**REBUPLIC OF TURKEY**

**AYDIN ADNAN MENDERES UNIVERSITY**

**HEALTH SCIENCE INSTITUTE**

**MOLECULAR BIOTECHNOLOGY DEPARTMENT**

**MASTER PROGRAMME**

# DEVELOPMENT OF APTAMER BASED CD62 DIAGNOSTIC KIT

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**MASTER THESIS**

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This thesis was supported by Aydın Adnan Menderes University Scientific Research Projects Unit with the project number ADU-TPF-19051.

**AYDIN-2020**

# ACCEPTANCE AND APPROVAL PAGE

The thesis titled "Development of Aptamer Based CD62 Diagnostic Kit" prepared by Mohammed Sattar Abduljabbar AL-SAADI within the Department of Molecular Biotechnology at Republic of Turkey, Aydın Adnan Menderes University Health Sciences Institute was accepted as a Master Thesis by the following jury.

Thesis Defense Date: 23/12/2020

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This thesis was approved by the jury above in accordance with the relevant articles of the Aydın Adnan Menderes University Graduate Education and Examination Regulations and was accepted by the Board of Directors decision number …….. taken at the session number…….. of the Institute of Health Sciences date on ……..

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# ACKNOWLEDGEMENTS

I am deeply grateful to my supervisor Prof. Dr. Özge ÇEVİK, for her guidance, encouragements, valuable comments and endless supports.

I would like to thank to all my thesis jury members, Prof. Dr. Özge ÇEVİK, Prof. Dr. Bülent BOZDOĞAN and Assist. Prof. Dr İsmail SARI for their suggestions and critics.

My special thanks go to Science, Technology Application and Research Center (ADU-BILTEM) members, especially to Res. Asst. Ömer ERDOĞAN and Res. Asst. Burçin İrem ABAS for their encouragements and sincere friendships.

I owe my sincere gratitude to my family for their endless love, thrust, encouragement and support at all stages of my life.

This project was supported by Aydin Adnan Menderes University Research Grant as a Graduate Research Project ADU-TPF-19051.

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# LIST OF ABBREVIATIONS

**ADP** : Adenosine diphosphate

**AMD** : Age-related macular degeneration

**ATF-2** : Activating transcription factor 2

**CD30** : Cell membrane protein of the tumor necrosis factor receptor family

**CD31** : Platelet endothelial cell adhesion molecule

**CD62** : Platelet Selectin protein

**CLEC-2** : C-type lectin like receptor 2

**CRD** : Carbohydrate recognition domain

**CRs** : Complement binding proteins

**CTEPH** : Chronic thromboembolic pulmonary hypertension

**DNA** : Deoxyribonucleic acid

**DVT** : Deep vein thrombosis

**EGF** : Epidermal growth factor

**ELISA** : Enzyme linked immunoassay

**ELONA** : Enzyme linked oligonucleotid assay

**E-selectin** : Endothelial cells Selectin protein

**FDA** : Food and Drug Administration

**FBS** : Fetal bovine serum

**GPCR** : G protein coupled receptors

**Gp2b/3a** : Glycoprotein 2b/3a

**ITAM** : Immunoreceptor tyrosine-based activation motif

**Kd** : Dissociation constants

**L-selectin** : Leukocytes Selectin protein

**MAb** : Monoclonal Antibody

**mTBA** : Modified thrombin binding aptamer

**N** : Nucleotides

**NF-κB** : Nuclear factor κ-light chain-enhancer of activated B cells

**PAR** : Protease-activated receptors

**PCR** : Polymerase chain reaction

**PE** **:** Pulmonary embolism

**PEG** **:** Polyethylene glycol

**PSGL-1** **:** P-selectin glycoprotein ligand- 1

**PTS** **:** Post-thrombotic syndrome

**RNA** **:** Ribonucleic acid

**REG1** **:** Resistance to glucose repression protein 1

**sLex** **:** Sialyl Lewisx

**siRNA** **:** Small interfering RNA

**ssDNA** **:** Single strand DNA

**TBA** **:** Thrombin binding aptamer

**TNT** **:** Tri-nitro toluene

**VEGF** **:** Vascular endothelial growth factor

**VTE** **:** Venous thromboembolism

**vWF** **:** Von Willebrand factor

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# ABSTRACT


## DEVELOPMENT OF APTAMER BASED CD62 DIAGNOSTIC KIT

**Sattar M. Aydin Adnan Menderes University Health Science Institute, Molecular Biotechnology Programme, Master Thesis, Aydin, 2020**

Disease-specific biomarkers consider as important tool for the pathological conditions efficient management, including susceptibility determination, diagnosis, and preventive monitoring efficacy. P-selectin is selectively expressed after platelets activation, and it is involved in formation of thrombus, and also in immune response. It is known that serum and plasma p-selectin (CD62) levels increase in some pathological conditions such as heart attack, stroke, some immune diseases and cancer. To date, a lot of methods have been developed to measure p-selectin level. Aptamers use for targeting specific biomarkers on their molecular shape the basis. SELEX (The Systematic Evolution of Ligands by EXponential enrichment) is a process which uses to choose aptamers with high affinity for targeting specific macromolecular. In this study, a new aptamer-based kit was developed to measure CD62. Firstly, aptamers that specifically bind to CD62 were isolated using the SELEX method. Among the used aptamers, Apt-1, Apt-2 and Apt-3 were bound to CD62 protein with high affinity. The Apt-2, which binds with the highest affinity to CD62, the binding constant is -9,6 kcal/mol and dissociation constant (Kd) is 18.15±2.36 nM. We used Bovine serum albumin in the specificity test as a negative control and it was determined that there is no binding between the selected aptamers did not and this protein. The Intra and Inter assay performing showed that the average of Intra-assay was CV % 6,52 and the average of Inter-assay CV % 3,96. As a result, a sensitive, specific, time-saving and low cost kit was developed for the measurement of CD62 with this study.



**Keywords:** Aptamer, CD62 protein, p-selectin, ELONA, platelet

# ÖZET

## APTAMER TABANLI CD62 TANI KİTİ GELİŞTİRİLMESİ

**Sattar M. Aydın Adnan Menderes Üniversitesi Sağlık Bilimleri Enstitüsü, Moleküler Biyoteknoloji Programı, Yüksek Lisans Tezi, Aydın, 2020.**

Hastalığa özgü biyobelirteçler, teşhis ve önleyici veya terapötik stratejilerin etkinliğinin izlenmesi dahil olmak üzere patolojik süreçlerin zamanında ve etkin yönetimi için önemli bir araçtır. P-selektin, aktive edilmiş trombositler ve endotel hücreleri tarafından spesifik olarak eksprese edilir ve trombüs oluşumu, immün yanıt oluşumu gibi birçok fizyolojik süreçte rol alır. Kalp krizi, felç, bazı immün hastalıklar ve kanser gibi bazı patolojik durumlarda serum ve plazma p-selektin (CD62) düzeylerinin arttığı bilinmektedir. Günümüze kadar, p-selektin düzeylerini ölçmek için birçok yöntem geliştirilmiştir. Aptamerler, moleküler şekilleri temelinde belirli biyomolekülleri hedeflemek için kullanılabilir. Eksponansiyel zenginleştirme ile Ligandların Sistematik Evrimi (SELEX), spesifik makromoleküler hedefler için yüksek afiniteli aptamerlerin seçilmesinde kullanılan yaklaşımdır. Bu çalışmada, CD62'nin ölçümü için aptamer tabanlı yeni bir kit geliştirilmiştir. İlk olarak, CD62'ye spesifik olarak bağlanan aptamerler, SELEX yöntemi kullanılarak izole edilmiştir. Kullanılan aptamerler arasında Apt-1, Apt-2 ve Apt-3 yüksek afinite ile CD62 proteinine bağlanmıştır. CD62'ye en yüksek afinite ile bağlanan Apt-2 için bağlanma sabiti -9,6 kcal/mol ve ayrılma sabiti (Kd) 18,15 ± 2,36 nM olarak bulunmuştur. Özgünlük testinde negatif kontrol olarak sığır serum albümini kullanılmış ve seçilen aptamerlerin bu proteine bağlanmadığı belirlenmiştir. Sonuç olarak, bu çalışma ile CD62'nin ölçümü için hassas, spesifik, zaman tasarruflu ve düşük maliyetli bir kit geliştirilmiştir.

**Anahtar Kelimeler:** Aptamer, CD62 protein, p-selektin, ELONA, trombosit

# 1. INTRODUCTION

## 1.1. Aptamers

Aptamers are oligonucleotides produced by using in vitro process which called SELEX (Systematic Evolution of Ligands by exponential Enrichment). Aptamers have been formed in order to bind with proteins which are associated with several disease. Aptamers are single-stranded nucleic acids that proceed for a certain task. SELEX starts with a big number of single stranded DNA or RNA oligonucleotides that limited with a certain function. Generally, the function is the binding with specific biomacromolecule, SELEX experiment sets a method for separating the DNA or RNA molecules that have done the function from the others that haven't, the chosen DNA molecules are amplified by PCR. But, if the library is RNA, it must firstly perform by reverse transcriptase to complementary DNA, after that by PCR. This DNA will be amplified. While ssDNA chains will be immediately amplified by PCR. The DNA group, which is produced by the first selection cycle, will be used to produce a new ssDNA and RNA library which will newly enter the new cycle procedure. This procedure will be repeated till a group of nucleic acids is chosen to perform the function sufficiently. Many studies about aptamers show that group of nucleic acids consider as an origin of a large group of three-dimensional forms. For this reason, it always finds SELEX in the first library, a group of chains that are responsible of the strongly binding with the proteins, and that make it regard as pharmaceutical and diagnostic agents (Brody and Gold, 2000).

In 1990, an oligonucleotide molecule was produced which with big affinity and specificity binds with T4 DNA polymerase, that screened oligonucleotide molecule considers as an aptamer, by using the SELEX procedure. Pegaptanib sodium (trade name: Macugen) considers as the first found aptamer medicine, FDA (Food and Drug Administration) confirmed it in 2004, it is used to age-related macular degeneration treatment. Today, the studies have discovered a number of aptamers produced by SELEX and these aptamers are three-dimensional shape molecule that have the ability for binding several macro biomolecules (Zhang et al, 2019).

The developing of aptamers have done to task several gene therapy. The nucleic acid aptamers have produced for large clinical targets number, include proteins of the viruses, the factors of transcription and growth, and the proteins of coagulation. Aptamer binding affinity to

the aimed protein molecule is with high Kd (dissociation constants), ranges from $1\times10^{-12}$ M to $1\times10^{-9}$ M. Aptamers have ability to distinguish between the coagulation factors (which are VIIa, IXa and Xa) that have the same structural domains set; between P selectin and Land E-selectin; and between reverse transcriptase of different HIV strains (White et al, 2001).

Aptamers are oligonucleotides (ssDNA or RNA), bases lengths range between 15 and 70 mers. The use of nucleic acid aptamers has been progressed, especially RNA aptamers, as therapeutic agents, ways for medicine delivery, analytical tools, diagnosis tools and also genetic instrument. Till the moment, though the only Macugen, Pfizer/Eyetech aptamer has been confirmed to the medicine task, there are another ten aptamers have been assessed for several levels of therapeutic experiments. Through 2015 the market of universal aptamers was evaluated with $ 107.56 million, and in 2020 it becomes $ 244.93 million. It is expected that the aptamers will hold the universal market biggest share in 2015, indicating increasing in nucleic acid aptamers applications (Zhu et al, 2015).

### 1.1.1. Characteristics of Aptamers

Aptamer gives great advantages if it is compare with the other targeting tools, where it binds in big selectivity and affinity, and they do not produce immunogenic. Moreover, they can be synthesized with easy and quick way by in-vitro procedures, are strong and stable, the previous features give these aptamers great attractive to peptides, also they consider as diagnostic and therapeutic too (Missailidis et al, 2005).

Aptamers have many advantages over monoclonal antibodies and the other medical tools; aptamers synthesis is much easier to scale up, also, aptamers are don't affect the thermal condition so they are stable, and there is the ability to be denatured several times without activity losses; moreover, they don't produce immunogenicity. While there are disadvantages of aptamers, like their quicker excretion because of their small size, some aptamers have weak binding with target molecules, sensitivity with serum digestion if the work was with an unmodified aptamer. During the last years since aptamer was choosen by SELEX, there is Pegaptanib (Pfizer/Eyetech) aptamer which has the ability in binding with VEGF (human vascular endothelial growth factor), and the Food and Drug Administration (FDA) confirmed this

aptamer which has been used in the treatment of AMD (age-related macular degeneration). There are many aptamers which are selected against several diseases that related molecular aims like vWF (von Willebrand factor) (figure 1), thrombin (figure 2), factor IX (figure 3) (Wang et al. 2011).

