled by monitoring the glycosyl profile during production.

In addition, bisecting GlcNAc modification which is GlcNAc transferred to the 4-position of the β-linked core, improves ADCC activity as it indirectly inhibits fucose addition (Hanashima, Suga, and Yamaguchi 2018). The addition of Bisecting GlcNAc increased the binding affinity of the antibodies to the FcγR3a receptor, causing 10-30 times more ADCC activity (Shinkawa et al., 2003; Davies et al. 2001). Terminal GlcNAc is a modification that affects the thermal stability of the CH2 domain. The removal of these residues resulted in a loss of thermal stability and decreased binding affinity for the FcγRIIb receptor (Mimura et al. 2001). Also, IgGs with terminal GlcNAc exhibited a higher serum half-life (Jones et al., 2007).

The high mannose content in the Fc glycans of IgG molecules is found in numbers from 5 to 9 bound to core GlcNAc. When Fc glycans are examined, they show significant

heterogeneity according to the number of terminal mannose residues, Man5GlcNAc2, Man6GlcNAc2, Man7GlcNAc2, etc. Typically, high mannose antibodies are 0.1% in human serum, while this rate can increase to 10% in recombinant proteins (Reusch and Tejada 2015). High mannose increases ADCC activity by increasing the binding affinity of IgG molecules to the FcγRIIIa (Zhou et al., 2008), while high mannose negatively decreases CDC activity by lowering the binding affinity of antibodies to C1q (Kanda et al. 2007). Circulating glycoproteins are recognized by specific glycan receptors and eliminated from the blood. Therefore, these receptors recognize antibodies containing glycan and rapidly remove them from the blood (M. Yu et al., 2012). It was observed that high mannose-containing IgG molecules have a shorter half-life in the circulation and are rapidly cleared in serum (Alessandri et al. 2012; Goetze et al. 2011; Wright et al. 2000). Therefore, the determination of terminal mannoses is considered a CQA because of their effects on the therapeutic activities of mAbs.

In addition, 20% of the IgGs found in the serum of healthy individuals end up with N acetylneuraminic acid (Neu3Ac or NANA), a type of sialic acid (Pučić et al. 2011). Negatively charged sialic acid added to the end of the glycans was also observed to affect the half-life of the antibody. It was observed that the half-life decreased when sialic acid was removed (L. Liu 2015). Antibodies containing terminal sialic acid have a longer half-life than non-sialylated forms. The underlying mechanism is this: the asialoglycoprotein receptor responsible for the endocytosis-mediated degradation of glycoproteins binds to terminal galactose and GlcNAc and functions. Sialic acid forms the surface of galactose like a mask, preventing this binding and thus protecting it from clearance (Lee et al., 2002). Terminal sialylation of IgGs is more sensitive to proteases than their asialylated forms (T.S. Raju and Scallon 2007). High terminal sialylation of IgGs causes lower affinity to the FcγRIIIa and decreases ADCC activity (Kaneko 2006). Large Neu5Ac groups restrict flexibility in the hinge region, which leads to limitations in ADCC activity and binding to antigen (Scallon et al., 2007). Therefore, because sialylation has both negative and positive effects, the control of this modification is essential in regulating ADCC activity and molecule structure.

Consequently, in mAbs produced for biotherapeutic purposes, many factors can affect the glycosylation of the antibody during production (Batra and Rathore 2016). The factors to be considered briefly are cell line, feed composition, cell viability, temperature, dissolved oxygen, ammonia, pH, and osmolarity. pH and ammonium are two fundamental parameters for a stable glycosylation pattern. Increasing ammonium concentration

increases the sialylation rate (Gawlitzek et al. 2000). In addition, pH changes in culture can also lead to changes in the sialylation profile (Borys, Linzer, and Papoutsakis 1993). As the ammonium concentration increases, IgG galactosylation decreases (M. Yang and Butler 2000).

### 1.5.2. Oxidation

Oxidation is a modification that has significant effects on antibodies, especially in methionine (Met) and less frequently in tryptophan (Trp), histidine (his), and other amino acids. Met oxidation can cause structural and functional changes (**Figure 16A**). But the effects of this modification vary according to which region of the antibody is oxidized (in the CDR region or the constant region?). Well-conserved Met252 and Met428 in the Fc region are susceptible residues to oxidation (Lam, Yang, and Cleland 1997; Chumsae et al. 2007; Gaza-Bulseco, Faldu, et al. 2008b; H. Liu, Gaza-Bulseco, Xiang, et al. 2008b; Wei et al. 2007). Oxidation in the Fc region disrupts FcRn binding and thus reduces the half-life of the antibody (Bertolotti-Ciarlet et al. 2009a; Weirong Wang et al. 2011). In addition, the yield is low since oxidized IgGs cannot be captured by protein A and Protein G chromographs during the bioprocess of IgG (H. Liu, Gaza-Bulseco, Xiang, et al. 2008b).

Although Met oxidation in the Fc region does not affect antigen binding (Lam, Yang, and Cleland 1997), it can cause a conformational change in the CH2 domain (H. Liu, Gaza-Bulseco, Xiang, et al. 2008b; Wei et al. 2007). The oxidation of Met causes the formation of a basic charge by changing the overall surface charge of the protein. The resulting charge variants can be analyzed by capillary isoelectric focusing (cIEF) or cation-exchange chromatography (CEX) (Salas-Solano et al. 2012; T. Zhang, Bourret, and Cano 2011).

However, oxidation of Trp residues can also have profound effects (J. Yang et al., 2007; Lam et al., 2011). For example, oxidation of Trp in the CDR region was found to cause a decrease in antigen binding affinity (Wei et al., 2007a) (**Figure 16B**).

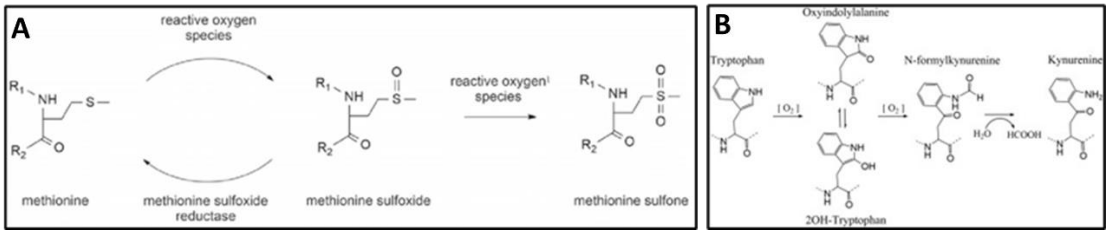



**Figure 16**: Oxidation Mechanism of IgGs

### 1.5.3. C-terminal Modifications

C-terminal Lys variants are frequently seen in mAbs produced by mammalian cells (**Figure 17**). mAbs are synthesized with Lys residues at the ends of their heavy chains. But some of these are clipped and discarded during cell culture (Dick et al. 2008; Harris 1995). The C-terminal Lys level remains constant since there is no carboxypeptidase activity in storage. However, these remaining C-terminal Lys are immediately removed in the circulation system (Cai, Pan, and Flynn 2011). Hence, C-terminal Lys does not seriously affect biological activity due to immediately being eliminated *in vivo* (Mvasi: EPAR - European Medicines Agency, n.d.). But it is still a modification that needs to be considered for mAb heterogeneity monitoring. Since lysine is a positively charged amino acid, its disappearance causes a decrease in the positive charge of the antibody, and the modified and non-modified can be easily separated by several charge-based separation methods (Yüce et al., 2021). In addition, since lysine loss at the C-terminal causes a mass shift of approximately 128 Da, they can also be separated by mass spectrometry (MS) methods, including intact mass and peptide mapping.

C-terminal Pro amidation was first characterized in recombinant IgG1 (Johnson et al. 2007). In this modification, Pro residue terminates with an amide group. This modification causes an increase in basic variants in the antibody (Hu et al., 2017). Subsequent studies showed that this modification was also present in IgG2, IgG3, and IgG4 (Tsubaki et al., 2013). No effect of amidation on Fc effector function was observed (Johnson et al., 2007) (**Figure 17**).

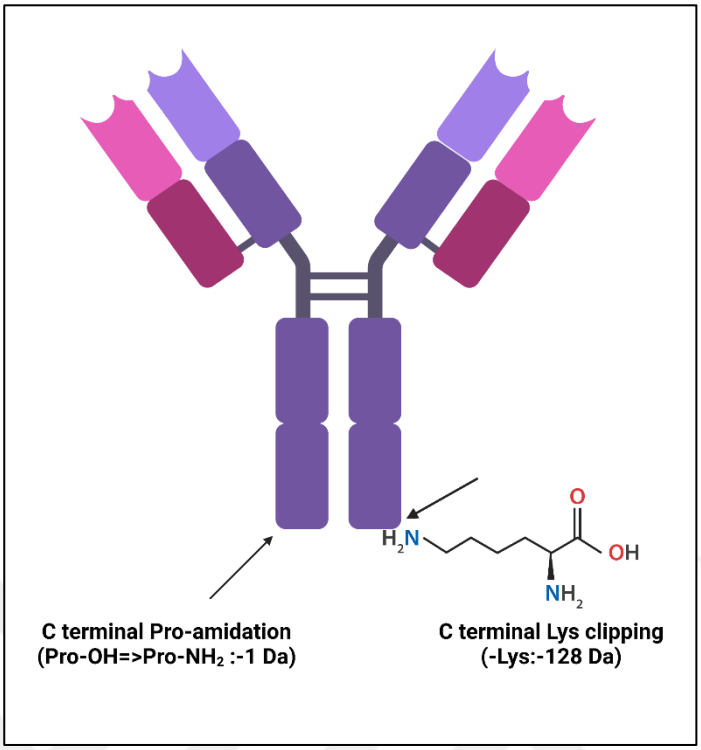



**Figure 17**: C-Terminal Modifications of IgGs

### 1.5.4. Glycation

Glycation is a non-enzymatic reaction between the N-terminal amino group of the protein or the side chains of Lys residues and reducing sugars (**Figure 18**). *In vivo*, these saccharides are usually glucose, but they can also be other reducing sugars such as fructose or galactose (Jairajpuri, Fatima, and Jairajpuri 2015). Glycation can generally occur during cell culture, formulation, and storage stages (Andya James D. et al. 1999; Goetze et al. 2012). However, increased glycation causes an increase in antibody aggregation (Yuk et al., 2011). Many studies have shown that glycation of lysines in the CDR region did not significantly affect antigen binding (Quan et al., 2008; K. Chen et al., 2009; Yan, Steen, et al. 2009a). In addition, glycation does not affect binding to FcRs (Goetze et al., 2012). It was observed that the half-life of mAbs in the blood increases with increasing glycation (Goetze et al., 2012). It either has a negative effect on antigen

binding (Kennedy, Skilbnn, and Self 2008; Dolhofer-Bliesener and Gerbitz 1990) or has no effect (Quan et al. 2008). Glycation is a common modification in the production of therapeutic antibodies. To reduce this situation and to increase safety, the treatment of reducing sugars should be restricted.

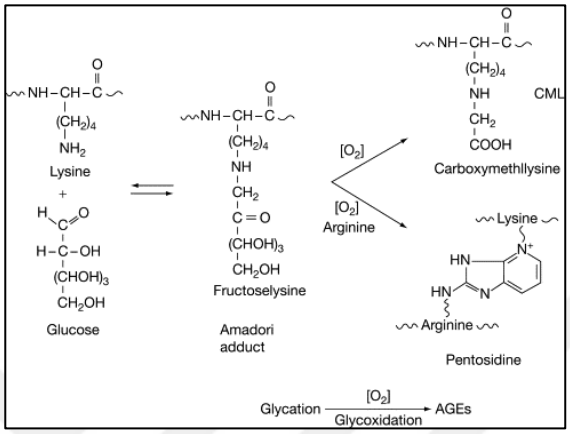



**Figure 18**: Glycation Mechanism of IgGs

### 1.5.5. Cysteine-related Variants

For each IgG subclass, cysteine residues form well-defined disulfide bonds. However, some cases may occur in different disulfide bond variants such as alternative disulfide bond, trisulfide bond, and racemization. However, they do not have a severe effect on the efficacy of the antibody (Gu et al. 2010; Aono et al. 2010; Q. Zhang et al. 2013). However, in the case of increasing free Cys, covalent aggregates form, thermal stability decreases (Huh et al., 2013), resulting in the antibody's potency decreasing.

### 1.5.6. Deamidation and isomerization of Asparagine

Deamidation is a chemical mechanism removing an amide functional group in the side chains of Asn or glutamine (Gln) or conversion to another functional group (**Figure 19**). Deamidation of Asn is a modification commonly observed in recombinant mAbs that affects their heterogeneity and stability (Terashima, Koga, and Nagai 2007; H. Liu, Caza-Bulseco, et al. 2008). Buffer, pH, and temperature are factors that affect the deamidation rate (Scotchler and Robinson 1974; Cournoyer et al. 2007). Deamidation of Asn in both the Fc portion of the antibody (Y. D. Liu, van Enk, and Flynn 2009; L. Wang et al. 2005; Chelius, Rehder, and Bondarenko 2005; Sinha et al. 2009) as well as in the CDR part (L. Huang et al. 2005a; Harris et al. 2001; Vlasak et al. 2009) can have some effect on antibody functions.

The thermal stability of the antibody decreases with Asn deamidation (Vlasak et al. 2009). While deamidation in the CDR region causes a decrease in the binding affinity of the antibody (Huang L, Lu J, Wroblewski VJ 2005; Harris RJ, Kabakoff B, Macchi FD 2001, Vlasak J, Bussat MC, Wang S 2009), deamidation in the Fc part has no an effect in the binding affinity of the antigen (Lyubarskaya et al. 2006; B. Yan, Steen, et al. 2009b).

Deamidation rate is high in oxidized and unfolded IgG molecules. This indicates that deamidation is related to protein conformation and solvent exposure (Chelius, Rehder, and Bondarenko 2005). Also, alkaline pH values promote deamidation, particularly the amino acids Asn331 and Asn360 (D. Liu et al. 2008). Some conserved Asn residues in the Fc region may also undergo deamidation. There is a highly conserved PENNY sequence in the Fc region, where Asn384 and Asn389 are amino acids that can be deamidated (Chelius, Rehder, and Bondarenko 2005). However, since the FcRn and FcγRs binding sites are far from these amino acids, a severe effect on effector function and clearance is not observed.

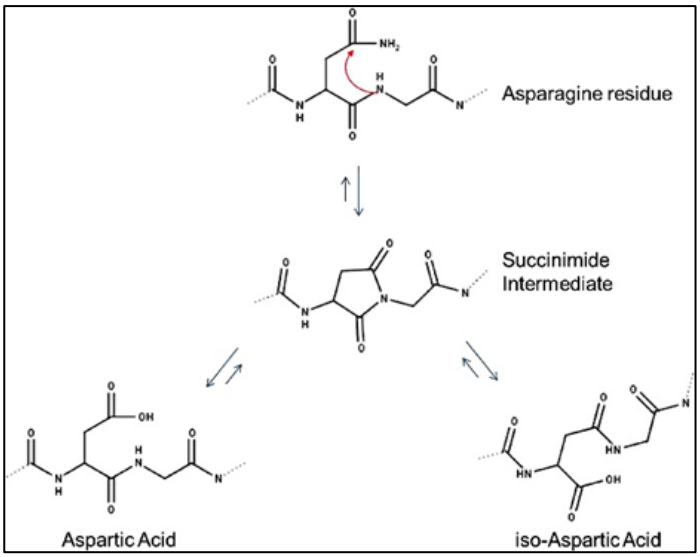



**Figure 19**: Deamidation and Isomerization of Asparagine

Native aspartic acid (Asp) can transform into isoaspartic acid (iso-Asp), which provides heterogeneity in the antibody by undergoing isomerization by itself (Sreedhara et al., 2012). Asp-Gly, Asp-Ser, and His-Asp are sequences most sensitive to isomerization (Brennan and Clarke 2009; Dick et al. 2007). Asp is first converted to the succinimide intermediate and converted back to Asp by hydrolysis or isomerized to iso-Asp. Although succinimide is unstable, it was detected in many recombinant mAbs (Ouellette et al., 2013; Yan, Steen, et al. 2009b; Cacia et al. 1996; X. C. Yu et al. 2011). When succinimide coming from Asp isomerization is in the CDR region of many antibodies, it causes a decrease in antigen binding and potency (Ouellette et al. 2013; B. Yan, Steen, et al. 2009b; Cacia et al. 1996; Valliere-Douglass et al. 2008) (**Figure 19**).

In one study, the isomerization of Asp 102 residue in the CDR3 region of the heavy chain of the Herceptin IgG molecule reduced the potency of the drug to 70% and had severe effects on drug efficacy (Harris et al. 2001). The addition of an extra methyl group to the backbone during isomerization affects the stability and structure of the protein. However, the antigen-binding capacity of many antibodies with Asp isomerization was also reduced (Cacia et al. 1996; Rehder et al. 2008; Wakankar et al. 2007). The heterogeneity of mAb resulting from deamidation and isomerization makes production consistency difficult (H. Liu, Gaza-Bulseco, and Lundell 2008). In addition, deamidation and isomerization can

occur at different stages of mAb production and are the main degradation reactions encountered during long-term storage. Therefore, the formulation strategies of therapeutic proteins need to be well evaluated to minimize these modifications and have an appropriate shelf life of the drug (Wakankar and Borchardt 2006).

### 1.5.7. The cyclization of Glutamine

The most common N-terminal modification is the cyclization of N-terminal Gln or glutamate (Glu) or pyroglutamate (PyroE). This modification occurs spontaneously or by the glutaminyl cyclase enzyme converted to the Gln or Glu cyclic form located at the antibodies' N-terminus (Dick et al. 2007; Gaza-Bulseco, Li et al. 2008; Terashima, Koga, and Nagai 2007) (**Figure 20).** The cyclization rate of Gln is faster than that of Glu. Cyclization occurs more spontaneously than enzymatic in the bioreactor during the mAb production, and this modification accelerated with increasing temperature. Although there is no significant change in the reaction rate in pH changes, it is known that sodium phosphate and ammonium carbonate in the cell culture medium and buffers increase PyroE modification (Dick et al., 2007). Conversion of Gln to PyroE makes antibodies more acidic, while converting Glu to PyroE makes antibodies more basic. Hence, this mAb heterogeneity can be observed and distinguished using charge-based methods such as CEX. In addition, they can be distinguished by MS methods as the conversion of Gln or Glu to PyroE causes mass shifts of 17 and 18 daltons, respectively.

The presence of PyroE did not show a severe effect on the antibody structure (L. Yu et al. 2006) and antigen-binding (Lyubarskaya et al. 2006). In addition, there was no effect on *in vivo* clearance when the N-terminus Glu and N-terminal pyroE were compared (Y. D. Liu et al. 2011). Sometimes part of the leader sequence may not be adequately removed and remain attached to the antibody (Khawli et al. 2010; Kaschak et al. 2011). its presence does not affect the binding of the antigen to the antigen (Khawli et al. 2010), its structure, FcRn binding, or pharmacokinetics (Khawli et al. 2010).

Since the reaction is non-enzymatic, PyroE formation can continue even in circulation after its production (Y. D. Liu et al., 2011). However, this modification does not cause a severe problem in terms of the efficacy and safety of the antibody.

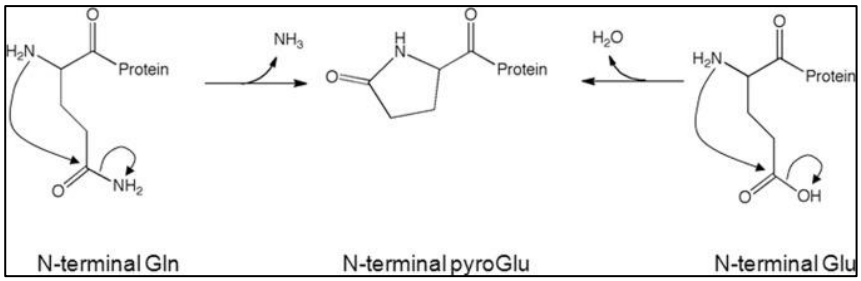



**Figure 20**: Pyroglutamate formation in IgGs

### 1.6. Forced Degradation Studies

Unlike generic drugs, the production of mAbs is challenging because of their complex protein structures. mAbs are exposed to many environmental stress factors such as temperature, light, mechanic stress, humidity, and pH during almost all production steps, from the upstream to post-development processes such as packaging, shipping, and storage (**Figure 21**). As a result of these environmental stresses, the biochemical structure of the antibodies can be severely affected. It may undergo various degradation pathways, which causes them to lose its biological activity. (Nowak, K. Cheung, et al. 2017) The degradation of recombinant mAbs is an important parameter affecting the product's quality, biological efficacy, and safety.

Forced degradation studies provide reliable data by imitating the potential stress factors via elevated conditions in a short time, instead of the more prolonged exposure to mild stress conditions usually seen in production or post-production (Chan 2016). In addition, forced degradation studies help understand the molecule's biophysical and biochemical properties, including the degradation mechanisms in depth (Blessy et al., 2014).

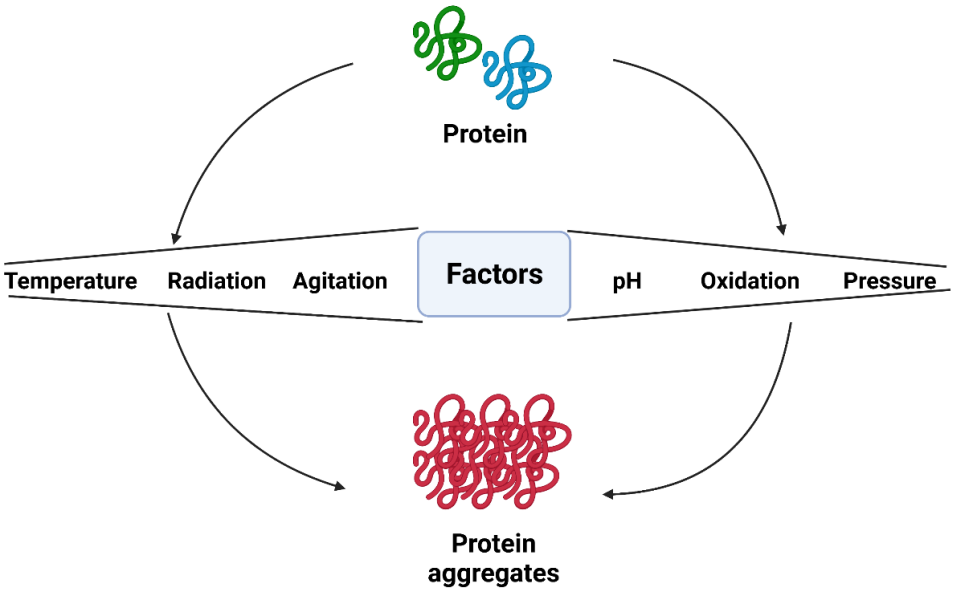



**Figure 21**: The Environmental Stress Factors affecting mAbs

The degradation pathways should be determined during the mAb development and production phase by conducting forced degradation studies. These studies are required to determine the formulation for mAbs and their biosimilar products and carry out stability studies. The factors that should be considered in evaluating the developed drugs' stability are stated in The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Q5C guideline (ICH 1996). In this guideline, the importance of developing and validating new methods in analyzing drug stability in the presence of stress factors, revealing possible degradation mechanisms, and developing more stable formulations are also mentioned. Therefore, the stability tests provide extensive information required to demonstrate the drug effectiveness, the appropriate formulation, and storage conditions to determine the shelf life. Consequently, to achieve the stability of mAbs-based drugs, it is essential to investigate and reveal the ways of degradation such as aggregation, fragmentation, and post-translational modifications that occur in the presence of the abovementioned stress factors (H. Liu, Gaza-Bulseco, and Lundell 2008; Banks et al. 2009).

### 1.6.1. Thermal stress

Temperature is one of the crucial parameters in changing the CQA of mAbs (Hawe et al. 2012). Therefore, this parameter occupies an essential place in forced degradation studies, and it is essential to examine the structural changes of the products in the presence of thermal stress. While these studies are carried out, the stability of the product can be determined in a short time following the treatment by high temperatures and normal storage temperatures. (Nowak, K. Cheung, et al. 2017) In addition, by speeding up the process, analysis of products exposed to thermal stress can be conducted in a short time, such as a week. (A. Zhang et al. 2012)

The mAbs can form aggregates by being exposed to many different temperatures (high and low values) during production, storage, and shipping. Especially high temperatures lead to irreversible aggregate structures in mAbs (Vermeer and Norde 2000). Since low temperature reduces hydrophobic interactions between solvent and protein (Dias et al. 2008), a vital force in protein folding, intermolecular hydrophobic regions between proteins interact, increasing aggregates' formation (Luo et al., 2011a). In a study by Hawe et al., high temperatures cause a severe amount of the aggregates of mAbs, which were observed by dynamic light scattering (DLS) and other light obscuration methods. (Hawe et al. 2009)

Stability studies are customarily carried out at storage or intermediate temperatures. However, in forced degradation studies, temperature values should exceed these to determine the temperature limits for aggregation or degradation. (Vermeer and Norde 2000) The temperature is set at 40 $^0$C to 80 $^0$C in forced degradation experiments; Jiskoot et al. (Hawe et al. 2009) showed that degradation at 37 $^0$C was higher than 4 $^0$C, Sharma (Sharma et al. 2010) showed that the incubation of drug products at 60 $^0$C and 80 $^0$C results in 5-20% degradation, and Tamizi (Tamizi and Jouyban 2016) also showed that mAbs showed degradation at 40 $^0$C. As can be understood from the mentioned studies, the thermal stress values should be higher than 4 $^0$C, the storage temperature of most of the mAbs, and 25 $^0$C, which is the accelerated temperature. For the thermal stress conditions, the temperature value must be at least 35 $^0$C and above (Shabestari, Mostafavi, and Malekzadeh 2018) because the stress aims to reveal the degradation profiles in a short time following the high temperatures. In addition, before determining the high-temperature limits, the unfolding temperature of the mAbs should be determined. For this

purpose, pre-screening studies should be done using other methods such as differential scanning calorimetry (DSC) and stability studies. (Hawe et al. 2012)

High-temperature conditions are the most critical parameters that provide crucial information about the potential degradation processes of mAbs under long storage conditions. According to the literature, high temperature triggers many different degradation pathways in mAbs. One of the main degradation mechanisms is the formation of protein aggregates which can be either soluble or precipitated. (Alexander and Hughes 1995; Banks et al. 2009; Fesinmeyer et al. 2009; Franey et al. 2010; Hawe et al. 2009; Jiskoot et al. 1990; Luo et al. 2011b; Islam 2016; H. Liu, Gaza-Bulseco, and Sun 2006; Telikepalli et al. 2014; Van Buren et al. 2009). Another way of degradation induced by temperature is the fragmentation formed by breaking peptide bonds. (Cordoba et al. 2005; Dillon et al. 2006; H. Liu, Gaza-Bulseco, and Sun 2006; H. Liu, Gaza-Bulseco, and Lundell 2008; Xiang et al. 2007) The main fragmentation sites are usually in the hinge region (Alexander and Hughes 1995; H. Liu, Gaza-Bulseco, and Sun 2006; Cordoba et al. 2005; Dillon et al. 2006; Xiang et al. 2007) and a little in the domain-domain surface. (H. Liu, Gaza-Bulseco, and Lundell 2008) However, fragmentation occurs between two Asp amino acids (Xiao and Bondarenko 2008) in CDR3 and between two Ser residues (Li et al. 2017). In addition, a temperature-dependent fragmentation study showed that a breakage between Pro and Asp in the CH2 domain had been demonstrated (Van Buren et al., 2009).

High temperature also causes the formation of some PTM in mABs. High temperature causes an increase in the amount of Asn (H. Liu, Gaza-Bulseco, and Sun 2006; Luo et al. 2011b; Kroon, Baldwin-Ferro, and Lalan 1992; B. Yan, Steen, et al. 2009a; Y. T. Zhang et al. 2014) and Gln (H. Liu, Gaza-Bulseco, and Sun 2006) deamidation, N-terminal PyroE formation (H. Liu, Gaza-Bulseco, and Sun 2006) and Met oxidation (Luo et al. 2011b; Lam, Yang, and Cleland 1997). Another PTM formed by high temperature is Asp isomerization (Chu et al. 2007; H. Z. Huang, Nichols, and Liu 2009; X. C. Yu et al. 2011), and succinimide is formed as an intermediate product of this isomerization (B. Yan, Steen, et al. 2009a; Cacia et al. 1996). It was also observed that the formation of many degradation profiles, including modifications such as deamidation of Asn or Gln, N terminal glutamine cyclization, and oxidation, increase the acidic type of mAbs at high temperatures (Jiskoot et al. 1990; Kroon, Baldwin-Ferro, and Lalan 1992; Y. T. Zhang et al. 2014; Chazin et al. 1989; Yüksel and Gracy 1986).

### 1.6.2. pH stress

Another critical parameter that can change the stability and structure of mAbs is pH (Dyck et al., 2019). In mAb production, during purification steps such as protein A chromatography and virus inactivation, mAbs are exposed to low pH solutions. Therefore, antibodies' structural changes and biological activities in a low pH environment should be determined. Besides that, low pH conditions during the purification stage can also be a reference point for forced degradation studies (Nowak, K. Cheung, et al., 2017).

