DEVELOPMENT OF DIAGNOSTIC PLATFORMS FOR SINGLE NUCLEOTIDE POLYMORPHISM DETECTION

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ABSTRACT

DEVELOPMENT OF DIAGNOSTIC PLATFORMS FOR SINGLE NUCLEOTIDE POLYMORPHISM DETECTION

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In this study, we report the results of different simple and cost effective approaches and platforms which were used to improve single nucleotide mismatch discrimination ability and investigation of the applicability of various materials and environments for the system.

As the solid support of the array platform both customly functionalized epoxy and amine combination and commercially purchased poly-L-lysine modified materials were used. Surface-probe interactions and immobilization efficiencies were investigated and affirmative results are gained with heterobifunctional cross linker mediated immobilization of polyethyleneglycol spacer functionalized surface probes. Glass capillary tubes were also used to develop a partially solution based approach that can detect single nucleotide polymorphisms (SNPs) through the Oligonucleotide Ligation Reaction. The

established sytem was capable of discriminating mistmatches at single nucleotide level by using unlabeled targets.

Mesoporous silica nanoparticles and nitrocellulose membrane strips were also used to develop a novel platform for SNP detection. Controlled release of chromogen molecules inside the pores of the silica nanoparticles were achieved related with the specific complementary probe hybridization events.

Use of unmodified target sequences in sandwich hybridization format permitted the usage of different detection signal probes throughout the study. Signal probes modified with 6-carboxyfluorescein or biotin at 3’ ends were successfully used to visualize the experimental results by using simple UV illumination and of color formation. This enables the platform to be followed by unaided eyes which makes it an ideal candidate for point of care detection purposes.

Keywords: nucleic acid, sandwich hybridization, single nucleotide polymorphism point of care, silica nanoparticles

ÖZ

TEK NÜKLEOTİT POLİMORFİZMİ TESPİTİNE YÖNELİK TANI PLATFORMLARININ GELİŞTİRİLMESİ

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Bu çalışmada, tek nükleotit farklılıklarını ayırt etme yeterliliğinin geliştirilmesi amacıyla kullanılan farklı tipteki basit ve uygun maliyetli yaklaşım ve platformlar ile farklı malzeme ve ortamların sisteme uygulanabilirlik araştırmalarının sonuçları sunulmaktadır.

Dizi platformlarında destek yüzey olarak, hem amaca özel olarak epoksi ve amin kombinasyonu ile fonksiyonelleştirilen hem de ticari olarak satılan poli-L-lizin ile kaplanan malzemeler kullanıldı. Yüzey-oligonükleotit dizisi (prob) etkileşimleri ve yüzeye bağlanma etkinlikleri araştırıldı ve polietilenglikol aralayıcı ile modifiye edilen yüzey oligonükleotit dizilerinin (yüzey probları) heterobifonksiyonel çapraz bağlayıcı aracılığıyla gerçekleştirilen yüzeye tutunma çalışmalarından olumlu sonuçlar alındı. Ayrıca tek nükleotit polimorfizmlerini oligonükleotit ligasyon reaksiyonu aracılığıyla tespit edebilen, kısmi çözelti

tabanlı bir yaklaşım geliştirmek için kapiler cam tüpler de kullanıldı. Oluşturulan sistem, işaretlenmemiş hedef oligonükleotitler kullanarak yanlış eşleşmeleri tek nükleotit seviyesinde ayırt edebilmiştir.

Tek nükleotit polimorfizmi tespiti için yeni bir platform geliştirmek amacıyla ayrıca mezoporlu yapıda silika nanoparçacıklar ve nitroselüloz membran yapıları kullanıldı. Silika nanoparçacıkların porlarının içindeki kromojen moleküllerin kontrollü salınımı, özgün tamamlayıcı dizilerin hibridizasyonu ile ilişkili olarak gerçekleştirildi.

İşaretlenmemiş hedef oligonükleotit dizilerinin sandviç hibridizasyon formatında kullanımı çalışma boyunca farklı tipteki tespit sinyal oligonükleotit dizilerinin (sinyal probları) kullanımına imkân vermiştir. 3' ucu 6-karboksifloresan veya biyotin ile işaretlenen sinyal dizileri, deneysel sonuçların basit UV ışıklandırma kullanılarak ya da renk oluşumunu izleyerek görüntülenmesinde başarılı şekilde kullanıldı. Platformun çıplak gözle izlenebilme özelliği, sistemi hasta-başı tespit amaçlı çalışmalar için ideal bir aday haline getirmektedir.

Anahtar Kelimeler: nükleik asit, sandviç hibridizasyon, tek nükleotid polimorfizmi, hasta başı sistemler, silika nanoparçacıklar

To My Beloved Grandparents

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LIST OF ABBREVIATIONS

APS	3-Aminopropyltrimethoxysilane
BSA	Bovine Serum Albumin
DNA	Deoxyribonucleic Acid
dsDNA	Double-stranded Deoxyribonucleic Acid
DTT	Dithiothreitol
EA	Epoxy-Amine
FAM	Carboxyfluorescein
GPS	3-Glycidoxylpropyltrimethoxysilane
HRP	Horseradish Peroxidase
OLA	Oligonucleotide Ligation Assay
OLR	Oligonucleotide Ligation Reaction
PBS	Phosphate Buffered Saline
PLL	Poly- L- Lysine
POC	Point of care
SDS	Sodium dodecyl sulfate
SM(PEG)$_n$	(succinimidyl-[(N-maleimidopropionamido)-diethyleneglycol]ester)
SSC	Saline-Sodium Citrate
ssDNA	Single-stranded Deoxyribonucleic Acid
Sulfo-EMCS	(N-[ε-maleimidocaproyloxy]sulfosuccinimide ester)

TCEP Tris(2-Carboxyethyl)phosphine

TMB 3,3',5,5'-Tetramethylbenzidine

CHAPTER 1

INTRODUCTION

1.1 Nucleic Acid Based Diagnostic Systems

Nucleic acid sequences which are unique to every living organism provide useful targets to identify an organism as well as the diagnosis of various diseases. Various genome projects were led to the completion and support the innovation for improved diagnostic methods for nucleic acid testing (Csako, 2006); (Rosi and Mirkin, 2005). Unique sequences of nucleic acid (either DNA or RNA) are used to reveal the existence of an organism in a given sample or a specific region related with a disease or feature in its genome via nucleic acid based diagnostics. Attitudes towards the reveal of nucleic acid sequences of interest can be mainly itemized in two categories as direct detection methods and amplification based methods. Direct detection methods use a probe to hybridize directly to the target sequence of interest. Amplification based methods run an in vitro amplification process to increase the amount of the target sequence followed by the amplified target detection. (O'Connor and Glynn, 2010).

Some of the common amplification techniques are; Polymerase Chain Reaction (PCR), oligonucleotide ligation reaction, branched DNA amplification and rolling circle amplification which relied on 'end point' analysis. Briefly, in the PCR process as an example, amplification reaction is followed by running the amplicon through an agarose gel. Exceptionally; real-time PCR applications, which are also important and high throughput techniques in nucleic acid based diagnostics,

permit the monitorization of amplification product accumulation while the reaction is taking place (Csordas *et al.*, 2004); (O'Connor and Glynn, 2010); (Raoult *et al.*, 2004).

Array platforms are generating the largest part in direct detection methods' area for nucleic acid based diagnostics. In general terms, arrays refer to a plenty of platforms in which thousands to tens of thousands of datapoints may be generated on a solid support in each experiment, (Allison *et al.*, 2005).