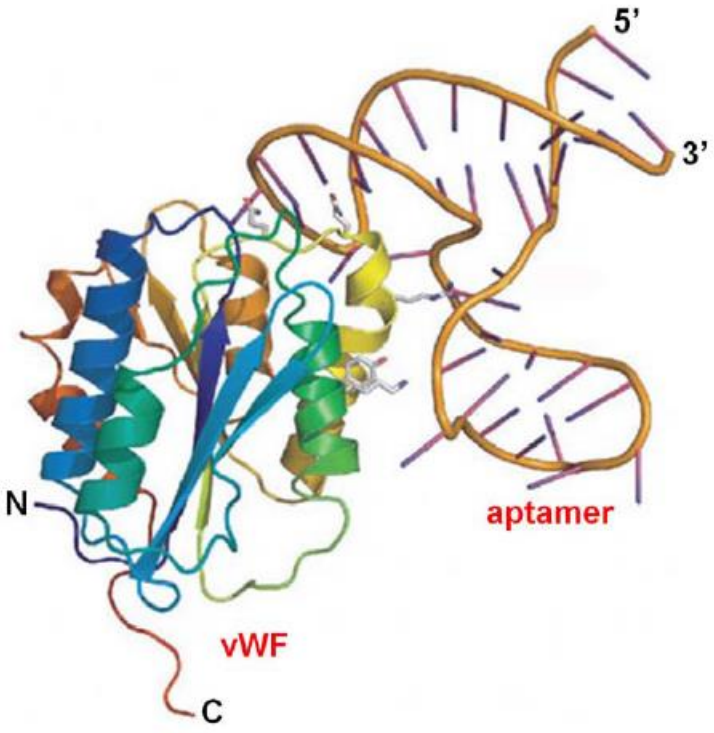



**Figure 1.** ARC1779 binding with vWF (von Willebrand factor) (Wang et al. 2011).

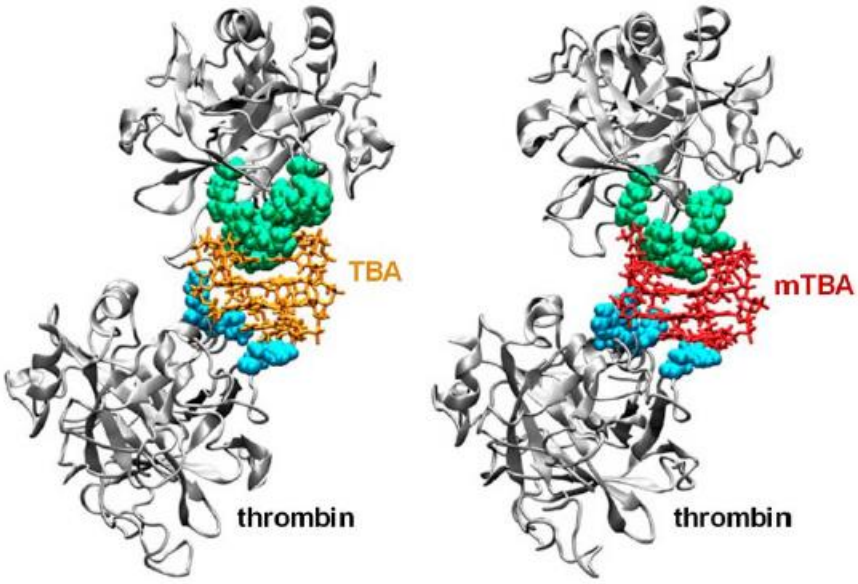



**Figure 2.** Illustration of the molecular interaction between thrombin and the thrombin binding aptamers (Wang et al, 2011).

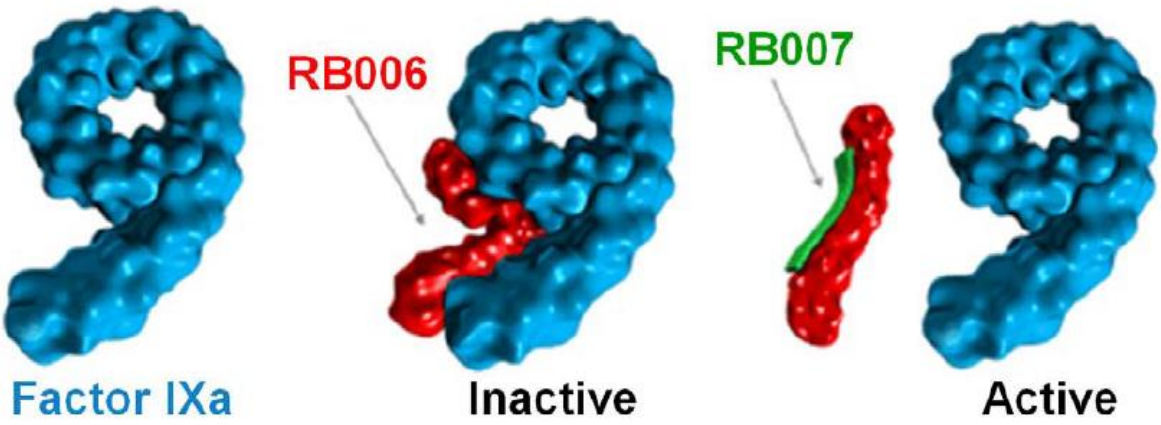



**Figure 3.** Illustration of antidotes that inactivate and activate Factor IX (Wang et al. 2011).

### 1.1.2. Structure and Function of Aptamers

Aptamers are a chains of nucleic acid oligonucleotides, they have size from 25 to 50 nucleic acid bases and they are formed by procedure called SELEX. The oligonucleotides that could produce a number of forms within random the used library considers as the base to get big success of aptamers producing process to a large group of targets, and this success can be occur with a process which includes producing a oligonucleotides library (Missailidis et al, 2005).

Aptamers are a three dimensional shapes produced through the folding of the nucleic acid (oligonucleotides). Its special three-dimensional structure, the aptamer bound with targets is occurred with lineaments like hairpins, internal loops, bulges, G-tetramers and pseudoknots. On other hand, aptamer bound with target effects with several factors like geometrical form, the electrostatic interactions of the charged groups and aromatic ring overlap, the van der Waals forces, the bases of the nucleic acid aptamer, and also the hydrogen bonds. Protein considers the most important target (Zhang et al, 2019).

There are a more of proved procedures to produce aptamer with high stability. Especially, SELEX have been used as new method to select aptamers for therapeutic and diagnostic applications. Until now, aptamers were chosen to detect large number of proteins, sugars, and all cells. Generally, aptamers classify to 2 major group: nucleic acid and peptide aptamers. Because the nucleic acids can be quickly digested by nuclease enzymes, many confirmed procedures were found to produce high stability aptamers, like the using of chemically modified oligonucleotides and PEG (polyethylene glycol) conjugation. The all peptide aptamers have a several loops to attach with protein. The variable loop consists of ten to twenty amino acids that give the good solubility properties of protein. The first peptide aptamer study did through 1996, in which aptamer recognizes several epitopes on Cdk2 (cyclin- dependent kinase 2) surface were chosen with K(d) (dissociation constants). If peptide Aptamer is compared with nucleic acid aptamer, the work on peptides aptamer is in the beginning, but step by step has confirmed as good region for chemistry scientist as also for the biology scientist since the last years (Wang et al, 2011).

Today, several aptamer-based analytical designs are progressed as appropriate instruments to detect and quantify several molecules. Aptamers using as way through the chemistry are an exciting researching area of on account of the binding abilities with the targets with the same of antibody affinities. Furthermore, aptamers give several benefits. The regeneration of Aptamer occurs in minutes by desaturate and renature them. The Selex conditions such as buffers, temp

and pH can be controlled to make the bound features better. It is also possible to change its sequence to regulate the binding selection. Aptamer can be synthesized by chemical procedures to increase the purity. It is produced through a laboratory process that doesn't require animal cells (Ravelet et al, 2006).

The optimization and the development of therapeutic properties such as; the stability, and kinetics, can make aptamer a effective and cost tool. Moreover, the aptamers techniques have also some advantage such as low toxicities, and increasing the half-live to long range (Gutsaeva et al, 2011).

There are another advantages of aptamer over antibody, includes the simple synthesis, the length of store time in r.t (room temperature), the aptamer stability within shipping, The variety of oligonucleotides that used for formation of aptamer gives the ability to select aptamer molecules to several targets. Consequently, aptamers consider as a good instrument in the medicine using within the therapy and diagnosis (Faryammanes et al, 2014).

Aptamer binds in specific way to the targets, include the high and low m.w (molecular weight) molecules. In biosensing field, aptamer is the identifying tools which can replace the antibody. anyway, the aptamer K(D) is higher than in value from antibodies K(D). Aptamer can be synthesized with the use of easy strategies (Cywinski et al, 2015).

### 1.1.3. DNA and RNA aptamers

DNA aptamer is look like the RNA aptamer, but there are some differences between them in the stabilities and accessibilities. RNA aptamer has less stability because it has reactive hydroxyl group (-OH) within its nucleotides (the 2 position of the ribose). The -OH can be separated within the solution (particularly in alkaline media). So, the 2-O- can react with the phosphorous atom causing the RNA hydrolyzing. If the OH group was removed, the nuclease resistance of the RNA molecule rise. ssDNA aptamer has more stable than the RNA aptamer because it has C-H bond within the nucleotide (in the deoxyribose sugar). These differences give ssDNA aptamer advantages in stability over the RNA aptamer. Various chemical modifications like the changing of the hydroxyl group by 2′-amino or 2′-fluoro, with the changing to the phosphodiester backbone with phosphorothioate or boranophosphate can be made to improve the chemical stability of RNA aptamers. More recently, new chemical modifications have been

found for increasing of siRNA stabilities, where the 2′-O-Methyl or phosphonodithioate were changed within the same nucleotides. These modification techniques which can increase siRNA stability consider as new way to use the techniques which based on RNA within the biology field. There is another confirmed modification with the using of locked nucleic acids method or produce RNA chains which called spiegelmer. This modification cause a structural change in the RNA chains. While these modifications protect RNA aptamers against nuclease attack, the activity of RNA nucleotide and non-base-pairing give the RNA the feature to form several and complex three-dimensional shapes. So, ssDNA aptamer is commonly chosen by libraries that have tall random areas for to obtain complex molecules. An advantage of ssDNA aptamer over RNA aptamer which is simple selection procedure. The select of RNA aptamers needs reverse transcription in every selection cycle. While the ssDNA aptamer synthesis procedure doesn't need this additional step. Also, DNA aptamer production is less costly than RNA aptamer production (Zhu et al., 2015).

### 1.1.4. Aptamer Differences with Monoclonal-Antibody

Antibodies target many molecules and are used to specifically recognize antigens and receptors. The applications which depend on working with antibody have been varied in many diseases such as; cancers, inflammations and autoimmune disorder. Like the using of antibody to treat lymphoma and leukemia. Aptamers are a new target strategy, it is able to make binding with several proteins and other molecule in high affinities and specificity (Missailidis et al, 2005).
The same in the antibody, aptamer binds with the target through its 3D structural shape which called "chemical antibodies". Aptamers technologies have been used with different medical and biological areas, such as; in vitro diagnosis, imaging, and targeting therapies. Aptamers is used in several fields, through the targeting therapeutic, aptamer-nanoparticle binding, drug conjugation, aptamers-mediated immunotherapy, genes therapies and aptamers-mediated biotherapy. The main feature during the use of aptamers, is their high targets selection. Also aptamers are different from nucleic acids probe, they have the ability for binding with the target by the structural recognitions (Figure 4). This procedure is the same in the antigens-antibodies reactions (Sun et al, 2014).

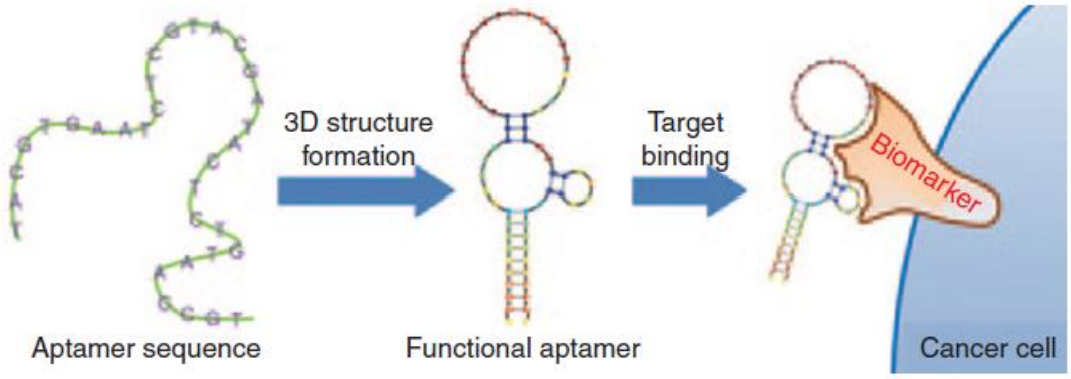



**Figure 4.** Pictorial representation of the interaction between aptamer and target biomarker (Sun et al., 2014).

Because of they have lower m.w (molecular weight) (8–25 kDa aptamers versus ~150 kDa of antibodies), aptamer has the ability for easily penetrating the tissue easily. There is study confirmed that aptamer didn't have the ability for immune system stimulating, while protein antibodies are highly immunogenic. Aptamers also are able for recognizing of several target molecules like ion, drug, proteins, bacteria, toxin, peptide, virus, cell, and tissue. clinically, there are some drugs based on aptamer such as; Macugen (modified RNA aptamer), which detects vascular endothelial growth factors, and The FDA (US Food and Drug Administration) has confirmed this aptamer. Within cancer treatment field, AS1411 aptamer can target nucleolins, proteins are expressed within the tumor cells. Table 1 shows some of therapeutic aptamers-based therapeutic (Sun et al, 2014).

