APTAMERS FOR BREAST CANCER PROTEIN MARKERS

By

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A Thesis

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DECLARATION

I do hereby declare that the content embodied in this thesis entitled **"Aptamers for Breast Cancer Protein Markers"** is the result of investigations carried out by me under the supervision of **Professor Utpal Bora** and is submitted to Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Assam, India for the award of degree of *Doctor of Philosophy in Biosciences and Bioengineering.* This work has not been submitted elsewhere for any degree or diploma or university to the best of my knowledge and belief.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators are referred, and copyright licenses have been taken from respective publishers.

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CERTIFICATE

This is to certify that the matter embodied in this thesis entitled **"Aptamers for Breast Cancer Protein Markers"** is the result of investigations carried out by **Mr. Arghya Sett** (Roll No.: 10610613) under my supervision, and is submitted to the Indian Institute of Technology Guwahati, Guwahati-781039, Assam, India for the award of degree of *Doctor of Philosophy in Biosciences and Bioengineering*. This work has not been submitted elsewhere for a degree.

UTPAL BORA, PhD

Professor

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August, 2016

DEDICATION

Dedicated to

Beloved Parents,

Respected Teachers,

Amrita

&

The ALMIGHTY

… Arghya

ACKNOWLEDGEMENTS

Time flies like an arrow, I have been at IIT Guwahati more than five years and I found those years as one of the most enriching moments of my life. I feel that I have matured as an individual identity and it couldn't be possible without the support of so many wonderful people around me and in my life. It's difficult, to put in words, how grateful, indebted I am to those people, but here is my modest attempt.

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"Where the mind is without fear and the head is held high;

Where the mind is lead forward by thee into

Ever-widening thought and action;

Into that heaven of freedom, my Father,

Let my country awake…" -Rabindranath Tagore

…….. Arghya Sett

SYNOPSIS

Breast cancer is a collection of clinically, histopathologically and molecularly heterogeneous diseases, with diverse outcomes and responses to treatment. One of the most prevalent invasive cancers forms in glandular tissues and supporting (stromal) tissues of the breast, usually the ducts (ductal carcinoma) and lobules (lobular carcinoma). Among several established biomarkers, Estrogen Receptor alpha (ERα) and Human Epidermal Growth factor receptor 2 (HER2) are the most prevalent biomarkers for diagnosis of breast cancer. These biomarkers are also important therapeutic targets for treatment of ERα, HER2 associated carcinomas. Traditional monoclonal antibody based diagnostics suffer from various drawbacks like high costs, complexity of production, batch to batch variability, cross-reactivity, contamination, and short shelf life. The substitute to conventional antibodies could be addressed by next generation affinity molecules that overcome the limitations of cost, synthesis, stability and specificity of target binding. Aptamers have gained significant importance as selective and affinity binding molecules due to the fact that they can be chemically synthesized with the added advantage of reduced time and production cost. Aptamers are short oligonucleotides that can be raised against almost every molecule of three dimensional structure. They offer great advantages due to their high target specificity, affinity, low molecular weights and the usual non-immunogenic nature. These aptamers can easily be modified, conjugated and developed to a sensor based system which will be more sensitive and specific to their targets.

Objectives: To develop a specific aptamer based electrochemical sensor for breast cancer detection, the following objectives were carried out:

I) Molecular cloning, over-expression and purification of recombinant marker proteins associated with breast cancer - a) Estrogen Receptor alpha , b) Extra cellular domain (ECD) of human epidermal growth factor receptor 2 (HER2).

II) Screening of DNA aptamer candidates specific for breast cancer biomarker proteins- ER α and HER2-ECD by *in-vitro* SELEX method.

III) Evaluation of binding specificity, characterization and sensitivity of candidate aptamers against ER α and HER2 positive cell lines and breast cancer tissue specimens.

IV) Modification of the enriched candidates and development of aptamer based electrochemical sensors to detect HER2 and ER associated carcinomas.

The thesis is organized into various chapters as described herein.

Chapter 1: Introduction and Review of Literatures:

A systematic review of literature was conducted to elaborate the existing technological advances in order to devise an effective plan of work to accomplish the chosen objectives of the research work. This chapter describes in detail the biomarkers of breast cancer, current trends of detection systems to diagnose the breast cancer. This chapter also discusses the oligonucleotide aptamers, various SELEX processes to screen the aptamers and advancements of SELEX screening technology. This chapter portrays in detail the various advantages of aptamers over traditional recognition molecules such as antibodies like ease of synthesis and modification, stability during long-term storage, nontoxicity, and lack of immunogenicity, which make them promising for various bioanalytical applications. It also briefs about the development of aptamers to analytical

device based various sensor platforms like optical, electrochemical, QCM and piezo electrical biosensors.

Chapter 2: Selection and Characterization of Specific Aptamers to Estrogen Receptor Alpha

This chapter describes the screening and selection of DNA aptamers specific to ER alpha, one of the pivotal biomarkers for breast carcinoma. To accomplish the objectives, we have successfully sub-cloned and expressed recombinant estrogen receptor alpha in pET28a. The specific DNA aptamer candidates were selected against NC-membrane immobilized ER alpha from a random pool of oligonucleotides after 14 iterative rounds of selection process. The sensitivity of the candidates were evaluated by isothermal calorimetry. Specificity of the aptamers toward the marker positive cell lines were tested by flow cytometry technique. To assess the biocompatibility of the selected aptamers, MTT assay was also performed both in cancer cell lines and normal cell lines. Immunocytochemistry with biotinylated aptamers suggests the specific binding of screened candidate aptamers to ER alpha positive cell lines. The specific aptamer stain the ERα-positive cells in breast tissues without cross-reacting to ERα-deficient fibroblasts, adipocytes, or inflammatory cells. To harness these characteristics of specific aptamers for detection of ERα expression in breast cancer samples, we performed the biotinylated aptamer-assisted histochemical analysis of ERα in tissue samples from breast cancer patients. The results were validated by performing immunohistochemistry on same samples with an ERα-antibody. All cytochemical and histochemical studies were performed considering scrambled aptamer sequence as a negative control. The study was aimed to screen novel DNA aptamer sequences against ER alpha and characterization of its specificity and sensitivity at the cellular and tissue level.

The chapter is further subdivided into separate sections detailing introduction, experimental sections, results, discussions and conclusions.

Chapter 3: Selection and Characterization of Aptamers Specific to Extra Cellular Domain (ECD) of Human Epidermal Growth factor Receptor 2 (HER2).

Human epidermal growth factor receptor 2 is a member of the EGFR family of receptors. Amplification of the HER2 gene and overexpression of its product induce cell transformation. Numerous studies have demonstrated the prognostic relevance of p185 (HER2), which is overexpressed in 10% to 40% of human breast tumors. Recent data suggest that p185 (HER2) is a ligand orphan receptor that amplifies the signal provided by other receptors of the HER family by heterodimerizing with them. Ligand-dependent activation of HER1, HER3, and HER4 by EGF or heregulin results in heterodimerization and, thereby, HER2 activation. Thus, HER2 is a critical biomarker for cancer diagnostics and an ideal target for therapeutics. In the present study, we developed novel DNA aptamers against the extra cellular domain (ECD) of HER2 through *in-vitro* Systematic Evaluation of Ligands by Exponential Enrichment (SELEX) process. The selected aptamer showed high affinity and selectivity towards the ECD domain of HER2, which plays a key role in target based cancer therapeutics. Isothermal calorimetry revealed the stronger binding affinity (K_d = 6.32±3.65nM) of thermodynamically stable aptamer candidates. Biotinylated DNA aptamer candidates showed stronger cytoplasmic staining in a HER2 positive breast cancer cell line (SKBR3) compared to HER2 negative cell (MDA MB 231). Cellular and histochemical studies revealed that this DNA aptamer could be used as a theranostic agent for HER2 positive carcinomas and could provide a novel cost effective alternate to conventional anti-HER2 antibody in solid phase immunoassays for cancer diagnosis and related applications.

This chapter is further subdivided into separate sections detailing introduction, experimental sections, results, discussions and conclusions.

Chapter 4: Aptamer Based Electrochemical Sensor System for Detection of Breast Cancer Protein Biomarkers.

This chapter describes a relatively general approach to the electrochemical aptamer based sensors that are based on the target binding-induced conformational change of the aptamers. We have employed the principle of target-induced folding or unfolding of electrode-bound oligonucleotides to fabricate electrochemical aptasensor. The specific aptamers were modified at both the end (redox group methylene blue at 3' end and thiol at the 5'end). The aptamers were attached to gold electrode surfaces by thiol chemistry and then analytes were detected by an electrochemical apparatus. All electrical measurements were conducted using alternating current voltammetry (ACV) at an AC frequency (frequency of 50 Hz) using a CHI 630 potentiostat (CH Instruments) in a standard 3 electrochemical cell system-Platinum wire was considered as counter electrode, where reference electrode (Ag/AgCl; saturated with 3 M KCl) and gold electrodes as working electrodes on which thiolated aptamers were immobilized. The aptamers can detect the target biomarkers in PBS at a nanomolar sensitivity level.

This chapter is also further subdivided into separate sections detailing introduction, experimental sections, results, discussions and conclusions

Chapter 5: Summary and Future Prospects:

This present thesis work describes the selection of aptamers specific to various breast cancer protein biomarkers like ER alpha and Her 2. These aptamers can lead to a sensor based system for detection of marker positive breast carcinomas.

Since ERα and Her2 is overexpressed in most carcinomas like breast cancer, gastric cancer, endometrial cancer, ovarian cancer, the ER α specific aptamers might have prospective as a

guiding ligand for targeted chemotherapy against a plethora of multiple malignancies. Nevertheless, extensive research with animal models and further clinical trials in patient samples is still required to validate the *in-vivo* binding specificity of the selected specific DNA aptamer candidate sequences.

The work presented in the thesis has been peer reviewed and resulted in the following international journal publications:

1) Ahirwar R, Vellarikkal SK, **Sett A**, Sivasubbu S, Scaria V, Bora U, et al. (2016) Aptamer-Assisted Detection of the Altered Expression of Estrogen Receptor Alpha in Human Breast Cancer. PLoS ONE 11(4): e0153001. doi:10.1371/journal.pone.0153001.

2) Sett, A., Das, S., & Bora, U. (2014). Functional Nucleic-Acid-Based Sensors for Environmental Monitoring. Applied Biochemistry and Biotechnology, 174(3):1073-91.doi: 10.1007/s12010-014-0990-3.

3) Bora U, Sett A, Singh D (2013) Nucleic Acid Based Biosensors for Clinical Applications. Biosens J 1: 104. doi: 10.4172/2090-4967.1000104.

4) Sett A., Das S., Sharma P. and Bora U. (2012) Aptasensors in Health, Environment and Food Safety Monitoring. Open Journal of Applied Biosensor, 1:9-19. doi: 10.4236/ojab.2012.12002.

Manuscripts communicated

1) Sett A., Borthakur B B, Sharma J D, Kataki A C, Bora U. (2016) DNA Aptamer Probes for Detection of Estrogen Receptor α Positive Carcinomas.

2) Sett A., Borthakur B B, Sharma J D, Kataki A C, Bora U. (2016) Selection of DNA Aptamers for detection of HER2 associated carcinomas.

3) Sett A., Mosahari V.P, Kalita J, Das D, Bora U. (2016) Development of an electrochemical aptamer based sensor system for breast cancer detection.

Manuscripts from collaborative work

1) **Sett A**, Gadewar M, Sharma P, Deka M, Bora U. (2016) Green synthesis of gold nanoparticles using aqueous extract of Dillenia indica. Adv. Nat. Sci.: Nanosci. Nanotechnol. 2(7).

2) Das R.K., **Sett A**, Kasoju N, Sumithra B, Bora U. (2011) Encapsulation of curcumin into poly-€-caprolactone nanoparticles and its physicochemical Characterization. International Journal of Biological Sciences and Engineering, 2(1), pp.1-8.

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Introduction and Review of Literature

Chapter 1

Introduction and Review of Literature

1.1 Introduction

Breast cancer is a collection of clinically, histopathologically and molecularly heterogeneous diseases, with diverse outcomes and responses to treatment. (Viale 2012) It is the one of the most prevalent invasive cancers which forms in tissues of the breast, usually the ducts (tubes that carry milk to the nipple) and lobules (glands that make milk). (Rivenbark et al. 2013) It occurs in both men and women, although male breast cancer is rare. In the past few decades, across the world, breast cancer has emerged to be one of the top cancer killers amongst women. Last year, one million new cases were diagnosed and more than five hundred thousand lives were claimed globally. Previously, USA had the worst breast cancer mortality statistics in the world but recently, India surpassed USA with more than fifty thousand mortalities due to breast cancer. WHO forecasts that by 2020, 70% of all breast-cancer cases worldwide will be in developing countries (http://www.notouchbreastscan.com/awareness-globaldisease.html).The prognosis and survival rate of breast cancer patients vary greatly depending on cancer type and staging. With best treatment and dependent on staging, 5-year relative survival varies from 98% to 23%, with an overall survival rate of 85 %.(Jemal et al. 1999) So as prevention is always better than cure, the early detection and diagnostics is a preeminent way to combat against breast carcinoma.

Classical clinico-pathological features indicating patient prognosis include tumor size, histological subtype and grade, lymph node metastases and lymphovascular invasion, which are derived from careful histological analysis of primary breast cancer samples. The TNM (tumor size, nodes, and

metastasis) system integrates these into tumor stages that have major prognostic value. (NCCN Clinical Practice Guidelines in Oncology). In this era of high-throughput methods, a plethora of novel biomarkers have been reported for prognostic and predictive purposes. However, only a few are considered in clinical routine due to the lack of sufficient validation to reach a Level of Evidence I or II according to the American Society of Clinical Oncology's Tumor Marker Utility Grading System (Harris et al. 2007; Hammond et al. 2010). Prognostic factors intend to predict objectively and independently patient clinical outcome independent of treatment, while predictive factors aim to forecast the response of a patient to a specific therapeutic intervention and are associated with tumor sensitivity or resistance to that therapy. Besides, prognostic factors necessarily require definition in patient cohorts that did not undergo systemic adjuvant treatment. Predictive markers may be the target of a specific therapy itself. Breast cancer treatment has experienced several changes in the past decades due to the discovery of specific prognostic and predictive biomarkers that enable the application of more individualized therapies to different molecular subgroups.(Williams et al. 2006) In patients with breast cancer, overexpression of protein biomarkers are associated with poor prognosis, aggressiveness of the disease, as well as resistance to chemotherapy and hormonal therapy. Thus, strategies to target these protein biomarkers (HER2, ER, and PR) are important in treating advanced breast cancer (Esteva *et al*. 2002).

1.2 Biomarkers and Disease management

Biological markers (biomarkers) can be defined as "cellular, biochemical or molecular alterations that are measurable in biological media such as human tissues, cells, or any biological fluids"(Naylor 2003). However, lately, the terminology has been broadened to include biological characteristics that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention. In clinical practice, biomarkers

include tools and technologies that can aid in understanding the prediction, cause, diagnosis, progression, regression, or management of any disease. At the onset of unveiling of entire human genome sequence, we are now capable of finding minor changes or alterations throughout the genome. These advances hold the promise of being able to tailor treatments to patients, and the potential benefits of clinically-relevant biomarkers would improve clinical efficacy and lower suffering by better treatment and patient selection. Biomarkers have been classified by Perera *et al* based on the sequence of outcomes from exposure to disease outset (Fig.1.1) (Perera and Weinstein 2000)

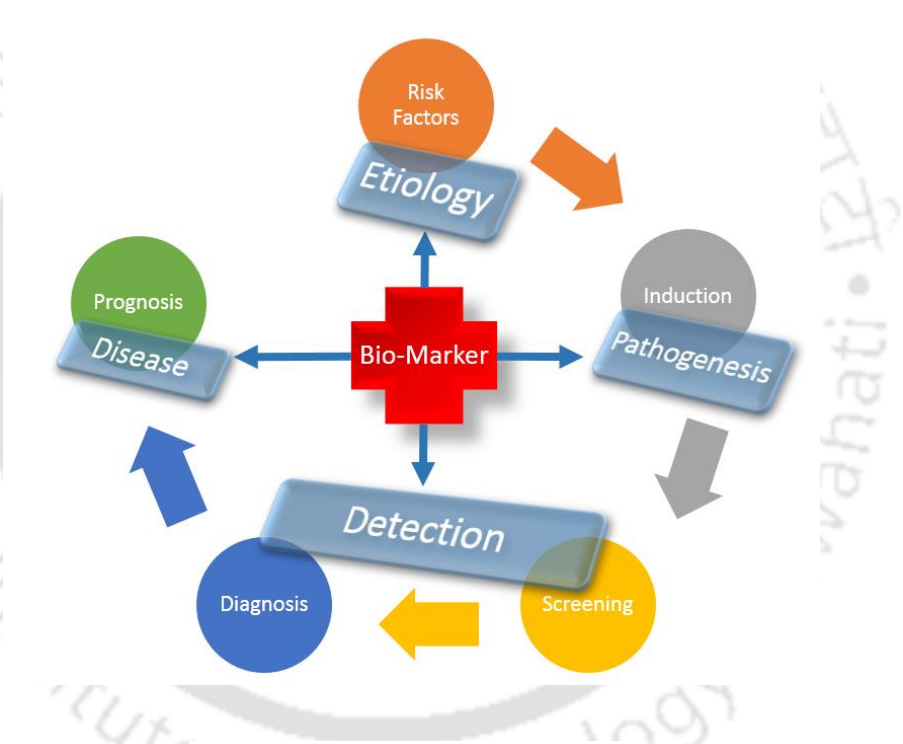

Fig 1.1 Application of Biomarkers

Although biomarkers readily offer epidemiological investigations, they are also useful in the investigation of the natural history and prognosis of a disease. Scientists have outlined the capabilities of biomarkers. In addition to defining the events between exposure and disease, biomarkers have the potential to identify the earliest events in the natural history, reducing the probability of misclassification of both disease and exposure, opening a window to potential mechanisms related to

the disease pathogenesis and accounting for some of the variability and effect modification of risk prediction. Biomarkers can also provide insight into disease progression, prognosis, and response to therapy. (Mayeux 2004)

Capabilities of Biomarkers include:

- Explanation of events between exposure and disease.
- Identification of early events in the natural history.
- Identification of mechanisms by which exposure and disease are related.
- Evaluation of dose-response.
- Reduction in misclassification of exposures or risk factors and disease.
- Establishment of variability and effect modification.
- Enhanced individual and group risk assessments.

Molecular biomarkers have the additional potential to identify individuals susceptible to various diseases or disorders (Galasko 2001). Molecular genetics already had a substantial impact on neurological practice, cancer detection and pathogenesis leading to improved diagnosis system. Classification of populations in terms of the degree of susceptibility on the basis of such biomarkers produces greater accuracy than relying on historical definitions of susceptibility(Muller and Graeber 1996). For example, a biomarker will allow the stratification of a population on the basis of a predisposed specific "genotype" associated with a specific illness rather than relying on a report of the "family history" of the disease. The capability to quantify "susceptibility" would be an extremely important protocol for estimating disease risk among various population.

Although some biomarkers are associated directly to the pathway of a disease, few also are related in some indirect way to the cause of disease. A biomarker could be dependent on another known or unknown factor to cause the disease. Thus, it is not the only determinant but it is in the causal pathway

and remains strongly related to the disease. Biomarkers showing prodromal signs enable earlier diagnosis or detect the outcome of interest to be determined at a more primitive stage of disease. Blood, urine, cerebrospinal fluid and serum provide the necessary biological information for the diagnosis. In these abnormal conditions, biomarkers are used as an indicator of a biological factor that represents either a subclinical manifestation, stage of the disorder, or a surrogate manifestation of the disease. Variability of biomarker expression is a major concern for which numerous statistical validation (multivariate analysis, Receiver Operating Characteristics (ROC) analysis) needs to be done. (Rotherham et al. 2012) The development of any biomarker should go in parallel with the standard design of any epidemiological project or clinical trials. The clinical trials of biomarker validations must be concluded with its accuracy, reliability, interpretability, and feasibility determination.

1.3 Biomarkers of Breast Cancer

Biomarkers are useful for diagnosis, monitoring disease progression, predicting disease recurrence, and therapeutic treatment efficacy. Predicting cancer outcome and influencing the cancer treatment will have a major role in determining the cost effectiveness in clinical cancer management.(Williams et al. 2006) With the advent of new advanced genomic and proteomic tools such as DNA & tissue microarray, 2D gel electrophoresis, mass spectrometry, protein assays coupled with bioinformatics analysis, it is possible to develop biomarkers that are capable to reliably & accurately predict outcomes during cancer management & treatment. (Baskın and Yiğitbaşı 2010; Beretov et al. 2014)In addition to the classical clinical prognostic factors of breast cancer, established molecular biomarkers such as estrogen receptor, human epidermal growth factor receptor 2, progesterone receptor have played a significant role in the selection of patients benefiting from endocrine therapy. There are three major protein biomarkers: human epidermal growth receptor2 (HER2), Estrogen receptor (ER) and

progesterone receptor (PR). Apart from these, there are several prognostic markers by which metastasis might be predicted or detected.

The marker of proliferation Ki67 was first identified by Gerdes *et al* in the 1980s using a mouse monoclonal antibody against a nuclear antigen from a Hodgkin's lymphoma cell line(Gerdes et al. 1984). Ki67 is a nuclear non-histone protein and was named after the University of Kiel, Germany. In early as well as locally advanced breast cancer, baseline Ki67 has been found to predict for response to chemotherapy, whereas this is not the case for endocrine treatment(Landberg and Roos 1993; Faneyte et al. 2003). In a study, it was reported that post-neoadjuvant chemotherapy measurement of Ki67 is a strong predictor for recurrence-free and overall survival(Ge et al. 2015). New approaches of combining established markers with novel factors are currently under evaluation. One of these is an immune-panel of ER, PgR, HER2, and Ki67, whose ability to differentiate between luminal A and B subtypes in a similar manner as the original 50-gene signature has been shown (Cheang et al. 2009). In this context, a cut-off of 13.25% Ki67 positive staining was used to discriminate between the subtypes, indicating that a higher score defines luminal subtype B tumors with a worse prognosis.

BRCA1 and BRCA2 are human genes located in chromosome 17 and 13, respectively,that produce tumor suppressor proteins. A person who inherits certain mutations (changes) in a BRCA1 gene has a higher risk of getting breast, ovarian, prostate, and other types of cancer. These proteins help repair damaged DNA and, therefore, play a role in ensuring the stability of the cell's genetic material. When either of these genes is mutated, or altered, such that its protein product either is not made or does not function correctly, DNA damage may not be repaired properly. As a result, cells are more likely to develop additional genetic alterations that can lead to cancer. Specific inherited mutations in BRCA1 and BRCA2 increase the risk of female breast and ovarian cancers, and they have been associated with increased risks of several additional types of cancer. Recently, researchers have studied possible

associations between lifestyle and reproductive factors and age at diagnosis. (Rieder et al. 2016) Together, BRCA1 and BRCA2 mutations account for about 20 to 25 percent of hereditary breast cancers and about 5 to 10 percent of all breast cancers(Claus et al. 1996; Berliner and Fay 2007; Meindl et al. 2011). In addition, mutations in BRCA1 and BRCA2 account for around 15 percent of ovarian cancers overall. (King et al. 2003) Breast and ovarian cancers associated with BRCA1 and BRCA2 mutations tend to develop at younger ages than their nonhereditary counterparts.

A harmful BRCA1 or BRCA2 mutation can be inherited from a person's mother or father. Each child of a parent who carries a mutation in one of these genes has a 50 percent chance of inheriting the mutation. The effects of mutations in BRCA1 and BRCA2 are seen even when a person's second copy of the gene is normal.

Nuclear Cyclin D1 is a key player in the human oncogenic pathway. (Kim and Diehl 2009) It is overexpressed at the mRNA and protein level in over 50% of breast cancer cases including 15% in which a gene amplification occurs.(Gillett et al. 1994; Courjal et al. 1996). Cells in the G1 phase of the cell cycle react to growth factor stimulation with the induction of D-type cyclins. (Reis-Filho et al. 2006). A possible predictive value of cyclin D1 in hormone receptor-positive patients has also been shown and recently the overexpression as well as amplification of the gene is a predictor of poor response to antiestrogen treatments. (Han et al. 2003; Kilker and Planas-Silva 2006). Further investigation is required to elucidate the specific role of cyclin D1 in breast tumor metastases.

1.3.1 Human Epidermal Growth Factor Receptor 2 (HER2)

Human epidermal growth factor receptor 2 (HER2) has been validated not only as a prognostic factor, but also a predictor of response to HER2 targeting therapy. The shift toward an earlier diagnosis of breast cancer due to improved imaging methods and screening programs highlights the need for new factors and combinations of biomarkers to quantify the residual risk of patients and also to indicate

the potential value of additional treatment strategies. With the introduction of high-throughput technologies, numerous multigene signatures have been identified that aim to outperform traditional markers: current prospective clinical trials are seeking evidence for their definitive role in breast cancer. There exist many more factors and approaches that have the potential to become relevant in the near future such as the detection of single disseminating and circulating tumor cells in blood and bone marrow as well as the detection of circulating cell-free DNA and microRNA (Aarthy *et al.* 2015). Randomized prospective testing and comparison with existing factors will be required to select those emerging biomarkers that offer substantial cost-effective benefit and thereby routine use for breast cancer diagnostics will be justified.

The HER2 proto oncogene is located on chromosome 17 and encodes a 185 kDa membrane-spanning protein composed of a ligand-binding extracellular domain (ECD), an alpha-helical transmembrane segment, and intracellular tyrosine kinase domain. HER2 is found in normal cells, but it is present in abnormally high quantities in a subset of breast carcinoma (BC) characterized by an aggressive natural history. (Bartlett *et al.* 2001; Weigelt *et al.* 2010). Although HER2-positive BCs usually display several genomic aberrations, they remain critically dependant on HER2 signalling for survival and growth. Blocking the HER2 receptor or its key downstream regulators can lead to cell apoptosis, often translating into striking clinical benefit. HER2 thus represents an important target which clearly drives breast oncogenesis and anti-HER2 therapy is the widely studied issue to combat against breast cancer. (Torti and Trusolino 2011).

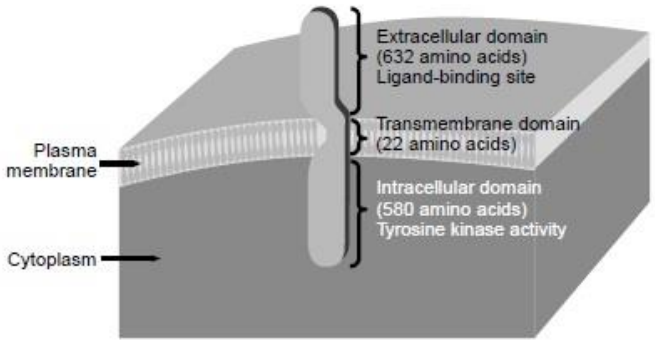

Fig 1.2. Three Domains of Her2 Protein (Ross et al. 2009)

HER2 is the second member of the human epidermal growth factor receptor (HER) family. (Cho et al. 2003) It is often called an orphan receptor as it does not bind to specific lgand for its activation. It plays a key role in the HER family, cooperating with other HER receptors via a complex signalling network to regulate cell growth, differentiation and survival. The HER2 proto-oncogene (also known as neu or c-erbB-2) encodes the transmembrane HER2 protein. HER2 is normally expressed in a variety of cell and tissue types excluding those of hematopoietic origin and importantly it is frequently overexpressed in a number of human cancers (Eccles 2001). Although HER2 is widely distributed, its signalling activity is regulated by the presence of HER agonists (ligands) and trans-modulating partners. (Rubin and Yarden 2001) The HER receptor family is involved in the regulation of normal breast growth and development and overexpression of HER2 is associated with breast cancer. (Arteaga and Engelman 2014). HER2 gene amplification (generation of more than the normal two gene copies) is the most common mechanism leading to breast cancer. (Yarden 2001) Approximately 15-20% of all BCs are HER2 positive and display HER2 protein overexpression (detected by IHC), which is always associated with HER2 gene amplification (detected by fluorescent in- situ hybridization). Of note, up to 40% of inflammatory breast cancers may be HER2- positive. Cleavage of the HER2 ECD results in a truncated version of the HER2 receptor that may be associated with resistance to trastuzumab.

HER2, a transmembrane protein of the Human epidermal growth factor receptor (hEGFR) family is a crucial predictive biomarker for various carcinomas (Tetu et al. 1996). (Fig. 1.3) Expression of HER2 is significantly related to positive lymph nodes, poor nuclear grade, lack of steroid receptors and high proliferative activity. It is a true predictive marker whose presence not only provides an indication of disease occurrence but also can lead to selection of appropriate cancer treatment such as the use of Herceptin® to treat patients with HER2 positive tumors. (Arteaga et al. 2011) The combination of HER2, estrogen and progesterone receptor status can provide a better indication of disease outcome in breast cancer.HER2 also plays an important role in gastric cancer. Though less is known about the molecular pathways induced by the HER2 receptor in gastric cancer compared to breast cancer, few researchers suggest that the HER2 receptor plays a similar role in gastric cancer (Gravalos and Jimeno 2008). There is mounting evidence of the role of HER2 overexpression in patients with gastric cancer, and it has been solely correlated to poor outcomes and a more aggressive disease. Additionally, preclinical data are showing significant antitumor efficacy of anti-HER2 therapies (particularly monoclonal antibodies directed towards the protein) in *in vitro* and *in vivo* models of gastric cancer.

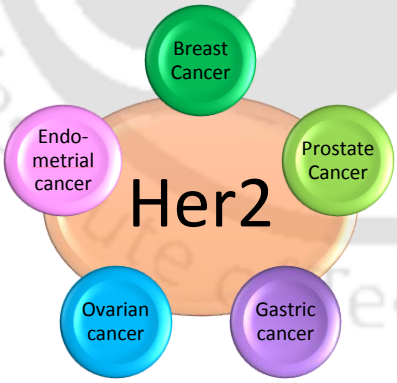

Fig1.3. Her2 as a biomarker in various carcinomas

1.3.2 Estrogen Hormone Receptor alpha (ERα)

For breast cancer management pathologic profile and particularly the expression of estrogen receptor (ER) and progesterone receptor (PR) are considered essential. In advanced cases, core biopsy results are the only data available. To evaluate reliability of data, results of ER, PR, MIB1, p53 and c-erbB2 on core biopsy were compared with those on surgical specimens. Results showed a statistically significant concordance for ER and PR in pT1 but not in pT2 tumours, possibly due to breast cancer heterogeneity. MIB1 results showed no significant concordance even for the pT1 group. There was statistically significant concordance even with pT1 and pT2 groups for p53 and c-erbB 2, probably due to the high number of negative cases for these markers. (Huang et al. 2014)

Steroid hormone receptors are multi-domain proteins composed of conserved well-structured regions, such as ligand binding domains (LBD) and DNA binding domains (DBD) along with other naturally unstructured regions including the amino-terminal domain (NTD) and the hinge region between the LBD and DBD. The hinge is more than just a flexible region between the DBD and LBD and is capable of binding co-regulatory proteins and the minor groove of DNA flanking hormone response elements. Since the hinge region can directly participate in DNA binding, it has also been termed the carboxyl terminal extension (CTE) of the DNA binding domain. The CTE and NTD are dynamic regions of the receptor that can adopt multiple conformations depending on the environment of interacting proteins and DNA. Both regions have important regulatory roles for multiple receptor functions that are related to the ability of the CTE and NTD to form multiple active conformations.

Estrogen hormone exert a wide variety of effects on growth, development, and differentiation, including important regulatory functions within the reproductive systems of both females and males, in mammary gland development and differentiation, as anti-atherosclerotic agents, in central nervous

system functions, and in the regulation of hypothalamic-gonadal axis (Htun et al. 1999). Estrogen hormone mediates these activities through binding to a specific intra-nuclear receptor protein, estrogen receptor (ER), encoded by two genes: alpha and beta (ERα and ERβ) that function both as signal transducers and transcription factors to modulate expression of target genes. While the action of ER alpha is already known, few fundamental questions are still unanswered. Since the initial cloning of the human estrogen receptor alpha (h-ERα) cDNA in 1986, there have been numerous attempts to express h-ERα in bacteria, yeast, baculovirus, and mammalian cells. (Zhang et al. 1999) However, it has been difficult to produce the relatively large amounts of ER required for biochemical, biophysical and structural studies. Zhang *et al* worked on genetic selection of steroid receptor DNA binding domain mutants with altered DNA binding specificity and enhanced affinity for the estrogen response element (ERE) and suggested that expression of a functional ER DNA binding domain might be toxic to *E.coli.* They had suggested that ER toxicity and loss of the ER expression plasmid, rather than the inability to fold the expressed ER, might be responsible for the low levels of ER expression in earlier works. The ER is believed to play an important role in the growth and development of a subset of hormone-dependent human breast cancers. Approximately one-third of all breast tumours contain significant amounts of ER and about two thirds of these are able to respond objectively to some form of anti-estrogen endocrine therapy. (Klinge 2000; Lonning 2012) Therefore, a better understanding either of the mechanism of estrogen action in these tumours or of the way in which their expression is regulated may lead to an improvement in the therapy of these cancers.