At low pH, the formation of soluble (Jiskoot et al. 1990) or insoluble aggregates (Jiskoot et al. 1990; Sharma et al. 2010; Arosio, Rima, and Morbidelli 2013) and fragmentation (Gaza-Bulseco and Liu 2008; Cohen, Price, and Vlasak 2007) is accelerated. Low pH leads to the fragmentation of antibodies generally in the hinge region and the formation of fragments of 40-50 kDa in non-reduced conditions and 20-30 kDa in reduced conditions (Cordoba et al. 2005; Xiang et al. 2007). However, in one study, it was shown that low pH values caused fragmentation by cutting between Asp and Pro in the CH2 domain of the antibody, which causes severe aggregation. Also, in low pH conditions, succinimide accumulation is observed due to Asp isomerization (Chu et al., 2007; Cacia et al., 1996; Nowak, Ponniah, et al. 2017). Theoretically, at low pH values, antibodies undergo precipitation at high concentrations, especially in drug substances. However, in addition to demonstrating fragmentation at low pH, the stability of the antibody in the case of high pH needs to be examined, and antibody-specific fragmentation pathways need to be analyzed.

The degradation profiles of the antibody should be evaluated at high pH and low pH. In the mAb purification process, antibodies are exposed to high pH conditions during the anion exchange chromatography (Nowak, K. Cheung, et al., 2017). In addition, antibodies are exposed to high pH during the pH neutralization steps following the protein A purification and virus inactivation steps. Antibodies form soluble and insoluble aggregates (Jiskoot et al. 1990; Joubert et al. 2011) and can undergo various fragmentation pathways (Jiskoot et al. 1990; Gaza-Bulseco and Liu 2008; Cohen, Price, and Vlasak 2007) in different pH conditions as well as in temperature. Peptide bonds on antibodies' hinge and domain-domain surfaces are very susceptible to hydrolysis (H. Liu,

Gaza-Bulseco, and Sun 2006; Dillon et al. 2006). High pH values can also be found to cause disulfide bonds to break (Jiskoot et al., 1990; Cohen, Price, and Vlasak 2007).

PTMs may alter mAbs' potency, immunogenicity, and even clinical activity during pH stress. These modifications cause degradation pathways via oxidation, deamidation, and isomerization, which affect the stability of the product (T. P. Patel et al. 1992; Beck, Sanglier-Cianférani, and Van Dorsselaer 2012; Serrato et al. 2007; J. Zhang and Robinson 2005; Beck et al. 2008). However, a study has also shown how Asp isomerization changes with pH and whether the biological activity is affected by this change (Dick et al. 2010). It was observed that Asp isomerization increases at low pH values. According to forced degradation studies with pH stress, if the Asp isomerization occurs in the CDR region, the antibody's binding affinity to the target molecule also decreases (Dick et al. 2010). The most common modification in this process is Asn deamidation and isomerization, occurring non-enzymatically (L. Huang et al. 2005a; Harris et al. 2001). For the effectiveness and stability of monoclonal antibodies, it is necessary to determine their susceptible sites to Asn deamidation and isomerization reactions observed at high pH (K. Patel and Borchardt 1990). It is necessary to enlighten the degradation processes in pH stress due to main chemical changes such as oxidation and deamidation, which change the drug's biological activity. (Daugherty and Mrsny 2006) Therefore, to develop the drug formulation, the most appropriate stability of the drug should be determined by testing various pH buffers.

### 1.6.3. Oxidative stress

Recombinant mAbs are exposed to many oxidizing environments such as impurities from mAbs production, dissolved oxygen, oxygen in the air, and free radicals formed due to reaction with metals. Forced oxidation studies are also a very effective method of understanding how sensitive mAbs are to oxidation and observing how their activities change. In particular, the investigation of antibodies' potency and binding properties by oxidizing residues in the CDR regions is the backbone of these studies. The most common approaches used for these studies are treating the antibody with hydrogen peroxide

($H_2O_2$) or tert-butyl hydrogen peroxide to investigate the oxidation of Met (folzer et al., 2015; Keck 1996) and incubation of antibodies with 2,2'-Azobis (2-amidinopropane) dihydrochloride for Trp oxidation (Bertolotti-Ciarlet et al. 2009a).

The most common degradation product in antibodies exposed to oxidizing agents in forced oxidation studies is Met oxidation, which causes the formation of methionine sulfoxide with a molecular weight increase of 16 Da and methionine sulfone with a weight increase of 32 Da (Luo et al. 2011b; Bertolotti-Ciarlet et al. 2009a; Gao et al. 2015; R. Yang et al. 2017; Weirong Wang et al. 2009). Studies have shown that Met252 residue in the DTLMISR peptide in the CH2 domain and Met428 residue in the SVMHEA peptide in the CH3 domain is susceptible to oxidation in the presence of oxidizing agents (D. Liu et al. 2008; Chumsae et al. 2007). Although their oxidation of Mets in the Fc region does not affect antigen binding, it may cause a conformational change of the CH2 domain. (H. Liu, Gaza-Bulseco, Xiang, et al. 2008a; Gaza-Bulseco, Faldu, et al. 2008a) As a result of conformational change, a decrease in binding to protein A (Weirong Wang et al. 2009; Gaza-Bulseco, Faldu, et al. 2008a) protein G, (Gaza-Bulseco, Faldu, et al. 2008a), and FcRn (Pan et al. 2009; Bertolotti-Ciarlet et al. 2009a) can be seen. High surface exposure is thought to be the reason for the oxidation of these Met amino acids. Besides Met oxidation, $H_2O_2$ also leads to antibody fragmentation (Yan, Yates, et al., 2009). Also, the construction of soluble and insoluble aggregates resulting from oxidation-induced conformational changes was shown in studies (Joubert et al., 2011).

# 2. AIM OF THE STUDY

Anti-VEGF IgG antibody is a mAb explicitly developed for targeting the vascular endothelial growth factor (VEGF) cytokine. VEGF is an important growth factor produced by many cells in the human body and triggers the formation of new blood vessels. Performing essential tasks such as vasculogenesis and angiogenesis provides the necessary oxygen and nutrients to the tissues in hypoxia conditions (Palmer and Clegg 2014).

Therefore, tumor cells secrete the VEGF molecule at a high level to provide the necessary nutrients and oxygen, and by stimulating the existing blood vessels, they provide vascularization towards themselves. As a result of this event, cancer cells grow and metastasize by spreading into other tissues (Senger et al. 1983).

VEGF growth factor interacts with VEGF receptors on the surface of vascular endothelial cells, initiating signaling that triggers the growth of vascular cells. Therefore, in tumor conditions, the interaction of VEGF with its receptor can be inhibited, thereby inhibiting the vascularization that develops into tumor cells (Karkkainen and Petrova 2000). mAbs developed against the VEGF molecule severely inhibit this interaction and block tumor neoangiogenesis (**Figure 22**). Tumor cells, which cannot receive nutrients and oxygen, die in a short time because they cannot perform their growth and spread activities. As tumor cells express more VEGF than normal cells and angiogenesis is controlled, anti-VEGF IgG does not have a very harmful effect on normal cells. For this purpose, mAbs used in the clinic and developed specifically for VEGF are produced. Additionally, biosimilars of these antibodies have also begun to be produced worldwide.

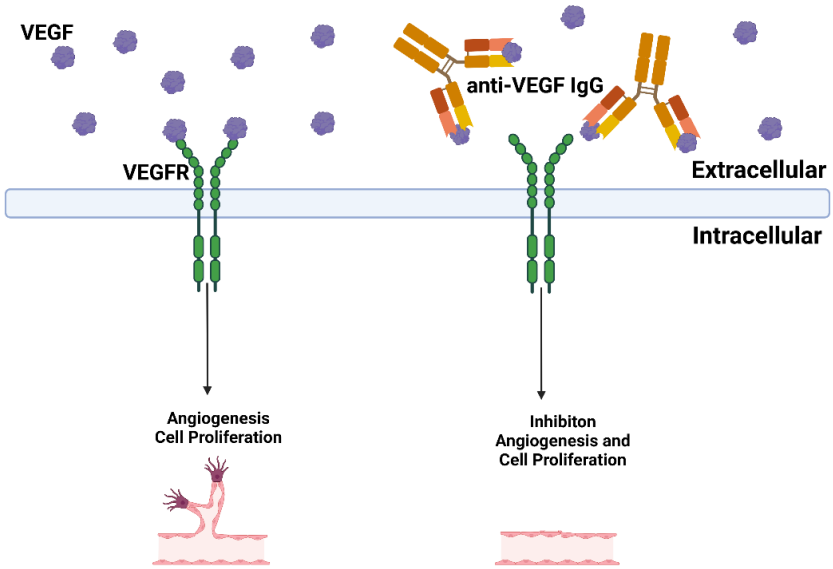



**Figure 22**: The Working Principle of Anti-VEGF IgG

These anti-VEGF IgG molecules are essential for cancer treatment, and quality controls during this mAb production are essential. In this thesis, the structural features (intact molecular weight, peptide map, PTMs, major glycoforms) and functional activities (proliferation performance, antigen-binding performance, response to receptors) of anti-VEGF IgG molecules exposed to certain environmental stress factors (thermal stress, oxidation stress, and pH stress) were investigated.

In the literature, structural changes in the anti-VEGF IgG molecules upon exposure to external stress conditions are usually investigated through structure-dependent techniques. The potential alterations in the biological activity of the molecules under the stress conditions have not been adequately investigated yet. Although such investigations are prerequisites for EMA and FDA filing, the methodological details and the full investigation reports of the biological responses against stress exposure are only briefly presented in these reports, preventing the analytical evaluation of the results in a comprehensive manner. When the FDA and EMA reports of the produced biosimilar drugs are examined, one can see that the *in vitro* biological activity investigations are

very limited, and the studies are not very detailed. Likewise, the changes in the biological activity of the mAb molecules after stress treatment are not reflected in the literature.

In this thesis, analytical and functional studies are conducted and shown separately. This study presents a multidisciplinary approach by evaluating the impact of the three most crucial stress factors on the structure and function of the selected drug molecules simultaneously.

In this study, tandem mass spectrometry (UPLC-MS/MS) was used to compare the similarity of the original sequence taken from databases of anti-VEGF IgG molecules exposed to stress factors and indicate the sequence. Whole protein intact analysis was performed by comparing glycoform profiles in the presence of stress factors using tandem MS. Since glycosylation modification causes a profound weight shift, the glycosylation profile can be characterized in intact analysis.

Moreover, the surface plasmon resonance (SPR) method was used to determine the binding affinity of mAbs exposed the stress factors to VEGF and FcRn receptors. The enzyme-linked immunosorbent assay (ELISA) method was used to determine the binding affinity to C1q protein. Finally, proliferation effects on Human Umbilical Vein Endothelial Cells (HUVECs) were checked to evaluate their *in vitro* biological activities. Eventually, the forced degradation studies of this antibody were completed by defining which modifications occurred against which stress factor and determining the functional changes caused by these modifications.

# 3. MATERIALS AND METHODS

## 3.1. Materials

### 3.1.1. Buffers, Solutions, and Chemicals

| Chemicals and Media Components | Company |
|---|---|
| HCI | Sigma, Germany |
| NaOH | Sigma, Germany |
| $H_2O_2$ | Sigma, Germany |
| Vascular Cell Basal Medium | ATCC PCS-100-030, USA |
| CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS) | Promega G3582, USA |
| VEGF 165A | Sigma, Germany |
| Ethanolamine | Cytiva, USA |
| NHS | GE Healthcare, Sweden |
| EDC | GE Healthcare, Sweden |
| Distilled Water | Merck Millipore, USA |
| HBS-EP Buffer | GE Healthcare, Sweden |
| DMSO | Sigma, Germany |
| Acetate Buffer | GE Healthcare, Sweden |
| DPBS | Sigma, Germany |
| EDTA | Applichem, Germany |
| Ethanol | Sigma, Germany |
| Glycine pH 1.5 Buffer | GE Healthcare, Sweden |
| Fetal Bovine Serum | Thermo Fischer Scientific, USA |
| HEPES Solution, 1 M | Sigma, Germany |
| AMBIC | Sigma, Germany |

| | |
|---|---|
| MS grade water | Merck, Germany |
| Isopropanol | Sigma, Germany |
| ACN | Merck, Germany |
| HRP-conjugated anti-C1q polyclonal Ab | Invitrogen, USA |
| L-glutamine, 200 mM | Thermo Fischer Scientific, USA |
| Formic Acid | Merck, Germany |
| NaCsI | Sigma, Germany |
| Methanol | Sigma, Germany |
| Anti-His IgG1 Ab | Cytiva, USA |
| His-tagged FcRn protein | Sigma, Germany |
| Glu-1-fibrinopeptide B | Waters, USA |
| NaCl | Sigma, Germany |
| Trypan blue dye | Sigma, Germany |
| SDS | Sigma, Germany |
| DTT | Sigma, Germany |
| IAA | Sigma, Germany |
| Urea | Sigma, Germany |
| RPMI 1640 | GIBCO, USA |
| Triton X-100 | Sigma, Germany |
| MS grade Trypsin | Sigma, Germany |

### 3.1.2. Equipment

| **Equipment** | **Company** |
|---|---|
| Autoclave | Hirayama, HiClave HV-110, Japan |
| Balance | ISOLAB, 302.31.002, Germany |
| Centrifuge | Eppendorf, 5415D, Germany<br>Eppendorf, 5702, Germany<br>VWR, MegaStar 3.0R, USA<br>Beckman Coulter, Allegra X-15R, USA |
| $CO_2$ Incubator | Thermo Fisher, Heracell Vios 160i, USA<br>Binder, Germany |
| Deep Freezer | -80 $^{o}$C, Forma, Thermo ElectronCorp., USA<br>-20 $^{o}$C, Bosch, Turkey |
| Microplate reader | Bio-Rad, USA |
| Filters (0.22 mm and 0.45mm) | Merck Millipore, USA |
| Biacore T200 SPR | Cytiva, USA |
| Freezing Container | Mr. Frosty, Thermo Fischer Scientific, USA |
| Acquity UPLC-ESI-Xevo G2-XS QTof | Waters, USA |
| Hemocytometer | ISOLAB, Neubauer, 075.03.001, Germany |
| Ice Machine | Scotsman Inc., AF20, USA |
| Laminar Flow | Heraeus, HeraSafe HS 12, Germany |

| | |
|---|---|
| | Heraeus, HeraSafe KS, Germany |
| NS300 Nanosight | Malvern Instruments, United Kingdom |
| Liquid Nitrogen Tank | Taylor-Wharton, 300RS, USA |
| Magnetic Stirrer | VELP Scientifica, Italy |
| Microliter Pipettes | Gilson, Pipetman, France |
| | ISOLAB, Germany |
| | Thermo Fisher Scientific, USA |
| Microscope | Zeiss, Primo Vert, Germany |
| | Zeiss Observer Z1, Germany |
| | Zeiss Confocal LSM 880, Germany |
| Microwave Oven | Bosch, Turkey |
| pH Meter | Mettler Toledo, USA |
| Refrigerator | Bosch, Turkey |
| Shaker Incubator | New Brunswick Sci., Innova 4330, USA |
| Spectrophotometer | New Brunswick Sci., USA |
| | NanoDrop 2000, Thermo Fischer Scientific, USA |
| Thermocycler | C1000 Touch, Biorad, USA |
| | Eppendorf, Mastercycler, Germany |
| | PTC-200, MJ Research Inc., Canada |
| Vortex | VELP Scientifica, Italy |

### 3.1.3. Mammalian Cell Lines, kits, and consumables

| **Materials** | **Company** |
|---|---|
| HUVEC cells | ATCC PCS-100-013, USA |
| Endothelial Cell Growth Kit | ATCC PCS-100-41, USA |
| Antibody Pair Buffer kit | Invitrogen, Austria |
| CM5 chip | Cytiva, USA |
| Acquity UPLC-BEH300 C4 1.7um column | Waters,USA |
| Nunc-Immuno 96-well plate | Thermo Scientific, Denmark |
| Amine coupling kit | Cytiva, USA |
| 96 well plate | Sarstedt, Germany |
| 24 well plate | Sarstedt, Germany |
| 6 well plate | Sarstedt, Germany |
| T75 flask | Sarstedt, Germany |
| T25 flask | Sarstedt, Germany |
| 1.5 ml Centrifuge tubes | Eppendorf, Germany |
| Micropipette tips | Eppendorf, Germany |
| 15 ml Falcon tube | Eppendorf, Germany |
| 50 ml Falcon tube | Eppendorf, Germany |
| Serological pipettes | Sarstedt, Germany |
| Lo-bind micro centrifuge tube | Eppendorf, Germany |

### 3.1.4. Software, Computer-based Programs, and Websites

| | |
|---|---|
| Biacore evaluation Software | Cytiva, USA |
| UNIFI MaxEnt1 Algorithm | Waters, USA |
| 1:1 Langmuir binding model | Cytiva, USA |
| UNIFI peptide mapping workflow | Waters, USA |
| Steady State Binding model | Cytiva, USA |
| Two State Binding model | Cytiva, USA |
| NanoSight NTA 3.1 software | Malvern, United Kingdom |
| Office 365 | Microsoft |
| GraphPad Prism 9 | GraphPad Software, Inc |

## 3.2. Methods

### 3.2.1. Degradation Conditions

The three most essential stress factors were evaluated for forced degradation studies.
In thermal stress experiments, 4 $^0$C (storage condition for control), 25 $^0$C (accelerated temperature) and elevated temperatures of 37 $^0$C, 45 $^0$C, 55 $^0$C and 70 $^0$C were used. 3 and 6 days were set as the incubation periods at these temperatures with 1mg/ml mAb concentration in 40 ul formulation buffer (α,α-trehalose dihydrate, sodium phosphate, polysorbate 20, pH 6.2)
In pH stress experiments, pH 6.2 (control pH), pH 3, pH 5, pH 7 and pH 9 were used. 1 day, 3 days, and 5 days were set as the incubation periods at these pH values. In pH stress studies, hydrochloric acid (HCI) is generally used for acid and sodium hydroxide (NaOH) for the base (ALSANTE et al., 2007). 0,1 M HCI (Sigma Aldrich) and 0,1 M NaOH (Sigma Aldrich) were prepared. 1mg/ml samples were incubated in 100 ul HCI and 100

ul NaOH solution. After incubation, buffer exchange was applied with 100 ul formulation buffer using centrifugal filter units (Millipore).

On oxidative stress experiments, According to the literature, the percentage of $H_2O_2$ (v/v) used for oxidation experiments is between 0.01 and 1, and incubation time can range from minutes to several hours. In these experiments, % of 0.05, 0.1, 0.3, 0.5 and 1 were used, starting from % 0.01. The incubation period was also 1 hour at room temperature. Thus, it can be properly observed that oxidation also increases with increasing $H_2O_2$ concentration. In addition, the oxidation rate due to incubation time was determined by incubating in 0.1% $H_2O_2$ for half an hour, 1 hour, 2 hours, and 4 hours. 1mg/ml samples were incubated in the indicated percentage of $H_2O_2$. After incubation, buffer exchange was applied with 100 ul formulation buffer.

### 3.2.2. Cell Proliferation Assay

Primary Umbilical Vein Endothelial Cells (HUVEC) (ATCC PCS-100-013) were used for cell proliferation studies. (H.-J. Park et al. 2006) Cells were grown at 37°C, 5% $CO_2$ in Vascular Cell Basal Medium (ATCC® PCS-100-030) supplemented with Endothelial Cell Growth Kit-VEGF (ATCC® PCS-100-41). In addition, a vascular cell basal medium containing no growth factors such as VEGF, endothelial growth factor (EGF), fibroblast growth factor (FGF) was prepared, which is called starvation medium. When the cells reached 80% confluency, subculture was performed. HUVEC cells are counted beforehand for cell proliferation studies, washed by centrifugation at 300 g for 5 minutes, and resuspended with a complete medium. $5x\ 10^3$ HUVEC cells/well are inoculated into 96 well plates in 200 ul complete media. It was incubated at 37 $^0$C overnight to allow the cells to adhere. The next day the complete medium is discarded, and the starvation medium that does not contain any growth factors is added. To lose the proliferative effect of the VEGF growth factor in the complete medium, cells were incubated at 37 $^0$C for 24 hours in a starvation medium. After 24 hours, monoclonal antibodies were introduced onto the cells at concentrations of 5000ng / ml, and VEGF was added at a concentration of 20ng / ml per well. However, the well in which no antibody was added but only the

VEGF growth factor was used to control the experiments. (Kim et al. 2018) Cells treated with antibodies and VEGFs were incubated at 37 $^{0}$C for 24 hours.

CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) (Promega G3582) was used to determine the proliferation rates of cells after 24 hours. The MTS assay is a commonly used test based on metabolic activity assay. (Maghni, Nicolescu, and Martin 1999) 20 µl of MTS reagent was added per well and incubated for 4 hours at 37 $^{0}$C. In addition, the MTS reagent was placed in an empty well and used as a blank during the measurement. After 4 hours of incubation, absorbance values at 490 nm were measured using an ELISA microplate reader (Biorad).

### 3.2.3. VEGF Binding Assay

The VEGF binding analyses of anti-VEGF IgG were performed on VEGF 165A-immobilized (Sigma-Aldrich) CM5 chips using a Biacore T200 SPR Instrument (Cytiva). (Handbook, n.d.) The chip surface was prepared with a well-optimized and standard 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide/N-hydroxysuccinimide (EDC-NHS) reaction. (de Mol and Fischer 2010) VEGF165A protein was diluted to 5 ng/ µL in pH 5.5 in 10 mM acetate buffer. The surface carboxylate groups activated due to the NHS / EDC reaction covalently interacted with the free amine groups of our target molecule VEGF 165A, effectively immobilizing the VEGF 165A protein on the chip surface. To block the activated carboxyl groups remaining after VEGF immobilization, 1M ethanolamine-HCl (Cytiva) was injected. The final immobilization level for the active flow cell was determined by approximately 500 response units (RU) for all experiments. An ethanolamine-immobilized flow channel was considered as the control surface in the experiments. The mAbs exposed to specified stress factors at three concentrations (15 nM, 5nM, 1.66 nM) were prepared in 1X HBS-EP buffer (10 mM HEPES, 15OmM NaCl, 3mM EDTA, 0.005% v/v polysorbate 20) at pH 7.4, which also works as a running buffer. Single-cycle kinetic analyses were conducted at the flow rate of 30 µL/min at 22 ºC. Analytes were injected for 120s in the association phase, followed by a dissociation phase of 1800s with the running buffer. Blank measurements were also performed on the active

and control flow channels by running buffer injections under identical conditions. The chip surface was regenerated by injecting 10 mM glycine pH 1.5 buffer for 90 s. Results were obtained by subtracting responses from blank flow cell and zero concentration analyte injection (running buffer). The SPR data were presented as the mean value, calculated from at least five measurements per sample. One-way analysis of variance, ANOVA test, revealed the statistically significant differences between the fractionated sample pairs ($p<0.05$ was considered significant). The equilibrium dissociation constants ($K_D$) were calculated by Biacore Evaluation Software using a 1:1 Langmuir binding model. (GE Healthcare 2013)

### 3.2.4. Intact protein Analysis

The monoclonal antibodies exposed to stress factors were firstly diluted to 0.5 mg/ml with 50 mM ammonium bicarbonate (AMBIC, Sigma-Aldrich) and injected directly into the LC-MS/MS system (Waters, ACQUITY UPLC-ESI-Xevo G2-XS QToF). Mobile phase A was MS grade water (Merck), mobile phase B was ACN (Merck), and mobile phase C was 1% formic acid (FA) (Merck). The reverse-phase separation was performed on ACQUITY UPLC-BEH300 C4 1.7 µm column (2.1mm x 50mm, Waters) using 1 min gradient (5-85% B). During the run, the flow rate and column temperatures were 0.4 µl/min and 80 ºC. Mass range was set to 500-4000 m/z and analyzed in Electrospray ionization (ESI)-positive and sensitivity mode. The instrument was calibrated using sodium cesium iodide (NaCsI) (Sigma-Aldrich), and Glu-1-fibrinopeptide B (Waters) was used as a lock-mass reference.

The deconvolution of raw mass spectra of intact mAb samples was performed by UNIFI MaxEnt1 algorithm (Waters) with the following parameters: input m/z range, 2400-3200; output mass range, 146500-150000; minimum intensity ratio left and right, 30%, FWHM, 0.73 (low m/z) and 0.92 (high m/z); the number of iterations, 20. Major glycoforms (G0F, G1F, G2F, G0) and C-terminal lysine were introduced as modifications, and only the components identified with <50 ppm mass error were accepted as glycoforms. The percentage of each glycoform was calculated using the formula: "Response % Glycoform

= (Response/Total Response of Glycoforms)*100".(Ladwig, Barnidge, and Willrich 2017)

### 3.2.5. Peptide mapping

The monoclonal antibodies exposed to stress factors in the forced degradation studies were treated with 1% sodium dodecyl sulfate (SDS) (Sigma-Aldrich) and 0.1M Dithiothreitol (DTT) (Sigma-Aldrich) in 50 mM AMBIC solution and incubated at $56^0$ C for 15 min with 50 µg per sample. After the reduction process, samples were treated with 20 mM Indole-3-acetic acid (IAA) (Sigma-Aldrich) for 30 min in the dark at room temperature for alkylation purposes. After the alkylation process, all samples were mixed with 8M urea and purified using 30 kDa Molecular weight cut-off (MWCO) disposable filter units (Millipore) at 14000g for 10 min, twice. The purified samples were treated with 1 ug trypsin (Pierce) in 75 ul AMBIC (1:50, w/w, enzyme to protein ratio) at 37 $^0$C for 1.5 hours. The tryptic peptides were collected by washing twice with 50 ul of 50 mM AMBIC at 14000g for 10 min. Finally, 1% formic acid was added to the samples before analysis. (Gundry et al. 2009)

The peptides obtained after the trypsin reaction were analyzed by ACQUITY UPLC-ESI-Xevo G2-XS QToF system (Waters). Mobile phase A, mobile phase B, and mobile phase C were M.S. grade water, acetonitrile (ACN), and 1% FA While the percentage of mobile C was fixed to 10%, and the percentage of mobile phase B was increased from 1 to 80% over an 85 which is total run time. The instrument was calibrated using NaCsI, and Glu-1-fibrinopeptide B (100 fmol/ul) was used as a lock-mass reference before running. Data-independent acquisition mode (DIA) was performed by sequential MS and MS/MS scans with 0.5 sec cycle time. Mass range was set to 50-2000 m/z, and all ions within the range were fragmented together without any precursor ion selection in sensitivity mode.

The raw data was processed by applying the UNIFI peptide mapping workflow parameters. The anti-VEGF IgG sequence was retrieved from http://www.drugbank.ca/ as a reference database. Trypsin was selected as a digesting reagent with one missed cleavage maximum. Carbamidomethyl-C was set as a fixed modification because of the

alkylation step in the sample preparation, while the other modifications (Oxidation-M, deamidation-N, succinimide intermediates, pyroglutamic Acid-N term) were set as a variable. The mass tolerance window was set within 10 ppm. The components greater than 10% matched primary ions (b/y ions), <10 ppm mass error, and no in-source fragment, were allowed for identification. The percentage of modifications was calculated using the following equation: "%peptide = (Response of modified peptide/Total response of the modified and unmodified peptides)*100".(Gundry et al. 2009)

### 3.2.6. C1q ELISA Assay

The direct ELISA method was used to evaluate the binding affinity of samples to the C1q molecule. In this method, an Antibody Pair Buffer kit containing coating buffer, blocking buffer (Assay buffer 5X), washing buffer (25X), chromogen, and stop solution was used (Invitrogen Cat. No. CNB0011, Austria). Antibodies (20-0,650 µg/ml) diluted in coating buffer (contains 50 mM carbonate buffer and 0.1% azide, pH 9.4) to Nunc-Immuno 96-well plate with high binding capacity (Thermo Scientific Cat. No.168194, Denmark) were placed in triplicate and incubated overnight at +4°C. After the plate was washed once with 1X wash buffer, blocking was done with 1X assay buffer by agitation at 500 rpm for one hour at room temperature (RT) to block the remaining binding sites. Then, human C1q protein (C1740-Sigma Aldrich) prepared in 1X assay buffer at a concentration of 2ug/ml was added to the plate and incubated for 2 hours at RT. After washing the plate five times with 1X wash buffer, it was treated with Horseradish Peroxidase (HRP)-conjugated anti C1q polyclonal antibody (Invitrogen Cat. No. 157277, USA) diluted 1:1000 at R.T. for 2 hours. After washing with 1 x wash buffer five times, the substrate/chromogen mixture was added. The reaction was stopped with a stop solution, and absorbance was measured at 450 nm using a microplate reader (Bio-Rad).

### 3.2.7. FcRn Binding Assay

FcRn binding analyses of the samples were carried out on Anti-His IgG1 antibody (Cytiva) immobilized CM5 chips (Cytiva). An amine coupling kit applied an anti-His IgG1 antibody immobilization procedure based on the manufacturer guide (Cytiva). His-Tagged FcRn (Sigma-Aldrich) protein and all other charge variant or whole molecule samples were prepared in 1X HBS-EP pH 6.0 running buffer with three-fold dilutions (15 nM, 5nM, 1.66 nM). Recombinant His-tagged FcRn molecule was captured on the active flow cell for 120s with 10 µL/min flow rate at 22 ºC. A blocked flow cell was used as a blank reference during all measurements. Samples were injected over both flow cells (active and blank) at 30 µL/min flow rate for 120s, followed by the dissociation phase of 900s with the running buffer. The chip surface was regenerated with 1X HBS-EP buffer (pH 7.4) for 60 s. Blank buffer injections were also performed on both flow channels, later subtracted from the active surface data before the fitting. The SPR data were presented as the mean value, calculated from at least three measurements per sample. One-way analysis of variance, ANOVA, was used to reveal the statistically significant differences between the fractionated sample pairs ($p<0.05$ was considered significant and $p<0.005$ was considered highly significant). The results were evaluated with Biacore Evaluation Software using the steady-state (X. Wang et al. 2017; Neuber et al. 2014).and two-state binding models (Bertolotti-Ciarlet et al. 2009b; Abdiche et al. 2015).