**Table 1.** Some of therapeutic aptamers-based therapeutics (Sun et al., 2014).

| Aptamers | Molecular targets | Sponsors | Medical indication |
|---|---|---|---|
| (REG1) | Coagulation factors (IX) | REGARDO Bioscience | Coronary-Artery Diseases |
| (NOX-H94) | Hepcidin | Noxxon P | Anemia |
| (NU172) | Thrombin Factor (Ila) | ARCA pharma | Heart Diseases |
| (ARC19499) | Tissue Factors Pathway Inhibitors (TFPI) | Baxter Corporations | Hemophilium |

Compared to monoclonal antibodies and other therapeutic agents, aptamer has considered as new ligand group for therapeutic and diagnostic, which is produced by SELEX, which is chosen for large number of target molecules like protein, sugar, nucleic acid, phospholipids, and many cells (Wang et al. 2011).

Aptamers also considers active more than antibody because of the high stability, easy modification, non-immunogenic molecule. Also, comparing to antibodies, aptamer also has other detecting features which made them a good way to treat cancers. Table 2 show the differences between aptamer and antibody (Nabavinia et al, 2017).

**Table 2.** The dissimilarity between aptamers and antibodies (Nabavinia et al., 2017).

| | Aptamers | Antibodies |
|---|---|---|
| History | In 1990 by Ellington and Szostack. | In 1975 by Kohler and Milstein. |
| Isolation processes | Invitro | Invivo |
| Production | Chemical production | Antibodies synthesis cell |
| Molecular target | For extracellular targets, nonimmunogenic molecules. | Intracellular and extracellular target, immunogenic molecules. |

## 1.2. The SELEX

Recently many researches have given the road to consider aptamer as suitable alternatives to antibody which cause the definition of newest regulations. Every selection SELEX cycle contains 3 steps, which are ordinary refined within every cycle. The first is to bind targets, the incubation of single-stranded oligonucleotides pool or random library with the targets. The second step, is the isolation of target-bound chains. and the third step, the reamplifying of the target-oligonucleotides by the PCR (polymerase chain reaction) for generating new ssDNA or RNA pool for using in next cycle (Nabavinia et al, 2017).

### 1.2.1. Principle of SELEX Process

The SELEX technology incorporates several fields, such as molecular biology, bioinformatics, and materials science, to improve aptamers for a several groups of targets. A large number (approximately 1014-15) of random DNA or RNA oligonucleotide library are firstly synthesized, and then several enrichment and screening cycles are carried out to select oligonucleotide chains from this library that can bind target molecules with high specificity and affinity. New base sequences will appear during the screening process, and the specificity and affinity with which the selected oligonucleotide strand binds the target molecule will gradually increase as progressing of the cycle. The final oligonucleotide chain will have the highest specificity and affinity for the target molecule and is termed as the aptamer of the specific target molecule (Zhang et al, 2019).

### 1.2.2. Synthesis of ssDNA and RNA Aptamers

Nucleic acid aptamers defined as single-stranded oligonucleotides which are ordinarily produced via SELEX process. The SELEX procedure is a combinatorial technique is used to screen large oligonucleotides libraries by repeated in vitro selection and amplification process. DNA and RNA libraries are easily produced by automated synthesis. Commonly, a randomized region (30 to 60 nucleotides (n) is surrounded on both sides by primer's regions for amplification. A library of individual molecules is then obtained ($N(max) = 4^{(n)}$). The library real degeneracy is dependent on the number of oligonucleotides rather than on N(max). In the

random libraries, the diversity of individual oligonucleotides can be as high as $10^{(15)}$–$10^{(18)}$ molecules. The general principle of the SELEX technology is schematically presented in Fig. 5. Briefly, the random sequence of the oligonucleotide library is incubated with a target molecule. Oligonucleotides which have affinity towards the target are isolated from the enormous number of species in the library. The binding oligonucleotides are then isolated and amplified in order to obtain an enriched library which is used for a next selection and amplification cycle. The binders enrichment efficiency is ruled by the stringency of the selection of each round. The enriched library is cloned and sequenced after around 10–15 cycles. The individual sequences are then analyzed in order to recognize the idea which agree with the minimal sequence required for target-specific binding (Ravelet et al, 2006).

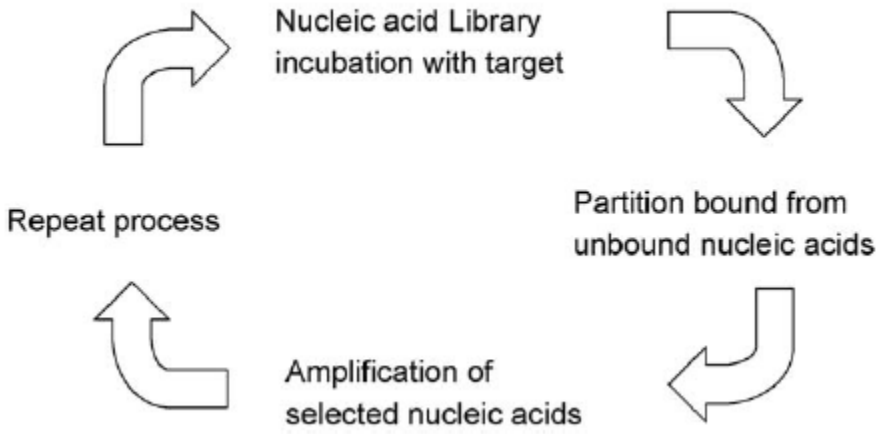



**Figure 5**. SELEX process (Ravelet et al, 2006).

### 1.2.3. Synthesis of Oligonucleotides Sequences Library

For aptamer screening, the random chains precisely have the ability for binding with targets, and these chains will be amplified and screened. ssDNA (or RNA) aptamer Screening begins with random oligonucleotides library building. In library building, firstly, sequences must be designed on the oligonucleotide chains (its length between 20-30 nucleotides). The sequence has restriction endonucleases and primers binding sites which needed for PCR reaction, while binding site contains RNA polymerase which required to prepare RNA aptamer. RNA chains

have to bind with reverse transcriptase. Short sequence is less expensive and it is easy to synthesize and design it, but if the sequence is too short, it will be difficult to build secondary structure that stably and specifically binds with the targets. An increase in the chain length will lead to increase libraries capacity. If the sequence is with 30-50 nucleotides, it can organize between $10^{14}$ to $10^{24}$ oligonucleotides sequences. The structure built by this nucleotide number will be enough for targets binding (Zhang et al, 2019).

### 1.2.4. Aptamer Selecting and Enrichments

After ssDNA oligonucleotides library building, oligonucleotides sequences must be enriched and screened for binding with targets in high affinity and specificity. Mature screening procedures contain affinity chromatography, capillary electrophoresis, magnetic separation, high-speed centrifugation, and nitrocellulose membrane filtration. There are many advanced screen techniques like photo SELEX, negative SELEX, counter SELEX, subtractive SELEX, which can progress aptamers selectivity. While Blended SELEX, and toggle SELEX develop the aptamers internationality. In addition, non-SELEX and automated SELEX can reduce cycle screening. Methods like tailored SELEX and genomic SELEX which are for combining different pools, increase the screening likelihood for aptamers. Today, cell-SELEX is openly utilized (Figure 6) (Zhang et al, 2019).

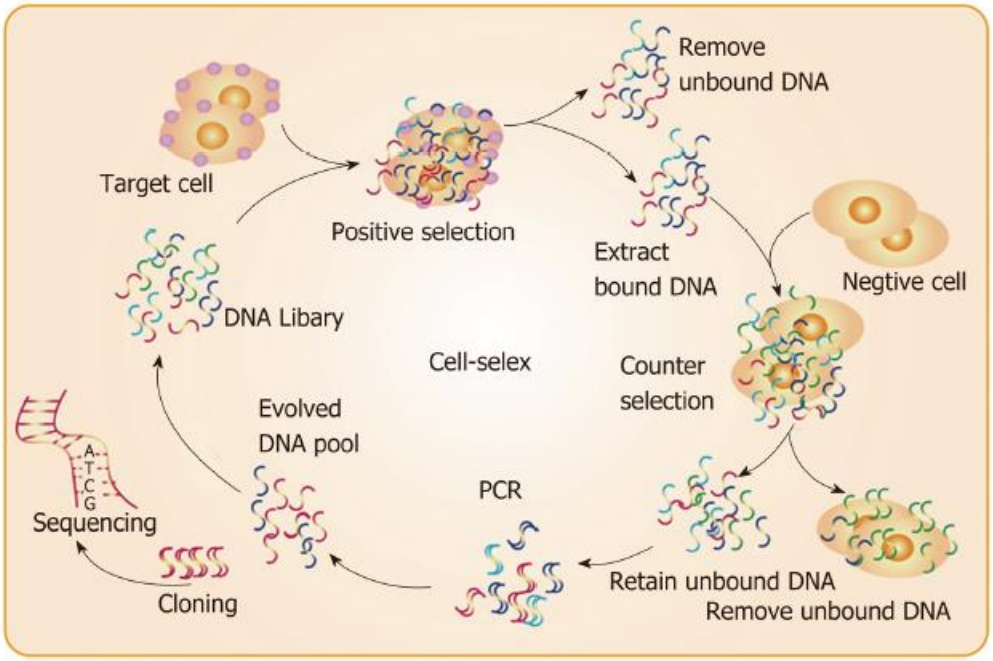



**Figure 6.** A brief summary of Cell-SELEX method (Zhang et al., 2019).

Cell-SELEX is fast method with high productivity, which shortens the screen time and reduces the screen cost. There is process called the chip cell-SELEX, which combine the microfluidic chip within the SELEX procedure, it needs about five screening cycles. (Hi-Fi)-SELEX is a rapid process to give active selection for ssDNA aptamers. the general amplifying procedure of ssDNA aptamer is like the follow: within first screen cycle, the using of targets for ssDNA oligonucleotide sequence library screening, while the oligonucleotides sequence ssDNA will amplify by PCR reaction; within second screen cycle, must rescreen the amplified product and the ssDNA will amplify by PCR reaction; after 8-16 screen cycles, clonal sequencing and flow-cytometry is carried out for obtaining the needed affinity and specificity aptamer. So, according to SELEX process utilized, the enrichment cycles number differs. In many situations, more cycles lead to consume reagents but not increasing for screen rates. Also, less cycles mayn't distinguish good aptamer. The suitable number of screen cycles is usually in it the affinity won't continue in the increasing (Zhang et al, 2019).

### 1.3. Aptamers Structure Modifications

For obtaining well-defined base sequences, Screening of enriched aptamers will be requiring for base sequencing. So, characterization and structural analysis of these screened aptamer require for the carrying out, factors estimating like the affinity and specificity. The aptamer chemical building is more stable than antibody. By the way, it may be extracted through the kidney in spite of the aptamer tolerance and stability to temperature and pH. Conventional optimization methods of aptamers structures contain the followings: (1) Replace the original nucleotides with the modified nucleotide in purpose of avoiding nucleic acids degradation; (2) Chemically building of an aptamer within high plasma and serum stability for increasing the aptamer stability to increase their therapeutic potent; (3) to increase their ribozyme resistance, aptamers are modified with locked nucleic acids. Methylene-linked 4'-carbon and 2'-oxygen in carbohydrates can hybridize with nucleic acids and aren't easily decayed with the enzymes; and (4) when aptamer attach to cholesterol, that will lead to extend its residence time inside the human body. Thus, by one or more of the above methods, the half-life survival of aptamer in human environment can be increase from minutes to hours, so the aptamer with modified features is better than antibody in the treatment and diagnosis of cancer (Zhang et al, 2019).

### 1.4. The Selectins

Selectins are cell membrane glycoproteins that show calcium dependent carbohydrate binding activity. This family has three members, whose names are Platelet selectin (P-selectin)-, Endothelial selectin (E-selectin) and Leukocytes selectin (L-selectin). They are named according to the type of cell from which they were first isolated (Figure 7).

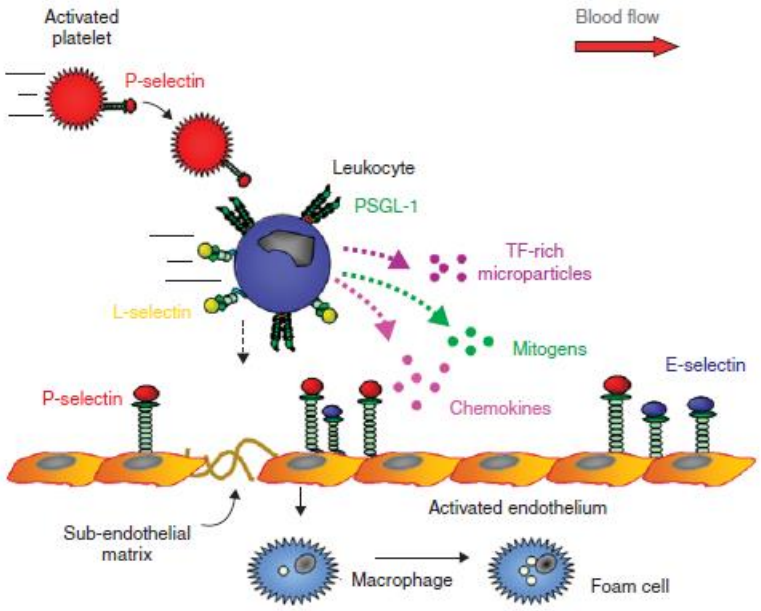



**Figure 7.** Illustration of selectins according to the isolated cell type (Bedard and Kaila, 2010).

P-selectin is synthesized mainly from granules of active platelets and Weibel bodies of endothelial cells. In addition, it is the type of selectin that is first released in the inflammation event. E selectin is also a glycoprotein released from activated endothelial cells. Unlike P- and E-Selectin, L-selectin is released from lymphocytes and is involved in the binding of lymphocytes with the endothelial capillaries of lymph nodes. Selectins contain five different sub compartments like N-terminal lectin-like zone, EGF-like zone, consensus repeats, C-terminal transmembrane zone which is different number of repeating regions entering the cell membrane and a cytoplasmic sequence EGF and CRD chains have almost the same molecular weight in all selectin species, while the lengths of extracellular consensus repeats differ from species to species (Figure 8). This structural difference enables all three types of selectin to undertake different roles in various biological processes (Silva et al, 2018).