1.1.1 Array Platforms

The use of ordered arrangements or 'arrays' of spatially localized molecules in parallel assays has already reached the large masses for studying interactions between biological molecules. These assays are commonly referred to as arrays, and the ones which are miniaturized to a small format are called microarrays (Falciani, 2007). Microarrays and macroarrays can be distinguished by the difference in the size of the immobilized spots. Microarray spots are represented in less than 200 microns while that of macroarrays are featured by a diameter of more than 300 microns (Bier *et al.*, 2008). Concurrent and parallel nature is the essence of the microarray approach. It is the fact that datum on a huge amount of elements is obtained in a single experiment, via a regular arrangement of "probes", which might have various molecular natures, on some kind of support and on assessment of signals detected after incubation with a usually labelled sample (Jordan, 2012). Nucleic acids, proteins or carbohydrates can be the biological materials used in these arrays, while arrays of chemicals and other small molecules have also been constructed. These materials are immobilized on the surface of a solid support in an ordered arrangement so that each positional coordinate where material has been deposited contains the material that represents a single gene, a gene region or any other molecule of interest (Falciani, 2007). There are various formats in array construction regarding their architecture, molecule types, used materials, signalling ways and many more. In spite of the diversity of formats, a common feature is shared by all arrays that they allow

multiple tests to be performed simultaneously. As an example; expression levels of thousands of genes in one sample or a specific antigen in hundreds of samples can be determined by DNA arrays in a single experiment (Nicolau and Müller, 2005). Some introduction is given in next paragraphs regarding different array types.

Protein/peptide arrays are developed research tools to study multiple protein interaction events and activity networks concurrently (Cutler, 2003); (Walter *et al.*, 2000). Interaction of proteins with antibodies, drugs, nucleic acids, small molecules or other proteins are among the interactions that can be monitorized via these arrays.

However, restricted availability and high expenditure of purged proteins for immobilization and the finite stability of immobilized proteins in their functional state create major bottlenecks in protein microarraying which further causes a challenging process for production of these arrays. Despite all, the technologies related to protein microarrays have been evolving in steady steps from the proof-of-concept to marketed products or services (Jordan, 2012); (Stoevesandt *et al.*, 2011)

Protein arrays might be divided into two groups: "protein expression or protein detection arrays" and "protein function arrays". Protein detection or expression arrays which are also called as "analytical", are typically used to profile a complex mixture of proteins in order to measure their affinity of binding specificity and expression levels. In this technique, the array, where a library of antibodies, affibodies or aptamers is immobilized on, is probed with the protein solution of interest (Bertone and Snyder, 2005). Related to protein detection or expression microarrays, reverse phase protein microarray (RPA) uses cells which are isolated from the targets of interest and lysed. Nitrocellulose slide is used to be arrayed with lysate and then probed with antibodies against the target protein and antibodies are typically detected with several signalling applications (Hall *et al.*, 2007). On the other side, protein function microarrays are used to study biological activities and several interactions of proteins with DNA, RNA, small molecule,

phospholipid and other proteins. When viewed from the health and medicinal issues many promising studies were conducted with protein chip platforms. They provide excellent ways to discover drug targets like the target of small molecule inhibitor of rapamycin (SMIR) which has been previously unknown (Huang *et al.*, 2004).

It has been declared that the direct study of the functional proteome has the potential to provide a wealth of information that complements and extends genomic, epigenomic and transcriptomic analysis in The Cancer Genome Atlas (TCGA) projects. In a very recent study, researchers used reverse-phase protein arrays to analyse patient samples from TCGA 'Pan-Cancer' diseases, using antibodies that target total proteins and post-translationally modified proteins (Akbani *et al.*, 2014). Acquired results were integrated with genomic and transcriptomic analyses of the same samples to identify commonalities, differences, emergent pathways and network biology within and across tumour lineages which further constructs a framework for the prognostic, predictive and therapeutic relevance of the functional proteome.

Ziauddin and Sabatini (2001) were the ones first described cell-based microarrays as an original method to perform high-throughput screening of gene function. In this method, nucleic acid (expression plasmid or RNA) are immobilized on array surface and utilised to transfect the cells with a proper transfection reagent. The addition of cultured cells in medium to the surface structures a cell microarray of transfected cells in a grid of nontransfected cells. Finally, the microarrays are fixed and prepared for immunofluorescence, DNA and F-actin staining, apoptosis detection, verification of gene silencing and many more other analysis. They have been also called as "reverse transfection cell microarrays" (Mishina *et al.*, 2004); (Wheeler *et al.*, 2005); (Ziauddin and Sabatini, 2001).

In a recent study (Kwon *et al.*, 2014) metabolism induced drug or drug candidate toxicity is predicted via developed "Transfected Enzyme and Metabolism Chip" (TeamChip) system. Gene transductions with recombinant adenoviruses that carry genes for drug metabolizing enzymes were done on the microarray platform

which is constructed with human cell culture. As a result, potential toxic responses of model compounds due to drug metabolism are identified.

Discovery of specific nucleic acid sequences, that bind to a various target molecules including small molecules, drugs, peptides, and hormones, and also complex objectives such as proteins, spores, and whole cells with high affinity and specificity via two and three dimensional positionings, had been evolved by "in vitro selection and amplification techniques". SELEX (systematic evolution of ligands by exponential enrichment) process is the technique to obtain those oligonucleotide ligands (Dong *et al.*, 2014); (Ellington and Szostak, 1990); (Tuerk and Gold, 1990). Higher affinity and specifities for their targets are reported for the aptamers in comparison with their equivalent antibodies (Baldrich, 2011). Ellington and colleagues reported number of arrays which had been constructed using the lysozyme, ricin, IgE, and thrombin RNA/DNA aptamers on streptavidin slides (Cho *et al.*, 2006); (Collett *et al.*, 2005). A relatively new study (Baird *et al.*, 2012) showed age-dependent changes in the cerebrospinal fluid proteome by an aptamer array. They used slow-off rate modified aptamers (SOMAmers) which contain novel chemically modified nucleotides. They are selected through a novel SELEX process to have slow dissociation rates. This study was a first wide survey of cerebrospinal fluid proteins by an aptamer array and had several advantages over mass spectrometry and immunoassays (Baird *et al.*, 2012); (Gold *et al.*, 2010).

Beginning from their first introduction in the mid-1990s, nucleic acid based microarrays have been the most commonly utilized application of microarray technology (Schena *et al.*, 1995). Comprehensive expression on fundamentals and details are given in following headings .

1.2 Nucleic acid based microarrays

Nucleic acid based microarrays, sometimes called DNA chips or DNA microarrays, are expansive platforms used in gene-expression analysis,

genotyping, and single nucleotide polymorphisms analysis (SNP) as well as other genomic or transcriptomic variations (Bier *et al.*, 2008).

Nucleic acid based microarrays (DNA microarrays as generally referred), are small, solid supports onto which the sequences from thousands of different genes are immobilized at fixed locations. Glass microscope slides, silicon chips, nylon membranes or any other material depending on the platform can be used as support material. The DNA, cDNA or oligonucleotide is printed, spotted, or synthesized onto the support. Sequence components (probes) are immobilized on the support in an orderly way, thus a researcher uses the location of each spot in the array to identify a particular gene sequence. The whole process is based on hybridization probing, a technique that uses fluorescently (or any other signalling molecule/system) labeled nucleic acid molecules as "mobile probes" or "signal probes" to identify complementary sequences that are able to base-pair with the ones previously immobilized on support surface. After this hybridization step is complete, one can place the microarray in a "reader" or "scanner" that may consist of some special equipment to receive and read the signals, like lasers, a special microscope, and a camera (NCBI, 2003).