### 3.2.8. Nanoparticle Track Analysis (NTA)

NTA device is used to measure the number of particles between certain sizes in liquid samples. During analysis, particles in liquid suspension are loaded into a sample cup illuminated by a laser of a specific wavelength. Particles in the light path are quickly detected with a 20x microscope and viewed with the help of a high-resolution digital camera. In contrast, the incoming laser light is scattered depending on the number and

size of the particles and reaches the detector. The camera records the video of particles moving with “Brownian motion” (Filipe, Hawe, and Jiskoot 2010).

NTA software analyzes many particles in the recorded images separately and simultaneously calculates their hydrodynamic diameters using the Stokes-Einstein equation. NanoSight device in high resolution; provides nanoparticle size and count-based concentration measurements. With real-time monitoring, small changes in the properties of particle populations become traceable, with all these analyzes confirmed by visual verification.

NS300 Nanosight (Malvern Instruments Ltd, Malvern, United Kingdom) was used to compare the aggregation profiles of the stressed antibodies. The PBS-P was filtered by syringe through 0.22 μm pore-sized filters into 15 ml falcon tubes. The samples were prepared using 250 μg protein in 1500 μl PBS-P. The samples were kept in an icebox to avoid conformational changes of proteins and taken out from the icebox only 20 minutes before the measurement. NanoSight NTA 3.1 software was used for NTA analysis. The sensitivity parameter is used as a high mode. The bandwidth is set at 1 nm. Continuous was chosen as the scanning mode, and wavelengths between 190 and 300 nm were scanned. The data pitch value was determined as 0.2 nm. Response 3 and accumulation parameter values were also determined as 10. Samples were loaded in a laser module (the sample should be loaded without any air pockets or bubbles). The laser module was mounted within the main instrument housing. The focus was optimized to give a clear, sharp image of the particles by adjusting both using the slider control in the NTA software and the focus dial on the right-hand side of the instrument. As a result of the camera recording, the results were obtained as pdf.

# 4. RESULTS

## 4.1. Thermal Stress

### 4.1.1. Determination of Glycosylation Patterns of mAbs by Intact Protein Analysis

Intact protein analysis is one of the essential methods used to elucidate the holistic structure of mAbs. It can help determine the modifications or fragmentations of the molecule by precisely measuring the molecular weight of the molecule (Ladwig, Barnidge, and Willrich 2017). It can show molecular weight shifts above 100 kilodaltons (kDa) with acceptable mass error. Glycosylation and C-terminal Lys cleavage are the most frequent modifications observed in mAbs. They can be detected by intact mass analysis because they cause a molecular weight shift greater than 100 Da (Domínguez-Vega et al., 2018). The glycosylation patterns of antibodies are essential for their biological activity (X. Yang and Bartlett 2019). Therefore, biological activity can also be affected when there is a difference in glycosylation profile.

The anti-VEGF IgG used in this study is glycosylated in the Asn303 residues (**Figure 4.1.1.1A**) (Seo et al. 2018). Also, the main N-linked glycoform structure of this mAb is both fucosylated and galactosylated in two chains (G0F N: G0F N=(G0F N(2)). Furthermore, lysines are clipped at the C terminal ends biantennary (G0F N(2)-**K(2)**). Therefore, an intact analysis can detect a molecular weight of about 149.2 kDa, the most dominant form. All samples were analyzed under the same conditions, and it is aimed to determine the glycoform differences that may occur due to temperature alterations at indicated incubation times.

According to the intact analysis of mAbs exposed to the indicated temperatures, it was observed that the dominant glycoform structure was G0F N(2)-K(2) at the 3rd and the 6th days for all samples. In **Figure 4.1.1.1B**, MS spectrums obtained from intact analysis of antibodies exposed to 4 $^{0}$C, which was considered as a control, 25 $^{0}$C and 37 $^{0}$C for 3 days and 6 days were shown. Each sample showed the same MS spectrum profile and did not have any unmatched peak. In **Figure 4.1.1.1A**, there is a schematized version of the glycoform structures found in the mAb samples. The observed and expected theoretical molecular weight matched with the error of 50 ppm, with each sample having the same single dominant peak (G0F N(2)-K(2)), which is around 40% of the total glycoforms. The relative percentage of the top 3 predominant glycoform species was given in the graph (**Figure 4.1.1.1C**), and G0F N(1), G1F N(1)-K, and G0 N(1), G0F N(1) forms were observed at a high rate. They were below 15% of total glycoform (The blue line shows 15%). Since antibodies have a heterogeneous structure, tiny proportions of other glycoforms were also observed. In general, rates above 5% in modification percentage indicate a significant change (C.-H. Chen et al. 2018). No significant changes were observed in glycoform structures following the temperature change at indicated incubation times in this experiment set. As expected, since glycosylation is formed enzymatically in the host cell, it is unlikely to be affected and changed by environmental factors. (Pisupati et al. 2017)

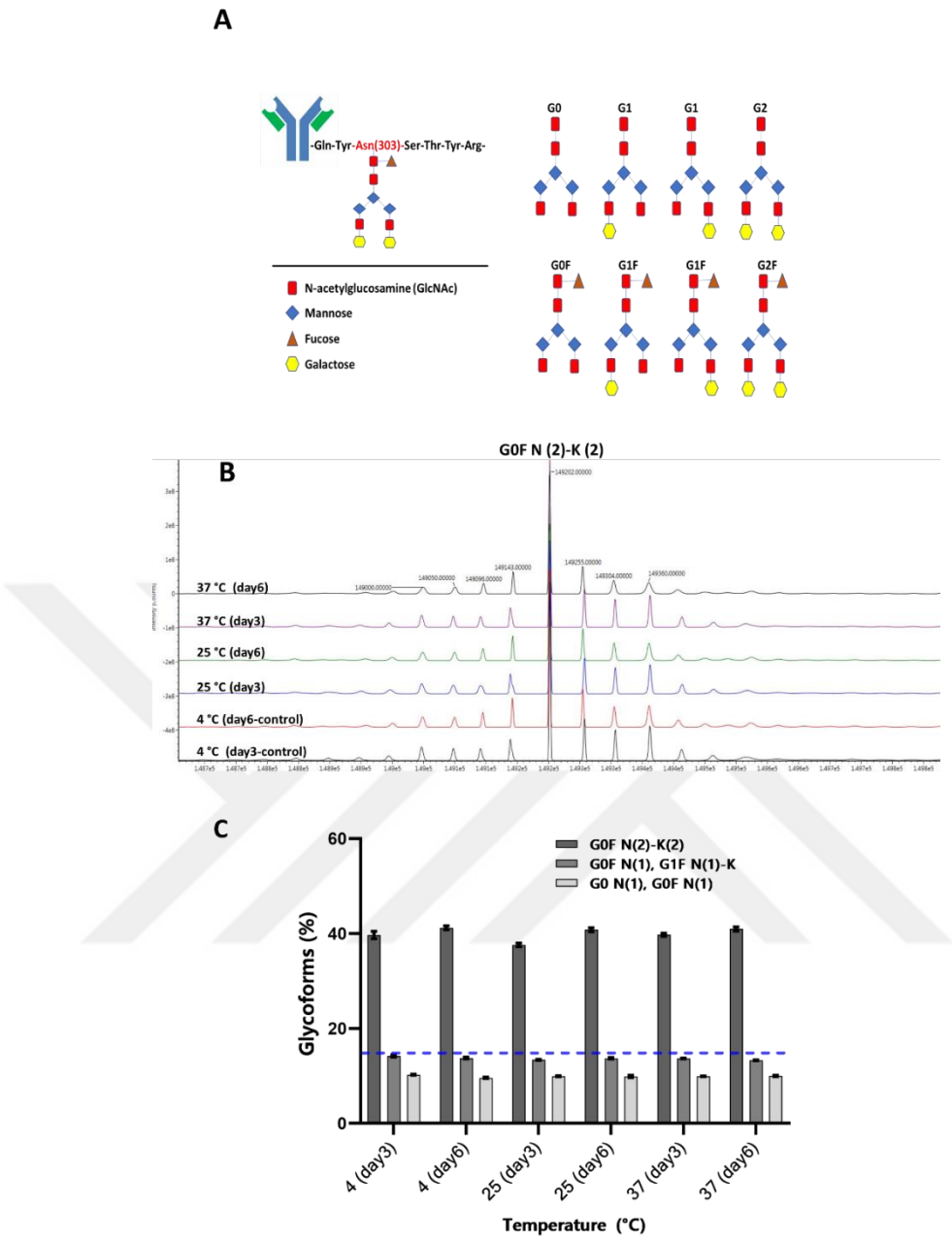



**Figure 4.1.1.1**: Intact protein analysis of mAbs expose to thermal stress by using mass spectrometry. (A) Representation of glycoforms that appear dominantly in mAbs. (B) Deconvoluted MS spectrums of mAbs exposed to different temperature values for 3 and 6 days (C) The graph shows the percentage of glycoforms obtained due to intact analysis of monoclonal antibodies in each group. The percentage values were calculated by averaging three separate injections. The standard deviation of all the values given in the table is less than 2. -K represents clipped lysine. The blue line shows a 15% value.

The glycoform distribution of mAbs was also investigated in extreme temperature conditions, where antibodies would not be exposed under normal conditions but may encounter in the laboratory, shipping, etc. In the extreme thermal stress study, antibodies were exposed to temperatures higher than 25 $^0$C and 37 $^0$C, including 45 $^0$C, 55 $^0$C, and 70 $^0$C. However, antibodies exposed to 70 $^0$C formed severe aggregation. This sample was not analyzed since it may prevent the proper operation of UPLC/MS-MS and SPR instruments. It was only used *in vitro* study of cell proliferation.

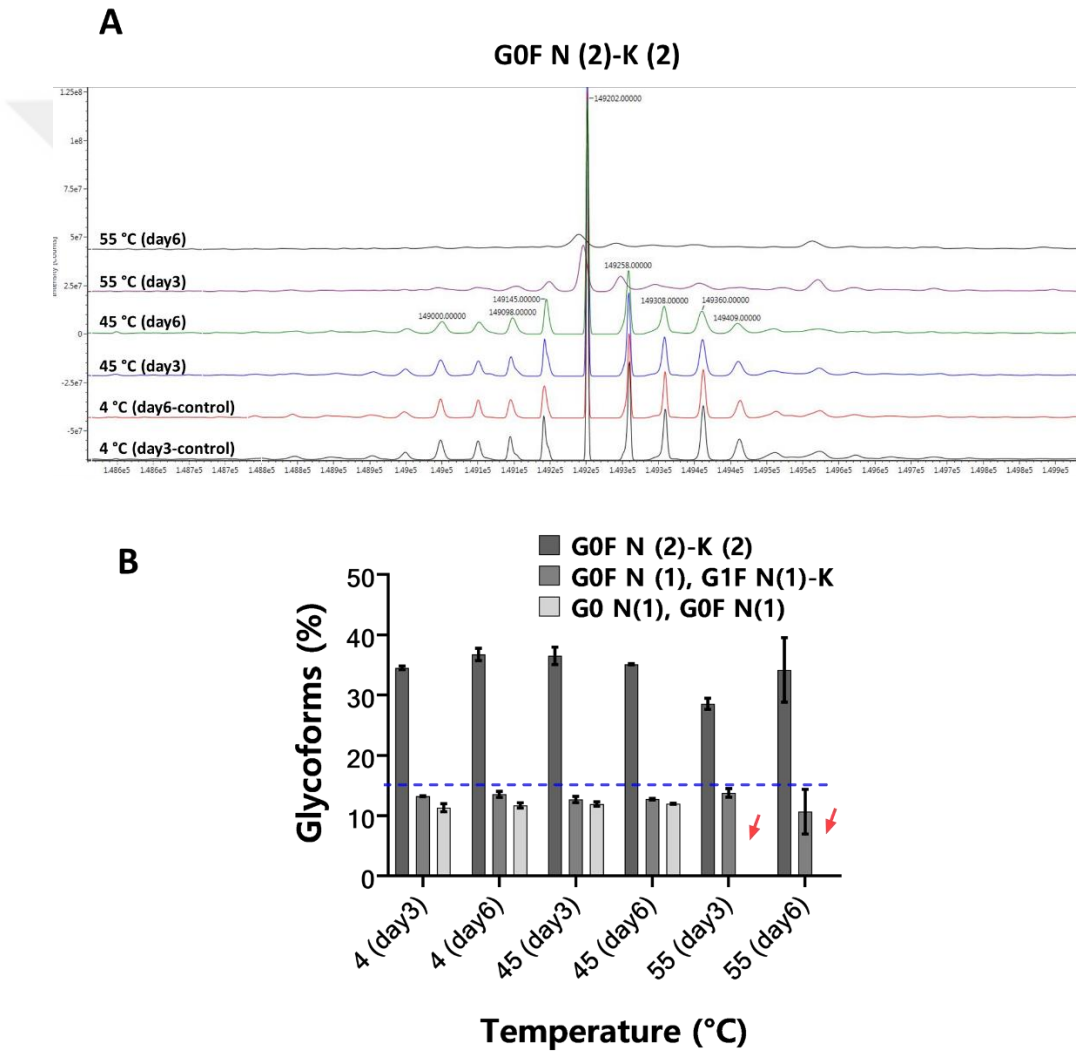



**Figure 4.1.1.2**: Intact protein analysis of mAbs expose to extreme thermal conditions by using mass spectrometry. (A) Deconvoluted MS spectrums of mAbs exposed to extreme temperature values for 3 and 6 days. (B) The graph shows the percentage of

glycoforms obtained due to intact deconvoluted analysis of monoclonal antibodies exposed to extreme temperature. The percentage values were calculated by averaging three separate injections. -K represents clipped lysine. The blue line shows a 15% value. The red arrow shows missing G0 N(1), G0F N(1) glycoforms.

By intact analysis, it was aimed to observe how the glycosylation profiles of antibodies incubated at 45 $^0$C and 55 $^0$C for 3 and 6 days would change. In **Figure 4.1.1.2A**, deconvoluted MS spectrums of all samples were shown resulting from intact analysis, and the relative percentage of 3 predominant glycoform species was given in the graph **(Figure 4.1.1.2B).** All samples were analyzed under the same conditions, and the samples incubated at 4 $^0$C were used as control. The dominant peak was determined as G0F N(2)-K(2) in each sample, and its ratio was between 30-35% in all samples. When the glycoform percentages were examined, G0F N(1), G1F N(1)-K, and G0 N(1), G0F N(1) forms were observed at a high rate, and they were below 15%. However, G0 N(1), G0F N(1) glycoform was not found in samples incubated at 55 $^0$C for 3 days and 6 days (red arrows). When the glycosylation distribution of mAbs incubated at 55 $^0$C for both 3 days and 6 days was examined, a decrease in the percentage of each glycoform was observed. These decreases were also clearly seen in MS spectrums (**Figure 4.1.1.2A)**. Most minor glycoform structures were seen in the samples incubated at 4 $^0$C, and 45 $^0$C decreased or even disappeared by 55 $^0$C.

Several studies showed that the monoclonal antibodies form an irreversible aggregate at high temperatures (Vermeer and Norde 2000; Luo et al. 2011a; Hawe et al. 2012; Shabestari, Mostafavi, and Malekzadeh 2018). Therefore, the temperature of 55 $^0$C may cause mAb aggregates in our study, and many glycoform structures may were lost due to the aggregation. Besides the disappearance of G0 N(1), G0F N(1) glycoform as a result of aggregation, relative percentages of the other glycoforms also decreased (data not shown). But the main glycoform G0F N(2)-K(2) was correctly defined at each temperature.

### 4.1.2. Determination of PTMs in mAbs by Peptide Mapping Analysis

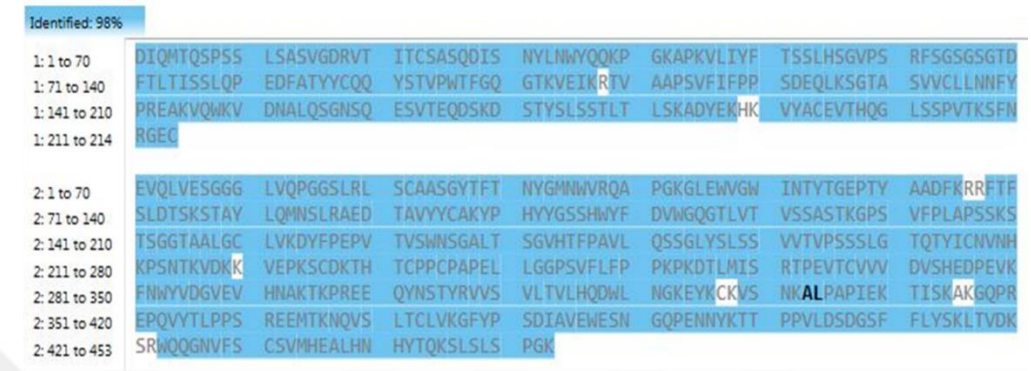
The mAbs can undergo many different post-translational modifications due to exposure to many different environmental stresses. It is necessary to investigate these PTMs, such as oxidation, deamidation, isomerization, and determine the drug's stability. (H. Liu, Gaza-Bulseco, and Lundell 2008; Banks et al. 2009) By performing peptide mapping analysis, both the sequences of the proteins and the PTMs can be determined. The modifications on each peptide can be identified by digesting mAbs into peptides by proteases such as trypsin before MS analysis.

Asn deamidation is highly associated with temperature. It was observed that the deamidation is increased at high temperatures, and the storage conditions should be at lower temperatures for drug stability. (K. Patel and Borchardt 1990) Additionally, many studies showed that high temperature increases the Asn deamidation (H. Liu, Gaza-Bulseco, and Sun 2006; Luo et al. 2011b; Kroon, Baldwin-Ferro, and Lalan 1992; B. Yan, Steen, et al. 2009a; Y. T. Zhang et al. 2014), Gln deamidation (H. Liu, Gaza-Bulseco, and Sun 2006), Met oxidation (Luo et al. 2011b; Lam, Yang, and Cleland 1997) and Asp isomerization. (Chu et al. 2007; H. Z. Huang, Nichols, and Liu 2009; X. C. Yu et al. 2011) Succinimide is also observed as an intermediate product of Asp isomerization. (B. Yan, Steen, et al. 2009a; Cacia et al. 1996)

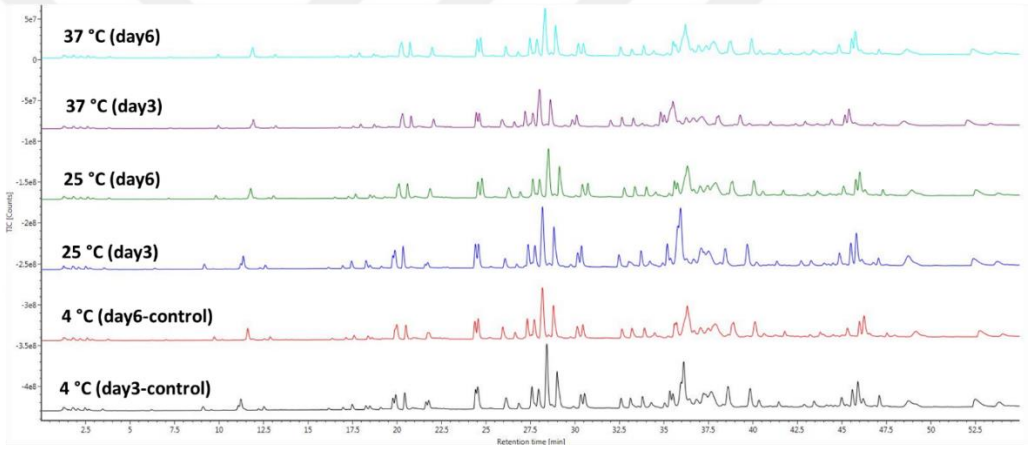
The studies showed previously that the *in vitro* stability and *in vivo* biological activity could change due to the deamidation of Asn and Asp residues. (McKerrow 1979; Cacia et al. 1996; Kroon, Baldwin-Ferro, and Lalan 1992) Asn deamidation and isomerization in the CDR regions of the heavy chain of many IgG type mAbs causes activity loss (Harris et al. 2001; L. Huang et al. 2005a; B. Yan, Steen, et al. 2009a; Haberger et al. 2014; Vlasak et al. 2009). Considering the recombinant mAb trastuzumab, isomerization of Asp 102 in the heavy chain in the CDR 3 region resulted in a decrease in the potency of the antibody. On the other hand, due to the deamidation of Asn 30 in the light chain in the CDR1 region, its biological activity was seriously affected. (Harris et al. 2001) In another study, Asn-55 in the heavy chain in the CDR 2 region of different IgG molecules was susceptible to deamidation *in vivo* (L. Huang et al. 2005a). In another study, ongoing succinimide formation was observed in acidic environments at high temperatures. (Stephenson and Clarke 1989) Chelius et al. identified 4 possible regions (SNG, ENN,

LNG, and LNN motifs) sensitive to deamidation in the conserved regions of IgG molecules in thermal stress by performing forced degradation studies (Chelius, Rehder, and Bondarenko 2005).

A




B




C

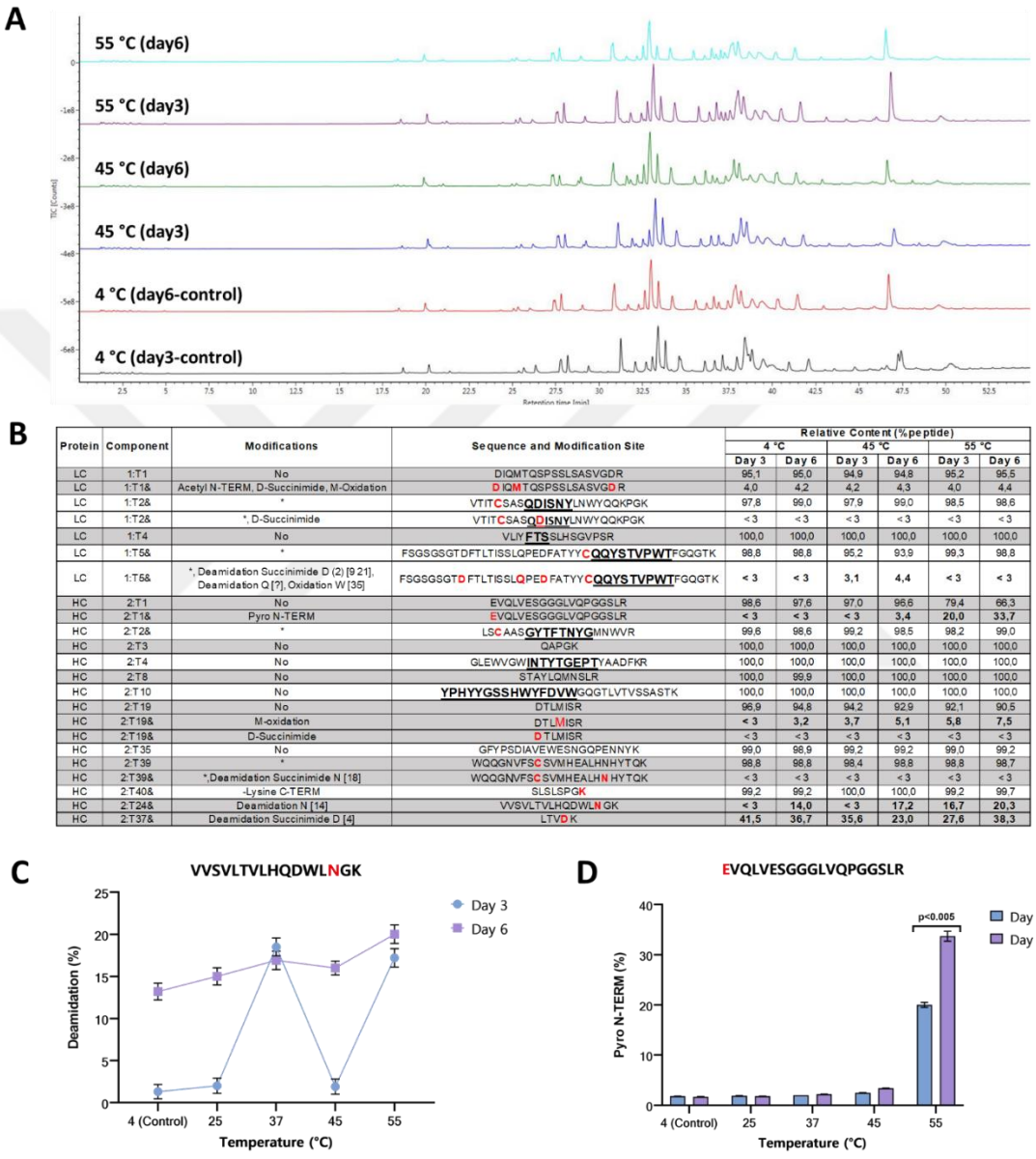
| Protein | Component | Modifications | Sequence and Modification Site | Relative Content (% peptide) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 4 °C | | 25 °C | | 37 °C | |
| | | | | Day 3 | Day 6 | Day 3 | Day 6 | Day 3 | Day 6 |
| LC | 1:T1 | No | DIQMTQSPSSLSASVGDR | 94,1 | 93,8 | 94,1 | 93,8 | 93,7 | 93,8 |
| LC | 1:T1& | Acetyl N-TERM, D-Succinimide, M-Oxidation | DIQMTQSPSSLSASVGDR | 5,5 | 5,5 | 5,4 | 5,5 | 5,7 | 5,5 |
| LC | 1:T2& | * | VTITCSASQDISNYLNWYQQKPGK | 97,9 | 98,2 | 97,8 | 98,2 | 98,1 | 98,3 |
| LC | 1:T2& | *, D-Succinimide | VTITCSASQDISNYLNWYQQKPGK | < 3 | < 3 | < 3 | < 3 | < 3 | < 3 |
| LC | 1:T4 | No | VLIYFTSSLHSGVPSR | 100,0 | 100,0 | 100,0 | 100,0 | 100,0 | 100,0 |
| LC | 1:T5& | * | FSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTK | 93,3 | 96,9 | 95,8 | 95,8 | 96,6 | 96,4 |
| LC | 1:T5& | *, Deamidation Succinimide D (2) [9 21], Deamidation Q [?], Oxidation W [35] | FSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTK | **5,6** | **2,0** | **2,2** | **3,0** | **2,2** | **2,4** |
| HC | 2:T1 | No | EVQLVESGGGLVQPGGSLR | 98,1 | 98,0 | 97,7 | 98,0 | 97,9 | 97,7 |
| HC | 2:T1& | Pyro N-TERM | EVQLVESGGGLVQPGGSLR | < 3 | < 3 | < 3 | < 3 | < 3 | < 3 |
| HC | 2:T2& | * | LSCAASGYTFTNYGMNWVR | 99,2 | 99,1 | 99,6 | 99,7 | 99,4 | 99,1 |
| HC | 2:T3 | No | QAPGK | 100,0 | 100,0 | 100,0 | 100,0 | 100,0 | 100,0 |
| HC | 2:T4 | No | GLEWVGWINTYTGEPTYAADFKR | 100,0 | 100,0 | 100,0 | 99,8 | 100,0 | 100,0 |
| HC | 2:T8 | No | STAYLQMNSLR | 99,9 | 99,7 | 99,7 | 99,3 | 99,5 | 99,8 |
| HC | 2:T10 | No | YPHYYGSSHWYFDVWGQGTLVTVSSASTK | 98,0 | 97,0 | 98,0 | 96,7 | 98,3 | 98,1 |
| HC | 2:T10& | Deamidation Succinimide D [13] | YPHYYGSSHWYFDVWGQGTLVTVSSASTK | < 3 | < 3 | < 3 | < 3 | < 3 | < 3 |
| HC | 2:T19 | No | DTLMISR | 95,4 | 95,3 | 95,4 | 95,5 | 95,3 | 95,0 |
| HC | 2:T19& | M-oxidation | DTLMISR | < 3 | < 3 | < 3 | < 3 | < 3 | < 3 |
| HC | 2:T19& | D-Succinimide | DTLMISR | < 3 | < 3 | < 3 | < 3 | < 3 | < 3 |
| HC | 2:T35 | No | GFYPSDIAVEWESNGQPENNYK | 99,5 | 99,1 | 100,0 | 99,5 | 99,5 | 99,5 |
| HC | 2:T39 | * | WQQGNVFSCSVMHEALHNHYTQK | 98,4 | 98,5 | 99,0 | 98,4 | 98,4 | 99,0 |
| HC | 2:T39& | *,Deamidation Succinimide N [18] | WQQGNVFSCSVMHEALHNHYTQK | < 3 | < 3 | < 3 | < 3 | < 3 | < 3 |
| HC | 2:T40& | -Lysine C-TERM | SLSLSPGK | 100,0 | 100,0 | 98,7 | 100,0 | 100,0 | 100,0 |
| HC | 2:T24& | Deamidation N [14] | VVSVLTVLHQDWLNGK | **< 3** | **13,2** | **< 3** | **15,1** | **18,5** | **16,9** |
| LC | 1:T9& | *, Deamidation N [11] | SGTASVVCLLNNFYPR | < 3 | < 3 | < 3 | < 3 | < 3 | < 3 |

**Figure 4.1.2.1**: Peptide mapping analysis of mAbs exposing thermal stress using mass spectrometry. (A) The coverage map of the anti-VEGF IgG protein sequence was

obtained by pep-map analysis (B) Total ion chromatograms (TIC) of mAbs exposed to different temperature values for 3 and 6 days. (C) Table showing the percentage of modified and unmodified tryptic peptides. The letters marked in red were the amino acids with the modification. Black underlined and bold sequences represent the CDR regions. The percentage values were calculated by averaging three separate injections. The standard deviation of all the values given in the table is less than 1. * Represents fixed carbamidomethyl C modification, which is formed due to iodoacetamide used in the alkylation step.