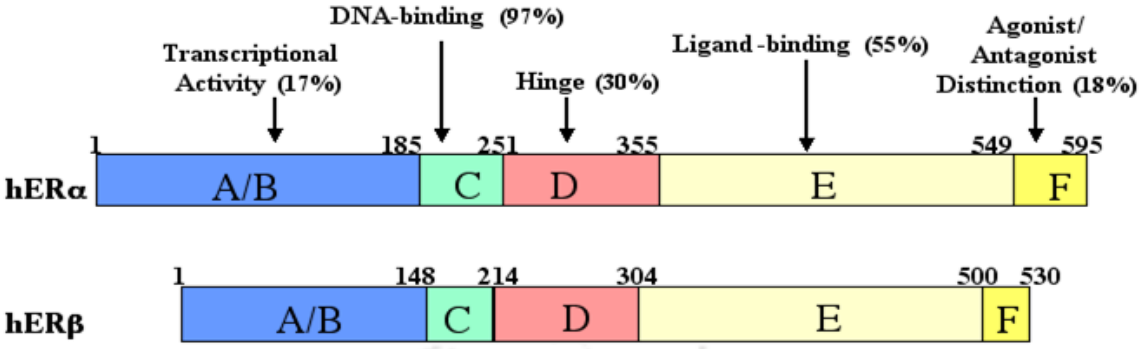

Fig 1.4: Schematic representation of the common structural and functional domains of ERα and β. (Klinge 2000)

Both ERs are widely expressed in different tissue types, however, there are some notable differences in their expression patterns. (Kumar et al. 2011) The ERα is found in endometrium, breast cancer cells, ovarian stroma cells, and the hypothalamus. (Brandenberger et al. 1997) In males, ERα protein is found in the epithelium of the efferent ducts. The expression of the ERβ protein has been documented in kidney, brain, bone, heart, lungs, intestinal mucosa, prostate, and endothelial cells. (Scobie et al. 2002) In terms of sequence homology, the ERβ shows a high homology to ERα in the DNA binding domain DBD (more than 95% amino acid identity) and in the ligand binding domain LBD (~55% amino acid identity). (Cowley et al. 1997) However, the N-terminal domain -NTD of ERβ is shorter than that of ERα with very poor sequence homology of only ~15% compared to that of ERα. (Fig.1.4)

Estrogen receptors are over-expressed in around 70% of breast cancer cases, referred to as "ER-positive", and can be demonstrated in such tissues using immunohistochemistry. Two hypotheses have been proposed to explain why ER causes tumorigenesis, and the available evidence suggests that both mechanisms contribute.

Firstly, binding of estrogen to the ER stimulates proliferation of mammary cells, with the resulting increase in cell division and DNA replication, leading to mutations. Secondly, estrogen metabolism produces genotoxic waste.

Both processes result in disruption of the cell cycle, apoptosis, DNA repair and therefore, tumour formation. ERα is certainly associated with more differentiated tumours, while evidence of ERβ involvement is controversial. (Shanle and Xu 2010) Different versions of the ESR1 gene have been identified (with single-nucleotide polymorphisms) and are associated with different risks of developing breast cancer. (Deroo and Korach 2006a; Deroo and Korach 2006b) Endocrine therapy for breast cancer involves selective estrogen receptor modulators (SERMS), such as tamoxifen, which behave as ER antagonists in breast tissue, or aromatase inhibitors such as anastrozole. ER status is used to determine sensitivity of breast cancer lesions to tamoxifen and aromatase inhibitors.(Dutertre and Smith 2000) Raloxifene (SERM molecule), has been used as a preventive chemotherapy for women judged to have a high risk of developing breast cancer. (Von Holst 2000; Ribo 2001). Another chemotherapeutic anti-estrogen, ICI 182,780 (Faslodex), which acts as a complete antagonist, also promotes degradation of the estrogen receptor. (Howell 2006) Results from two Phase III trials revealed that fulvestrant (Faslodex) is as effective as the third-generation selective aromatase inhibitor anastrozole in postmenopausal women with advanced breast cancer following progression on anti-estrogen therapy. In clinical practice, fulvestrant is well tolerated and much prescribed, with good rates of clinical benefit observed as first-line therapy and following progression on prior endocrine agents.

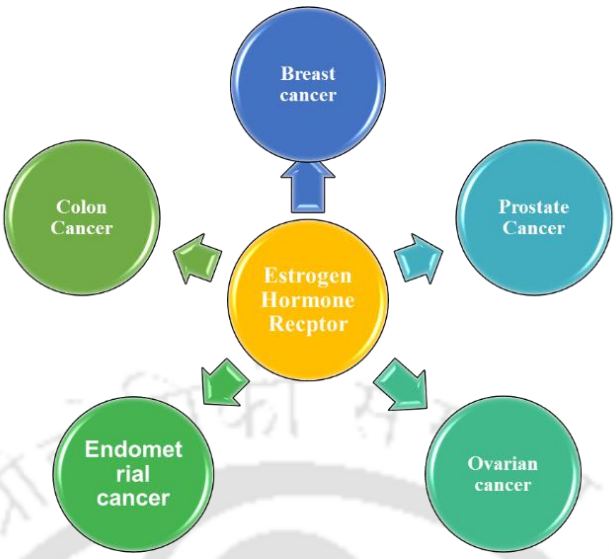

Fig 1.5: Role of ER alpha in various cancers

Estrogen and the ERs have also been implicated in breast cancer, ovarian cancer, colon cancer, prostate cancer, and endometrial cancer. Advanced colon cancer is associated with a loss of ERβ, the predominant ER in colon tissue, and colon cancer is treated with ERβ-specific agonists. (Schleipen et al. 2011) Phytoestrogens such as quercetin can modulate estrogen receptor activities in such a way that it may prevent cancers including breasts, prostate, colon, colorectal and others by stimulating apoptosis. (Caiazza et al. 2015) Being ERβ a ligand-activated transcription factor, it binds to estrogen response elements located in the promoter region of the gene. (Klinge 2000) This was tested in HeLa cells treated with a pure estrogen receptor antagonist which blocked both estradiol and quercetin from inducing caspase-3 activation. ERβ is expressed in the human colon and activates a specific signal transduction pathway that controls apoptosis in the colon. Generally ERβ gets activated by estradiol and recently found to be activated by quercetin.

1.3.4 Progesterone Hormone Receptor (PR)

In light of recent clinical trials, the debate concerning the risks and benefits of progestin-based postmenopausal hormone replacement therapy (HRT) has reached a renewed level of urgency. (Ismail et al. 2003) Irrespective of any hypothesis, the need of the hour is to perform more basic research to address progesterone's fundamental role in mammary development and tumorigenesis. Towards this end, the progesterone receptor knockout (PRKO) mouse demonstrated that progesterone is essential for pregnancy-associated mammary gland ductal side-branching and alveologenesis and that these morphological changes are dependent on progesterone-induced mammary epithelial proliferation. Importantly, the PRKO mouse showed that the progesterone-proliferative signal significantly contributes to mammary tumour susceptibility in an established mammary tumour model. Insight into the cellular mechanism(s) by which progesterone affects mammary morphogenesis has been disclosed by a new PR-LacZ knockin mouse, which revealed that PR's spatial expression pattern undergoes precise choreographed distributional changes that precede key stages in postnatal mammary development.(Graham and Clarke 2002) In the case of early pregnancy, the segregation of cells undergoing progesterone-induced proliferation from those that express PR implicates a paracrine mode of action for progesterone-induced mammary epithelial proliferation, whereas the pre-parturient decline of PR expression underscores the need to remove this signal for full functional differentiation of this tissue. Our findings support the proposal that the mammary gland's normal response to the progesterone-signal is dependent upon specific spatial organizational patterns of PR expression and that derailment in these cellular processes may contribute to abnormal mammary development, including cancer. To identify the downstream molecular targets that mediate progesterone's effects in this tissue and further investigation of such targets will not only enhance our mechanistic understanding of progesterone's role in mammary

development and cancer, but may also facilitate the formulation of new design strategies in breast cancer diagnosis and/or treatment. Based in part on progesterone's established role in countering estrogen induced epithelial proliferation in the endometrium (the rationale for the inclusion of progestins in postmenopausal hormone replacement therapy (HRT)), this steroid was assumed to elicit similar anti-proliferative effects in the mammary epithelium and, therefore, exert negligible contributions toward mammary tumour initiation and/or progression.(Hill et al. 2012)

1.4 Triple Negative Breast Cancer

Triple negative breast cancer (TNBC) - negative for estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) is a distinct breast cancer subtype, which remains a great clinical challenge. TNBC is an immunohistochemical subtype of breast cancers with a triplet of negative staining for ER, PR and HER2 (Lehmann et al. 2011). In contrast, the basal-like breast cancer phenotype is molecularly defined. (Ismail-khan and Bui 2010)Critical to this immunohistochemical definition of TNBC is clarity regarding 'negative' staining of all three biomarkers. Prior arbitrary thresholds for discriminating between positive and negative ER and PR status include 1%, 10% and 20%. Diagnostic thresholds vary between laboratories and clinical trials, making cross trial comparison and application of results difficult. Therapeutic efficacy for TNBC patients is also very poor. (Ismail-khan and Bui; Gelmon et al. 2012). In TNBC, majority is grouped as infiltrating ductal carcinoma, otherwise not categorized. Prototypical histopathological features of TNBC are high grade, poor tubule formation, high mitotic count, a pushing border, central fibrosis and lymphocytic infiltrate. Prognosis and response to treatment vary depending on the marker expression. It is essential to identify subtypes with a better prognosis for those who may be spared intensive adjuvant therapy, and equally to identify those in greatest need of systemic intervention. This biological diversity within TNBC has been explored by gene expression profiling. Five main

clusters were discriminated by gene expression of epidermal growth factor receptor (EGFR), CK5/6, c-KIT, the androgen receptor and interferon/immunoglobulin related-genes. Particularly the immune features were strongly associated with prognosis.

Assessment of biomarkers by traditional monoclonal antibodies face great challenge in the past few years. To circumvent these issues, a specific class of biomolecules have emerged as a 'Magic Bullet'.(Jayasena 1999). In the year 1990, Gold and Szostak identified a class of oligonucleotides 'Aptamers' from randomly synthesized oligonucleotide libraries, following a process known as SELEX (Systematic Evolution of Ligands by Exponential enrichment). (Tuerk and Gold 1990; Aquino-Jarquin and Toscano-Garibay 2011). The most important property of an aptamer, from the Latin aptus (to fit), is its high target selectivity. These short, chemically synthesized, single-stranded (ss) RNA or DNA oligonucleotides fold into specific three-dimensional (3D) structures with dissociation constants usually in the pico- to nano-molar range. Moreover, in contrast to other nucleic acid molecular probes, aptamers interact with their targets through structural recognition, a process similar to that of an antigen-antibody reaction. Thus, aptamers are also referred to as "chemical antibodies." These multifaceted molecules play a pivotal role in modern disease diagnosis and therapeutics. (Santosh and Yadava 2014)

1.5 Aptamers-A Novel class of oligonucleotides for disease management

Aptamers are small oligonucleotides, peptides and peptide nucleic acids that can bind with high affinity and selectivity to diverse targets like small and macromolecules, from organic, inorganic and biological origin. Aptamers can fold into distinct secondary and tertiary structures, bind to their targets with high affinity (dissociation constants on the order of nano- to picomolar) and recognize their targets with a specificity that challenges antibodies and other biological ligands. Nucleic acid aptamers are selected from a random pool by an iterative process called SELEX (**S**ystematic **E**volution of

Ligands by **Ex**ponential Enrichment Analysis), whose principle is similar to that of natural selection in evolution proposed by Charles Darwin over 150 years ago. SELEX process combines aptamer enrichment through iterative selection process of affinity binding and polymerase chain reaction (PCR) to select high affinity, feature-specific single-stranded RNA, DNA or PNA (peptide nucleic acid) aptamers. The selection of ligands beyond natural systems emanates from a chemically produced oligonucleotide library with the variety of sequences e.g. 10^{13-15} different oligonucleotides. The number of variation depends on the length of the variable region. With a variable region of 40 oligonucleotides, theoretically, there are possibilities of approximately $4^{40}(\cong 10^{15})$ random oligonucleotide sequences. Diversity of the oligonucleotide library and the amplification steps of target-binding oligonucleotides during the selection process considerably facilitates the selection of ligands with highest affinity compared to the natural selection process. The SELEX process can be carried out under conditions similar to such used in the assay for which the aptamer is being developed. As a consequence, the aptamer will maintain its structure and will function in the final assay. The aptamer will not dissociate or otherwise change its physical characteristics, which might be a problem with antibodies.

Antibody based treatment suffers from various drawbacks. They are highly immunogenic, laborious and expensive to produce and with high batch to batch variation. To circumvent antibody issues, nucleic acid ligands "Aptamer" based technologies are emerging as a novel candidate in diagnostic and treatment of various diseases. These synthetically enriched molecules are simple and economic to synthesize, easy to modify and are more thermostable than antibodies.

Table 1.1 Comparison chart for Monoclonal antibody vs. Aptamer:

Monoclonal Antibody	**Aptamer**
Stability: Proteins, the substance that make up mAbs, are notoriously known to be easily denatured. mAbs require refrigeration to avoid denaturation. Furthermore, proteins degrade over time - resulting in a limited shelf life.	**Stability:** Aptamers can withstand repeated rounds of denaturation/renaturation, and retain their structures. They are also temperature resistant - enabling them to be stable at room temperature and do not require refrigeration. These properties result in an indefinite shelf life
Immunogenicity: Antibodies are typically recognized by the body's immune system as foreign and dangerous, evoking an undesired immune response.	**Immunogenicity:** Aptamers are not recognized by the body's immune system as foreign, and do not evoke a negative immune response.
Production: laborious, expensive, high batch to batch variation, week to month to develop and subject to contamination.	**Production:** simpler and controlled chemical reactions, little to no variation, automated, chemical synthesis, no contamination.
Size: Antibodies are large molecules. They can resist filtration by the kidneys, leading to longer half-lives. However, their size prevents access to smaller areas.	**Size:** Aptamers are small molecules. They are especially subject to kidney filtration, resulting in short half-lives. However, they can bind to smaller targets that antibodies cannot reach.
Ability to modification: Antibodies cannot accommodate conjugates without negative consequences such as reduced activity.	**Ability to modification:** Aptamers can readily accept conjugates. Modifications can also be incorporated during synthesis to prevent kidney filtration.

In the decade or so since the SELEX procedure was first introduced, an abundant literature (Brody et al. 1999; Stoltenburg et al. 2007; Keefe et al. 2010) has reinforced the initial insight that single-stranded nucleic acids are a source of a vast number of three-dimensional shape and structure. This binding equilibrium compares favourably with the binding strength of most antigen antibody

complexes (Zhu et al. 2015). Moreover, the comparison made is usually between a monovalent aptamer and a divalent antibody. Using two different multimeric proteins, dimerization of the cognate aptamer has led to a large increase in binding strength. In such cases, this increase is due to a decreased dissociation rate constant for the dimerized aptamer from the protein.

In the last decade, there has been a renaissance in the field of nucleic acid probes-aptamers, which can bind to a broad range target molecules (proteins, peptides, drugs, small molecules, organic or inorganic elements like metal ions, and even whole cells, etc.) with high affinity and specificity. Particularly, aptamers which are screened and isolated using the iterative technique SELEX have been developed for a number of disease-associated proteins, and many of them are currently in clinical trials as therapeutic moieties or as tools for imaging or drug delivery (Phillips et al. 2008; Lee et al. 2015b). In recent years, aptamers have been studied as therapeutic tools both for treatment and for prevention, with the first aptamer-based drug recently approved by the U.S. FDA in treatment for age-related macular degeneration (AMD). (Khati 2010; Smuc et al. 2013)

Several aptamer-based bio-detection approaches have been reported where the aptamers are labeled with molecules like redox probes, fluorescent dyes, metallic, non-metallic nano particles or nanocrystals or quantum-dots as an integral part of the signal transduction module (Levy-Nissenbaum et al. 2008; Strehlitz et al. 2008; Lee et al. 2015a)(Levy M 2005, Strehlitz B 2008).

In addition, in the past several years there has been increased use of aptamers as chemical antibodies. (Phillips et al. 2008). There are several reports that describe methods for diagnosing and staging diseases by detecting or measuring proteins associated with certain clinical conditions by aptamers that recognize oligopeptide epitopes on a target protein.

1.6 Various SELEX Process

Isolation of aptamers specific for the target of interest can be achieved via SELEX from a large pool (> 1 x 10^{14}) of single-stranded oligonucleotides with random sequences. (Jayasena 1999; Keefe et al. 2010) After the incubation of the random aptamer pool with the target followed by the removal of non-binding aptamers, the bound aptamer species are recovered. These recovered nucleic acid sequences are amplified with PCR (in the case of DNA aptamer) or RT-PCR (for RNA aptamers). An enriched pool of potential binding aptamers generated from PCR amplification is used in the subsequent selection rounds (typically 6-10 rounds). At the last round of SELEX, the resulting highly enriched pool of aptamers are cloned or subjected to next generation sequencing followed by further characterization and engineering of individual cloned aptamers. In addition to selection against a purified or highly enriched target molecule, SELEX also can be performed using live cells and even in a whole mouse to isolate cell or organ-specific aptamers against a specific type of cells or an organ/tissue. (Cho et al. 2009; Li et al. 2009) Therefore, the power of SELEX enables one to generate specific aptamers against an inorganic or organic molecule, a protein, a cell and even an organ of interest.

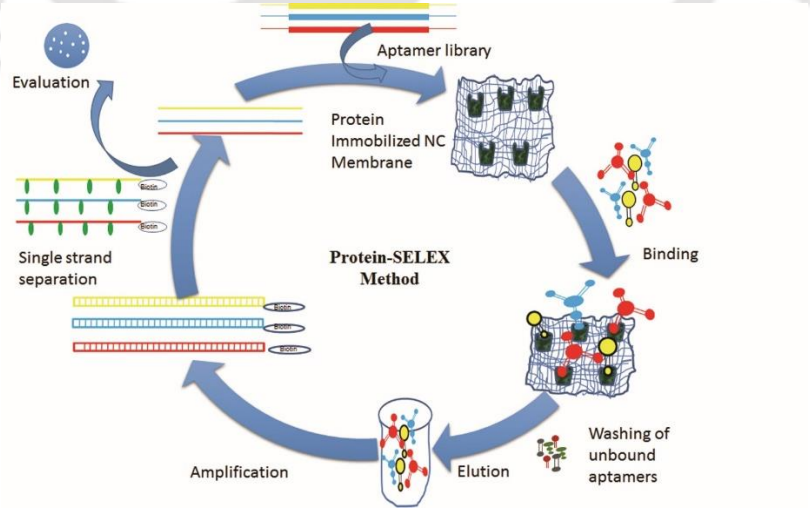

Fig 1.6: A Typical schema of Protein-SELEX process

A giant leap in improvement of SELEX technology has been made since its discovery in 1990. Conventional SELEX is a well-established and effective method, but due to its immense time- and labor-consumption, continuous improvement of alternative methods for aptamer selection has been inevitable.

1.6.1 Capillary Electrophoresis-SELEX

One of the most frequently used variants of SELEX methods is CE- SELEX (capillary electrophoresis SELEX), first published in 2004. (Mendonsa and Bowser 2004a; Mendonsa and Bowser 2004b)This process helped in reduction of the number of rounds of aptamer selection from generally 15- 16 rounds, in conventional SELEX, to 4 rounds in CE-SELEX while retaining the affinity and specificity of the aptamer to its target. The principle of this method is based on separation of an aptamer–target complex from unbound oligonucleotide pool according to their electrophoretic mobility. The oligonucleotides bound to its target have lesser mobility than free oligonucleotides, which pass through a capillary channel into the waste. Then, pressure is applied to collect the aptamer/protein complex. Many aptamers have been published so far which were screened by CE-SELEX. They include the following: aptamers against IgE ,(Mendonsa and Bowser 2004a) neuropeptide Y (ssDNA) (Mendonsa and Bowser 2005), human immunodeficiency virus reverse transcriptase (HIV-1 RT, ssDNA aptamer),(Mosing et al. 2005) ricin toxin (ssRNA) (Tang et al. 2006),and anthrax protective antigen (ssDNA modified into nano aptasensor). (Choi et al. 2011) Another techniques primarily based on CE is the kinetic capillary electrophoresis (KCE) methods of aptamer selection. Others include NECEEM (non-equilibrium capillary electrophoresis of equilibrium mixtures) (Blind and Blank 2015) ,(ECEEM (equilibrium capillary electrophoresis of equilibrium mixtures) and Sweep-CE (sweeping capillary electrophoresis).(Berezovski et al. 2006)

1.6.2 Magnetic Bead Based SELEX Process

A SELEX method based on magnetic beads for immobilization of the target was published in 1997. (Bruno 1997) In this method, magnetic bead-based SELEX, the target protein was immobilized on magnetic beads, incubated with an oligonucleotide library and the resulted target–aptamer complex was separated from a pool of unbound oligonucleotides by a magnetic separator. (Suh and Jaykus 2013) Binding of an aptamer to its target was qualitatively evaluated by epifluorescence microscopy. This approach provided several DNA aptamers including those against 4-chloroaniline, 2, 4, 6-trichloroaniline and pentachlorophenol. At a later stage, flow cytometry was introduced for DNA quantification instead of fluorescence microscopy. (Sun et al. 2014) A more convenient and relatively effortless, FluMag-SELEX method was published in 2005 by Stoltenburg and co-workers. (Stoltenburg et al. 2005; Wu et al. 2015) The principle of FluMag-SELEX is based on immobilization of the target on magnetic beads and after the first round of selection, ssDNA was amplified by PCR and labeled using fluorescein-modified primers for quantification of selected aptamers in further selection rounds. The obtained oligonucleotides are then cloned and sequenced. The dissociation constants of the enriched aptamers can be determined by fluorescence signal measurement. Aptamers against streptavidin, ibuprofen and polychlorinated biphenyls were generated using this approach. (Zhu et al. 2013; Stoltenburg et al. 2015)

Research of magnetic bead based SELEX has led to the development of microfluidic-based SELEX technology. (Qian et al. 2009) For example, continuous-flow magnetic activated chip-based separation (CMACS), which contains micro fabricated nickel ferromagnetic structures, creates a magnetic field within the micro channel. The hydrodynamic and magnetophoretic forces were then applied for partitioning of aptamers to protein immobilized on magnetic beads. Such microfluidic-based methods for aptamer selection are called M-SELEX. Although the CMACS device performed well in high-

throughput, it was difficult to handle and any obstacles in the micro channel caused bead aggregation resulting in low purity of aptamers reported later by the same group.

1.6.3 Cell-SELEX Process

All previously described SELEX processes are based on prior knowledge of the target for aptamer selection. Cell-SELEX is one of the exceptional cases. This well-developed method does not require prior target knowledge. Whole cells, both eukaryotic and prokaryotic, can be used to generate highly specific aptamers using this process.(Ohuchi 2012) In this process a randomized oligonucleotide library is incubated with the whole cells to fish out the aptamers specific to unknown cell surface receptors. A series of aptamers against various cancer cell types were generated by this method. Some of them interact with cells derived from human hepatocarcinoma, (Ninomiya et al. 2013) glioblastoma multiforme, (Gao et al. 2012) mouse tumor endothelial cells, (Ara et al. 2012) small cell lung cancer, (Kunii et al. 2011) prostate cancer, (Wang et al. 2014) lung adenocarcinoma and colorectal cancer. (Zhang et al. 2010) Specific aptamers were also selected against bacterial cells, such as *Salmonella typhimurium, Escherichia coli, Vibrio parahaemolyticus, and Trypanosoma cruzi* using cell-SELEX technology. (Nagarkatti et al. 2012; Dwivedi et al. 2013; Moon et al. 2013; Suh et al. 2014)

Another distinctive SELEX method using whole cells is a combination of cell-internalization SELEX with high-throughput screening. After each round, all internalized RNA was isolated, transcribed into DNA and amplified by PCR. Concentrated salt solution was used to remove the unbound and non-internalized RNA. The obtained aptamers were analyzed by high throughput sequencing and bioinformatics assays. (Thiel et al. 2012)

1.6.4 *In-vivo* SELEX Process

In vivo SELEX is a technique that generates aptamers in living organisms. In the first *in vivo* SELEX experiment, an infectious pool of HIV-1 DNA genomes with random mutations was transfected into

CD4+ T cells and the protocol proceeded with multiple rounds of viral replication followed by cloning and sequencing. (van Bel et al. 2014) This work led to selection of replication-competent viruses from the initial library. A similar approach was used later for identification of Rous sarcoma virus mutants by using randomized sequences and domains of the HIV-1 5′ leader RNA. Another variant of in vivo SELEX was developed to select aptamers capable of specific localization inside a tumor of a living organism. (Cheng et al. 2013) The overall selection process in this *in vivo* SELEX is very similar to conventional SELEX; with the exception of using a live organism for the selection process instead of using isolated targets which are employed in the conventional SELEX method.

1.6.5 Mirror-image SELEX or Spiegelmers

In this technology, aptamers against an enantiomer form of the target are selected through the standard SELEX protocol. After several selection rounds, enriched aptamers are sequenced. Obtained sequences are chemically synthesized using unnatural L-nucleotides that form an aptamer binding the native form of the target protein. Such L-oligonucleotides exhibit superior properties over aptamers consisting of natural oligonucleotides due to their increased stability to nuclease degradation. (Pestourie et al. 2005)

1.6.6 Deconvolution SELEX Process

In this method different aptamers selected for the various targets are separated from the complex mixture. Initially, human erythrocyte ghost cells were selected as a complex target. In this approach the standard SELEX protocol is applied first to enrich the aptamers against the complex target. Aptamers from the last round of selection are then cloned and the sequences of the obtained clones are determined. The sequences are then divided into families. Aptamers from each family are labeled with a photo reactive molecule at the 5′ end and radiolabeled at the 3′ end. Such aptamers are incubated with the complex target. After aptamer binding, proteins of the target are visualized by SDS-PAGE

and autoradiography. In this manner an aptamer that binds to a certain protein of a complex target is identified. (Berezovski et al. 2008)

1.7 Aptamers as Therapeutics

Aptamers fold into tertiary conformations and bind to their targets through shape complementarity at the aptamer-target interface. (Khati 2010) An aptamer bound to a protein can modulate protein functions by interfering with protein interaction with natural partners. Similar to antibodies, aptamers gain entrance to target cells via receptor-mediated endocytosis upon binding to cell surface ligands. (Cibiel et al. 2011) In addition, the *in vitro* generation of aptamers via SELEX confers a low-cost advantage over the long and arduous development process of antibodies. (Keefe et al. 2010) Importantly, aptamers can penetrate into tumor cores much more efficiently than antibodies due to their ~20-25-fold smaller sizes compared with full sized monoclonal antibodies. (Brody et al. 1999; Soper et al. 2006) Given that the nucleic acid aptamers function *in vivo* through the blood plasma, several limitations of aptamers should be considered. Being polynucleotides, nucleic acid aptamers are naturally susceptible to enzymes degradation by exo and/or endo-nucleases, leading to a reduced in vivo circulatory half-life. This drawback can be alleviated by side chain chemical modifications to aptamers, incorporating unnatural nucleotide bases (locked and unlocked nucleic acids) and capping the aptamer ends, thus minimizing the susceptibility to exonuclease attack. (Barciszewski et al. 2009; Veedu and Wengel 2010) Short blood residence time is another challenge with in vivo aptamer applications, which is due to fast removal of the aptamer from the circulation by renal filtration as most aptamers have a size smaller than the renal filtration threshold of 40 kDa. (Ahirwar et al. 2016) To achieve desired serum half-life, aptamers can be engineered by conjugation with a terminal polyethylene glycol (PEG), although this may compromise the extent of tumor penetration (Ng et al. 2006). It is worth noting that post-SELEX modifications following the selection of aptamers may alter

the 3-D structure of the aptamers, leading to the loss or altered binding affinity and specificity. Such problems can be prevented by using random aptamer pools containing modified nucleotides during the SELEX selection. (Sampson 2003; Shamah et al. 2008) In addition, the ability of aptamers to interact with cells may decrease due to repulsion of nucleic acids by negatively-charged cell membranes. This can be refuted by increasing the binding affinity and specificity of aptamers toward their cell surface receptors to trigger receptor-mediated endocytosis.

In the field of oncology, two aptamers, namely, AS1411 (35) and NOX-A12, have entered clinical trials. (Keefe et al. 2010) AS1411 (formerly ARGO100; Antisoma) is a guanine quadruplex aptamer obtained from a guanine-rich oligonucleotide library in the anti-proliferation screen, which is not a typical SELEX process. The guanine quadruplex structure benefits AS1411 because it is resistant to nuclease degradation and enhances cell uptake. In *in-vitro* validations, AS1411 inhibits more than 80 types of cancer cell lines. In addition, the target of AS1411 has been verified to be nucleolin, and the relevant mechanism and specificity against cancer cells have also been described. In preclinical tests, AS1411 shows efficacy toward xenograft models, including non-small cell lung, renal, and breast cancers. It entered clinical trials in 2003 and passed phase II trials for acute myeloid leukemia. A subsequent phase II trial for renal cell carcinoma was started in 2008 (clinical trial ID NCT00740441).(Rosenberg et al. 2014) NOXA12 (Olaptesed pegol; Noxxon) is an L-form RNA aptamer known as Spiegelmer and is used for cancer therapy. NOX-A12 targets the chemokine CXCL-12 and blocks its binding to its receptor. (Hoellenriegel et al. 2014) This disrupts the tissue gradient of CXCL-12 and reduces the cancer cell homing that might lead to tumor metastasis and drug resistance. (Kruschinski 2013) Currently, phase II clinical trials for NOX-A12 are underway for the treatment of chronic lymphocytic leukemia and refractory multiple myeloma (clinical trial IDs NCT01486797 and NCT01521533).

1.8 Nucleic acid Based Biosensors

A biosensor is an integrated device capable of providing specific quantitative or semi-quantitative analytical information using biological or biomimetic recognition elements. The bio-recognition layer is intrinsically designed to interact with the target. Due to any sort of biochemical reaction, a series of physicochemical changes in the medium are detected by the transducer and converted into a discrete or continuous digital signal. The design and integration of biosensors offer unique features to improve current analytical methods.

The timely monitoring and regulation of various parameters is important to many applications in food industry, clinical diagnoses, hygiene, environmental protection, drug development and forensics, etc. (Barthelmebs et al. 2010; Sett 2012; Bora et al. 2013) Therefore, there is a requirement to have reliable analytical devices, which can perform rapid and accurate analyses. As per definition of IUPAC, a biosensor is an integrated receptor-transducer device, which is capable of detecting a biological or biochemical reaction and provide quantitative or semi-quantitative analytical information. The biosensor consists of a biological recognition element, which acts upon a biochemical mechanism, and, on the other hand, of a transducer relying on electrochemical, mass, optical or thermal principles. The characteristic trait of a biosensor is the direct spatial contact between the receptor and the transducing element. Biosensors have been developed using the specificity of a biological molecule integrated to a physicochemical transducer to convert a perceived signal (usually very weak) into a comprehensible electrical signal (Turner, Anthony PF; Wilson 1989). Apart from a genetic material, the use of nucleic acids as recognition molecule in biosensor design is relatively a new and exciting area in analytics. (Wang et al. 1997) Nucleic acid based biosensors (NABs) have applications in (i) clinical diagnostics of inherited diseases and pathogenic infections, (ii) laboratory analysis like probe development for different immunodetection techniques and microarray systems.

NABs primarily use deoxyribonucleic acid (DNA), ribonucucleic acid (RNA), peptide nucleic acid (PNA), and aptamers (both DNA and RNA) as oligonucleotide probes. The fundamental principle behind NABs depend on sequence complementarity as per Chargaff's rules of base pairing (for DNA, A=T, G≡ C) except in the case of aptamers. Principle of aptamer based detection is more akin to antigen-antibody or receptor-ligand interactions.