1.2.1 Printing Technologies and Microarray Manufacturing Techniques

Various established formats of DNA microarray platforms are present in research areas and market. In order to categorize them in terms of production area, one can say that they fall into two categories: those that are constructed within laboratories, and those that are produced under industrial manufacturing conditions by commercial companies. Utilization of non-porous solid supports and methods for high-density spatial synthesis of oligonucleotides caused a rise of interest in these areas. Pat Brown and colleagues, who are pioneers of the platform, can robotically spotted about 10,000 cDNAs onto a glass support and hybridized them with a double-labelled probe These are also called "home brew" or "roll your own" glass slide microarrays which are produced in-house, often in a core facility. Steve Fodor and colleagues have adapted photolithographic

masking techniques to produce arrays with 400,000 distinct oligonucleotides, each in its own 20 mm^2 region. Best known example for the second format is the Affymetrix GeneChip™ format. Other companies are developing in situ synthesis with reagents delivered by ink-jet printer devices. (Schena *et al.*, 1995); (STRYER *et al.*, 1991); (Falciani, 2007); (Lander, 1999).

Synthesis on the chip or the photolithographic technique is a kind of process production which is similar to wafer production in microelectronics (Figure 1.1). Chemically activated silicon wafer surface is covered with photo-labile protecting groups. Following UV irradiation through a mask, the activated groups are reactive at certain localities and a nucleotide, that again resists the same photo-labile protecting group at its 3' end, may couple with its 5' end. Repetition of this procedure with all four bases will end up with defined sequences in the array. However, the method is limited to oligonucleotides. On the chip surface, the length of the oligonucleotides is limited up to 25 nucleotides since there is no 100% fruitful chemical reaction and thus the addition of incomplete reactions results in a loss of sequence properties. Name of the company "Affymetrix" is very much linked to this method being one of the market leaders (Bier *et al.*, 2008).

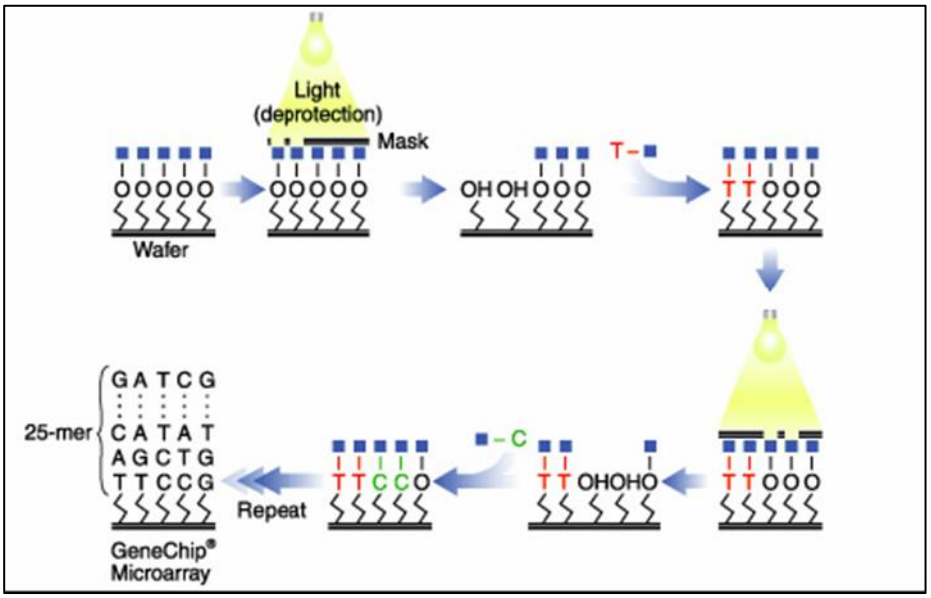

Figure 1. 1 Photolithographic synthesis of oligonucleotide on microarrays (Affymetrix)

Depositing tiny amounts of oligonucleotides by microdispensing is an alternative way of creating a microarray other than photolithography. There are two major techniques used for printing glass slide microarrays: noncontact deposition methods, and contact deposition methods.

In non contact methods small droplets of liquid onto a solid surface are deposited by ink-jet printing technology. Technique is used either to print presynthesized oligonucleotides or other biological materials, or for in situ synthesis of oligonucleotides on a solid surface. Low and medium density microarrays can be manufactured by this type of dispensing method. The company "Agilent" is perhaps the most well known using this technique. On the other hand, contact printing technique uses pin tools, or needles, which are dipped into the probe solution and dispense a certain amount of material by physically contacting the support material (Bier *et al.*, 2008); (Falciani, 2007).

1.2.2 Surface Functionalization and DNA Immobilization Chemistries

The classical structure of a DNA microarray platform can fundamentally be itemized into three key components which were also indicated by (Conzone and Pantano, 2004) as; a solid, inert, impermeable support, a coating that links the inorganic glass to the organic biological molecules and an array of biomolecular probes suited for hybridization. In this study, our diagnostic platforms are also covering these clauses.

The composition and morphology of the solid support and the choice of surface chemistry affect several critical requirements for a succesful array platform. These requirements include the controlled and reproducible deposition of small amounts of sample on a surface, stable immobilization of probes or biomolecules to the surface at high density and consistent surface concentration and detection methods that will provide a quantitative measure of the interaction (Nicolau and Müller, 2005).

1.2.2.1 Solid Support and Its Preliminary Preparation

The most commonly used support material for DNA arrays is “glass”. It is a durable material and can survive at high temperatures and washes of high ionic strength. Besides, it is flat, transparent and easy to handle. Glass surface can be commonly represented as in Figure 1.2. This simple representation is ideal for conceptualization however the surface of glass is not that much simple as the figure suggests. Glass material contains layers of adsorbed water and hill and valley topology in nanometer-sizes. Highly polar surface groups and structural roughness together makes the glass an ideal substrate to bind a variety of materials by adsorption .

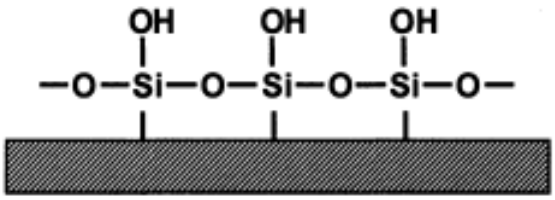

Figure 1. 2 Common representation of a glass surface (Aboytes *et al.*, 2003)

Techniques for modifying glass substrates are also well developed so thus the DNA samples can be covalently attached onto a treated glass surface. It has low fluorescence property thus it does not contribute to background noise (Cheung *et al.*, 1999); (Nicolau and Müller, 2005).

Preparing a clean surface is a prerequisite for generating a microarray slide. Standard glass microscope slides may possibly contain some environmental contaminants simply like dust on the surface which need to be excessively cleaned before coating processes. Those irregularities may arise on surface coating and they might affect all the upstream processes. Removing glass contaminants can be achieved by many methods but generally classified as acid, alkali or physical cleaning approaches (Table 1.1). Usage of physical cleaning methods like ultrasonic and spray cleaning, plasma, pyrolysis, UV/ozone and laser applications, are limited by the availability of specialized equipment. Besides, alkaline solutions are readily available to most labs and their disposal is less of an issue than with acidic effluents. Alkaline glass cleaning works by etching and incubation time is dependent on the level of glass contamination. Alcohol presence in the alkaline solution also helps removing the adsorbed organics from the surface, thus increasing the efficiency of the alkaline cleaning application(Aboytes *et al.*, 2003).