PTMs following exposure to thermal stress were demonstrated by peptide mapping. Firstly, the protein sequence of each sample was obtained with a 98% identity rate to the original sequence **Figure 4.1.2.1A,** and over 95% similarity in the coverage map is an acceptable score (Xie et al., n.d.). The tryptic peptides' total ion chromatograms (TIC) obtained from each sample were shown in **Figure 4.1.2.1B,** and there were minor differences in several peak heights. However, the general peptide profile was similar. The differences can be observed by newly formed or the altered amount of the modifications. The algorithm identified the peaks in UNIFI based on several data like molecular weight of the precursor and product ions, -b and -y ion sequencing, and fragmentation pattern. In **Figure 4.1.2.1C**, it was shown that the list of several identified tryptic peptides, the modification type, and percentages. The peptides and the modification in the table were found predominantly in the CDR region. These peptides were specifically examined because the modification in the CDR region alters the efficacy of biological activity. The modifications that may be changed in other protein regions were also included. According to the modification percentages, there were not too many significant alterations among the samples. It should be noted that the alterations above 5% must be considered in terms of the CQAs. Interestingly, it was observed that Asn deamidation increased in HC/T24 peptide (VVSVLTVLHQDWL**N**GK) with increasing temperature **(Figure 4.1.2.1C)**.
While there is a deamidation rate below 3% in the control sample at 4 $^{0}$C for 3 days, this rate increased to 18.5% when incubated at 37 $^{0}$C for 3 days; the same trend was observed for 6 days at the same temperatures. While there was a deamidation rate 13,2 % in the control sample at 4 $^{0}$C for 6 days, this rate increased to 15,1 % at 25 $^{0}$C and 16,9 % at 37 $^{0}$C for 6 days. This result showed that the deamidation increased with elevated

temperature, which is in line with the literature. (Kroon, Baldwin-Ferro, and Lalan 1992; B. Yan, Steen, et al. 2009a; Y. T. Zhang et al. 2014) No major modifications were identified in other fragments. The pep-map analysis of mAbs was also investigated in extreme temperatures of 45 $^{0}$C and 55 $^{0}$C.




| Protein | Component | Modifications | Sequence and Modification Site | Relative Content (%peptide) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 4 °C | | 45 °C | | 55 °C | |
| | | | | Day 3 | Day 6 | Day 3 | Day 6 | Day 3 | Day 6 |
| LC | 1:T1 | No | DIQMTQSPSSLSASVGDR | 95,1 | 95,0 | 94,9 | 94,8 | 95,2 | 95,5 |
| LC | 1:T1& | Acetyl N-TERM, D-Succinimide, M-Oxidation | DIQMTQSPSSLSASVGDR | 4,0 | 4,2 | 4,2 | 4,3 | 4,0 | 4,4 |
| LC | 1:T2& | * | VTITCSASQDISNYLNWYQQKPGK | 97,8 | 99,0 | 97,9 | 99,0 | 98,5 | 98,6 |
| LC | 1:T2& | *, D-Succinimide | VTITCSASQDISNYLNWYQQKPGK | < 3 | < 3 | < 3 | < 3 | < 3 | < 3 |
| LC | 1:T4 | No | VLIYFTSSLHSGVPSR | 100,0 | 100,0 | 100,0 | 100,0 | 100,0 | 100,0 |
| LC | 1:T5& | * | FSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTK | 98,8 | 98,8 | 95,2 | 93,9 | 99,3 | 98,8 |
| LC | 1:T5& | *, Deamidation Succinimide D (2) [9 21], Deamidation Q [?], Oxidation W [35] | FSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTK | < 3 | < 3 | 3,1 | 4,4 | < 3 | < 3 |
| HC | 2:T1 | No | EVQLVESGGGLVQPGGSLR | 98,6 | 97,6 | 97,0 | 96,6 | 79,4 | 66,3 |
| HC | 2:T1& | Pyro N-TERM | EVQLVESGGGLVQPGGSLR | < 3 | < 3 | < 3 | 3,4 | 20,0 | 33,7 |
| HC | 2:T2& | * | LSCAASGYTFTNYGMNWVR | 99,6 | 98,6 | 99,2 | 98,5 | 98,2 | 99,0 |
| HC | 2:T3 | No | QAPGK | 100,0 | 100,0 | 100,0 | 100,0 | 100,0 | 100,0 |
| HC | 2:T4 | No | GLEWVGWINTYTGEPTYAADFKR | 100,0 | 100,0 | 100,0 | 100,0 | 100,0 | 100,0 |
| HC | 2:T8 | No | STAYLQMNSLR | 100,0 | 99,9 | 100,0 | 100,0 | 100,0 | 100,0 |
| HC | 2:T10 | No | YPHYYGSSHWYFDVWGQGTLVTVSSASTK | 100,0 | 100,0 | 100,0 | 100,0 | 100,0 | 100,0 |
| HC | 2:T19 | No | DTLMISR | 96,9 | 94,8 | 94,2 | 92,9 | 92,1 | 90,5 |
| HC | 2:T19& | M-oxidation | DTLMISR | < 3 | 3,2 | 3,7 | 5,1 | 5,8 | 7,5 |
| HC | 2:T19& | D-Succinimide | DTLMISR | < 3 | < 3 | < 3 | < 3 | < 3 | < 3 |
| HC | 2:T35 | No | GFYPSDIAVEWESNGQPENNYK | 99,0 | 98,9 | 99,2 | 99,2 | 99,0 | 99,2 |
| HC | 2:T39 | * | WQQGNVFSCSVMHEALHNHYTQK | 98,8 | 98,8 | 98,4 | 98,8 | 98,8 | 98,7 |
| HC | 2:T39& | *,Deamidation Succinimide N [18] | WQQGNVFSCSVMHEALHNHYTQK | < 3 | < 3 | < 3 | < 3 | < 3 | < 3 |
| HC | 2:T40& | -Lysine C-TERM | SLSLSPGK | 99,2 | 99,2 | 100,0 | 100,0 | 99,2 | 99,7 |
| HC | 2:T24& | Deamidation N [14] | VVSVLTVLHQDWLNGK | < 3 | 14,0 | < 3 | 17,2 | 16,7 | 20,3 |
| HC | 2:T37& | Deamidation Succinimide D [4] | LTVDK | 41,5 | 36,7 | 35,6 | 23,0 | 27,6 | 38,3 |

**Figure 4.1.2.2**: Peptide mapping analysis of mAbs exposed to extreme thermal stress by using mass spectrometry. (A) Total ion chromatograms of mAbs exposed to extreme temperature values for 3 and 6 days. (B) Table showing the percentage of modified and unmodified peptides (C) Deamidation rate on VVSVLTVLHQDWLNGK peptide. (D) Pyroglutamic acid rate on EVQLVESGGGLVQPGGSLR peptide. The letters marked in

red were the amino acids with the modification. Black underlined and bold sequences represent the CDR regions. The percentage values were calculated by averaging three separate injections. The standard deviation of all the values given in the table is less than 1. * Represents fixed carbamidomethyl C modification, which is formed due to iodoacetamide used in the alkylation step. The data were analyzed statistically using the Single-way ANOVA test.

The mAbs were incubated at 45 $^0$C and 55 $^0$C for 3 and 6 days to determine possible alterations in modifications at higher temperatures. The protein sequence of the samples was determined, and the coverage score was 98% by peptide mapping analysis. In **Figure 4.1.2.2A**, the tryptic peptide's TIC chromatograms seemed similar to the control sample except for minor differences among the samples. Also, in **Figure 4.1.2.2B,** the modification percentages on the peptides were listed as shown in the previous temperature experiment set. It was observed that asparagine deamidation in the HC / T24 peptide (VVSVLTVLHQDWL**N**GK) increased with the elevated temperature, as was observed in the previous experiment set. When the 3-day incubation results were examined, the deamidation rate increased at 37 °C, while no deamidation was observed at 45 °C. However, an increase was seen at 55 °C again. The possible reason for these results is an unforeseen deficit in MS analysis because no deamidation was observed at 4 and 25 °C, but a significant increase was observed at 37 and 55 °C. Figure 4.1.2.2C, the deamidation rate on the VVSVLTVLHQDWLNGK peptide for all temperature conditions tested.

Apart from this, a modification was detected, which was not observed at 37 $^0$C in the previous experiment set. PyroE formation at the HC/2T1 peptide (**E**VQLVESGGGLVQPGGSLR) in the N terminus increased significantly with increasing temperature. When looking at the results of both 3 and 6 days of treatment, there was no high percentage of PyroE at 4 $^0$C and 45 $^0$C, but when it was increased to 55 $^0$C, this rate dramatically increased to 20% in 3 days and 34% in 6 days (**Figure 4.1.2.2D**). When a literature review is shown, increasing the temperature accelerates the conversion of the N-terminus Gln to PyroE, which is commonly observed in mAbs (Du et al., 2012). In elevated temperatures, many studies showed N-terminal PyroE formation (H. Liu,

Gaza-Bulseco, and Sun 2006). Therefore, this result was found consistent with the literature.

Moreover, a slight increase was observed in the Met oxidation in HC / 2T19 peptide (DTL**M**ISR) with increasing temperature. In 3-day and 6-day incubations, oxidation was less than 3% at 4 ° C, and these values increased to 6% and 7% at 55 °C, respectively. Again, when examined in the literature, studies show that oxidation also increases at high temperatures.(Luo et al. 2011b; Lam, Yang, and Cleland 1997) But our data did not show a significant increase.

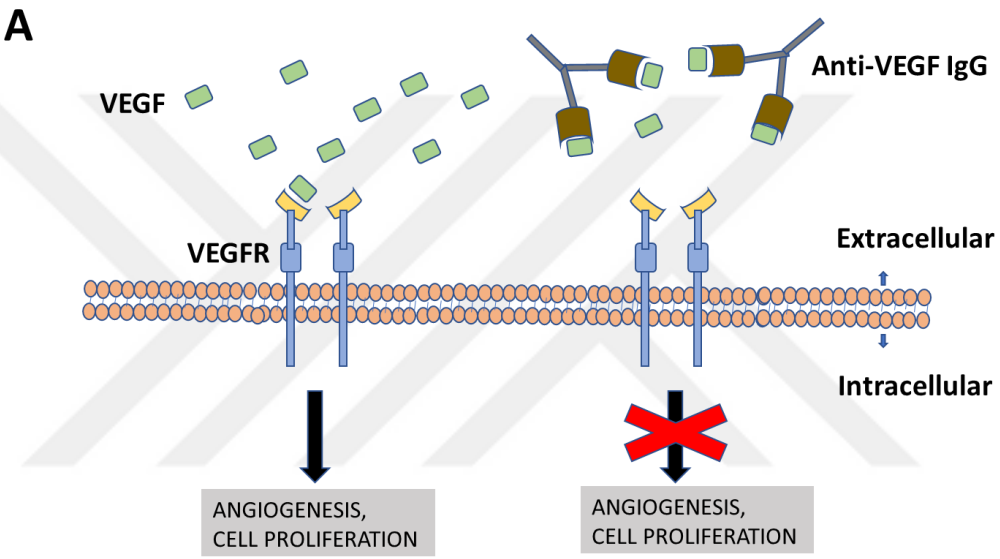
### 4.1.3. Cell Proliferation assay with mAbs exposed to thermal stress

*In vitro* proliferation assay in HUVEC cells is a commonly used method to show the biological activity of the developed anti-VEGF IgG mAbs. HUVEC are endothelium cells isolated from the umbilical cord's vein (H.-J. Park et al. 2006). It is a cell type commonly used in physiological and pharmacological research, such as angiogenesis, fibrinolysis, macromolecule transfer, and blood coagulation (Jiménez, Krouwer, and Post 2013). The studies that used this cell significantly contributed to the literature in tumor angiogenesis, especially in angiogenesis studies (Z. Chen et al. 2009). Because HUVEC cells are an endothelial cell line, they can respond to the VEGF molecule by VEGF receptor and increase the proliferation of the cells by interacting with the growth factor **(Figure 4.1.3 A**) (C. Yu et al. 2020). In the presence of anti-VEGF IgG, VEGF growth factors cannot interact with their receptors resulting in inhibition of cell proliferation. Therefore, to show the biological activity of mAbs against the VEGF growth factor, HUVEC proliferation is routinely analyzed *in vitro.*

In this study, the proliferation assay with HUVEC cells was conducted to determine the changes in the biological activity of anti-VEGF recombinant IgGs under various thermal stress conditions. Generally, two methods are used to determine proliferation based on the determination of DNA molecules or the metabolic activity (Maghni, Nicolescu, and Martin 1999). DNA is stained with thymidine analogs and analyzed calorimetrically by an ELISA reader to determine the number of DNA methods (Taupin 2007).

In this study, proliferation experiments were carried out by determining metabolic activity, MTS assay (Aslantürk 2018). The MTS assay (5- (3-carboxymethoxyphenyl) -

2- (4,5-dimethyl-thiazolyl) -3- (4-sulfophenyl) is a colorimetric method commonly used in cell proliferation analysis (Stone, Johnston, and Schins 2009). The principle of this method is that mitochondrial activity increases due to increased proliferation, and the function of mitochondrial enzymes such as dehydrogenase is analyzed (Mosmann 1983). Tetrazolium salts turn into formazan as a result of mitochondrial activity. This conversion causes a color change that can be measured in the spectrophotometer, providing information about the proliferation of the cell.



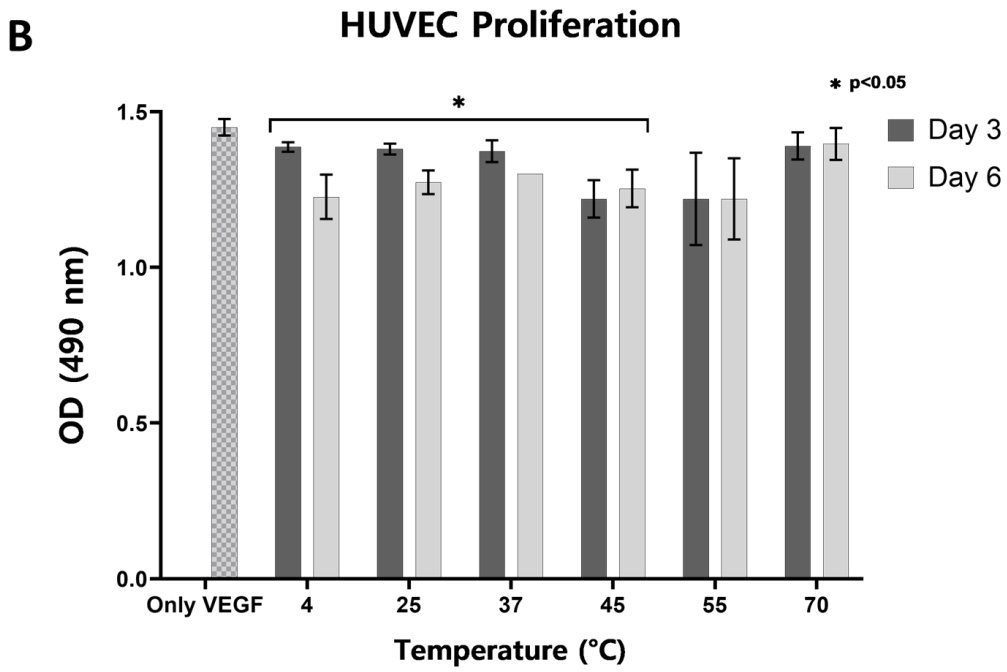



**Figure 4.1.3**: Cell proliferation effects of mAbs expose to thermal stress by MTS. (A) The biological mechanism of VEGF growth factor in HUVEC cells. (B)The anti-proliferative effect of mAbs exposed to temperature stress was observed by applying the MTS cell proliferation assay on HUVEC cells. A microplate reader measured Absorbance values at a wavelength of 490 nm. Experiments were carried out in triplicate. To interpret the results statistically, Single-way ANOVA was applied, and (*) indicates $p<0.05$.

The effect of antibodies exposed to different temperature conditions for 3 and 6 days on the proliferation of HUVEC cells was tested using the MTS method. In **Figure 4.1.3B**, the absorbance values of all samples at 490 nm wavelength were shown. Cells showed the highest proliferation rate in the sample with only VEGF (no mAbs) growth factor, the control group. A decrease in cell proliferation relative to the control was observed in other samples. As a result of the statistical analysis using the ANOVA test, HUVEC proliferation is decreased significantly ($p<0.05$) in other samples, except samples at 55 $^0$C and 70 $^0$C. In addition, antibodies incubated for 3 and 6 days inhibited cell proliferation in the same trend. The VEGF neutralization function of mAbs incubated at 55 $^0$C and 70 $^0$C was ineffective as other parameters in reducing cell proliferation. Although there was a slight decrease compared to the control sample, there was no significant decrease in terms of statistical analysis. This may suggest that high temperature caused a decrease in the number of antibodies binding to VEGF due to the increased protein aggregation. This could explain why cell proliferation is not seen as low as other temperatures.

Considering the literature, it was shown that biological activity might decrease due to the increase in aggregation and fragmentation of mAbs at high temperatures (Alexander and Hughes 1995; H. Liu, Gaza-Bulseco, and Sun 2006), which has not been observed significantly in our studies. Biological activity can be seriously affected due to fragmentations and modifications, especially in the CDR region (Cordoba et al. 2005; B. Yan, Steen, et al. 2009a). However, in our study, the lack of any unexpected modification in CDR regions as shown in peptide mapping data is consistent with the no observation of a dramatic loss of biological activity.

### 4.1.4. VEGF Binding Analysis of mAbs exposed to thermal stress

The anti-VEGF IgG recombinant antibody can bind the VEGF growth factor via the Fab region and prevents VEGF from interacting with its receptor VEGFR on cells (C. Yu et al. 2020). As a result of the recognition of the target molecule by mAbs, the biological activity of that molecule is inhibited. This has led to the consideration of mAbs as unique drug candidates for treating many diseases (Singh et al. 2018). Therefore, determining the binding kinetics of the newly developed monoclonal antibody drug candidates against the target molecule has great importance.

Many methods were used to determine the binding kinetics of the recombinant antibodies produced. ELISA (Raghava and Agrewala 1994; Liang et al. 2007), Biolayer interferometry (BLI) (Kamat and Rafique 2017; Estep et al. 2013), KinExA (Estep et al. 2013), and SPR-based methods (Estep et al. 2013; Hearty, Leonard, and O'Kennedy 2012; de Mol and Fischer 2010) are commonly used for investigating antibody-antigen binding affinity. The SPR method used in this study is a method that is used in the pharmaceutical industry and many antibody research laboratories for revealing real-time binding kinetics and affinity.(Beeg et al. 2019; Malmqvist 1993; Hearty, Leonard, and O'Kennedy 2012; Estep et al. 2013)

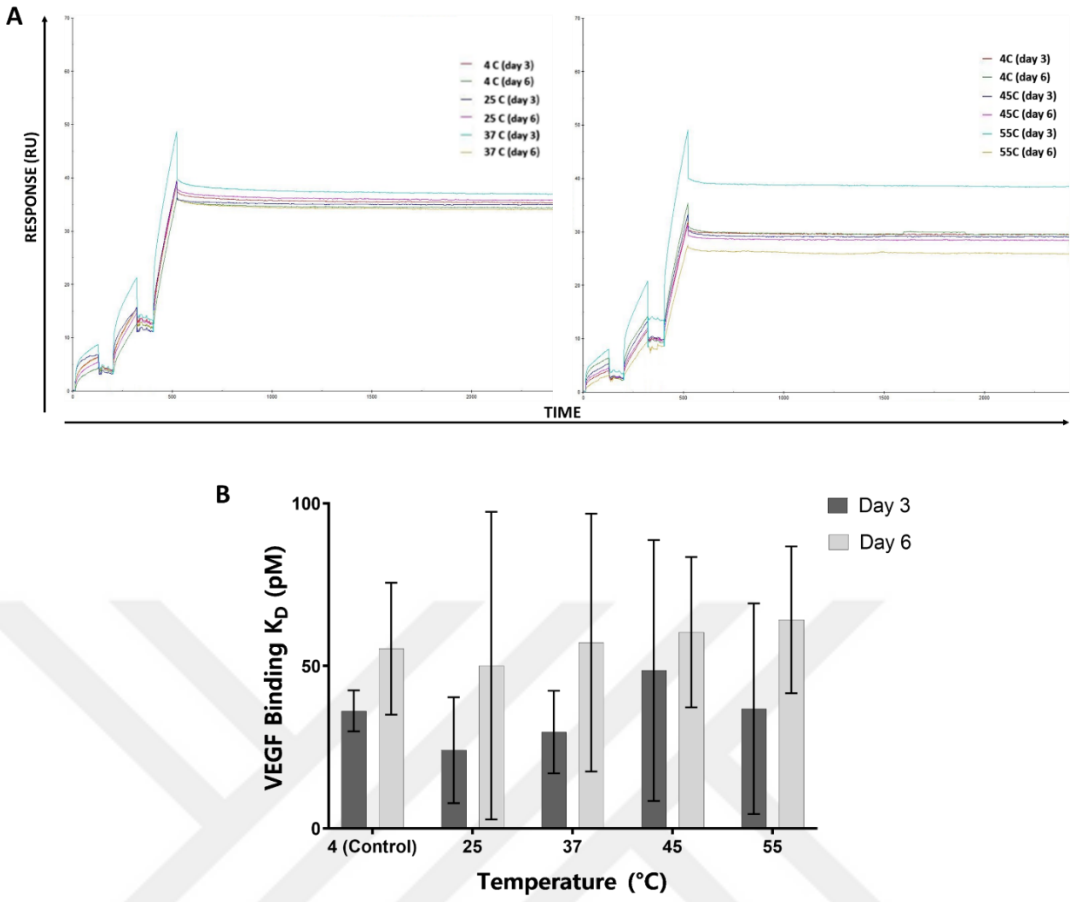



**Figure 4.1.4.1**: VEGF Binding Analysis of mAbs exposed to thermal stress by surface plasmon resonance (SPR). (A) Sensogram images of each sample were given. Time-dependent response units were shown in these sensorgrams. (B) The VEGF binding affinity values of each sample were plotted with standard deviations. Experiments were carried out in triplicate. To interpret the results statistically, Single-way ANOVA was applied. Langmuir 1:1 binding model was applied.

The binding affinities of mAbs, which were exposed to different temperatures for 3 and 6 days, to VEGF protein were determined using the SPR method. Sensorgrams of each sample were shown in **Figure 4.1.4.1A**. Binding responses to VEGF did not change significantly over time. Sensorgram profiles were similar for each sample. In **Figure 4.1.4.1B**, the binding affinities ($K_D$) in the picomolar level were given. Control samples incubated for 3 and 6 days at 4 $^0$C have a $K_D$ value of 36.17±5.9 and 55.29±19.24 pM against the VEGF molecule, respectively. Binding kinetics of antibodies incubated at

different temperature conditions were calculated by comparing them to the control group. Binding affinities of antibodies incubated at 25 $^0$C for 3 and 6 days were 24.07 ± 14.58 pM and 50.10±38.61 pM, respectively. These values were 29.68 ± 11.36 pM and 57.17 ± 34.35 pM when incubated at 37 $^0$C for 3 and 6 days. When the samples were incubated at 45 $^0$C, the $K_D$ values were determined as 48.61 ± 28.38 pM and 60.36 ± 18.85 pM for 3. and 6. days, respectively. Finally, the binding affinities of antibodies exposed to 55 $^0$C, the highest temperature in the experiment, were 36.84 ± 26.44 pM and 64.17 ± 19.56 pM for 3. and 6. days. ANOVA test was used for statistical analysis of the alteration, and no significant difference was observed between the samples. The $K_D$ values of all samples were close to the control. In addition, there was no statistically significant difference again when looking at the effect of incubation time on binding affinity. As a result, the binding affinities did not change with either temperature shifts or incubation duration.

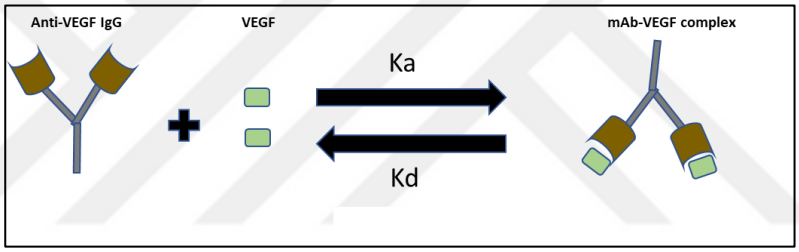



**Figure 4.1.4.2.** $K_D$ value is related to association rate constant (Ka) and dissociation rate constant ($K_d$).

While performing SPR experiments, the equilibrium constant ($K_D$) is calculated to determine affinity. In equilibrium, $K_D$ value is associated with the complex formation, which is the association rate constant ($K_a$), and the complex breakdown, which is described as dissociation rate constant ($K_d$) (**Figure 4.1.4.2)**. A High $K_a$ value indicates a high affinity between molecules. At the same time, the high $K_d$ value gives information about how stable the complex is. Therefore, with the determination of affinity, these parameters were also controlled. When the $K_a$ and $K_d$ values of the samples were compared, it was not observed that there was a significant difference (data not shown). Therefore, no change in affinity was observed due to these parameters.

Many studies in the literature have shown that antibodies can lose their functions due to structural degradation at high temperatures (Alexander and Hughes 1995; B. Yan, Steen, et al. 2009a; Fesinmeyer et al. 2009). It was observed that PTMs in the antibody, mainly due to high temperatures, can change the binding affinities on target molecules (X. Lu et al. 2020; Jiskoot et al. 1990; Y. D. Liu, van Enk, and Flynn 2009). However, it is also essential where the modification occurs in the antibody (Cordoba et al. 2005; Dillon et al. 2006; Xiang et al. 2007). Many studies showed that the antibody could lose its interaction with the antigen due to the modifications in the CDR parts of the Fab regions that allow the antibodies to bind to the target molecule (Schmid et al. 2018; Xiao and Bondarenko 2008; Sˇkulj et al. 2014). Additionally, loss of affinity is supported by *in vitro* studies (Kroon, Baldwin-Ferro, and Lalan 1992). High temperatures did not affect both binding affinity and in vitro cell proliferation in our studies. However, no statistically significant inhibition of cell proliferation was observed in 55 $^{o}$C and 70 $^{o}$C samples due to aggregation formation. The pep-map analysis also supports this by showing that no modification occurred in the CDR region.

### 4.1.5. FcRn Binding Analysis of anti-VEGF IgG exposed to Thermal stress

The FcRn is an essential immunological receptor that ensures long half-lives of IgG and Fc-conjugated proteins in serum. In addition, FcRn is also responsible for the bidirectional transcytosis of IgG molecules in epithelial cells (Kuo and Aveson 2011). Thus, it ensures the bioavailability of the antibody by ensuring that IgG is delivered to many parts of the body (Deng et al., 2012).

The FcRn receptor consists of a transmembrane protein and a soluble B2 microglobulin non-covalently bound to it (Simister and Mostov 1989). FcRn binds to CH2-CH3 sites in the Fc domain of IgG under acidic pH conditions (pH 6.0) in the endosome. Then, this interaction disappears at neutral pH, allowing the antibody to be released back into the blood. Thus, the catabolism of IgG was prevented, and its half-life in the blood increased (Roopenian and Akilesh 2007). Therefore, characterizing the interactions of therapeutic

IgG molecules with FcRn is an essential parameter for predicting the *in vivo* pharmacokinetic properties of the mAbs.

Both *in vivo* and *in vitro* assays are used to analyze FcRn interaction. Recent studies have shown that the FcRn interaction demonstrated by *in vitro* methods is coordinated with the IgG half-life in serum (Suzuki et al. 2010; Dall'Acqua, Kiener, and Wu 2006). Therefore, *in vitro* affinity analyzes have gained significant importance, and many methods were developed to demonstrate this interaction (Yeung et al., 2009; Mathur et al., 2013).

Pharmacokinetic properties such as the half-life of the antibody in the serum can be determined using animal studies to demonstrate the indirect FcRn interaction. However, due to the *in vitro* data being compatible with *in vivo*, the importance of *in vitro* methods has increased due to the workload and cost reduction by reducing animal experiments (Schlothauer et al., 2013).