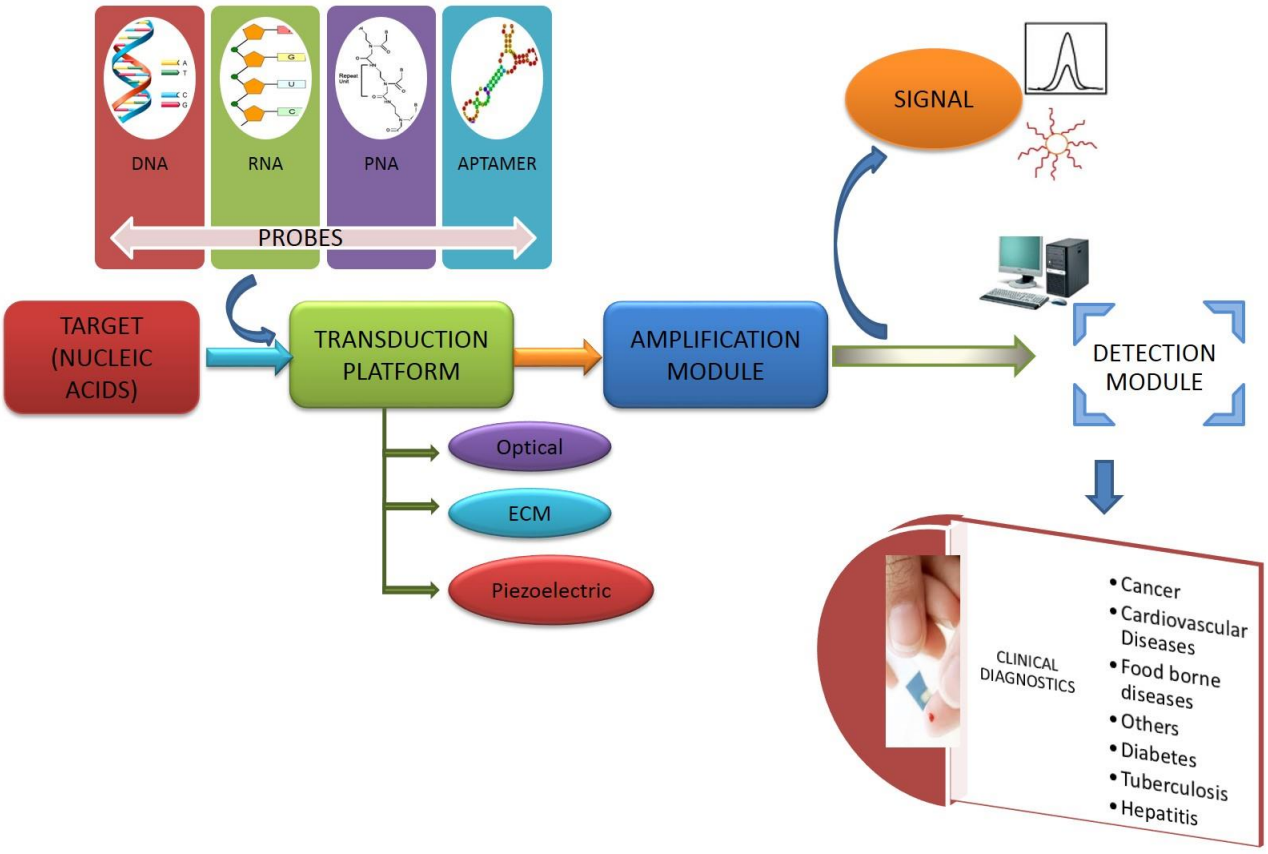

Fig 1.7: Schema of Aptamer based biosensor system

NABs are developed by immobilization of nucleic acids (DNA, RNA, PNA, oligonucleotides) to a solid support by adsorption, covalent bonding or ionic interaction and coupled to a physico-chemical transducer. (Wang 2000) There are various methods available for immobilization like adsorption to inert carriers, chemical cross-linking to macroscopic beads or magnetic particles, physical entrapment

in gel-like lattice and microencapsulation in nanospheres, etc. The immobilization process also aids in probe orientation and ready accessibility to target element. (Herne and Tarlov 1997)

In case of physico chemical method, various fabrication strategies are available to attach a DNA probe to transducer surface. These include use of thiolated DNA for self-assembly onto gold plated transducers (electrodes, piezoelectric crystals), covalent linkages to gold surface by stabilizer molecules like 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) HCl-mediated reaction, use of biotinylated DNA to form conjugate with surface immobilized avidin or streptavidin, and carbodiimide coupling to functional groups on carbon nanotubes (SWCNTs, MWCNTs) (Sperling et al. 2006). For solution-based hybridization assays, different conditions (ionic strength, temperature, solubility, catalyst concentration) are taken into considerations which have to be optimized in the later stage.

Using smart and an advanced nucleic acid recognition process involving Peptide Nucleic Acid (PNA), aptamers can augment the efficiency of DNA based biosensors. PNA is a DNA analogue in which the sugar phosphate backbone is replaced with pseudo peptide. PNA is composed of repeating N-(2-aminoethyl)-glycine units linked by peptide bonds. (Ray and Norden 2000; Raoof et al. 2011) The unique structural hybridization and sensing feature of solution phase PNA can be readily extrapolated onto transducer surface to fabricate a highly sensitive biosensor. Recently scientists have developed supramolecular ionic liquids grafted on nitrogen-doped graphene aerogels (SIL-g-(N)GAs), which used as a novel electrochemical DNA biosensor for the direct detection of the breast cancer-related BRCA1 gene. The sensor performance was monitored by cyclic voltammetry (CV) and differential pulse voltammetry (DPV) and limit of detection (LOD) was found at the 2 pM range. The detection was done without any kind of enzyme, label, or sophisticated equipment. In another study, another group developed an electrochemical DNA biosensor for the rapid detection of sequence (5’ AAT GGA

TTT ATC TGC TCT TCG 3') specific for the breast cancer 1 (BRCA1) gene. The proposed electrochemical genosensor is primarily dependent on a short oligonucleotide DNA probe immobilized onto chemically synthesized zinc oxide nanowires on a gold electrode via the hydrothermal technique. Although there are numerous examples where oligonucleotide strands were used as an effective tool for bio sensing application in breast cancer detection. (Soper et al. 2006; Lu et al. 2008)

In 90's a class of short oligonucleotides termed Aptamers emerge as a unique molecular probe, which can be chemically synthesized, modified and easily integrated with a variety of nanomaterials, such as gold nanoparticles, quantum dots, carbon nanotubes, and iron oxide nanoparticles and other fluorescent modules. The application of these magic bullet 'Aptamers' as fluorescent, colorimetric, and electrochemical sensors in recent medical diagnostics is quite promising. (Bora et al. 2013)

1.9 Aptamer based Biosensors

Typical recognition elements in biosensors are enzymes, antibodies, nucleic acids or any microorganisms. Aptamers are a new promising class of bio-receptors, because of their selectivity, sensitivity and stability, the reproducibility of the target binding reaction and their production by chemical synthesis ensuring a constant lot-to-lot quality, and the ease of regeneration of aptamer derived surfaces.

Proteins are mostly detected by monoclonal antibodies in analytical platforms like ELISA, immunoblotting assay, microarrays and in some biosensors also. However, aptamers (chemical antibodies) are equivalent to monoclonal antibodies pertaining to binding affinities, specificity and moreover they offer significant advantages. They are more resistant to high temperature denaturation and degradation and their affinity and specificity can easily be enhanced by rational design or by techniques of molecular evolution. Furthermore, they can easily be tailor-made and modified with

functional groups (amine, thiol), fluorescent tags, and redox moieties (methylene blue, ferrocene) that allow covalent, directed immobilization on biochips, resulting in highly ordered receptor layers that lead to development of various platform (optical, opto-electrical, electrochemical, piezo-electrical, etc.) based aptasensors. Aptamers can also distinguish between chiral molecules and are able to recognize a distinct epitope of a target molecule.

Biosensors for protein and other molecule detection mainly involve antibodies and enzymes but lately modified aptamers like SOMAmers (slow off-rate modified aptamers) are emerging as highly efficient probe molecules for specific detection of target analytes. (S.K. et al. 2011; Gupta et al. 2014) Aptamers can rival antibodies in various applications. They are very small in size (40-100 nucleotide bases) in comparison to other bio-recognition elements. This allows efficient immobilization, miniaturization, integration, and automation of biosensors. Once selected, aptamers can be chemically synthesized with high reproducibility and purity. DNA aptamers are usually chemically more stable than its RNA counterpart, facilitating reusability of the biosensors. In contrast, RNA aptamers are susceptible to degradation by the ribonucleases typically found in cell lysates and serum. To circumvent this problem, modifications of the 2' positions of pyrimidine nucleotides with amino/fluoro groups have been introduced. (Kujau and Wölfl 1998) Another possibility is the use of RNase inhibitors. (Minunni et al. 2004)

Aptasensor based analysis is continuously evolving with various detection schemes ranging from label-free methods such as surface plasmon resonance (SPR) and quartz crystal microbalance (QCM) measurements to label dependent methods such as electrochemistry, fluorescence and chemiluminescence field effect transistors, etc. (Li et al. 2006; Dong and Zhao 2012; Kwon et al. 2012) However currently electrochemical and optical aptasensors constitute the two predominant

types under development.(Sassolas et al. 2011) These can even distinguish between chiral molecules and are able to recognize a distinct epitope of a target molecule. (Michaud et al. 2003)

Aptamers are preferentially used in field-effect transistor (FET)-type sensor formats because of their small sizes. Aptamers are smaller in size than the Debye length of the double layer (i.e. 3 nm at 10 mM ionic concentrations), so that the binding event with a protein can occur within the electrical double layer in buffer solution. Changes in the charge distribution can thus easily be detected by FETs. On the contrary, antibodies (10 – 15 nm) are not as appropriate as the corresponding significantly smaller sized aptamers. Scientists also employed aptamers with carbon nanotubes (CNTs) to develop a single-walled carbon nanotube (SWCNT)-FET aptasensor. An anti-thrombin DNA aptamer was covalently linked to the side wall of CNTs that were assembled between source and drain electrodes. Target binding to aptamers led to measurable decrease in conductance. Detection limit was around 10 nM , but the sensitivity could be improved up to sub-nanomolar range by using high-quality SWCNT-FETs (with high on/off ratio). The same group also demonstrated SWCNT-FETs based aptasensor for the detection of *E. coli*. (Mao et al. 2006)

A label-free amperometric aptasensor based on thrombin acting both as a protein to be detected and as an enzyme catalyst has also been described by Mir and co-workers (Mir et al. 2006). They actually developed several strategies to develop anti-thrombin electrochemical aptasensors. Thiolated aptamers were immobilized on a gold electrode and incubated with thrombin. The electrochemical measurement was performed in the presence of ß-Ala-Gly-Arg-pNA as the thrombin substrate, thus leading to p-nitroaniline production that could be detected. The electrochemical detection was faster and more sensitive than the optical one.

A potentiometric aptasensor has been developed by Evtugyn with the anti-thrombin aptamers. (Evtugyn et al. 2008)This simple and cheap device was based on poly(phenothiazine) conducting

polymers electro-polymerized on a glassy carbon electrode. Avidin-modified polymer surfaces obtained by direct electrostatic precipitation was used to immobilize biotinylated anti-thrombin DNA aptamers. The difference in the potential of the sensor in pH 3 and in pH 7.6 media indicated potentiometric thrombin detection in the concentration range from 10^{-9} to 10^{-6} M.

Traditional analysis detection techniques, such as immunohistochemistry, flow cytometry, Chemical in-situ hybridization (CISH) however do not meet the necessity for point-of-care (POC) diagnostics because they usually require expensive instruments, complicated operations, long analytical time and skilled personnel (Liu et al. 2009). One of the most extensively used method for HER2 expression analysis is based on immunohistochemistry (IHC). This particular method, however, has major technical limitations with the analytical sensitivity, target specificity, capacity to multiplex, and subjectivity in image understanding (Rhodes et al. 2000; Gown 2008). Considerable discordance has been reported between the results of HER2 studies performed in diverse laboratories. (Reddy et al. 2006; Kaufman et al. 2014) Hence, a Fluorescence in situ Hybridization (FISH) technology is currently used to detect HER2 gene amplification in place of the ambiguous IHC based results. (Sapino et al. 2013) Although, all these traditional methods mentioned above are in practice in a smaller or larger way to diagnose breast cancer through the detection of HER2, ER, PR or other biomarkers, their miniaturization and onsite analysis is the major concern for the point-of care diagnosis of breast cancer. In this regard, newer methods based on sensor systems have been attempted by various researchers for quick, sensitive, and selective detection of well-established biomarkers.(Hayat et al. 2014) Among the entire sensor read out devices, an electrochemical system method is usually preferred due to the advantages of being portable, simple, easy to use, cost-effective, disposable method for the lab-on-a-chip diagnostic system. (Zhu et al. 2008; Gruhl et al. 2010; Chandra 2013). With aptamers, the approach should be affordable to screen more people, especially in developing countries and detect

cancer at early stages that would increase the survival rate. Coupling nano-materials and bio recognition molecules with aptamers represent new directions toward a novel diagnostic platform for detection of breast cancers. Therefore, aptamers are key in developing an electrochemical sensor based breast cancer detection method. Recently there has been a study of development of the novel nanomaterial based electrochemical detection of BRCA1 gene.

A novel aptamer biosensor for cancer cell assay has been reported on the basis of ultrasensitive electrochemical detection. The assay uses the aptamer as a capture probe to recognize and bind the tumor marker on the surface of the cancer cells, forming an aptamer-based sandwich structure. Functionalized nanoporous materials, porous graphene oxide/Au composites (GO/Au composites) and porous Pt-Fe alloy have been introduced into the biosensor. (Yi et al. 2013)

Scientists have reported an electrochemical sensor that can detect label-free cancer cells using the first clinical trial II used aptamer AS1411 and functionalized graphene. By taking advantages of AS1411 aptamer high binding affinity and specificity to the overexpressed nucleolin on the cancer cell surface, they have developed an electrochemical aptasensor that can distinguish cancer cells from normal ones and detect less amount of cancer cells also. EIS data showed that the detection is ranged from 1×10^3 to 1×10^6 cells/mL, with a correlation coefficient R of 0.988 and the limit of detection is calculated to be 794 cells/mL, which was better than the chemiluminescence detection of HeLa cells using AS1411. With DNA hybridization technique, this E-DNA sensor can be regenerated and reusable for cancer cell detection. This work might be a good lead for label-free cancer cell detection based on the aptamer and graphene-modified electrode. (Feng et al. 2011)

In a study, scientists have developed a "signal-on" electrochemical aptasensor for simultaneous determination of two tumor markers MUC1 and VEGF-165 in cancer cells, by using a ferrocene-labeled aptamer-complementary DNA (cDNA) as probe. cDNA complimentary to both MUC1

aptamer and VEGF-165 aptamer immobilized onto an electrode surface formed a long double strand with ferrocene far away from the electrode surface and thus the probe could not able to provide any electrochemical signal. Nevertheless, the presence of the two tumor markers will inhibit the hybridization of cDNA with the aptamers, thus the distance between ferrocene and the electrode is changed, and a "signal-on" electrochemical method to detect two tumor markers simultaneously. Results show that the electrochemical signal increases with the addition of either tumor markers, but the biggest electrochemical signal can only be obtained when both tumor markers are present. Electrochemical aptasensor can not only detect the two markers, but also distinguish their co-existence. That also displays high selectivity and sensitivity towards the detection of the tumor markers, provides it immense potential for clinical applications in the future.(Zhao et al. 2012)

In another study, a simple and sensitive electrochemical impedimetric aptasensor based on gold nanoparticles (AuNPs) signal amplification was developed for ultrasensitive detection of tumor markers (mucin 1 protein, MUC1). The designed cDNA, which is partly complementary with the MUC1-aptamer was immobilized on a gold electrode. The detection of MUC1 could be carried out by means of switching structures of aptamers from DNA/DNA duplex to DNA/target complex. The change of the interfacial feature of the electrode was characterized by electrochemical impedance analysis (EIS) with the redox probe ferrocene. Moreover, as the signal enhancer, the aptamer-modified AuNPs conjugates was introduced on the electrode by the hybridization of cDNA with aptamer. This aptasensor has a low detection limit of 0.1 nM, and also exhibits several advantages of high sensitivity and good selectivity. Thus, it would provide a general model for the detection of tumor marker based on an impedimetric aptasensor. (Thiel et al. 2012)

Recently, it has been shown that detection of breast cancer tumor cells outside the primary tumor is an effective early diagnosis with great prognostic and clinical utility. One group has developed a

signal-on fluorescence aptasensor for label-free, facile and sensitive detection of MCF-7 breast cancer cells.(Cai et al. 2014) Due to target–aptamer specific recognition and single-stranded DNA-sensitized luminescence of terbium (III), the proposed aptasensor showed excellent sensitivity with a detection limit as low as 70 cells mL^{-1}. Compared with other common organic dyes and the emerging nano-technological probes, the combination of terbium (III) and single-stranded DNA signal probes serve as more powerful bio-probes because of their stable optical property, good biocompatibility and free from complex synthesis. The feasibility investigations have illustrated the potential applicability of this aptasensor for selective and sensitive detection of MCF-7 breast cancer cells. Moreover, this proposed aptasensor can be also extended for the determination of other tumor cancers or bio-molecules by altering corresponding aptamers specific to other cancer biomarkers.

In another study, a label-free capacitive aptasensor was developed based on capturing of human epidermal growth factor receptor 2 (HER2) protein by anti-HER2 ssDNA aptamers as a bio-recognition element functionalized on interdigitated microelectrodes of a capacitor.(Qureshi et al. 2015) The aptasensor response was measured by the non-Faradaic Impedance Spectroscopy (nFIS) method. Capacitance signal specific to target HER2 protein is based on changes that occurred due to charge distribution upon interaction of aptamer–protein molecules against the applied AC frequency. Spiked HER2 protein expression in human serum was successfully detected through concentration-dependent changes in impedance/capacitance values as a result of the formation of aptamer–HER2 protein complexes on capacitor microelectrodes. The label-free capacitive aptamers based sensory system is a versatile and promising approach for early detection of cancer biomarkers in dilute human serum and the method potentially can be extended to a wide variety of diseases biomarkers.

1.10 Conclusion

Aptamers are a promising class of molecular agents for biomarker detection due to their small size, chemical stability and cost effectiveness over conventional bio-receptors such as antibodies. Recent progresses in micro/nano-fabrication technology have driven researchers to develop aptamer based biosensors. The factors that affect accuracy, sensitivity and stability of aptasensor are also addressed in this section. Despite recent advances in developing electrochemical and other aptasensor, the search for label-free and reagent less devices remains a challenge, especially for detection of small molecules.

As the aptamers are very much specific, sensitive to their targets and cost-effective than antibodies, they could easily be exploited as a diagnostic tool. The specific aptamers that are screened against the well-known established protein biomarkers of breast cancer (HER2, ER), would be used as a sensing probe to develop a biosensor for detection of breast carcinoma.

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Chapter 2

Selection and Characterization of Aptamers Specific to Estrogen Receptor Alpha

Chapter 2

SELECTION AND CHARACTERIZATION OF APTAMERS SPECIFIC TO ESTROGEN RECEPTOR ALPHA

2.1 INTRODUCTION

Estrogen hormones play a pivotal role in growth, differentiation and developmental functions of reproductive systems of both females and males. (Klinge 2000) It also acts as an anti-atherosclerotic agent (Chiong and Miller 2003) in central nervous system functions, and in the regulation of endocrine glands in the hypothalamic- gonadal axis. Estrogens accomplish these activities binding to specific estrogen receptor (ER) which is encoded by two gene isoforms: alpha and beta (ERα and ER β). Both isoforms can act as signal transducers and transcription factors to modulate further downstream signal cascade. Human estrogen receptor α, a 67 kDa ligand- inducible transcription factor is the key mediator of 17 β-estradiol (E2) induced proliferation, differentiation and development of breast and uterine tissue. It is also one of the most pivotal biomarkers in breast cancer, gastric cancer diagnostics and targeted therapeutics. (Tonetti and Jordan 1999; Ariazi et al. 2006) Structurally, this receptor contains three conserved domains; N-terminal domain (NTD; 1-180 aa), central DNA binding domain (DBD; 181-263 aa) and C-terminal ligand binding domain (LBD; 303-552 aa). Binding of estrogen to the ER triggers its release from molecular chaperones, causing receptor dimerization and subsequent expression of target genes through recruitment of transcription factors and DNA binding proteins. (Marino et al. 2006) Estrogen receptors and its signaling pathways play an important role in breast cancer progression and metastasis. More than half of the breast cancers overexpress ER alpha and around 70% of ER alpha positive cases respond to anti-estrogen (for

example tamoxifen) therapy. (Ali and Coombes 2000; Saha Roy and Vadlamudi 2012) The presence of the estrogen hormone stimulates the cells grow faster and quicker compared to ER deficient cells. Presence of this receptor in almost two-thirds of breast tumors and their subsequent treatment by hormonal therapy establishes ERα as a useful target for further diagnostic method development (Lonning 2012). Guideline of the American Society of Clinical Oncology (ASCO) and the College of American Pathologist (CAP) has aimed to improve the hormone receptor testing methods in breast cancer patients. (Hammond et al. 2010) Thus, an ER targeting molecule could provide a promising alternative to antibody -based diagnostics and related applications. Treatment regimen depends on breast cancer nomenclature to define specific subgroups, which are treated according to the best data available. Following the ASCO-CAP guidelines for breast carcinoma, cancer cells with nuclear staining > 1% were interpreted as positive.

In 1990 a new category of high affinity oligonucleotides named ‘aptamers’ was discovered, which can bind selectively and specifically to any target. (Tuerk and Gold 1990; Brody et al. 1999) The targets can range from simple inorganic molecules to larger proteins or even cells. These high affinity nucleic acid binding agents named ‘aptamers’ can be identified by screening a diverse pool (10^{14}-10^{18} molecules) of synthetic oligonucleotide libraries through an iterative SELEX method. (Stoltenburg et al. 2007) Systematic Evaluation of Ligands by Exponential Enrichment Analysis (SELEX) involves multiple rounds of selection and amplification. Each round of selection remove weaker binding molecules and enriches strong binding oligonucleotide sequences, which finally develop into candidate aptamer sequences. Aptamers are gaining promising importance in clinical applications due to their high target specificity, affinity, smaller size and usual non-immunogenic characteristics.(Smuc et al. 2013) Biosensors based on nucleotide aptamers have a greater potential than antibodies and can be useful for

biomarker detection, cancer diagnosis, detection of pathogens, use as small molecules to assure food safety, and to monitor other hazardous agents. (Sett 2012)

The expression and expansion of ER positive cells is a common observation in breast tumors and the ER expression level diverges among normal and cancerous mammary epithelial. Selective inhibition of the ER action by selective estrogen receptor modulators (SERMs) provides an approach to treat or restrict the growth of receptor positive cancers (Dutertre and Smith 2000). The phenomenon however requires the precise quantification of ER expression status to categorize the tumors as ER alpha positive. Several assays have been developed to achieve this task. Radiolabeled agonist/antagonists or antibodies are essential to enumerate precisely and accurately the ER content of human breast tissues. Among these, the histopathological studies using antibodies is the most preferred choice, but the concomitant drawbacks of conventional antibodies, such as production cost, production time, inconsistencies in assay results (batch to batch variation), lead to frequent false positive estimations. Such system has evoked concerns over future perspective of these affinity reagents in histochemical and other diagnostic assays. Alternatively, the evolving translational research on affinity reagents (recombinant or engineered antibodies and chemical antibodies 'aptamers') offer therapeutic and diagnostic potential as well apart from the ambiguities of antibodies. Having geometrical conformational flexibility and synthesis dynamics, aptamers can easily be tagged or conjugated with a detection module to detect cancer metastasis. In this study, we developed a novel DNA aptamer based detection system for ERα positive carcinoma.

2.2 OUTLINE OF THE RESEARCH STUDY

i) Molecular cloning, over-expression and purification of recombinant marker proteins associated with breast cancer-estrogen receptor alpha.

ii) Selection of DNA aptamer candidates for breast cancer biomarker protein ER alpha by *in-vitro* protein-SELEX method.

iii) Determination of various binding parameters of screened selected aptamers.

iv) Determination of specificity and characterization of candidate aptamers against ER alpha positive and negative cell lines.

v) Evaluation of ER alpha expression pattern using specific candidate aptamers in breast cancer patient tissue samples.

2.3 EXPERIMENTAL SECTION

2.3.1 Cloning and transformation of recombinant ER α in bacterial system

pVP16- ER α construct (plasmid no.# 11351) was obtained from Addgene ® consisting of the full estrogen receptor α gene. The full domain of nuclear receptor estrogen receptor α (ER α) was amplified by specifically designed primers containing restriction enzyme sites Nhe I-Sal I for pET 28a vector. The primers used to amplify the ER α gene were: 5'GC*GCTAGC*ATGACCATGACCCTC3' (Forward primer containing NheI site), 5'GC*GTCGAC*TCAGACTGTGGCAGG 3' (Reverse primer containing SalI site). PCR reaction volume of 50µl contained: Mg2+ ions (2.5mM), dNTPs (0.2mM), primers (1.5µM), 1 unit of Taq DNA polymerase (1µl of 1 Unit/µl) and 5µl of pVP16-ER α (45ng) construct. The PCR thermal profile was: initial denaturation at 94°C for 4 min for 1 cycle, then denaturation at 94°C for 30 sec, annealing at 61°C for 45 sec, elongation at 72°C for 50 sec for 30 cycles and final extension at 72°C for 10 minutes. The amplified ER α gene and pET28a vector were both digested with NheI and SalI to form sticky ends. Then, digested ER α (165 ng) was ligated with digested pET28a vector (55ng) in a 10 µl total ligation mixture at a molar ratio of 3:1(insert: vector). The ligation mix was incubated overnight at 16ºC. Ligated product was transformed into 100µl of chemically competent *E.coli* DH5α cells. Transformed kanamycin resistant positive colonies were screened by PCR amplification with ER α gene specific forward and

reverse primers. The positive colonies (pET28a-ERα) were again transformed into the *E.coli* BL21 expression system.

2.3.2 Expression and purification of recombinant ER α protein:

The expression of positive clones containing pET28a-ERα construct were analyzed under various induction condition (temperature, induction period, shaking speed) with varying concentration of 0.1mM-1.0mM IPTG. After induction with IPTG, the cells were harvested at 8,000 rpm for 15 minutes and stored at 4°C. The cells were lysed with an ultra-sonicator (8 sec on, 15 sec off pulse for 40-45 cycles) by dissolving them in 50mM Tris-Cl buffer pH 8.3 and 300mM NaCl containing 10mg/mL of lysozyme, 1mM PMSF. The cell-free extract was obtained after centrifugation for 20 minutes at 10,000 rpm at 4° C. His-tagged recombinant ER α protein was purified by nickel affinity chromatography (GE His Trap FF Crude 5ml column). The binding buffer constitutes 50mM Tris, 150 mM Imidazole and 150 mM NaCl and the purified fractions were eluted with 300 mM Imidazole. All purified fractions were pooled and the buffer was exchanged with 50mM Tris (pH 8.3) containing 300 mM sodium chloride using Amicon ultra-centrifugation filters (cutoff size 10kDa, Merck ®). The purity and molecular weight of recombinant ERα was checked by 12% SDS-PAGE and western blotting.

2.3.3 *In vitro* selection of DNA aptamers against recombinant ERα

An oligonucleotide library of 76-nt length, comprising a central random region of 40 nt long and flanking region of 18nt at both 5’ and 3’ end (5’ ATACCAGTCTATTCAATT–N40-AGATAGTATGTGCAATCA 3’), forward primer (5’-ATACCAGTCTATTCAATT-3’ conjugated with 6-FAM at 5’ end) and reverse primer (5’-TGATTGCACATACTATCT-3’ conjugated with biotin at 5’end) were purchased from IDT Technologies (USA).The aptamer library was synthesized at 1 µM synthesis scale and PAGE purified whereas labeled and unlabeled primers were synthesized at 100 nM synthesis scale. Unlabeled primers were also

synthesized to clone the enriched sequences in the T/A cloning vector pTZ57R/T (Invitrogen, USA) to obtain unique candidate sequences in separate clones.

500 picomoles of DNA aptamer library was first heated at 95°C for 10 min and immediately kept on ice for 5 minutes. Then, the DNA aptamer library was kept at room temperature to equilibrate. Counter SELEX was performed by incubating library in aptamer binding buffer (20 mM Tris-Cl, pH 7.5, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$) against only nitrocellulose membrane for 1 hour at room temperature. Simultaneously, 1µg of purified ER α protein was also immobilized on NC membrane. (Nonaka et al. 2010) The unbound aptamer library obtained after counter SELEX, was incubated with protein immobilized on NC membrane for 1hour at room temperature. After incubation, the membrane was washed thrice with TBSTE (0.1% Tween-20 in Tris-buffered saline). The aptamer bound to protein NC membrane was eluted by heating at 95°C for 8 minutes. The eluted aptamer pool was amplified with unlabeled forward primer and biotinylated reverse primers using the following thermal cycling: one cycle of 94 °C for 5 min and 14 thermal repetitive cycles of 94°C for 30s, 45°C for 30s, and 72°C for 50 s and a final extension of 72 °C for 5 min. PCR-amplified sequence was mixed with pre-equilibrated streptavidin magnetic beads followed by strand separation using alkaline denaturation (0.1 M NaOH).(Wilson 2011) Separated non-biotinylated sense strand in the supernatant was collected and utilized for the next round of screening. To monitor the enrichment of the sequences we performed agarose gel electrophoresis (2% AGE) of the eluted aptamer pool after every two rounds. After iterative SELEX cycles, the enriched pool was PCR amplified with the unmodified forward and reverse primers and ligated to the pTZ57R/T vector which has 3'-ddT overhangs. Chemically competent *E.coli* XL10 cells were used for transformation, and transformed colonies were primarily selected based on blue-white screening. Positively transformed white colonies were picked and cultured overnight and plasmids were isolated using a miniprep plasmid isolation kit (SIGMA). The presence of

candidate sequences in isolated plasmids were further verified by PCR amplification with specific primers. The positively transformed plasmids were further sequenced by Sanger's method.

2.3.4 *In-silico* analysis of the candidate sequences

In-silico analysis was performed with the enriched sequences to find the most potent ER α binding DNA aptamer. To find the most probable ER α-aptamer we carried out preliminary analyses such as free energy comparisons and structural stability of the selected candidates. Secondary structures of the aptamer sequences were determined through a free energy minimization algorithm using Mfold software (http://frontend. ioinfo.rpi.edu/applications/mfold/cgi-bin/dna-form1.cgi). (Zuker 2003)

2.3.5 Binding Analysis by Isothermal Calorimetry

Chemical and thermodynamic parameters of aptamer binding have been studied by isothermal titration calorimetry (ITC) analyses, suggesting that the anti-ERα aptamer undergoes the enthalpy-driven conformational change of its 3D structure. (Potty et al. 2009; Shui et al. 2012) All calorimetry experiments were performed at 25°C using a MicroCal VP-ITC (Micro Cal Inc., Northampton, MA, USA). ERα protein and aptamers were prepared in phosphate buffered saline (pH 7.4). The sample cell holder contained 300µL of 1.5µM recombinant purified ER α protein. The thermal equilibration step at 25◦C was followed by an initial 1200-s delay step and, subsequently 30 injections. Typically, 30 serial injections (1.5µL per one injection) of 20 µM aptamer candidate at spacing of 130 sec were made with continuous stirring of the solution (at 210 rpm) in the sample cell. Each injection generated a heat-burst curve (µcal/sec) versus time.

2.3.6 Aptamer binding assay by Chemiluminescence

To determine the affinity of the aptamer-ERα complex, an assay for ligand–receptor interactions was performed.(Xu et al. 2014; He et al. 2015) The aptamer was biotinylated at the 5' end.

Next, the purified recombinant ER α protein was coated on 96-well microtitre plates (2 µg/well), and incubated with increasing concentrations (0-1000nM) of the biotinylated ER_Apt1. All points were performed in triplicate. The assay was performed using an anti-ER α monoclonal antibody as positive control. Non-specific binding of aptamer was confirmed by performing an experiment wherein BSA was used as a target protein. Incubations were performed at 37°C for 1 hour. The unbound aptamer was removed by washing the well four times with PBST (0.1% Tween-20) solution, while specific binding was quantified with streptavidin–HRP conjugate (1:1000 dilution from 1mg/ml) as a probe. The intensity of developed chemiluminescence was estimated at 490 nm (Tecan Microplate Reader) after addition of 100 µl chemiluminescent peroxidase substrate (CPS 350, SIGMA).

2.3.7 Competitive Binding Assay

For the homologous competition binding assay, the immobilized ERα in wells (1.0 µg/well) of a microtiter plate was co-incubated with biotinylated and non-biotinylated ER_Apt1. In the mixture of two sequences, the concentration of biotinylated ER_Apt1 (100 nM) was kept constant, while it was varied in a range of 0-100 nM for non-biotinylated sequences. The incubation was done at room temperature for 1 hour and later signal of bound labeled aptamer was recorded at 490nm (Tecan Microplate Reader).