Table 1. 1 Comparison of various slide cleaning approaches (Aboytes *et al.*, 2003).

Method	Advantage	Disadvantage
Chromerge	Consistently effective cleaning.	Banned in many locations because of toxicity. High disposal expense.
Piranha Solution	Consistently effective cleaning.	Very reactive, and high disposal expense.
Hydrofluoric Acid	Very effective at removing contaminants. Fast reaction time.	Highly toxic, and slide damage can occur if process goes for an extended period.
Alkaline	Consistently effective cleaning.	Slower reaction, requiring longer incubation periods.
Ultrasonication	Clean surface with little strong solvent disposal.	Initial equipment requirement, and not as effective as the Piranha solution (Shirai 2000).
Plasma	Very effective cleaning method with a fast reaction time.	Requires a reactor
Pyrolysis	No solvent requirement.	Reactor/High heat environment. Large contaminants may produce soot residue.
Solution Sprayer	Effective for macromolecule removal only.	Not effective against smaller particle/organic contamination.
UV/ozone	Effective at removing thin film contaminants, very fast reaction time.	Thicker layers of contaminants will not be effectively cleaned. Initial equipment cost is high.
Laser	Effective cleaning method with a fast reaction time.	Initial equipment requirement, and possible recontamination if vacuum not applied.

1.2.2.2 Surface Coating Chemistries

Surface chemistry of the support material is a determinant factor for the probe immobilization chemistry, hybridization, and spot-size in the performance of a microarray study.

Glass can be coated either adsorptively, as with poly-I-lysine, or covalently, as with functionalized silanes. Glass surface interacts with the coating agents via combination of H-bonds, coulombic interactions and van der Waals' forces.

Functionalized silanes are used to introduce a variety of functional groups to glass slides with epoxy-silane and amino-silane being the most common (Guo et al., 1994; Lamture et al., 1994; Schena et al., 1995). These and other alkoxysilanes such as (3-mercaptopropyl)trimethoxysilane (Moller et al., 2000), bind covalently to glass via silylethers, leaving the alkylfunctional group available for further manipulation. The resulting silanized slides can be used directly to bind nucleic acids or be further reacted with a variety of homo- or heterobifunctional cross-linkers to introduce a variety of surface chemistries.

1.2.2.3 DNA Immobilization Chemistries

For specific detection, realized by a base-pair interaction with targets, the probes must be placed on the surface in an addressable structure (Heise and Bier, 2005). This addressable structure has great importance while designing the experimental strategy. Many factors like scope of the assay, support's surface structure, binding interactions with target, labeling strategy, limit of detection are building a matrix together to define the addressable structure. Nucleic acids can be immobilized onto glass surfaces in terms of covalent and/or non-covalent (ionic intercations) ways.

Ionic interaction strategy is the simplest method for surface immobilization of oligonucleotides as there is no requirement for any terminal or internal modification. Immobilization is based on electrostatic interactions occurring between the negatively charged phosphate groups present on oligonucleotide probe and positive charges on the surface (Sassolas *et al.*, 2008). Nucleic acid probes can bind via ionic interactions to PLL or APS coated surfaces that have positively charged amine groups (Figure 1.3). Surface binding of nucleic acids at multiple attachment points causes them to be conformationally restricted so thus they may tend to be less available for hybridization than covalent end bindings (Aboytes *et al.*, 2003).

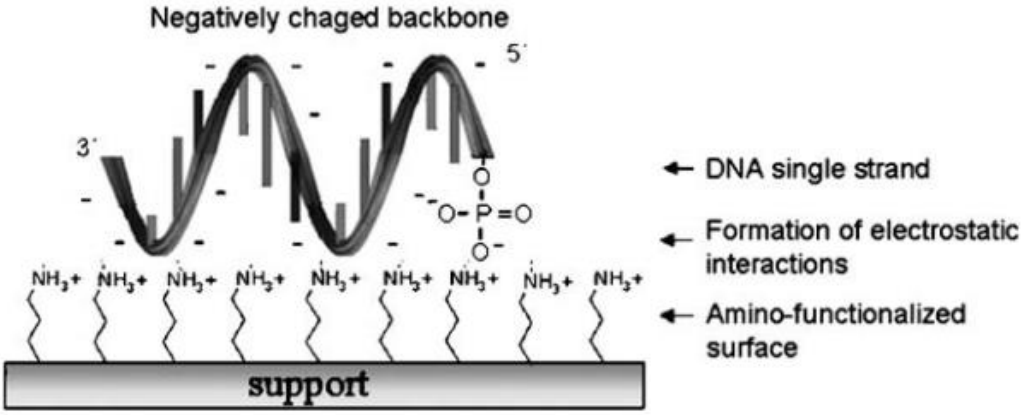

Figure 1. 3 Electrostatic interactions between DNA and charged surface (Heise and Bier, 2005).

On the other hand, covalent binding provides a more stable method for immobilizing DNA to surfaces. A covalent bond is formed by sharing of electrons between two atoms. The dissociation energy for a typical covalent bond is 100 kcal/mol and by far the strongest in biochemistry (Heise and Bier, 2005). This approach requires modification of oligonucleotide at its 5' or 3' end and so thus the surface, such that each partner presents a reactive pair of functional groups. The coupling reaction between the modified nucleic acid and the activated surface can be carried out directly or via a crosslinker moiety (Tjong *et al.*, 2014). Oligonucleotide probe can orient itself in reference to an engineered oligonucleotide end modification. This orientation increases the availability of slide-immobilized (probe) sequences for hybridization with target as the binding is not happening through the oligonucleotide's nucleotide bases or backbone. This kind of attachment also allows for more stringent washing so thus the less non specific binding (Aboytes *et al.*, 2003); (Beier and Hoheisel, 1999).

Various types of 3' or 5' oligonucleotide modifications are commercially available for covalently attaching DNAs to support platforms. These modifications include amine-oligos covalently linked to an activated carboxylate group or succinimidyl ester, thiol-oligos covalently linked via an alkylating

reagent such as an iodoacetamide or maleimide, acrydite-oligos covalently linked through a thioether, Biotin-oligos captured by immobilized streptavidin (Devor and Behlke, 2005). Chemistry of surface and DNA functionalizations must be appropriately matched to start an effective array platform.

Most commonly used surface modifications to immobilize DNA or oligonucleotides onto glass slides are: poly-L-lysine (PLL), 3-aminopropyltrimethoxysilane (APS), 3-glycidoxy-propyltrimethoxysilane (GPS), aldehyde or carboxylic acid.

Aminosilane coatings offer high concentration of available primary amino groups which become protonated so thus the positively charged when placed in contact with a near-neutral, aqueous solution. Negatively charged DNA probes during the are ionically attracted to this positively charged aminosilane surface. If covalent modification is required, ultraviolet (UV) cross-linking is generally used to achieve covalent linkages (Conzone and Pantano, 2004). On the other side cross linker molecules can be also used to acquire a covalent binding between amino modified surface and an oligonucleotide probe with various end modifications.

