NAZARBAYEV
UNIVERSITY

CHARACTERIZATION OF SINGLE-STRANDED DNA APTAMERS AGAINST CARCINOEMBRYONIC ANTIGEN

Nigara Yunussova
(M.Sc., Nazarbayev University)

A THESIS SUBMITTED
FOR THE DEGREE OF MASTER OF SCIENCE IN BIOLOGICAL SCIENCES
DEPARTMENT OF BIOLOGY
SCHOOL OF SCIENCES AND HUMANITIES
NAZARBAYEV UNIVERSITY
2020

DECLARATION

I hereby declare that the thesis is my original work, and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis. This thesis has also not been submitted for any degree in any university previously.

Nigara Yunussova

_____

10 May 2020

ACKNOWLEDGEMENTS

Foremost, I would like to express my sincere gratitude to my advisor Dr. Damira Kanayeva for her support of my study and research, for her patience and motivation. Her instructions guided me throughout my work. Special thanks for her sensitivity and attention to me. She taught me a lot.

Besides my advisor, I would like to thank Marzhan Sypabekova for helping me with SPR and for her powerful support anytime, anywhere.

My sincere thanks go to my friends. I am very grateful that I met them at this university. They made my student's life unforgettable and bright.

Last but not the least, I would like to thank my family members, my parents for spiritual support throughout my life.

TABLE OF CONTENTS

Nazarbayev University, School of Sciences and Humanities, Department of Biology
Master´s Degree Program in Biology

Nigara Yunussova: Characterization of single-stranded DNA aptamers against carcinoebryonic antigen
Master of Science thesis; 52 pages, 3 appendices
Supervisor: Damira Kanayeva, Department of Biology, School of Sciences and Humanities, Nazarbayev University, Nur-Sultan 010000, Kazakhstan
10.05.2020

Keywords: carcinoembryonic antigen, aptamers, ELONA, SPR.

ABSTRACT

Cancer biomarkers play a crucial role in the development of diagnostics tools for cancer detection. Carcinoembryonic antigen (CEA) is a well-known cancer biomarker that is a cell surface glycoprotein, critical in fetal development. Its expression in adult healthy cells is highly associated with colorectal carcinoma and metastasis. Colorectal cancer (bowel cancer or colon cancer) is the third death causing type of cancer, both worldwide and in Kazakhstan. Therefore, there is an urgent need to develop diagnostic assays where aptamers can be employed. Aptamers are artificial recognition agents that have advantages over antibodies because of smaller size, simpler to synthesize and incorporation of modifications, and higher stability allowing high selectivity during detection. Previously, single stranded DNA (ssDNA) aptamers against CEA were selected by SELEX method (Systematic Evolution of Ligands by Exponential enrichment). As a result of twelve SELEX cycles, six aptamer candidates were selected from a pool of ssDNA library with a central 40 nt random region. Predicted secondary structures of selected six aptamers showed similarities in structure between some of them as well as distinct structures. In this study, aptamers against CEA were evaluated based on the binding of the ssDNA aptamers against CEA using ELONA. Obtained results show that the selected aptamers showed a solid specificity and sensitivity values comparing to the non-target proteins (IL-6 and HSA) as well as controls. Future work will be concentrated on the SPR protocol optimization to improve the data regarding affinity. In the case of low signal, aptamers could be modified easily with self-assembled monolayers (SAMs), such as HS groups or magnetic/gold nanoparticles or any other fluorescent reporting molecules. These could be further used in the application of characterized aptamers in the electrochemical impedance spectroscopy (EIS) based aptasensor for the detection of cancer biomarkers. In addition, CEA aptamers on CEA spiked human serum samples will be tested in order to observe how a protein rich medium can affect the detection of the target.

LIST OF TABLES

LIST OF FIGURES AND ILLUSTRATIONS

ABBREVIATIONS

BSA – bovine serum albumin
CAP - capture biotinylated analytes sensor chip
CEA – carcinoembryonic antigen
CRC – colorectal cancer
ssDNA – single-stranded deoxyribonucleic acid
ELISA – Enzyme Linked Immunosorbent Assay
ELONA - Enzyme Linked Oligonucleotide Assay
FDA – Food and Drug Administration
FOBT - fecal occult blood testing
LOD – limit of detection
NAs – nucleic acids
NP - nanoparticles
PCR - polymerase chain reaction
RNA – ribonucleic acid
sHRP - streptavidin-conjugated horseradish peroxidase
SPR – Surface Plasmon Resonance
SELEX - Systematic Evolution of Ligands by EXponential enrichment
TAMs - tumor-associated macrophages

1 INTRODUCTION

Nowadays, cancer is defined as a driving cause of death worldwide and the most significant barrier to increasing life expectancy in each nation (Bray et al., 2018). In 2018, 18.1 million new cases and 9.6 million deaths were recorded globally (Who.int, 2020), and an increase up to 3,093 million cases is expected by 2040 (Who.int, 2020). Lung, breast, and colon cancers are the most three widespread types in terms of incidence and are within the highest five in terms of mortality (Who.int, 2020). In Kazakhstan, there were 33,949 new cases registered in 2018 (Gco.iarc.fr, 2020), where lung, breast, stomach, and colon cancers are the most common ones (Gco.iarc.fr, 2020). Colon cancer is the third most common worldwide, being the third and second most commonly diagnosed both in males and females, respectively (Favoriti et al., 2016). The worldwide burden of colorectal cancer (CRC) is anticipated to increase by 60% to 2.2 million new cases and 1.1 million deaths by 2030 (Arnold et al., 2017). Colon cancer is known as a "silent disease" which usually starts as noncancerous growths, known as polyps, and numerous patients do not complaint until the disease is troublesome to remedy (Vatandoost et al., 2015). Diagnosis of the disease at its early stages with high sensitivity and specificity is one of the fundamental necessities for colon cancer screening (Salehi et al., 2012). As of now, medicine offers several screening techniques for colon cancer detection, including fecal occult blood testing (FOBT), flexible sigmoidoscopy, and colonoscopy (Itzkowitz et al., 2008; Franz et al., 2013). These methods have long been rooted in the world medical practice. However, fear of invasive procedures and abhorrence to bowel preparations present serious barriers to CRC screening (Young & Womeldorph, 2013). Colonoscopy is known as a gold-standard procedure, but its function can be limited by invasiveness and its high cost (Berg & Soreide, 2011; Carmona et al., 2013). Other limitations of the colonoscopy technique are bleeding, the chance of colonic perforation, and erroneousness in the detection of small adenomas (Young & Womeldorph, 2013). The disadvantage of FOBT is a low sensitivity and specificity towards small polyps (Bretthauer, 2011). For these reasons, minimum invasive and most importantly fast and accurate diagnostic methods for cancer detection need to be developed (Vatandoost et al., 2015). Serum biomarkers in this sense are non-invasive screening strategies for the detection of CRC (Vatandoost et al., 2015). Biomarkers are quantifiable characteristics of biological processes that are estimated as signs of different pathogenic processes or pharmacological responses to therapeutic approaches that can be eventually measured (Strimbu & Tavel, 2010). Nucleic acid-based biomarkers such as gene mutations or polymorphisms and quantitative gene

expression analysis, peptides, proteins, and other small molecules can be used as non-imaging biomarkers, which enable them to be measured in biological samples (Huss, 2015). Different types of biomarkers can be used for a specific purpose, for example, disease prognosis biomarkers (cancer biomarkers), biomarkers for monitoring the clinical response to intervention and staging of disease biomarkers (Kroll, 2008).

Serum biomarkers can direct numerous neglected needs in CRC screening, like high sensitivity, safeness, availability, convenience, and cost of the procedures (Creeden et al., 2011). New technologies allow the detection of tumour markers in blood or other body fluids. Critical pathways in the human organisms, such as cell growth, apoptosis and inflammation include proteins that can be used as biomarkers for colon cancer detection (Creeden et al., 2011). Carcinoembryonic antigen (CEA) is a biomarker as of now acknowledged for the detection of CRC in patients (Compton et al., 2000). CEA is hence broadly used as a marker of epithelial malignancies, particularly for colon tumours (Ilantzis et al., 2002).

Nowadays, modern medical technologies have established devices known as biosensors. These devices catch the biological signal and translate it into a measurable electrical signal (Ali et al., 2017). Biosensors can distinguish certain tumor biomarkers by way of measuring the level of some proteins highly expressed by tumors (Bohunicky & Mousa, 2011). The device establishes a few focal points: firstly, it is specific due to the immobilized system used in it; secondly, rapid and continuous control is possible; thirdly, response time is short (Koyun et al., 2012). Indeed, the fact that the complexity and differing qualities of cancer have postured numerous challenges within the clinical field, biosensor technological know-how can grant rapid and accurate results, whilst maintaining its cost-effectiveness (Bohunicky & Mousa, 2011).

This study reports findings on the characterization of the previously selected aptamers against CEA and their further application in the development of an electrochemical impedance aptasensor for the rapid detection of the CEA biomarker. Among different types of biosensors, an electrochemical biosensor offers significant advantages, being easily miniaturized, portable, low cost, and simple-to-operate (Grieshaber et al., 2008). A biosensor, where aptamers are used as bio-recognition molecules is known as an aptasensor. In this study, characterization of ssDNA aptamers against CEA was carried out using an Enzyme-Linked Oligonucleotide Assay (ELONA) and a Surface Plasmon Resonance (SPR) technique. RNA nature aptamers permit single-shot measurements, whereas ssDNA ones are excellent for designing reusable aptasensors (Sassolas et al., 2009). Artificial affinity reagents based on oligonucleotides – aptamers have progressively been used as diagnostic tools and can be

easily obtained by the *in vitro* or *in vivo* selection procedure, named as the systematic evolution of ligands by exponential enrichment (SELEX). Being 10-100 smaller than antibodies, aptamers offer a great opportunity to place a significantly larger number of them on the surface of an aptasensor in a smaller area (Debnath et al., 2010). It increases the effectiveness of the detection of various biomarker proteins using a significantly smaller amount of diagnosed fluid, such as, for example, blood serum. Because of exclusive three-dimensional conformation, aptamers can attach to any biological molecules, which creates the basis for the development of effective diagnostic tools (Yang et al., 2011). Due to implicit capacity of all nucleic acids to denature and re-nature, aptasensors can be used more than once, repeatedly, without loss of its sensitivity (Feng et al., 2011; Ocana et al., 2012).

It becomes clear that we need to improve diagnostic assays, where aptamers can be employed. Optimization of the assay and characterisation of the sensitivity of CEA aptamer were carried out, which will allow further use of selected aptamer in the development of an electrochemical impedance aptasensor resulting in an abundant contribution to the field of biosensors for the detection of CRC serum biomarkers.

1.1 Carcinoembryonic antigen (CEA)

An antigen that was discovered for the first time both in fetal colon and colon adenocarcinoma in 1965 by Gold and Freedman is now known as the carcinoembryonic antigen (CEA) (Gold & Freedman, 1965). Since the protein was found only in the tumor and embryonic tissues, it was termed as the carcinoembryonic antigen (Duffy, 2001). A radioimmune test for measuring serum CEA concentration was proposed four years after the discovery of protein by Thomson et al. (1969). When researchers concluded that the CEA level in blood was around corresponding to the size of the tumour, efforts were made to show that it can be used for the colon cancer detection and other tumours of the gastrointestinal tract (Jessup & Thomas, 1989). However, a consequent work revealed that CEA is also produced in healthy tissues (Clifford et al., 1989). CEA is overexpressed in numerous gastrointestinal cancers, such as colorectal (Ning et al., 2018), esophageal (Yi et al., 2009), gastric (Feng et al., 2017), pancreatic (Imaoka et al., 2016), hepatocellular (Edoo et al., 2019) and gallbladder (Wen et al., 2017) carcinomas, breast (Wang et al., 2017) cancer, lung (Rakhra et al., 2017) cancer, the female reproductive tract cancer (ovarian, cervical and endometrial carcinomas) (Guo et al., 2017), and prostate cancer (Ordònez, 2003). The most important reason why CEA is an effective biomarker for colon cancer and other malignancies,

possibly because it has a reasonably confined expression in healthy tissues, it is a stable molecule and it appears in excessive levels in malignancies (Hammarström, 1999).

CEA encoding gene is presently classified as a part of the immunoglobulin supergene family (Thompson et al., 1991) and includes 29 genes, being clustered on chromosome 19q (Duffy, 2001).

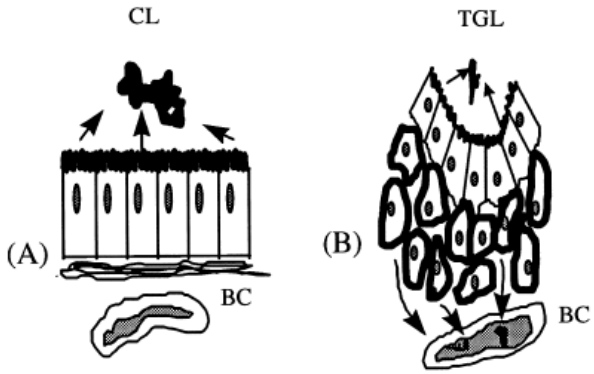

Figure 1. CEA excretion pathways in normal and cancer colon. (A). In the normal colon polarized columnar epithelial cells express exclusively CEA on the apical surface and release it into the colon lumen (CL). CEA has no access to blood capillaries (BC). (B). In colon cancer, epithelial cells encountering 'blind' tumor gland lumens (TGL) are partially polarized and release CEA into the lumens. Adopted from Hammarström, 1999.

Analysis of the liver metastasis revealed that CEA has a molecular weight of 180-200 kDa and it is a glycoprotein containing approximately 60% carbohydrates (Thomas et al., 1990). CEA might act as an adhesion molecule, equally to both ICAM-1 and ICAM-2, because of structural similarities to certain immunoglobulin-related proteins (Duffy, 2001) and, thus, mediates homotypic cell aggregation (Benchimol et al., 1989). In healthy human colon, CEA is localized on the apical surface of mature enterocytes, and the majority of it is produced in the colon (**Figure 1A**) (Hammarström, 1999). CEA normally disappears with feces, hence, only a low level of it is seen in healthy individuals (Hammarström, 1999). In the case of CRC, tumor cells which usually lost polarity and do not have the basal lamina, begin uncontrolled multiplication and as a result, CEA is collected on the cell surface (**Figure 1B**) (Hammarström, 1999). Normal ranges of CEA levels in healthy people are up to 2.5 ng/mL and 5 ng/mL for nonsmokers and smokers, respectively (Tothill, 2009). CEA can be found in columnar epithelial cells and goblet cells in the colon (Hammarstrom, 1997), in mucous neck cells (Hammarstrom, 1997) and pyloric mucous cells (Vetter et al., 2015) in the stomach, in squamous epithelial cells of the tongue, esophagus and cervix, in secretory epithelia and duct

cells of sweat glands and epithelial cells of the prostate (Lamerz et al., 1992; Hammarström et al., 1997).