2.3.8 Electrophoretic Mobility Shift Assay (EMSA)

The binding of ER_Apt1 to its target ER alpha protein in solution was analyzed by EMSA. Prior to incubation with protein, aptamer was denatured at 95 °C for 10 minutes and the sample was kept to room temperature (slow annealing). (Sosic et al. 2011) Then folded aptamer (10nM) was incubated with increasing concentrations of ERα (from 0 to 800 nM) and ERβ (0nM to 800 nM) in a total reaction mixture of 20 µL at 25 °C for 30 minutes. After incubation, free aptamer and protein-aptamer complexes were resolved by 9% polyacrylamide gel containing 0.5X TBE

(Tris-borate-EDTA buffer) and the aptamers were stained with the fluorescent DNA binding dye SybrGreen (Invitrogen). In another experimental setup, 5% native PAGE was run to observe the shift of aptamer-protein complexes and further migration of protein-aptamer complex was visualized with coomassie blue staining.

2.3.9 Cell viability assay

Human breast cancer cell line MCF7 (ERα positive), MDA MB231 (ERα negative) were cultured in a CO_2 incubator (5% CO_2) and maintained at 37° C and 80-85% humidity. The cell viability of the selected aptamer was determined by the MTT (3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyl tetrazolium bromide) dye conversion assay. (Forrest et al. 2013) Cells were seeded at 10^4 per well (counted using a hemocytometer after staining with trypan blue) in a 96-well cell culture plate (Corning ®) and are maintained with cell culture medium (DMEM: Dulbecco's Modified Eagle Medium). Cells were treated with an increasing concentrations (0 to 200nM) of ER_Apt1 and non-specific scrambled aptamer for 24 hrs and 48 hrs. Cells were then incubated with 20 µl of 5mg/ml of MTT dissolved in PBS (pH 7.4) for 4 hours at 37°C in CO_2 incubator only. Purple colored formazan crystals were dissolved by adding them to 100 µl of dimethylsulfoxide (DMSO) and incubated for 30 minutes in the dark. Absorbance was measured at 570 nm using a microplate reader (Tecan) with background subtraction at 630 nm.

2.3.10 Analysis of aptamer binding affinity using flow cytometry

To determine the specificity of selected aptamers against ERα overexpressing cell line, 6-fluorescein (FAM) labeled aptamers were incubated with ERα positive and negative cell lines. 100nM of ER_Apt1 was added to $1x10^4$ MCF7 cells (ERα positive) grown for 24-hour after removing the DMEM media. After 30 minutes of incubation at room temperature, the cells were washed twice with 0.5 mL of aptamer binding buffer and then analyzed by flow cytometry (BD FACS Calibur Flow Cytometer, BD). (Liu et al. 2012) For assessing aptamer binding on cells,

the cells were scraped off the culture plates and dissolved in 500 µL cold PBS buffer. The FAM-labeled aptamers were incubated separately with 1x 10^4 of MDA MB-231 cells (ER α negative) in binding buffer at RT for 30 min. Cells were then washed twice with aptamer binding buffer prior to final resuspension in 500 µl of PBS and analyzed by flow cytometry (n=20,000 count). To evaluate the binding characteristics of the candidate aptamer to ERα structure, we incubated ER α positive MCF7 cells with FAM-labeled ER_Apt1 and scrambled aptamer in 200 µL binding buffer at 37°C for 30 min. Mononucleotide scrambled aptamer was used as non-specific binding controls.

2.3.11 Immuno-staining of ER α+ breast cancer cells by selected candidates

An immunofluorescence (IF) assay of the selected candidate was performed against ERα positive (MCF7) and negative cell lines (MDA MB 231). Sub-confluent cultures of MCF7 and MDA MB 231 cells on Poly-L-Lysine (PLL) coated coverslip were fixed using 100% chilled methanol. Cells were then incubated with nuclear permeabilizing buffer (0.25% Triton X-100, 0.2 µg/ml EDTA and 1% BSA in PBS) for 30 minutes. After blocking (10% BSA, 0.3% triton X-100 in 1X PBS), FAM labeled aptamer candidates were added and an ERα specific mouse monoclonal antibody (ab9269, Abcam, USA) was used as a positive control. Polyclonal FITC conjugated secondary antibody (ab6785, Abcam) was used against the mouse monoclonal antibody.

An immunocytochemistry (ICC) assay was performed on formalin-fixed monolayer cultures of MCF7, MDA MB 231 breast tumor cells and the control normal cell line L929. Sub-confluent cultures of MCF7 and MDA MB 231 were grown and fixed on coverslips as mentioned earlier. The nonspecific sites and endogenous peroxidase activity of cells were blocked and ICC profiling was performed with 100 nM conc. of biotinylated ER_Apt1 and with monoclonal ERα-antibody (ab9269, Abcam, USA) as positive control. HRP conjugated secondary antibody

(ab 6789, Abcam, USA) was used to capture the ERα specific monoclonal antibody.(Petersen et al. 1987) Scrambled mononucleotide aptamer as a negative control was also treated with all the three cell lines.

2.3.12 Assessment of ER α expression in breast tissue samples using specific aptamer

Four mm-thick sections of paraffin-embedded tissue blocks were cut and mounted on polylysine-coated slides for immunostaining of ER α on breast cancer tissues by using the ER_Apt1. The tissue sections were dewaxed in xylene and rehydrated through a descending gradient of ethanol (100 %, 90%, 70%, and 50% respectively). After rehydration, an antigen retrieval treatment was performed in Tris-EDTA buffer (pH 9.0) by microwave irradiation for two cycles (900 W, 720 W) each of 5 minutes. Endogenous peroxidase activity was blocked by using a 0.03% hydrogen peroxide solution (dissolved in PBS) at room temperature for 30 minutes. Next, the biotinylated ER aptamer (1µM) was added on tissue sections and incubated at 4°C overnight in aptamer binding buffer. After a thorough washing in 0.1M PBS solution, antigenic epitopes of the tissue sections were visualized using a streptavidin–HRP conjugate (1:500) (S911, Invitrogen) and DAB plus substrate system (Invitrogen). Finally, sections were counterstained with haematoxylin and mounted with DPX (Sigma).

2.4 RESULTS

2.4.1 Cloning, expression and purification of recombinant ER α protein in E.coli BL21 cells

The ERα domain was amplified from pVP16-ERα construct, with specifically designed primers. The molecular architecture on the basis of DNA sequence shows 1800 bp long ER α isoform (whereas estrogen receptor beta isoform 1500 bp), which was confirmed by PCR amplified products on 0.8% agarose gel. Amplified product was ligated with double digested pET28a vector and transformed in *E.coli* DH5α cells. After overnight incubation, we obtained

kanamycin resistant transformed colonies from which we screened positive colonies containing desired insert by PCR amplification with gene-specific primers. Then, plasmid DNA was isolated from PCR positive colonies and digested with NheI and SalI at 37°C for 3 hours. The successful cloning was confirmed by 0.8% agarose gel electrophoresis where vector backbone and insert ERα domain shows two distinct bands of 5.3kb and 1.8kb respectively. (Fig. 2.1) The positive clones were sequenced to verify the insertion of ERα in the pET28a vector backbone.

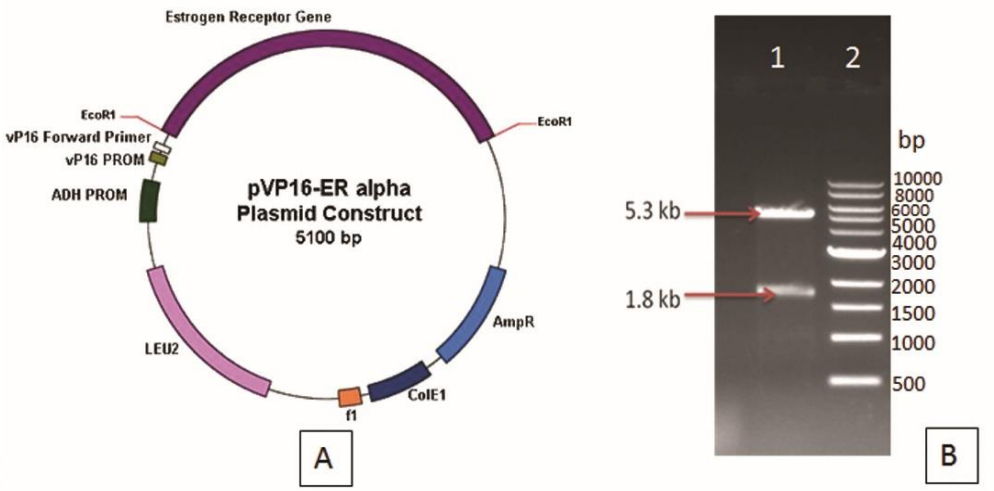

Fig. 2.1 Sub-cloning of ER alpha in pET28a expression vector**.** (A) Map of pVP16-ER alpha plasmid construct. (B) Confirmation of cloning into pET28a after restriction digestion by Nhe I and Sal I. Lane 1 shows release of insert (1.8kb) after restriction enzyme digestion where pET28a vector backbone is 5.3kb. Lane2. NEB 1kb Ladder.

Sequenced positive clones were again transformed into *E.coli* BL21 expression cells and induction parameters were optimized to attain highest yield of ERα in bacterial system. PET28a-ERα recombinant clones were over-expressed in *E.coli BL21* cells with 0.1mM IPTG, only when the optical density of the culture has reached 0.5-0.6. The optimum expression was found at 30°C after induced with 0.1mM IPTG at 180 rpm for 6 hours. After harvesting the cell lysate, the recombinant protein containing the N-terminal His_6-tag was purified by immobilized metal ion affinity chromatography. The expression and purification of ERα protein was analyzed by SDS-PAGE shown in Fig.2.2 confirming its molecular size of approximately 68kDa. The purity

of the protein was also confirmed by western blot against a monoclonal antibody specific to human estrogen receptor α (ab9269, Abcam, USA).

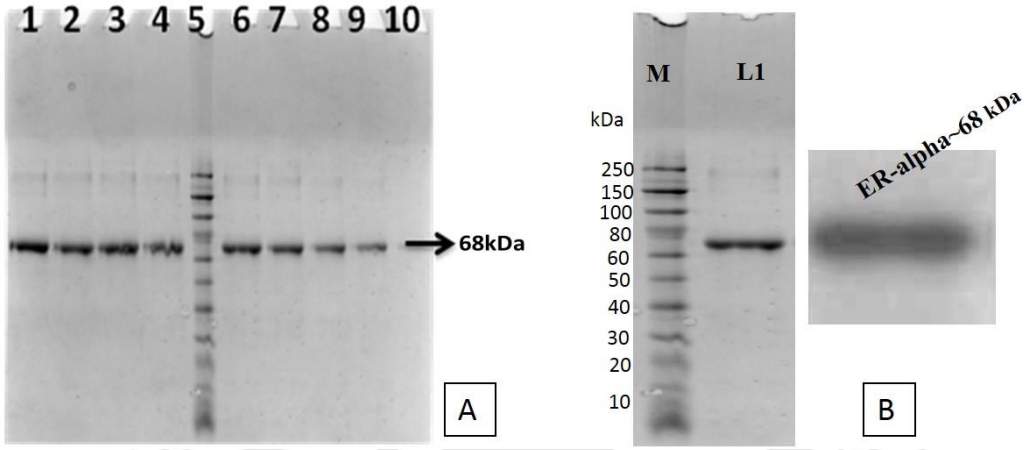

Fig. 2.2 SDS-PAGE and Western Blot of ER alpha. (A) Purified his tagged ERα fractions were run on 12%SDS-PAGE after eluted with 300 mM immidazole from batch culture. Lane 5: NEB Broad range protein Ladder (10-250kDa). (B) Western blot analysis to verify with anti-ERα monoclonal antibody.

2.4.2 Screening of enriched DNA aptamer candidates by Protein-SELEX process

To find ERα specific aptamer, from a library of $10^{14\text{-}15}$ random oligonucleotides of 40 random bases was taken as a starting pool. The purity of the synthesized library was checked and a distinct band of 80 bases in 2% agarose gel was evident. The amount of oligonucleotides in the first round of SELEX screening was kept to 500 picomoles to ensure a sufficient number of sequences during subsequent rounds of selection. Prior to SELEX, the aptamer library was denatured and renatured to unfold any compact structure. NC membrane bound sequences were removed from the initial pool library during the counter selection step. Furthermore, a selection pressure was applied to SELEX enrichment by decreasing the molar ratio of ER α to DNA from 1:1 to 1:10 following the fifth round and then to1:20 after the tenth round of SELEX onwards. The amount of protein was reduced to 0.5 µg (cycles 5-10) and to 0.25 µg (cycles 10-14) in a binding volume of 500 µl. As the SELEX process progressed, the non-specific sequences were excluded and the eluted products were checked by 2% AGE. (Soo et al. 2010) As the cycle

number increased, the band intensity increases commensurately. After 14 rounds, the intensity did not increase significantly. (Fig 2.3)

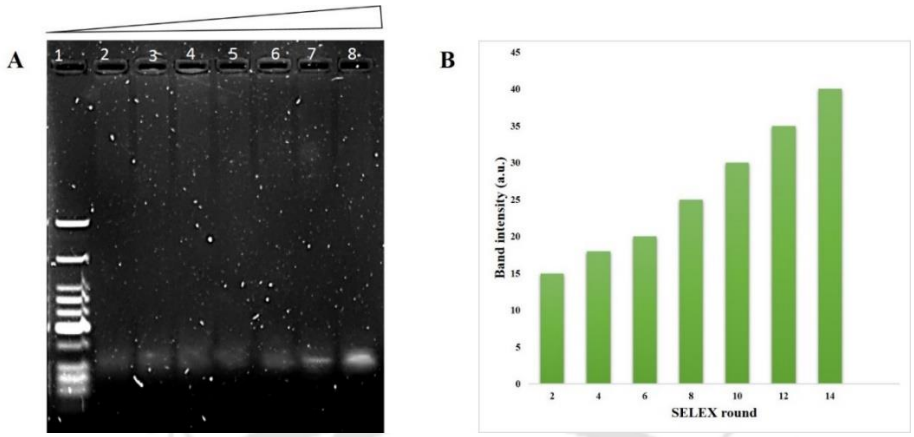

Fig. 2.3 Enrichment of the specific aptamers: (A) 2% Agarose gel electrophoresis showing the evolution of enriched aptamers as the SELEX round increases (Lane 2 to 8, lane 1: NEB low molecular weight ladder), (B) Histogram showing the increased intensity of eluted aptamers (intensity of the band calculated by ImageJ)

The SELEX process was restricted to fourteen rounds and the entire enriched pool was cloned in a TA cloning vector. After successful transformation and blue-white screening, we found 45 white colonies in LB-ampicillin plates. Among them, thirty colonies were found PCR positive and then sequenced. After sequencing analysis, we found 4 enriched aptamer sequences having different copy number. (Table 2.1) ER_Apt1 with the highest copy number was the most enriched sequences among the pool.

Table 2.1: Enriched Aptamer sequences obtained against ERα after Sangers sequencing

Seq. ID	Sequence	Length	Copy no
ER_Apt1	CCCGGCATGGTTGCGGAGCAGGAGTATAACACTACCATTG	40	15
ER_Apt2	TAATTAAGCTAGTGGGTGCTCTTAACGGAAGCATATCCCG	40	8
ER_Apt3	ACAGGACCATCGCAACCGATTGATACGGGTGAGCATGTGG	40	4
ER_Apt4	GGACGCCGTTGTCACCATTTGCTTCTGGATGCGTCGTTGC	40	4

Table 2.2: Thermal Profile of Enriched DNA aptamer candidate sequences

Seq. ID	Sequence	*Free Gibbs energy (ΔG) (kcal/mol at 37 °C)*	*Enthalpy change(ΔH) (kcal/mol)*	*Entropy change (ΔS) (cal/K.mol)*
ER_Apt1	CCCGGCATGGTTGCGGAGCA GGAGTATAACACTACCATTG	-4.16	-40.40	-116.7
ER_Apt2	TAATTAAGCTAGTGGGTGCT CTTAACGGAAGCATATCCCG	-2.32	-50.60	-155.6
ER_Apt3	ACAGGACCATCGCAACCGAT TGATACGGGTGAGCATGTGG	-3.30	-45.80	-134.2
ER_Apt4	GGACGCCGTTGTCACCATTT GCTTCTGGATGCGTCGTTGC	-2.82	-46.50	-140.8

We also performed homology similarity assessments to find base similarities in the enriched sequences. Enriched sequences however are unique in primary structure, and no consensus motif was present among candidate sequences. Although some three base repeats (CCG, GGA, and CAT) are present in all four sequences, the secondary structures are different. Further *in silico* analyses such as free energy comparisons and structural stability was performed by Mfold analysis tool to assess the best candidate for the ERα protein. Several secondary structures were obtained for a single candidate depending on their free Gibbs energy. The most stable structure having minimum free Gibbs energy is shown in the Figure 2.4. Unique candidates with the lowest minimum free Gibbs energy (ΔG) and other thermodynamic parameters is presented in the Table 2.2. Notably, single stranded aptamers can form intra-molecular base-pairing in portions of aptamer sequences in the bound state with the target molecule. As calculated in Table, ER_Apt1 showed the minimum free Gibbs energy (ΔG) of -4.16 compared to other binding candidates. Therefore, based on the *in-silico* thermodynamic analyses, we selected ER_Apt1 as the most promising aptamer for ERα.

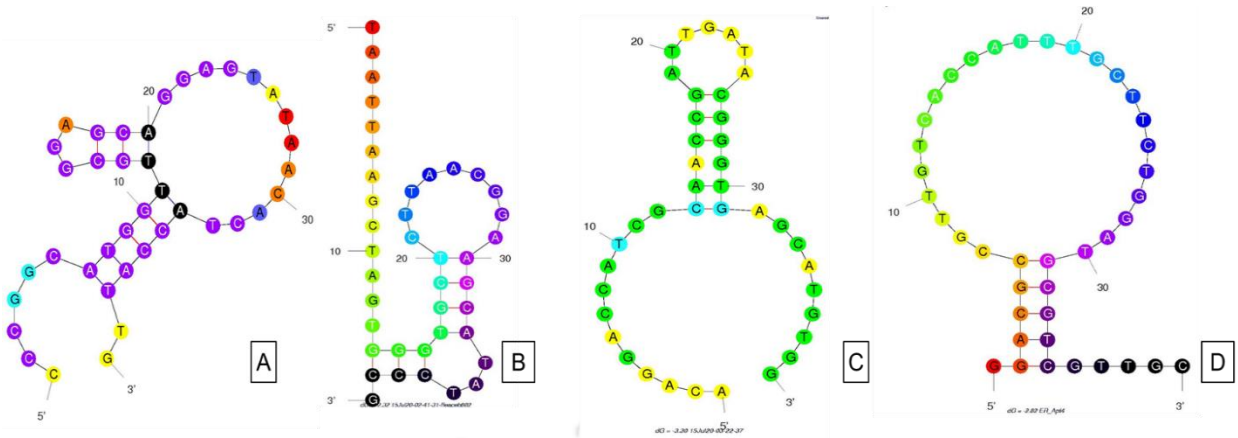

Fig. 2.4 Predicted secondary structures of enriched DNA aptamer sequences by Mfold. (A) ER_Apt1 aptamer form dumbbell-shaped structure with extending hairpin loop (B) ER_Apt2 aptamer form bi-hairpin loop structures (C) ER_Apt3 form bulged loop structure with hairpin loop extending outwards (D) ER_Apt4 aptamer form one bulged hairpin loop structure.

2.4.3 Binding assessment of the enriched sequences

Isothermal calorimetry (ITC) was carried out to confirm the *in silico* predictions and subsequently determine the sequence with best binding credentials. The resulting associated temperature (kcal per mole of injectant) was plotted against the molar ratio of the reactants. The binding affinity and thermodynamic parameters: association constant (Ka), binding enthalpy (H), entropy (S) were obtained from the aptamer-protein binding reaction by fitting one site binding model. Consequently, the changes in Gibbs free energy (ΔG) and the changes in entropy (ΔS) can be calculated using following equation of ΔG, where T is reaction temperature (in K) and R is the gas constant (1.986 cal K^{-1} mol^{-1}).

$\Delta G = -RT \ln K_a$ and $\Delta G = \Delta H - T\Delta S$

Therefore, this binding mechanism reveals a highly favorable enthalpy of reaction (ΔH) at 25 °C of –255.5±0.2 kcal mol^{-1} and unfavorable entropy of reaction (TΔS) -14.6 kcal $mol^{-1}deg^{-1}$ as shown in figure 2.5. These thermodynamic and conformational data indicates the local conformation change of the aptamer that occur during ligand binding. (Pagano et al. 2009) The saturation curve for molar ratio vs. heat exchanged during the specific binding process of the

ER aptamer is presented in figure 2.5. The area under each peak was integrated and fit to a one-site independent binding model to determine the binding affinity and thermodynamic parameters enthalpy and stoichiometry (Origin 5.0 software (Microcal, Inc.). As calculated by the isotherm model, the affinity constant (Ka) between selected candidate ER_Apt1 and ER alpha protein is $1.15 \pm 1.01 \times 10^{8}\ M^{-1}$.

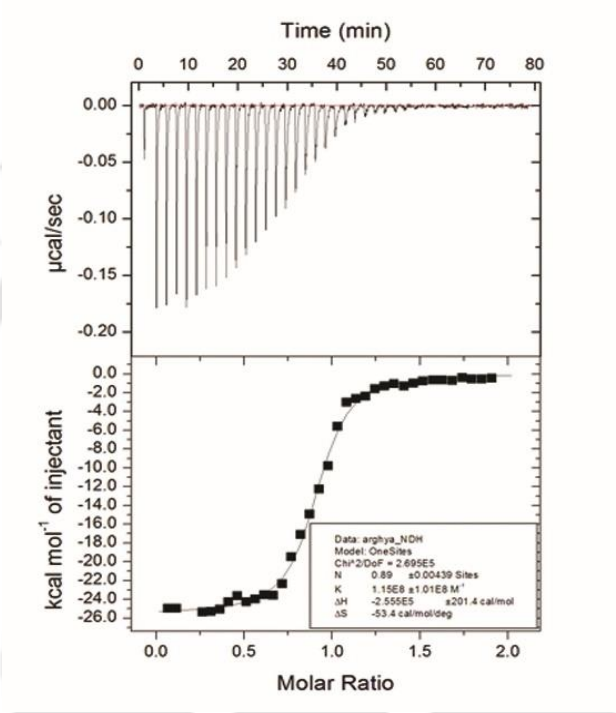

Fig 2.5 Isothermal titration calorimetry: Isotherms were fitted to a one-site independent binding model. The thermodynamic parameters are shown in the diagram.

A simple binding equilibrium with 1:1 stoichiometry can be described by:

A+P⇀↽AP in which A is the aptamer, P is the ERα Protein and AP is the target complex. The equilibrium can be described using either a dissociation constant K_d, or association constant Ka

$$Kd = \frac{[A][P]}{[AP]}$$

$$Ka = \frac{1}{Kd} \quad Or \quad \frac{[AP]}{[A][P]}$$

2.4.4 Competition analysis between labelled and unlabeled candidates

In a homologous competitive binding assay, a constant amount of labeled ER_Apt1 with a gradient of unlabeled aptamers were incubated with purified receptor. The maximum binding was calculated and the data was fit to the following equation:

$$Y = B\max(\frac{[E]}{[E]+[U]+Kd}) - b$$

where Y is the measured signal, B_{max} is the maximum binding, [U] is the concentration of unlabeled competitor, [E] is the biotinylated aptamer concentration, b is the background binding, and K_d is the dissociation constant.(Waybrant *et al*. 2012). The data was fit using non-linear regression analysis with the program Origin 8 and the dissociation constant was determined to be 8.4 ± 1.3 nM based on the binding curve shown in Figure 2.6.

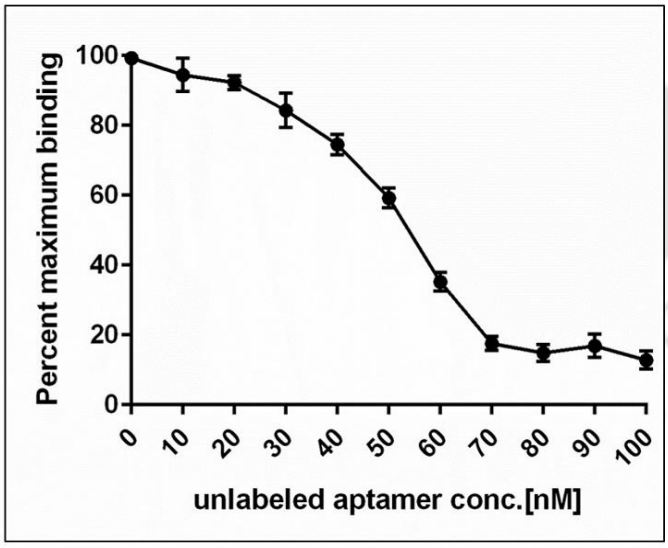

Fig 2.6 Homologous competition curve for ER_Apt1: 0-100nM of unlabeled ER_Apt1 with fix 100nM of biotinylated aptamers co-incubated with ER alpha in microtitre plates.

2.4.5 Electrophoretic Mobility Assay of Aptamer-protein complex

We observed the amount of free aptamers in the 9% native poly acrylamide gel and the shift of aptamer-protein complex with reference to free protein in 5% gel electrophoresis. When the aptamer was incubated with ER alpha protein, the band intensity of free aptamer gradually decreases with respect to free aptamer. At the highest protein conc. 800nM of ER alpha, faint band of free aptamer is visible. But when ER_Apt1 was treated with ER beta, the band intensity did not alter much. But in the 9% gel, the aptamer-protein complex did not resolve due to tiny pore size. It is difficult to enter inside gel matrix for the complex due to its higher molecular weight (aptamer and protein in complex around 80kDa). But in lower percentage of PAGE, the aptamer-protein complex enters into the gel and shift of aptamer bound protein complex was clearly visible with respect to free protein. (Fig 2.7) Free protein migrated longer distance as the high conc. ER alpha protein –aptamer complex migrated least. However, in case of ER beta, the shift of complex is not significant.

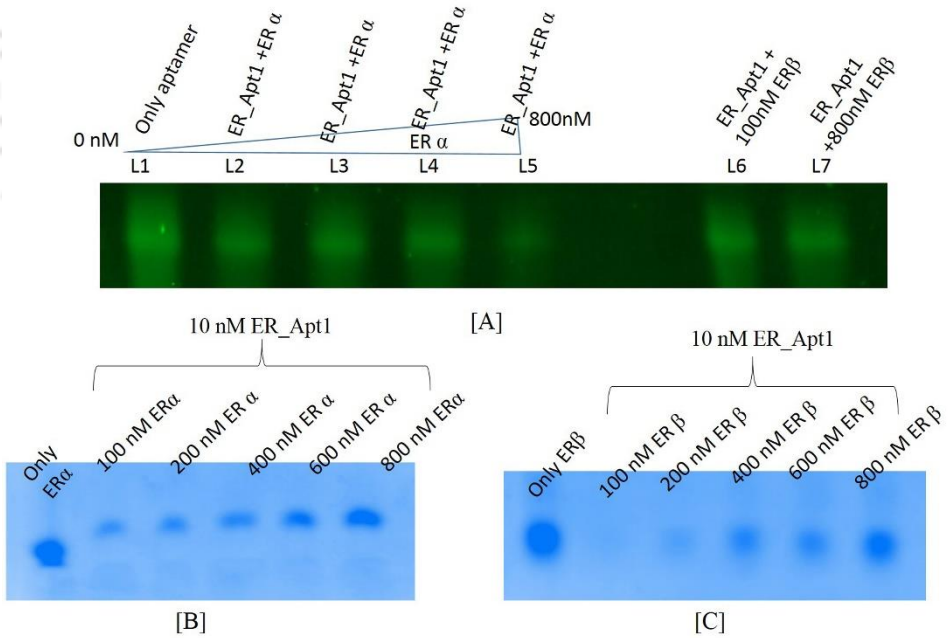

Fig. 2.7 Migration study of protein-aptamer complexes. (A) Band intensity pattern of free aptamers when treated with ERα and ERβ, (B) After treatment with various concentrations of ERα shift of band pattern is visible upon binding with ER_Apt1, (C) After treatment with various concentrations of ERβ, no major shift of band pattern was observed.

2.4.6 Flow cytometry analysis of the aptamer candidate

6-FAM labeled ER_Apt1 was co-incubated with 24-hour grown ERα positive and negative cell lines. The histogram profile (Fig. 2.8) showed the significant shift of fluorescence signal obtained from the FL1 channel when ER_Apt1 was subjected to the human breast adenocarcinoma MCF7 cell line (ER α positive). However when incubation with the ER α negative cell line MDA MB 231, FAM labeled ER_apt1 resulted in a fluorescence signal that was similar to treatment with random oligonucleotides. (Fig. 2.8C) The results indicated that ER_Apt1 specifically binds with the ER α positive breast cancer cell line.

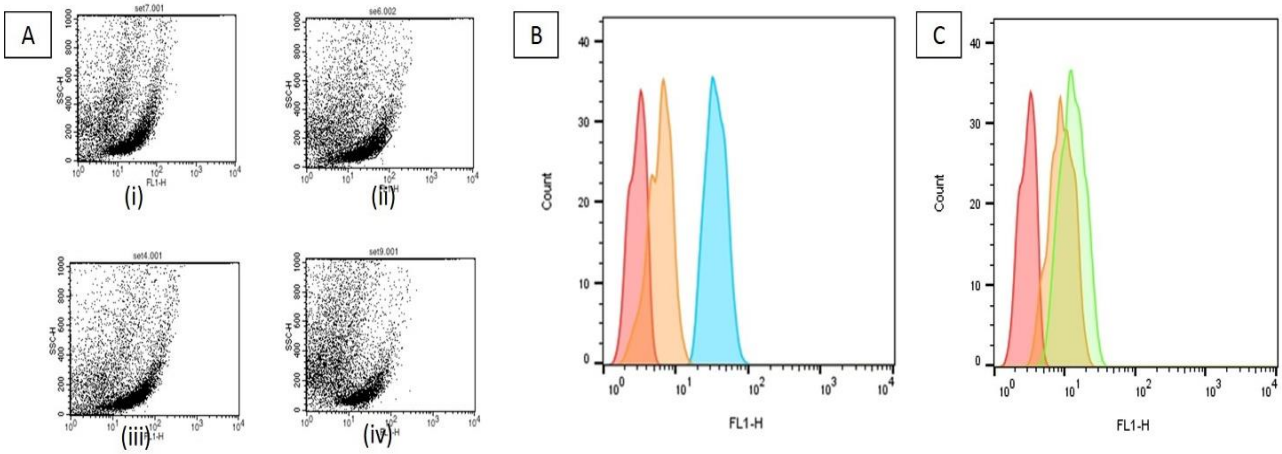

Fig 2.8 Aptamer binding study by FACS analysis (A) Dot plot of SSC-H vs FL1-H depicts that treatment with various concentrations of FITC-ER_Apt1 do not alter the granularity of the ERα positive MCF7cell line (i-ii) and ERα negative cell line MDA MB231 (iii-iv). (B) Histogram Overlay Plots of aptamers treatment on MCF7 cells: Red spectra represents MCF7 cells without any treatment, yellow spectra represents cells treated with scrambled aptamer and blue spectra represents treatment with ER_Apt1. (C) Histogram Overlay Plots of aptamers treatment with MDA MB231 cells: Red spectra represents only MDA MB 231 cell without any treatment, yellow spectra represents cells treated with scrambled aptamer and green spectra represents treatment with ER_Apt1.

2.4.7 Binding assessment of ER_Apt1 by chemiluminescent assay

The detection range of the aptamer-based protein detection assay was quantitated by plotting a standard curve for ER_Apt1 in the working range of 0−1000 nM. The ER α protein linked aptamer assay was performed in a sandwich format. The assay showed a linear rise in chemiluminescence (at 490nm wavelength) in the dynamic range of 100−800 nM of ER_Apt1 with a limit of detection of 13.2±2.5 ng/mL. (Fig 2.9) The linear regression analysis was performed and R^2 value was found to be 0.9212. The precision of the assay is expressed as the coefficient of variation with intra-assay CV <12% (GraphPad Prism 5.03, GraphPad Software, San Diego, CA, USA). A 95% confidential interval (slope 0.0008918 to 0.001106) proved that the regression analysis is reliable.