Similar with the aminosilane coated surface, poly-L-lysine (PLL) modified surfaces also provide ionic interaction of nucleic acid probes. PLL is a basic, synthetic, positively charged, poly(aminoacid) polyelectrolyte that adsorbs from aqueous solution to the negatively charged surface of glass. PLL coatings generally can be gained with budget prices than any other silane coatings but lack of longer shelf lives (Rampal, 2007).

Epoxysilane coated substrates are instead based on the irreversible, covalent, ring opening interaction between epoxide end groups in the coating and nucleophilic groups, such as amino groups, on the DNA probe (Conzone and Pantano, 2004). (Figure 1.4). The reaction can take place with primary amines, sulfhydryls, or hydroxyl groups to create secondary amine, thioether, or ether bonds, respectively. During the coupling process, ring opening forms a β-hydroxy group on the epoxy compound.

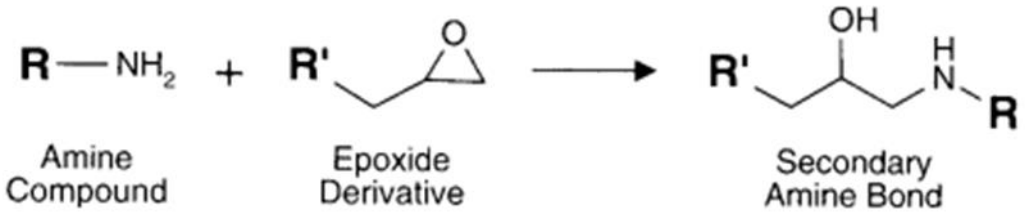

Figure 1. 4 Nucleophilic attack of epoxides by amines to form amino alcohols. R is epoxide modified glass and R' is 5' modified DNA (Hermanson, 2013).

The reaction of the epoxide functionalities with hydroxyls requires high pH conditions, usually in the range of pH 11–12. Amine nucleophiles react at more moderate alkaline pH values of at least pH 9.0. Amine functionalization of 3’ or 5’ ends of an oligonucleotide probe provides a binding parameter for an epoxy coated surface. Epoxy groups on the surface also form a covalent linkage with the primary amine groups on the bases of the DNA. Therefore, epoxy functionalized surfaces are preferred for the covalent immobilization of unmodified or amino modified oligonucleotides. Sulfhydryl groups are the most highly reactive nucleophiles with epoxides, requiring a buffered system closer to the physiological pH range of 7.5–8.5 for efficient coupling (Hermanson, 2013). The main advantages of epoxide chemistry are its relative stability and generally lower background than aminosilane (Taylor *et al.*, 2003).

Aldehyde-terminated silanes are another class of active chemistries used for DNA immobilization. Aldehyde functionalized surfaces selectively react with amine-terminated oligos through a well-known Schiff base reaction, which is often driven to completion through the addition of a reducing agent to the reaction solution.

1.2.2.3.1 Cross Linker Reagents

It is already known that covalent immobilization has a greatest advantage over ionic interactions with support surface. Covalent immobilization is stable under a

variety of denaturing and harsh conditions over long periods of time (Lim *et al.*, 2010). Silanized surfaces can be used directly to covalently bind nucleic acids or be further reacted with a variety crosslinkers having homo or heterobifunctional properties to introduce a variety of surface chemistries. (Sorribas *et al.*, 2001) (Blalock, 2003), but there have been also oligonucleotide immobilization ways on PLL coated slides via crosslinkers in a similar way like the silanized ones.

Surface activation can be done either by the use of zero-length crosslinkers, homobifunctional and heterobifunctional linkers, or trifunctional linkers (Figure 1.5).

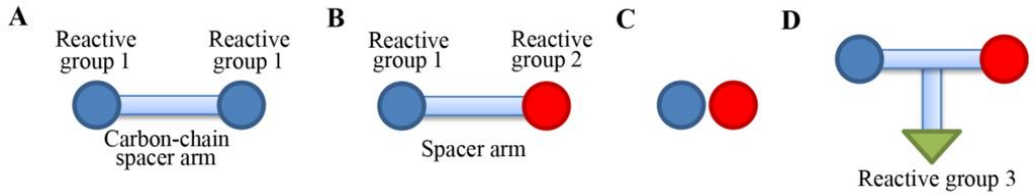

Figure 1. 5 Four types of cross links' applications (A) Homo-bifunctional; (B) Hetero-bifunctional; (C) zero-length; and (D) hetero-trifunctional cross-link (Pi and Sael, 2013).

Zero length cross-linkers activate functional groups on surfaces without any chain elongation, thus one atom of a molecule is covalently attached to an atom of a second molecule with no linker or spacer. All reactive surface modifications, for example aldehydes, epoxy-groups, and halogenated surfaces may also be termed as zero length crosslinkers in this approach.

Homobifunctional crosslinkers are generally symmetrical in design with a carbon chain spacer connecting the two identical reactive ends and they can conjugate two equal functional groups of reactants (Figure 1.6). The most often employed homobifunctional crosslinkers are 1,4-phenylene diisothiocyanate, pentanedial,

1,4-butanediole diglycidyl ether, disuccinimidyl carbonate, dithio bis (sulfosuccinimidyl propionate) and dimetylsuberimidate (Heise and Bier, 2005); (Hermanson, 2013).

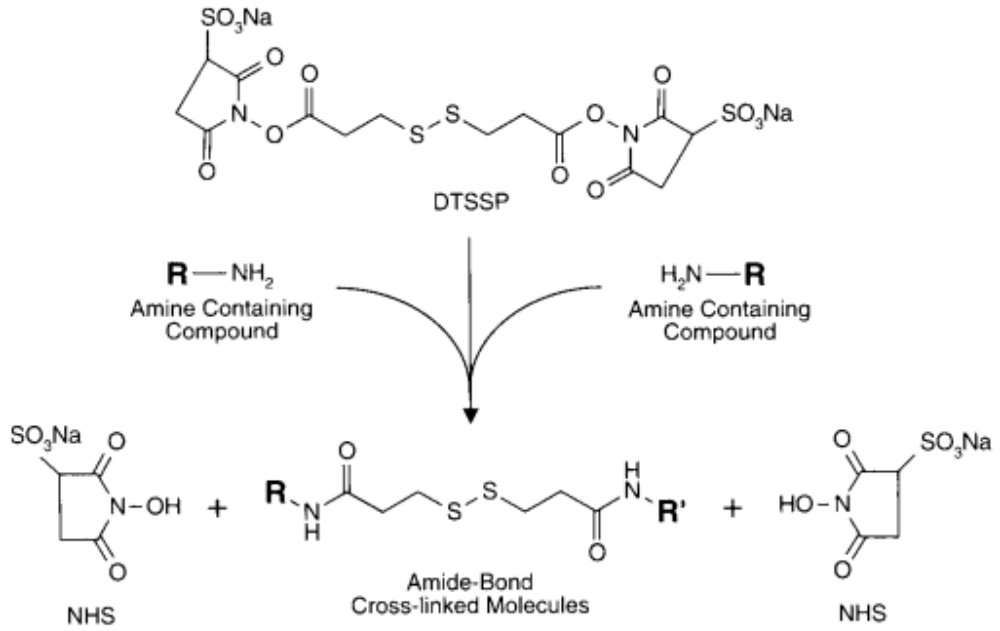

Figure 1. 6 DTSSP (dithio bis (sulfosuccinimidyl propionate)) can form crosslinks between two amine-containing molecules through amide linkages (Hermanson, 2013).