Additional evidence has shown that enforced CEA overexpression is related to anoikis, which is a form of apoptosis induced by detachment from cell-matrix, and, thus, reinforces a process of metastasis (Yan et al., 2016). The liver is the main site of metastasis (Wu et al., 2010); hence, prognostic data may also be provided by CEA in patients whose liver metastasis is developing, and following curative resection for colorectal cancer (Duffy, 2001). CEA levels should return to a normal range within 4-6 weeks after the successful surgical elimination of colon cancer (Filella et al., 1994). Permanent measurement and monitoring of circulating CEA level are valuable in patient's condition diagnosis and prognosis due to its drug resistance, strong correlation with cancer progression and metastasis (Lee, 2017). However, provided data do not indicate whether CEA can be associated with metastasis, since serum CEA could be related to other factors, as a differentiation state, which may define whether CRCs will metastasize (Jessup & Thomas, 1989). Jessup and Thomas (1989) demonstrated in their study that CEA strengthens the metastatic potential of two weak metastatic CRCs in the nude mouse, nevertheless, it did not make a non-metastatic CRC metastatic either a highly metastatic CRC more aggressive. Further, since microscopic metastases are localized in the organs which eliminate CEA from the blood, the elevations of serum CEA may happen in patients without clinically obvious metastases (Thomas et al., 1975; Toth et al., 1989).

1.2 Aptamers

Long-time, nucleic acids (NAs) were considered only as chemical compounds whose principal functions are the storage of hereditary material (DNA), also the transfer of this information from gene to a protein product (RNA). A significant contribution to the confirmation of nucleic acids' multi-functionality (Ferreira & Missailidis, 2007) has been the discovery of oligonucleotides, which specifically are capable of catching various target molecules (Ni et al., 2017). Now, these oligonucleotides are known as aptamers. Aptamers are RNA or single-stranded DNA (ssDNA) nature molecules that can bind to any target with high affinity and specificity by folding into a three-dimensional structure (Nezlin, 2016). The target molecules can be either complex protein structures or simple inorganic molecules and even intact cells (Lakhin et al., 2013). The reason for high affinity and specificity (Ikebukuro et al., 2004) of synthetic oligonucleotides to various target molecules is their three-dimensional structure (**Figure 2**) (Darmostuk et al., 2015).

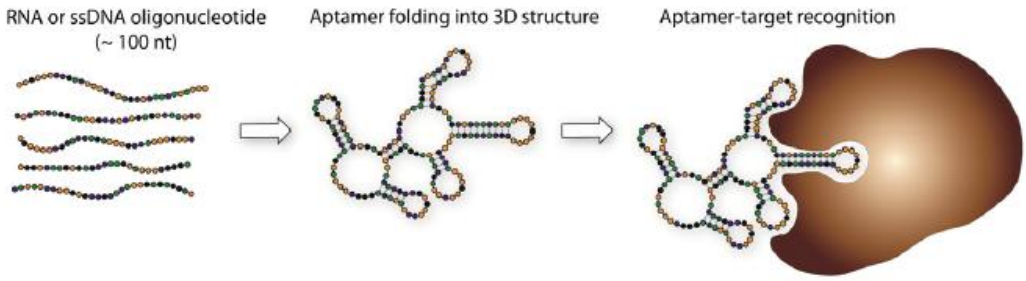

Figure 2. A graphical representation of an aptamer – target interaction. Adopted from Darmostuk et al., 2015.

Aptamer molecules can be synthesized by *in vitro* selection or evolution processes (Romero-López & Berzal-Herranz, 2017). In 1990, the bases of a new *in vitro* technology for the identification of RNA molecules, which are able to specifically bind to a target was described by two independent research groups (Ellington & Szostak, 1990). The principals of the *in vitro* selection process were established by Tuerk and Gold and then was called SELEX (Systematic Evolution of Ligands by Exponential enrichment) (Tuerk & Gold, 1990).

The SELEX method principally contains three steps: selection, partitioning, and amplification (Zhuo et al., 2017). A library of synthetic molecules consists of up to 60 oligonucleotides in a random area, flanked by short invariable regions, usually used to anneal primers during the PCR (Darmostuk et al., 2015). A schematic overview of an *in vitro* aptamer selection using a SELEX technique is depicted in **Figure 3**. Step one allows binding of high-affinity aptamers to the target molecules and for these several incubations of a specific target with a library is needed; the second step proceeds with the elution of high-affinity from low-affinity binders; and the last third step involves amplification of high-affinity candidates by polymerase chain reaction (PCR) (Mallikaratchy, 2017). Potential aptamer candidates, which are collected from several selection series, consequently, are sequenced and their binding kinetics can be assessed by different methods (Darmostuk et al., 2015). It was originally successful to establish the SELEX method in developing hundreds of aptamers for usage in diagnostics and therapeutics aims (Jayasena, 1999). However, the only one aptamer for neovascular age-related macular degeneration - Macugen (pegaptanib) has been approved by the FDA (Shima et al., 2006).

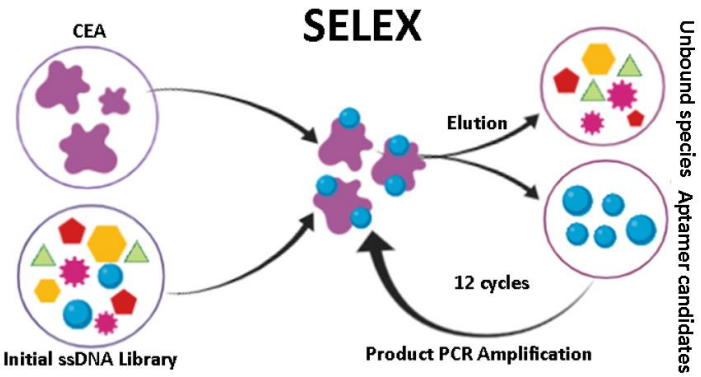

Figure 3. A schematic overview of an *in vitro* aptamer selection using a SELEX technique. *In vitro* selection starts with the generation of a random ssDNA molecules library. Then target ligand is introduced to a library and sequences demonstrate affinity towards the target molecule. High-affinity binders are consequently isolated from any unbound sequences. The potential aptamer candidates are then collected and amplified by PCR. Multiple rounds are completed until the library converges on to a collection of sequences with high affinity for the target molecule.

Ellington and Szostak coined the term aptamer to define the selected molecules by the application of this method (Ellington & Szostak, 1990). The term aptamer was coined by Ellington and Szostak and it defines the selected molecules by the application of this technique (Ellington & Szostak, 1990). For their mode of action, aptamers are also called chemical antibodies (Cerchia, 2018); however, unlike antibodies, they exhibit several advantages: aptamers are stable molecules; aptamers production is comparatively cheap; aptamers can be regenerated, without damage to the specificity and they are smaller in size (~3 nm) than antibodies (10–15 nm) (Que-Gewirth & Sullenger, 2007). Despite these benefits, the fact that the composition of nucleic acid-based aptamers (ssDNA, RNA) is receptive to nuclease-mediated cleavage, and the polyamide backbone of peptide aptamers is subjected to protease directed degradations, hence the lack of stability of aptamers in biofluids severely disrupt their practical application (Wang et al., 2011). In order to overcome this problem, conjugation with different functional groups such as biotin or fluorophores, without loss of their specific activity, can be used to modify aptamers (Nezlin, 2016). The use of aptamers instead of antibodies in ELISA procedures not only simplifies detection of a protein but also provides much greater sensitivity (Lakhin et al., 2013).

Aptamers can be widely used as bio-recognition elements in the development of the aptasensors (Meirinho et al, 2017). The size of aptamers (10 - 100 times smaller than antibodies) makes it possible to place a significantly larger number of them on the surface of

an aptasensor in a smaller area (Lakhin et al., 2013). It increases the effectiveness of the detection of various biomarker proteins using significantly less diagnosed fluid, for example, blood serum. In addition, aptasensors can be used more than once, but repeatedly, without loss of sensitivity, due to the denaturation inherent in all nucleic acids and renaturation (Feng et al., 2011; Ocana et al., 2012). Several conducted studies have shown that sensitivity of an aptasensors can be improved by the way of chemical modification of aptamers. For example, in the study conducted by Sassolas et. al. (2009), catalytic labels, metal nanoparticles (NP) such as Pt NPs (Polsky et al., 2006) or Au NPs (Wang et al., 2009) were used for signal amplification in the fabrication of the amperometric aptasensors for the detection of thrombin. Thus, the sensitivity of an aptasensor could be increased by the presence of nanoparticles on the electrode surface to produce a signal enhancement (Lohndorf et al., 2005; Li et al., 2008). The use of aptamers in diagnostics has significantly fewer restrictions than in therapy. Taking into account all the advantages of aptamers, we can say that they will soon take a worthy place both in the tool kit of a modern scientist and among therapeutic and diagnostic drugs.

no.	Sequence	Sensitivity/Specificity	Methods and results in brief	Reference
1	many aptamers: CEA8, CEA59, CEA56-1 , CEA54, CEA65, CEA57-1 , CEA57-2, CEA42-2, CEA47, and CEA 49	n/a	ELONA - binding specificity to CEA EMSA – confirm specific binding to CEA SPR – the affinity of CEA aptamers Flow cytometry - binding to CEA+ and CEA- cells using Cy5 labelled aptamers Disclosed are novel aptamers to TNFα and CEA, and their use in a variety of therapeutic and diagnostic methods and uses.	Gariepy et al., 2013
2	a.TACCAGCTTATT CAATT (original Left primer) b.AGGGGGTGAAG GGATACCC (G rich motif)	n/a	SELEX – aptamer selection PCR – amplification FP - test the affinity of several oligonucleotide sequences to CEA Disclosed herein are aptamers that comprise a nucleic acid sequence that has a specific affinity for a target.	Smith CL, 2013
3	not given (3 aptamers)	n/a	SELEX – aptamer selection CI - investigation of cell bound with aptamer, PCR - cloning and sequencing Three aptamers were selected to binding assay with target cells. These aptamers were confirmed to have affinity and specific binding for T84 cell line (target cell), showed by confocal imaging.	Correa et al., 2011

4	not given	n/a	SELEX – aptamer selection Ligand mediated target recognition - recognition and binding of polyclonal oligobodies against purified protein Western blot – recognition and binding of polyclonal oligobodies against native protein The CEA-specific aptamer which can bind both the purified and native protein with the high specificity was obtained	Wang et al., 2007
5	a.TCCCGCATCCTCCGCCGTGCCGACCC b.CCCCAGGAAGAACCTACTCACTG	n/a	Flow cytometry -binding specificity of aptamers xCELLigence RTCA SP label-free -ability of aptamers to inhibit CEA-dependent cellular adhesion ELISA- identify aptamers capable of inhibiting homotypic interactions The results highlight the effectiveness of targeting the cell adhesion properties of cancer cells with aptamers in preventing tumor implantation.	Orava et al., 2013
6	not given	n/a	SELEX – aptamer selection SPR - specificity and affinity of the aptamer for CEA protein Using SELEX, RNA aptamer that bound to the PELPK sequence in CEA with high affinity and specificity was identified. The isolated aptamer bound specifically to CEA-positive cells and inhibited interactions between CEA and heterogeneous nuclear ribonucleoprotein M4.	Lee et al., 2012
7	not given	n/a	SELEX – aptamer selection TLC- radiochemical purity and stability of aptamers Radiolabeled aptamer Apt3-amine showed the highest affinity to T84 cells. When evaluated with HeLa cells (CEA−), lower uptake was observed, suggesting high specificity for this aptamer. These results suggest that the Apt3-amine aptamer directly labeled with 99mTc could be considered a promising agent capable of identifying the carcinoembryonic antigen (CEA) present in tumor cells.	Correa et al., 2014
8	5′-SH-ATACCAGCTTATTCAATT-3′	The resulting aptasensor, best operated at a voltage as low as 0.18 V vs. Ag/AgCl, is highly sensitive and has a wide linear range that extends from 0.1 pg·mL−1 to 10 ng·mL−1 of CEA.	TEM- image of the synthesized GNRs CV - electrochemical measurements ELISA - serum samples investigation To enhance sensitivity, gold nanorods (GNRs) were selected as a support for the immobilization of aptamers. The aptasensor was applied to the determination of the cancer biomarker CEA in a sandwich format.	Si et al., 2017

9	not given	n/a	RNAComposer web server - bioinformatic analysis of reported ssDNA aptamer sequences Impedance electrochemical spectroscopy - electrochemical measurements ZDOCK - selection of aptamer among the created library Based on ZDOCK scores, the interaction domain of CEA, and steric hindrance due to glycosylation, two aptamer sequences (G3S1.5 and G2S2.2) were selected. The result showed that the interaction ability of selected aptamers was about 13.5 fold higher than the control. It can be concluded that the selected aptamers have good potential for detection of carcinoembryonic antigen biomarker.	Yarizadeh et al., 2019
10	5′-SH-ATACCAGCTTATT CAATTTTTTTTTTG TCCGTGCTAGAAG GAAACAGT-TAC-3′	Wider linear range was obtained for the determination of CEA (from 0.5 pg mL−1 to 1 ng mL−1) with a detection limit as low as 0.1 pg mL−1.	RCA - signal amplification Cyclic Voltammetry - electrochemical testing The practical applicability of the method was studied by analyzing CEA in human serum samples.	Jiang et al., 2017
11	RNA aptamers a. 5′-GCG GAA GCG UGC UGG GCU AGA AUA AUA AUA and b. 5′-GCG GAA GCG UGC UGG GCU AGG GCG GCG GCG GGA AAA CCA GUA CUU UCG U-3′ AGA AAA CCA GUA CUU UCG U-3	n/a	SELEX – aptamer selection PCR - cloning and sequencing Animal study - tumor volumes measurements BCA assay -total protein concentration measurements Immunoprecipitation–immunoblot assay - bound proteins elution CEA-specific aptamer improved 5-FU sensitivity in chemoresistant colon cancer cells in vitro and in vivo, and thus represents a novel 5-FU adjuvant to overcome the chemoresistance in CRC patients.	Lee JH & Lee S-W, 2019
12	ATACCAGCTTATT CAATT	n/a	Fluorescence Microscopy - detect the binding of DNA aptamers to CEA Aptamers bind specifically to CEA on the surface of cancerous cell line: MCF7 (human breast adenocarcinoma) by fluorescence microscopy. CEA specific DNA aptamer does not bind to the surface of a CEA negative cell line: COLO320DM (human colon adenocarcinoma).	Tabar GH & Smith C, 2010
13	5’-NH2-ATA CCA GCT TAT TCA ATT-3’	n/a	Preparation of dsDNA Immobilized MBs Amplification Reaction MCE-LIF Procedure Aptamer-based MCE assays for amplification detection of carcinoembryonic antigen (CEA) in human serum were described.	Zhao S, 2019