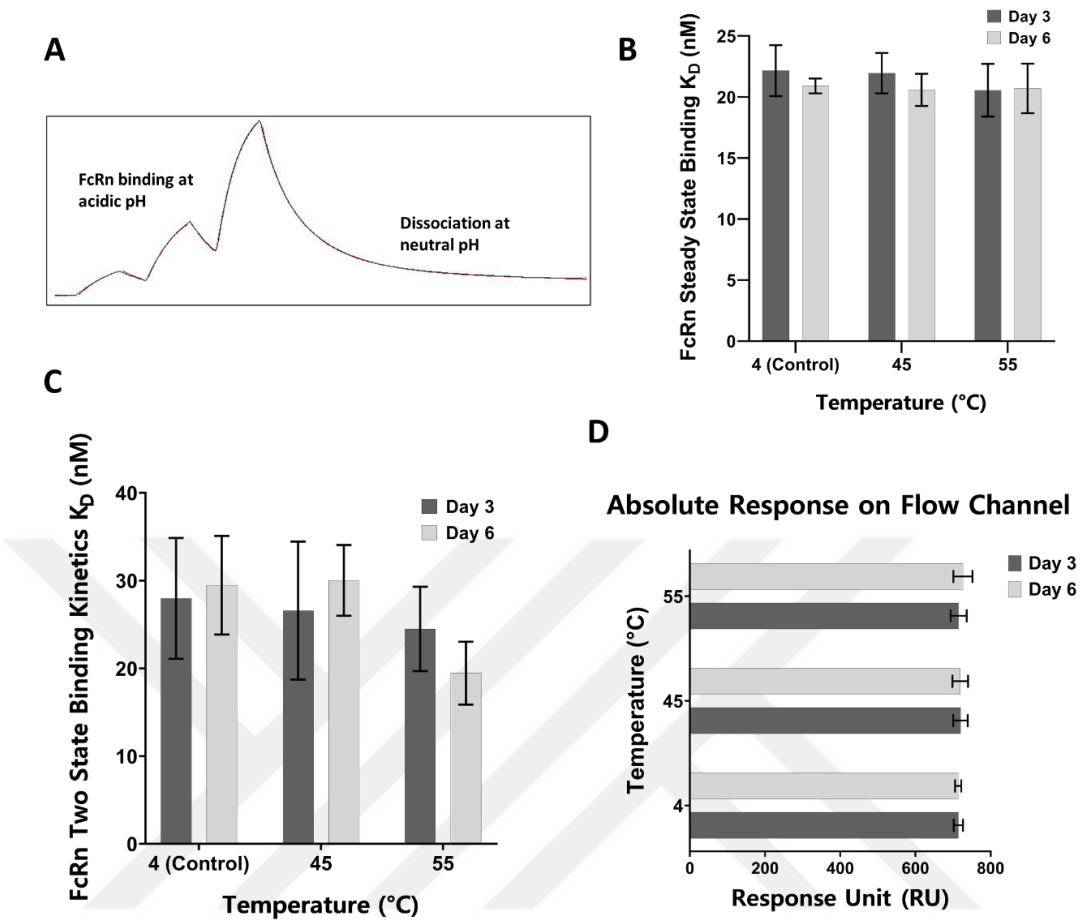



**Figure 4.1.5**: FcRn binding analysis of the samples using SPR: (A) Typical sensorgram of IgG and immobilized FcRn interaction was shown. FcRn binds the IgG molecules at acidic pH and releases them at neutral pH. (B) Steady-state interaction of anti-VEGF IgG with immobilized FcRn was represented as the mean of at least three measurements. (C) Two-state binding interaction of anti-VEGF IgG with immobilized FcRn was represented as the mean of at least three measurements. (D) Absolute response values of the samples on the flow channel. There were no significant differences between the samples based on the single-way ANOVA analysis.

Many *in vitro* methods are used to demonstrate the FcRn interaction. These methods are generally divided into two, using cells or being cell-free. In the FcRn interaction performed using the cell, the interaction can be effectively demonstrated by imitating the

physiological conditions (Mathur et al., 2013). The most commonly used cell-free methods are SPR, Fluorescence Resonance Energy Transfer-based assays (FRET), amplified luminescent proximity homogenous assay (Alpha screen), ELISA, and BLI (Y. Lu et al. 2011; A. Zhang et al. 2012; Wozniak-Knopp, Stadlmann, and Rüker 2012; Wu et al. 2015).

In this thesis, FcRn binding analyzes were performed using the SPR method. Many studies have shown that FcRn and anti-VEGF IgG binding affinities range from 6 nM to 2500 nM (Neuber et al. 2014; Gao et al. 2015; Visser et al. 2013). In studies using SPR, these affinity values were reported between 6.58±0.12-49.6±1.78 nM for monoclonal antibodies, while it is between 9.99±0.43-71.9±15.7 nM for human IgG1 (Szikora et al. 2017).

While performing FcRn binding affinity experiments, anti-VEGF IgG molecules incubated for 3 and 6 days at 45 and 55 $^{o}$C were used. Looking at the pep-map data, no PTM formation was observed at 25 and 37 $^{o}$C, which were other temperature parameters, and the samples showed the same profile as the control sample. Because PTMs were only seen in the samples exposed to 45 and 55 $^{o}$C, experiments were carried out with these samples, with the thought that 25 and 37 $^{o}$C samples could show the same results as the control sample in FcRn binding affinity.

Antibodies incubated at 4 degrees were again accepted as a control sample, and the results were evaluated by comparing the results of other samples concerning the reference. Two different fitting models were applied while determining the FcRn binding affinity. Thus, the differences that may occur by applying two different models to the same data will be revealed. Many studies have provided results by applying these models (Miao et al. 2017; Neuber et al. 2014; Bertolotti-Ciarlet et al. 2009b; Abdiche et al. 2015). In this experiment, the overlaid sensorgrams obtained from all samples at different pH conditions were represented in **Figure 4.1.5A**. Evaluating the comparative steady-state FcRn binding data, the $K_D$ values of the control sample at 3 and 6 days were calculated as 22.15±1.18 and 20.90±0.52, respectively. The $K_D$ values of the samples incubated at 45 $^{o}$C for 3 and 6 days were 21.95±1.43 and 20.58±1.14 nM, respectively. Finally, the $K_D$ values of the antibodies kept at 55 $^{o}$C for 3 and 6 days were found to be 20.55±1.87 and 20.70±1.76 nM, respectively.

When analyzed according to the two-state kinetics model, the $K_D$ values of the control sample at 3 and 6 days were calculated as 27.97±5.96 and 29.47±4.87, respectively. The

$K_D$ values of the samples incubated at 45 $^oC$ for 3 and 6 days were 26.58±6.81 and 30.02±3.49 nM, respectively. Finally, the $K_D$ values of the antibodies kept at 55 $^oC$ for 3 and 6 days were found to be 24.50±4.16 and 19.45±3.10 nM, respectively. When both models were evaluated statistically, they did not result significantly.

The risk of aggregation increases by changing the thermal stability of the Fc part of the antibody (D. Liu et al., 2008). In addition to decreasing its binding affinity for FcRn, its binding affinity for protein A and protein G also decreases (Bertolotti-Ciarlet et al. 2009a; D. Liu et al. 2008). Due to the decreased affinity of antibodies for FcRn, their half-life in serum is also reduced (Weirong Wang et al., 2011). In addition, its adverse effects, such as rapid clearance in serum, were demonstrated in vivo experiments (Stracke et al., 2014; Suzuki et al., 2010). However, since FcRn binding is related to the Fc region of the antibody, modifications in this region can seriously affect FcRn binding (Weirong Wang et al., 2011; S. Wang et al. 2016; Tsuchida, Yamazaki, and Akashi 2016). Since there was no PTM in these regions in our studies, there was no difference in FcRn binding according to temperature change.

### 4.1.6. C1q ELISA Binding

The complement system is an ensemble made up of many small proteins belonging to the innate immune system. Complement system members play a significant role in the ability of antibodies and phagocytic cells to more effectively eliminate microbes and damaged cells. In addition, it increases inflammation and provides the formation of a MAC on the target cell (**Figure 4.1.6A**).

The complement system uses the cooperation of antibodies while performing these critical tasks (Merle, Noe, et al., 2015). IgG and IgM bind to the antigens on the target cell and call the complement system members. They bind to target molecules on the target cell membrane and open lytic pores. This event is accomplished by forming a MAC, resulting in the cell being destroyed by apoptosis (Merle, Church, et al., 2015). This

killing mechanism is called CDC. It is at the forefront of effector functions that effectively uses mAbs for therapeutic purposes (Meyer, Leusen, and Boross 2014).

The event that initiates the complement cascade required for CDC is the interaction between the complement system member, C1q, and the CH2 domain in the Fc region of the IgG molecule (B. Wang et al. 2019) (**Figure 4.1.6A)**. Therefore, showing the C1q interaction with IgG, meaning CDC activity. But this is not the case with all antibodies. For example, in the case of bevacizumab, there is C1q binding but no CDC activity. Because VEGF, the target molecule of bevacizumab, is a soluble protein and not attached to the cell membrane (Y. Wang et al. 2004). Since the anti-VEGF IgGs we use were bound to VEGF as a target molecule, they were not expected to show CDC activity, and only C1q bindings were analyzed. When examining the literature for C1q binding analysis, it is seen that ELISA and SPR methods were generally used (Zeitlin et al. 2011b; He et al. 2014; Jovic and Cymer 2019). In this thesis, the ELISA method was used in C1q binding studies. When the literature was examined again, it was shown how C1q binding changes with increasing or decreasing antibody concentration using many different antibody concentrations (Tammen et al. 2017; Idusogie et al. 2000; Strasser et al. 2019).

When thermal stress samples were examined in the pep-map analysis, no PTM was observed in any other sample except the samples treated at 45 and 55 $^{o}$C for 3 and 6 days. 4 $^{o}$C was used as the control sample 25, 37 $^{o}$C samples gave results like the control. Therefore, for C1q binding experiments, samples treated at 45 and 55 $^{o}$C for 3 and 6 days were examined. While evaluating the results, the absorbance value of the C1q-IgG structure formed due to the bonding was measured at 450 nm using different antibody concentrations (0.6, 1.25, 2.5, and 5 ug/ml). When the results were examined, it was observed that the absorbance value decreased with the decrease of the antibody concentration, representing decreasing C1q binding. As the control sample was referenced, it was seen that the C1q binding of all samples at each concentration was the same as the reference. However, it was observed that C1q binding decreased with decreasing antibody concentration in each sample. Consequently, it was observed that there was no change in the C1q binding of antibodies exposed to thermal stress (**Figure 4.1.6B)**.

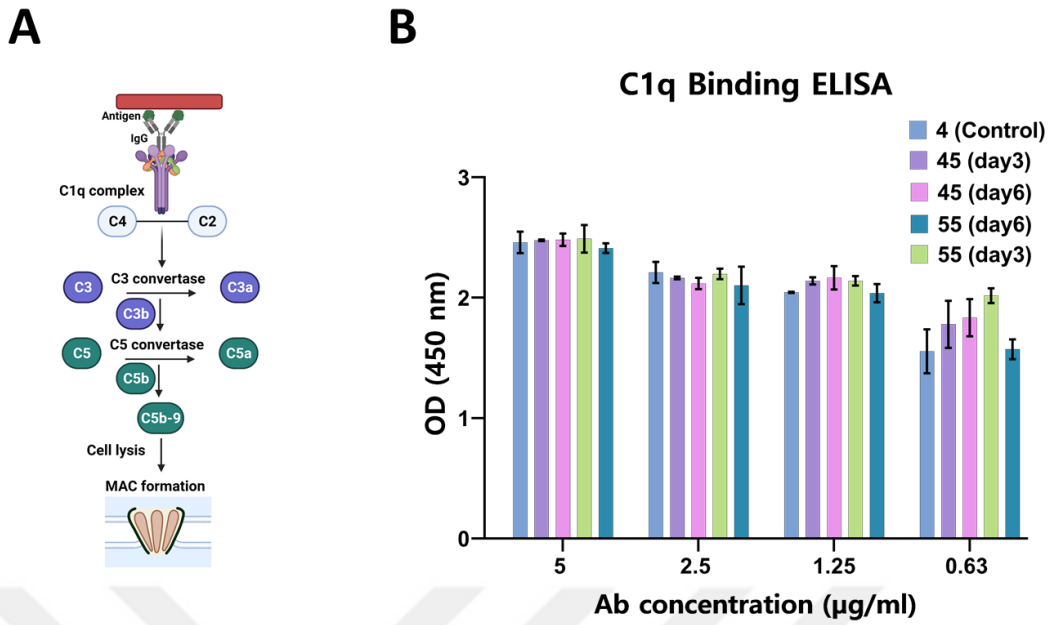



**Figure 4.1.6**: C1q ELISA binding analysis of the samples. (A) Schematic illustration of C1q complex, complement cascade and its interaction with antibody. (B) the results of C1q ELISA binding of thermal stress exposed anti-VEGF IgG at different concentrations. The data represented the mean of at least three independent measurements. There was no significant difference between the samples compared control sample.

The literature showed that the most significant factor affecting C1q binding is the glycosylation profile of the antibody (Jez et al., 2012). Here, experiments have shown that CDC activity increases with terminal galactose (Seo et al. 2018). Although studies showing how C1q binding is affected by temperature were limited in the literature, it was shown that CDC activity decreases due to aggregation at high temperatures, especially at temperatures above 65 degrees Celsius (Xu et al. 2019). Some studies have shown that these temperatures were ineffective (Bansal, Dash, and Rathore 2020).

## 4.2. pH stress

### 4.2.1. Investigation of Glycosylation Patterns of mAbs exposed to various PH stress conditions by Intact Protein Analysis

To compare the glycosyl profiles of anti-VEGF IgGs exposed to different pH values at different incubation times, intact protein analysis was performed using a validated UPLC/MS-MS protocol. The control sample was anti-VEGF IgG that was not exposed to any pH stress and was kept in its formulation buffer (pH 6.2). The samples exposed to pH stress were compared with the control sample, and their glycosyl profiles were determined. According to the results, the dominant glycoform structure was G0F N(2)-K (2) both in the control sample and in all other samples exposed to pH stress. When the MS spectrums of antibodies exposed to specific pH values for 1,3, and 5 days were examined separately, it was seen that all of them had similar spectra **(Figure 4.2.1A, B, C**). In addition to the dominant glycosyl structure, two more dominant structures were encountered in all antibodies together with the control sample. These were G0F N (1), G1F N(1)-K, and G0 N(1), G0F N(1) structures. But these were not even 15% of the total glycoform percentages (**Figure 4.2.1D**) (blue line indicates 15 % value). Because our dominant glycosyl form, G0F N (2)-K (2), was present at a rate of 40-45% in all samples. This ratio was a result we expected from our previous works, and this experiment confirmed it. Since antibodies have a heterogeneous structure, glycosyl forms have also been detected apart from these three dominant glycosyl forms (data not shown). However, their presence is not essential because they represent a tiny proportion of the total percentage of glycoforms. Demonstrating the three dominant glycosyl forms was sufficient for our antibody's structural and functional properties. When comparing with the control sample, if a change of more than 5% is detected, we accept this as a significant change (C.-H. Chen et al. 2018). However, when the glycoform profile of the samples exposed to different pH values on different days was compared with the control sample, no significant change was encountered.

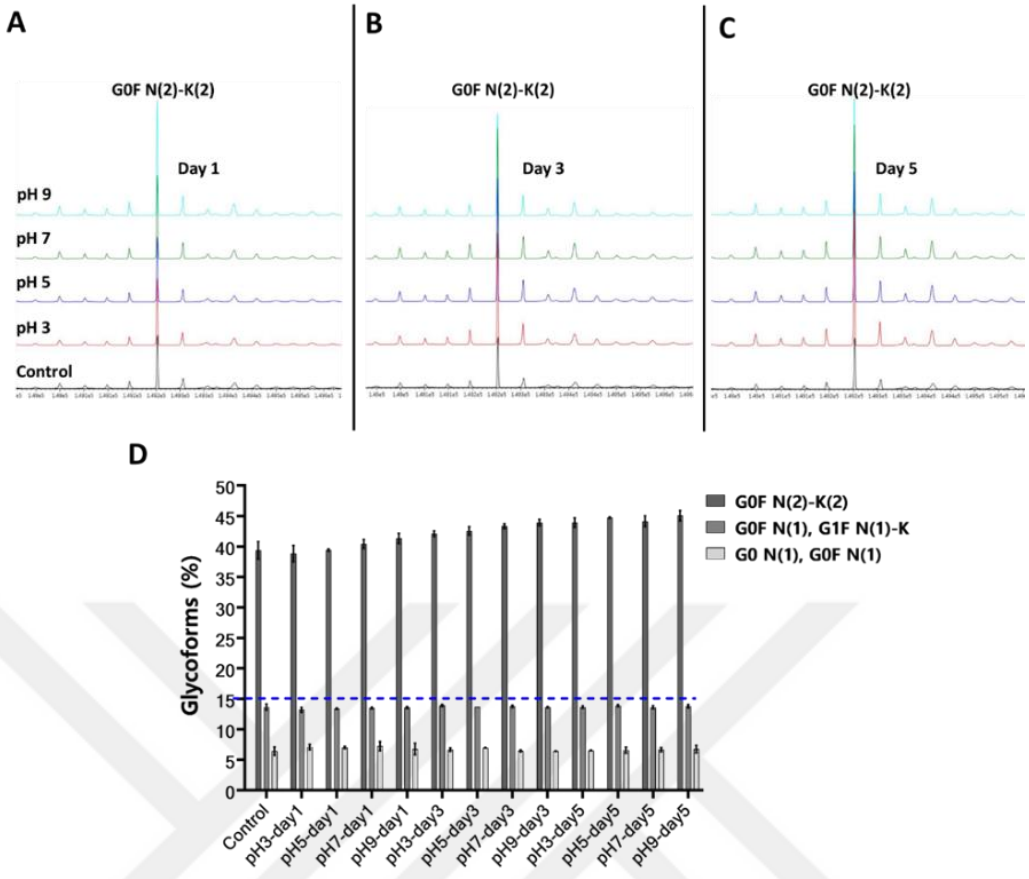



**Figure 4.2.1**:Intact Protein analysis of mAbs expose to pH stress by using mass spectrometry. (A) Deconvoluted MS spectrums of one day incubated mAbs in pH. (B) Deconvoluted MS spectrums of three days incubated mAbs in pH. (C) Deconvoluted MS spectrums of five days incubated mAbs in pH. (D) The graph shows the percentage of glycoforms obtained in the intact analysis of mAbs for all samples. The percentage values were calculated by averaging three separate injections. -K represents clipped lysine. The blue line shows a 15 % value.

Therefore, as expected, glycosylation of an antibody occurs under the control of enzymatic processes within the cell during its synthesis (Pisupati et al., 2017). Therefore, we did not expect to see any significant change in glycosyl profiles as there was no enzymatic process in pH stress.

### 4.2.2. Determination of PTMs on mAbs by Peptide-Mapping Analysis

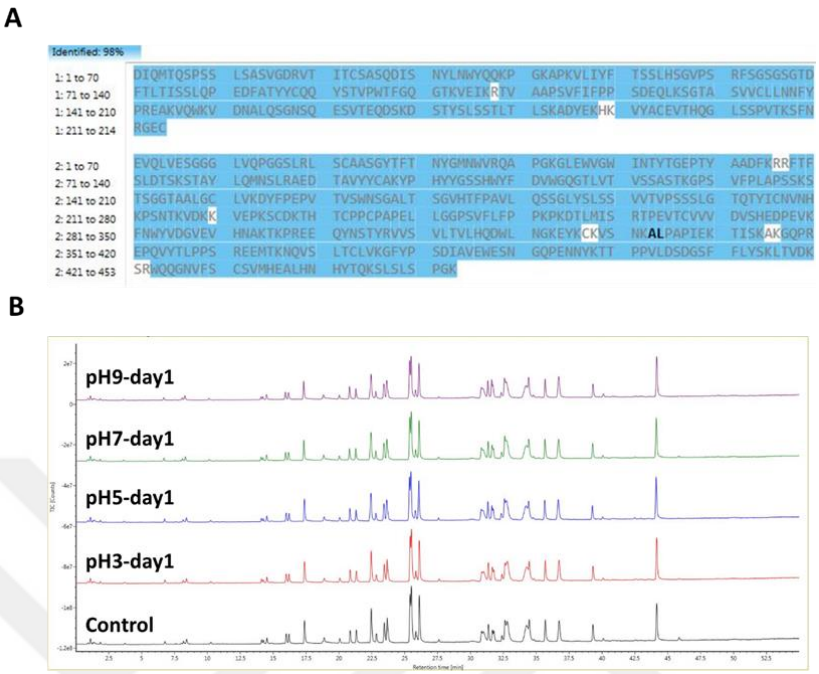



**Figure 4.2.2.1**: Peptide mapping, ng analysis of mAbs exposing pH stress using MS. (A) The anti-VEGF IgG protein sequence coverage map was obtained by pep-map analysis. (B) Total ion chromatograms of mAbs exposing different pH values for one day. The percentage values were calculated by averaging three separate injections.

Another critical parameter that affects the stability of mAbs is pH. The studies showed that the degradation pathways might be triggered against the pH changes (H. Liu, Gaza-Bulseco, Faldu, et al. 2008). In addition to fragmentation and aggregation, PTMs have also been identified in the presence of different pH environments (Chu et al., 2007; Cacia et al., 1996; Nowak, K. Cheung, et al., 2017; H. Z. Huang, Nichols, and Liu 2009). Additionally, the antibody's biological activity may be affected due to such PTMs, which mainly occur in the CDR region (Cacia et al., 1996; Du et al., 2012).

Therefore, it is crucial to show the effects of the pH stress, which has such significant effects in samples. PTMs formed in anti-VEGF IgG molecules exposed to different pH values for different days were identified using pep-map analysis. The antibodies shown

here as control examples represent antibodies that were not exposed to any pH stress and were in their formulation buffer (pH 6.2). First of all, the protein sequence of all samples was determined (**Figure 4.2.2.1A**). The coverage percentage for all samples was over 95%. Therefore, the sequence of our antibody was correctly identified in all products. According to the TIC, the peptide profiles of the samples were highly similar with minor differences (**Figure 4.2.2.1B)**. As a result of the Pep-map analysis, deamidation was observed in two peptides when all peptides were examined in PTM. These peptides were HC / T24 peptide (VVSVLTVLHQDWLNGK) and HC/T8 peptide (STAYLQMNSLR).

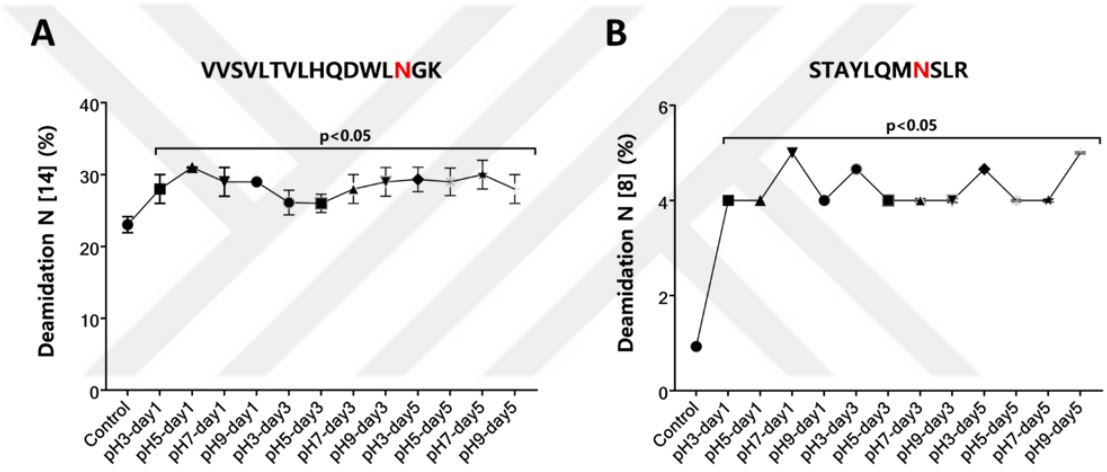



**Figure 4.2.2.2**: Deamidation rate on VVSVLTVLHQDWLNGK peptide (A). Deamidation rate on STAYLQMNSLR peptide (B) The letters marked in red were the amino acids with the modification. The percentage values were calculated by averaging their separate injections, and the data was analyzed statically using the Single-way ANOVA test.

When the ratio of VVSVLTVLHQDWLNGK peptide to deamidation due to pH change in the Asn amino acid (N [14]), a deamidation of around 23% was observed in our control sample, the deamidation rate of the samples remaining at pH3, pH5, pH7, and pH9 for one day was determined as 28%, 31%, 29%, and 29%, respectively (**Figure 4.2.2.2A**). Likewise, the deamidation rate of the samples remaining at pH3, pH5, pH7, and pH9 for 3 days was determined as 26%, 26%, 28%, and 29%, respectively. The deamidation rate of antibodies exposed to the same pH values for 5 days was 29%, 29%, 30%, and 28%,

respectively. As can be seen from the control example, the HC/T24 peptide was highly susceptible to deamidation. This peptide can undergo deamidation even during the peptide mapping sample preparation step. An increase in deamidation was also observed in our samples exposed to pH stress compared to the control sample. The change in pH, rather than the increase or decrease in pH, caused deamidation. In addition, exposure to different incubation times at different pH values did not cause an increase in deamidation. Deamidation appears to be the same at all incubation times. However, both the pH change and the different incubation times at these pH values increased the deamidation rate of the antibodies compared to the control sample. Therefore, it can be said that the deamidation rate of this peptide increased with pH change.

When we examined the STAYLQMNSLR peptide, an increase in deamidation was observed in the Asn amino acid (N [8]) pH change **Figure (4.2.2.2B)**. According to the deamidation rates, a deamidation of around 0.9% was observed in our control sample. The deamidation rate of the samples remaining at pH3, pH5, pH7, and pH9 for one day was determined as 4%, 4%, 5%, and 4%, respectively. Likewise, the deamidation rate of the samples remaining at pH3, pH5, pH7, and pH9 for 3 days was determined as 4%, 5%, 4%, and 5%, respectively. The deamidation rate in antibodies exposed to the same pH values for 5 days was 5%, 4%, 4%, and 5%, respectively. As seen in this peptide, pH change causes deamidation, not pH increase or decrease. The incubation period did not make any difference in the deamidation trend. However, when the antibodies were exposed to pH stress, there was an increase in deamidation compared to the control sample.

Deamidation is a non-enzymatic post-translational modification of monoclonal antibodies that converts amino acids Asn and Gln to Asp and Glu, respectively. Asn is more susceptible to deamidation than Gln. Deamidation is severely affected by the pH of the environment (Chelius, Rehder, and Bondarenko 2005). In neutral and basic pH (pH> 6) conditions, the amide groups undergo cyclization in the molecule and cause the formation of the intermediate product succinimide, which causes the formation of degradation products (Manning et al., 2010). In acidic conditions, amide groups undergo direct hydrolysis and only trigger the formation of Asp (J. Y. Zheng and Janis 2006). At the same time, the deamidation effect of the buffer type and the temperature on Asn was demonstrated by studying an IgG molecule (J. Y. Zheng and Janis 2006).

When evaluating high pH values, pH 8 and pH9 are generally studied. However, in some cases, the pH value of 10 can reveal the deamidation susceptibility of Asn residues on the antibody (Jiskoot et al. 1990; H. Liu, Gaza-Bulseco, and Sun 2006). The deamidation prone site commonly observed in mAbs is SNGQPENNY peptide located in the Fc part, and it is generally abbreviated as "PENNY" (Y. D. Liu, van Enk, and Flynn 2009; L. Wang et al. 2005; Chelius, Rehder, and Bondarenko 2005; Sinha et al. 2009). In addition, deamidations were also observed in the CDR region at high pH conditions (L. Huang et al. 2005a; Harris et al. 2001; Bertolotti-Ciarlet et al. 2009b). Hence, deamidation of this PENNY peptide is an essential reference in determining the susceptibility of antibodies to deamidation under specific conditions. Oliyai (Oliyai et al. 1994) and Patel (K. Patel and Borchardt 1990) proved how these reactions change depending on pH with their studies. Asn deamidation increased when the pH was kept higher than 5. The pH range of 3 and 5 is generally the optimum Asn deamidation range for proteins (K. Patel and Borchardt 1990). Therefore, according to the literature, deamidation against pH change can occur, as seen in our experiments.

### 4.2.3. Cell Proliferation Assay with Anti-VEGF IgG exposed to pH stress

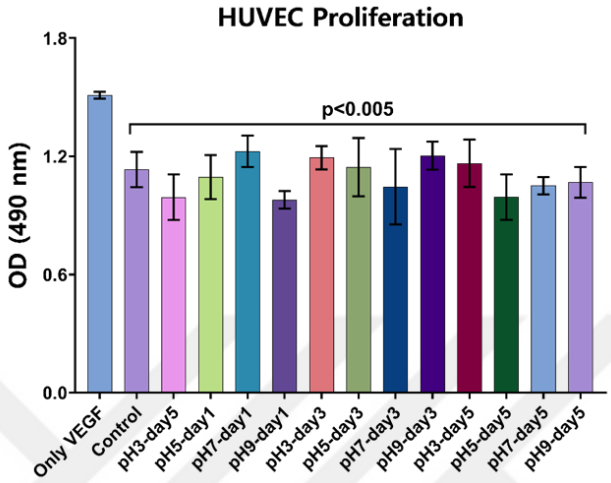



**Figure 4.2.3**: Cell proliferation effects of mAbs expose to pH stress by MTS. The antiproliferative effects of anti-VEGF IgG were observed by applying the MTS cell proliferation assay on UVEC cells. Absorbance values were measured at a wavelength of 490 nm by a microplate reader. Experiments were carried out in triplicate. To interpret the results statistically, single-way ANOVA was applied.

Proliferation effects were investigated using HUVEC cells to determine the biological activity of anti-VEGF IgGs exposed to various pH stress conditions. Since VEGF is a growth factor and its receptor VEGFR is also highly expressed in HUVEC cells, the biological activity of VEGF can be monitored well in HUVEC cells. It was analyzed how the anti-VEGF IgG binding to VEGF changes its binding with its target molecule in the presence of pH stress and how HUVEC proliferation was affected due to this change.

In the proliferation experiment, the MTS method was used as previously described, and the proliferation values were compared by measuring the OD values at 490 nm (Maghni, Nicolescu, and Martin 1999). The example we call “only VEGF” represents the sample in which no antibody was added to the medium during the cell culture, and only the VEGF growth factor was present in this sample. Therefore, with this sample, the biological

activity of VEGF was expected to be the highest, and thus cell proliferation should be at the maximum level. Other samples were compared with the VEGF-only sample, and their effects on cell growth can be compared. As expected, the highest absorbance value was found only in the VEGF sample when the results were evaluated (**Figure 4.2.3**). Therefore, cell proliferation was at its maximum in this example. The control sample represents the anti-VEGF IgG molecule that was exposed to any stress and was observed to inhibit VEGF's biological activity and decrease cell proliferation severely. When the effects on cell proliferation of all antibodies exposed to different pH values on different days were examined (**Figure 4.2.3**), it was observed that all antibodies statistically significantly reduced cell proliferation compared to the VEGF sample only. In addition, it was observed that all antibodies exposed to pH stress could inhibit cell proliferation almost as much as the control sample.