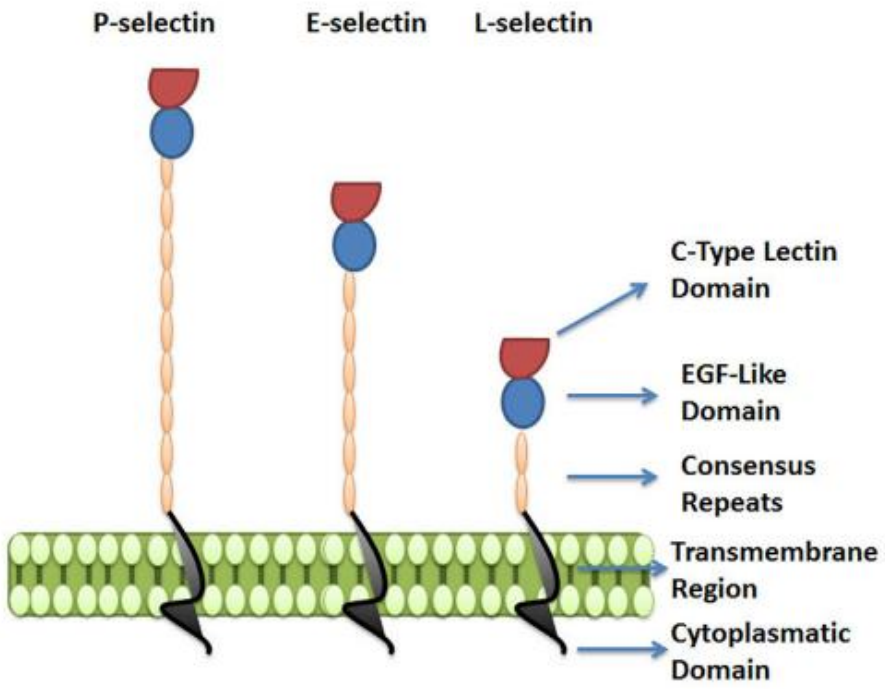



**Figure 8**. The regions of selectin family (*Silva et al., 2018).*

The three-dimensional crystal structure of P- and E-selectins has been illuminated by X-ray crystallography. And thus, it gives the opportunity to determine from which region the ligands can be attached to the structure. All selectins contain a calcium-binding site, which contains a sequence to which sLex can bind, enabling inflammation processes to be triggered (Figure 9C). Also, P-selectin glycoprotein ligand-1(PSGL1) is a transmembrane protein that can bind to all three types of selectin with different affinities (Figure 9B). It has been shown that the metastatic properties of cancer cells increase with the expression level of PSGL-1 (Bedard and Kaila, 2010).

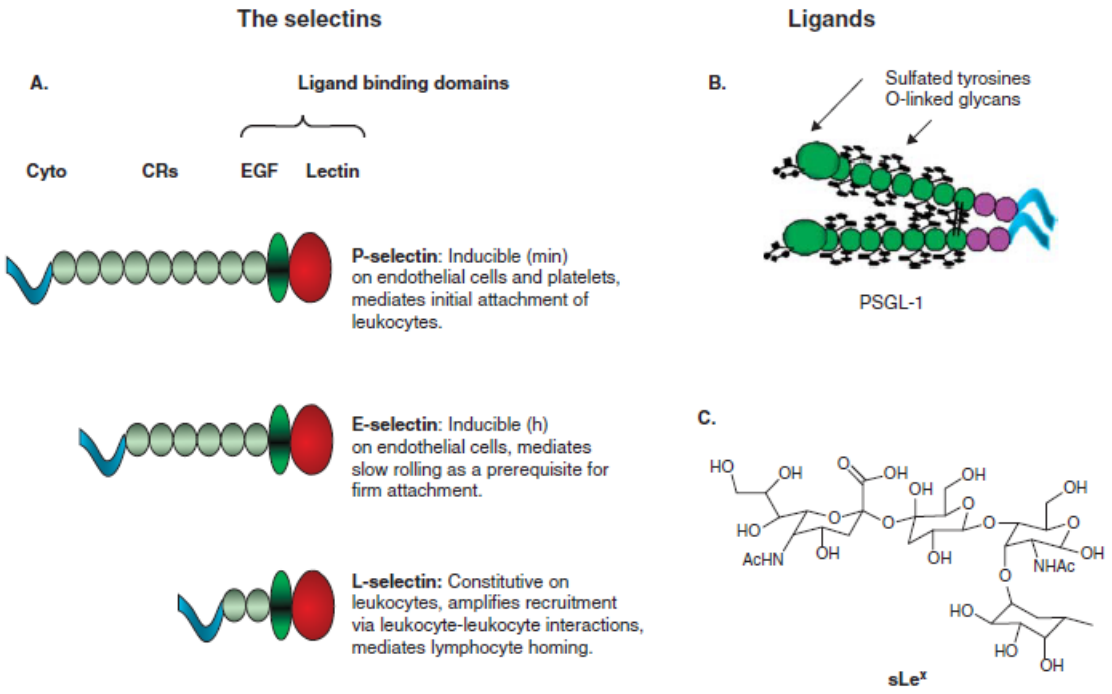



**Figure 9.** The structure of selectins (A) and its ligands (B,C) (Bedard and Kaila, 2010).

### 1.4.1. L-selectin

L-selectin considers as one of the vascular cell adhesion proteins, it locates in the leukocyte. After its expression, leukocyte leaves the blood and will be able for binding with the endothelial cell. In inflamed tissues, when leukocyte moves within layer of the endothelial cells, it will be able to expose their antigens and this transient endothelial migration considers as the base of the inflammatory responses. L-selectin is one of the main biomarkers for several diseases based on this migration mechanism like leukemia and systemic sclerosis. L-selectin develops tumors metastasis. However, selectins are not established and classical biomarkers, whereas on white blood cells, they are expressed and start to produce the adhesion when suitable vascular molecule is expressed on endothelial cells. The concentration the ranges of L-selectin within healthy person blood is between (0.5-1.5) µg/ml. while its concentration in patient will be much lower (Cywinski et al, 2015).

### 1.4.2. P-selectin

P-selectin is expressed on activated platelet cells surfaces as a glycoprotein by the endothelial cell and platelet, it's stored in the α-granules, respectively. Because of its expressing in endothelial cell, both of the P-selectin with E-selectin had the term of "vascular selectins". P-selectin can be stimulated by several stimulants like thrombin and histamine lead to fuse the storage chambers with the plasma membrane that make P-selectin more selectin protein translocate from granule to the cells surfaces (Silva et al., 2018).

### 1.4.3. E-selectin

E-selectin is molecule adhere on the leukocyte on activated endothelial cell and is expressed by the bone marrow. Within hours of exposure, these cytokines potently stimulate the E-selectin mRNA transient transcription within human endothelial cell. E-selectin decreases the leukocyte movement to lower velocity than L-selectin and P-selectin. E-selectin plays main role in cells escape to inflammation site within the human, and in the immune effector staffing for inflammatory site targeting. (Silva et al., 2018).

## 1.5. Platelets

Platelets are created from the megakaryocytes and get through the blood without a nucleus, in hemostasis platelets play key roles. Megakaryopoiesis is a complicated process in which megakaryocytes grow and create polypoid megakaryocyte. Mature megakaryocytes restructure their cytoplasm through the endothelial cell and transfer the platelet to the blood stream. Like any small anucleate cells, platelets contain several organelles like golgi apparatus and mitochondria. Platelet has many receptor and adhesion molecules and many granules. It has the ability for protein synthesizing because of the presence of its mRNA. The main platelets function is to bind with damaged blood vessels, so it will aggregate for thrombin forming and preventing the bleed. Furthermore, the platelets aggregate at endothelial cell erosion sites, inducing formation of thrombus and promoting atherothrombotic disease. Many studies showed that antiplatelet drugs affect immunity of host and modulate platelets response to inflammations,

decreasing the infection and mortality. Thus, there are more evidences that platelet has a main role within the inflammations and immune response (Yun et al, 2016.)

### 1.5.1. Platelet Activation

Platelet activation is related with to the CD62p (P-selectin) expression, which binds to monocyte and granulocyte involved in subendothelial layers degradations. Platelet is activated in blood circulation by several stimulants like collagen, epinephrine, ADP and fibrin. The activation of platelet has been settled with CD62p exposing (Cevik et al, 2013).

Various conditions can affect platelets in the circulation and these variations can lead to thrombin formation. Platelet generates microparticles, many pathophysiological functions appear, such as stroke. Each marker of platelets activation is related to the platelets surface through the activation like p-selectin. Glycoprotein 2b/3a which is common as integrin αIIbβ3, is one of the main receptors of the membrane and plays a main role through activation of the calcium dependent platelet (Cevik et al, 2016).

### 1.5.2. Platelet Activation Pathways

The platelet activation is mainly related with the coagulation cascades opening. Platelet adhesion to the extracellular matrix considers as the first step within the hemostasis. With high shear condition, on the platelet membrane vWF (von Willebrand factor) composes a bridges between the collagen and glycoprotein platelets receptors. Exposed collagen has the ability of binding with GP VI receptors platelet GP Ia/IIa. during activation, platelets excrete the content of the granules and change shape. Active GP IIb/IIIa receptors plays main role to mediate the platelet aggregation. The activation of platelet is stimulated by the secretion of platelets product. On exposed collagen, GP VI is considered as one of the main signaling receptors engaged within the activation of platelet. After the GP VI interaction with collagen, platelet starts activation and releases dense granules and the ($\alpha$-) contents. Thrombin considers as one of the strong agonists to platelet, it converts fibrinogen to fibrin for platelet plugs stabilizing. By PAR (protease-activated receptor), Thrombin activates platelets by GPCR. At low thrombin concentration, PAR1 mediates the activation of human platelet, while PAR4 needs a high amount of thrombin concentrations for platelet activation. For contributing to arrest bleeding and in situations such as

trauma, signaling by PAR4 is available to the protective mechanisms. Agonists like epinephrine, and serotonin can use GPCR for potentiating the platelet response. These events of platelet meet with the platelet activation final common pathway, and that is the functional regulation for adhesion receptors of integrin. The activation of the GP IIb/IIIa receptors lead to the cross-linking of vWF between receptors or fibrinogen, resulting in the aggregation of the platelet. This boosts additional platelet recruitment to the vascular injuries sites, lead to allow the formation of thrombin (Yun et al, 2016).

### 1.6. Cellular Interactions

A number of inflammatory mediators are secreted by activated platelets and these mediators don't have apparent role in hemostasis. With the conditions of hemostatic, platelet doesn't have the ability for binding with the leukocytes. After platelet activation, it adheres to monocytes and neutrophils, and its interaction with lymphocyte has been identified. Platelet interacts with the leukocytes and binds thrombosis, inflammation and atherogenesis. P-selectin (or CD62p) mediates the bind between the platelet and other cells. After the platelet's activation, it expresses amounts of P-selectin that is moved to the surfaces of platelets from $\alpha$-granules. P-selectin proteins with their ligands, PSGL-1 (P-selectin glycoprotein ligand-1), play a main role in platelet interaction with endothelial cells and leukocytes. Both of the neutrophil, monocytes and eosinophil have been notified for the expressing of PSGL-1. P-selectin binds the platelet to leukocyte and consider as the main mediator for the formation of platelet-leukocyte aggregate that leads to release cytokine and adhere it to endothelium (Yun et al, 2016).

### 1.7. Thrombosis

Thrombosis is a blood clot formation in blood vascular, occurs in venous and arterial system. Thrombosis participates in several conditions such as stroke, myocardial infarctions. (Oklu, 2017).

Thrombosis is blood localized clotting, occurs within venous and arterial circulations and has a big medical effect. Acute arterial thrombosis is the close reason of several myocardial infarction cases (heart attack) and of about 80% of strokes, in the developed world collectively it

considers as the most common death cause. Arterial thrombosis can be treated with platelets target drug, but the venous thrombosis can be treated with proteins target drug (Mackman, 2008).

### 1.8. Aptamer–Protein Interactions

Aptamers are oligonucleotide sequences and have the ability for binding with targets. The targets may be protein, cell and tissue. Aptamer interaction with the target is based on the events of molecular recognitions because of the aptamer 3D structure which based on their sequences. This interaction is dependent on Van der Waals force, electrostatic forces and the hydrogen binding between targets and their aptamers. Aptamers differ from antibodies; they are the best alternative to identify targets because of their easy classified through the chemical building. Unlike antibody, aptamer considers ad signal-transduction agent and also a selective target-capture agent. According to that, aptamer, which has an unlimited shelf life and batch-to-batch reproducibility, can be produced for target molecules that don't produce the immune responses, like a toxin and explosive like TNT (tri-nitro toluene) and ricin (Parekh et al, 2008).