14	not given	n/a	Recent progress and challenges in aptamer and SELEX technology are presented and highlighted some representative applications of aptamers in cancer therapy	Hori et al., 2018
15	a. 5′–NH2–ATACCAGCTTATTCAATT–3′ b. 5′–NH2–CCCATAGGGAAGTG GGGGA–3′	Under optimized conditions, the assay has a linear response in the 10.0 fg mL−1 to 200.0 ng mL−1 CEA concentration range and a lower detection limit of 3.2 fg mL−1.	Preparation of the graphene oxide (GO) and nitrogen–doped graphene (NG) Synthesis of the graphene quantum dots (GQDs) Preparation of the sandwich–type electrochemical aptasensor. The quantitative determination of CEA was achieved by differential pulse voltammetry, best at a working potential of around −0.27 V vs. Ag/AgCl.	Shekari et al., 2019
16	CGA TAC CAG CTT ATT CAA TTC CTC AGC GCT GGT ATC G	The newly developed method achieved a wide linear range of 10 fg/mL to 10 ng/mL with low detection limit of 3.5 fg/mL.	Preparation of gold nanoparticles Preparation of Fe3O4Au Preparation of carboxyl functionalized RuSiO2 nanocomposites ECL detection of target CEA Taking advantages of the magnetic Fe3O4@Au NPs for carring abundant signal probes, sensing target and ECL detection, the developed ECL strategy is convenient, rapid and displayed high sensitivity for CEA detection, which has great potential for analyzing the clinical samples in practical disease diagnosis applications.	Jie et al., 2018

Table 1. A list of published aptamer sequences against CEA.

1.4 Enzyme Linked Oligonucleotide Assay

An Enzyme-Linked Immunosorbent Assay (ELISA) was developed in 1971 as a replacement for the radioimmunoassay (RIA) and since then has been in use as a basic research tool of biochemistry, medicine, and immunology the (Gan & Patel, 2013). ELISA is a plate-based assay and it works on the principle of antigen-antibody interactions in combination with photometric visualization of the binding (Stoltenburg et al., 2016). Application of aptamers in ELISA presented an ELISA-derived assay named as an Enzyme-Linked Apta-sorbent Assay (ELASA) or Enzyme-Linked Oligonucleotide Assay (ELONA) (Toh et al., 2015). In this assay, an aptamer immobilization on the solid support surface is the major criterion, which enables efficient capture of the target molecule (Toh et al., 2015). The immobilization procedure can be facilitated by an aptamer functionalization with a terminal functional group such as amine or biotin (Balamurugan et al., 2006). Immobilization of the DNA probe on the microtitre plates surface is the most extended strategy in the interaction of biotinylated probes with streptavidin (or avidin)-coated surfaces (Landgraf et al., 1991; Lazaro et al., 1993;

Kostyu et al., 1993; Sanchez et al., 2010) because this type of surface retains the biological activity of the immobilized molecule better than direct passive adsorption. Moreover, the high biotin-streptavidin affinity constant which is (K_d~10^{-14}M) a robust system as compared with direct passive adsorption (Välimaa & Laurikainen, 2006). To accomplish flexibility between the aptamer and functional group, an oligonucleotide spacer is added to the terminal functional groups (Toh et al., 2015). As thymidine has a lower probability of non-specific binding to different immobilization surfaces, the spacer commonly is from a thymidine nucleotides string (Kimura-Suda et al., 2003). As a result, target protein binding and the dissociation constant can be increased and improved by these spacers (Liss et al., 2002; Balamurugan et al., 2006; Centi et al., 2007). In this study, ELONA was performed for the characterization purposes of selected aptamers against CEA. CEA was immobilized onto the surface of the platform followed by blocking with bovine serum albumin (BSA) with the next addition of biotinylated aptamers and streptavidin-conjugated horseradish peroxidase (sHRP) (Toh et al., 2015).

1.5 Surface Plasmon Resonance (SPR)

Surface plasmon resonance (SPR) was firstly introduced in the early 1990s, it has been proven to be considerably powerful technology in determining affinity, specificity and kinetics parameters during the macromolecules binding in many types of bonds, including protein-DNA (Majka et al., 2007; The et al., 2007), protein-protein (Kim et al., 2006; Madeira et al., 2011), enzyme-substrate (Geitmann et al., 2004; Fong et al., 2002), and receptor-drug (Nguyen et al., 2015) interactions. The technology of SPR is a real-time, high-throughput, and also label-free platform which characterizes both the kinetic properties and affinity of a selected aptamer and its target (Zhuo et al., 2017). The evanescent wave may excite the surface plasmon, and that is why the name of this phenomenon is known as "surface plasmon resonance" (SPR) (Tang & Zeng, 2010). Generally, either aptamer or target is initially immobilized onto an electrode surface, then a variety of non-tethered analyte concentrations are flowed through (Zhuo et al., 2017). SPR based instruments monitor changes happening on certain surfaces of a metal sensor, such as copper, silver, or gold in an optical way (Abadian et al., 2014). Surface plasmons on a metallic film can be excited by photons at particular conditions, transforming a photon into a surface plasmon and it depends on the refractive index of the adsorbate (Markey, 1999). Commonly, a glass prism presented in all SPR instruments couples the polarized light into the sensor which is coated with a thin gold layer

(**Figure 4**) (Brogioni & Berti, 2014). Ligand immobilization and binding events occur on the metal layer with a flow channel, where reactants flow in a buffer (Brogioni & Berti, 2014). SPR angle changes if binding occurs and it is monitored in real-time by detecting changes in the intensity of the reflected light, which is recorded in a sensorgram (**Figure 4**) (Pattnaik, 2005). Resonance units (RU) are used to record the refractive index variations (Brogioni & Berti, 2014). For the majority of proteins, 1 RU equals to 1 pg mm^{-2} concentration on the electrode surface (Stenberg et al., 1991). In the case of a biosensor, the molecule immobilized onto the sensor surface is called a ligand, and the molecule injected over the coated surface in a continuous flow is called an analyte (Brogioni & Berti, 2014).

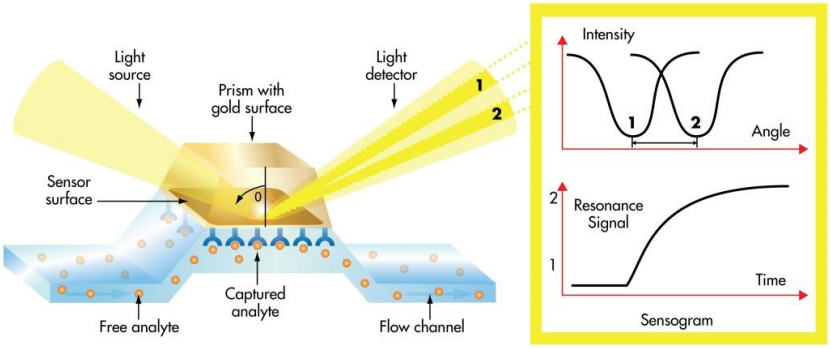

Figure 4. A schematic configuration of an SPR detector. The polarized light is coupled by a glass prism on the biosensor gold layer chip surface integrated with a flow channel for continuous flow of buffer. At a certain incidence angle, the SPR phenomenon is seen as a dip in the intensity of the reflected light. The shift of the reflection angle from position 1 to position 2 determines a change in the structure of the medium near the gold film as a result of the binding event between the ligand and the analyte molecules. This angular variation is recorded in resonance units (RU) and plotted *versus* time in a sensorgram. Adopted from the Bruker Corporation.

In the sensorgram depicted below (**Figure 5**), after a stable baseline is reached, a positive response can be viewed during the sample injection, since an analyte binds to a ligand (GE Healthcare). During the dissociation phase, the response decreases, and after completion of an analysis cycle, regeneration solution passes over the sensor surface to clean from the bound analyte and also preparing for the next cycle (**Figure 5**) (GE Healthcare).

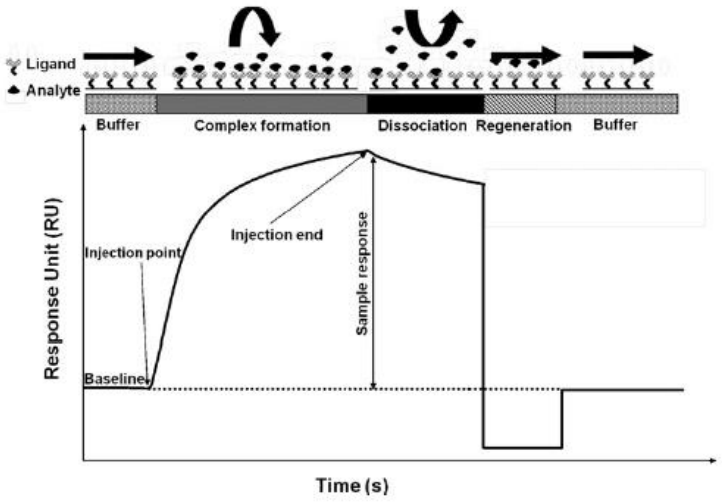

Figure 5. Surface chemistry with corresponding changes in an SPR sensorgram. The changes in the optical waves are determined on the surface. Association and dissociation kinetics during biomolecular interactions are shown. Start and end of the injection, sample response is shown with arrows. Adopted from Gopinath (2010).

Biacore is one of SPR instruments that allows to observe affinity and kinetics interaction studies between proteins and even small analytes (< 1000 Da) with high sensitivity and further data analysis with a special software (Jason-Moller et al., 2006). There are two ways on how to increase the sensitivity of an SPR instrument: first is to reduce the noise, second is to increase the signal (Tanaka et al., 2009). A variety of methods have been developed to increase the sensitivity of SPR to enable researchers to detect analytes even at enormously low concentrations (Springer et al., 2014). Such methods use antibodies labelled with enzymes (Kim et al., 2005; Cao et al., 2007), secondary and tertiary antibodies (Chung et al., 2006; Su et al., 2008) and metallic NPs. Particularly, AuNPs with diameters division from 5 to 40 nm have been extensively used to reinforce the SPR response (Springer et al., 2014). The nanoparticle-enhanced surface plasmon resonance (SPR) was used in the method described by Kim et al. (2010) to detect immunoglobulin E (IgE) proteins, which could potentially be useful is the allergic diseases diagnosing. Two various approaches that are based on the surface formed sandwich complexes with biofunctionalized Au-NPs to improve the SPR detection signal for an IgE and, then, a comparison of their detection performances resulted that both detection schemes were capable to directly measure IgE at femtomolar concentrations (Kim et al., 2010). A biotinylated secondary antibody with 20 nm AuNPs coated with streptavidin was used in the study of Huang et al. (2005) to improve the limit of detection (LOD) for prostate-specific antigen. These research data show that the sensitivity and specificity of an aptasensor can be significantly improved by means of different NPs.

There are several commercially available SPR instruments that can be used to monitor real-time interaction between label-free molecules (Nagata & Handa, 2000; Schasfoort & Tudos, 2008). One of them is Biacore systems which supports scientists to solve the intricacies of protein interactions for more than 15 years (Jason-Moller et al., 2006). Biacore SPR technology is a Sweden based company created in the 1984 year (Gopinath, 2010). These established SPR systems are popular primarily in pharmaceutical development, quality control, and basic life science researches (GE Healthcare). Originally, Biacore technology was done with monoclonal antibodies to exemplify characteristic functional properties such as concentration (Löfas et al., 1991), specificity, affinity, kinetics (Karlsson et al., 1991) and epitope mapping (Fägerstam et al., 1990). Nowadays a wide range of label-free sensor biomolecules in a real-time fashion with only small amounts of samples (μg or sub-μg) can be tested by a Biacore (Berggard et al., 2007). The nature of molecules of interest is not limited, which is from organic compounds to proteins, nucleic acids, glycoproteins, and even viruses and whole cells can be studied by Biacore systems (GE Healthcare). The main trademark of the Biacore's SPR biosensors is the ability to analyze kinetic association and dissociation rate constants from the real-time measurement of binding interactions, hence, providing valuable information about the complex formation and complex stability that cannot be revealed through affinity measurements (Jason-Moller et al., 2006). The SPR machine is equipped with different types of sensor chips with different surfaces and they are available for the Biacore systems (**Table 2**) to accommodate different biomolecules and surfaces of the electrodes are either pre-immobilized with ligands to capture biomolecules or modified by standard immobilization techniques (Gopinath, 2010). For example, CM5, CM4, and CM3 chips are covered with a hydrogel matrix of flexible, unbranched carboxymethylated dextran covalently attached to the surface alkanethiol monolayer, which provides a hydrophilic, solution-like environment favorable for the most interactions between proteins or other biomolecules (Jason-Moller et al., 2006).

For the experiments where nucleic acids are coupled with biotin, SA chip from Biacore is routinely used (Gopinath et al., 2006; Sekiya et al., 2006). In studies of Gopinath et. al. (2010), streptavidin pre-immobilized SA-sensor chip was used and poly (T) linked biotin molecules were subsequently were attached to it (Gopinath et al., 2006). They have prepared RNA aptamers 24-mer poly (A) nucleotides at the 3'- end which was annealed to the complementary biotinylated oligo (dT) [5'-biotin-(T)24-3'] (**Figure 6**). In this setting, RNA aptamers against Haemagglutinin (HA) of influenza A and B were tested (Gopinath et al., 2006).