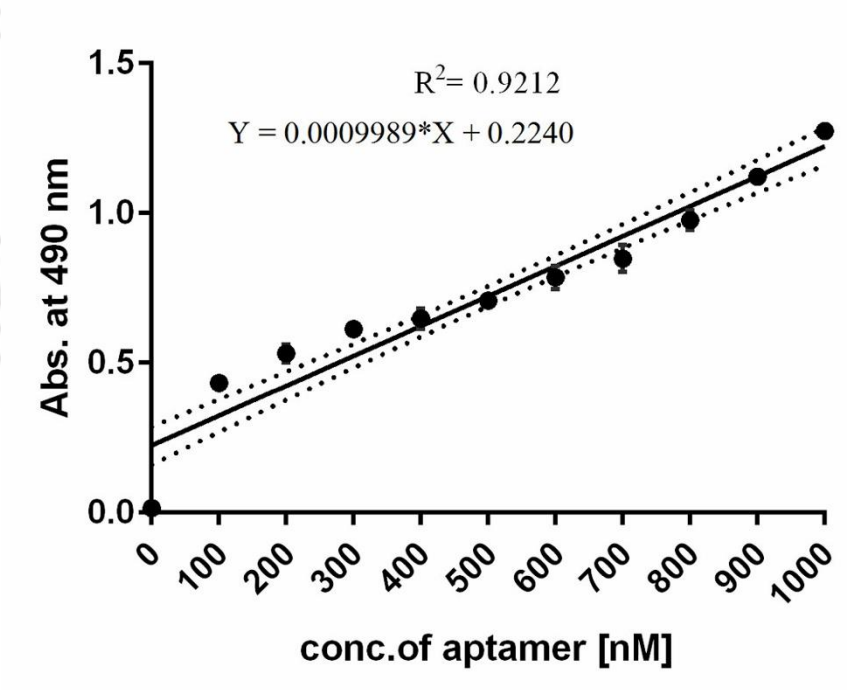

Fig 2.9 Aptamer Binding assay by Chemiluminescence analysis. Binding of enriched aptamer ER_Apt1 (0-1000 nM) with immobilized ERα (2µg) was measured by colorimetric saturation binding assays. Linear regression analysis was performed by a Graph Pad Prism statistical analysis tool.

2.4.8 Cytotoxicity assay

MCF-7 cells (10^4/well) were cultured in DMEM supplemented with different concentrations of aptamers. MTT reagent was added at a final concentration of 0.1 mg/mL to both 24hr and 48hr treated cells and the absorbance of reduced MTT measured at 570 nm. Cell viability was expressed as a percentage of the control by the following equation: cell viability (%) = ($A_{treated}$-$A_{control}$) x 100. where $A_{treated}$ and $A_{control}$ is absorbance of treated cells and untreated cells respectively. (Lee et al. 2015) A two tailed t-test with unequal variance was performed to determine significance between two groups (0 nM and 200 nM treatment with ER_Apt1) and results showed that the difference between two treatment groups was significant ($p<0.0001$). (Fig 2.10)

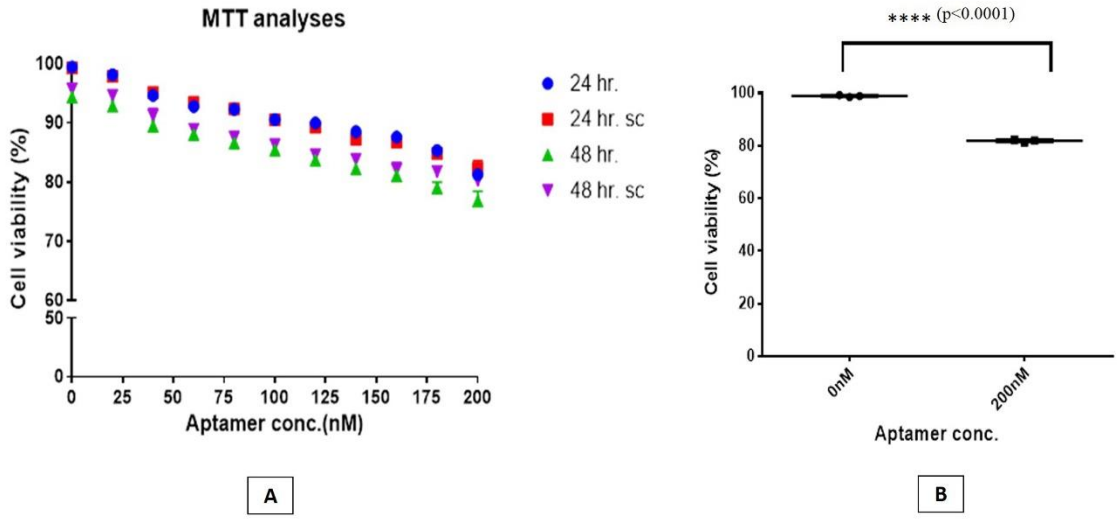

Fig 2.10 MTT cell viability assay. (A) Cell viability of MCF-7 cells exposed to different concentrations of ER_Apt1 and scrambled non-binding sequence (0–200 nM) over a period of 24-hr and 48 hr treatment. (B) Significance test between 0nM and 200nM of ER_Apt1 treated group ($p<0.0001$).

2.4.9 Immunocytochemistry against breast carcinoma cell lines

Chromogenic immunocytochemistry (ICC) assay was performed on formalin-fixed 24-hour grown monolayer cultures of MCF7 (ERα+), MDA MB 231 (ERα -) breast tumor cells and L929 normal cell line. ICC profiling was performed using biotinylated ER_Apt1 comparing it

to the ERα specific monoclonal antibody (ab9269) as positive control and scrambled sequence considering as a negative control. The ligand molecules binds to the ER structure of MCF7 cell lines after the incubation of the cells with permeabilizing buffer. Nonspecific sites and endogenous peroxidase activity of cells were also blocked prior to the addition of candidate molecules which reduced the background noise. The nuclei of MCF7 cells were found to stain dark brown by developing with the DAB chromogen in aptamer based ICC assays (Fig.2.11). In contrast, no nuclear staining was seen in MDA MB231 and L929 with either of the affinity molecules. However, weak staining in MDA MB 231 cells were evident which could be attributed by cytosolic expression of ERα.

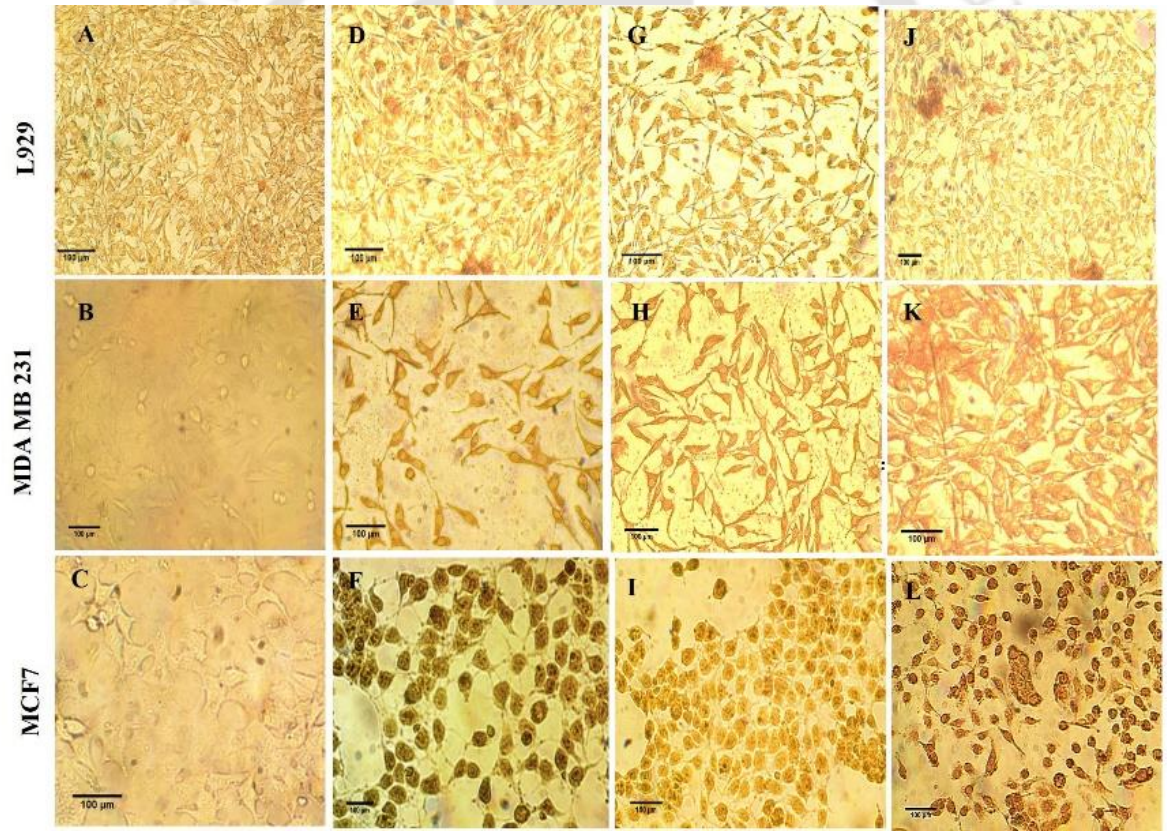

Fig. 2.11. Immunocytochemistry study with aptamers. Bright field images of L929 (A), MDA MB231 (B), MCF7 (C) cells respectively. L929, MDA MB231, MCF7 cells treated respectively with biotinylated ER_Apt1 (D, E, F), biotinylated scramble sequence as a negative control (G, H, I) and with ER specific mAb as positive control respectively (J, K, L).

In a cell based immunofluorescence assay, FAM labeled candidate ER_apt1 binds particularly to the nuclei of MCF7 cells. The nuclei appeared green when it was excited at 488nm after addition of FAM labeled candidates. (Fig. 2.12) Overall, these observations suggest that the aptamer binds ER alpha, which was similar to the antibody and further provided evidence its utility in cell imaging.

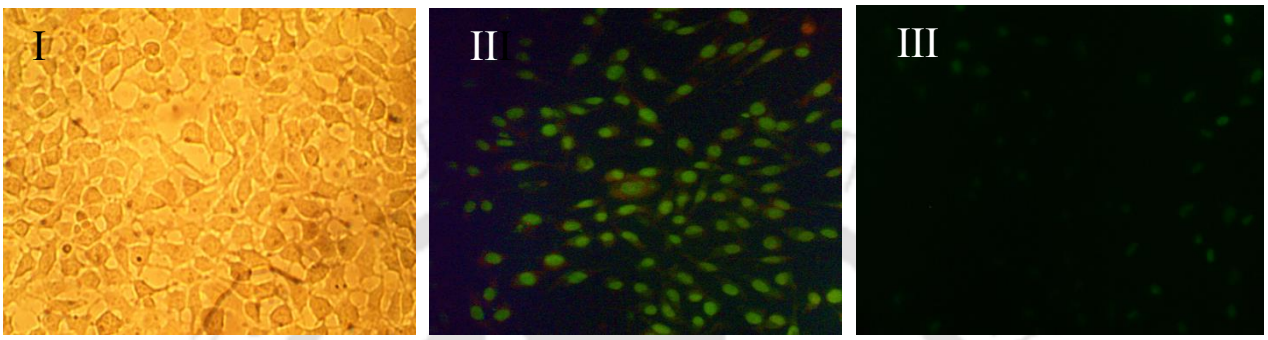

Fig. 2.12. Immunofluorescence study of ER_Apt1. I- Bright field image of MCF7. II- MCF7 cells treated with FAM labeled ER_Apt1. III- MCF7 cells treated with scramble sequence.

In this study, we have tried the immunocytochemistry assay to locate estrogen receptor in ER alpha positive MCF7 cells using ER_Apt1 specific aptamer. In ICC assay, aptamers are biotinylated and then chromogenic DAB based system was applied for microscopy visualization under normal light. (Bright field image)

However, in immunofluorescence based assay (IF), aptamers are modified with FAM (a fluorescence probe) at 5' end and the nuclei of MCF7 cells appeared green under fluorescence microscopy when FAM labeled ER_Apt1 enters into cells and specifically binds to nuclei.

2.4.10 Immunohistochemistry against ER α overexpressing Human breast cancer tissue samples

In the final stage of aptamer validation and utility in clinical application, we have carried out the detection of ER expression in tissue specimens using newly identified candidate ER_Apt1. Histochemical studies were performed on formalin preserved paraffin embedded (FPPE) tissue

specimens of human breast cancer where histopathology (infiltrating duct carcinoma) and immunohistochemistry for ER (strongly positive 90% and above duct carcinoma cells stained, moderately positive 50-90% cells stained, weakly positive <50% cells stained and negative <5% cells stained) was known. Immunohistochemistry with the aptamer showed specific localization in the nucleus of malignant duct cancer cells. The aptamer showed no cross reactivity with ER alpha negative tissue samples. (Fig 2.13) Biotinylated non-specific sequence showed some nuclear staining as it can form random secondary structure which binds to ER alpha like structure.

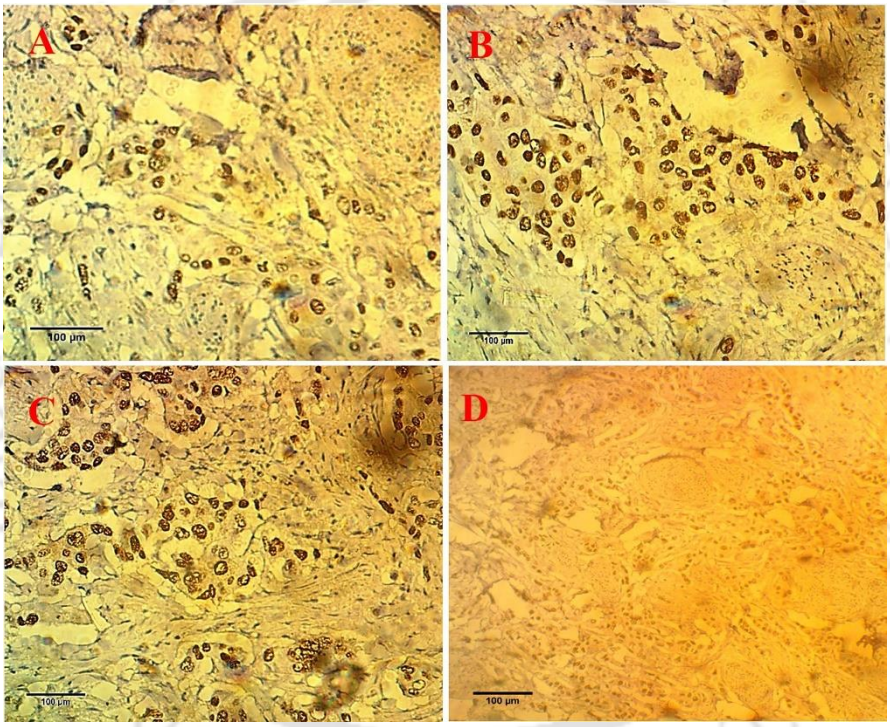

Fig. 2.13. Immunohistochemistry of paraffin embedded tissue sections. After antigen retrieval of breast cancer tissue specimens (A) stained with Non-specific sequence, (B) stained with biotinylated ER_Apt1 (C) stained with monoclonal antibody specific to ER alpha, (D) without treatment of any aptamer/antibody.

2.5 DISCUSSION

The focus of this work was to develop an aptamer based ERα detection system. For this we started with an ERα protein-SELEX process and aptamer based ERα assessments. Screened DNA aptamer sequences against human ERα were found to bind the target at nanomolar sensitivity. These aptamers can be exploited as a molecular probe in detection of ERα overexpressing cell lines and ERα positive cancer tissue specimens. Testing ERα specific aptamer to profile ERα expression in breast tumors resulted in an alternative histochemistry assay for breast cancer assessments. The DNA aptamer candidate ER_Apt1 showed specific nuclear localization of ERα in malignant duct cancer tissues with no cross-reactivity to ER-deficient fibroblasts, inflammatory cells or adipocytes. These specific aptamers can be exploited as a component of biosensor for detection of various types of estrogen hormone dependent cancers (breast, endometrial, ovarian, colorectal, prostate), other estrogen hormone dependent disorders or dysfunctions of Central Nervous System (CNS), skeletal system and more specifically osteoporosis, cardio vascular system, hematological system, inflammation and immune system, and reproductive system.

To accomplish the objectives of this study, we have amplified the full ERα domain from a pVP16- ERα construct (Addgene), with gene specific primers containing Eco RI and Sal I restriction enzyme sites. Amplified product was ligated with double digested pET28a vector and transformed in *E.coli DH5α* cells. Initially codon degeneracy was performed to select the clone sequence that would code for humanized recombinant ERα protein. After overnight incubation, we obtained kanamycin resistant transformed colonies from which positive clones were screened by PCR amplification containing an ERα insert. The successful directional cloning was confirmed after plasmid DNA digestion with both restriction enzymes Nhe I and Sal I. Two distinct bands of 5.3kb and 1.8kb were clearly visible due to the pET28a backbone and ER alpha insert respectively. (Fig 2.1)

Overexpression of ERα protein in the *E.coli* bacterial system and its subsequent purification was analyzed by 12% SDS-PAGE as shown in Fig.2.2 confirming its molecular mass of approximately 68kDa. The purity of the protein (almost 95%) was also confirmed by western blot analysis compared against the monoclonal antibody specific to the human ERα (ab9269, Abcam, USA).

To obtain ERα specific aptamers, we carried out protein-SELEX against the ER alpha protein. Although two isoforms (ERα and ERβ) display distinct regions of sequence homology, crystal structures of their DNA-binding domains showed 'not significant' similarity (17% identity). (Kumar et al. 2011)With this information, we did not carry out the SELEX process against ERβ structure.

We started with an initial pool of an aptamer library of 76 nucleotides. Nitrocellulose membranes were used as an immobilization platform where ER α protein was immobilized. Blocking the protein-bound surfaces prior to the selection process of aptamers is a common practice to counteract nonspecific enrichment. However, such experiments lead to the enrichment of "blocking agent-binding sequences" and thus loss of probable aptamer candidates that might bind with target ERα protein in subsequent steps. In this study, we excluded the blocking step of NC membrane with BSA protein. Instead, we introduced a counter selection step wherein the starting DNA library was incubated only with NC membrane (without immobilized ERα) to remove the membrane bound sequences from the initial pool library. After fourteen rounds of enrichment, the entire enriched pool of the last SELEX round was cloned and sequenced using Sangers' capillary sequencing method. Analysis of the sequencing data showed the enrichment of 4 different sequences that bind specifically to ERα protein. Further *in silico* analyses such as free energy comparisons and structural stability was performed to assess the aptamers with best binding affinity to ERα protein. We selected an aptamer candidate with the most thermodynamically stable predicted structure after *in silico* analyses. As calculated in Table 2.2,

ER_Apt1 showed a minimum free Gibbs energy (ΔG) of -4.16, demonstrating better structural folding stability of this sequence over other same-length random sequences. Therefore, on the basis of structural analysis and free energy comparisons we carried out further binding analysis with ER_Apt1 having minimum ΔG and with the most stable structure.

Isothermal calorimetry (ITC) was carried out to confirm the *in silico* predictions and subsequently determine the sequence with best binding affinity**.** ITC study explains the binding isotherm of the aptamer-protein complex. The saturation curve for molar ratio vs. heat exchanged during the specific binding process of the ER aptamer is presented in the saturation curve seen in Fig.2.3. The area under each peak was integrated and fit to a one-site independent binding model to determine the binding affinity and thermodynamic parameters enthalpy and stoichiometry. The dissociation constant (K_d) of the ER_Apt1-protein complex was found to be in the range of 8.69 ± 1.1 nM, which was confirmed by ITC analyses. Thus, ER_Apt1 can be considered as a promising aptamer probe along with aptamers reported earlier. (He et al. 2015) In an earlier study, RNA aptamers were selected to find a novel drug site in the estrogen receptor although they do not bind specifically to the alpha or beta isoform (Xu et al. 2014). In addition, DNA aptamers are more stable than their RNA counterparts and they possess higher systematic retention values. So, ER_Apt1 shows more potency as detection molecule than the candidates discussed in earlier studies. *In-vivo* generation of complex monoclonal antibody incurs various issues like batch to batch variation, expertise personnel, sacrifice of animals and thus resulting in high costs for antibody-based assays. Moreover, aptamer production cost is also 10-50 times less than antibody(Low et al. 2009)

We have performed the protein-aptamer gel shift assay of ER_Apt1 treated with specific target ERα and non-specific protein ERβ. From the gel shift assay, it has been shown that increasing concentration of ER alpha protein, the aptamer-protein complex stays near the wells, migration get retarded compared to free protein due to bulky nature of the aptamer-protein complex. The

amount of free aptamer also decreases with increasing conc. of ERα as they form complex with the target molecule ERα. However, when the aptamers treated with ERβ, the aptamer-protein complex would not form as they are not cognate partners and thus the intensity of free aptamers remain unaltered. In another experiment, the shift of aptamer-protein complex with respect to only ERα protein is significant but after treatment with ER beta, there is no shift of band pattern.

The specific candidate ER_Apt1 was labeled with 6-FAM at 5' end for its fluorescence study. The FAM labeled candidate was co-incubated with 24-hour grown ERα positive human breast adenocarcinoma MCF7 cells and the fluorescence intensity was analyzed by flow cytometry. The histogram profile (Fig.2.8) showed a significant shift (Geometrical mean 28.72) of fluorescence signal in the FL1 channel. However, the ER α negative cell line (MDA MB 231) showed no significant shift after treatment with fluorescence labeled candidates. These results indicated that ER_Apt1 specifically binds to the ERα containing cell lines but not others. (Liu et al. 2012) The signals obtained from side-scattered light (SSC) was proportional to the cell granularity or internal complexity of the cells after treatment with the reporter molecule. The granularity of MCF7 cells (pattern obtained of the SSC channel) was unaltered after incubation with different concentrations of aptamers which reveal that selected aptamer candidates do not cause any form of perturbation to the human breast cancer cell line. (Fig. 2.8A) Fluorescence imaging analysis also showed the nuclear localization in ER positive MCF7 cells when they were stained with FAM labeled ER_Apt1. For all experiments, mononucleotide scrambled aptamer was used as non-specific binding control. The nucleotide composition of the test and control aptamers were the same, however, due to the scrambled primary sequence of the control aptamers, they will not fold into the exact same tertiary structure as the test aptamers and therefore will not bind to the target molecule. (Nagarkatti et al. 2012) In another cell based immunofluorescence assay, FAM conjugated aptamers were incubated with MCF7 cells and as a positive control, HRP conjugated polyclonal secondary antibody (ab6728) was used for antibody based detection. Overall, these

observations suggest that the aptamer could be as useful as specific monoclonal antibody in cell imaging purposes. In spite of the structural complexity of cells or even tissues, ER_Apt1 was found to bind ERα precisely and sensitively.

ER_Apt1 was labeled with biotin at 5'end to determine the binding ability at the cellular and tissue level. Chromogenic immunocytochemistry (ICC) assay was performed on 24-hour grown monolayer cultures of breast tumor cells (MCF7, MDA MB 231) and normal cells (L929). MCF7 cells are ERα positive and MDA MB 231 are ERα negative, respectively. In immunocytochemistry and immunofluorescence assays, the addition of Triton-x 100 is essential for nuclear permeabilization and only then can the epitopes be exposed to bind to ligands. Due to the enhanced cell penetration ability, DNA aptamer can enter into the cell and attach to the nuclear receptor. The nuclei of MCF7 cells stained dark brown by developing with DAB chromogen in aptamer based ICC assays (Fig. 2.11). In both the study, two-step method was mandatory for antibody based assays although, only single step is required in case of detection with labeled aptamer ER_Apt1. Further, this study also proves that labelling of ER_Apt1 with biotin (chemical) or FAM (fluorescence) do not alter its specificity toward its target ERα protein.

From the MTT study, it was found that ER_Apt1 and scrambled aptamer both inhibited the proliferation of breast cancer cells at the 200nM conc. Results did not show any noticeable cytotoxicity at lesser concentration against the treatment with the breast carcinoma cell line. The candidates also showed no significant toxicity toward the control normal cell line L929.

In paraffin embedded tissue sections, biotinylated ER_Apt1 showed specific and ER alpha-selective localization in the nuclei of malignant duct cancer cells. Cross reactivity to fibroblasts, inflammatory cells and adipocytes or non-specific binding to cytosolic or extracellular components were minimal. Due to the smaller size of the DNA aptamer (6-30kDa)compared to the antibody (125-150kDa), the aptamer can penetrate tissue more easily.(Ku et al. 2015) In

addition, this DNA candidate can fold spontaneously into unique three dimensional conformations which make them suitable for resistance to pH, temperature and other surrounding changes in the process of tissue immunohistochemistry. Overall, ER_Apt1 showed more potency than an ERα specific monoclonal antibody as a diagnostic agent against ER alpha malignancies.

2.6 CONCLUSION

In brief, we have successfully cloned and expressed recombinant estrogen receptor alpha in a bacterial system. We demonstrated that the specific aptamer candidates selected after iterative rounds of a selection process showed a high affinity for estrogen receptor α molecules during *in vitro* DNA-protein binding studies. The biotinylated aptamer detected ERα expression in paraffin-embedded breast cancer tissue samples, which was comparable with established immunohistochemistry results with an ERα specific monoclonal antibody. FITC conjugated aptamer candidates showed no cytotoxicity, thus making them suitable candidates for bio-diagnostics and drug delivery. Therefore, the described DNA aptamer can be exploited as a promising diagnostic module for non-invasive cancer diagnosis. It can also provide a novel cost effective alternate to conventional ER antibody screening in solid phase immunoassays for cancer diagnosis and related applications.

Since ERα is overexpressed in most carcinomas including breast cancer, gastric cancer, endometrial cancer, ovarian cancer, the ER α specific aptamers might have prospective as a guiding ligand for targeted chemotherapy against a plethora of multiple malignancies. Nevertheless, extensive research with animal models and further clinical trials in patient samples is still required to validate the *in vivo* binding specificity of the selected ER alpha specific DNA aptamer candidate.

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Chapter 3

Selection and Characterization of Aptamers Specific to the Extra Cellular Domain of Human Epidermal Growth Factor Receptor 2.

Chapter 3

SELECTION AND CHARACTERIZATION OF APTAMERS SPECIFIC TO THE EXTRA-CELLULAR DOMAIN OF HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR 2

3.1 INTRODUCTION

The Epidermal Growth Factor Receptors (EGFR/ErbB) belongs to a family of receptor tyrosine kinases (RTK), and comprise of four members HER1/ ErbB1, ErbB2/HER2, ErbB3/HER3 and ErbB4/HER4. Epidermal growth factor receptors play a pivotal role in diseases including cancer, neurodegenerative diseases and cardio vascular diseases. Among the four EGFR family members (HER1-4), Human Epidermal Growth factor Receptor 2 (HER2) is the most well-known biomarker linked to breast cancer, gastric cancer (Gravalos and Jimeno 2008),ovarian cancer (Tuefferd et al. 2007; Wang et al. 2016) and other carcinomas including lung and oral cancer. HER2 overexpression has been increasingly recognized as a frequent molecular abnormality in cell signaling pathways. In case of various carcinomas, *HER2* gene amplification has been associated with uncontrolled cell proliferation, tumor metastases, reduced apoptosis and increased angiogenesis. The association between HER2 overexpression and a poor prognosis is supported by significantly shorter overall survival rate and time to relapse in patients with HER2-overexpressing breast or ovarian cancer relative to patients with tumors without HER2 overexpression. (Berchuck et al. 1990)

Akin to other members, HER2 has three domains, the extra-cellular domain (ECD), transmembrane domain (TM) and intra-cellular tyrosine kinase (TK) domain. The extra-cellular

domain has four sub-domains I-IV. Unlike other receptors in the HER family, sub-domain I on HER2 is constantly in contact with sub-domain III. Interaction between these two sub-domains keep the receptor in an always open conformation, exposing sub-domain II, and ensuring the receptor is always ready to dimerize with other human epidermal growth receptors. (Sliwkowski 2003) Sub-domain II is the dimerization domain which helps HER2 to bind with other receptors of the HER family to initiate the Her2 mediated pathway. While the exact role of sub-domain IV in HER2 functioning is still not fully elucidated, it is believed to stabilize and lock the receptor in an open conformation and is not directly involved with dimerization (Landgraf 2007). The intra-cellular domain has a cytoplasmic linker, a tyrosine kinase component, and a tail that phosphorylates and recruits adapter proteins following dimerization. These adapter proteins can then initiate cell proliferation signaling, leading to cell growth, differentiation, and angiogenesis. Thus, the extra-cellular domain could be the primarily target to assess HER2 positive carcinomas. HER2- targeted therapy with monoclonal antibodies against the ECD of HER2 (e.g., Trastuzumab) has achieved promising results against in clinical trials HER2-expressing tumors. (Vogel et al. 2003)

Following the identification of biomarkers, a sensitive and precise method is required for both the disease diagnosis and prognosis. Mostly monoclonal antibodies are used for bio-molecular recognition. However, antibodies suffer from limitations in numerous biological and biomedical applications such as they are sensitive to temperature, prone to irreversible denaturation, expensive and show batch to batch variation.(Xiang et al. 2015) Existing methods for detecting HER2 are based on biopsies followed by fluorescence *in situ* hybridization (FISH) at genetic level and immunohistochemistry (IHC) techniques for detecting HER2 at protein levels. In contrast to antibodies, aptamers may serve as an alternative therapeutic modality. As alternative ligands,

chemical antibodies "aptamers" can recognize a large range of molecules and possess unique properties for diagnostic and therapeutic applications. Aptamers are chemically synthesized single stranded DNA, RNA or modified nucleic acids. The specific candidates are selected from a large pool of nucleic acids through Systematic Evolution of Ligands by Exponential Enrichment (SELEX). This iterative process was first developed in the early 1990s by two laboratories (Tuerk and Gold 1990; Ellington 1996). To date, various aptamers have been screened against diverse targets ranging from circulating tumor cells (CTCs), acute lymphoblastic leukemia cells (CCRF-CEM cells) to various prognostic markers PTK7, c-myc7, EGFR, ER and HER family of receptors. Similar to monoclonal antibodies, aptamers can also bind to targets with high affinity and specificity. Previous studies have also reported high-affinity aptamers for Her2 protein detection with dissociation constants of picomolar to sub-nanomolar range. (Bora et al. 2013).

In this study we report specific deoxyribonucleic acid aptamers against the ECD of the HER2 receptor, which would be useful for detection of HER2 positive carcinomas. Combination of aptamers with nano theranostic modality will aid in non-invasive cancer diagnostics and therapeutics.

3.2 OUTLINE OF THE RESEARCH STUDY

i) Sub-cloning, over-expression and purification of recombinant extra cellular domain (ECD) of human epidermal growth factor receptor 2 (Her2) protein, which is a pivotal biomarker associated with breast cancer.

ii) Selection of specific DNA aptamer candidates for Her2-ECD by *in vitro* protein-SELEX method.

iii) *In vitro* binding characterization of screened aptamers by ITC.

iv) Evaluation of specificity and characterization of candidate aptamers against Her2 positive and negative cell lines.

v) Detection of Her2 expression pattern using specific candidate aptamers in breast cancer patient tissue samples.

3.3. EXPERIMENTAL SECTION

3.3.1 Cloning and purification of recombinant extracellular domain of Her2 protein

The nucleotide region encoding the extracellular domain (ECD) of Her2/ ErbB2 protein was amplified from pSV2-c-erbB2 plasmid construct (Plasmid# 16250). Plasmid costruct was obtained from Addgene, a plasmid repository. The region of the ECD in the Her2 gene was amplified by specifically designed primers fabricated with restriction enzyme sites Sal I-HindIII (for pET 28a vector , forward primer containing Sal I site 5'GC*GTCGAC*ATGGAGCTGGCGGCCTTGT3' and reverse primer containing Hind III site 5' CG*AAGCTT*TGACAGAGATGAAGGACGTC3'). The amplicon was first sub-cloned in the TA cloning vector (pTZ57 R/T, Fermentas) to obtain the properly digested sticky ends of the insert.

The amplified PCR product was extracted from the agarose gel using a gel extraction kit. (Sigma)Then 10µl of ligation mixture was made following the below mentioned protocol at a molar ratio of 3:1 or 6:1 of amplicons and pTZ57R/T vector.

Table3.1 Ligation reaction setup for TA cloning at 3:1 ratio of insert to vector

Components	volume
10x Rapid ligation buffer	5µl
pTZ57R/T vector	1µl (50ng)
PCR product (ECD region)	2.5µl (123ng)
T_4 DNA ligase	1µl
H_2O (Sigma)	0.5 µl
	10 µl

The ligation mix was incubated overnight at 16ºC. Ligated product was transformed into 100µl of *E. coli DH5* alpha competent cells. Then bacterial cells were plated on LB agar plate containing Ampicillin (100µg/ml), IPTG (100mM) and X-gal (50µg/ml), then incubated overnight at 37ºC.