Several reports have shown that mAbs can undergo many PTMs due to the pH change and undergo fragmentation and aggregation processes (Dick et al. 2010; Kroon, Baldwin-Ferro, and Lalan 1992; McKerrow 1979). In particular, modification in the CDR region has a critical role in losing biological activity (B. Yan, Steen, et al. 2009b; Hawe et al. 2009; Harris et al. 2001). However, we did not observe a severe loss of biological activity in the pH stress experiment since we did not find any modification in the CDR regions, as shown in the pep-map data. As a result, following the literature, the absence of any PTM formation in the CDR regions did not affect the binding of the antibody to the VEGF target in our experiments. As a result, it did not cause a change in its *in vitro* biological activity. No problem was encountered in the biological activities of antibodies, both exposed to different pH values and exposed to these pH values at different times.

### 4.2.4. Characterization of Aggregation profiles of mABs

Enormous difficulties are encountered during the formulation, storage, and administration of mAbs. These difficulties are physical and chemical instability that cause denaturation and aggregation (Oliva, Llabrés, and Fariña 2015). Aggregates of mAbs potentially trigger immunogenicity in patients and cause severe effects such as anaphylactic reactions after administration of them (Ratanji et al., 2014). However, aggregates in the mAbs

formulation are considered a CQA in the FDA (Rathore and Winkle 2009). Therefore, analyzing how the structural stability of the mAb changes in the presence of many stress factors is of great importance in applying therapeutic antibodies.

When formulating new biotherapeutic antibodies, the stress parameters that create the aggregation conditions are generally temperature, extreme pH values, UV exposure, sonication, and organic solvents. Aggregations due to pH have significant effects on the formulation of antibodies (Jiskoot et al. 1990; Joubert et al. 2011; Arosio, Rima, and Morbidelli 2013; Sharma et al. 2010). It is crucial to determine which pH values aggregation occurs and the amount of this aggregation to prevent this issue. With the formation of aggregation, the antibody loses its physical stability and its bioactivity (Singla et al., 2016). Aggregation occurs due to significant changes in antibodies' secondary and tertiary structures.

The chemical reasons for the aggregation of therapeutic antibodies are PTMs of the antibody. These are incomplete disulfide bonds, C-terminal Lys processing, deamidation, isomerization, and oxidation (Joshi et al., 2014).

Deamidation is a chemical modification that can alter both the structure and biological activity of the mAb due to generating unwanted negative charges (L. Huang et al. 2005b; Robinson and Robinson 2001; Kosky et al. 2008; Geiger and Clarke 1987; Y. D. Liu, van Enk, and Flynn 2009). Therefore, deamidation of some specific Asn residues in the presence of stress parameters such as pH, temperature, and oxidation may cause a change in the tertiary structure of the antibody (Robinson and Robinson 2001; Kosky et al. 2008).

Commonly used methods for tracking and analyzing insoluble aggregations are size exclusion-high performance liquid chromatography (SEC), DLS, field flow fractionation (FFF), MS, transmission electron microscopy (TEM), intrinsic and extrinsic fluorescence, and NTA (Beck et al. 2013; Berkowitz et al. 2012; Vasudev, Mathew, and Afonina 2015; Bickel et al. 2016; Mahler et al. 2005).

The hydrodynamic size distribution in the solution can be measured effectively using DLS without any labeling process (Murphy and Pallitto 2000). However, DLS cannot measure the number of aggregate concentrations and can give average properties. Other methods such as TEM and the atomic forced microscope may not provide detailed information about the aggregation size, structure, and mechanical characteristics of the solution (Adamcik and Mezzenga 2012). Also, quantitation of an entire population is very

difficult. Although SEC is a very effective method for measuring monomer losses, it is less sensitive to a small number of aggregations (Carpenter et al., 2010). Although fluorescence-based methods were used effectively in aggregation characterization, chemical labeling is required (Crick et al., 2013).

NTA is a scattering technique that independently monitors the diffusion of each particle in the solution. In addition, unlike DLS, it can provide particle number, hydrodynamic size, and particle by particle size distribution information (D. T. Yang et al., 2014). Recently, NTA was used very effectively to characterize spherical and globular morphologies such as aggregation of biotherapeutic molecules (Filipe, Hawe, and Jiskoot 2010; C. Chen et al. 2013; Sediq et al. 2016; Bickel et al. 2016; Filipe et al. 2012). According to the literature, the highest aggregation rate was observed in antibodies exposed to pH stress at low and high pH values (Bansal, Dash, and Rathore 2020; Saito et al. 2019). In addition, it was shown in the study that the formation of insoluble aggregates was observed to be very high at pH values close to the pI value of the antibody. Since antibodies are exposed to low pH values in protein A chromatography and virus inactivation processes during mAb production, they tend to form aggregation (Saito et al. 2020).

Fragmentation may also occur due to pH changes in therapeutic antibodies (Gaza-Bulseco and Liu 2008). Fragmentation rate increases, especially at low pH (Morris, Watzky, and Finke 2009). Low pH causes the CH2 domain to unfold and increases the surface accessibility of the antibody. Thus, the antibody can undergo fragmentation (S. Zheng et al., 2017).

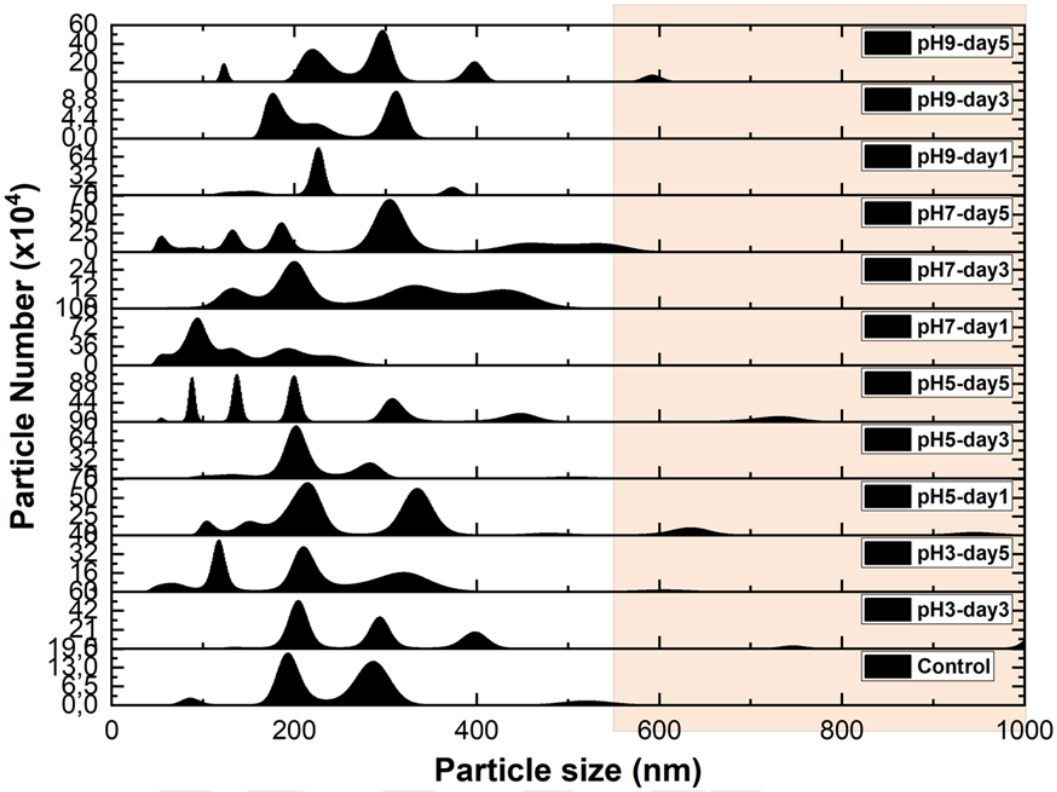



**Figure 4.2.4**: Characterization of aggregation profiles of mAbs exposed the pH stress. The samples were prepared in 250 ug protein in 1500 ul PBS-P buffer. Particle movements in all samples were monitored for 1 minute to determine their size and numbers.

NTA analysis was performed to compare the aggregation profiles of anti-VEGF IgGs exposed to pH stress. When we examined the aggregation profiles of the antibody samples exposed to different incubation times at different pH values, the antibodies that were not exposed to any pH value, which was our control sample, were accepted as reference. Our control sample is stored in the formulation buffer at +4 C$^{o}$. When the aggregation profile of the control sample was examined, different particles were observed in the size of 200 nm, 300 nm, and approximately 550 nm (**Figure 4.2.4**). Since these particles are in our reference sample, they are not seen as severe aggregation. Therefore, particles with a size greater than 550 nm are considered aggregation. A 550 nm scratch (Pink region) was made in our control sample, and the particle sizes after this line were evaluated as aggregation. When samples exposed to pH stress were examined, large particles were

found in many samples. Particles of approximately 750 nm in size in pH 3-day 5 samples, around 650 nm in pH5 day 1 sample, around 750 nm in the pH5-day5 sample, and around 600 nm in pH9-day5 sample were found. The absence of any of these in our control sample indicates that these particles were formed by pH stress. However, since these aggregates are not at a severe level, they do not significantly affect the drug's effectiveness because most of the particles formed in all samples were generally observed in the particle size range of the control sample. Depending on the pH change, particles of different sizes were encountered in many samples, and aggregation formation was observed.

### 4.2.5. VEGF Binding Analysis of mAbs exposed to pH stress

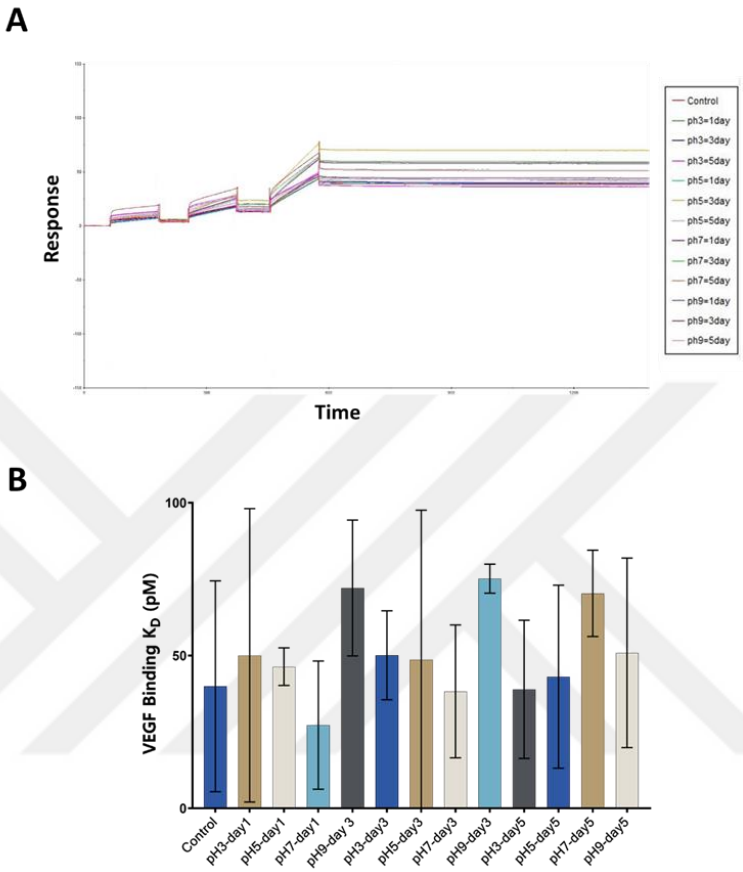



**Figure 4.3.5**: VEGF binding analysis of mAbs exposed to the pH stress by SPR. (A) Sensongram images of each sample are given. Time-dependent response units are shown in these sensorgrams. (B) the VEGF binding affinity values of each sample are plotted with standard deviations. Experiments were carried out in triplicate. For statistical analysis, single-way ANOVA was applied. Langmuir 1:1 binding model was applied.

The SPR method evaluated the binding affinities of antibodies exposed to pH stress to VEGF, the target molecule. Our control sample represents our reference sample, the anti-VEGF IgG molecule that was not exposed to pH stress. VEGF binding affinities of antibodies exposed to pH stress were statistically compared with the control sample. Sensorgrams of each sample are shown in **Figure 4.3.5A**. Binding responses to VEGF

did not change significantly over time. Sensorgram profiles are similar for each sample. In **Figure 4.3.5B**, the binding affinities in the picomolar level are given. The $K_D$ value of our control sample was found to be 39.93±28.17 pM. When the $K_D$ values of the antibodies exposed to pH stress were examined, the $K_D$ values of the antibodies exposed to pH3, pH5, pH7, and pH9 for 1 day were determined as 50.05±33.95, 46.35±4.35, 27.23±17.12, 72.1±19.22 pM, respectively. Likewise, looking at the antibodies exposed to pH stress for 3 days, the $K_D$ value of pH3 was determined as 50.1±10.3, pH5 48.6±42.3, pH7 38.2±18.82, and pH9 75.15±3.35 pM. Finally, the $K_D$ values of the antibodies exposed to pH stress for 5 days were found to be 38.9±19.5 at pH3, 43.2±24.5 at pH5, 70.3±12.21 at pH7 and 50.9±25.1 pM at pH9. When the results were examined, there was no statistically significant increase or decrease compared to the control sample.

The binding affinity of the antibodies to the target molecule is an indicator of the biological activities of the antibodies. This study has shown that changes in the pH environment did not significantly affect the binding of the antibody to the target molecule. A similar result was observed in the in vitro proliferation experiment. The binding affinity modifications must be in the CDR region where the antibody interacts with the target molecule (B. Yan, Steen, et al. 2009b; Hawe et al. 2009; Harris et al. 2001; Vlasak et al. 2009). Many studies have shown that antibodies can lose their functions due to degradation and modifications at pH changes (Alexander and Hughes 1995; B. Yan, Steen, et al. 2009b; Fesinmeyer et al. 2009). It was observed that PTMs might occur in the antibody CDR region and change the binding affinities on target molecules (Dick et al., 2010; Kroon, Baldwin-Ferro, and Lalan 1992; McKerrow 1979). However, there was no difference in both in vitro cell activity and VEGF binding assay in our study.

### 4.2.6. FcRn Binding Analysis of Anti-VEGF IgG exposed to pH stress

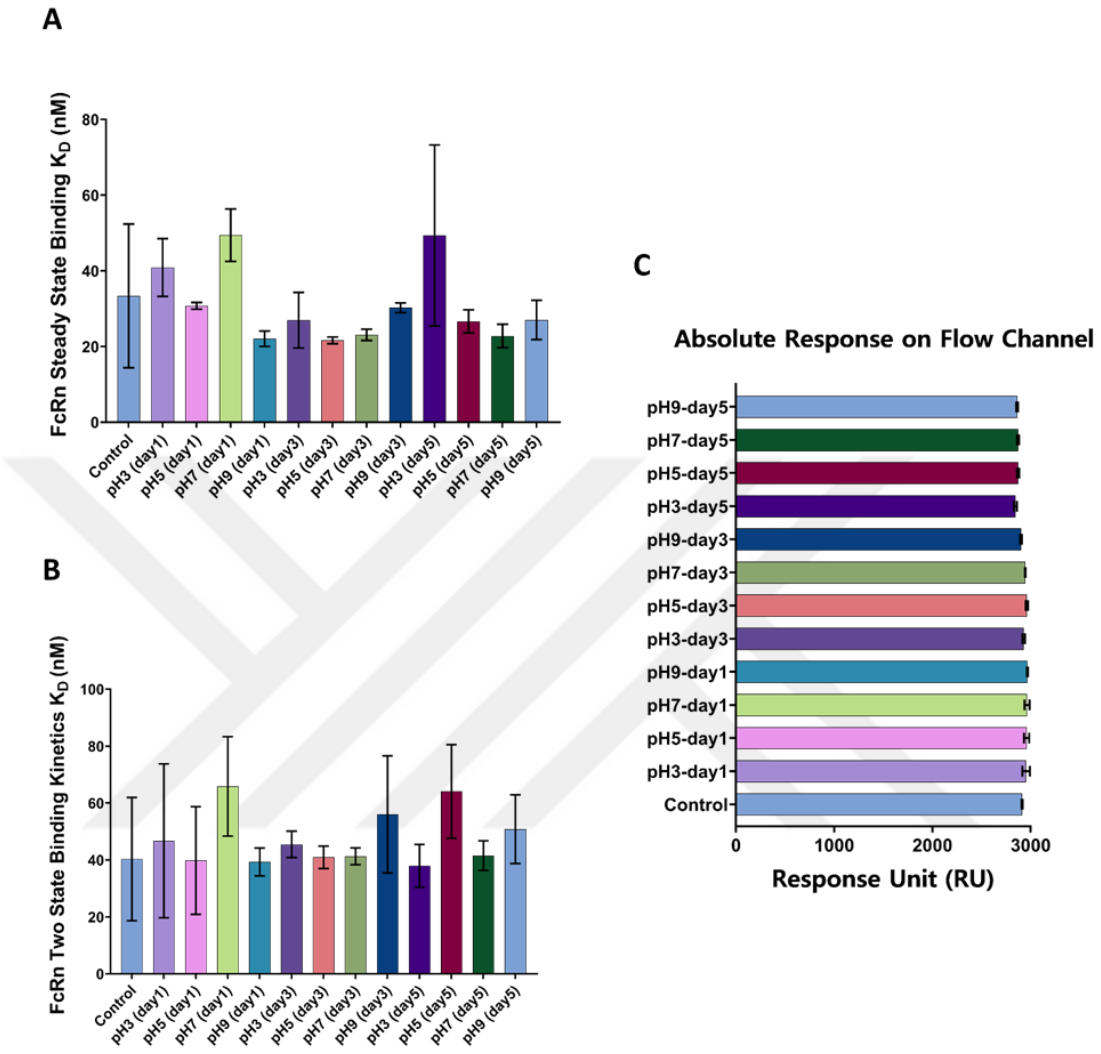



**Figure 4.2.6**: FcRn binding analysis of the samples using SPR: (A) Steady-state interaction of anti-VEGF IgG with immobilized FcRn was represented as the mean of at least three measurements. (B) Two-state binding interaction of anti-VEGF IgG with immobilized FcRn was represented as the mean of at least three measurements. (C) Absolute response values of the samples on the flow channel. There were no significant differences between the samples based on the single-way ANOVA analysis.

The SPR method was used to determine the binding affinities of antibodies exposed to pH stress with the FcRn receptor. When using the SPR method, analyses were carried out

according to the two models, steady-state binding and two-state binding kinetic models. Therefore, the binding affinity was compared with the two models and analyzed. When the results were examined according to the Steady-state binding model (**Figure 4.2.6A)**, the anti-VEGF IgG molecule that was not exposed to pH stress was accepted as a control sample. Its $K_D$ value was determined as 33.4±16.45 nM. Statistical analyzes were made by comparing the samples exposed to pH stress with the control sample. The $K_D$ values of the samples exposed to pH stress for 1 day were determined as 40.8±6.62 at pH3, 30.75±0.75 at pH5, 49.43±5.99 at pH7 and 22.05±1.76 nM at pH9. The $K_D$ values of the antibodies remaining in the 3-day incubation were measured as 26.95±6.32 nM at pH3, 21.68±0.77 at pH5, 23.08±1.23 at pH7 and 30.25±1.05 nM at pH9. Finally, the $K_D$ values of the antibodies remaining in the 5-day incubation were measured as 49.35±20.72 nM at pH3, 26.65±2.65 at pH5, 22.78±2.68 at pH7, and 27.03±4.24 nM at pH9. Compared to the control sample, there was no statistically significant increase or decrease in FcRn binding in the samples exposed to pH stress according to the steady-state model.

When the analysis was performed according to the two-state model with the same samples (**Figure 4.2.6B)**, the $K_D$ value of the anti-VEGF IgG control sample, which was not exposed to pH stress, was determined as 40.31±18.74 nM. Statistical analyzes were made by comparing the samples exposed to pH stress with the control sample. The $K_D$ values of the samples exposed to pH stress for 1 day were determined as 46.73±22.06 at pH3, 39.82±16.37 at pH5, 65.83±14.25 at pH7, and 39.29±4.25 nM at pH9. The $K_D$ values of the antibodies remaining in the 3-day incubation were measured as 45.51±4.01 at pH3, 40.95±3.40 at pH5, 41.28±2.54 at pH7 and 56.03±17.84 nM at pH9. Finally, the $K_D$ values of the antibodies remaining in the 5-day incubation were measured as 37.95±6.14 nM at pH3, 64.08±14.23 at pH5, 41.52±4.50 at pH7, and 50.83±9.88 nM at pH9. Compared to the control sample, there was no statistically significant increase or decrease in FcRn binding in the samples exposed to pH stress according to the two-state model.

In addition to these results, absolute response values of the samples were determined in the flow channel. Examining the absolute response values confirms both steady-state and two-state binding results (**Figure 4.2.6C)**. The absolute response indicated the presence of bound molecules on the chip surface; the number of molecules was high due to the FcRn interaction. Therefore, the molecule with the highest absolute response shows the best affinity. As a result, absolute response results showed similarity in all examples and proved valid for both models tested.

No results were shown in the literature on the FcRn binding of PTMs formed in the Fc region in the presence of pH stress. It was reported that some deamidation modification might be encountered in the Fc region in the presence of pH stress (Y. D. Liu, van Enk, and Flynn 2009; L. Wang et al. 2005; Chelius, Rehder, and Bondarenko 2005; Sinha et al. 2009). However, it has not been shown whether these modifications have any effect on FcRn binding. Since the effect of Met252 oxidation in the CH2 domain on FcRn binding was determined as the PTM with the most significant effect, it is accepted that other PTMs do not have much effect.

In some studies, it was observed that FcRn binding increased with increased aggregation (Bajardi-Taccioli et al., 2015; Wu et al., 2015). AlphaScreen method was used for FcRn binding in this study. Other methods were tried to understand whether the increase in FcRn binding is related to the method (Y. Lu et al., 2011; Schlothauer et al. 2013). An increase in Fcrn binding was also observed in these studies. In another study, the effect of aggregation on binding affinities with FcRn was investigated using the BLI method (Bajardi-Taccioli et al., 2015). According to the results of this study, the dissociation constant of antibodies with a high aggregation rate was slower than products with low aggregation. Another study reported that this increase was an avidity effect (Geuijen et al., 2017).

The CH2 domain has relatively lower thermal stability (Ionescu et al., 2008). Especially in low pH conditions (Martsev et al. 1995; Kamerzell, Ramsey, and Middaugh 2008). In environments with a pH lower than 3, conformational changes in the CH2 domain and its interaction with the CH3 domain weakens. Therefore, the CH2 domain loses its stability (Tischenko, Abramov, and Zav'yalov 1998). Therefore, PTMs occurring in this domain have severe effects on the stability of the CH2 domain.

### 4.2.7. C1q ELISA Binding

The c1q ELISA method was used to show how the binding affinities of anti-VEGF IgG molecules exposed to pH stress to the C1q molecule changed. While performing the C1q

ELISA experiment, the samples were incubated at different pH values and periods, and antibody concentrations of 2.5 ug/ml, 1.25 ug/ml, and 0.63 ug/ml were tested starting from 5 ug/ml. Thus, the decreasing concentration of the antibody was expected to decrease if there was an affinity for c1q binding. Assessing the results, the control sample was not exposed to pH stress, and the binding affinity to C1q protein was found to be the highest (**Figure 4.2.7A**). While it had the highest absorbance value at 5 ug/ml antibody concentration, the absorbance value, its binding to C1q, decreased as the antibody concentration decreased.

Interestingly, when the values of antibodies exposed to pH stress were examined, a significant decrease was observed compared to the control sample. Compared to the control antibody, the absorbance values of the antibodies exposed to all stress decreased, and it was observed that the C1q binding values decreased when the antibody concentrations decreased. However, c1q binding was statistically significantly decreased at each antibody concentration compared to the control sample.

When the absorbance values of the samples were examined closely at 1.25 ug/ml antibody concentration, a statistically significant decrease was observed in the C1q binding of all the antibodies exposed to pH stress compared to the control sample (**Figure 4.2.7B**).

No results were shown in the literature on the C1q binding of PTMs formed in the Fc region in the presence of Ph stress. It was reported that some deamidation modification might be encountered in the Fc region in the presence of pH stress (Y. D. Liu, van Enk, and Flynn 2009; L. Wang et al. 2005; Chelius, Rehder, and Bondarenko 2005; Sinha et al. 2009). However, it was not shown whether these modifications had any effect on C1q binding.

A study showed that CDC activity decreased due to aggregation in the presence of temperature, oxidation, and pH stress (Bansal, Dash, and Rathore 2020). Evaluating the results found in this study, 20% aggregate formation was observed in the antibody in pH and oxidative stress. As a result, it was observed that CDC activity decreased by 20%.

The literature has also observed that the aggregation rate increases with the increase of Asn deamidation in the presence of pH stress (Geuijen et al., 2017). Due to the formation

of aggregation, the CDC activity of antibodies exposed for a long time in the presence of pH stress may decrease, albeit slightly (Xu et al. 2019).

When our results were compared to the literature, no PTM in the Fc region that affected C1q binding was encountered. It is accepted as the most effective PTM oxidation, which seriously affects C1q binding. The reason for the decrease in c1q binding in our studies may be the presence of aggregates samples, causing a conformational change in the binding regions.

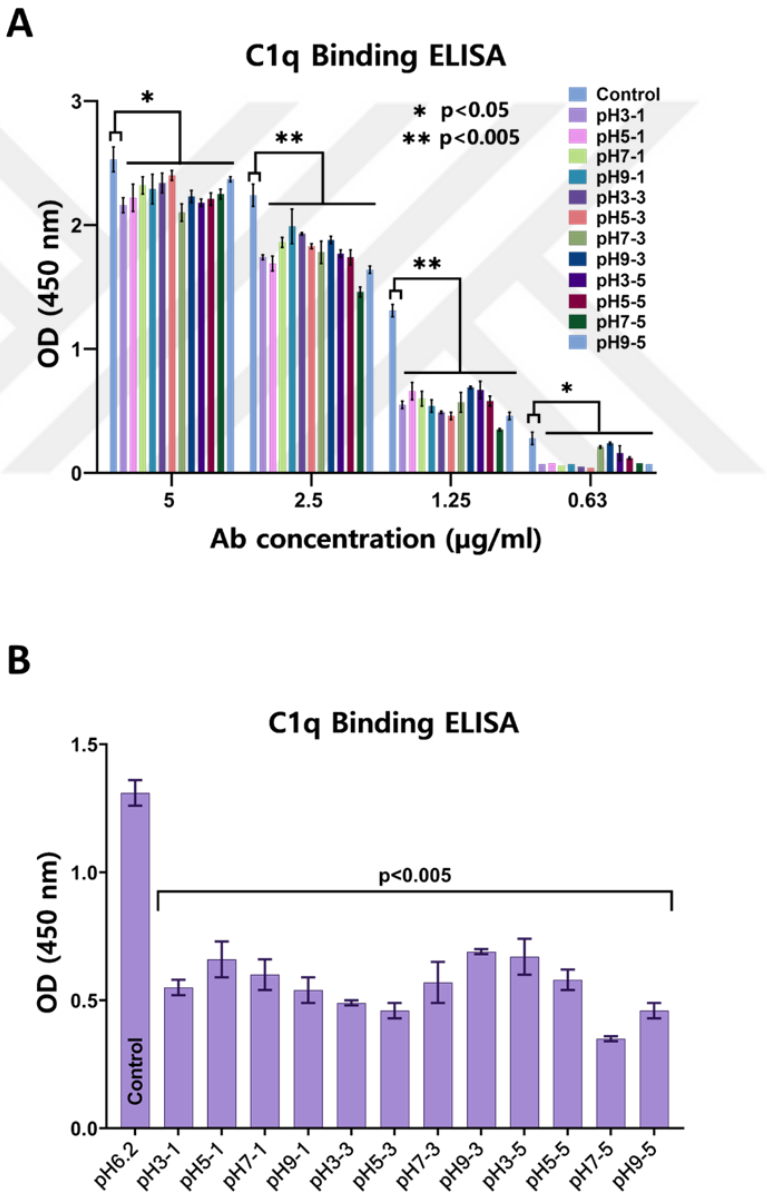



**Figure 4.2.7**: C1q ELISA binding analysis of the mAbs. (A) The results of C1q ELISA binding of pH stress expose anti-VEGF IgG at different concentrations. (B) The results

of C1q ELISA binding of pH stress expose anti-VEGF IgG at 1.25 ug/ml Ab concentration. The data represented the mean of at least three independent measurements. For statistical analysis, single-way ANOVA was applied.

## 4.3. Oxidative stress

### 4.3.1. Investigation of PTMs in mAbs exposed to various oxidation conditions by peptide mapping analysis

Firstly, the intact analysis of anti-VEGF IgG molecules exposed to oxidation stress was not shown in the results because a proper glycosyl profiling of antibodies by MS could not be elicited as a result of stress. Samples were prepared and processed by MS. However, the mass values of the glycoform structures could not be adequately defined due to the change in mass shifts in the presence of oxidation. However, as stated in the literature, the glycosyl profiles of antibodies do not change in the presence of stress factors (Pisupati et al., 2017). Therefore, we moved on with other experiments to acknowledge no change in glycosyl profiles in anti-VEGF IgGs exposed to oxidative stress.