Chip name	Dextran, nm	Modification	Application
CM7	100	100% carboxylation	High density CM-dextran- high capacity
CM5	100	100% carboxylation	General
CM4	100	30% carboxylation	Cell extracts/serum and membranes
CM3	30	100% carboxylation	Cell extracts/serum and membranes
C1	none	100% carboxylation	Phage binding
NTA	100	NTA	Capturing poly HIS-groups
HPA	none	hydrophobic	Lipid capturing
L1	100	lipophilic substances	Bilayers to mimic membranes
SA	100	streptavidin	Capturing of biotin
CAP	100	oligopeptide	Oligopeptide mediated capture of biotin
AU	none	none	User defined surface chemistry
SIA	none	none	Surface interaction analysis in material science

CM-carboxymethylated dextran, NTA-nitrilotriacetic acid, HPA- hydrophobic surface, L1- lipophilic, SA-streptavidin, CAP- capture biotinylated analytes, AU- plain gold surface, SIA- Surface Interaction Analysis

Table 2. An overview of available Biacore sensor chips with their corresponding surface modifications and potential applications. Adopted from SPR-Pages.

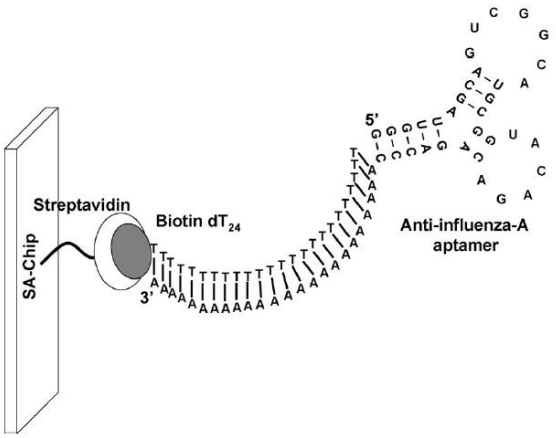

Figure 6. A method of an aptamer attachment onto a biotinylated oligo. Biotin dT(24) and poly-A (24) tail hybridization of an aptamer made on a SA chip. Aptamer is synthesized against HA of an influenza A. Adopted from Gopinath (2010).

An SPR has made considerable progress in becoming a powerful technology (Brogioni & Berti, 2014) and a wide range of biotechnological problems can be solved with the help of label-free, fast-response, and sensitive SPR techniques (Silin & Plant, 1997). SPR based experiments can significantly improve the biosensor data quality by their accurate design and data processing (Brogioni & Berti, 2014).

2 MATERIALS AND METHODS

2.1 Reagents

Item	Use	Source
CEA	Target protein	Cat. No. C4835, Sigma-Aldrich, MO, USA
Monoclonal Anti-Human IgG1−Biotin antibody produced in mouse	Positive control, ELONA	Cat. No. B6775, Sigma-Aldrich, MO, USA
5'- aptamer [BtnTg] – 3' (aptamer sequence 1, 2, 3, and 7)	ELONA	Synthesized by Lumiprobe, Moscow, Russia
5'- aptamer [BtnTg] – 3' (aptamer sequence 6)	ELONA, SPR	Synthesized by Sigma-Aldrich
CEA aptamer 5'-TTAACTTATTCGACCATA[BtnTg]-3'	Positive control, ELONA	Adopted from Shu et al., 2013 synthesized by Sigma-Aldrich
Human serum albumin (HSA)	Non – target protein	Cat. No. SRP6182, Sigma-Aldrich Co., MO, USA
Interleukin-6 (IL-6)	Non – target protein	Cat. No. SRP3096, Sigma-Aldrich Co., MO, USA
96 – well plates	ELONA	Cat. No. 655061, Greiner bio-one, Kremsmünster, Austria
Sodium carbonate (Na_2CO_3)	Coating buffer	Cat. No. 497-19-8, Sigma-Aldrich Co., MO, USA
Tris buffer saline (TBS)	Buffer	In house made: 8.69 g NaCl, 6.05 g Tris Base, 1 L of sterile water pH was adjusted to 7.3 - 7.4
Sodium chloride (NaCl)	Buffer component	Cat. No. CAS 7647-14-5, Fisher Scientific, PA, USA
Tris base	Buffer component	Cat. No. BP152, Fisher Scientific, PA, USA
Sterile water	Buffer component	Cat. No. BP2484, Fisher Scientific, PA, USA
Bovine serum albumin (BSA)	Blocking Buffer	Cat. No. BPE1600, Fisher Scientific, Loughboroug, UK
Streptavidin-HRP	Enzyme	Cat. No. N204, Thermo Scientific, MA, USA
Sensor chips SA	SPR	Cat. No. BR-1000-32, GE Healthcare, Uppsala, Sweden
Biotin CAPture Kit	SPR	Cat. No. 28920233, GE Healthcare, Uppsala, Sweden
HBS-EP+	Buffer	Cat. No. BR-1008-26, GE Healthcare, Uppsala, Sweden

Sodium hydroxide (NaOH)	Buffer	Cat. No. BP359, Fisher Scientific, Loughboroug, UK
Isopropanol	Buffer component	Cat. No. 383920010, Acros Organics, Geel, Belgium

Table 3. A list of reagents and consumables used in this study.

2.2 Evaluation of relative binding events (OD_{450}) of ssDNA aptamers against CEA using ELONA

Six aptamers were previously selected against CEA using the SELEX method by Dr. Damira Kanayeva's research group. For further characterization of CEA aptamers purposes, an ELONA was employed, which is based on a ninety-six well plate that was coated with a 500 ng of CEA in a 100 µL of 100 mM Na_2CO_3 buffer overnight at 4 ˚C. After overnight incubation, the wells were washed 3 times with 1× TBS (pH = 7.36 - 7.4). After an unbound CEA was washed away, the wells were blocked with freshly prepared 5% BSA for 1 h at 4 ˚C. The wells were then washed 3 times with TBS. 100 µL of biotinylated aptamer sequences (1), (2), (3), (6), and (7) in a concentration of 500 nM diluted in water were added into each well and incubated for 2 h at room temperature. After incubation, the wells were washed 3 times with 1× TBS. Next, streptavidin-horseradish peroxidase (sHRP) was diluted 1:10000 in 1× TBS, and, then, a 100 µL of this solution was added into each well, and the plate was incubated for another 15 min at room temperature. Then, the plate was washed 3 times with 1× TBS to remove any unbound sHRP. Next, a 100 µL of sterile H_2O was applied into each well, followed by addition of a 50 µL of TMB in H_2O solution (1:1). The addition of TMB dilution was performed under dark conditions. The plates were then incubated for 30 min at room temperature covered by an aluminum foil. After incubation, a 50 µL of 1M H_2SO_4 was added into each well to stop the reaction. The optical density (OD) value was measured at 450 nm by a spectrophotometer **(Figure 7)** (BioTek Instruments, USA). The results were analyzed by Gen5 2.0 data analysis software. The experiment was conducted in three technical replicates.

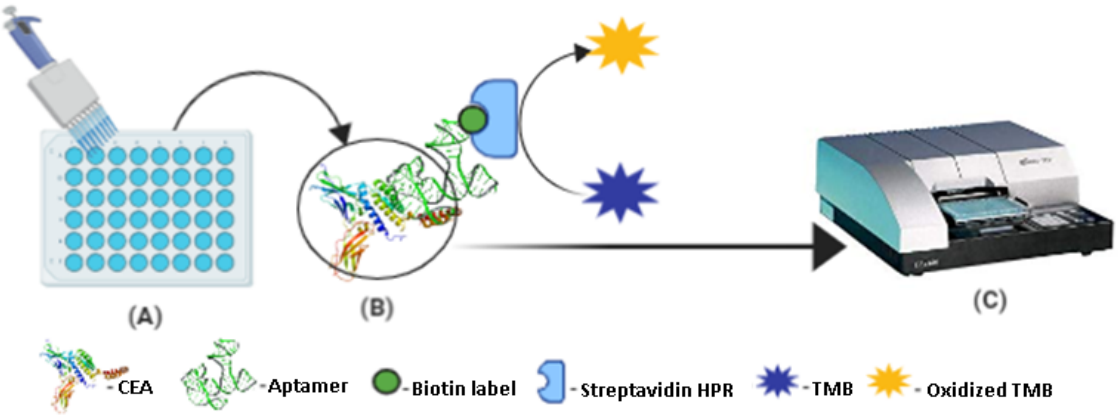

Figure 7. A schematic overview of ELONA: (A) a ninety-six well plate coated with a 500 ng of CEA target protein; (B) a binding event of the biotin-labelled aptamer sequence (6) with CEA, sHRP and followed by TMB; (C) an OD value measurement at 450 nm of an aptamer (6) – CEA binding event with a spectrophotometer.

In this study, there were two positive controls, one was a 500 nM biotinylated CEA aptamer sequence, 5'-TTAACTTATTCGACCATA[BtnTg]-3', obtained from Shu et. al. (2013), and a 30 ng/ml monoclonal anti-human IgG1-biotin was used as the second positive control. H_2O was used as the background, and a well with the CEA but without any aptamer was used as a negative control. H_2O and CEA with no aptamer were used as background controls. All OD values were subtracted from the background. First, a screening of relative binding events of all six CEA aptamer sequences using ELONA was done. Next, an aptamer with the highest OD_{450nm} value, aptamer sequence (6), was further evaluated using ELONA in a concentration range of 0, 62.5, 125, 250, 500, 750 and 1000 nM to the target CEA and non-target proteins IL-6 and HSA at a concentration of 500 ng/mL.

2.3 Surface Plasmon Resonance experiments for target validation

BIAcore X100 SPR instrument (GE Healthcare, Austria, Vienna) equipped with SA **(Figure S1)** and CAP **(Figure 8)** sensor chips from Biotin CAPture Kit (GE Healthcare, Austria, Vienna) was used to determine the affinity of the ssDNA aptamer sequence to CEA protein in kinetics studies. Following procedure was used for SPR experiments adopted from Fechter et al. (2019) and Biotin CAPture Kit Instructions (GE Healthcare) with slight modifications. All experiments were conducted at 25°C with a flow rate of 10 ml/min, unless specified differently. CEA (2.5, 5, 10, 20, 40 ug/mL) samples were diluted in HBS-EP+ buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v Surfactant P20). Aptamer (6) (1µM)

was diluted in SELEX buffer (50 mM Tris-HCl; 25 mM NaCl; 5 mM $MgCl_2$). Flow cell 1(Fc-1) was used as a control and Fc-2 was used as an active cell, where 3'- biotinylated aptamer sequence (6) was immobilized.

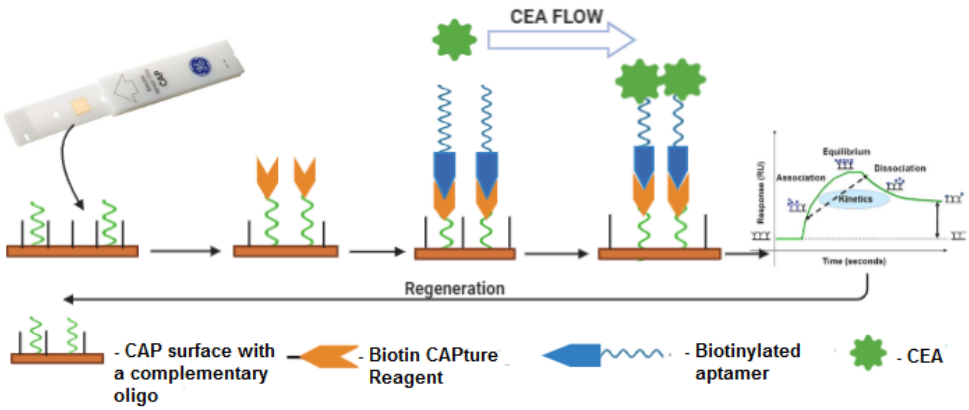

Figure 8. A schematic overview of a CAP functionalized gold surface used in the SPR for the affinity binding characteristics of the aptamer (6) against CEA. The Biotin CAPture Reagent composed of streptavidin conjugated with an oligonucleotide was hybridized to a complementary sequence immobilized on the sensor chip. Biotinylated aptamer sequence (6) against CEA was captured to the Biotin CAPture Reagent. CEA was used as a target analyte. The interactions between CEA and captured aptamer was explored. The functionalized surface was then regenerated and was reused with the new Biotin CAPture Reagent in the next round.

Biacore X100 was first cleaned with desorb (removes adsorbed proteins from the autosampler) before docking the sensor chip. After docking the CAP sensor chip into the instrument, it was left on a stab by mode with the running buffer HBS-EP+ (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v Surfactant P20) overnight. The CAP surface was conditioned after that with three 60 s injections of a regeneration solution (3 parts of regeneration stock 1 solution (8 M guanidine-HCl) and 1 part of regeneration stock 2 solution (1 M NaOH)) when the chip was docked for the first time with the flow rate 10 uL/min and contact time 120 s. The surface functionalization of the CAP chip was initiated when Biotin CAPture Reagent with streptavidin (SA) conjugated oligonucleotides was hybridized to the complementary sequence immobilized on a sensor chip at a 2 uL/min flow rate and contact time 300 s. After successful hybridization of Biotin CAPture Reagent with streptavidin (SA) conjugated oligonucleotides onto the complementary sequence immobilized on a sensor chip, response level should reach 2,500-5,000 RU. Prior to the injection of 5'-aptamer (6)-[BtnTg]-3', it was denatured at 95ºC for 3 min followed by an incubation on ice for 4 min. 5'-aptamer

(6)-[BtnTg]-3'was introduced onto the functionalized CAP surface chip at a flow rate 10 uL/min for 300 s in the Fc-2. A reference cell (Fc-1) without an aptamer was blocked with 1 M ethanolamine-HCl (pH 8.5) for 600 s, which did not require regeneration after ethanolamine-HCl blocking. Fc-2 active flow well was blocked with a 10 mM of biotin for 5 min at a flow rate 10 uL/min with no regeneration after blocking. Target CEA in different concentrations (2.5, 5, 10, 20, 40 ug/ml) was injected over the aptamer functionalized CAP sensor chip surface, and the affinity binding was monitored for 300 s followed by washing with running buffer at a flow rate of 10 uL/min. Dissociation time was 600 s. The surface was subsequently regenerated with 6 M guanidine hydrochloride in 0.25 M NaOH at a flow rate of 10uL/min for 120s. The evaluation of obtained results was done using BiaEvaluation software (Biacore) by 1:1 [Langmuir] fitting model.

2.4 Data analysis

All statistical analyses were performed by Microsoft Excel 2010 and GraphPad Prism 8 (GraphPad Software, San Diego, California). Differences of OD across aptamers were tested by two-tailed *t*-test, ANOVA and Kruskal - Wallis test. An SPR analysis was performed using the BiaEvaluation software (Biacore Software, Uppsala, Sweden) in 1:1 [Langmuir] fitting model. Data were expressed as mean ± standard deviation. The values $P < 0.05$ were considered statistically significant.