Heterobifunctional crosslinkers have reactive centers capable of reacting with two chemically unequal functional groups. For example, one part of a crosslinker may contain an amine-reactive group, while another portion may has a thiol-reactive group. The linkers are beneficial in immobilization approaches because they can covalently bind two distinct chemical entities which otherwise would remain unreactive towards one another, and they behave as physical spacers which provide greater accessibility to each of the linked biomolecules against steric interferences (Chrisey *et al.*, 1996a); (Chrisey *et al.*, 1996b); (Hermanson, 2013).

SM(PEG)n reagents and EMCS and its water-soluble analog Sulfo-EMCS are heterobifunctional crosslinkers with N-hydroxysuccinimide (NHS) ester and maleimide groups that allow covalent conjugation of amine and sulfhydryl groups. NHS esters react with primary amines at pH 7-9 to form amide bonds, while maleimides react with sulfhydryl groups at pH 6.5-7.5 to form stable thioether bonds (Figure 1.9). Conjugation experiments involving this type of heterobifunctional crosslinker are usually performed at pH 7.2-7.5, with the NHS-ester (amine-targeted) reaction being accomplished before or simultaneous with the maleimide (sulfhydryl-targeted) reaction. SM(PEG)n reagents have soluble polyethylene glycol (PEG) spacer arms. These spacer arms improve water solubility of reagent and conjugate, reduce the agglomeration potential of the conjugate, increases flexibility, and so thus reducing the negative effects of steric hindrance on immobilization kinetics. Spacer arm lengths are remarked as "n" and they vary from 2 to 24 (Figure1.7) (Pierce, 2009).

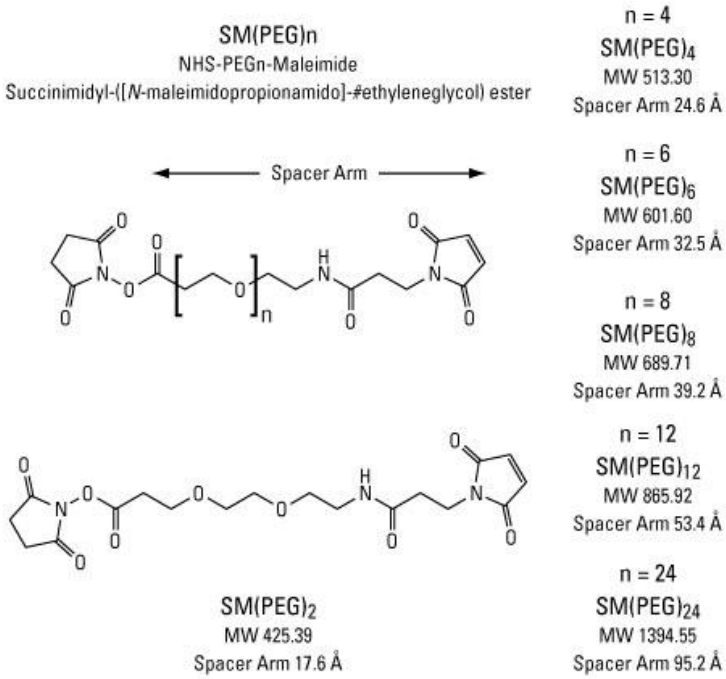

Figure 1. 7 Chemical structures of SM(PEG)n crosslinking reagents (Pierce, 2009).

Sulfo-EMCS has solubility in water and many other aqueous buffers. On the other hand, EMCS must be dissolved first in an organic solvent such as dimethylsulfoxide (DMSO) or dimethylformamide (DMF) (Figure 1.8)

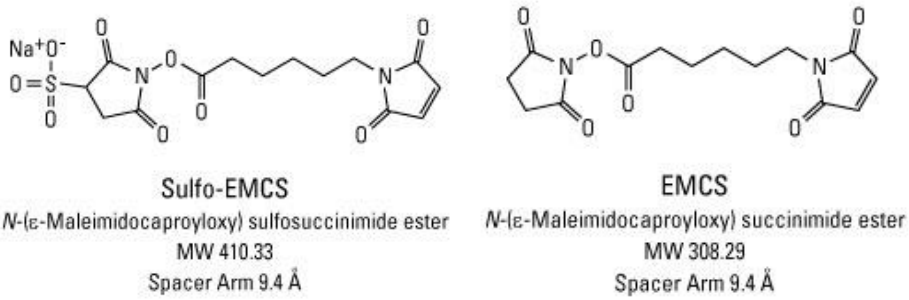

Figure 1. 8 Chemical structure of Sulfo-EMCS and EMCS crosslinking reagents (Pierce, 2009).

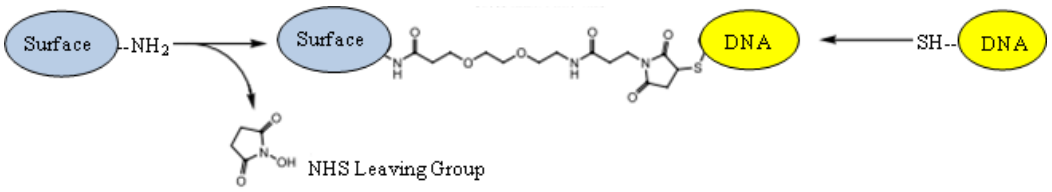

Figure 1. 9 Crosslink formation by reaction of $SM(PEG)_2$ with amine modified surface and thiol modified oligonucleotide probe

1.2.2.3.2 Steric Hindrance and Spacer Molecules

Steric hindrance, which may vary with probe density, spacer length, as well as hydrophobicity and charge of the solid support, is one of the main parameters that affect the efficiency of hybridization between target DNA and immobilized probes (Chizhikov *et al.*, 2001). It is one of the main constraints induced by attaching one end of the surface probe to a solid support thus preventing the target in solution to make close approach to the immobilized surface probe.

Spacer molecules, which can be mainly itemized as polyethylene glycol based, alkyl (poly carbon) based ones (Figure 1.10) and poly thymine (poly T) types, have been generally used in array studies to ease the interfering effects of the surface.

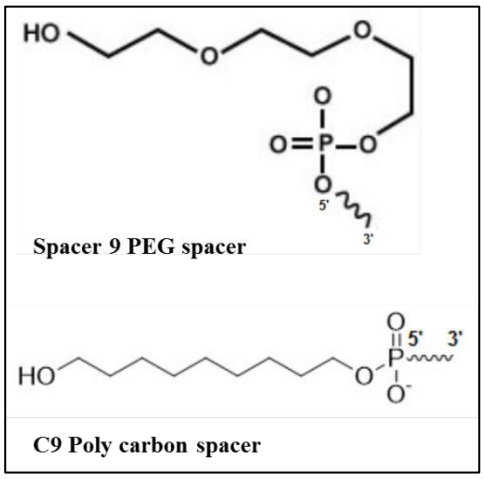

Figure 1. 10 Molecular structure of PEG and poly carbon spacers

Each of them has different characteristics. PEG spacers are hydrophilic and flexible, the poly-C spacer is hydrophobic, and the poly T spacer is negatively charged, flexible, and hydrophilic. PEG8 (8 PEG repeats), C 24 (24 carbons long),

and T 10 (10 thymine nucleic acids) spacers have roughly identical lengths (Waybrant *et al.*, 2014).