The pep-map method was used to characterize PTMs in anti-VEGF IgG molecules in the presence of oxidative stress. For this purpose, when the literature is examined, it was seen that different percentages of $H_2O_2$ are used effectively (Nowak, K. Cheung, et al., 2017). Within this project's scope, 0.05, 0.1, 0.3, 0.5, and 1 percentage were determined starting from 0.01 % $H_2O_2$. Antibodies were incubated for 1 hour at room temperature in each percent of $H_2O_2$. Afterward, all the samples were exchanged with the formulation buffer, and the experiments were carried out.

First of all, the amino acid sequence of anti-VEGF IgG molecules was determined by pep-map analysis. As seen in **Figure 4.3.1.1A**, the obtained sequence was 100% compatible with the theoretical sequence. After the sequence was determined, the characterization of the PTMs in the peptides was performed. As a result of pep-map analysis, PTMs in all tryptic peptides were analyzed. PTM was not found in any peptide

except DTLMISR. Therefore, it was not necessary to show the table containing all peptides. The oxidation rate of the DTLMSIR peptide on the Met residue is given in **Figure 4.3.1.2A**.

When the total ion chromatography of antibodies exposed to different $H_2O_2$ percentages is examined, it is seen that they have a very similar profile. When the chromatogram is examined, the oxidation of this peptide is seen at the retention time of the DTLMISR peptide (**Figure 4.3.1.1B)**.

When the results were examined, no oxidation PTM was encountered in this peptide in the anti-VEGF IgG molecule that we used as a control, not exposed to any oxidative stress. However, there was no oxidation in our smallest $H_2O_2$ percentage, 0.01. Oxidation may not have been encountered since this percentage's $H_2O_2$ ratio was relatively low. In addition, incubation at room temperature for 1 hour may not have been sufficient in 0.01 % $H_2O_2$ (**Figure 4.3.1.2A)**.

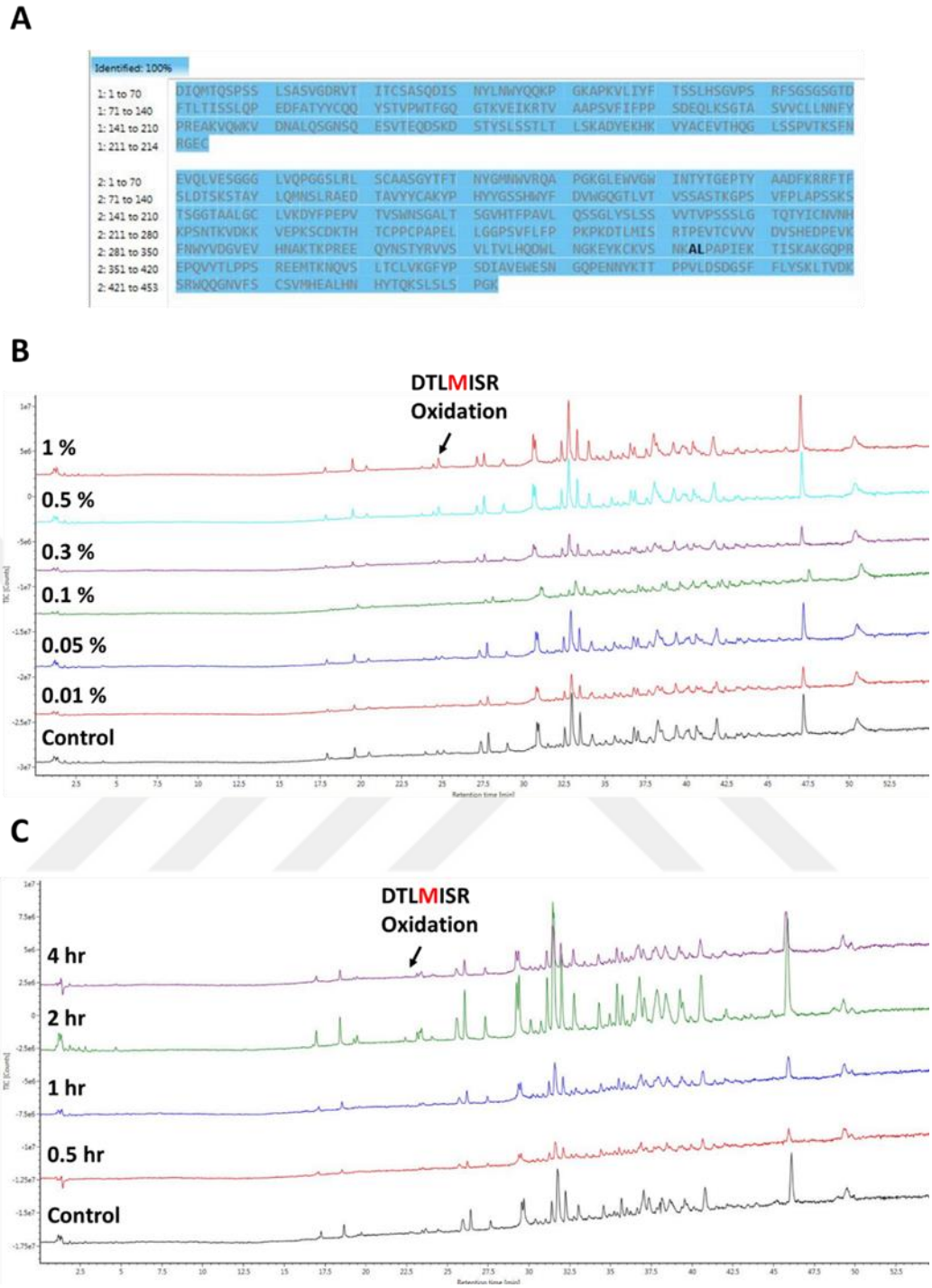



**Figure 4.3.1.1**: Peptide mapping analysis of mAbs exposing oxidative stress using MS. (A) The anti-VEGF IgG protein sequence coverage map was obtained by pep-map analysis. (B) Total ion chromatograms of mAbs exposing different percentages of $H_2O_2$. (C) Total ion chromatograms of mAbs exposing 0.1 % of $H_2O_2$ in different incubation times. The letters marked in red are the amino acids with the modification. The oxidation of the DTLMISR peptide is seen at a particular retention time.

Oxidation started to show itself at 0.05 percent $H_2O_2$ (**Figure 4.3.1.2A)**. At 0.05%, 20% of the DTLMSIR peptides were oxidized. Looking at the following percentages, Met oxidation of almost all DTLMSIR peptides occurred in 32% in 0.1% H2O2, 62% in 0.3% $H_2O_2$, 80% in 0.5% $H_2O_2$ and 99% in 1% $H_2O_2$ (**Figure 4.3.1.2A)**. Therefore, it was observed that Met oxidation increased smoothly with increasing $H_2O_2$ percentage in DTLMISR. In addition, oxidation of peptides was examined in all $H_2O_2$ percentages since Trp, and Met residues are the most common in antibodies. This oxidation was observed only in DTLMISR in our case.

In addition to these studies, antibodies were incubated in 0.1% $H_2O_2$ for 0.5, 1, 2, and 4 hours at room temperature to investigate the effect of incubation time on oxidation at a certain percentage of hydrogen peroxide. In ta previous study, we had observed that approximately 32% of the DTLMSIR peptide was oxidized when we incubated for one hour in the presence of 0.1%$H_2O_2$. Therefore, this ratio was chosen considering that 0.1 percent $H_2O_2$ antibody is ideal for showing the impact of oxidation stress in the samples.

When the TIC of antibodies exposed to different incubation times in 0.1% $H_2O_2$ was examined, it was seen that they had a very similar profile. When the chromatogram was examined, the oxidation of this peptide was seen at the retention time of the DTLMISR peptide (**Figure 4.3.1.1C)**.

When the results were examined, it was observed that Met oxidation increased only in DTLMISR with the increase of the incubation time, just as with the increase in percent concentration (**Figure 4.3.1.2B)**. When all peptides were examined again, we observed that DTLMISR was the only peptide that underwent oxidation compared to the control sample. Approximately 23% of DTLMSIR peptide oxidation was observed in 0.5h incubation, compared to 31% at 1 hour, 42% at two hours, and 64% at 4 hours. Therefore, it was shown in this study that oxidation can increase depending on the waiting time. In addition, 31% DTLMISR oxidation showed that the MS protocol developed performed well.

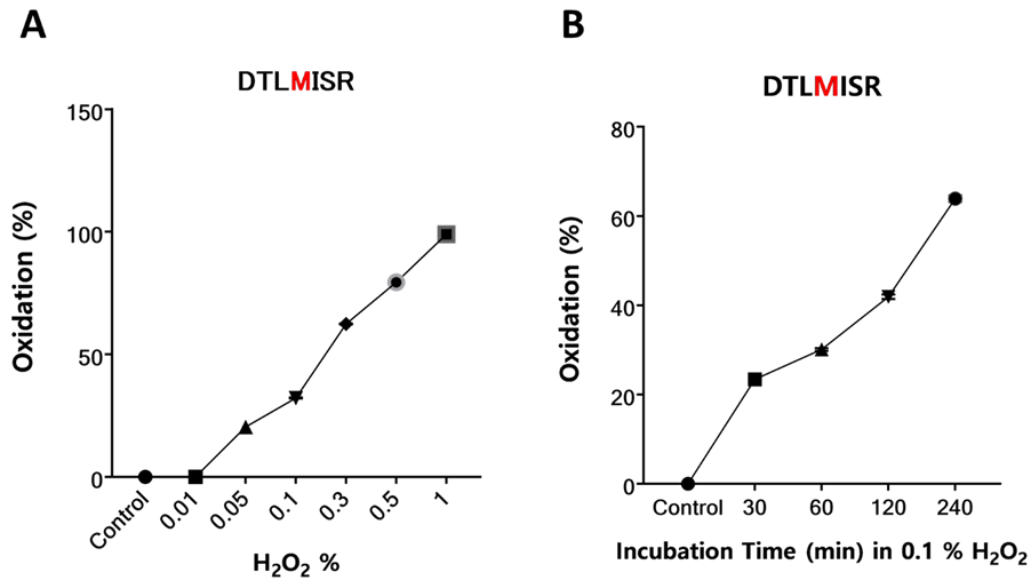



**Figure 4.3.1.2**: Oxidation rate on DTLMISR peptide according to different percentages of $H_2O_2$ (A). Oxidation rate on DTLMISR peptide according to different incubation times in 0.1% $H_2O_2$ (B) The letters marked in red are the amino acids with the modification. The percentage values were calculated by averaging their separate injections.

When we examined the literature, it was stated that the Met252 residue in the CH2 domain of the antibody, which means Met in the DTLMISR, and the Met428 residue in the SVMHEA peptide in the CH3 domain are the amino acids most sensitive to oxidation (Chumsae et al. 2007; H. Liu, Gaza-Bulseco, Xiang, et al. 2008a).

In addition to the fact that the Fc regions of the antibodies are susceptible to oxidation, oxidation can also occur, especially in the CDR region (Roberts et al. 1995; Shen et al. 1996). For example, in the case of the orthoclone OKT3 antibody, it was shown that the Met34 residue in the CDR region is oxidized in the presence of oxidative stress (Kroon, Baldwin-Ferro, and Lalan 1992). As a result, the biological activity of the antibody was affected. In these studies, it was shown that the most oxidized region was the Met252

residue. Therefore, Met252 is the primary antibody oxidation site (Lam, Yang, and Cleland 1997). However, in our studies, no oxidation was found in the CDR regions.

Therefore, in the presence of oxidative stress, anti-VEGF IgG undergoes oxidation from the Fc region. This oxidation rate increases as the percentage of $H_2O_2$ increases and the incubation time increases. Subsequent experiments were to investigate whether this oxidation had any effect on the function of the antibody.

### 4.3.2. Investigation of Cell Proliferation Performances of mAbs exposed to $H_2O_2$

The first experiment to investigate whether there is any effect on the biological activity of the anti-VEGF IgG molecule in the presence of oxidative stress was to show a proliferation effect on HUVEC cells. Because HUVEC cells carry a high degree of VEGF receptor on their surface, they can generate a proliferation signal in the presence of VEGF cytokine, and the cells begin to grow. Therefore, anti-VEGF IgGs that bind to the VEGF molecule in the medium prevent this interaction, and cell growth slows down. Therefore, the effect of antibodies exposed to oxidative stress on proliferation by binding to VEGF molecule was investigated.

For this purpose, it was decided to use 0.1% and 0.5% $H_2O_2$, and antibodies were incubated at these percentages at room temperature. Therefore, it was desired to investigate cell proliferation by using two different percentages. The growth rates of cells treated with VEGF and oxidized antibodies were determined by measuring the absorbance values by the MTS method.

The Only VEGF sample represents cells with no antibodies added and only VEGF cytokine added. Since there is no antibody binding to VEGF in this sample, we see the proliferation effect of VEGF at maximum. Considering the results, the only VEGF sample showed the maximum OD value, representing the maximum cell proliferation (**Figure 4.3.2**).

In the control sample, the cells were treated with anti-VEGF IgG molecules that were not exposed to oxidative stress. Here, the molecule was in its natural therapeutic form. Therefore, proliferation was restricted with this sample, and thus, the method's accuracy

was proven. In addition, the effect of anti-VEGF IgGs exposed to oxidative stress on cell proliferation was determined by comparing them with both control and only VEGF samples.

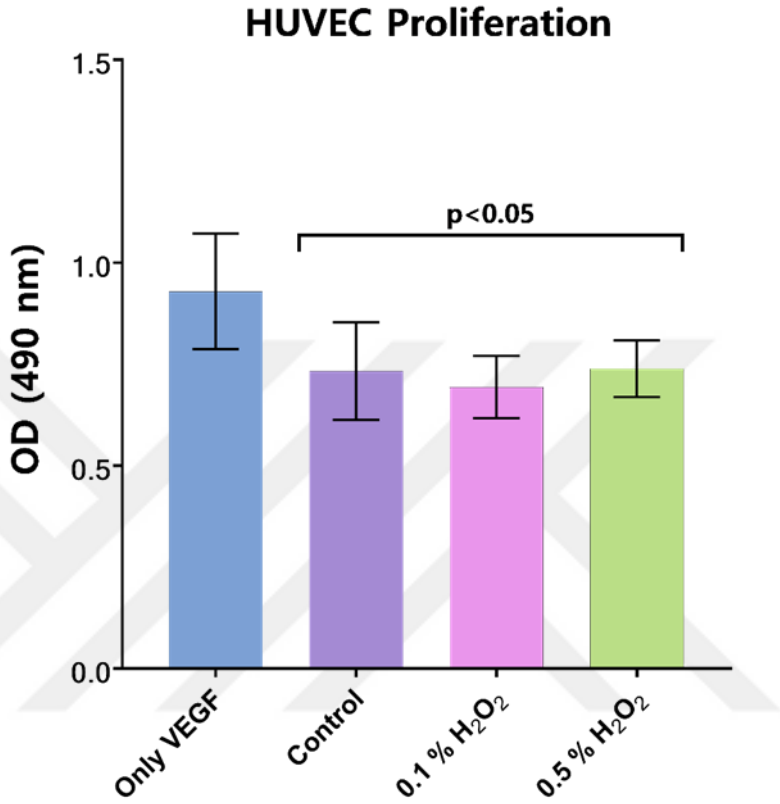



**Figure 4.3.2**: Cell proliferation effects of mAbs expose the $H_2O_2$ by MTS. Absorbance values were measured at a wavelength of 490 nm by a microplate reader. Experiments were carried out in triplicate. To interpret the results statistically, ANOVA was applied

When the results were examined, it was observed that only VEGF had the highest absorbance value among the samples, and the sample with the highest cell proliferation was observed in **Figure 4.3.2**. In addition, the control sample gave a low absorbance value by inhibiting cell proliferation. When the cells treated with antibodies exposed to 0.1% and 0.5% $H_2O_2$ were examined, it was observed that it affected cell proliferation just as much as the control sample compared to the only VEGF sample. In addition, it was observed that increasing the oxidative stress surface did not have a severe effect on

proliferation. Therefore, considering the statistical values of the results, it was found that antibodies exposed to oxidative stress did not affect cell proliferation.

As can be understood from the MS results, there was no inhibition of VEGF binding since Met252 was oxidized only in the Fc part of the antibody, and there was no modification in the CDR region. The literature also shows that increased oxidation in Met252 did not lose the ability of the antibody to bind and neutralize VEGF (D. Park et al. 2020). Our results confirmed this and showed that increased oxidation in Met252 did not affect VEGF neutralization.

### 4.3.3. C1q ELISA Binding

ELISA method was used to reveal the interaction of anti-VEGF IgGs exposed to oxidative stress with c1q protein. It was used starting from 5 µg/ml antibody concentration and up to 0.6 µl/ml with 1:2 dilution, and c1q binding at decreasing concentration was determined by measuring the absorbance value. When the C1q binding affinities of antibodies treated with specific percentages of $H_2O_2$ for 1 hour at room temperature were examined, it was observed that c1q binding decreased in all samples with the decrease of antibody concentration (**Figure 4.3.3.1A**). Anti-VEGF IgG molecule that was not exposed to $H_2O_2$ was used as a control sample, and samples exposed to oxidative stress were evaluated according to this reference. Antibodies exposed to all percentages of $H_2O_2$ had decreased c1q binding affinities relative to the reference antibody at all antibody concentrations. The control sample showed maximum c1q binding absorbance, while all other samples showed a statistically significant decrease compared to the control.

Evaluating the antibody concentration values of only 1.25 ug/ml, it was seen that the affinity of antibodies exposed to all $H_2O_2$ percentages to C1q decreased significantly compared to the control (**Figure 4.3.3.1B)**.

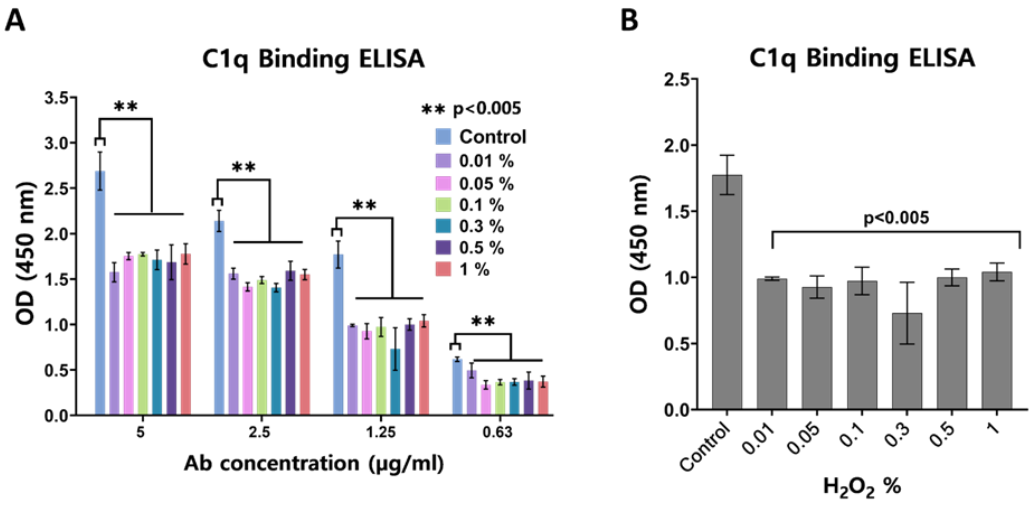



**Figure 4.3.3.1**: C1q ELISA binding analysis of the mAbs exposed to different $H_2O_2$ percentages. (A) The results of C1q ELISA binding of oxidative stress expose anti-VEGF IgG at different Ab concentrations. (B) The results of C1q ELISA binding of oxidative stress expose anti-VEGF IgG at 1.25 ug/ml Ab concentration. The data represented the mean of at least three independent measurements. For statistical analysis, single-way ANOVA was applied.

However, when samples incubated with 0.1% $H_2O_2$ at room temperature for specific times were examined, even half an hour of exposure caused a significant decrease in the C1q binding affinity of the antibodies (**Figure 4.3.3.2A**). Here, it was observed that C1q binding decreased with decreasing antibody concentration. As a result of each determined incubation, C1q binding affinity decreased drastically. Again, if we examine the 1.25 ug/ml antibody concentration example, it is seen that the decrease of c1q binding affinity compared to the control occurs effectively (**Figure 4.3.3.2B)**.

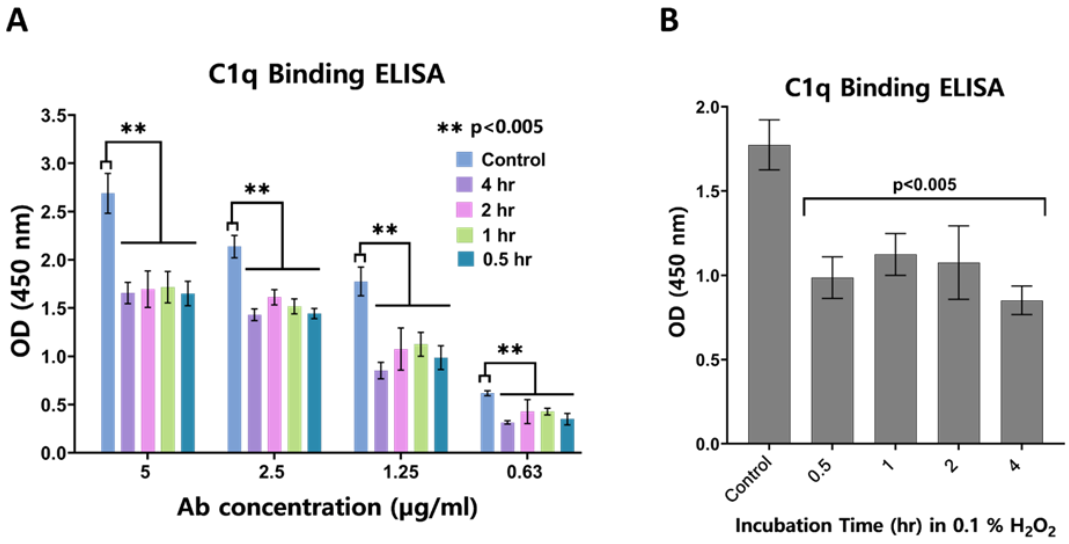



**Figure 4.3.3.2**: C1q ELISA binding analysis of the mAbs exposed to 0.1 % H2O2 at different incubation times. (A) The results of C1q ELISA binding of oxidative stress expose anti-VEGF IgG at different Ab concentrations. (B) The results of C1q ELISA binding of oxidative stress expose anti-VEGF IgG at 1.25 ug/ml Ab concentration. The data represented the mean of at least three independent measurements. For statistical analysis, single-way ANOVA was applied.

As a result, the C1q binding affinity of antibodies exposed to both specific percentages of $H_2O_2$ and the same percentage of $H_2O_2$ at different times decreased. When the literature is examined, since C1q interacts with the CH2 domain in the Fc part of the antibody, the PTMs that will form here may affect the C1q interaction (Ambrogelly et al. 2018). Studies show that Met252 oxidation in the Fc part reduces C1q binding affinity and directly decreases CDC activity (S. Wang et al. 2016). With the oxidation of the CH2-CH3 interface region, a conformational change occurs in this region, affecting the binding of C1q. Our pep-map data observed that Met252 was oxidized in the DTLMSIR peptide. Oxidation of the Met252 residue in the Fc region affects C1q binding in our studies. However, the decrease in c1q binding affinity did not increase proportionally with the increase of hydrogen peroxide concentration, and even at 0.01%, a decrease of 1% was observed. But we observed a decrease in c1q binding affinity due to oxidation stress. Both

pep-map and C1q ELISA experiments were consistent with the results stated in the literature.

### 4.3.4. FcRn Binding Analysis of anti-VEGF IgG exposed to Oxidation

When the literature is examined, it was shown that oxidative stress has severe effects on mAbs. In this stress, especially Met and Trp, undergo oxidation, adverse effects can be observed (Lam et al., 2011; S. Wang et al. 2016). Met 252 and Met 428 in the Fc part of the antibody are the two most oxidized residues. Their oxidation reduces the interaction of FcRn with IgG (Bertolotti-Ciarlet et al., 2009a; Weirong Wang et al., 2011). The risk of aggregation increases by changing the thermal stability of the Fc part of the antibody with the oxidation of Met252 and met428 (Gaza-Bulseco, Faldu, et al. 2008b). In addition to decreasing its binding affinity for FcRn, its binding affinity for protein a and protein G also decreases (Bertolotti-Ciarlet et al. 2009a; H. Liu, Gaza-Bulseco, Faldu, et al. 2008). Due to the decreased affinity of antibodies for FcRn, their half-life in serum is also reduced (Weirong Wang et al., 2011). In addition, its adverse effects, such as rapid clearance in serum caused by the oxidation of the Fc region, were demonstrated *in vivo* experiments (Stracke et al., 2014; Suzuki et al., 2010).

Met252 and Met 428 residues are also present at the CH2 and CH3 interfaces in the Fc part. When the crystal structure of the Fc region and its interaction with FcRn is examined, this interaction occurs in the CH2-CH3 interface region (Martin et al. 2001). In mouse experiments, it was shown that when 79% of Met252 and 57% of Met428 are oxidized, the serum half-life of antibodies decreases by approximately 20% (Weirong Wang et al., 2011). In addition, when the oxidation of met252 and met428 was evaluated separately, it was observed that the clearance of the antibody in serum increased more than the oxidation of met428 in M252 oxidation (Xu et al. 2019). Therefore, the oxidation of met252 is the oxidant with the most harmful effect among these residues.

This study used the SPR method to determine the binding affinities of anti-VEGF IgGs exposed to oxidative stress to FcRn. To determine the affinity, $K_D$ values were determined with two different models, steady-state affinity and two-state binding. First of all, when we look at the steady-state results of FcRn binding affinity at different $H_2O_2$ percentages,

anti-VEGF IgG, our control sample, which has not been exposed to any stress, has the lowest $K_D$ value, and its value was calculated as 13.15±0.36 nM (**Figure 4.3.4.1A**). So its interaction with FcRn appears to be quite good. Interestingly, it was observed that the $K_D$ value increased as the percentage of $H_2O_2$ increased. Therefore, the binding affinity of FcRn statistically decreased significantly. Considering the $K_D$ values, 19.9±1.06 at 0.01% $H_2O_2$, 23.48±3.79 at 0.05%, 24.05±3.62 at 0.1%, 55.78±8.74 at 0.3%, 52.13±12.01 at 0.5% and 70.01±1.8 nM at 1%, $K_D$ values were calculated. Therefore, the $K_D$ value increases with the increase in the percentage of $H_2O_2$ compared to the steady-state model, and thus the binding affinity for FcRn decreases. When the same results were examined according to the two-state model, no statistically significant results were observed (**Figure 4.3.4.1B)**. According to this model, the $K_D$ value of each sample is almost equal to each other. Considering the $K_D$ values, 24.31±1.77 for control, 24.17±14.21 at 0.01% $H_2O_2$, 30.31±9.77 at 0.05%, 31.62±9.69 at 0.1%, 65.29±13.80 at 0.3%, 50.71±24.64 at 0.5% $K_D$ values were calculated. In addition, the increase in the $K_D$ value with the increase in the percentage we see in the steady-state does not occur in this model. Therefore, the steady-state model is seen as a suitable model for our experiments.

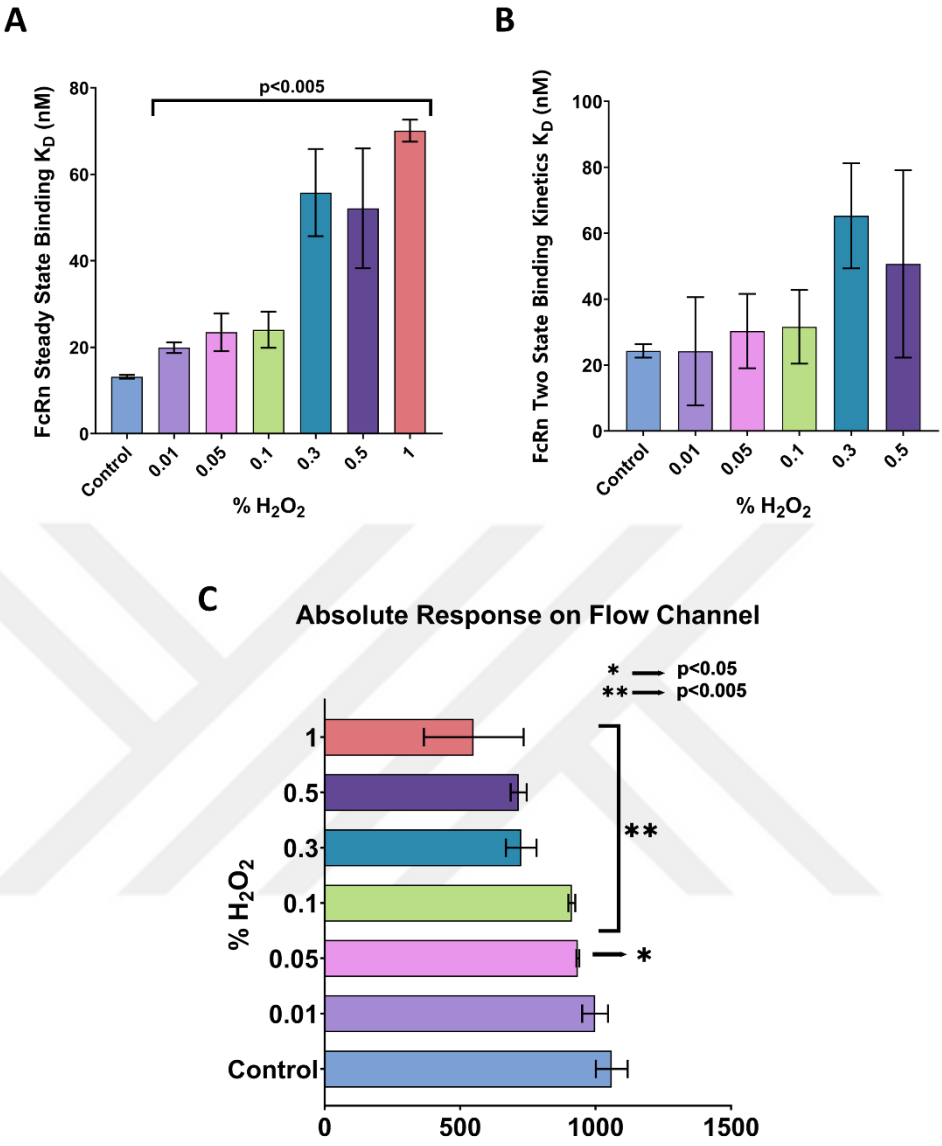



**Figure 4.3.4.1**: FcRn binding analysis of the oxidized samples using SPR: (A) Steady-state interaction of anti-VEGF IgG with immobilized FcRn was represented as the mean of at least three measurements. (B) Two-state binding interaction of anti-VEGF IgG with immobilized FcRn was represented as the mean of at least three measurements. (C) Absolute response values of the samples on the flow channel. For statistical analysis, the single-way ANOVA analysis was applied.