3 HYPOTHESIS AND AIMS OF THE THESIS

Following is the hypothesis of the thesis:

The hypothesis of the thesis is whether ssDNA aptamers will have a high affinity and specificity to CEA which will be addressed by the following aims:

1. Evaluation of relative binding events (OD_{450}) of ssDNA aptamers against CEA using ELONA;
2. To determine a binding affinity of the most sensitive aptamer to the target CEA and non-target proteins;
3. The use of surface plasmon resonance experiments for target validation.

4 RESULTS

4.1 Evaluation of the binding event of ssDNA aptamers and CEA using ELONA

Previously, the research group of Dr. Damira Kanayeva selected six CEA aptamer sequences as a result of the twelve SELEX cycles. In this study, these aptamer sequences were screened using ELONA that could be observed on **Figure 9**. A commercially available highly purified CEA (stock concentration 1.0 mg/ml) was used as the target. The wells were coated with protein overnight using a coating buffer – sodium carbonate, followed by an immobilization of aptamer sequences in a 500 nM concentration. There were two positive controls, where a known biotinylated CEA aptamer sequence 5'-TTAACTTATTCGACCATA[BtnTg]-3' (Shu et al., 2013) was used as the first positive control, and a 30 ng/mL monoclonal anti-human IgG1-biotin was used as the second positive control since it is the most common type of antibody found in human's blood circulation. Screening of relative binding events of biotinylated aptamer sequences (1)-(4), (6)-(7) against the target CEA using ELONA was conducted (**Figure 9**).

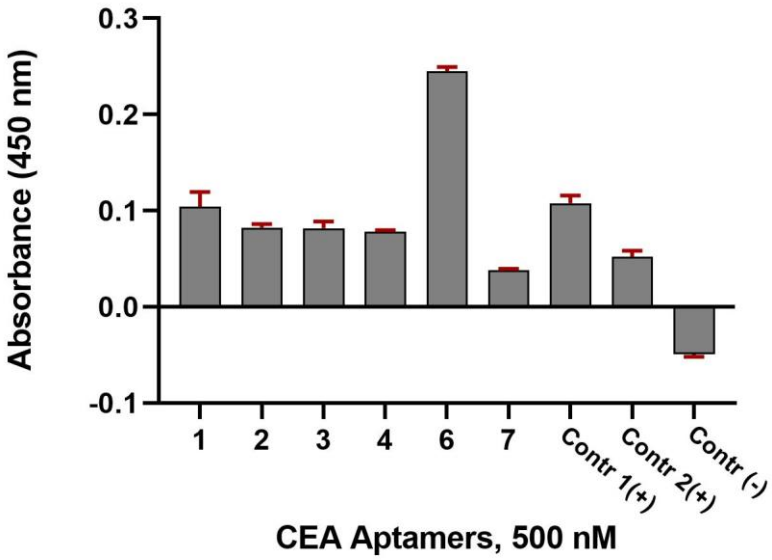

Figure 9. Screening of relative binding events of biotinylated aptamers against the target CEA using ELONA. A CEA aptamer (Shu et al., 2013) was used as the first positive control, a 30 ng/mL monoclonal anti-human IgG1-biotin was used as the second positive control, and a well with CEA but without any aptamer was used as a negative control. H_2O was used as a background. All OD values were subtracted from the background. All data are shown as the means of ±SEMs (n=3).

As it can be seen from **Figure 9** screened aptamer sequences had a better binding affinity towards the target CEA than Control 1 (+), the CEA aptamer sequence obtained from Shu et al. (2013). Among six screened sequences, aptamer sequence (6) showed the highest OD value (OD_{450}= 0.245), this means the higher affinity to the target molecule – CEA. On the contrary aptamer sequence (7) showed the lowest binding affinity with OD_{450} = 0.038. Kruskal-Wallis test was performed to see whether there was a statistically significant difference in the distribution of OD across categories of screened aptamers. Results gave a p-value = 0.01, which is acceptable to conclude that there was a statistically significant difference in the distribution of OD values among six tested aptamers. H_2O was used as a background, and all OD values were subtracted from the background. This experiment was conducted in three technical replicates.

4.2 Binding affinity of the most sensitive aptamer to the target CEA and non-target proteins, a specificity study

Aptamer sequence (6) was chosen for further assessment since it showed the highest binding to the target CEA. A concentration dependent analysis of aptamer sequence (6) (**Figure 10**) revealed an increase in absorbance signal (OD_{450}) form concentration ranging from 0 nM up to 1,000 nM. Obtained concentration dependent results were compared to two positive controls. A CEA aptamer sequence (500 nM) (Shu et al., 2013) was used as the first positive control, a 30 ng/mL monoclonal anti-human IgG1-biotin was used as the second positive control, and a well with the CEA but without any aptamer was used as a negative control. A two-tailed *t*-test was performed in GraphPad Prism 8 to find statistically significant difference between aptamer (6) in 500 nM and Cntrl (+) 1 in 500 nM, which was aptamer sequence adopted from Shu et al. (2013). The results showed that aptamer (6) worked better than Cntrl (+) 1, since p-value was 0.007. H_2O was used as a background. All OD values were subtracted from the background. The experiment was conducted in six technical replicates.

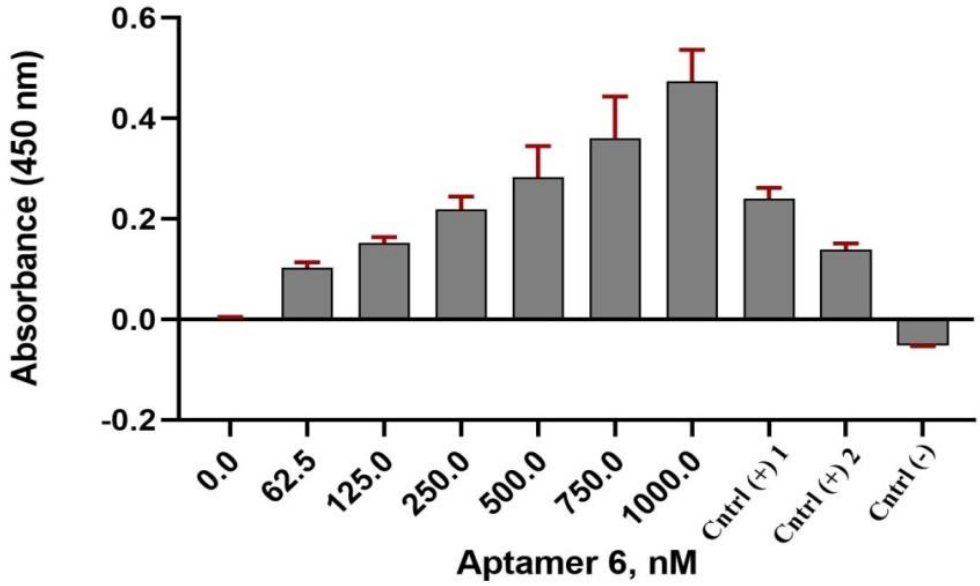

Figure 10. A screening of the relative binding events of the biotinylated aptamer sequence (6) to the target CEA using ELONA. A 500 nM CEA aptamer sequence (Shu et al., 2013) was used as the first positive control, a 30 ng/mL monoclonal anti-human IgG1-biotin was used as the second positive control, and a well with the CEA but without any aptamer was used as a negative control. H_2O was used as a background. All OD values were subtracted from the background. All data are shown as the means of ±SEMs (n=6).

In order to see a relationship between CEA and six different aptamer (6) concentrations, linear regression model was built, which is depicted in **Figure 11**. The coefficient of determination (R^2) for aptamer sequence (6) was 0.9508, which indicates that 95% of OD values fit the model. The regression equation for aptamer sequence (6) was y = 0,1416ln(x) - 0,5161, where x is the aptamer sequence (6) concentration and y is the OD value. K_d of aptamer (6) was also evaluated by measuring the absorbance at 450 nm. GraphPad Prism 8 was used to perform a nonlinear curve fitting analysis for the K_d calculation according to the equation $Y = B_{max} \times X/(K_d + X)$, where B_{max} is the maximal binding, and K_d is the concentration of ligand required to reach half-maximal binding. The aptamer (6) showed K_d values in the nanomolar range that equals to 91.96 nM (10.93 to 418.6 nM (95% CI)). The experiment was conducted in three technical replicates.

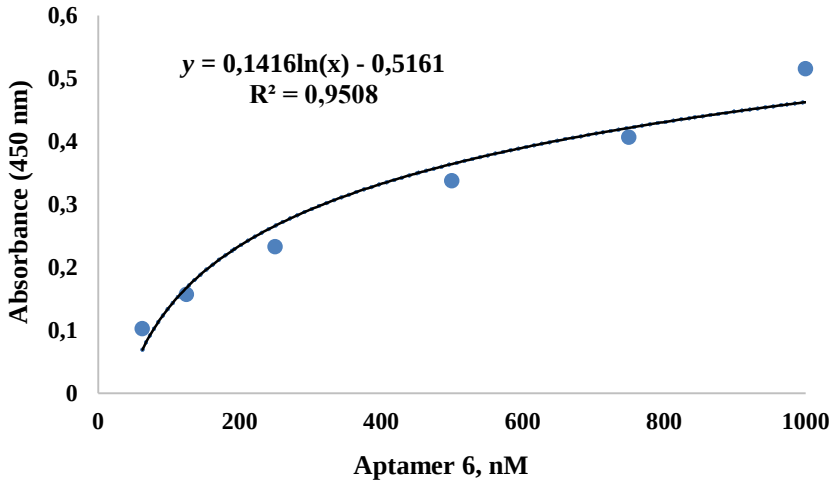

Figure 11. Binding of the aptamer sequence (6) in the concentration range of 0 – 1,000 nM to the target CEA (500 ng/mL) with the regression equation for OD_{450} difference versus aptamer sequence (6). All data are shown as the means of ±SEMs (n=3).

Further, a specificity study was performed, where other proteins present in human blood were tested, using ELONA. In this assay, a ninety-six well based plate was incubated with three different proteins **(Figure 12)**, where CEA is the target protein (500 ng/mL), and IL-6 and HSA were used as non-target ones in a concentration of 500 ng/mL. In this experiment, we also applied controls, where a 500 nM CEA aptamer (Shu et al., 2013) was used as the first positive control, a 30 ng/mL monoclonal anti-human IgG1-biotin was used as a second positive control, and a well with CEA but without any aptamer was used as a negative control. H_2O was used as a background. All OD values were subtracted from the background. The experiment was conducted in three technical replicates. As can be observed from **Figure 12**, a general concentration dependent pattern (0 – 1,000 nM) to the aptamer sequence (6) was discovered with the highest binding to the target CEA. ANOVA was performed to see statistically significant difference between those tested three proteins. As a result p-value was 0.04, which allows conclude that there was a statistically significant difference in OD values among CEA, IL-6, and HSA.

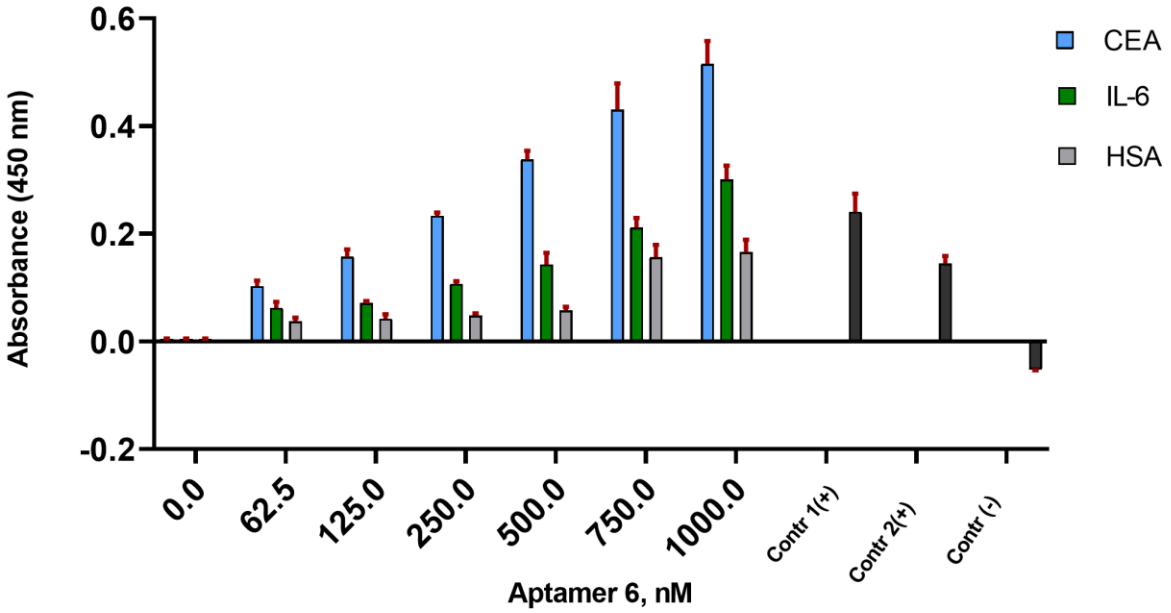

Figure 12. Binding affinity of the aptamer sequence (6) to proteins CEA, IL-6, and HSA. Binding of the biotinylated aptamer sequence (6) to the target CEA and non-target IL-6 and HSA was determined by ELONA. A 500 nM CEA aptamer (Shu et al., 2013) was used as the first positive control, a 30 ng/mL monoclonal anti-human IgG1-biotin was used as the second positive control, and a well with CEA but without any aptamer was used as a negative control. H_2O was used as a background. All OD values were subtracted from the background. All data are shown as the means of ±SEMs (n=3).

4.3 Surface Plasmon Resonance Experiments for Target Validation

SPR technique measures live interactions of protein-protein or protein-DNA interaction by providing information on the rate constant of dissociation of the analyte from the ligand (k_{off}), the rate constant of association of the analyte to the ligand (k_{on}) and the rate constant of dissociation at equilibrium defined as the ration k_{off}/ k_{on} (K_D). In this study, SPR was used for further evaluation of the aptamer sequence (6) against the target CEA. Immobilization of the aptamer sequence (6) was based on the surface functionalization of the CAP chip when Biotin CAPture Reagent with streptavidin (SA) conjugated oligonucleotides was hybridized to the complementary sequence immobilized on a sensor chip and biotinylated aptamer sequence (6) against CEA was captured to the Biotin CAPture Reagent surface (**Figure 13**). Commercially available CAP chip is designed to capture biotinylated analytes on a streptavidin surface. Aptamer (6) was also immobilized onto CAP electrode. A stable baseline was obtained by the flow of the HBS-EP+ running buffer over the sensor surface. CAPture Kit includes biotin CAPture reagent, which was used for the SA hybridization. This step was successful and reached the target – 3000 RU, as it was recommended in the CAPture Kit protocol. As soon as

the electrode surface with streptavidin was ready, aptamer (6) was immobilized onto the CAP surface and reached the target 1500 RU. After aptamer (6) immobilization step finished, CEA ran over the surface of the chip.