After digestion with Hind III and Sal I, Her2-ECD fragment is obtained with sticky overhangs for further sub-cloning. Now, the pET28a (+) vector has HindIII and Sal I specific restriction sites in its MCS region. The digested Her2-ECD insert was ligated with double digested pET28a vector in a 10 µl total ligation mixture at a molar ratio of 3:1. The ligation mix was incubated overnight at 16ºC and later the ligated product was transformed into 100µl of *E. coli* BL21 competent cells. Transformed cells were plated on LB agar plates containing kanamycin (25µg/ml) and incubated at 37ºC overnight. The cloned construct was verified by Sanger sequencing. Expression of the HER2-ECD protein was performed in *E. coli* BL21 (DE3) by growing the cells in LB media supplemented with kanamycin (25µg/ml) at 37 °C until an OD at 600 nm reached 0.6. The expression conditions were optimized for various temperatures, shaking conditions and isopropyl ß-D-1-thiogalactopyranoside (IPTG) concentrations. The cells were harvested by centrifugation and resuspended in lysis buffer A containing 50 mM sodium phosphate buffer (pH 7.3), 300 mM NaCl, 10mg/ml lysozyme and 1 mM phenyl methane sulfonyl fluoride (PMSF). Protease inhibitor PMSF was added to prevent degradation of the induced protein. After sonication, the lysate was clarified by centrifugation at 10000 rpm for 30 min. The supernatant was filtered and loaded onto a 5 ml Hi Trap IMAC HP column (GE Healthcare). The column was pre-equilibrated with binding buffer B containing 50 mM sodium phosphate buffer (pH 7.3), 50mM imidazole and 300 mM NaCl. After washing the column with buffer B, the bound protein was eluted using a linear gradient of imidazole (up to 500 mM) in buffer C containing 50 mM sodium phosphate buffer (pH 7.3). All the eluted fractions were pooled and dialyzed to remove the imidazole. The dialyzed sample was concentrated

and its molecular weight was verified in 12% SDS-PAGE. After SDS PAGE, the protein was transferred to NC membrane and was analyzed by western blot technique with a monoclonal antibody specific to Her2 (ab16899).

3.3.2 Preparation of DNA aptamer library and in-vitro selection process (SELEX)

Prior to the beginning of the SELEX process, a 86-mer combinatorial DNA library consisting of 40 nucleotide long random region (ATACCAGTCTATTCAATT –N40-AGATAGTATGTGCAATCA) and forward primer F1 (5'-ATACCAGTCTATTCAATT-3') and reverse primer R1 (5'-TGATTGCACATACTATCT-3' conjugated with biotin at the 5'end) was obtained from Integrated DNA Technologies (IDT, USA). A typical combinatorial library of 1 µmol scale was synthesized where the pool is limited to 10^{14}–10^{15} number of sequences (Jayasena 1999). The concentration of the ssDNA library was determined by a Nanodrop UV spectrophotometer (Eppendorf, USA) and adjusted to 500 picomoles for the first round of selection.

The aptamer selection protocol against the ECD of HER2 followed in this study was adapted from earlier study with some modifications(Nonaka et al. 2010). For the initial SELEX round, the ssDNA pool (500 picomoles, corresponding to $1.8 \sim 3.0 \times 10^{14}$ ssDNA copies) was first heated at 95°C for 10 minutes and then cooled rapidly on ice for 10 minutes to allow intra-strand base pairing. Fifteen rounds of positive-SELEX targeting the ECD of HER2 protein was performed, while two rounds of negative (counter) SELEX were also done in between the SELEX process. Counter SELEX was performed by incubating the ssDNA library in aptamer binding buffer (20 mM Tris-Cl, pH 7.5, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$) against only the nitrocellulose membrane for 1 hour at room temperature. Initially, 1µg of purified HER2-ECD protein was immobilized on NC membranes(Rotherham et al. 2012). The unbound aptamer library obtained after counter SELEX, was incubated with protein immobilized on NC membranes for

1hour at room temperature. After incubation, membrane was washed three times with TBST (0.1% Tween-20 in Tris-buffered saline). The ssDNA aptamers bound to protein immobilized on NC membranes were eluted by heating at 95°C for 10 minutes. Then the pool was amplified by PCR using specific thermal conditions: one cycle of 95 °C for 6 min and 14 thermal repetitive cycles of 95°C for 30s, 45°C for 30s, and 72°C for 60 s and a final extension of 72 °C for 7 min with unlabeled F1 and biotinylated R1 primers. Following PCR, the double-stranded product was separated to ssDNA with streptavidin-magnetic beads (New England Biolabs) for the of SELEX process as described earlier. After each round of SELEX, the enriched aptamer library was confirmed by 2% gel electrophoresis and the concentration was checked using a Nanodrop UV spectrophotometer (Eppendorf, USA). Following the entire SELEX processes, the enriched aptamer candidates were amplified with unlabeled primers and cloned into pTZ57R/T cloning vector (InsTA Cloning kit, Life Technologies, USA). After transformation, positively transformed white colonies were selected. Plasmid DNA was isolated from the positive clones (Sigma Plamid Miniprep Kit) and verified with the F1, R1 primers. The selected clones were sequenced by the Sanger sequencing protocol (Scigenome Labs Pvt. Ltd, India.). Analysis and processing of the obtained raw sequencing data (electropherogram) was done using the BioEdit® tool.

3.3.3 *In silico* analysis of the candidate sequences

After obtaining the candidate sequences, *in silico* analysis was performed to find the most structurally favorable DNA aptamer that will bind to HER2-ECD protein. We carried out computational analyses such as free energy comparisons and structural stability of the selected candidates to determine the most potent ECD-aptamer. We had followed the Mfold analysis tool to predict most stable secondary structures of the aptamer sequences, which can be accessed at http://frontend.ioinfo.rpi.edu/applications/mfold/cgi-bin/dna-form1.cgi web link. (Zuker 2003)

3.3.4 Binding study by Isothermal Calorimetry (ITC)

The binding affinity and binding parameters (entropy, enthalpy) of the aptamers to the Her2-ECD protein were measured by ITC. All the calorimetry experiments were performed at 25°C using a MicroCal VP-ITC (Micro Cal Inc., Northampton, MA, USA). The protein and aptamers were prepared in phosphate buffered saline (pH 7.5). In a typical ITC experiment, the sample cell holder contained 300µL of 1.2µM recombinant purified Her2-ECD protein. The thermal equilibration step at 25◦C was followed by an initial 1100-sec delay step and, subsequently fifteen injections. Typically, 15 serial injections (1.5µL per one injection) of 15 µM aptamer candidate at a time spacing of 120 sec were set with continuous stirring of the reaction mixture (at 220 rpm) in the sample cell holder. Each injection generated a heat-burst curve (µcal/sec) versus time (min). Data were analyzed using MicroCal Origin software by fitting to a single-site binding model.(Brown et al. 2010)

3.3.5 Aptamer affinity study by flow cytometry

Unique 40-deoxyribonucleotide long sequences obtained after sequencing the SELEX pools were selected for further characterization. The FAM-labeled aptamers were incubated separately with 1x 10^4 SKBR-3 cells (HER2 overexpressing cell line) and MDA MB-231 cells (HER2 negative breast cancer cell line).

Before each treatment, the aptamers were subjected to a short denaturation-renaturation cycle. The assay was performed using 250nM of FAM labeled aptamer and control scrambled candidates with 10^4 SK-BR3 cells in a total volume of 1 ml. The DNA aptamer candidate was first denatured by heating at 90°C and renatured by flash cooling on ice prior to incubation with monolayer of SK-BR3 cells grown overnight at 37°C. The incubation was done for 60 minutes at RT with gentle

rotation. The aptamer-exposed cells were then pelleted by centrifugation (2500x g for 10 min) and washed three times prior to being resuspended in 200 µl of PBS. Flow cytometry was performed to measure the attachment of FAM labeled ECD_Apt1 using FACS Calibur™ flow cytometer (BD Biosciences, USA). The data (cell count = 10,000) were analyzed using the BD Cell Quest™ Pro software (BD Biosciences).

3.3.6 Predicting binding affinity of aptamer by Chemiluminescence

Affinity and specificity measurements of the enriched sequences were carried out using an enzyme-linked aptamer assay. For affinity determination, 1.5 µg of recombinant ECD protein was coated to the activated wells of a 96-well microtiter plate by 2hrs of incubation at 37 °C. After the unbound surface had been blocked with 2% BSA, the wells were titrated with 500 nM labeled sequences and incubated for 2 h at room temperature. To determine the affinity of the aptamer-ECD complex, an enzyme-linked immunosorbent assay for ligand–receptor interactions was performed as reported earlier (Xu et al. 2014).The aptamer was biotinylated at the 5' end. Next, the purified recombinant HER2-ECD protein was coated on 96-well microtitre plates (2 µg/well), and incubated with increasing concentrations (0-1000nM) of the biotinylated candidate. All points were performed in triplicate. The assay was performed using an anti-HER2 monoclonal antibody as positive control. Non-specific binding of aptamer was confirmed by performing an experiment wherein BSA was used as a target protein. Incubations were performed at 37°C for 1 hour. The unbound aptamer was removed by washing the well four times with PBST (0.1% Tween-20) solution, while specific binding was quantified with streptavidin–HRP conjugate (1:1000 dilution from 1mg/ml). The intensity of developed chemiluminescence was recorded at 490 nm (Tecan Microplate Reader) after addition of 100 µl chemiluminescent peroxidase substrate (CPS 350, SIGMA).

3.3.7 MTT cytotoxicity Assay

HER2 positive human breast cancer cells(SKBR3) , HER2 negative MDA MB231 cells, MCF7 cells and the fibroblast cell line L929 (as negative control) were cultured in a CO_2 incubator (5% CO_2) and maintained at 37°C and 80-85% relative humidity. The cell viability of the selected aptamer was determined by a MTT (3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyl tetrazolium bromide) dye conversion assay. (Nicol et al. 2013) Cells were seeded at 10^4 per well (counted using a Haemocytometer after viability staining with trypan blue) in a 96-well cell culture plate (Corning ®) and are maintained with cell culture medium (SKBR3 cell line was maintained in McCoy5a medium and DMEM: Dulbecco's Modified Eagle Medium for other cell lines). Cells were treated with an increasing concentration of aptamers (0 to 200nM) for 24 hrs and 48 hrs. Cells were then incubated with 20 µl of 5mg/ml of MTT dissolved in PBS (pH 7.4) for 4 hours at 37°C in a CO_2 incubator. Purple colored formazan crystals were dissolved by adding 100 µl of dimethylsulfoxide (DMSO) and incubated for 30 minutes under dark condition. The absorbance was measured at 570 nm using a microplate reader (Tecan) with background subtraction at 630 nm.

3.3.8 Immuno-staining of Her2 positive breast cancer cells by selected aptamer

An immunofluorescence (IF) assay of the selected candidate was performed against HER2 overexpressing SKBR3 cell line and HER2 negative cell lines (MDA MB 231, MCF7). Sub-confluent cultures of SKBR3 and MDA MB 231, grown on Poly-L-Lysine (PLL) coated coverslip were fixed using 100% chilled methanol. The cells were incubated with nuclear permeabilizing buffer (0.25% Triton X-100, 0.2 µg/ml EDTA and 1% BSA in PBS) for 30 minutes. After blocking (10% BSA, 0.3% Triton X-100 in 1X PBS), FAM labeled aptamer candidates were added staining with the HER2 specific mouse monoclonal antibody (ab16899, Abcam, USA) served as a positive

control. Polyclonal FITC conjugated secondary antibody (ab6785, Abcam) was used against the mouse monoclonal antibody.

An immunocytochemistry (ICC) assay was performed on formalin-fixed monolayer cultures of SKBR3 and MDA MB 231 breast tumor cells. These two cell lines were used as HER2 positive and negative cells, respectively. Sub-confluent cultures of SKBR3 and MDA MB 231were grown on sterile poly-L-lysine coated coverslips then fixed using 4% para-formaldehyde. The nonspecific sites and endogenous peroxidase activity within cells were blocked with 0.03% H_2O_2 (diluted in PBS, pH 7.2). ICC profiling was performed with 100 nM conc. of biotinylated ECD_Apt1 and also with monoclonal HER2-antibody (ab9269, Abcam, USA) as positive control. HRP conjugated secondary antibody (ab 6789, Abcam, USA) was used to detect the HER2-ECD specific monoclonal antibody.

3.3.9 Assessment of HER2 expression in breast tissue samples using specific aptamer

Four-mm-thick sections of paraffin-embedded tissue blocks were cut and mounted on PLL coated slides for immunostaining of the HER2 receptor on breast cancer tissues using the ECD_Apt1. The tissue sections were dewaxed in xylene and rehydrated through a descendant gradient of ethanol (100 %, 90%, 70% and 50%, respectively). After rehydration, antigen retrieval treatment was performed in Tris-EDTA buffer (pH 9.0) by microwave irradiation for 3 cycles (900 W, 720 W and 540 W) each of 5 minutes. Endogenous peroxidase activity was blocked by using a 0.03% hydrogen peroxide solution (dissolved in PBS) at room temperature for 30 minutes. Next, the biotinylated aptamer candidate (1µM) was added on tissue sections and incubated at 4°C overnight in aptamer binding buffer. After a thorough washing in 0.1M PBS solution, antigenic epitopes of the tissue sections were visualized using a streptavidin–HRP conjugate (1:500) (S911, Invitrogen)

and DAB plus substrate (Invitrogen). Finally, sections were counterstained with haematoxylin and mounted with DPX (Sigma).

3.4 RESULTS

3.4.1 Molecular cloning, expression and purification of recombinant Her2-ECD protein in *E. coli BL21*

3.4.1.1 Screening and selection of positive clone from TA cloning

Transformed colonies were picked on the basis of blue-white colony selection, and grown on 5ml LB broth containing Ampicillin at 37ºC 180rpm for overnight. Cultures were pelleted and plasmid was isolated. 10µl of the DNA was subjected to sequential restriction digestion by *Sal I* and *Hind III*, respectively. Digested product run on a 0.8% agarose gel showed a corresponding band of pTZ57R/T vector 2886bp and the *ECD region (~1.8 kb).*The digested DNA was gel extracted for further cloning into the pET28a(+) vector. Digested product run on a 0.8% agarose gel showed a corresponding band of pTZ57R/T vector 2886bp and Hind III-Sal I digested ECD region (~1.8 kb).(Fig3.1)

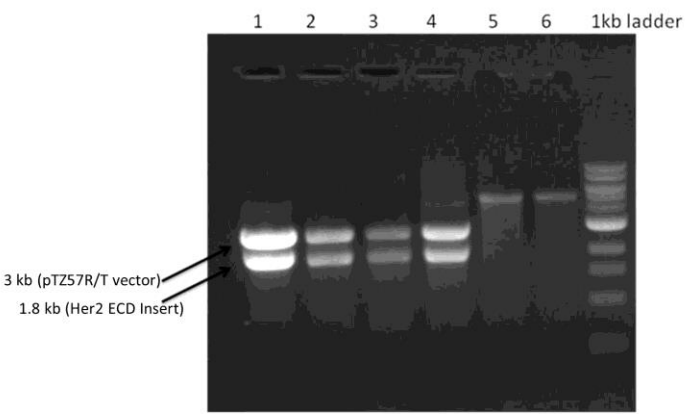

Fig 3.1. Confirmation of cloning into pTZ57R/T after restriction digestion by Sal I and Hind III, 0.8% agarose gel showing restriction digestion of pTZ57-ECD, where pTZ57R/T and ECD are around 3kb and 1.8kb, respectively.

3.4.1.2 Screening and selection of positive clones from pET28a

The sequence encoding the extracellular domain of *HER2* (1.8Kb) was sub-cloned in the pET28a vector, which harbors an N-terminal His-tag, with specifically designed primers from the pSV2-c-erbB2 construct. The double-digested amplicon was ligated with Sal I and Hind III double-digested pET28a vector and transformed in *E. coli DH5* alpha cells. After an overnight incubation, we obtained kanamycin resistant transformed colonies. Positive colonies were screened containing the desired insert by PCR amplification with gene-specific primers. After digestion with Sal I and Hind III restriction enzymes, successful cloning was confirmed using 0.8% agarose gel electrophoresis, where vector backbone and insert ECD domain shows two distinct bands of 5.3kb and 1.8kb, respectively (Fig 3.2).

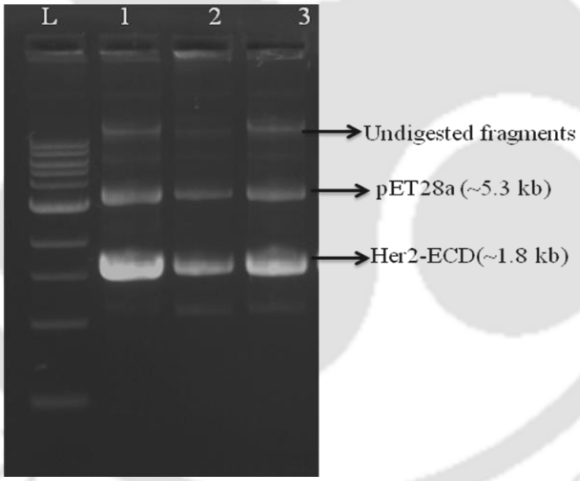

Fig 3.2 Confirmation of cloning into pET28a after restriction digestion by Sal I , Hind III, 0.8% agarose gel showing restriction digestion of positive clones, where pET28a and ECD are approx.. 5.3kb and 1.8kb, respectively. Lane 1-3: After digestion of Her2-ECD-pET28a constructs with Sal I –Hind III showing insert Her2-ECD (~1.8 kb).

3.4.1.3 Expression and purification of Her2-ECD protein in pET28a vector

Positive clones were transformed into *E. coli* BL21 expression cells and induction conditions were optimized to attain highest expression. The maximum yield in the soluble fraction was achieved when the overexpression was performed at 16 °C for 12 to 16 hours at 140 rpm with the induction of 0.1mM IPTG. The recombinant protein containing the His_6-tags was purified by immobilized metal ion affinity chromatography. The maximum yield of the HER2-ECD fraction was obtained when eluted with 300 mM concentration of imidazole. The expression and purification of the extra cellular domain of the HER2 protein was analyzed by SDS-PAGE as shown in Fig.3.3 confirming its molecular size of approximately 73 kDa. The purity of the protein was also confirmed by western blot analysis using a monoclonal antibody specific to Her2 (ab16899, Abcam, USA).

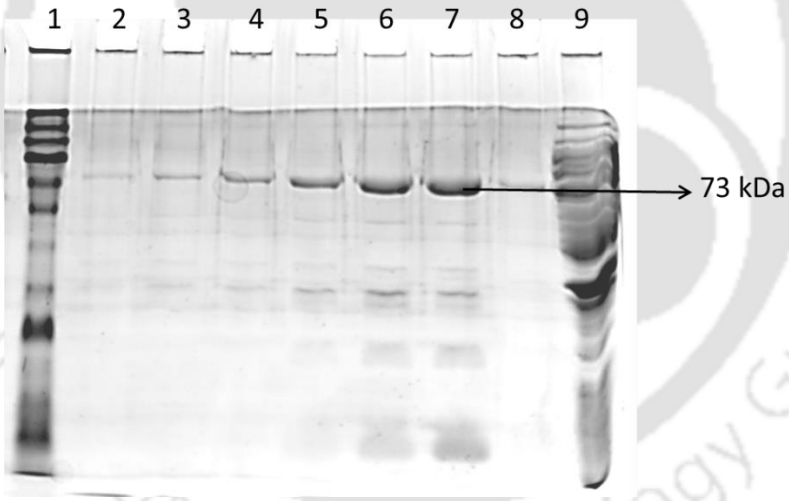

Fig 3.3 SDS PAGE of purified Her2-ECD fractions. Lane 1: NEB Broad range protein ladder Lane 2-8: E2-E8 purified fractions of his tag Her2 ECD proteins after eluted with 300 mM imidazole. Lane 9- whole cell fraction.

3.4.2 Selection of specific DNA aptamer candidate sequences for Her2-ECD by *in-vitro* SELEX process

An aptamer library containing 40 random oligonucleotides was considered as a starting pool for our protein-SELEX process to screen for Her2-ECD specific aptamer candidate sequences. In the first round of SELEX screening the library amount was kept to 500 picomoles to ensure a sufficient number of available sequences during following selections. Blocking the protein-bound membrane surfaces prior to oligonucleotide binding is a common practice to avoid nonspecific enrichment. However, such practices also result in enrichment of "blocking agent-binding sequences". To overcome this, we did not perform the BSA blocking and have introduced a negative selection step wherein the starting aptamer pool is first incubated with only NC membrane (without immobilized ECD protein) and the membrane bound sequences were removed from the initial pool library. Furthermore, a selection pressure was applied to SELEX enrichment by lowering the molar ratio of protein to DNA from 1:1 to 1:10 following the fifth round and to 1:20 after the tenth round of the process. The amount of recombinant pure protein was reduced to 0.55 µg (cycles 5-10) and to 0.20 µg (cycles 10-15) in a binding volume of 500 µl. After amplification and cloning the entire enriched pool of the last SELEX round in pTZ57R/T vector, the positive transformed clones were sequenced by Sanger's capillary method. Analysis of the sequencing data by BioEdit® tool showed the enrichment of 4 different sequences, which will bind specifically to HER2-ECD protein. After *in silico* analyses, we selected aptamers with the most thermodynamically stable predicted secondary structure. As calculated in Table 3.2, ECD_Apt1 showed lowest free Gibbs energy (ΔG) of -3.24, having better structural folding stability among other screened DNA aptamer sequences.

Table 3.2 Thermal Profile of Enriched Sequences Obtained through Sanger's sequencing

Seq. ID	Sequence	*Free Gibbs energy (ΔG) (kcal/mol, 37 °C)*	*Enthalpy change(ΔH) (kcal/mol)*	*Entropy change (ΔS) (cal/K.mol)*
ECD_Apt1	**CCGCAACCACGACCGAAAGAC AACGCAATCTGACACGTGG**	**-3.24**	**-67.90**	**-208.4**
ECD_Apt2	**GGAGACTCAATTAATTTCAGTG CCGCAACGATGATAGAT**	**-0.13**	**-33.60**	**-107.9**
ECD_Apt3	**ACGGCCGGTTTACTCGTGAGG GCTTTCTCTGTGGGTCTTG**	**-1.46**	**-29.80**	**-91.3**
ECD_Apt4	**ACATCGCAATTGGGACTTGAA TCACCTACGCATGCTTG**	**-0.09**	**-26.40**	**-84.8**

Further *in-silico* secondary structure analyses was performed by Mfold. Various secondary structures were obtained for a single aptamer candidate depending on their free energy. The most stable structure of the four candidate sequences was considered having minimum free energy. (Fig. 3.4)

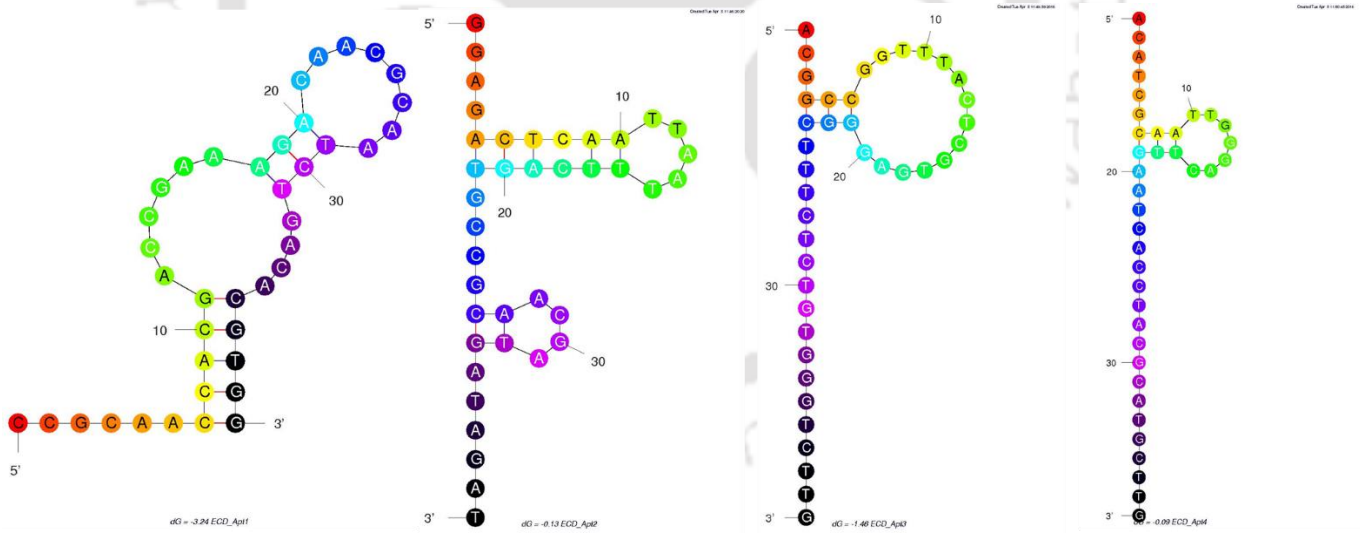

Fig 3.4 Secondary structure predictions of the candidate aptamers by Mfold

3.4.3 Thermodynamic analyses of candidate aptamer sequence

To further investigate the *in silico* predictions and determine the sequence with best binding efficiency, isothermal calorimetry (ITC) was carried out between titration of the pure protein and

enriched candidate sequences**.** The saturation curve for molar ratio vs. heat exchanged during the specific binding process of the ECD_Apt1 is presented in fig 3.5 and the resulting associated temperature (kcal per mole of injectant) was also plotted against the molar ratio of the reactants. The area under each peak was integrated to determine the binding affinity and thermodynamic parameters enthalpy and stoichiometry of the candidate sequence, a one-site independent binding model was considered (Origin 5.0 software Microcal, Inc.). As shown in Fig.3.5, ECD_Apt1 showed an affinity constant (Ka) between selected candidate ECD_Apt1 and ECD protein is1.58 ± 1.16x10^{8} M^{-1} .Therefore, this binding mechanism reveals a highly favorable enthalpy of reaction (ΔH) at 25 °C of –3.346±0.135 kcal mol-1 and favorable entropy of reaction (TΔS) 4.668 kcal $mol^{-1}deg^{-1}$ as shown in Figure 3.5. These thermodynamic and conformational data indicates the local conformation change of the aptamer that occurs during ligand binding. As calculated by the isotherm model, we found the dissociation constant (K_d) to be 6.33 ± 0.86 nM, which was further conferred by following aptamer specific binding assays.

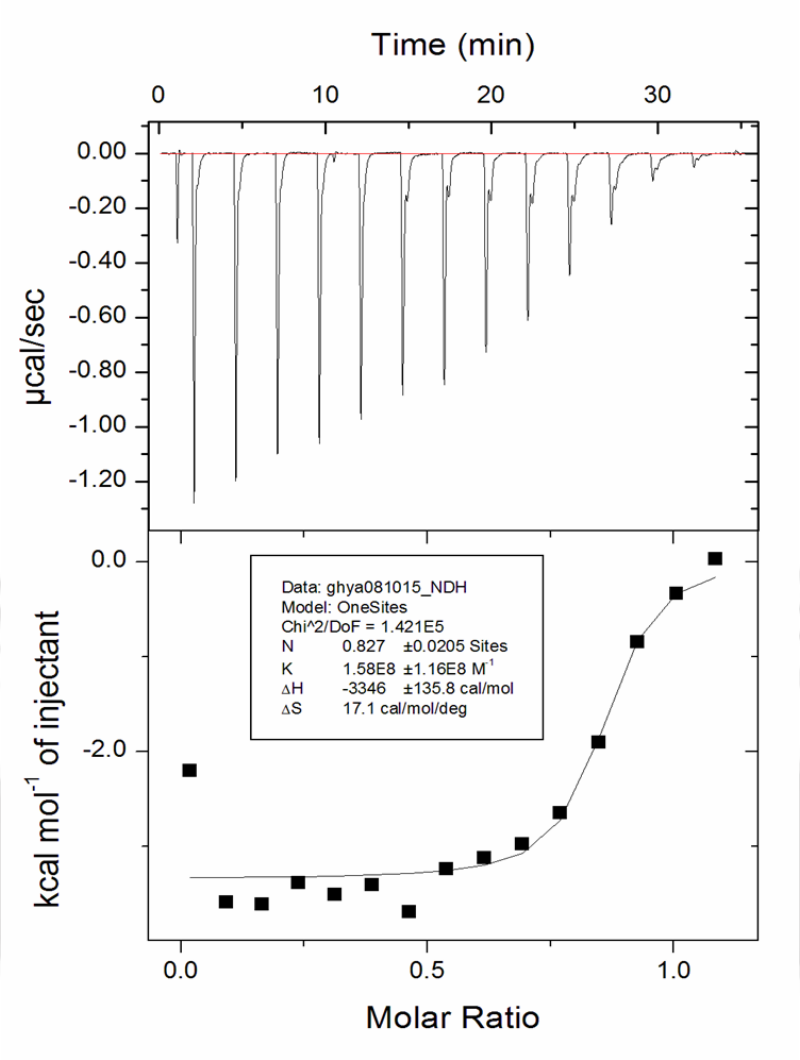

Fig.3.5 Isothermal titration calorimetry (ITC) graph of ECD_Apt1 when titrated with 300µL of 1.2µM recombinant purified Her2-ECD protein: Isotherms were fitted to a one-to-one binding model. The thermodynamic parameters (K, ΔS, ΔH) are shown in the diagram.

3.4.4 Nanomolar Affinity of the selected aptamers:

The detection range of the aptamer-based protein detection assay was quantitated by plotting a curve for ECD_Apt1 in the working range of 0−1000 ng/mL. ECD of HER2 linked aptamer assay was performed in a sandwich format. The assay showed a linear rise in chemiluminescence (at

490nm wavelength) in the dynamic range of 100−700 ng/mL ECD_Apt1 with a limit of detection of 12.5±2.5 ng/mL. (Fig.3.6)

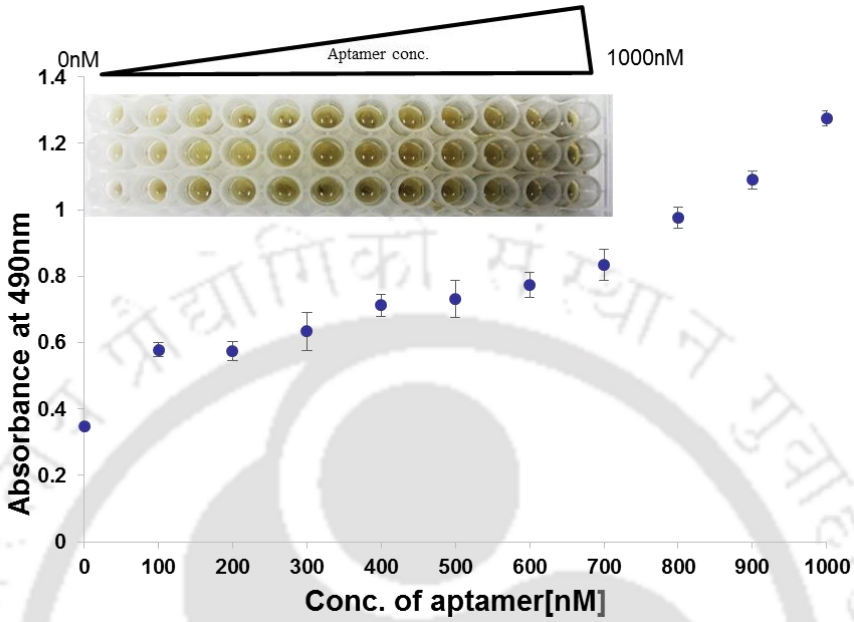

Fig.3.6 Binding of enriched aptamer ECD_Apt1 (0-1000 nM) with immobilized ECD protein (2µg) was measured by colorimetric saturation binding assays. Results show the linear rise in the dynamic range of 100−700 ng/mL of ECD_Apt1 with a limit of detection of 12.5 ±2.5 ng/mL.

3.4.5 Flow cytometry analysis of ECD specific aptamers with breast cancer cell lines

6-FAM labeled ECD_Apt1 was co-incubated with a 24-hour grown HER2 overexpressing human breast adenocarcinoma SKBR3 cell line and the fluorescence intensity was analyzed by flow cytometry. The histogram profile (Fig.3.7) showed the significant shift of fluorescence signal obtained from FL1 channel. However, the HER2 negative cell line (MCF7) resulted in negligible fluorescence signal. No significant shift was visible when both the cells were treated with random oligonucleotides. The results indicated that ECD_Apt1 specifically binds with the HER2 positive breast cancer cell line.