In organisms, the pair of aptamer interaction with protein plays a role in several therapeutic potentials and physiological functions. The quickly predicting of the pair of Aptamer interaction with protein is important for design aptamer able to bind with specific protein (figure 10), that give a clear idea to understand the mechanism of the pair of aptamer interaction with protein and develops therapy based on aptamer (Zhang et al, 2016). There are many studies in the literature on specific binding of aptamers to proteins and the number of studies on aptamer is increasing every day (Liu et al, 2011).

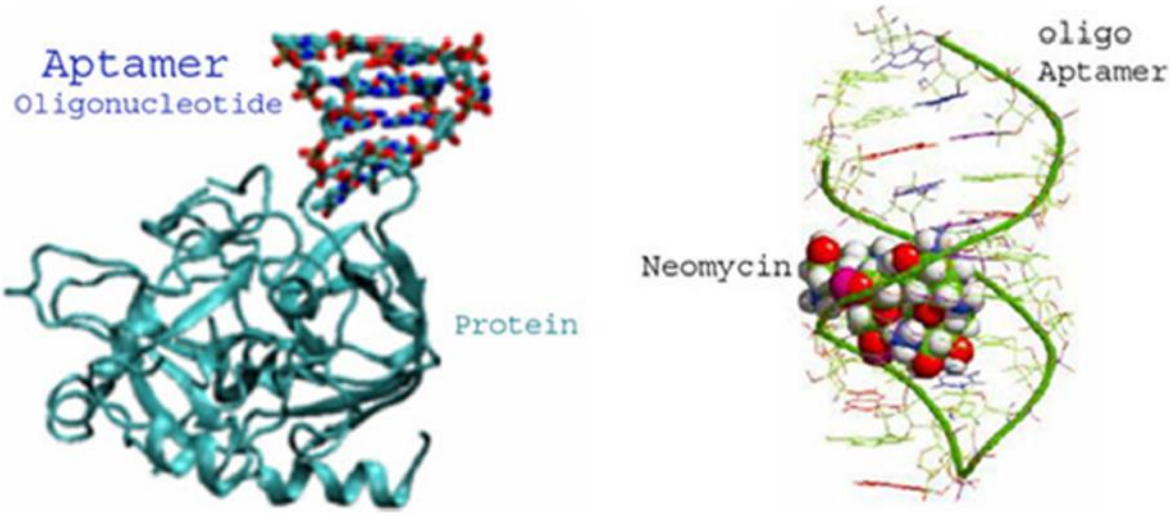



**Figure 10**. The structures of aptamers binding to specific targets. (Zhang et al., 2016)

# 2. MATERIALS AND METHODS

## 2.1. Materials

### 2.1.1. Equipments

- Cell Culture Flasks T75, T25 (Corning)
- 6,12,24,96-Well Plate (Corning)
- Micropipette 1000µl, 200µl, 20µl, 2µl (Rainin)
- Micropipette tips 1000µl, 200µl, 10µl (Rainin)
- Serological Pipette 25ml, 10ml, 5ml (SPL, 91005,10,25)
- Microcentrifuge tubes 2ml, 1.5ml (Isolab)
- Cryotube 2ml (Isolab)
- Falcon Tubes 50ml, 15ml (Corning)
- PCR Tubes (Thermo Fisher)
- PCR Plate and Seal (Isolab)
- Western Blot Apparatus (Biorad)

### 2.1.2. Instruments

- Sterile Biosafety Cabinet (Heraeus HS12)
- Centrifuge (Eppendorf 5415R)
-Inverted Microscope (Olympus CK40)
- Vortex (Isolab IP42)
- Ice machine (Uğur Buzal 60)
- pH Meter (Mettler Toledo S220)
- $CO_2$ Incubator (Nuaire NU5500)
- Centrifuge (Hettich Universal 320R)
- Centrifuge (Hettich Universal 32R)
- Orbital Shaker (Thermo Scientific 88882004)
- Ultra centrifuge (Beckman Coulter Optima L-100 XP)
- Distilled Water Device (Nüve ND12)

- Ultra Distilled Water Device (Milipore Simplicity 185)
- Ultrasonic bath (Bandelin RK 100)
- Precision balance (Shimadzu ATX224)
- Water bath (Isolab I602.01.001)
- Refrigator (Samsung RT59QMSW)
-80 $^{o}$C refrigator (Nüve NF 190)
- Spectrophotometer (Microplate Reader) (Biotech Epoch)
- Thermal cycler (BioRad T100)
- Nanodrop (Nabi NB1-A-190301)
- PCR Device (Applied Biosystems StepOnePlus Real-Time PCR System)
- Blok heater (Jeio Tech AAHJ111K05)
- Magnetic stirrer (Benchmark H4000 HSE)
- Liquid Nitrogen Tank (Nuaire NU7105)
- Transfer Device for western blotting (BioRad Trans Blot Turbo System)
- İmaging systems (Syngene GBox Chemi XRQ)
- Fluorescence Microscope (Zeiss Axio Vert A1)

### 2.1.3. Chemicals

All chemicals used in this thesis were commercially purchased from Sigma and Merck companies.

## 2.2. Methods

The procedure can be summarized in the specific aptamer selection studies and experimental processes for CD62.

### 2.2.1. Building Aptamer by SELEX

Oligonucleotides library and primers were purchased from Integrated DNA Technologies (USA). Also we get recombinant p-selectin (rCD62,) protein by using commercial (228-11332-2 Raybiotech). All procedures were done at room temperature. ssDNA aptamer library was

selected as 5′- GATAGAATTCGAGCTCGGGC-N25- GCGGGTCGACAAGCTTTAAT- 3′) at 95 °C for 5 min was heated and then at room temperature for 30 min cooled for refolding in 200 µL of SELEX buffer (20 mM Tris at pH 7.5 with 150 mM NaCl, 1 mM MgCl2, 2.7 mM KCl and 0.005% NP-40). The aptamer library was incubated with 1 µg of 6X histidine tagged rCD62 and of Ni-NTA beads for 30 min with shaking, after that a centrifuge is used to remove unbounded ssDNAs which bind to beads and 6X histidine tag rCD62. After that, the pellet was incubated for 30 min with appropriate concentrations (500 nM for cycle 1, 100 nM for cycles 2−5, 10 nM of cycle 6 and 1 nM for cycle 7) of 6X histidine-tagged rCD26 protein. Followed by washing them three times with SELEX buffer (1 mL), then the beads that contain the rCD62-bound sequences were incubated with a solution containing 1 M NaCl and 10 mM NaOH for 5 min with shaking and undergoing to phenol extraction. PCR the eluted samples were amplified, using Taq polymerase in 2X PCR master mix using forward (5′-GGAGCGTTAGCCAAGGC-3′) and reverse (5′-AACCGCGGACAAAGTGCTCC-3′) primers (95 °C for 5 min, 20 cycles (95 °C for 30 s, 58 °C for 30 s and 72 °C 30 for seconds) and 72 °C for 3 min). After the precipitation and centrifugation of ethanol to recover PCR product, ssDNA aptamers were prepared. After that ssDNAs were purified by ethanol precipitation and phenol extraction. Aptamer quantitation was made using nanodrope and propagation procedures were repeated. After quantification it used for the next SELEX selection cycle.

### 2.2.2. Determination of Binding Affinity (KD)

Selection Buffer (SB: 100 mM NaCl, 20 mM Tris-HCl [pH 7.6], 2 mM MgCl2, 5 mM KCl, 1 mM CaCl2) Each aptamer candidate was heated, To examine the aptamer binding ability of candidates, a premix containing 1 nM of the aptamer and 250 nM of the extra MgCl2, in SB, was prepared for each oligonucleotide. After that 85 µL of the premix was added to the microplate wells containing 10 µL of SB, or 1 nM rCD62p solution in SB, each in triplicates. And this mix was incubated at room temperature for 30 min, followed by adding 5 µL of the SYBR Green I solution in SB, to each well. Incubation for 2 h. After that, and by using the microplate reader, the fluorescence of the SYBR Green I (excitation at 480 nm, emission at 520 nm) was recorded. Measurements were performed in duplicates. The DNA concentration of 5 nM was fixed. The bank library was used as the non-binding control. Measurements were

performed in triplicates. Concentration of rCD62p for the Kd measurement was varied from 0.5 to 300 nM, the fluorescent signal was plotted against the rCD62p concentration. And by fitting the binding data to a one-site saturation equation in the Graphpad software 7.04, Kd was calculated.

### 2.2.3. Direct ELONA TEST Protocol

This process was performed by steps:

**Coating antigen to microplate**

**1.** rCD62 and commercial human plasma (purchasing) were diluted to a final concentration of 20 µg/ml in PBS (1.16 g $Na_2HPO_4$, 0.1 g KCl, 0.1 g $K_3PO_4$, 4.0 g NaCl in 500 ml distilled water pH 7.4) or other carbonate buffer (3.03 g $Na_2CO_3$, 6.0 g $NaHCO_3$ in 1000 ml distilled water pH 9.6). and by pipetting, 50 µl of the CD62 dilution was added in the wells of the plate.

**2.** Plate was covered with an adhesive plastic and incubated at room temperature for 2 h.

**3.** After discharged in to the wells, it was washed with 200 µl PBS.

**Blocking**

**4.** Remaining protein-binding sites in the coated wells were blocked by adding 200 µl blocking buffer (serum, non-fat dry milk, casein, gelatin in 1% PBS).

**5.** Plate was covered with an adhesive plastic and incubated for 2 h at room temperature.

**6.** After discharged in to the wells, it was washed with 200 µl PBS.

**Incubation with the Aptamer**

**7.** By adding 100 µl of the Aptamer, plate was covered with an adhesive plastic and incubated for 2 h at room temperature.

**8.** After discharged in to the wells, it was washed four times with 200 µl PBS.

**Detection**

**9.** 100 µl of the HRP solution were added per well.

**10.** 100 µl of stop solution were added to all wells.

**11.** The absorbance was taken 450 nm wavelength.

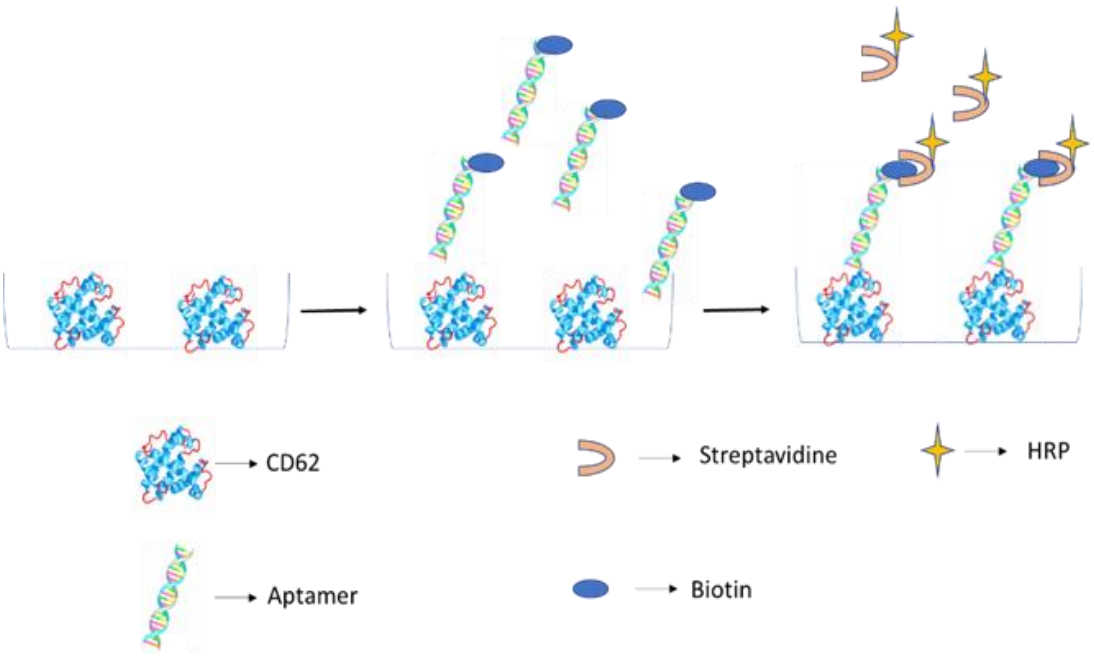



**Figure 11.** The binding between CD62 and the aptamer within ELONA test.

### 2.2.4. Western Blotting Protocol

This process was performed by steps:

#### 2.2.4.1 BCA Protein Determination

Samples collected with the blasting solution were loaded in 2 replicates into a 96-well ELISA plate in accordance with the BCA protein assay kit. The prepared BCA solution was added on them and left for 30 minutes incubation at 37 ° C. At the end of the time, absorbance were taken at 562 nm.

Prepared solutions:

BCA solution: Reagent A and Reagent B were prepared in a ratio of 1:50.

BCA standard solution: 2 mg / ml stock solution, batch with PBS as 1000 µg / ml, 500 µg / ml, 250 µg / ml, 125 µg / ml, 62.5 µg / ml and 31.25 µg / ml dilution was made.

25 µl of sample or 25 µl of standards were loaded into a 96-well elisa plate. 200 µl BCA solution

was added onto the loaded wells. It was incubated at 37 $^{0}$C for 30 minutes. After incubation, measurement was taken at 562 nm in Termo Labsystems - Multiskan Spectrum plate reader.