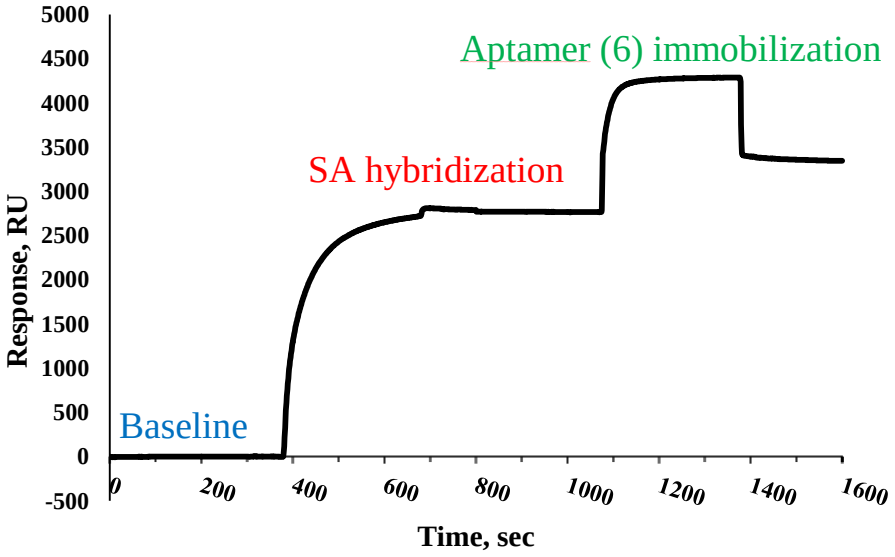

Figure 13. A typical SPR sensor response to the surface functionalization of the CAP chip when Biotin CAPture Reagent with streptavidin (SA) conjugated oligonucleotides was hybridized to the complementary sequence immobilized on a sensor chip and biotinylated aptamer sequence (6) against CEA was captured to the Biotin CAPture Reagent surface.

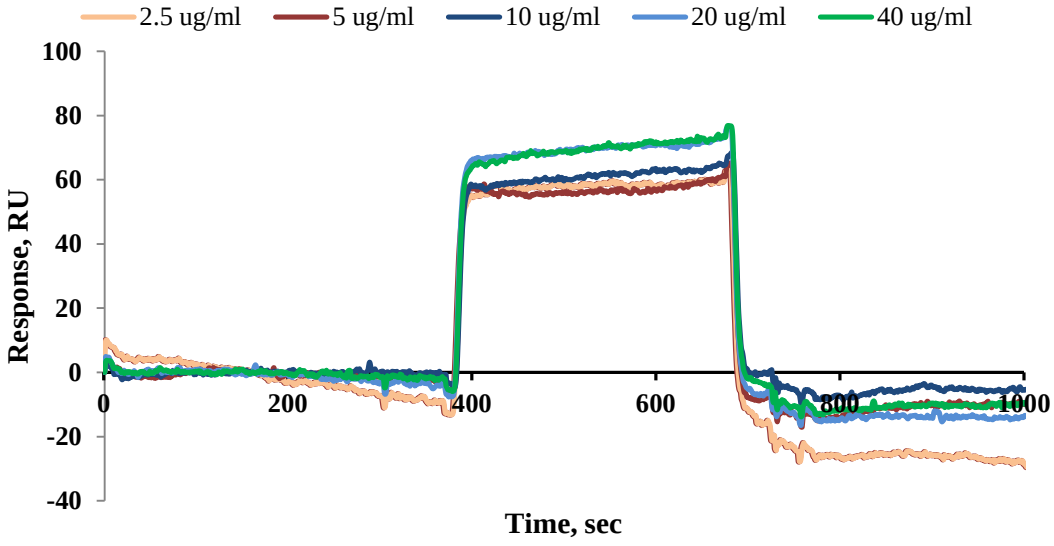

Figure 14. SPR sensorgram obtained from the interaction between the target CEA and biotinylated aptamer sequence (6). In each case, the protein was injected over immobilized aptamer (6) with an initial concentration of 2.5 ug/ ml, followed by 5, 10, 20, 40 ug/ml.

Figure 14 shows a SPR sensorgram obtained from the interaction between the target CEA and biotinylated aptamer sequence (6). In each case, the protein was injected over immobilized aptamer (6) with an initial concentration of 2.5 ug/ml, followed by 5, 10, 20, 40 ug/ml .The sensorgram in **Figure 14** particularly shows the event occurring on the Fc-2. Fc - 1 was used as a reference cell. It demonstrates the direct interaction between CEA and aptamer (6). The first phase in biomolecular interaction experiment is association phase, when analyte and ligand bind to one another as it can be seen from the sensorgram in **Figure 14**. However, dissociation phase is unstable and there are negative values. Dissociation phase describes the stability of the analyte-ligand complex. The reason of obtaining the negative values is that CEA more strongly binds to the reference channel, since the surface of the electrode is damaged somehow. Due to Covid-19 pandemic, it was not unfortunately possible to further perform SPR optimization studies to improve the binding affinity of an aptamer-protein complex.

5 DISCUSSION

5.1 Evaluation of the binding of the ssDNA aptamers against CEA by ELONA and binding affinity of the most sensitive aptamer to the target CEA and non-target proteins, a specificity study

ELONA method was used to evaluate the binding event between aptamers and target molecule – CEA. In this assay, ninety-six wells are coated overnight with CEA protein and next immobilized with 3'-end biotin labelled aptamers. Since it is known that interaction of biotin and streptavidin is the strongest non-covalent bindings, with an association constant 10^{15} (Gonzalez et al., 1997; Chivers et al., 2011), sHRP was added to the system and resulted to the interaction with biotin. Next TMB added to sHRP resulted into oxidation of TMB, turning in blue color product (Olucha, Matrinez-Garcia, Lopez-Garcia, 1985). The reaction stopped by the next addition of sulfuric acid, which turned the product into yellow color resulting stable diimine (Fanjul-Bolado, Gonzales-Garcia, Costa-Garcia, 2005). The absorbance of the final solution is measured at 450 nm since at this wavelength yellow color has the highest absorbance peak.

Six different aptamer sequences were immobilized in a concentration of 500 nM and CEA was in a 500 ng/mL. Aptamer sequence (Shu et al., 2013) was used as a first positive control and a 30 ng/mL biotin was used as a second positive control, and CEA without aptamer was used as a negative control. H_2O was used as a background. Screening of six biotinylated aptamers by ELONA **(Figure 9)** showed that aptamer sequence (6) has the highest affinity towards CEA, since it has the highest OD value in comparison with others. The rest of the aptamers showed non-significant binding to the target molecule – CEA.

After screening of all selected aptamers, sequence (6) was tested in a concentration-dependent manner **(Figure 10)** again by ELONA. A range of concentrations of aptamer (6) (0 – 1,000 nM) were incubated with CEA. The results revealed that aptamer sequence (6) shows the positive correlation between aptamer concentrations and OD values (**Figure 11**). Aptamer sequence (Shu et al., 2013) was used as a first positive control and a 30 ng/mL biotin was used as a second positive control, and CEA without aptamer was used as a negative control. H_2O was used as a background.

Next, specificity test was done using two non-target proteins: Interleukin-6 (IL-6) and Human serum albumin (HSA) (**Figure 12**). IL-6 is a glycosylated polypeptide having a molecular weight of nearly 25 kDa and is one of the major cytokines in the tumor microenvironment, is

an important factor which is found at high concentrations and known to be deregulated in cancer (Kumari et al., 2016). IL-6 appears to be part of a cytokine network that regulates inflammation by triggering a consolidated phase through monocyte recruitment with a direct effect on the organization and integration of tumor-associated macrophages (TAMs), which are essential components of the tumor structure (Lippitz & Harris, 2016). HSA is the most abundant protein in plasma, is a monomeric multi-domain macromolecule, representing the main determinant of plasma oncotic pressure and the main modulator of fluid distribution between body compartments (Peters, 1996; Evans, 2002; Mendez et al., 2005a). HSA is a valuable biomarker of many diseases, including cancer, rheumatoid arthritis, ischemia, post-menopausal obesity, severe acute graft-versus-host disease, and diseases that need monitoring of the glycemic control (Gupta & Lis, 2010; Koga & Kasayama, 2010; Sbarouni et al., 2011).
Planned comparisons revealed that aptamer (6) has high specificity to the CEA, rather than to the IL-6 and HSA proteins. Also the test was done in dose-response analysis manner and still there is positive correlation between concentration and OD values.

5.2 Surface Plasmon Resonance Experiments for Target Validation

The results obtained from ELONA allowed select aptamer (6) as one with the highest affinity towards CEA and the next step was to move to the more sensitive technique. And in our case it was an SPR. Surface Plasmon Resonance was conducted to find the K_D value of the aptamer (6). This value can be calculated using formula k_{off}/k_{on}. Calculated high K_D value indicates low affinity between the ligand and the analyte and low K_D *vice versa* describes high affinity between the ligand and the analyte.
Here, ELONA results were used to quantify the binding constants, K_d and B_{max} of aptamer (6). The affinity curve obtained using the ELONA showed that the best fit curve corresponded to a one site-specific binding model (with R_2 value 0.95), from which the values of K_d and B_{max} were computed.
Biacore X100 (GE Healthcare) is equipped with different types of sensor chips. In this experiment two different sensor chips were used - SA chip and CAP chip. SA sensor chip pre-coated with streptavidin was used to directly immobilize biotinylated aptamer (6) and CAP pre-coated with an oligo sequence was immobilized with SA by injecting CAP-reagent from the Biotin CAPture Kit (GE Healthcare) followed with immobilization of aptamer (6).
Before starting the experiment, stable baseline was obtained, since it is important for accurate kinetic data. In the experiment with SA electrode **(Figure S2)** four steps are illustrated. This

was an “Automatic Run” by Biacore, where values, such as flow rate, contact time and concentration of protein are adjusted by Biacore itself. The machine also offers “Manual Run” option, where user can change these values manually. First of all, stable baseline was obtained by flow of the running buffer – HBS-EP+ over the sensor surface. Next, regeneration buffer – 1M NaCl with 12 mM NaOH was injected to activate the sensor surface. Then, biotin tagged aptamer (6) was immobilized on SA chip with a flow rate 10 uL/min. Biacore offers three types of flow rate: 5, 10 and 30 uL/min. In the experiment 10 uL/min was used as an optimal flow rate, since it allows efficient interaction between the ligand and the analyte. Before injection the CEA, blocking procedures were done. Flow cell-1 was used as a reference cell, since it is not pre-coated with streptavidin, was blocked by 1M ethanolamine-HCl. Flow cell-2 was blocked with biotin in order to reduce non-specific binding. The result of this experiment was not successful. As it can be seen from the sensorgram, there is no RU change in baseline and this shows no binding. One of the problems was inappropriate biotin product used for blocking. Biotin (CAS 58-85-5) from Sigma-Aldrich was not suitable for use as a blocking reagent. D-biotin is suitable for use in such experiments, but the reagent was found late and unfortunately, the laboratory was out of SA chips.

Next, the experiment was performed with CAP chips. CAP electrode is a good alternative to the SA chip, since it offers reversible ligand capture. The results of the sensorgram demonstrate **(Figure 13, 14)** successful SA hybridization and aptamer (6) immobilization procedures. It should be noted that SA hybridization, as well as aptamer immobilization steps were successful and reached target RU, 3000 and 1500 RU, respectively.

6 CONCLUSION AND FUTURE WORK

In this study, aptamers against CEA were evaluated based on the binding of the ssDNA aptamers against CEA using ELONA. Obtained results show that the selected aptamers have a good specificity and sensitivity values comparing to the non-target proteins (IL-6 and HSA) as well as controls. Aptamer (6) showed the highest affinity against CEA among all screened six aptamer sequences. Future work will be concentrated on the SPR protocol optimization to improve binding affinity results. In case of a low signal, aptamers could be modified easily with self-assembled monolayers (SAMs) such as HS groups or magnetic/gold nanoparticles or any other fluorescent reporting molecules that could be further used in the application of characterized aptamers in the electrochemical impedance spectroscopy (EIS) based aptasensor for the detection of a cancer biomarker. We also plan on testing CEA aptamers on CEA spiked human serum samples to observe how a protein rich medium will affect the detection of the target.