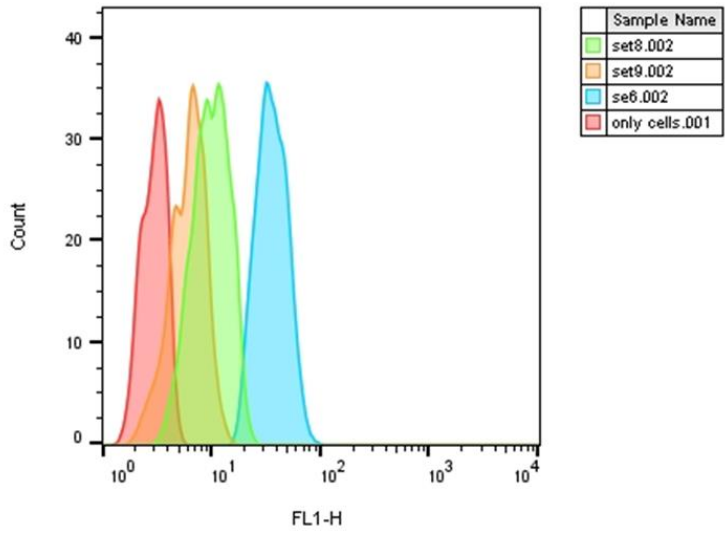

Fig. 3.7 Histogram Overlay Plots of aptamers treatment to SKBR3: Red spectra represents cells without any treatment, the yellow spectra represents cells treated with scrambled aptamer and green and blue spectra, respectively cells treated with 50 and 100 nM ECD_Apt1.

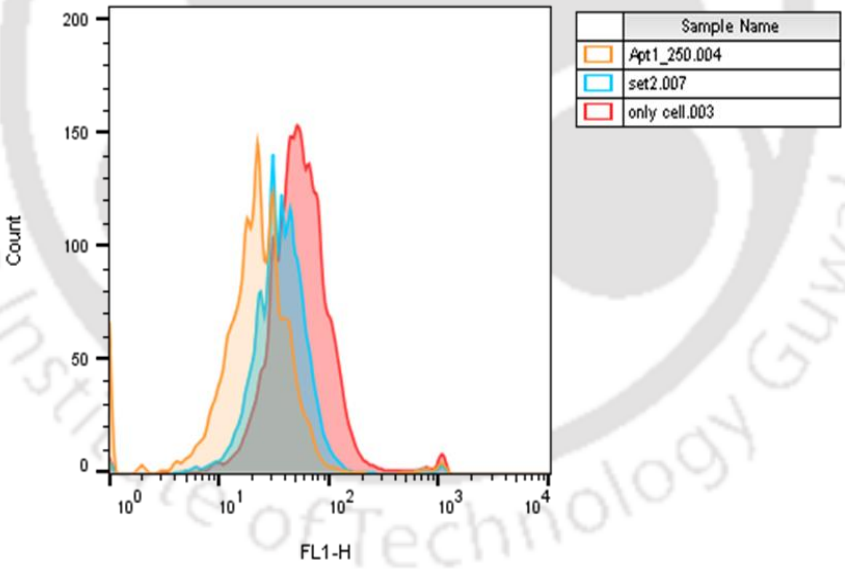

Fig. 3.8 Histogram Overlay Plots of aptamers treatment with MCF7 : Red spectra represents cells without any treatment, the yellow spectra represents cells treated with scrambled aptamer , and blue spectra represents treatment with ECD_Apt1.

3.4.6 Immunocytochemistry against breast carcinoma cell lines

A chromogenic immunocytochemistry (ICC) assay was performed on formalin-fixed 24-hour grown monolayer cultures of SKBR3, MCF7, MDA MB 231 breast tumor cells and a L929 normal cell line. The SKBR3 cell line was used as HER2 positive and MDA MB 231, MCF7 as HER2 negative cells. The cytoplasm of SKBR3 was found to stain dark brown with DAB chromogen in aptamer based ICC assays (Fig. 3.9) The HER2 expression was also studied with a monoclonal HER2 antibody (ab9269) as a positive control. No cytoplasmic staining was seen in MDA MB231 and L929 with either of the affinity molecules.

In another cell based immunofluorescence assay, positive control, HRP-conjugated polyclonal secondary antibody (ab6728) was used to label the primary HER2 antibody. Overall these observations suggest that the aptamer could be as useful in cell imaging purposes.

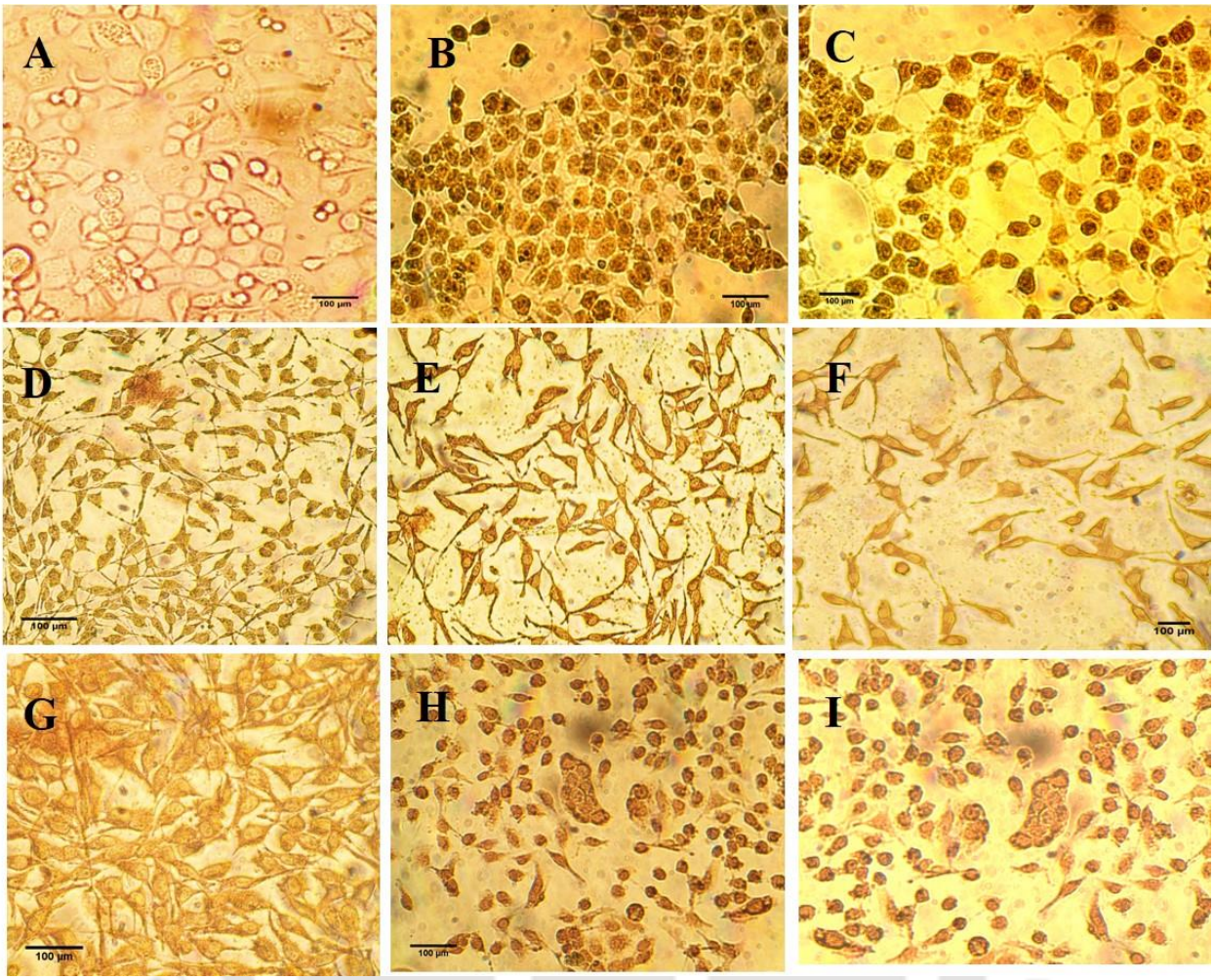

Fig. 3.9 Immunocytochemistry study of ECD_Apt1 aptamer when treated with a panel of control, Her2 positive and negative cell lines.
SK-BR3 (A), L929 (D), MCF7 (G) cells treated with scramble aptamer sequence.
SK-BR3 (B), L929 (E), MCF7 (H) cells treated with biotinylated ECD_Apt1.
SK-BR3 (C), L929 (F), MCF7 (I) cells treated with mAb specific to the ECD of Her2.

3.4.7 Effect of aptamers on cell viability

SKBR3 cells (10^4/well) were cultured in DMEM supplemented with different concentrations of aptamers. After 24 hours, MTT reagent was added to final concentration of 0.5 mg/mL and the absorbance of reduced MTT measured at 570 nm. Percent survival of aptamer was calculated by taking untreated cells as 100%. Cell viability was expressed as a percentage of the control by the

following equation: cell viability (%) = (Nc-Nt) / Nc x 100. where Nt and Nc are number of treated cells and control cells, respectively. (Forrest et al. 2013) After 48 hours, ECD_Apt1 candidate showed no cytotoxicity to malignant cells (SKBR3, MCF7) or even control normal cell line (L929) (Fig 3.10)

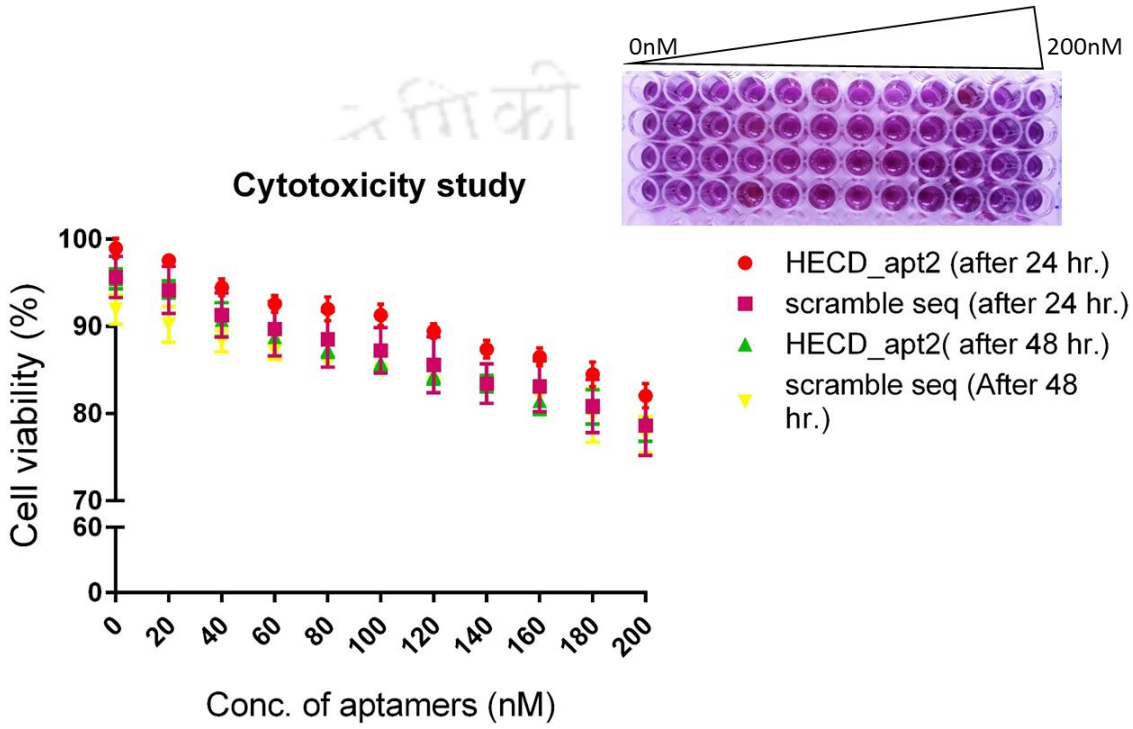

Fig.3.10 MTT cytotoxicity study of the aptamers. Upto 150nM the candidates showed no significant cytotoxicity, but higher than 150 nM conc. it showed toxicity when treated with 48 hrs.

3.4.8 Immunohistochemistry against HER2 overexpressing Human breast cancer tissue samples

The diagnostic potential of ECD_Apt1 was evaluated in tissue histochemistry-based profiling studies for Her2 expression status in human breast cancer specimens. IHC staining for HER2 was performed on invasive ductal carcinoma cells. A positive control was used with a commercial anti-

HER2 antibody. The biotinylated ECD_Apt1 was shown to bind specifically in the cytoplasm of FPPE human breast tissue samples. (Fig 3.11)

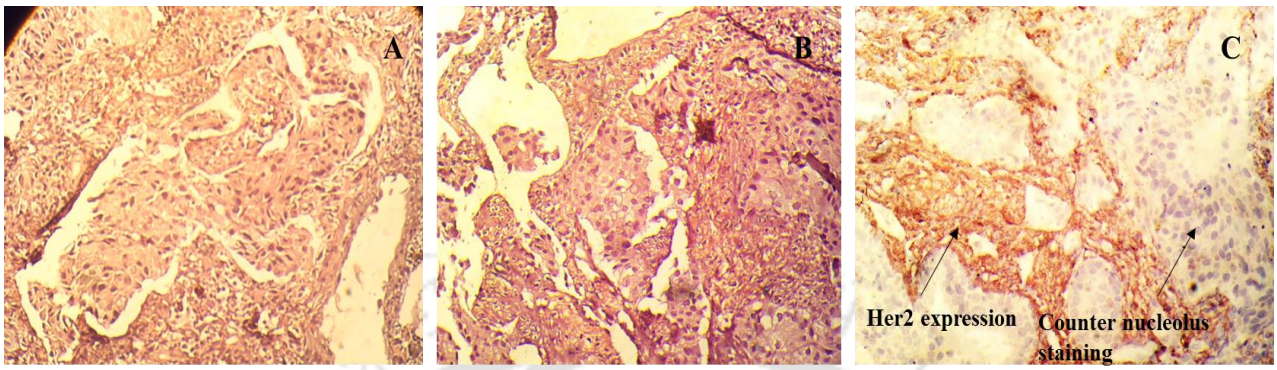

Fig. 3.11 Apta-Immunohistochemistry with Biotinylated ECD_Apt1.

A- Her2 positive breast cancer tissue specimens stained with biotinylated scramble aptamer sequence.

B- . Her2 positive breast cancer tissue specimens stained with biotinylated ECD_Apt1

C- Her2 positive breast cancer tissue specimens stained with commercially available specific monoclonal antibody**.**

3.5 DISCUSSION

The goal of this work was to develop an aptamer based HER2 detection method. For this, we carried out SELEX process with the ECD of HER2 as a bait followed by assessment of screened aptamers. Selected DNA aptamer sequences were found to bind to target Her2 at nanomolar sensitivity. The testing of the HER2 specific aptamers to profile the status of Her2 expression in breast tumors showed them as promising probes for breast cancer diagnostics. The DNA aptamer candidate ECD_Apt1 showed specific cytoplasmic staining due to Her2 receptor expression in malignant duct cancer tissues with no cross-reactivity to HER-deficient fibroblasts, inflammatory cells or adipocytes.

To accomplish the objectives of this study, we amplified the extracellular domain of Her2 from pSV2-cerbB2 construct (Addgene), with gene specific primers containing Sal I and Hind III restriction enzyme sites. Initially codon degeneracy was performed to select the clone sequence that would code for humanized recombinant extra cellular domain of HER2 in bacterial system. The sequence was extracted from NCBI entry NM_004448 (*Homo sapiens* ErbB2 receptor tyrosine kinase 2, transcript variant 1). We selected kanamycin resistant transformed colonies and further positive clones were screened by PCR amplification. Cloning was confirmed after double digestion of plasmid DNA with Sal I and Hind III. Two distinct bands of 5.3kb and 1.8kb were clearly visible and represent the pET28a backbone and Her2-ECD insert, respectively. (Fig 3.2) The directional cloning was confirmed by Sanger dideoxy sequencing.

Overexpression of Her2-ECD protein in an *E. coli* bacterial system and its subsequent purification was analyzed by 12% SDS-PAGE as shown in Fig. 3.3. The purity of the protein (almost 95%) was also confirmed by western blot analysis using a monoclonal antibody specific to human epidermal growth receptor 2 (ab16899, Abcam, USA).

To obtain an ECD-specific DNA aptamer, we started with an initial pool of an aptamer library of 76 nucleotide length. Nitrocellulose membrane was used as an immobilization platform where protein was immobilized by covalent interaction. Blocking the protein-bound surfaces prior to the selection process of aptamers is a common practice to counteract nonspecific enrichment. However, such experiments lead to enrichment of “blocking agent-binding sequences” and thus loss of probable aptamer candidates that which might bind with target in subsequent steps. In this study, we excluded the blocking step of NC membrane with BSA protein. Instead, we introduced a counter selection step wherein the starting DNA library is incubated only with NC membrane (without immobilized Her2-ECD protein) to remove the membrane bound sequences from the initial pool.

Notably, we gradually decreased the concentration of protein to be immobilized to increase the stringency of selection process. As the round of selection process progressed, the diversity of the aptamer pool also decreased. After 15 rounds of iteration, the enriched selected pool of the last SELEX round was cloned and sequenced using Sangers' capillary method. Analysis of the sequencing data showed the enrichment of 4 different sequences that binds specifically to Her2-ECD. The protein-SELEX method is primarily focused on repeated iterative incubations of a random DNA library with the target protein, followed by repeated amplifications of the target-bound nucleic acids by PCR. Following the iteration cycles, screened candidates can be enriched with higher specificity and sensitivity against the target.

Further *in silico* analyses such as free energy comparisons and structural stability was performed to assess the aptamers with the best binding affinity to the Her2-ECD. We selected an aptamer candidate with the most thermodynamically stable structure predicted after *in silico* analyses. As calculated in Table 3.2, ECD_Apt1 showed a minimum free Gibbs energy (ΔG) of -3.24, having highest structural stability. This sequence can also form unique secondary structure in a stipulated ionic environment. On the basis of structural analysis, we carried out further binding analysis with the ECD_Apt1 having minimum ΔG and the most stable structure among other candidates. ITC can elaborate on the binding stoichiometry of the aptamer-protein with high precision due to computer-controlled stepping motor driven injections. Due to the the fact that the reactants are analyzed in their native forms, the irreproducible ambiguous result inherent to chemical modification or immobilization procedure is avoided. The estimation of binding stoichiometry is important in the characterization of binding mechanisms of the aptamer and its target HER2-ECD.

ITC experiments were carried out to identify the aptamers binding strongly to Her2-ECD. For binding thermodynamics of a reaction (like ΔG, ΔH, ΔS) it is important to understand the

molecular mechanism of interaction between target receptor and binding aptamer molecules. Chemical and thermodynamic parameters of aptamer binding have been studied by isothermal titration calorimetry (ITC) analyses, suggesting that the anti-HER2-ECD aptamer undergoes the enthalpy-driven conformational change of its 3D structure. (Pagano et al. 2009). Binding thermodynamics reveal the driving molecular forces such as entropic reaction of uptake/release of water and ion molecules, the limitation of degree of freedom of polypeptide main chain and side groups, enthalpy change during bond formation, and the burial of water-accessible surface area. Typical ITC data analysis involves fitting the data to single-site or two-site binding model, which assumes that macromolecules are not undergoing ligand-induced changes in self-association. (Perozzo et al. 2004) Here we assume a one-to-one site binding model where structure of the aptamer was unaltered upon binding of its target protein. The measurement of ΔH is useful for ITC experiments, which measures the heat changes due to buffer ionization.

The specific candidate ECD_Apt1 was labeled with 6-FAM at the 5' end for fluorescence studies with the ECD structure. The FAM labeled candidate was co-incubated with a Her2 positive human breast adenocarcinoma SKBR3 cell line and the fluorescence intensity was analyzed by flow cytometry. The histogram profile (Fig.3.7) showed a significant shift of fluorescence signal obtained from the FL1 channel. However the HER2 negative cell line MCF7 and control normal cell line (L929) showed no significant shift after treatment with the fluorescence labeled candidates. This indicated that ECD_Apt1 specifically and precisely binds with HER2 positive cell lines, but not others. (Moosavian et al. 1990)The signals obtained from side-scattered light (SSC) is proportional to the cell granularity or internal complexity of the cells after treatment with the reporter molecule. The granularity of SKBR3 cells (pattern obtained at SSC channel) was unaltered after incubation with different concentrations (0-200nM) of aptamers, which reveals that selected

aptamer candidates do not cause any form of perturbation to the malignant cell line or to a control normal cell line. Fluorescence imaging analysis also showed that ECD_Apt1 stained the cytoplasm of SKBR3 cells. FAM labeled random ssDNA was used as negative control for all fluorescence study.

To estimate the binding at cellular and tissue level, ECD_Apt1 was labeled with biotin at the 5'end to of the molecule. Chromogenic immunocytochemistry (ICC) assay was performed on monolayer cultures of breast tumor cells (SKBR3, MCF7, MDA MB 231) and normal cells (L929). SKBR3 cells considered as Her2 positive and MDA MB 231, MCF7 as Her2 negative cells. Due to enhanced cell penetration, DNA aptamers can enter into the cell and attach to Her2. The cytoplasm of SKBR3 cells (HER2 positive cell line) stained dark brown by developing with DAB chromogen (Fig.3.9). In both studies, a two-step method was mandatory for antibody reaction although with the labeled ECD_Apt1, only a single step was required to detect HER2. Further, this study also showed that labelling of ECD_Apt1 with biotin (chemical) or FAM (fluorescence) did not alter its specificity toward its target.

Recent studies have identified several RNA and DNA HER2 aptamers. In one study a RNA aptamer was selected against HER2 protein and proposed that it could potentially be utilized in constructing novel imaging agents for HER2-positive tumors(Kang et al. 2009; Kim and Jeong 2011). *In vitro* SELEX was performed using a library of DNA aptamers against HER2-positive cells as target. The HY5 aptamer was found to bind strongly, but it failed to attain specificity in HER2 positive tissue sections. (Dastjerdi et al. 2011) Another DNA aptamer had been found that could selectively deliver doxorubicin to HER2-positive cells *in vitro*.(Liu et al. 2012) However, in this present study, ECD_Apt1 screened against the extra cellular domain of HER2, showed greater potential as an alternative nucleotide binder. HER2-affinity of ECD_Apt1 is weaker than the monoclonal antibody

Trastuzumab due to the potential structural complexity of the antibody. Antigen-binding sites of antibodies are composed of the highly variable regions of the heavy and the light chains of immunoglobulin, and presumably may form numerous intricate structures that can establish strong binding with the HER2 protein. It is possible the longer DNA sequences of ECD_Apt1 would allow for the generation of more complicated 3-D structures, which may contribute to tighter binding with the target HER2. Our protein-linked aptamer assay was performed in a sandwich format, and the assay showed a linear rise in color absorbance in the dynamic range of 100-750 ng/mL of HER2-ECD protein with a limit of detection of 15.5±2.5 ng/mL. From the MTT study, the aptamers did not show any noticeable cytotoxicity up to 150nM concentration against the treatment with the breast carcinoma cell line. The aptamer candidates (both the ECD_apt1 and scrambled sequence) also showed no toxicity to the cultured control normal cell line L929.

In paraffin embedded tissue sections, biotinylated ECD_Apt1 showed specific and Her2-selective localization in the nucleus of malignant duct cancer cells. Cross reactivity to fibroblasts, inflammatory cells and adipocytes or non-specific binding to cytosolic or extracellular components was minimal. Due to smaller size of the DNA aptamer (6-30kDa) compared to the specific monoclonal antibody (125-150kDa), aptamers can penetrate tissue more readily.(Ku et al. 2015) In addition, aptamers can fold into unique three dimensional conformations spontaneously which make them suitable for resistance to pH, temperature and other surrounding changes during immunohistochemistry. ECD_Apt1 showed potential like the HER2 specific monoclonal antibody as a diagnostic agent for detection of HER2 positive malignancies.

3.6 CONCLUSION

In conclusion, we demonstrated that the specific aptamer candidates selected after iterative rounds of selection process showed high affinity towards the extracellular domain of HER2 during *in vitro* DNA-protein binding studies. The aptamer ECD_Apt1 also exhibited HER2 marker status in paraffin-embedded breast cancer tissue samples, which was comparable with established immunohistochemistry with a HER2 specific monoclonal antibody. These aptamers can be exploited as molecular probes in the detection of Her2 overexpressing cell lines and HER2 positive cancer tissue specimens. Therefore, they can be exploited as a promising diagnostic module for non-invasive cancer diagnosis and can provide a novel cost effective alternative to conventional antibody in solid phase immunoassays. Since HER2 overexpression is abundant in most carcinomas the HER2 specific aptamers are promising as a guiding ligand for targeted chemotherapy against a plethora of multiple malignancies. The DNA aptamer against HER2 could be exploited to fabricate an aptamer based sensor, which is amenable for monitoring cancer patient compliance and facilitating the evaluation of therapeutic agents in clinical trials. Nevertheless, extensive research with animal models is still required to evaluate the *in vivo* binding specificity of the aptamers.

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Chapter 4

Aptamer Based Electrochemical Sensor System for Detection of Breast Cancer Protein Biomarkers

Chapter 4

APTAMER BASED ELECTROCHEMICAL SENSOR SYSTEM FOR DETECTION OF BREAST CANCER PROTEIN BIOMARKERS

4.1. INTRODUCTION

The early detection or diagnosis of cancer can significantly lower the mortality rate and save human lives. Enormous effort has been ardently dedicated to the exploration of new technologies to detect early signs and symptoms of the diseases. (Subramanian et al. 2015) Biomarkers are defined as characteristics or indicators that provide insight information about biological conditions whether normal or pathological.(Naylor 2003) Thus, detection of biomarkers at the earliest stage of the cancer is of utmost importance for clinical prognosis. A biosensor is a device that is capable of providing qualitative and/or quantitative information of biomolecular interactions via coupling of a biological recognition element to a transducer. A biosensor is made up of two major components: a bio receptor and a transducer. The bio receptor is a biomolecule that binds specifically to the analyte of interest and the transducer translates the chemical/biochemical event into detectable and measurable signals. Bio receptors are made up of any proteins, antibodies, aptamers, peptides or any other biomolecules. The compelling features of aptamers, however, include lower susceptibility to denaturation, low cost and less batch variation, ease of modification to facilitate covalent bonding to material surfaces as well as long storage life. There are numerous ways to introduce labels into aptamers, and the nature of the label and the site of introduction strongly influence the mechanism of signal transduction. These advantages have spurred advancements in aptamer-based sensors that are also referred to as aptasensors.(Cho et al. 2009). Aptasensors can be categorized according to

the mode of signal transduction, which includes optical, electrochemical, mass-sensitive and micromechanical sensing.

Electrochemical measurement based detection is gaining much attention in biosensor technology due to its ease of fabrication and integration of electrochemical cells with lab-on-a-chip devices. It also offers a simple, fast and inexpensive sensing platform as well as simultaneous multi-analyte detection. A commonly used electrochemical detection setup consists of three electrodes, including the working electrode, auxiliary electrode and reference electrode. Biomolecular interactions occurring at the electrode interface will generate a change in current or potentials which can be detected by the electrochemical aptasensor. Various approaches have been employed in electrochemical detection for aptamer sensing. These include the use of catalytic labels such as enzymes or redox probes, inorganic or organic catalysts as well as nanoparticles. Electroactive labels such as ferricyanide, ferrocene, methylene blue (MB), bis-anthraquinone-modified propanediol are also employed as the redox moiety or reporting units of aptasensor. (Song et al. 2006; Kim et al. 2007; Yoshizumi et al. 2008)

This study describes a relatively general approach to the electrochemical aptasensor, which is based on the target binding-induced conformational change of the aptamers. We have employed the principle of target-induced folding or unfolding of electrode-bound oligonucleotides to fabricate electrochemical aptasensors. This device, which are often termed E-Apt (electrochemical, aptamer-based) sensors, are comprised of an oligonucleotide probe modified with a redox reporter (in this study methylene blue) at one terminus and attached to a gold electrode via a thiol-gold bond at the other. Binding of an analyte (here protein biomarkers) to the oligonucleotide probe changes its structure and dynamics, which in turn influences the efficiency of electron transfer to the interrogating gold working electrode. This class of sensors perform well even when challenged

directly with cell extracts, blood serum and other complex multicomponent sample matrices. Among both the electrochemical DNA and aptamer based sensors reported to date are examples of 'signal-off' architectures in which target binding sequesters the redox moiety from the electrode, reducing the observed Faradic current (Fan et al. 2003) and 'signal on' sensors in which target binding enhances the rate at which the redox moiety strikes the electrode, leading to increased current. (Immoos et al. 2004).

Based on previous work with several aptamer based breast cancer biomarker detection systems, we wanted to develop a redox-labeled aptamer for electrochemical sensing of Her2 and ER. Earlier, scientists had developed a simple and reproducible electrochemical biosensor based on a gold electrode (GE) to detect DNA. (Benvidi et al. 2015) Self-assembled monolayers (SAMs) of thiolated single-stranded DNA (SH-ssDNA), or BRCA1 5382 ins C mutation detection (ssDNA), were immobilized on the electrode for a specific time. Then, measurements of complementary DNA strains in the genome samples were done by electrochemical impedance spectroscopy. To develop an electrochemical aptasensor, design criteria like using short bridging DNA, complimentary sequence and the comparison of their performances by using both electrochemical and biochemical methods are critical. (Chakraborty et al. 2009) In another study, a new kind of aptamer-based electrochemical sensor was introduced, which is not based on the target binding-induced conformational change of the aptamers but by using a 15-mer thrombin-binding aptamer (5'-GGTTGGTGTGGTTGG-3') as the model oligonucleotide. The sensor was developed by first self-assembling the aptamer (i.e. a thrombin-binding aptamer) onto an Au electrode and then hybridizing the assembled aptamer with a ferrocene (Fc)-labeled short aptamer-complementary DNA oligonucleotide to form an electroactive double-stranded DNA (ds-DNA) oligonucleotide. The binding of the aptamer with its target thrombin interrupts the Watson-Crick base pairing of ds-DNA

oligonucleotide which is attached to the electrode. It also leads to the dissociation of Fc-labeled short complementary DNA oligonucleotide from the electrode surface, resulting in a decrement of current signal, which can be used for the analyte detection. (Lu et al. 2008) Electrochemical aptasensors may offer greater signal stability, sensitivity, and ease of calibration compared to fluorescence aptasensors.(Song et al. 2006) Different electrochemically active labels (e.g., redox compounds, enzymes, or metal nanoparticles) have been employed to relay the electrical signal resulting from aptamer-analyte binding. (Yuan et al. 2010; Roushani and Shahdost-Fard 2015). In a study, Xiao and colleagues modified electrodes with a redox tag containing aptamers and demonstrated the electrochemical detection of a range of analytes. (Lai et al. 2007; Xiao et al. 2007)

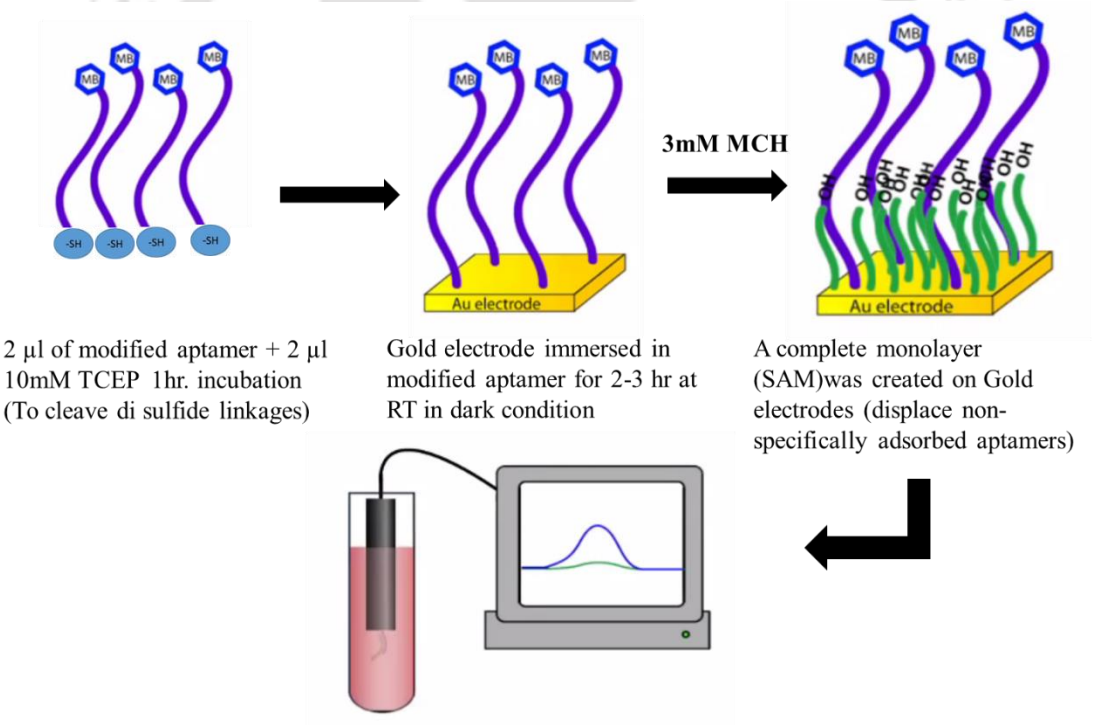

Fig. 4.1 Fabrication schema of the developed aptasensor

The hindrance of the redox reaction of methylene blue on a gold surface, due to an increased density of the covering layer by binding of the immobilized DNA aptamer with its target protein

(estrogen receptor/ ECD of Her2 protein) was used as signal for the binding reaction. The signal was measured by cyclic voltammetry. This aptasensor for the detection of biomarkers is reusable and allows measurements in the relevant analytical range for clinical applications.