### 2.2.4.2. Immunoblotting Assay

Prepared solutions:

4X stacking gel solution: 0.5 M Tris, 0.4% SDS, pH: 6.8

4X Separating gel solution: 1.5 M Tris, 0.4% SDS, pH: 8.8

30% Acrylamide-bis solution

10% Ammonium Persulphate solution (APS)

10% SDS solution

TEMED

2X Sample loading buffer: 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.004% bromphenol blue and 0.125 M TrisHCl, (pH 6.8)

Electrophoresis running solution: 5 m MTris, 38.4 mM Glycine, 1% SDS

Transfer solution: 5 mM Tris, 38.4 mM Glycine, 20% Methanol

Blocking solution: 2.5% BSA solution

TBST: 20 mM Tris, 154 mM NaCl, 0.1% Tween 20

Primary antibody (1: 1000): CD62

Secondary antibody (1: 3000): (HRP conjugated sc-2030 (R), sc-2020 (G), sc-516102 (M)

Chemiluminescence Reagent: Santa Cruz (sc-2048)

Film Development solutions

Item 4% Stacking Gel 12% Separation Gel 15% Separation Gel

4X batching solution 1.25 ml

4X Separation solution - 2.5 ml 2.5 ml

30% Acrylamide-bis 0.65 ml 4 ml 5 ml

10% SDS 50 µL 100 µL 100 µL

APS 25 µL 50 µL 50 µL

TEMED 5 µL 10 µL 10 µL

Distilled water 3.05 ml 3.4 ml 2.4 ml

After the gels are prepared and polymerized between the glasses, they are placed in the tank. The protein (CD62) samples was determined were boiled at 95$^{o}$C for 5 minutes with sample loading buffer prepared at a ratio of 1: 1. Samples containing protein at the rate of 25 µg / mL were loaded into each well. 5 µl of standard (Intron, 24052) was loaded into one well. In electrophoresis, samples were first run at 100 Volt current for 1 hour.

Immunoblotting was performed with a semi-dry system in order to pass the proteins in the gel to the membrane after electrophoresis. PVDF membrane was placed on blotting paper. By carefully placing the gel on the membrane, blotting was carried out at 25 Volt current and 1 amp resistance for 30 minutes.

After the PVDF membrane transfer of the proteins, the membrane was blocked with BSA at room temperature for 2 hours. After 2 hours, it was incubated with the CD62 Aptamer at + 4$^{0}$C overnight on the shaker. After the incubation, it was washed 3 times with TBST. The membrane was incubated with secondary antibody (HRP) for 2 hours on the mixer at R.T. After the incubation, it was washed 3 times with TBST. It was then incubated with the solution containing the chemiluminescence substrate (Santa Cruz Immuno Cruz ™ Western Blotting Luminol Reagent: sc-2048) in the dark for 1 minute. The bands were analyzed using the Syngene imaging system.

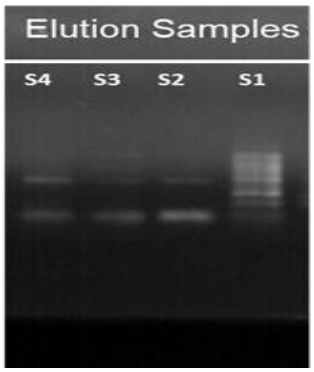
### 2.2.5 ELONA Kit Validation and Quality Testing

Validation tests, which are generally found in kit studies, were applied for the aptamer-based CD62 test kit developed in this thesis. ELONA validation tests are based on the methods valid for ELISA tests. Sensitivity, specificity, intra and inter-assay coefficient, recovery and linearity of variation were also calculated. Validation tests were performed using recombinant rCD62 and it was checked whether the diagnostic kit works in a commercially purchased real human sample (Human Sodium citrate plasma, Sigma P9523, USA).

# 3. RESULTS

## 3.1. Cell SELEX Studies

After fifteen cycles of positive negative selection we identified three molecular probes that were highly specific for the p-selectin. The elutions used in making the selection were controlled by running on agarose gel. In these elections, the specific band S3 elution was choosen and used for aptamer designs after sequencing (Picture 1). After sequencing, 3 different aptamer for CD62 have been designed by bioinformatic analysis and the sequences belonging to the designed aptamers are given table 3. We sequenced these three aptamer candidates and studies. The sequences of aptamers APT1, APT 2 and APT 3 that bind to the p-selectin protein are respectively (Table 3).




**Picture 1.** Agarose gel images of aptamers specific bonded to CD62

**Table 3:** The sequences of aptamers for CD62

| **Aptamer Number** | **Sequences (5'-3')** |
|---|---|
| APT1 | 5'-GGAGCGTTAGCCAAGGCCGCTTGATTAACCGCGGACAAAGTGCTCC-3' |
| APT2 | 5'-GGAGCGTTAGCCAAGGCCGCGGCGTAAACCGCGGACAAAGTGCTCC-3' |
| APT3 | 5'-GGAGCGTTAGCCAAGGCTACGGGATTAACCGCGGACAAAGTGCTCC-3' |

Since aptamers can be thought of as comprising ‘‘shape’’ libraries, we generated potential secondary structures for each of the selected aptamers and drawn for each of the predicted structures (Figure 12, 13, 14).

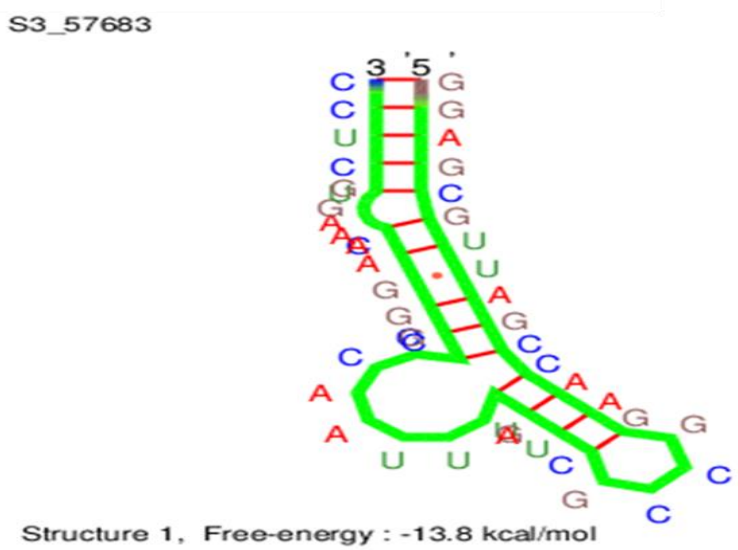



**Figure 12.** The genetic map of Aptamer 1.

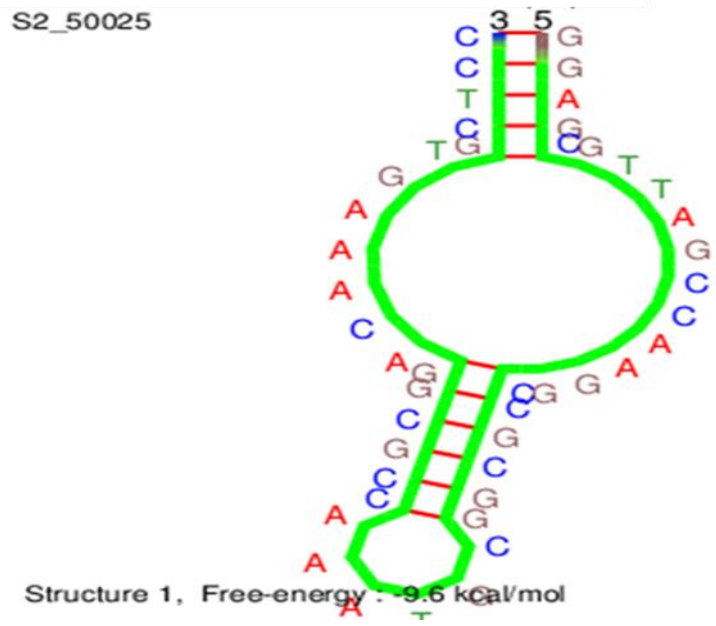



**Figure 13.** The genetic map of Aptamer 2.

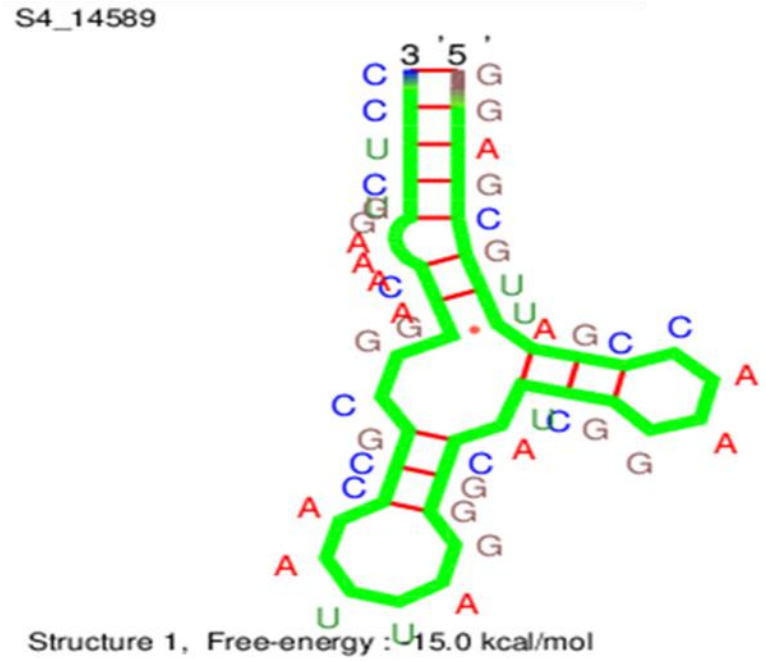



**Figure 14.** The genetic map of Aptamer 3.

### 3.2. Aptamer Binding Affinity Characteristics

To determine the binding equilibrium of the aptamers to rCD62, we incubated rCD62 with incremental concentrations of the aptamers and measured the fluorescence of individual well by Sybr Green I (Figure 15, 16, 17). Based on our analysis, all aptamers evaluated showed high affinity binding to their epitopes in Table 4: Aptamer S1 (Kd = 41.99±6.96 nM); Aptamer S2 (Kd = 18.15±2.36 nM); and Aptamer S3 (Kd = 64.18±8.33 nM). In addition, Gibbs energy, which is a measure of the energy required to separate from the bonded structure, was respectively found to be -13.8 kcal/mol, -9.6 kcal/mol, -15.0 kcal/mol for S1,S2 and S3 (Table 4).