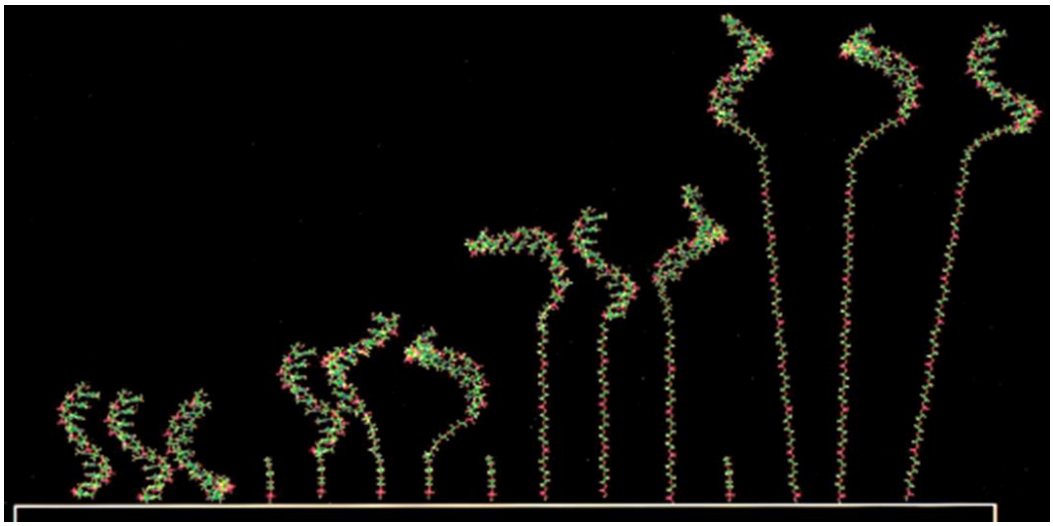

Figure 1. 11 Theoretical view for spacer (oligoethylene glycols 26, 60 and 105 atoms in length, respectively) effects on the accessibility of oligonucleotide probes on surface (Southern *et al.*, 1999)

Spacers help to overcome steric interference, and this effect can be examined in Figure 1.11 that the ends of the probes closest to the surface are less accessible than the ends furthest away; immobilized molecules are crowding each other. More effective interaction with the target can be achieved when the oligonucleotides are better able to extend away from their neighbours via their long spacers (Southern *et al.*, 1999). This case also described by Shchepinov *et al.* (1997) with the effect of solution state on molecular interactions. They basically said that the more an immobilised molecule is spatially removed from the solid support the closer it is to the solution state and the more likely it is to react freely with dissolved molecules.

1.2.2.3.3 Surface Blocking Strategies

Blocking reactions are typically used to prevent labeled probes and target products from unspecific attachment to the surface of the array (surface probe immobilized) during the hybridization reaction. Blocking methods also provide the advantage of washing away unbound oligonucleotides from the surface that would otherwise compete with the labeled species (Hegde *et al.*, 2000); (Taylor *et al.*, 2003). Some of the blocking reagents are Tween 20, polyethylene glycol, casein, milk proteins and serum albumins. Bovine serum albumin (BSA), is the most preferred and commonly used reagent mainly because of its low cost and easy to prepare in solution phase. BSA is generally used at a 1 to 3% concentration in neutral buffers. It is inexpensive and can be stored dry or as a sterile solution at 4°C (Brorson, 1997); (Heller, 1995); (Jeyachandran *et al.*, 2010); (Reimhult *et al.*, 2008).

1.2.3 Solid-Phase Hybridization of Oligonucleotide Probes

In nucleic acid hybridization, an oligonucleotide probe is allowed to anneal to its complementary strand which possibly is present in the sample. This offers an extremely specific way to identify and quantify the gene or gene region in the sample of interest (Söderlund, 1989). This phenomenon is well understood in solution state. However, adequately understanding solid phase hybridization is much more complex. Interactions of immobilized strands, referred to as 'probes', with 'target' sequences from solution occurs at a solid liquid interface in solid-phase or 'surface' hybridization systems. The interfacial area is distinct from the bulk solution; thus, surface hybridization might be reckoned to deviate from expectations merely based on acquintance of solution hybridization (Levicky and Horgan, 2005).

Surface chemistry, interaction area of surface and probe and also surface probe density are the site specific influencing factors for solid phase hybridization. Salt concentration, pH, temperature, GC content, and probe length are the common

factors that influence both the solid phase and solution based hybridization systems.

It has been experimentally shown that high probe coverage suppresses surface hybridization and also decreases the rate of hybridization (Henry *et al.*, 1999); (Peterson *et al.*, 2001); (Steel *et al.*, 2000); (Steel *et al.*, 1998); (Walsh *et al.*, 2001). However, when probes are too much aparted to come into contact, parts of bare surface will be accessible to binding of target strands thus it is also assumed that targets might first bind and then diffuse along the solid support before hybridizing to a probe. It was also shown that the hybridization rate increases with strand length, as might be expected from longer probe-target pairs having more sites for initiation of duplex formation. However, a delay in initiation of hybridization is also observed for longer pairs. By contrast, it needs significantly less time for shorter pairs to be arranged (Chan *et al.*, 1995); (Levicky and Horgan, 2005)

Proximity to a solid support affects the probe accessibility to hybridization with the target. Efficiency is improved with increased distance from the surface, consistent with others' studies in which incorporation of molecular spacers to displace probes from the solid support led to higher hybridization yields (Guo *et al.*, 1994); (Shchepinov *et al.*, 1997). Strong and undesired probe-surface interactions, much likely electrostatic events, also suppresses the hybridization efficiencies (Levicky *et al.*, 1998)

In the context of these topics, it is clearly seen that the probe and target sequence and functionalization designs together with the surface chemistry is much of an issue to be challenged about hybridization events in realising the specific objectives of the platform to be developed.

Electrostatic effects are also playing important roles in addition to steric effect in the solid-phase hybridization processes because of the negative charge of oligonucleotides. Cations compensate for the negative charge of the oligonucleotide backbone and stabilize the oligonucleotide complexes. Their shielding effects are more pronounced in solid-phase hybridization in comparison

with hybridization in a solution due to the high surface density of negatively charged probes in the layer arrangement (Špringer *et al.*, 2010). That is the reason to use salt including buffers in immobilization and hybridization events of oligonucleotides. (Fotin *et al.*, 1998); (Gao *et al.*, 2006); (Levicky and Horgan, 2005). It is observed that the yield of the probe immobilization on the surface increases with an increase of the concentration of monovalent sodium in the immobilization buffer (Peterson *et al.*, 2001), however, increment in salt content reduces the stringency of discrimination between mismatched sequences. Cho *et al.* (2004) showed that the determinant parameter influencing the probe density and subsequent hybridization is the total concentration of sodium, whereas the type of buffer had only a minor effect. Even more, Springer *et al*. (2010) states that the divalent magnesium is found to be much more effective in hybrid stabilization than monovalent sodium which is the generally used one in immobilization and hybridization buffers.

Higher GC content increases the stability of DNA duplexes as the melting temperature increases with GC%. Melting temperature (Tm) is a practical measure of the stability of a nucleic acid duplex which corresponds to the midpoint in the observed transition from double-stranded to single-stranded form. Hybridization conditions are chosen to promote heteroduplex formation and the hybridization temperature is often as much as 25°C below the Tm. Besides, for oligonucleotide probes, hybridization temperature nearly equals 5°C-10°C below the Tm. Probe-target heteroduplexes are most stable thermodynamically when the region of duplex formation contains perfect base matching. Increasing the concentration of cations in buffer systems and reducing the temperature reduces the hybridization stringency, and enhances the stability of mismatched heteroduplexes. On the other hand, lowering the concentration of cations and increasing the temperature maximizes hybridization stringency via facilitating the denaturation of mismatched duplexes (Keller and Manak, 1993); (Strachan and Read, 2010).