7 REFERENCES

1. (2020) *Gco.iarc.fr* Available at: http://gco.iarc.fr/today/data/factsheets/populations/398-kazakhstan-fact-sheets.pdf [Accessed January 30, 2020]
2. *(2020). Who.int.* [online] Available at: https://www.who.int/cancer/PRGlobocanFinal.pdf [Accessed 30 Jan. 2020].
3. Abadian PN, Kelley CP & Goluch ED (2014) Cellular Analysis and Detection Using Surface Plasmon Resonance Techniques. *Analytical Chemistry* **86:** 2799–2812
4. Ali J, Najeeb J, Ali MA, Aslam MF & Raza A (2017) Biosensors: Their Fundamentals, Designs, Types and Most Recent Impactful Applications: A Review. *Journal of Biosensors & Bioelectronics* **08**
5. Arnold M, Sierra M, Laversanne M, Soerjomataram I, Jemal A & Bray F (2017) Global patterns and trends in colorectal cancer incidence and mortality. *Gut* **66:** 683-691
6. Balamurugan V, Singh RP, Saravanan P, Sen A, Sarkar J, Sahay B, Rasool TJ & Singh RK (2006) Development of an Indirect ELISA for the Detection of Antibodies against Peste-des-petits-ruminants Virus in Small Ruminants. *Veterinary Research Communications* **31:** 355–364
7. Berg M & Søreide K (2011) Genetic and Epigenetic Traits as Biomarkers in Colorectal Cancer. *International Journal of Molecular Sciences* **12:** 9426-9439
8. Berggård T, Linse S & James P (2007) Methods for the detection and analysis of protein–protein interactions. *Proteomics* **7:** 2833–2842
9. Bohunicky & Mousa S (2010) Biosensors: the new wave in cancer diagnosis. *Nanotechnology, Science and Applications:* 1
10. Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R., Torre, L. and Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: *A Cancer Journal for Clinicians*, **68(6),** pp.394-424.
11. Bretthauer M (2011) Colorectal cancer screening. *Journal of Internal Medicine* **270:** 87–98
12. Brogioni B & Berti F (2014) Surface plasmon resonance for the characterization of bacterial polysaccharide antigens: a review. *MedChemComm* **5:** 1058
13. Cao, Cuong & Sang Jun Sim (2007) Double-Enhancement Strategy: A Practical Approach to a Femto-Molar Level Detection of Prostate Specific Antigen-α1-Antichymotrypsin (PSA/ ACT Complex) for SPR Immunosensing. *J. Microbiol. Biotechnol* **17:** 1031–1035
14. Carmona F, Azuara D, Berenguer-Llergo A, Fernandez A, Biondo S, de Oca J, Rodriguez-Moranta F, Salazar R, Villanueva A, Fraga M, Guardiola J, Capella G, Esteller M & Moreno V (2013) DNA Methylation Biomarkers for Noninvasive Diagnosis of Colorectal Cancer. *Cancer Prevention Research* **6:** 656-665
15. Centi S, Tombelli S, Minunni M & Mascini M (2007) Aptamer-Based Detection of Plasma Proteins by an Electrochemical Assay Coupled to Magnetic Beads. *Analytical Chemistry* **79:** 1466–1473
16. Cerchia L (2018) Aptamers: Promising Tools for Cancer Diagnosis and Therapy. *Cancers* **10:** 132
17. Chung J, Park J, Bernhardt R & Pyun J (2006) Immunosensor with a controlled orientation of antibodies by using NeutrAvidin–protein A complex at immunoaffinity layer. Journal of Biotechnology 126: 325–333
18. Clifford P. Stanners DBDC (1989) Studies on the Control of Gene Expression of the Carcinoembryonic Antigen Family in Human Tissue. *Basic Sciences* **49:**4
19. Compton C, Fenoglio-Preiser CM, Pettigrew N & Fielding LP (2000) American Joint Committee on Cancer prognostic factors consensus conference. *Cancer* **88:** 1739–1757
20. Creeden J, Junker F, Vogel-Ziebolz S & Rex D (2011) Serum Tests for Colorectal Cancer Screening. *Molecular Diagnosis & Therapy* **15:** 129–141
21. Darmostuk M, Rimpelova S, Gbelcova H & Ruml T (2015) Current approaches in SELEX: An update to aptamer selection technology. *Biotechnology Advances* **33:** 1141–1161
22. Debnath M, Prasad G & Bisen P (2009) Food Diagnostics. *Molecular Diagnostics: Promises and Possibilities*: 467-482
23. Duffy MJ (2001) Carcinoembryonic Antigen as a Marker for Colorectal Cancer: Is It Clinically Useful? *Clinical Chemistry* **47:** 624–630

24. Gold P & Freedman SO (1965) Demonstration of Tumor-Specific Antigens In Human Colonic Carcinomata By Immunological Tolerance And Absorption Techniques. *The Journal of Experimental Medicine* **121:** 439–462
25. Edoo MIA, Chutturghoon VK, Wusu-Ansah GK, Zhu H, Zhen TY, Xie HY & Zheng S-S (2019) Serum Biomarkers AFP, CEA And CA19-9 Combined Detection for Early Diagnosis of Hepatocellular Carcinoma. *Iranian Journal of Public Health*
26. Ellington AD & Szostak JW (1990) In vitro selection of RNA molecules that bind specific ligands. *Nature* **346:** 818–822
27. Fägerstam LG, Frostell Å, Karlsson R, Kullman M, Larsson A, Malmqvist M & Butt H (1990) Detection of antigen—antibody interactions by surface plasmon resonance. Application to Epitope Mapping. *Journal of Molecular Recognition* **3:** 208–214
28. Favoriti P, Carbone G, Greco M, Pirozzi F, Pirozzi R & Corcione F (2016) Worldwide burden of colorectal cancer: a review. *Updates in Surgery* **68:** 7-11
29. Feng F, Tian Y, Xu G, Liu Z, Liu S, Zheng G, Guo M, Lian X, Fan D & Zhang H (2017) Diagnostic and prognostic value of CEA, CA19–9, AFP and CA125 for early gastric cancer. *BMC Cancer* **17:**737
30. Ferreira C & Missailidis S (2007) Aptamer-based therapeutics and their potential in radiopharmaceutical design. *Brazilian Archives of Biology and Technology* 50: 63-76
31. Filella X, Molina R, Pique JM, Grau JJ, Garcia-Valdecasas JC, Biete A, et al (1994) CEA as a prognostic factor in colorectal cancer. *Anticancer Research* **14**: 705–708
32. Fong KE & Yung L-YL (2013) Localized surface plasmon resonance: a unique property of plasmonic nanoparticles for nucleic acid detection. *Nanoscale* **5:** 12043
33. Franz M, Scholz M, Henze I, Röckl S & Gomez L (2013) Detection of colon polyps by a novel, polymer pattern-based full blood test. *Journal of Translational Medicine* **11:** 278
34. Gan SD & Patel KR (2013) Enzyme Immunoassay and Enzyme-Linked Immunosorbent Assay. *Journal of Investigative Dermatology* **133:** 1–3
35. Gao S, Zheng X, Jiao B & Wang L (2016) Post-SELEX optimization of aptamers. *Analytical and Bioanalytical Chemistry* **408:** 4567–4573
36. Geitmann M & Danielson UH (2004) Studies of substrate-induced conformational changes in human cytomegalovirus protease using optical biosensor technology. *Analytical Biochemistry* **332:** 203–214
37. Gold P & Freedman SO (1965) Specific Carcinoembryonic Antigens Of The Human Digestive System. *The Journal of Experimental Medicine* **122:** 467–481.
38. Gopinath SC (2010) Biosensing applications of surface plasmon resonance-based Biacore technology. *Sensors and Actuators B: Chemical* **150:** 722–733
39. Grieshaber D, Mackenzie R, Vörös J & Reimhult E (2008) Electrochemical Biosensors - Sensor Principles and Architectures. *Sensors* **8:** 1400–1458
40. Guo J, Yu J, Song X & Mi H (2017) Serum CA125, CA199 and CEA combined detection for epithelial ovarian cancer diagnosis: A meta-analysis. *Open Medicine* **12:** 859-864
41. Hammarström S (1999) The carcinoembryonic antigen (CEA) family: structures, suggested functions and expression in normal and malignant tissues. *Seminars in Cancer Biology* **9:** 67–81
42. Hao Tang, Jinhua Chen, Lihua Nie, Yafei Kuang, Shouzhuo Yao (2007) A label-free electrochemical immunoassay for carcinoembryonic antigen (CEA) based on gold nanoparticles (AuNPs) and nonconductive polymer film. *Biosensors and Bioelectronics* **22**: 1061–1067
43. Huang L, Reekmans G, Saerens D, Friedt J-M, Frederix F, Francis L, Muyldermans S, Campitelli A & Hoof CV (2005) Prostate-specific antigen immunosensing based on mixed self-assembled monolayers, camel antibodies and colloidal gold enhanced sandwich assays. *Biosensors and Bioelectronics* **21:** 483–490
44. Huss R (2015) Biomarkers. *Translational Regenerative Medicine*: 235–241
45. Ikebukuro K, Kiyohara C & Sode K (2004) Electrochemical Detection of Protein Using a Double Aptamer Sandwich. *Analytical Letters* 37: 2901-2909
46. Ilantzis C, Demarte L, Screaton RA & Stanners CP (2002) Deregulated Expression of the Human Tumor Marker CEA and CEA Family Member CEACAM6 Disrupts Tissue Architecture and Blocks Colonocyte Differentiation. *Neoplasia* **4:** 151–163

47. Imaoka H, Mizuno N, Hara K, Hijioka S, Tajika M, Tanaka T, Ishihara M, Hirayama Y, Hieda N, Yoshida T, Okuno N, Shimizu Y, Niwa Y & Yamao K (2016) Prognostic impact of carcinoembryonic antigen (CEA) on patients with metastatic pancreatic cancer: A retrospective cohort study. *Pancreatology* **16:** 859–864
48. Itzkowitz S, Brand R, Jandorf L, Durkee K, Millholland J, Rabeneck L, Schroy P, Sontag S, Johnson D, Markowitz S, Paszat L & Berger B (2008) A Simplified, Noninvasive Stool DNA Test for Colorectal Cancer Detection. *The American Journal of Gastroenterology* **103:** 2862-2870
49. Jason-Moller L, Murphy M & Bruno J (2006) Overview of Biacore Systems and Their Applications. *Current Protocols in Protein Science* **45**
50. Jayasena SD (1999) Aptamers: An Emerging Class of Molecules That Rival Antibodies in Diagnostics. *Clinical Chemistry* **45:** 1628–1650
51. Jessup M & Thomas P (1989) Carcinoembryonic antigen: Function in metastasis by human colorectal carcinoma. *Cancer And Metastasis Review* **8:** 263–280
52. Karlsson R (2004) SPR for molecular interaction analysis: a review of emerging application areas. *Journal of Molecular Recognition* **17:** 151–161
53. Kim M-G, Shin Y-B, Jung J-M, Ro H-S & Chung BH (2005) Enhanced sensitivity of surface plasmon resonance (SPR) immunoassays using a peroxidase-catalyzed precipitation reaction and its application to a protein microarray. *Journal of Immunological Methods* **297:** 125–132
54. Kim N, Gan HH & Schlick T (2007) A computational proposal for designing structured RNA pools for in vitro selection of RNAs. *Rna* **13:** 478–492
55. Kimura-Suda H, Petrovykh DY, Tarlov MJ & Whitman LJ (2003) Base-Dependent Competitive Adsorption of Single-Stranded DNA on Gold. *Journal of the American Chemical Society* **125:** 9014–9015
56. Kostyu DD, Pfohl J, Ward FE, Lee J, Murray A & Amos DB (1993) Rapid HLA-DR oligotyping by an enzyme-linked immunosorbent assay performed in microtiter trays. *Human Immunology* **38:** 148–158
57. Koyun A, Ahlatcolu E & Koca Y (2012) Biosensors and Their Principles. *A Roadmap of Biomedical Engineers and Milestones*
58. Kroll W. (2008). Biomarkers− predictors, surrogate parameters− a concept definition. In: Schmitz G, Endres S, Götte D, eds. *Biomarker*. Stuttgart: Schattauer, 1–14.
59. Lakhin, V.Z. Tarantula, L.V. Gening (2013) Aptamers: problems, solutions and prospects. *Acta Naturae* **5**:4-19
60. Lamerz R (1992) CA 19–9, GICA (Gastrointestinal Cancer Antigen). *Serological Cancer Markers*: 309-339
61. Landgraf A, Reckmann B & Pingoud A (1991) Direct analysis of polymerase chain reaction products using enzyme-linked immunosorbent assay techniques. *Analytical Biochemistry* **198:** 86–91
62. Lazaro AM, Fernandez-Vin˜a MA, Liu Z & Stastny P (1993) Enzyme-linked DNA oligotyping. *Human Immunology* **36:** 243–248
63. Li F, Chen W & Zhang S (2008) Development of DNA electrochemical biosensor based on covalent immobilization of probe DNA by direct coupling of sol–gel and self-assembly technologies. *Biosensors and Bioelectronics* **24:** 781–786
64. Li B, Wang Y, Wei H & Dong S (2008) Amplified electrochemical aptasensor taking AuNPs based sandwich sensing platform as a model. *Biosensors and Bioelectronics* 23: 965-970
65. Liss M, Petersen B, Wolf H & Prohaska E (2002) An Aptamer-Based Quartz Crystal Protein Biosensor. *Analytical Chemistry* **74:** 4488–4495
66. Löfås S, Malmqvist M, Rönnberg I, Stenberg E, Liedberg B & Lundström I (1991) Bioanalysis with surface plasmon resonance. *Sensors and Actuators B: Chemical* **5:** 79–84
67. Löhndorf M, Schlecht U, Gronewold TMA, Malavé A & Tewes M (2005) Microfabricated high-performance microwave impedance biosensors for detection of aptamer-protein interactions. *Applied Physics Letters* **87:** 243902
68. Madeira A, Vikeved E, Nilsson A, Sjögren B, Andrén PE & Svenningsson P (2011) Identification of Protein-Protein Interactions by Surface Plasmon Resonance followed by Mass Spectrometry. *Current Protocols in Protein Science*
69. Majka J & Speck C Analysis of Protein–DNA Interactions Using Surface Plasmon Resonance. *Analytics of Protein–DNA Interactions Advances in Biochemical Engineering/Biotechnology*: 13–36

70. Mallikaratchy P (2017) Evolution of Complex Target SELEX to Identify Aptamers against Mammalian Cell-Surface Antigens. *Molecules* **22:** 215
71. Markey F (1999) What is SPR anyway? *BIA Journal* **6:** 14–17