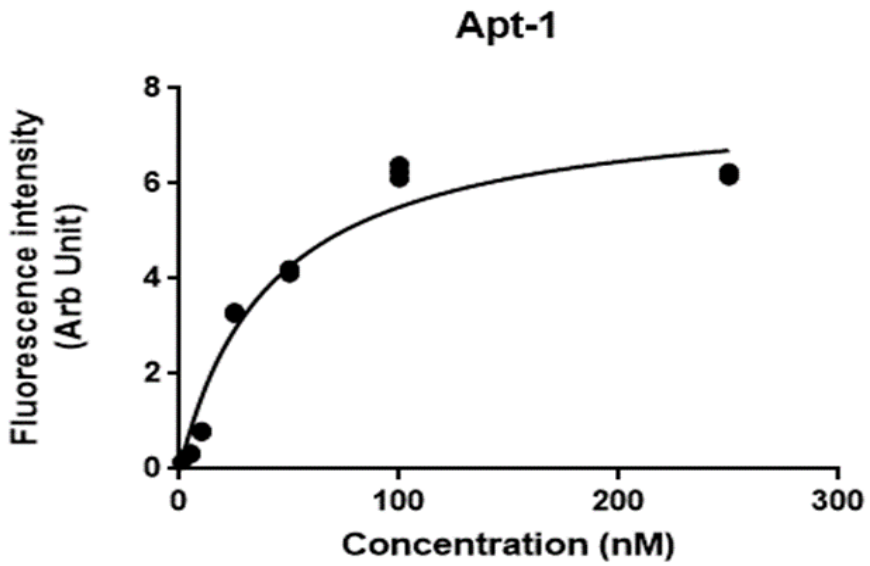



**Figure 15.** Binding equilibrium for Aptamer 1 (Apt-1)

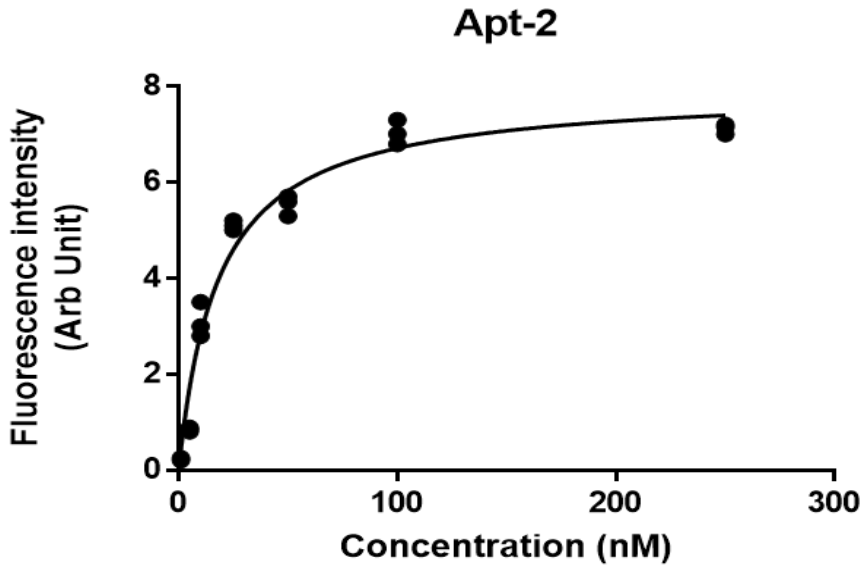



**Figure 16.** Binding equilibrium for Aptamer 2 (Apt-2)

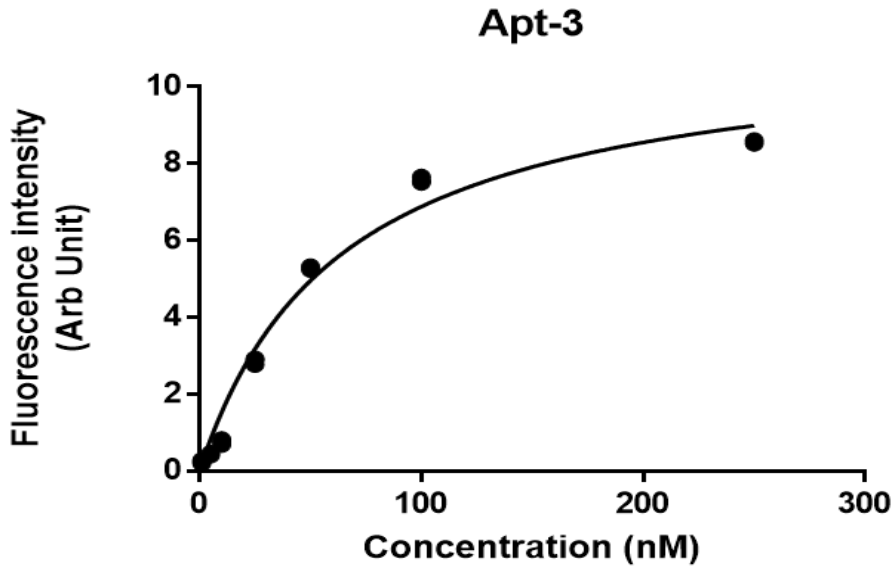



**Figure 17.** Binding equilibrium for Aptamer 3 (Apt-3)

**Table 4.** The range of Kd and ΔG values for the three Aptamers.

| Aptamer Number | Sequences (5'-3') | ΔG Value (kcal/mol) | Kd Value (nM) |
|---|---|---|---|
| APT1 | 5'-GGAGCGTTAGCCAAGGCCGCTTGATTAACCGCGGACAAAGTGCTCC-3' | -13.8 | 41.99±6.96 |
| APT2 | 5'-GGAGCGTTAGCCAAGGCCGCGGCGTAAACCGCGGACAAAGTGCTCC-3' | -9.6 | 18.15±2.36 |
| APT3 | 5'-GGAGCGTTAGCCAAGGCTACGGGATTAACCGCGGACAAAGTGCTCC-3' | -15.0 | 64.18±8.33 |

As seen in Table 4, the aptamer with the lowest KD value is Apt-2. Apt-2 was selected for kit development studies and was used as a candidate aptamer because of its high binding affinity to CD62.

### 3.3. ELONA Kit Validation and Quality Tests Results

#### 3.3.1. Sensitivity and Spesifity Tests

By applying the sybr green method, the binding affinity of the three different CD62 aptamers Apt-1 Apt-2 and Apt3 with BSA as negative control was measured separately in equal

concentrations. The aptamers we selected were specific for CD62 and not specific for BSA by comparing the signals on the fluorometer. Unlike BSA, it showed high binding between CD62 protein and aptamers. The highest attachment tendency was found to be Apt-2 and the results were statistically significant.

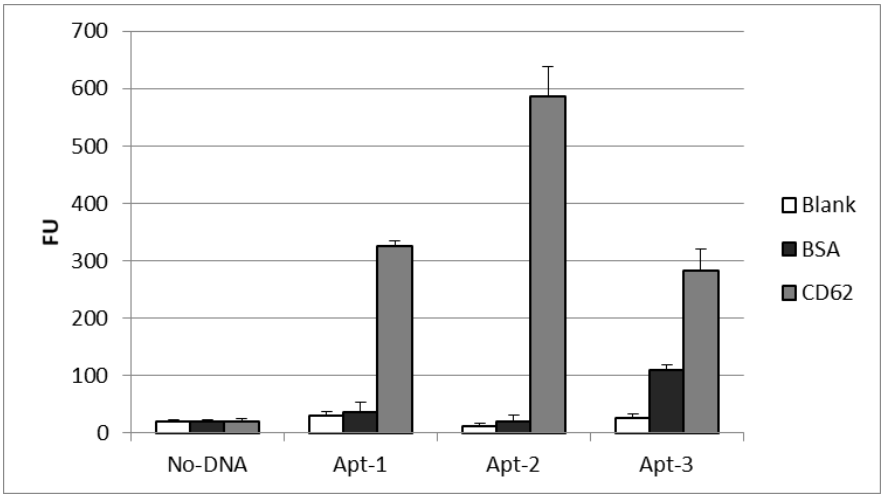
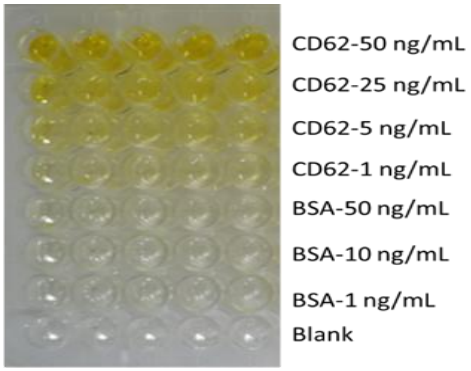



**Figure 18.** Aptamer binding with BSA and CD62 via SYBR green methods.

In the aptamer-based CD62 diagnostic kit, Specificity and Sensitivity tests were performed in the first state with a constant concentration of Apt-2 and different concentrations of recombinant rCD62 and BSA. Then we performed the procedure with different concentrations of Apt-2 and constant concentration of CD62 and BSA, the color change in the samples were measured in the diagnostic kit. In the both states showed a high Specificity and Sensitivity for Apt-2 binding CD62 unlike to Apt-2 binding BSA. BSA and CD62 concentration was used 0.5-50 ng/mL. Sensitivity was calculated 0,30 ng/mL of CD62.




**Picture 2.** The well images of ELONA methods for specificity and sensitivity tests

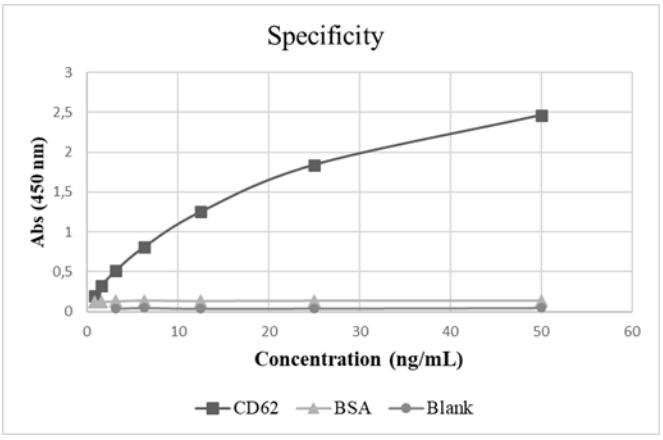



**Figure 19.** Specificity test (Constant Apt-2 concentration 25 nM with changing protein concentration)

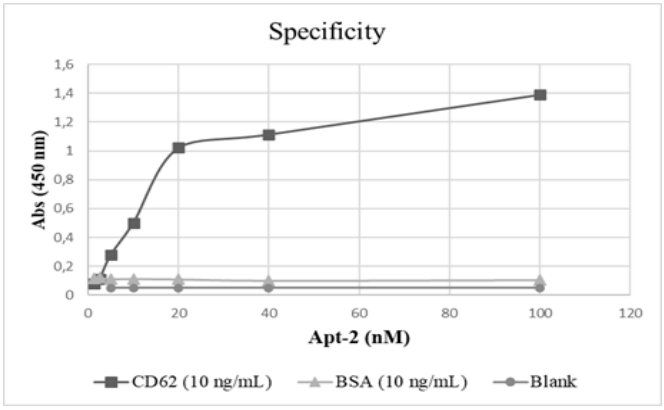



**Figure 20.** Specificity test (Constant CD62 and BSA concentration 10 ng/mL with changing aptamer concentration)

### 3.3.2. Intra and Inter Assay Tests Results

CD62 ELONA kit was evaluated by testing 6 replicates each for three samples in the same assay. The Intra and Inter assay performing showed that the average of Intra-assay was CV % 6,52 and the average of Inter-assay CV % 3,96 (Table 5). As a result of these intra-assay and inter-assay evaluations, the intra-assay results were found to be quite low and effective when compared to the accepted rates in general ELISA tests. (Ratios for general ELISA Intra-Assay:

CV <10% and Inter-Assay: CV <12%). The results of this test show that the samples run in different wells of the plate and comparable results are obtained.

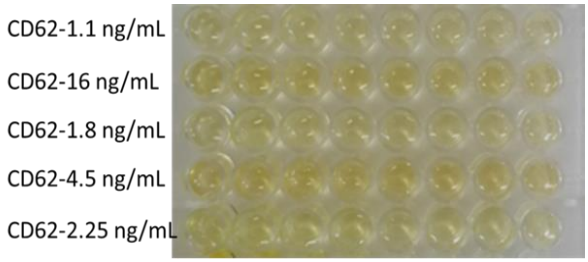
**Table 5.** Intra and Inter assay value of prepared ELONA based test (Accuracy and Precision Value).

| | Intra-assay | | | Inter-assay | | |
|---|---|---|---|---|---|---|
| Sample | 1 | 2 | 3 | 1 | 2 | 3 |
| Measurements Number | 6 | 6 | 6 | 6 | 6 | 6 |
| Average ±SD (ng/mL) | 6.023±0.241 | 9.014±0.625 | 2.146±0.187 | 6.162±0.407 | 9.224±0.240 | 2.676±0.072 |
| %CV | 4.00 | 6.86 | 8.71 | 6.60 | 2.60 | 2.69 |

### 3.3.3. Recovery Tests Results

The recovery test is used to determine whether the indicator to be measured is affected by different sample matrices. Protein-rich matrices such as serum and plasma can affect the ability to accurately measure a marker. In this study, BSA, which is abundant in serum and plasma, was used as matrix. The results of the measurements of rCD62 placed in BSA were calculated (Table 6).

The Recovery test was done by using known concentration with several diluted of rCD62 to show if the analyte detection effected by dilution or not, the test showed that the analyte detection was little affected from dilution to another (Picture 3).




**Picture 3.** The well images of ELONA methods for recovery test

**Table 6.** Recovery results of prepared ELONA based test for CD62 measurement

| **Sample** | 1 | 2 | 3 | 4 | 5 | Negative |
|---|---|---|---|---|---|---|
| **Obtained Value (ng/mL)** | 15,84±0,43 | 4,66±0,14 | 2,36±0,17 | 1,95±0,09 | 1,09±0,05 | 0,004 |
| **Expected Value (ng/mL)** | 16 | 4,5 | 2,25 | 1,8 | 1,1 | 0 |
| **%Recovery** | 95,06±1,81 | 103,58±3,12 | 104,84±7,55 | 107,95±5,05 | 98,94±4,80 | NA |

### 3.3.4. Linearity Test Results

Linear dilution experiments are used to demonstrate the operability of the test kit at different dilution rates. It helps to measure the dynamic range for test kits to be measured in human samples. A well-developed diagnostic test kit should provide information about the precision of the results, even when samples are diluted. In this study, we prepared a sample dilution buffer for dilution of the samples (1, ½, ¼, 1/8). Linearity is important for accurate measurement of its concentration across the dynamic range of the CD62 assay (Table 7). Linearity values in ELISA tests can vary between 70-130% and can be accepted within these ranges.

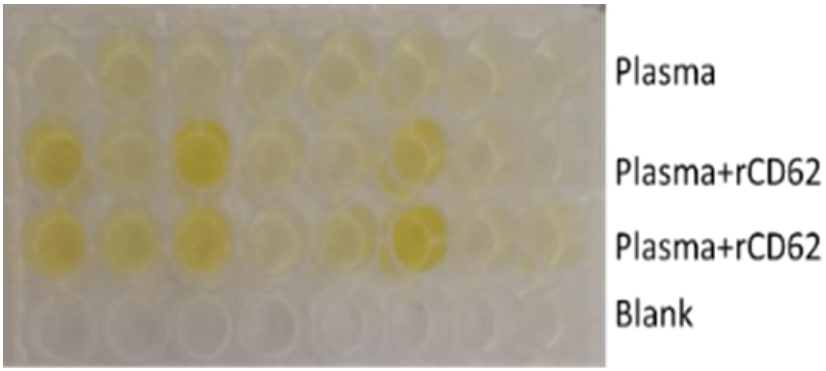
**Table 7.** Linearity values of ELONA test prepared for CD62 measurement

| **Dilution** | **Obtained Value (ng/mL** | **Expected Value (ng/mL)** | **% Expected** |
|---|---|---|---|
| 1 | 24,87 | 25 | 99,49 |
| 1/2 | 12,39 | 12,5 | 99,10 |
| 1/4 | 7,18 | 6,25 | 114,88 |
| 1/8 | 3,54 | 3,125 | 113,17 |

### 3.4. CD62 Detection Results in Real Human Sample

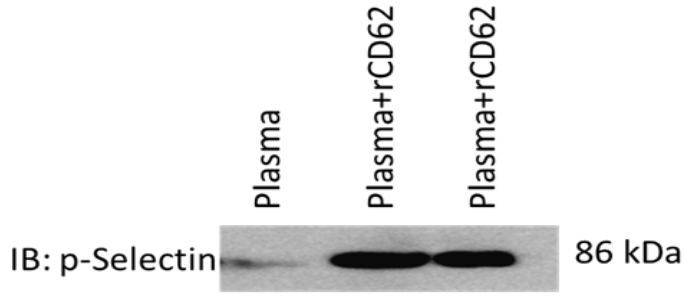
CD62 ELONA kit has been performed in commercial plasma of human samples (Human Sodium citrate plasma, Sigma P9523, USA). Since commercial healthy human samples are used in this study, CD62 levels will be low. In real human samples, CD62 levels increase only with the activation of platelets. A clinically high patient modeling has been performed by adding

rCD62 to plasma test samples. Plasma was used instead of serum because the p-selectin (CD62) is released from platelets. Commercial human plasma contains 4% trisodium citrate as anticoagulant for platelet function. The color changes were observed in samples by adding recombinant rCD62 to the commercial control plasma.