However, the FcRn binding affinity of antibodies incubated in 0.1% $H_2O_2$ at room temperature at different times was also investigated using two models. First of all, it was observed that our control sample showed the highest binding affinity with the lowest $K_D$ value in the steady-state, and this value was determined as 13.15±0.36 mM (**Figure 4.3.4.2A)**. The $K_D$ of FcRn binding affinity of antibodies exposed to 0.1% $H_2O_2$ for half an hour was 44.3±4.86, 30.65±11.70 for 1 hour, 31.45±7.69 for 2 hours, and 30.25±2.26 mM for 4 hours incubation. Therefore, the FcRn binding affinities of the oxidized samples showed a statistically significant decrease compared to the control sample. And again, when the two-state model is evaluated, we cannot correctly see the low affinity in the steady-state (**Figure 4.3.4.2B)**. The control $K_D$ 24.13±1.77 nM. The $K_D$ of FcRn binding affinity of antibodies exposed to 0.1% $H_2O_2$ for half an hour was 186.79±87.77, 63.14±14.45 for 1 hour, 31.38±8.59 for 2 hours, and 30.60±3.5 mM for 4 hours incubation. There is a slight increase in the Kd value, but this is not statistically significant.

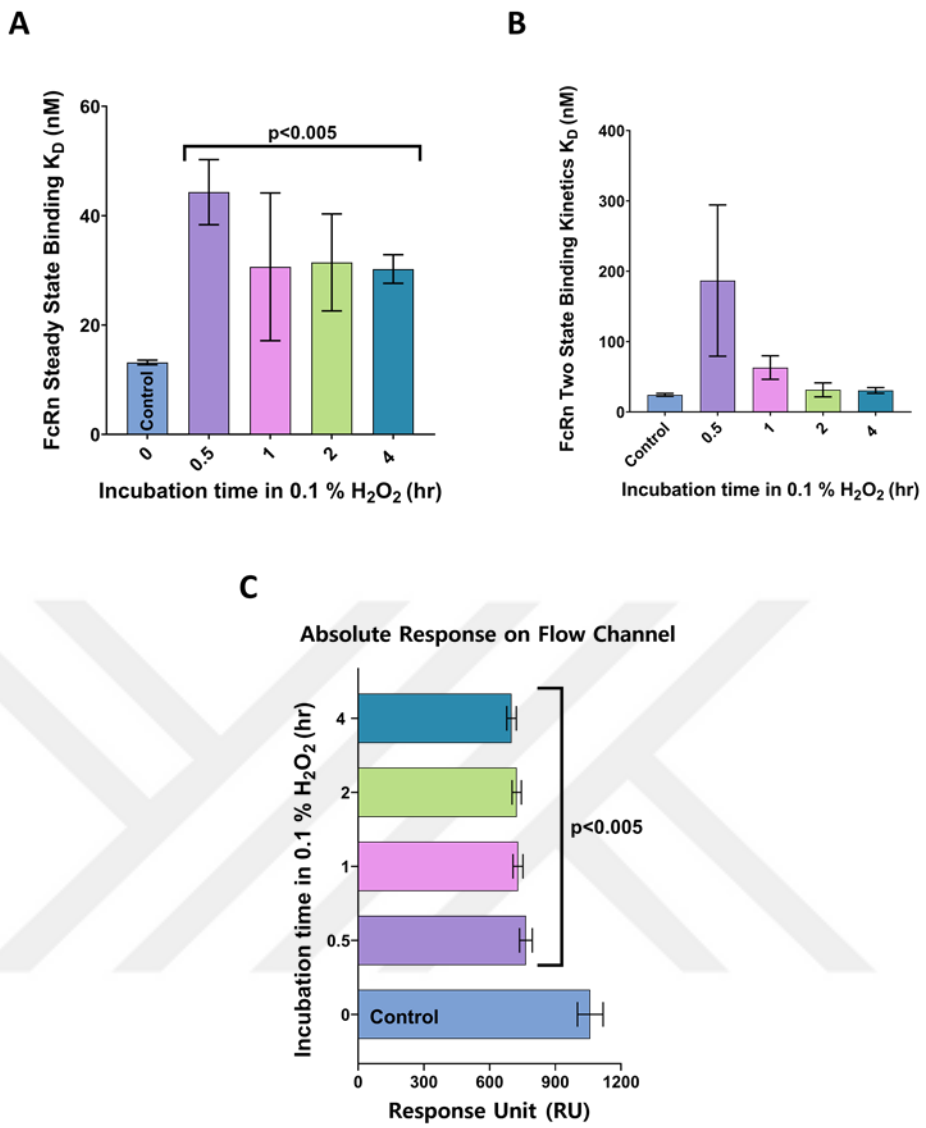



**Figure 4.3.4.2**: FcRn binding analysis of the oxidized samples using SPR: (A) Steady-state interaction of anti-VEGF IgG with immobilized FcRn was represented as the mean of at least three measurements. (B) Two-state binding interaction of anti-VEGF IgG with immobilized FcRn was represented as the mean of at least three measurements. (C) Absolute response values of the samples on the flow channel. For statistical analysis, the single-way ANOVA analysis was applied.

In addition to these results, absolute response values of the samples were determined in the flow channel. When the absolute response values were examined, it was confirmed the steady-state binding interaction results. When looking at different $H_2O_2$ values, the control example showed the highest absolute response value (**Figure 4.3.4.1C)**. This

indicates that there was a very high number of bound molecules on the chip surface; the number of molecules was high due to high FcRn interaction. Therefore, the sample with the highest absolute response showed the best affinity. The absolute response rate decreased with the increase in the percentage rates, and therefore the FcRn binding affinity also decreased. The same results were obtained in the samples that were incubated at different times. The absolute responses of the samples exposed to oxidative stress decreased compared to the control sample (**Figure 4.3.4.2C)**. Therefore, their FcRn Binding affinity was reduced. As a result, the absolute response results confirmed the results we obtained in the steady-state binding model.

As a result, Met252 oxidation, which we saw in the peptide mapping study, decreased FcRn binding affinity. These results confirm the data in the literature. In addition, only Met252 oxidation can harm FcRn binding alone.

### 4.3.5. VEGF Binding Analysis

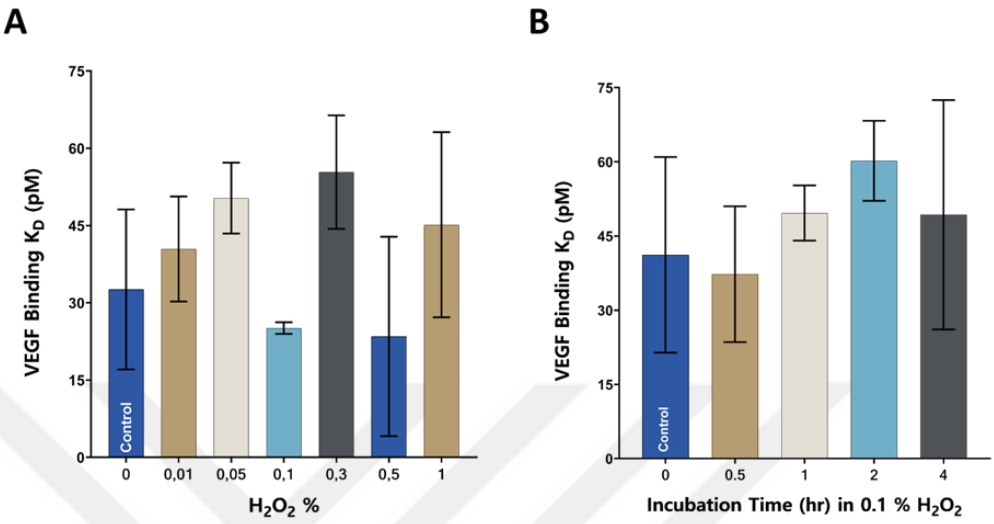



**Figure 4.3.5**: VEGF Binding Analysis of mABS exposed to oxidative stress by SPR. (A) The VEGF binding affinity values of mAbs exposed to different % $H_2O_2$ (B) The VEGF binding affinity values of mAbs exposed to 0.1 % $H_2O_2$ in different incubation times. Experiments were carried out in triplicate. To interpret the results statistically, an ANOVA test was applied. Langmuir 1:1 binding model was used.

Oxidative stress can have severe effects on the stability and function of mAbs. The negative effect of oxidation on the activity of the antibody may vary depending on which region of the antibody it is. In particular, the oxidation that may occur in the CDR regions that enable the antibody to show the ability to recognize the target molecule can significantly affect the activity of the antibody. When the literature was examined, it was observed that methionine oxidation in the CDR3 region decreased the target molecule binding capacity as a result of a study (Burkitt et al., 2017). In addition, in another study, it was observed that methionine in the CDR region was not oxidized in the presence of oxidative stress, and thus, the activity did not decrease (Shen et al. 1996). Suppose the amino acid Trp, which tends to oxidize, is localized in the CDR region. In that case, it may undergo oxidation when exposed to oxidative stress and cause low activation. A

study determined that the oxidation of Trp in the CDR3 region could seriously reduce the binding of the antibody to the target molecule. As a result, the antibody's biological activity decreased (Wei et al., 2007). Another study showed that Trp in the CDR1 region of the recombinant IgG1 molecule is very sensitive to oxidation (Hensel et al. 2011). As can be seen from the results, Met oxidation is less in the CDR regions and more in the Fc region (Jarasch et al., 2015). Therefore, the oxidation of Trp in the CDR regions is higher than that of Met amino acids. As a result, it was shown that biological activity was more effective (Y. Yan et al. 2016; Pavon et al. 2016). Therefore, although there are few studies on the oxidation of the CDR region, the target molecule binding property of the antibody may change as a result of this event (Hensel et al., 2011; Y. Yan et al. 2016; D. Boyd, Kaschak, and Yan 2011).

In this thesis, the SPR method was used to determine the affinities of anti-VEGF-IgG molecules exposed to oxidative stress to VEGF, the target molecule. First, VEGF binding analyzes of antibodies exposed to different $H_2O_2$ percentages were performed (**Figure 4.3.5A**). The $K_D$ value of our control sample, which was not exposed to any oxidative stress, was determined as 32.60±12.69 pM. At 0.01% H2O2 this value is 40.45±8.84, at 0.05% 50.33±5.6, at 0.1% 25.1±0.8, at 0.3% 55.36±8.9, at 0.5% 23.47±15.8 and finally at 1% $H_2O_2$ 45.16±14.68 pM. When the results were interpreted statistically, there was no significant increase or decrease compared to the control sample.

In addition, the binding affinities of antibodies to VEGF, which were incubated at different incubation times in 0.1% $H_2O_2$, were also evaluated (**Figure 4.3.5B**). The $K_D$ value of our control sample was determined as 41.2±17.2 pM. The $K_D$ value of the antibodies exposed to oxidation for half an hour was determined as 37.33±11.20, 49.6±4.5 in 1-hour incubation, 60.2±10.75 in 2 hours incubation, and 49.3±20.07 pM in 4 hours incubation. Just as we saw with antibodies exposed to different percentages of $H_2O_2$, the affinities for VEGF of antibodies exposed to oxidative stress at different incubation times were not statistically changed compared to the control sample.

When our pep-map results were examined, it was analyzed that antibodies exposed to oxidative stress could oxidize only the Met252 residue in the Fc region. No oxidation or other modification of the CDR regions took place. Therefore, as stated in the literature, oxidation that does not occur in the CDR regions does not have any negative consequences on the binding affinity of the antibody, the target molecule. Our results

confirmed the absence of this affinity loss in parallel with the absence of PTM formation in the CDR.

### 4.3.6. Aggregation profiles of Oxidized mAbs

In the presence of many stress factors, antibodies can enter the aggregation and fragmentation process. One of these factors is oxidative stress. Oxidation can trigger the formation of insoluble or soluble aggregates by creating conformational changes in the mAbs (Bansal, Dash, and Rathore 2020). The oxidation of Met and Trp amino acids causes aggregation by changing the stability and structure of mAbs (Wei Wang, Nema, and Teagarden 2010). Oxidation also causes fragmentation of the mAbs (Bansal, Dash, and Rathore 2020). Remarkably, this fragmentation in the hinge region has adverse effects on the stability and activity of the antibody (Glover et al., 2015).

In this study, aggregation profiles of anti-VEGF IgGs in the presence of oxidative stress were analyzed by the NTA method. First, the aggregation profiles of antibodies exposed to different $H_2O_2$ percentages were examined (**Figure 4.3.6.1**). The aggregation profile in control antibody samples that were not exposed to any stress was taken as a reference when looking at these profiles. Control samples are in formulation buffer and contain particles of different sizes. The control sample showed particles around 200 nm, 300 nm, and 550 nm. After 600 nm, the control sample does not contain any particles. Therefore, particles with a size greater than 600 nm indicate the formation of aggregates. When all samples were examined by drawing a line (Pink region) after 600 nm, aggregation formation was observed in some samples. Particles larger than 600 nm were found in the samples exposed to 0.1%, 0.3%, 0.5% and 1% $H_2O_2$. Therefore, aggregations are formed by oxidation stress. However, these aggregation profiles are insufficient to reduce the drug's effectiveness.

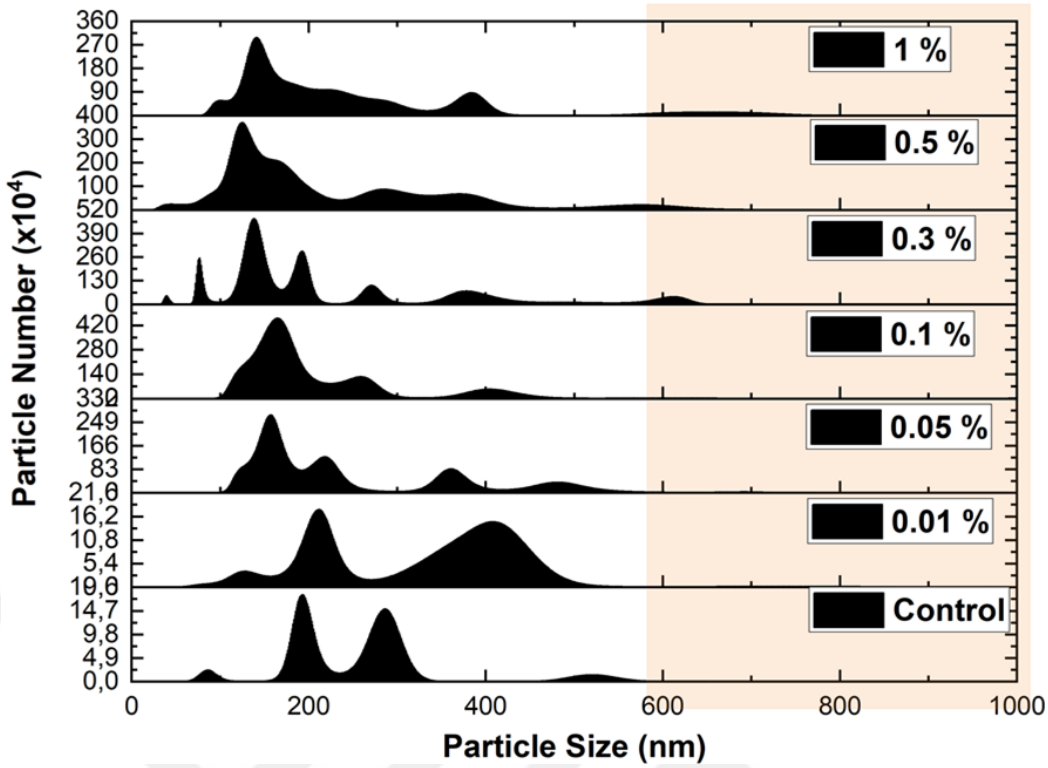



**Figure 4.3.6.1**: Comparative analysis of aggregation profiles in oxidized mAbs. The samples were prepared in 250 ug protein in 1500 ul PBS-P buffer. Particle movements in all samples were monitored for 1 minute to determine their size and numbers.

Likewise, aggregation profiles of our antibody samples exposed to 0.1% $H_2O_2$ at different incubation times were also examined (**Figure 4.3.6.2)**. Again, our control sample was accepted as a reference, and particles larger than 600 nm were followed. Interestingly, no particles larger than 600 nm were detected at each incubation time. All samples exhibited particles within the particle size range of the control sample. In the previous experiment, no aggregation was observed in the samples exposed to 0.1% $H_2O_2$. Maybe this percentage value cannot trigger the formation of aggregation. In addition, aggregation may occur if an incubation time of more than 4 hours is used.

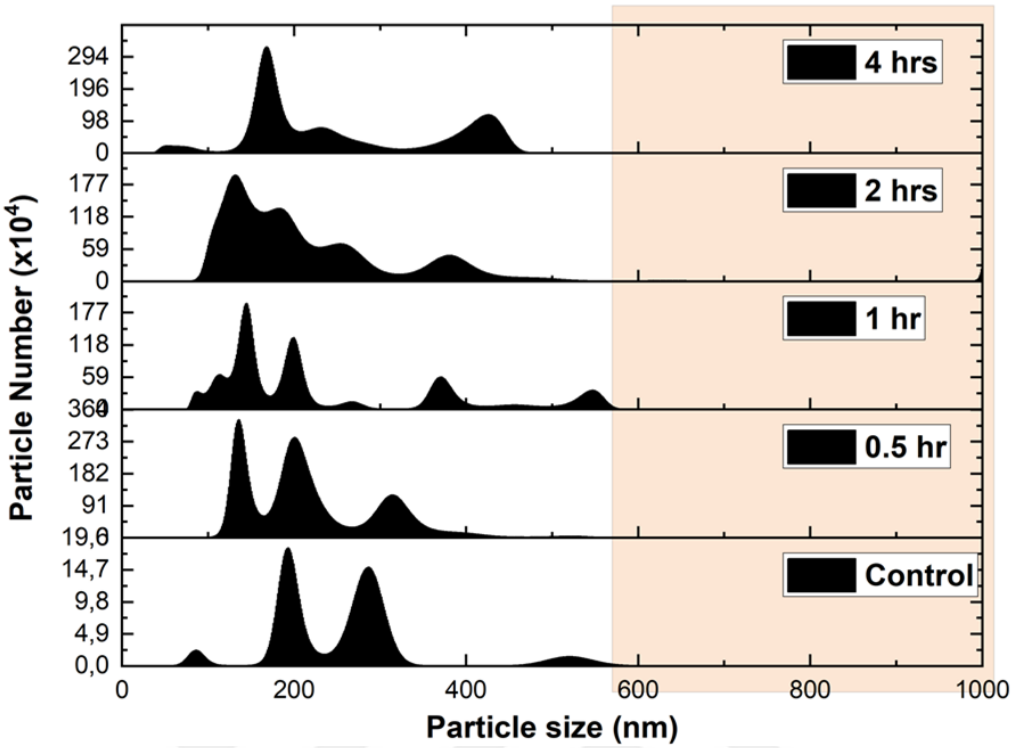



**Figure 4.3.6.2**: Comparative analysis of aggregation profiles in oxidized mAbs related incubation time. The samples were prepared in 250 ug protein in 1500 ul PBS-P buffer. Particle movements in all samples were monitored for 1 minute to determine their size and numbers.

As a result, in the presence of oxidative stress, the formation of large particles was observed in our samples compared to the control sample. Therefore, it was understood that oxidation causes changes in the conformational structure of the antibody.

# 5. CONCLUSION

The production of recombinant mAbs has gained a prominent place in the pharmaceutical industry (T Shantha Raju 2010). However, the production and post-production processes of such a critical drug molecule are very demanding. Since antibodies have a protein structure, they undergo structural changes by being affected by many environmental factors. Especially during and after production, it is exposed to many environmental stress factors causing either structural or functional degradation. Forced degradation studies aim to provide these environmental stress factors in the laboratory environment, and all stability studies of the antibody can be performed in a shorter time period (Nowak, K. Cheung, et al. 2017). These studies are required by authorities such as the FDA to demonstrate the therapeutic product's stability, structure, and biological activity.

In this study, we investigated the structural and functional changes in anti-VEGF IgG molecules in response to various temperatures, pH, and oxidation conditions. Although the structural alterations in mAbs upon environmental stress conditions have been well reported in the literature, many of those were based on only MS studies, constituting only a small portion of the required quality assessment criteria. In this thesis, many functional studies such as antigen-binding capacity, receptor binding performance, indirect CDC activity, aggregate formation, and cell proliferation monitoring studies were performed to provide comprehensive data about the model mAb exposed to the three most important environmental factors stress conditions.

Antibodies are protein structures and large complex structures formed by adding carbohydrate molecules. The addition of carbohydrates to antibodies is called glycosylation. This event is responsible for maintaining the antibody's stability and

performing effector functions such as ADCC and CDC (T. Shantha Raju 2008). Because antibody molecules bind to the target molecule, neutralize it, and coordinate with other immune system elements. Therefore, we need to examine the change of the glycosyl profile of the antibody in the presence of the stress factors that we determined. The anti-VEGF IgG molecule was glycosylated at the Asn303 position in the Fc region (Seo et al. 2018). We determined three dominant glycosyl profiles in the anti-VEGF IgG molecule (Seo et al. 2018) that were G0F N(2)-K(2), G0F N(1) G1F N(1)-K, and G0 N(1) G0F N(1). But our dominant glycosyl profile was G0F N(2)-K(2). And this accounts for about 40% of all glycosyl profiles. It was observed in these studies that the main glycosyl profile of the antibody was G0F N(2)-K(2) at all temperatures determined in the presence of thermal stress. However, when the temperatures reached extreme conditions, we could not detect some glycosyl profiles due to aggregation in the antibody. The main glycosyl profile was again defined as G0F N(2)-K(2) in antibodies incubated at 55 °C for both 3 days and 6 days. However, our other dominant glycosyl profiles, G0F N(1) G1F N(1)-K, and G0 N(1) G0F N(1), could not be defined at this temperature because many glycoform structures could not be defined due to aggregation. In the literature, Many studies have shown that protein-structured antibodies undergo aggregation and fragmentation at high temperatures (Shabestari, Mostafavi, and Malekzadeh 2018). As a result, the glycosyl profile of the antibody did not change when many different temperatures and incubation times were evaluated.

When the glycosyl profiles of the antibodies exposed on different days at different pH values were examined, three dominant glycosyl forms were detected again. Therefore, the glycosyl profile did not change again. Finally, when we looked at the glycosyl profile of antibodies exposed to oxidative stress, we could not detect glycosyl profiles due to oxidation in MS because intact protein analysis is performed to determine the glycosyl profile. Since this showed us the mass increase and decrease in the molecule, it could not correctly define the glycosyl profiles due to oxidation. But following the literature, no difference was observed in the glycosylation distribution in the presence of stress factors. Because glycosylation is an enzymatic reaction and takes place inside the cell during antibody production (Pisupati et al., 2017).

The reason for the degradation profiles is chemical modifications. Therefore, it is imperative to analyze these modifications, also known as PTM. For this purpose, we first separated the antibodies exposed to stress factors into peptides by treating them with

trypsin. Then, these peptides were analyzed by MS, and the modifications were characterized. We saw two peptide modifications with a temperature increase. These were PyroE formation at the N terminus of EVQLVESGGGLVQPGGSLR and deamidation of the VVSVLTVLHQDWLNGK peptide. Deamidation increased in a coordinated manner with increasing temperature. The formation of PyroE was observed only in extreme temperature condition, at 55 $^{o}$C. These were also observed in the literature and are consistent with our data (Kroon, Baldwin-Ferro, and Lalan 1992; Du et al. 2012). An increase in deamidation was observed in the peptides VVSVLTVLHQDWLNGK and STAYLQMNSLR in antibodies exposed to pH stress. But when the results were examined, the pH change triggered these modifications. The increase or decrease in pH or the presence of incubation time induced deamidation, showing no increasing or decreasing trend. Finally, when the antibodies were oxidized, Met oxidation was found only in the DTLMISR peptide. In addition, it was observed that the oxidation of Met increased in a coordinated manner with the increase in the percentage of $H_2O_2$. No other modifications were found. According to the literature, met 252 and met 428 are the most oxidized residues in the Fc region, and our results have confirmed this (Gaza-Bulseco, Faldu, et al. 2008a; Chumsae et al. 2007). In our results, oxidation was seen only in met252 and not in met428. In addition, the VVSVLTVLHQDWLNGK peptide is highly sensitive to deamidation, according to the literature (Mukherjee et al. 2010).

The mechanism of HUVEC cell proliferation was used to control the biological activities of these modifications. Here, when VEGF binds to the HUVEC cell, the cell begins to divide and grow. Anti-VEGF IgG molecule does not prevent this interaction by binding to VEGF (Papadopoulos et al. 2012). Therefore, the cell divides less. Considering this feature of the antibodies exposed to stress, cell proliferation was inhibited to the same degree compared to the control sample. And this was the same for all stress factors. Likewise, when the binding affinities of antibodies exposed to all stress factors with the SPR method with VEGF were examined, there was no problem in the binding affinity of any of them. As stated in the literature, if there is no modification in the CDR region, the interaction with the target molecule is not impaired (Neuber et al. 2014; Dick et al. 2010). Since no modification was found in the CDR region in our studies, the affinity did not

decrease either. Therefore, cell culture studies could inhibit proliferation as it effectively binds to VEGF.

FcRn is an essential receptor in maintaining the half-life of antibodies in serum (Abdiche et al. 2015). The interaction of therapeutic antibodies with FcRn is of great importance in demonstrating the drug's efficacy. Therefore, it is also of great importance to show whether the interaction of antibodies exposed to stress with FcRn changes. For this purpose, the SPR method was used in our studies. This method used two different models, steady-state and two-state binding, to show the affinities. Different groups used different models when the literature was examined (X. Wang et al. 2017; Neuber et al. 2014; Abdiche et al. 2015). Using both models, we analyzed the results of the two models comparatively. When the results were examined, there was no change in thermal and pH stresses in both models. However, Met252 oxidation severely inhibited FcRn binding compared to the steady-state model. The results are also verified with the absolute response received in the flow channel. As is known, the CH2-CH3 domain in the Fc region is responsible for FcRn binding (Gao et al. 2015). Modifications taking place here also prevent this interaction. This interaction is decreased because Met252 is in the CH2 domain.

The C1q protein is responsible for the CDC activity, which is one of the vital effector functions of the antibody (Hong et al. 2017). Therefore, it is essential to demonstrate the interaction of antibodies with C1q. Our studies determined the interaction of antibodies exposed to stress with C1q using the ELISA method. No change in the C1q binding of antibodies exposed to thermal stress was observed. However, it was observed that C1q binding affinities of antibodies decreased when exposed to pH and oxidative stress. It was shown in literature studies that Met252 oxidation reduces C1q affinity (Mo et al. 2016). Because C1q interacts with the antibody from the CH2 domain in the Fc region, modifications may limit the interaction. However, when c1q binding of antibodies exposed to different percentages of $H_2O_2$ is examined closely, there is no decrease in C1q binding with increasing percentage. Even at 0.01, the lowest $H_2O_2$ percentage, c1q binding, could reduce as much as the $H_2O_2$ percentage. Here, something other than oxidation modification might have affected C1q binding. One of these reasons may be the conformational change of the CH2 domain under oxidation stress (K. Zheng et al. 2021). However, although antibodies exposed to pH stress did not undergo a modification in their Fc region, their C1q binding affinity decreased. When the literature is examined,

it was stated that the CH2 and CH3 domains might undergo conformational changes in the presence of pH (S. Wang et al. 2016). Maybe a structural change in our studies could prevent C1q interaction. Because of this, C1q interaction may have decreased.

Finally, the NTA method determined aggregation profiles of antibodies exposed to stress factors. It is known that stress factors can create aggregation and fragmentation (Nowak, Ponniah, et al. 2017). Our studies could not analyze the aggregate profiles of antibodies exposed to thermal stress because we had no samples left. When the aggregation profiles of the samples exposed to pH and oxidative stress were examined, it was seen that both stress factors caused the aggregation. When the samples were compared with the control that was not exposed to any stress, large-sized particles were found in the oxidized samples.

As a result, the structure and biological activity of the anti-VEGF IgG molecule against stress factors were characterized by the studies carried out within the scope of this thesis. With this study, preliminary studies were carried out in realizing such drug candidates, and it will be a pioneer for the continuation of the studies. However, the limited number of studies in the literature and the fact that these studies are not multidisciplinary shows the novelty of this thesis. With this study, a multidisciplinary forced degradation study will participate in the literature.

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