Marquart JA Biacore Available at: https://www.sprpages.nl/sensor-chips-intro/biacore-sensor-chips [Accessed April 12, 2020]
72. Meirinho S, Dias L, Peres A & Rodrigues L (2017) Electrochemical aptasensor for human osteopontin detection using a DNA aptamer selected by SELEX. *Analytica Chimica Acta* 987: 25-37
73. Nagata K & Handa H (2000) Real-time analysis of biomolecular interactions: applications of BIACORE Tokyo: *Springer*
74. Nezlin R (2016) Use of aptamers in immunoassays. *Molecular Immunology* **70:** 149–154
75. Nguyen H, Park J, Kang S & Kim M (2015) Surface Plasmon Resonance: A Versatile Technique for Biosensor Applications. *Sensors* **15:** 10481–10510
76. Ni S, Yao H, Wang L, Lu J, Jiang F, Lu A & Zhang G (2017) Chemical Modifications of Nucleic Acid Aptamers for Therapeutic Purposes. *International Journal of Molecular Sciences* 18: 1683
77. Ning S, Wei W, Li J, Hou B, Zhong J, Xie Y, Liu H, Mo X, Chen J & Zhang L (2018) Clinical significance and diagnostic capacity of serum TK1, CEA, CA 19-9 and CA 72-4 levels in gastric and colorectal cancer patients. *Journal of Cancer* **9:** 494–501
78. Ocaña C, Pacios M & Valle MD (2012) A Reusable Impedimetric Aptasensor for Detection of Thrombin Employing a Graphite-Epoxy Composite Electrode. *Sensors* **12:** 3037–3048
79. Ordóñez NG (2003) The Immunohistochemical Diagnosis of Mesothelioma. *The American Journal of Surgical Pathology* **27:** 1031–1051
80. Pattnaik P (2005) Surface Plasmon Resonance: Applications in Understanding Receptor–Ligand Interaction. *Applied Biochemistry and Biotechnology* **126:** 079–092
81. Polsky R, Gill R, Kaganovsky L & Willner I (2006) Nucleic Acid-Functionalized Pt Nanoparticles: Catalytic Labels for the Amplified Electrochemical Detection of Biomolecules. *Analytical Chemistry* **78:** 2268–2271
82. Que-Gewirth NS & Sullenger BA (2007) Gene therapy progress and prospects: RNA aptamers. *Gene Therapy* **14:** 283–291
83. Rakhra K, Zhu EF, Abraham W, Moynihan KD, Mehta N, Wittrup KD & Irvine DJ (2017) Abstract A42: Combination immunotherapy of an autochthonous murine lung cancer model expressing human CEA as a tumor-associated self-antigen. *Immunomodulation*
84. Romero-López C & Berzal-Herranz A (2017) Aptamers: Biomedical Interest and Applications. *Pharmaceuticals* **10:** 32
85. Salehi R, Salehi A, Emami M & Mohammadi M (2012) Methylation pattern of SFRP1 promoter in stool sample is a potential marker for early detection of colorectal cancer. *Advanced Biomedical Research* **1:** 87
86. Sanchez JLA, Henry OYF, Mairal T, Laddach N, Nygren A, Hauch S, Fetisch J & O'Sullivan CK (2010) Colorimetric quantification of mRNA expression in rare tumour cells amplified by multiple ligation-dependent probe amplification. *Analytical and Bioanalytical Chemistry* **397:** 2325–2334
87. Sassolas A, Blum LJ & Leca-Bouvier BD (2009) Electrochemical Aptasensors. *Electroanalysis* **21:** 1237–1250
88. Schasfoort RBM & Tudos J Chapter 1. Introduction to Surface Plasmon Resonance. *Handbook of Surface Plasmon Resonance*: 1–26
89. Sekiya S, Noda K, Nishikawa F, Yokoyama T, Kumar PK & Nishikawa S (2006) Characterization and Application of a Novel RNA Aptamer against the Mouse Prion Protein. The Journal of Biochemistry 139: 383–390
90. Shu H, Wen W, Xiong H, Zhang X & Wang S (2013) Novel electrochemical aptamer biosensor based on gold nanoparticles signal amplification for the detection of carcinoembryonic antigen. Electrochemistry Communications 37: 15–19
91. Silin V & Plant A (1997) Biotechnological applications of surface plasmon resonance. *Trends in Biotechnology* **15:** 353–359
92. Ng E, Shima D, Calias P, Cunningham E, Guyer D & Adamis A (2006) Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease. *Nature Reviews Drug Discovery* 5: 123-132

93. Surface Plasmon Resonance - Unlocking the analytical power (2018) Bruker.com Available at: https://www.bruker.com/products/surface-plasmon-resonance.html [Accessed April 12, 2020]
94. Surface plasmon resonance GE Healthcare Life Sciences Available at: https://www.gelifesciences.com/en/us/solutions/protein-research/knowledge-center/surface-plasmon-resonance [Accessed April 12, 2020]
95. Špringer T, Ermini ML, Špačková B, Jabloňků J & Homola J (2014) Enhancing Sensitivity of Surface Plasmon Resonance Biosensors by Functionalized Gold Nanoparticles: Size Matters. *Analytical Chemistry* **86:** 10350–10356
96. Stenberg E, Persson B, Roos H & Urbaniczky C (1991) Quantitative determination of surface concentration of protein with surface plasmon resonance using radiolabeled proteins. *Journal of Colloid and Interface Science* **143:** 513–526
97. Stoltenburg R, Krafčiková P, Víglaský V & Strehlitz B (2016) G-quadruplex aptamer targeting Protein A and its capability to detect Staphylococcus aureus demonstrated by ELONA. *Scientific Reports* **6**
98. Strimbu K & Tavel JA (2010) What are biomarkers? Current Opinion in HIV and *AIDS* **5:** 463–466
99. Surface plasmon resonance GE Healthcare Life Sciences Available at: https://www.gelifesciences.com/en/us/solutions/protein-research/knowledge-center/surface-plasmon-resonance/surface-plasmon-resonance [Accessed February 2, 2020]
100. Sypabekova M, Bekmurzayeva A, Wang R, Li Y, Nogues C & Kanayeva D (2017) Selection, characterization, and application of DNA aptamers for detection of Mycobacterium tuberculosis secreted protein MPT64. *Tuberculosis* **104:** 70–78
101. Tanaka Y, Yoshikawa H, Itoh T & Ishikawa M (2009) Laser-induced self-assembly of silver nanoparticles via plasmonic interactions. *Optics Express* **17:** 18760
102. Tang Y, Zeng X & Liang J (2010) Surface Plasmon Resonance: An Introduction to a Surface Spectroscopy Technique. *Journal of Chemical Education* **87:** 742–746
103. Thomas P, Toth CA, Saini KS, Jessup JM & Steele G (1990) The structure, metabolism and function of the carcinoembryonic antigen gene family. Biochimica et Biophysica Acta (BBA) - *Reviews on Cancer* **1032:** 177–189
104. Thompson JA, Grunert F & Zimmermann W (1991) Carcinoembryonic antigen gene family: Molecular biology and clinical perspectives. *Journal of Clinical Laboratory Analysis* **5:** 344–366
105. Toh SY, Citartan M, Gopinath SC & Tang T-H (2015) Aptamers as a replacement for antibodies in enzyme-linked immunosorbent assay. *Biosensors and Bioelectronics* **64:** 392–403
106. Tomáš Špringer & Jiří Homola (2012) Biofunctionalized gold nanoparticles for SPR-biosensor-based detection of CEA in blood plasma. *Anal Bioanal Chem* **404:** 2869–2875
107. Tothill IE (2009) Biosensors for cancer markers diagnosis. *Seminars in Cell & Developmental Biology* **20:** 55–62
108. Tuerk C & Gold L (1990) Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* **249:** 505–510
109. Välimaa L & Laurikainen K (2006) Comparison study of streptavidin-coated microtitration plates. *Journal of Immunological Methods* 308: 203-215
110. Vatandoost N, Ghanbari J, Mojaver M, Avan A, Ghayour-Mobarhan M, Nedaeinia R & Salehi R (2015) Early detection of colorectal cancer: from conventional methods to novel biomarkers. *Journal of Cancer Research and Clinical Oncology* **142:** 341-351
111. Vetter E, Kronast M, Tölge M & Zimmermann W (2015) Lgr5-expressing stem cells are not the cells of origin of pyloric neuroendocrine carcinomas in mice. The Journal of Pathology 238: 42-51
112. Wang W, Xu X, Tian B, Wang Y, Du L, Sun T, Shi Y, Zhao X & Jing J (2017) The diagnostic value of serum tumor markers CEA, CA19-9, CA125, CA15-3, and TPS in metastatic breast cancer. *Clinica Chimica Acta* **470:** 51–55
113. Wen Z, Si A, Yang J, Yang P, Yang X, Liu H, Yan X, Li W & Zhang B (2017) Elevation of CA19-9 and CEA is associated with a poor prognosis in patients with resectable gallbladder carcinoma. *Hpb* **19:** 951–956
114. Wu X-Z (2010) Serological diagnostic factors for liver metastasis in patients with colorectal cancer. *World Journal of Gastroenterology* **16:** 4084

115. Yan C, Hu Y, Zhang B, Mu L, Huang K, Zhao H, Ma C, Li X, Tao D, Gong J & Qin J (2016) The CEA^{-/lo} colorectal cancer cell population harbors cancer stem cells and metastatic cells. *Oncotarget* 7
116. Yang X, Li N & Gorenstein D (2010) Strategies for the discovery of therapeutic aptamers. *Expert Opinion on Drug Discovery* 6: 75-87
117. Yi Y, Li B, Wang Z, Sun H, Gong H & Zhang Z (2009) CYFRA21-1 and CEA are useful markers for predicting the sensitivity to chemoradiotherapy of esophageal squamous cell carcinoma. *Biomarkers* **14:** 480–485
118. Young P & Womeldorph C (2013) Colonoscopy for Colorectal Cancer Screening. *Journal of Cancer* **4:** 217-226
119. Zhuo Z, Yu Y, Wang M, Li J, Zhang Z, Liu J, Wu X, Lu A, Zhang G & Zhang B (2017) Recent Advances in SELEX Technology and Aptamer Applications in Biomedicine. *International Journal of Molecular Sciences* **18:** 2142

8 APPENDICES

Appendix 1.

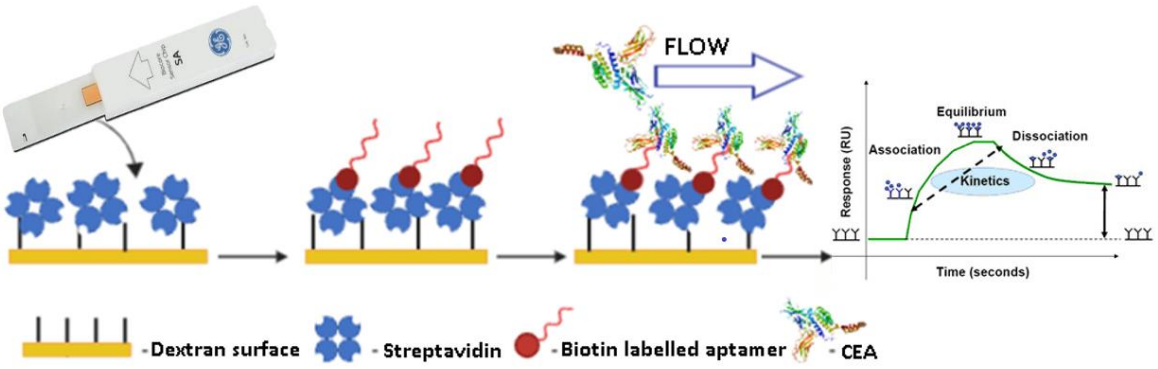

Figure S1. A schematic overview of a surface chemistry employed in the SPR technique using a streptavidin (SA) functionalized electrode for the detection of binding affinity of the aptamer (6) against CEA.

The results obtained from ELONA are bulk and need to be tested by more sensitive method. Aptamer (6) was immobilized onto SA electrode **(Figure S1)**, which is pre-coated with streptavidin, hence allows direct binding between biotinylated aptamer and streptavidin molecule. The surface of the chip was activated by regeneration buffer. There are two flow cells on the sensor chip. Flow cell - 1 was blocked by ethanolamine-HCl and was used as a reference cell. Flow cell - 2 was blocked by biotin to reduce non-specific binding. The Biacore provides automatic in-line reference subtraction.

Appendix 2.

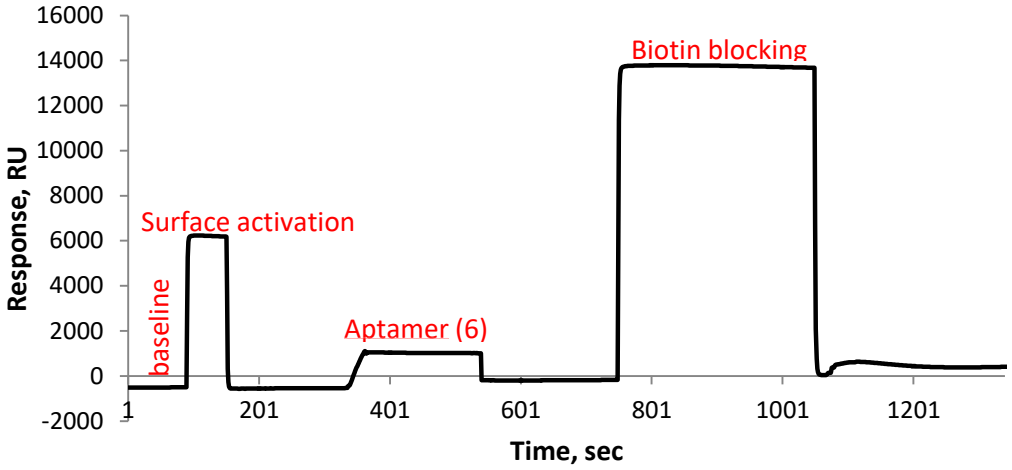

Figure S2. A typical SPR sensor response to the surface functionalization of the SA chip when the biotinylated aptamer sequence (6) was captured on the SA surface followed by the blocking with biotin.

Appendix 3.

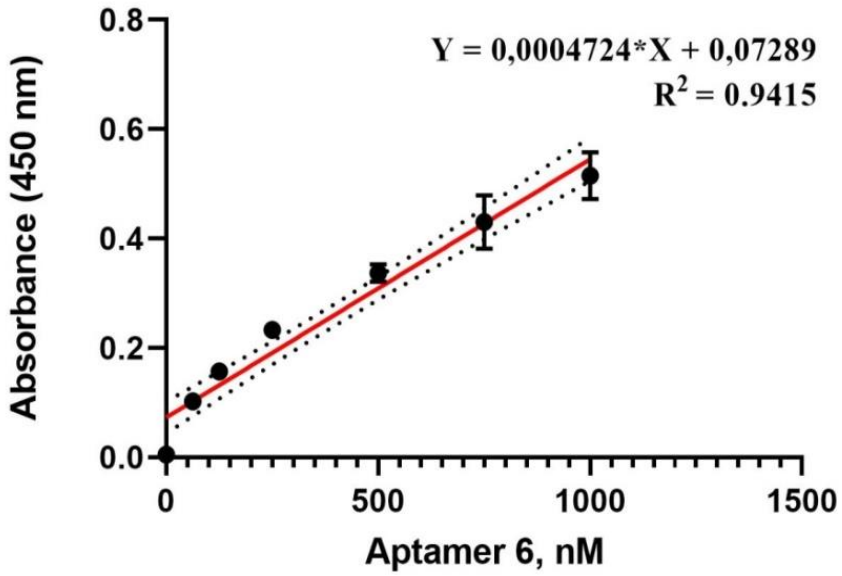

Figure S3. Binding of the aptamer sequence (6) in the concentration range of 0 – 1,000 nM to the target CEA (500 ng/mL) with the regression equation for OD_{450} difference versus aptamer sequence (6). All data are shown as the means of ±SEMs (n=3).