**Picture 4.** The well images of ELONA test for measurement of human plasma CD62 level

We applied the western blot method to show that the proteins that bind to the wells in Elisa are not specific. Plasma samples used here were loaded into wells and western blot was performed using the p-selectin antibody. We have shown that the specific p-selectin antibody detected CD62 in the samples and is accurate in terms of molecular weight. This test showed that the binding between the rCD62 protein and the aptamer in the tests we performed in the ELONA kit will also be in real human samples and is correct.




**Picture 5.** Western blot analysis of expression levels of rCD62 binding in plasma samples

# 4. DISCUSSION

Selectins are member of calcium dependent type-I transmembrane glycoproteins consisting of two to nine consensus repeats, a transmembrane domain, a cytoplasmic tail, an extracellular C-type lectin domain, and an EGF domain (Kansas, 1996). Shortly after their isolation and characterization, selectins were identified as adhesion molecules that play a key role in leukocyte trafficking and hemostasis. P-selectin is continuosly syntesized in endothelial cells of the lung and the choroid plexus, megakaryocytes, thrombocytes and is stocked within Weibel-Pallade bodies of endothelial cells or alpha-granules of platelets, respectively. P-selectin is offered on the surface of activated endothelial cells or platelets through exocytosis of storage granules and their fusion with the cell membrane (Mcever, 2002; Laubli and Borsig, 2010). Expression of P-selectin on platelet surfaces induces recruitment of leukocytes to platelet aggregates, formation of platelet–leukocyte aggregates and has an important role in vascular homeostasis, atherosclerosis and inflammatory leukocyte extravasation.

A soluble form of P-selectin, which is regarded as a proteolytic fragment or a soluble splice variant absent the transmembrane domain, is found in serum and plasma. Over expression of P-selectin has been brought about several inflammatory disorders, including adult respiratory distress syndrome, acute lung injury, ischemia-reperfusion injury, Gram-negative septic shock, thrombotic diseases, malaria, systemic sclerosis, connective tissue disease and rheumatoid arthritis. In addition to these inflammatory diseases, increased plasma levels of p-selectin have been detected in cardiovascular diseases, stroke, diabetes and cancer. Measurement of serum and plasma p-selectin levels is very important for the diagnosis, treatment and follow-up of these diseases (Kneuer et al, 2006; Kappelmayer et al, 2004; Blann et al, 2003).

The first method developed for P-selectin measurement is flow cytometry. This method which is developed in the second half of the 1980s, is used very limitedly due to the need for expensive equipment and specialized personnel (Shattil et al, 1987). Later, Whiss et al, (1997) developed an elisa-based method for p-selectin measurement. In their study, the bottom of the 96-well plates was coated with monoclonal p-selectin antibody and bound the p-selectins released by the plates to these antibodies as antigens. They preferred to use p-nitrophenyl phosphatase as secondary antibody. Nowadays, modern analytical methods such as Maldi-TOF MS, high performance liquid chromatography and surface plasmon resonance are also used for

p-selectin measurement. However, these methods involve some parameters that need to be improved, such as the use of expensive equipment, pre-treatment process, excessive time consumption and low precision (Murray et al, 2001; Gestwicki et al, 2002). All of the described these methods have been developed based on protein-protein interactions.

In recent years, a new measurement method has been developed using the aptamer-protein interaction. Development of aptamer-based methods to measure disease-related biomarkers has gained momentum in recent years. Compared to protein-protein interaction-based methods, aptamer-protein interaction based methods have many superior features such as low molecular weight, high specifity, selectivity, excellent thermal properties, appropriate chemical synthesis in absence of the need for experimental animals, easy chemical alteration with different chemical or florescence probes (Kordasht HK and Hasanzadeh M, 2020).

For the detection of a biomarker by aptamer-based methods, firstly, aptamers that specifically bind to that biomarker must be selected from the aptamer pool. The most commonly used guideline method for this purpose is systemic evolution of ligands by exponential enrichment (SELEX). In the SELEX method, aptamers that specifically bind to a biomolecule are extracted from the aptamer pool. This process is carried out by changing various physical parameters such as pH, temperature, salt concentration. There are several studies in the literature in which DNA and RNA based aptamers that bind specifically to p-selectin have been developed. Faryammanesh et al, (2014) have been isolated the DNA aptamer, which selectively binds to the P and E selectin, using the SELEX method in order to monitor the metastasis formation of cancer cells. The sequence of this aptamer which contains 89 bases is 5′-GCCTGTTGTGAGCCTCCTAACGATTTGGATTTGGGGTGGAGGGTATGGTTTGTGCTG CGTTCATTTCCCATGCTTATTCTTGTCTC-3′ and the binding constant for p-selectin is Kd=95±18 nM. In the other study, Gutsaeva et al, (2011) is find an anti–P-selectin RNA based aptamer (ARC 5690) for the treatment of cell sickle disease via decrease adhesive interactions of blood cells. ARC 5690 contains 33 bases and the sequence is 5’-CUCGCAGACAACCGGAU GAAAUCGACCGGAG-3’. Binding constant of ARC5690 for p-selectin is approximately15 picomolar. For monitoring tumor vascularity, Mann et al, (2010) have been designed an thiosulphate modified aptamer (ESTA-1) that specifically binds to E-Selectin with a different approach. ESTA-1 contains 73 bases and the sequence is 5’-CGCTCGGATCGATAAGC TTCGATCCCACTCTCCCGTTCACTTCTCCTCACGTCACGGATCCTCTAGAGCACTG-3’.

Binding constant of ESTA-1 for E-selectin is 47 nM. Studies on the use of p-selectin-specific aptamers in medicine are increasing day by day (Hahn, 2018; Mercier et al, 2017; Morita et al, 2016). Diaz and coworkers (2015) are used the ARC5692 aptamer as a P-selectin inhibitor to prevent the formation of venous thrombosis. In our study, aptamer-2 which binds CD-62 with the highest affinity, contains 48 bases and the sequence is 5'-GGAGCGTTAGCCAAGGCCGCG GCGTAAACCGCGGACAAAGTGCTCC-3'. Binding constant of aptamer-2 for p-selectin is 18.15±2.36 nM. Aptamer 2 appears to have a lower equilibrium constant, especially when compared to the aptamer designed by Faryammanesh et al. Therefore, with this study, an aptamer that binds to p-selectin with higher affinity was developed.

Sensitivity and specificity are the main parameters used to measure the effectiveness of a test for the measurement of a developed biomarker. If the biomarker to be measured is found in serum or plasma, a specificity test is usually performed against the most abundant protein in serum and plasma, which is mostly serum albumin. For this developed CD62 kit, sensitivity can be expressed as the minimum amount of CD62 that can be measured (Altman and Bland, 1994). In this study, three aptamers were identified that specifically bind to the CD62 protein in the Aptamer pool. In specificity studies, it was determined that these three aptamers, which bind CD62 with high affinity, do not bind bovine to serum albumin. Thus, it was confirmed that specific aptamers were selected to measure CD62 levels in the serum and plasma. When the sensitivity values of elisa-based commercial kits sold for P-selectin measurement (MyBioSource MBS722270, Elabscience E-EL-H0917, Thermo Fisher Scientific BMS-219-4) are examined, it is seen that it is in the range of 0.1-0.2 ng/mL. In our study, the sensitivity value is 0.3 ng/mL and this value is very close to the sensitivity values of commercially available kits.

Precision can be defined as the power of the method to give the same result in replicates of the same sample. Precision is calculated as inter-assay and intra-assay. Acceptable values in precision studies are less than CVs for inter-assay 15% and for intra-assay 10%. Intra-assay averages of commercially available kits (MyBioSource MBS722270, Elabscience E-EL-H0917, Thermo Fisher Scientific BMS-219-4) are less than CVs 5%, 10% and 7.8%, respectively. The intra-assay precision value of the CD62 measurement kit developed by us is CV 6.52%. In the same way, inter-assay averages of commercially available kits (MyBioSource MBS722270, Elabscience E-EL-H0917, Thermo Fisher Scientific BMS-219-4) are less than CVs 7.25%, 10% and 5.4%, respectively. The inter-assay precision value of the CD62 measurement kit developed

by us is CV 3.96%. The CD62 measurement kit developed by us has superior values than standard Elisa kits in the market in terms of both inter-assay and intra-assay.

Linearity is to obtain results that are directly proportional to the amount of analyte in the test sample within a specified range. In linearity measurements, samples are generally diluted in proportions such as 1/2, 1/4, 1/8, 1/16 and necessary calculations are made. The desired linearity values for an optimal kit are between 70% and 130% (Jhang et al, 2004). Linearity values of commercially available kits in the market (MyBioSource MBS722270, Elabscience E-EL-H0917, Thermo Fisher Scientific BMS-219-4) range from 96-108%, 93-109% and 81.3-108.9%, respectively. The linearity values of the CD62 measurement kit developed with this study are between 99.49% and 113.17%. These results are within the limits found appropriate in the literature.

Recovery test is one of the parameters that determine the analytical performance of a measurement. The recovery test is performed to determine how the measurement of analyte will affect in complex fluids such as serum or plasma (Kościelniak and Wietecha, 2002). Recovery values of commercially available kits in the market (MyBioSource MBS722270, Elabscience E-EL-H0917, Thermo Fisher Scientific BMS-219-4) range from 94-103%, 93-109% and 69-81%, respectively. The recovery values of the CD62 measurement kit developed with this study are between 95.06% and 107.95% and these values are very close to the recovery values of commercially available kits.

On the other hand, apart from analytical performance effectiveness, low cost and short-term analysis are the main features that should be found in a test. The average analysis time of the Elisa-based 96-well kits on the market is approximately 5 hours and the average price is approximately $ 600. It is seen that developed this CD62 kit is more advantageous than eliza-based kits with an average of less measurement time and low cost.

As a result, this study was conducted on CD62 protein due to its clinical importance, as P-selectin plays a role in causing widespread complications and disorders including coronary artery disease, stroke, diabetes and malignancies. Early detection of P-selectin CD62 leads to more treatments or longer survival against certain chronic diseases, such as cancer, cardiovascular disease and diabetes. P-selectin CD62 is clinically detected with using antibody by performing Human P-Selectin solid-phase sandwich ELISA (enzyme-linked immunosorbent assay), it will measure the amount of the target-specific antibody pair. The disadvantage of

sandwich ELISA is the difficulties of the antibody optimization. Since each antibody should reacts with a specific epitope on the target protein and does should not react with its partner, so only products specifically tested for sandwich ELISA must be used. Also, antibodies are thermally not stable molecules and can be denatured multiple times with big loss of activity. The other disadvantage of antibody using is that antibody can produce immunogenicity. In antibody-based systems, every production process is both expensive and does not have the same specificity. Today, it is important to develop tests that can be measured directly and obtain the same results continuously. So Aptamer-based test ELONA is very important because it lacks all these defects and is thus able to detect protein with accuracy, specificity, and high sensitivity, in addition to being non-immunogenic and more stable with temperature.

# 5. CONCLUSION AND RECOMMENDATION

Innovative devices and analysis methods have been used in the diagnosis and treatment of various diseases in the world, and researchers have focused on systems with high sensitivity and that will be tested in a short time. Since aptamers are specific and continuously produced oligonucleotides, they have become popular in the development of diagnostic kits in recent years. Methods used to measure thrombocyte functions are expensive and usually give long-term results. In this study, we developed an aptamer-based diagnostic kit that can detect platelet activation in a short time and can be used in clinical studies. With this aptamer-based kit, accurate and early detection of p-selectin molecules involved in many chronic and acute diseases will be possible. The diagnostic kit developed in this study will contribute to the development of innovative methods and will benefit the development of new diagnostic kits with high accuracy, high specificity and sensitivity in world.

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# CURRICULUM VITAE


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**EDUCATİON**

| Degree | Institution | Date of graduation |
|---|---|---|
| Doctora Degree | xxx | xxx |
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**SCHOLARSHİPS and AWARDS**

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**WORK EXPERIENCE**

| Year | Place/Instution | Title |
|---|---|---|
| 2014-2016 | JOSWE Pharmaceutical company | Medical Representative |
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**ACADEMIC PUBLICATIONS**

**1. ARTICLES**

xxx

## 2. PROJECTS

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## 3. CONGRESS PAPERS

xxx

### A) Papers Presented at International Congresses

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### B) Papers Presented at National Congresses

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