4.2. OUTLINE OF THE RESEARCH STUDY

i) Modification of selected aptamers specific to ER alpha and Her2 markers to make them suitable for sensor fabrication.

ii) Attachment of the modified aptamers to Gold electrodes to develop electrochemical aptasensor.

iii) Analysis of various electrochemical measurements (cyclic voltammetry, square wave voltammetry) when the aptasensor was tested with breast cancer associated marker proteins.

4.3. EXPERIMENTAL SECTION

4.3.1 Modification of specific aptamer candidate sequences

We have screened DNA aptamer candidates ER_Apt1 and ECD_Apt1 against breast cancer marker proteins ER alpha and Her2-ECD. To attach them on gold electrodes, the selected candidates were modified at both ends. The redox probe methylene blue (MB) was covalently attached to 3' end of DNA candidates, whereas, the 5' end was modified with a thiol(-SH) group. We have obtained dual modified oligomer probes from IDT technologies at 1mM synthesis scale. The specification of those probes are as follows:

4.3.2 Preparation of gold electrodes

Gold working electrodes with the specification (outer dia-6.35mm, inner dia. - 2 mm, Cat. no. CHI 101) were purchased from M/s CH Instruments, Inc, USA. At first the gold electrodes were polished with micro polish powder (0.05µm alumina powder) and rinsed with deionized water.

Then the electrodes were sonicated for 15-20 minutes to remove any traces of alumina. A smooth and clean surface is required to support sufficient aptamer binding to gold electrodes.

4.3.3 Preparation of the modified aptamer solution

5 µl of 200 µM thiolated and methylene blue (MB) modified DNA aptamers were mixed with 10 µl of 10mM TCEP (Tris-(2-carboxyethyl) phosphine hydrochloride). The mixture was incubated in the dark for 60 minutes at room temperature (25 °C). TCEP selectively and completely reduces water-soluble alkyl di sulfide linkages present in the aptamer solution. As MB is light-sensitive, the incubation of the modified aptamer with TCEP solution was performed in the dark. The reduction of thiolated MB-aptamers by TCEP was visualized by loss of the blue color of MB. The loss of the blue color indicates the reduction of MB to leuco MB, which is colorless. The solution was then diluted in 200 µl with PBS.

4.3.4 Modification of the gold electrodes with the thiolated and MB conjugated aptamer

After TCEP treatment, freshly cleared gold electrodes were immersed into 200 µl of aptamer solution and kept in an eppendorf tube sealed with parafilm. The gold electrodes were incubated for 2 hour at room temperature in the dark. Following incubation and self-assembled monolayer (SAM) formation, excess aptamer probes physically adsorbed on the electrode surface were removed by rinsing with deionized water. Immediately before use, 0.4ml of 6-mercaptohexanol (MCH) was mixed with 1.46 ml of PBS to achieve a final concentration of 2 mM. Modified electrodes were dried using nitrogen and immediately transferred to 2 mM 6-mercaptohexanol solution. The incubation was performed for 3-5 hr at room temp in the dark. Excess 6-MCH, which was physically adsorbed on the electrode surface was removed by room temperature-deionized water rinses (~1min). Excess water was gently wicked off from the edge of the electrode using a

laboratory wipe (no nitrogen needed) and the modified gold electrodes were directly transferred to PBS for storage. The modified electrode can be stored in PBS in the dark at 4°C for up to 1 week.

4.3.5 Electrochemical apparatus set-up

All measurements were conducted using alternating current voltammetry (ACV) at an AC frequency (50 Hz) using a CHI 630 potentiostat (CH Instruments) in a standard electrochemical cell. We typically employed a platinum (Pt) wire as the counter electrode (CH Instruments, cat. no. CHI115) and an Ag/AgCl (saturated with 3 M NaCl; CH Instruments, cat. no. CH111**)** as a reference electrode. These two electrodes were set into a 3-electrode electrochemical cell with the MB-aptamer modified gold working electrode.

4.3.6 Signal measurements by aptasensor

Initially a background scan was recorded in PBS before analyte was added. All three electrodes were properly connected and all immersed in the buffer/ electrolyte solution before initiating measurements. The signal gain of E-AB sensors (i.e., the change in current observed at a given target concentration) spiked suddenly as the frequency increased above ~1 Hz, until it plateau, typically at ~50 Hz (Ricci et al. 2007). The optimal frequency of E-AB sensors, in contrast, vary from aptamer to aptamer depending on its loop structure. E-AB sensor measurements were generally conducted using alternating current voltammetry (ACV) with a step potential of 10mV, an amplitude of 25mV and at an AC frequency of ~ 50 Hz. Target protein ER alpha or Her2-ECD were added in PBS and signals were recorded with ER_Apt1 and ECD_Apt1 modified E-AB, respectively. Cyclic voltammetry (CV) and square-wave voltammetry (SWV) readings were recorded corresponding to different concentration of analytes (0mM to 25mM).

4.4. RESULTS AND DISCUSSION

4.4.1 Electrochemical detection of ER alpha using aptamer modified electrodes

The C6- thiol and MB-modified DNA probe was chemically synthesized at 1 µM scale (probe DNAs modified with 11-carbon (C11) alkane thiols form more stable sensors, leading to significantly improved shelf life and supporting operation at higher temperatures where specificity and equilibration times are improved). Assembly of modified aptamers were done on gold electrode as described by Liu and colleagues. (Liu et al. 2010).

Given its excellent affinity towards ER alpha, we sought to convert ER_Apt1 carrying hairpin into an electrochemical biosensor. This was done by conjugating a redox tag (MB) at the 3' end of the DNA aptamer sequence. The choice of the redox tag depends on earlier reports describing MB conjugated nucleic acid molecules as offering better shelf life and gain of electron transfer to other redox moieties (e.g. ferrocene).(Ferapontova and Gothelf 2009; Kang et al. 2012). In the absence of target ER alpha, hairpin molecules were closed and MB redox tags were positioned next to the electrode surface. Binding of ER alpha molecule to the loop caused hairpins to open, thus moving MB tags away from the electrode surface and decreasing electron transfer or current at the electrode. Cyclic voltammetry recording was measured to monitor changes in the intensity of the reduction current of MB as shown in Figure 4.2.

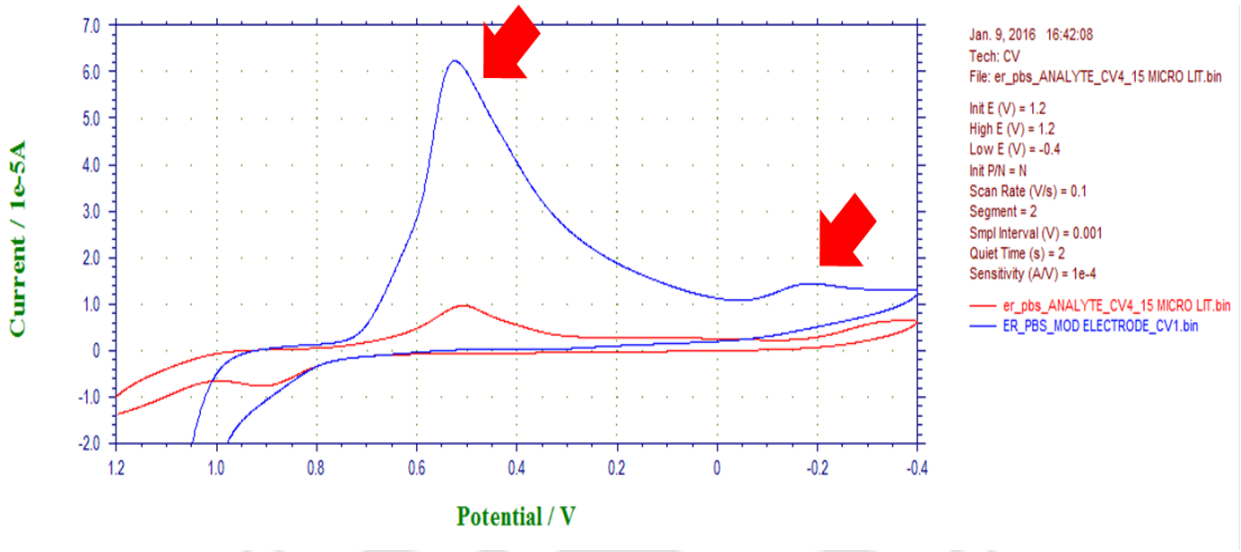

Fig 4.2. Cyclic Voltammetry measurements of ER alpha by ER_Apt1 modified electrode.

The peak current intensity dropped as a function of increasing analyte ER alpha concentration. Fig. 4.2 depicts typical cyclic voltammograms (CVs) obtained from MB labeled ER_Apt1 modified gold electrodes in 50mM PBS. The electrodes showed well-defined redox waves (one at +0.50V, another short peak at - 0.2 V) when the modified aptamer was only in PBS (Blue curve). However, when ER alpha was added, a typical redox wave was recorded, but the anode current decreased drastically. As the ER alpha concentration was gradually increased, the anode peak current decreased.

As shown in Figure 4.3, the loss in signal due to binding of aptamer molecules was proportional to the ER alpha concentration. This change in the signal could be plotted as the change in reduction current (ΔI) before and after addition of ER alpha. The calibration curve of signal change vs ER alpha concentration showed that the aptasensor had a limit of detection of 1mM with linear range extending to 25 mM. The detection limit of this aptasensor correlates with the concentration of ER alpha when it was conjugated with gold electrodes. The binding efficiency of the DNA aptamer probe does not change appreciably after being conjugated with gold electrodes.

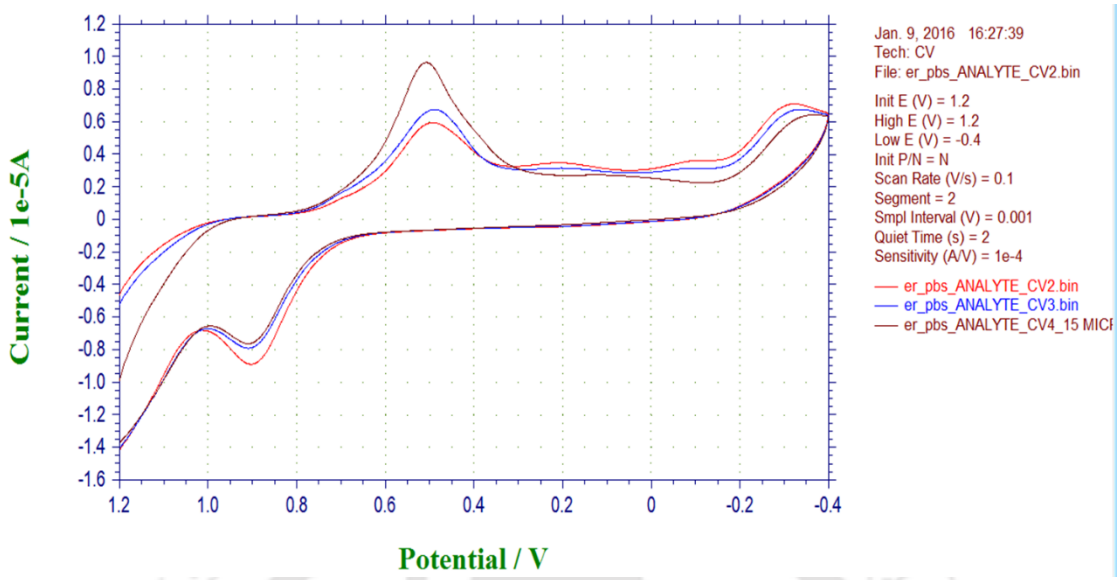

Fig 4.3. Cyclic voltammograms corresponding to the analysis of different concentrations of ER alpha protein (from top to bottom) (a) 1mM, (b) 10 mM, (c) 25 mM.

The frequency range for square wave voltammetry (SWV) analysis has been noted as an important parameter in defining the sensitivity of the aptasensor.(White and Plaxco 2010) We characterized behavior of aptamer-containing electrodes at frequencies ranging from almost 5 to 1000 Hz in the presence of 15µM ER alpha (concentration) and determined that the signal change was highest for frequencies ranging from 50 to 60 Hz. Therefore, all subsequent SWV experiments were performed at a 50 Hz sampling frequency. Figure 4.4 shows a typical SWV voltammograms with MB reduction peak appearing at -0.25 V (vs Ag/AgCl). Upon increasing the concentration of ER alpha analyte in the buffer, the peak current also uniformly further decreased, suggesting unfolding of the hairpin molecules.

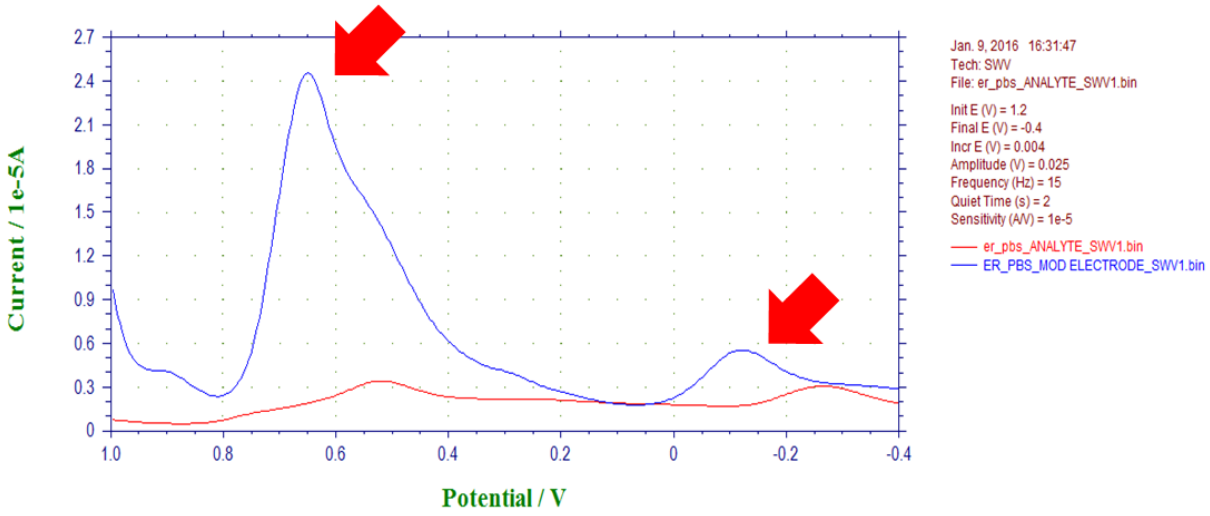

Fig. 4.4 SWV plot showed the decrease in Faradaic current when analyte ER alpha was added.

4.4.2 Electrochemical detection of Her2 using aptamer modified electrodes:

We have characterized the response of MB-modified aptamer based sensor when it was treated with specific analyte Her2-ECD protein. Figure 4.5 shows a typical cyclic voltammograms with MB reduction peak appearing at-0.2 V and +0.5 Volts (vs Ag/AgCl). Upon increasing the concentration of Her2-ECD, the peak current uniformly decreased, suggesting unfolding of the hairpin molecules. The square wave voltammetry (SWV) plot showed that decrease of current when the analyte ECD was added into the buffer. Both the peaks at 0.6V, -0.1V plateaued when the analyte was added and shift of the peak was also observed which signifies the use of aptamers as a sensor.

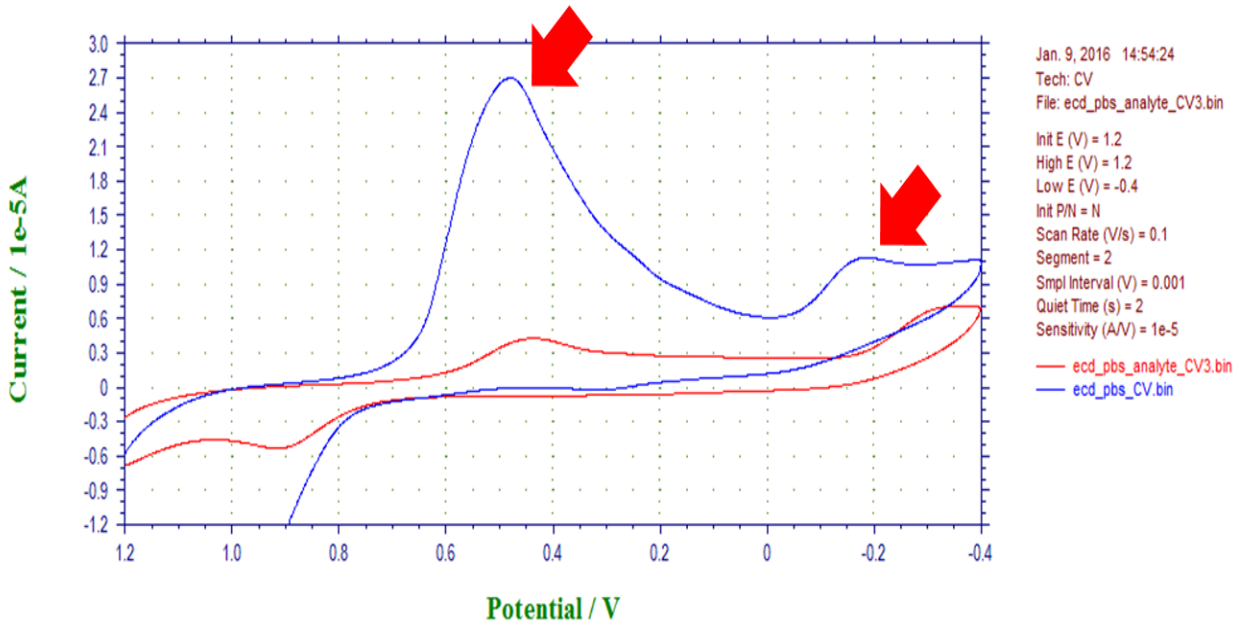

Fig. 4.5. Cyclic Voltammetry measurements of Her2-ECD by ECD_Apt1 modified electrode**.**

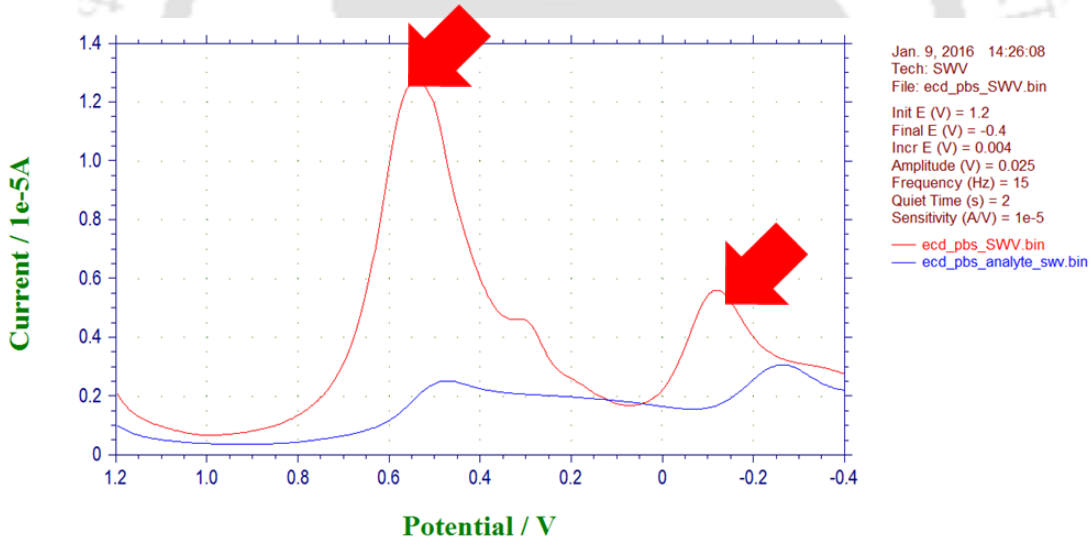

Fig. 4.6. SWV plot showed that decrease in faradaic current when analyte Her2-ECD was added into the PBS.

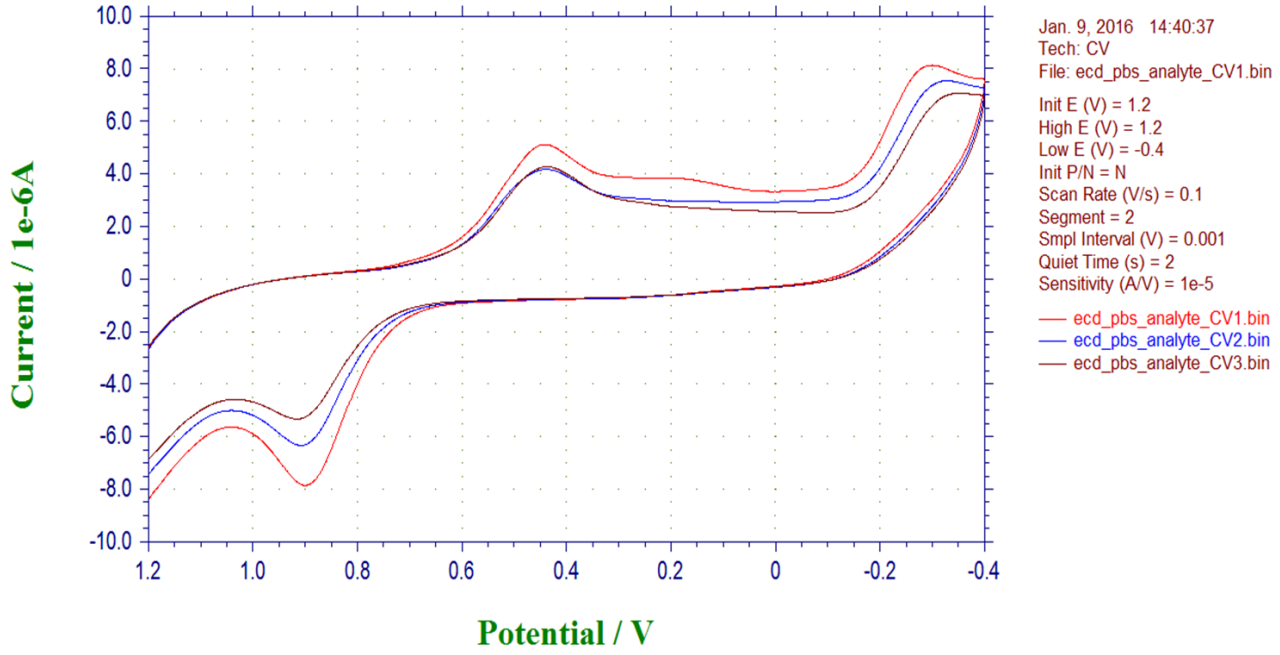

Fig 4.7. Cyclic voltammograms corresponding to the analysis of different concentrations of HER2-ECD protein (from top to bottom) (a) 1mM, (b) 10 mM, (c) 25 mM

As shown in Figure 4.7, the loss in signal due to binding of aptamer molecules was proportional to the HER2 concentration. From the calibration curve of signal change vs Her2-ECD concentration, results show that the aptasensor had a limit of detection of 1.2 mM with linear range extending to 26 mM. The detection limit of the aptasensor corresponds with the concentration of ECD protein.

4.4.3 Effects of aptamer surface density on biosensor performance

An important factor affecting sensitivity and performance of an aptasensor is the packing density of aptamer molecules on the surface.(Ricci et al. 2007; White et al. 2008)Given that the binding of analytes likely induced the conformational changes in the hairpin structure of the aptamer, the surface density of aptamers attached to gold electrodes was hypothesized to be related to sensitivity of the aptasensor. The MB-aptamer surface density was calculated on the basis of the intensity of

the reduction peaks collected during SWV experiments. Such an approach is frequently used to determine grafting density of DNA strands. (Yang et al. 2002; Zhu et al. 2008) In our calculations, it has been further assumed that one MB molecule was conjugated to one aptamer. (Liu et al. 2010) Another point to note is that MB labeling efficiency was ~30%, so that the surface density values reported below refer only to MB-modified aptamer molecules.

The first reported example of this class of biosensors, the aptamer based electrochemical sensor (E-AB), consists of a self-complementary DNA probe that forms a stable stem-loop structure (Fan et al. 2003). One terminus of the stem-loop probe DNA is covalently modified with a redox reporter, usually ferrocene and methylene blue (Drummond et al. 2003; Liu et al. 2010). The opposite end is modified with a long-chain alkane thiol, which enables chemi-absorption to the surface of a gold electrode via standard gold-thiol self-assembled monolayer (SAM) chemistry. In the absence of target, the probe's stem loop structure forces the redox reporter into proximity with the electrode, supporting efficient electron transfer. A number of E-AB sensors have been reported that employ the principle of target binding-induced folding of a redox-modified DNA aptamer to modulate electron transfer and signal the presence of a range of non-nucleic acid targets (Qing et al. 2014). Among both the E-DNA and E-AB sensors reported to date are examples of 'signal-off' architectures in which target binding sequesters the redox moiety from the electrode, reducing the observed Faradic current ,and 'signal on' sensors in which target binding enhances the rate at which the redox moiety strikes the electrode, leading to increased current. (Liu et al. 2010)

4.5 CONCLUSION

Electrochemical biosensors are portable, low cost, simple-to-operate, easily miniaturized and would also be used in turbid media with optically absorbing and fluorescent compounds. Electrochemical aptasensors appear as promising devices, based on aptamers that are very small in size compared to other bio recognition molecules like antibodies or enzymes. Nonspecific adsorption phenomena are usually less pronounced on nucleic acid conjugated surfaces as compared to other protein derivative counterparts. In this chapter, we described the development of an electrochemical aptamer-based biosensor for detection of breast cancer biomarkers. Bio recognition elements of the biosensor consisted of a thiolated DNA hairpin carrying a redox tag. Gold electrodes modified with aptamer probes showed Faradaic current signatures consistent with reduction potential of MB. Binding of ER alpha and Her2-ECD caused the current to decrease, thus providing a basis for detection. The biosensor was specific to ER alpha and Her2-ECD and showed a limit of detection of 1mM and 1.2 mM, respectively.

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Chapter 5

Summary and Future Prospects

Chapter 5

5.1 SUMMARY

This present study demonstrates the selection of aptamers specific to various breast cancer protein biomarkers like ER alpha and HER2. These aptamers can lead to a sensor based system for detection of marker positive breast carcinomas.

5.1.1 Selection and Characterization of Specific Aptamers to Estrogen Receptor Alpha

In brief, we have successfully sub-cloned and expressed recombinant estrogen receptor alpha in bacterial system. We demonstrated that the specific aptamer candidates selected after iterative rounds of selection process showed a high affinity for estrogen receptor α molecules during *in vitro* DNA-protein binding studies. The biotinylated aptamer can detect ERα expression in paraffin-embedded breast cancer tissue samples, which was comparable with established immunohistochemistry methods with ERα specific monoclonal antibodies. FITC conjugated aptamer candidates showed no cytotoxicity thus making them suitable candidates for bio-diagnostics. Therefore, the developed DNA aptamer can be exploited as a promising diagnostic module for non-invasive cancer diagnosis. It can also provide a novel cost effective alternate to conventional ER antibody in solid phase immunoassays for cancer diagnosis and related applications.

5.1.2 Selection and Characterization of Aptamers Specific to Extra cellular domain (ECD) of Human Epidermal Growth Factor Receptor 2 (Her2)

In summary, we demonstrated that the specific aptamer candidates selected after iterative rounds of selection process showed high affinity towards the extra cellular domain of Her2 during *in vitro* DNA-protein binding studies. The aptamer ECD_Apt1 also exhibited Her2 marker status in paraffin-embedded breast cancer tissue samples, which was comparable with established immunohistochemistry methods with a Her2-specific monoclonal antibody. These

aptamers can be exploited as a molecular probe in detection of Her2 overexpressing cell lines and Her2 positive cancer tissue specimens. Therefore, aptamers can be exploited as a promising diagnostic module for non-invasive cancer diagnosis and it can provide a novel cost effective alternative to conventional antibody in solid phase immunoassays for cancer diagnosis and related applications. Since Her2 overexpression is abundant in most carcinomas, the Her2 specific aptamers are promising as a guiding ligand for targeted chemotherapy against a plethora of multiple malignancies. The DNA aptamer against HER2 could be exploited to fabricate an aptamer based sensor, which is amenable for monitoring cancer patient compliance and facilitating the evaluation of therapeutic agents in clinical trials. Nevertheless, extensive research with animal models is still required to evaluate the *in-vivo* binding specificity of the aptamers.

5.1.3 Aptamer Based Electrochemical Sensor System for Detection of Breast Cancer Protein Biomarkers

We have developed an aptamer based electrochemical sensor system that will be able to detect various breast cancer biomarkers. We have modified the selected aptamers specific to ER alpha and Her2 markers to make them suitable for sensor fabrication. We then modified aptamers by chemically attaching them by thiol-chemistry to gold electrodes to develop an electrochemical aptasensor. With the help of the developed aptasensor system, the presence of various breast cancer associated marker proteins were analyzed by various electrochemical parameters (cyclic voltammetry, square wave voltammetry).

5.2 FUTURE PROSPECTS OF THIS STUDY

We have developed an aptamer based electrochemical detection system for Her 2 and ER alpha positive breast carcinomas. We have successfully displayed the specificity and sensitivity of specific aptamers at the *in vitro* level and validated in breast cancer tissue specimens. Based on this knowledge, further studies might be performed on *in vivo* models.

With more focus on *in vivo* studies for potential clinical applications, aptamers can be developed in combination with DNA nanostructures, nanomaterials, or microfluidic devices as diagnostic probes or therapeutic agents for cancers and several other diseases. The use of aptamers as targeting agents in drug delivery might also be explored. To realize the full potential, aptamers might be exploited to develop diagnostic kits that will be more portable, cost-effective and robust using simple devices for real-time and on-site detection and monitoring, instead of the laborious and time-consuming diagnostic tests currently available in clinical labs. Regarding a therapeutics approach, there is still untapped potential in the combination of the target recognition and strong binding property of aptamers with exquisitely designed nanomaterials that can be used as effective drug delivery platforms. Many nanomaterials, such as liposomes, polymer vesicles and silica nanoparticles, combined with DNA aptamers, have shown feasibility for use in *in vivo* targeted drug delivery. The integration of diagnostic capabilities with therapeutic interventions, termed as “Theranostics” is critical to address the challenges of cancer heterogeneity and adaptation. Although having immense potential of these theranostic agents, tailor-made modifications and validation experiments are needed to be executed before aptamer-based drug delivery can reach clinical trials and eventually the day-to-day management of cancer patients.

List of Publications:

1) Ahirwar R, Vellarikkal SK, Sett A, Sivasubbu S, Scaria V, Bora U, et al. (2016) Aptamer-Assisted Detection of the Altered Expression of Estrogen Receptor Alpha in Human Breast Cancer. PLoS ONE 11(4): e0153001. doi:10.1371/journal.pone.0153001.

2) Sett A, Das S, & Bora U. (2014). Functional Nucleic-Acid-Based Sensors for Environmental Monitoring. Applied Biochemistry and Biotechnology, 174(3):1073-91.doi: 10.1007/s12010-014-0990-3.

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4) Sett A, Das S, Sharma P. and Bora U. (2012) Aptasensors in Health, Environment and Food Safety Monitoring. Open Journal of Applied Biosensor, 1:9-19. doi: 10.4236/ojab.2012.12002**.**

Manuscripts communicated

1) Sett A, Borthakur B B, Sharma J D, Kataki A C, Bora U. (2016) DNA Aptamer Probes for Detection of Estrogen Receptor α Positive Carcinomas.

2) Sett A, Borthakur B B, Bora U. (2016) In-vitro Selection of DNA Aptamers against Extra Cellular Domain of Human Epidermal Growth Receptor 2 in HER2 Positive Carcinomas.

3) Sett A, Mosahari V P, Kalita J, Das D, Bora U. (2016) Development of an electrochemical aptamer based sensor system for breast cancer detection.

Manuscripts from collaborative work

1) **Sett A**, Gadewar M, Sharma P, Deka M, Bora U. (2016) Green synthesis of gold nanoparticles using aqueous extract of Dillenia indica. Adv. Nat. Sci.: Nanosci. Nanotechnol. 2(7).

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VITAE

Arghya Sett was born on December 26, 1985 in the city of joy, Kolkata, (West Bengal). He completed his Secondary Examination conducted by West Bengal Board of Secondary Education (10th Class), West Bengal in 2001 and Higher Secondary Examination conducted by West Bengal Council of Higher Secondary Education (12th Class), West Bengal in 2003. He qualified West Bengal Joint Entrance Examination and completed his Bachelor of Technology in Biotechnology in 2008 from Heritage Institute of Technology, Kolkata under West Bengal University of Technology in 2010. He completed his M.Tech. (Biotechnology) from VIT University, Vellore, Tamilnadu, India in July, 2010.

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