1.2.4 Sandwich Hybridization

Direct hybridization or sandwich hybridization are the two different strategies that can be employed when performing nucleic acid hybridization assays. Direct hybridization method involves the hybridization of surface attached oligonucleotide probe with the previously labeled target molecule. On the other hand, partial hybridization of immobilized oligonucleotide probe can be achieved to a part of the target whereas the other part of the target is complementary to another oligonucleotide probe which is actually the reporter or the signalling part of the system (Cansız, 2010). In sandwich hybridization format, one universal probe is labeled or functionalized for a specific signalling reaction rather than labeling the many targets coming from any source (Figure 1.12) This technique reduces the time, cost and challenging steps to prepare individually labeled targets to be detected and so thus enhances the overall condition of the assays in terms of time, cost, and specificity.

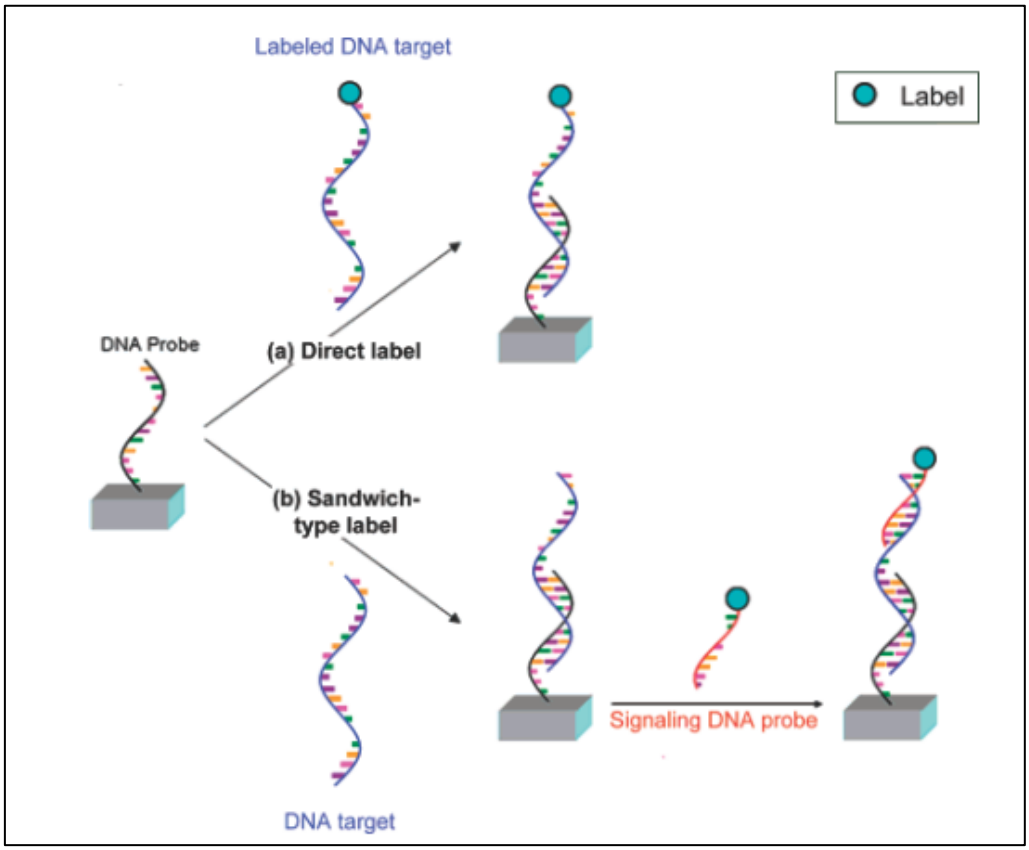

Figure 1. 12 Schematic representation for labeled probes in DNA biosensors and microarrays. (a) direct hybridization (b) sandwich hybridization (Sassolas *et al.*, 2008).

In addition to convenience in labeling related signal acquirement strategies, sandwich hybridization is more specific than direct hybridization assays because two hybridization events must occur in order to generate a signal.

In 1977, nucleic acid sequence analysis with sandwich hybridization was first studied (Dunn and Hassell, 1977). It has since been modified and used not only for the detection of pathogens, bacteria, viruses etc., but also for the analysis of sequence polymorphisms and genetic modifications in targets of interest.

Detection specificity, cost and reliability of the detection equipment and methods and amount of the target needed, are the common factors that limits the usage of the microarrays in the research and diagnostic applications. Labeling and

detection methods are the critical factors for these parameters. In sandwich hybridization based formats, no requirement for target to have a label, provides flexibility in the signal development strategy and so thus the detection equipment for signal acquiring. In this context, a signal probe can be labeled in a variety of ways like metal nanoparticles, fluorescence, radiolabelling, biotin, or an enzyme linked assay such as the oxidation reaction of TMB which gives blue coloured visual products (Storhoff *et al.*, 2005).

1.2.5 Application Areas for Nucleic Acid Based Arrays

Nucleic acid based microarrays are a variety of platforms used in gene-expression analysis, genotyping of individuals, analysis of point mutations and single nucleotide polymorphisms (SNP) as well as other genomic or transcriptomic variations (Bier *et al.*, 2008). Research and development processes, manufacturing and usage of these platforms are exponentially growing against or together with the culture based, sequencing and amplification based methods especially in the areas of medicine, pathogen detection, contaminant and genetic modification analysis in food and feed.

In public health, it is essential to minimize the prevalence of food borne diseases and reduce microbial contaminations in foods. Currently, conventional methods are based on cultivation of target pathogens or indicator microorganisms on specific media which are time consuming and sometimes lacking specificity in selecting or identifying unknown pathogens in foods (Abubakar *et al.*, 2007)(Abubakar *et al.*, 2007); (Suo *et al.*, 2010). There have been also high throughput sequencing or quantitative PCR based methods which have high costs and need for specialized equipment and personnel. On the other hand, developing array based methods are open to modifications to be used as hand held devices for point-of-care diagnostics or field assays. Many platforms are also under study to use cheaper materials, reducing the need for specialized costly equipments and specific, expert users. Recently, Huang *et al.* (2014) established a sensitive and specific oligonucleotide microarray using quantum dots as labels to detect food-

borne pathogenic bacteria.Test was performed on glass slides and signals gathered from QDs were red by a scanner, even it is also possible to visualize by a common UV transilluminator.

Cervical cancer is the second most frequent female malignancy worldwide in which the main cause is human papilloma virus (HPV). The careHPV test which is being developed by qiagen, is a powerful, rapid, and precise HPV-DNA detection method in which antibodies bind to magnetic beads, rapidly capturing specific target HPV nucleic acid sequences, which are then detected using a chemiluminescence signal. The test can be performed without electricity or running water and offers HPV detection results in a matter of hours specifically for application in low resource public health settings to screen women (Qiao *et al.*, 2008); (Ying *et al.*, 2014). Besides, Genomica company improved "clinical arrays® human papillomavirus" low density array system for the detection 35 HPV genotypes. The system has the steps of obtaining samples, DNA extraction and purification, amplification, hybridization and automated detection and interpretation of results. The most interesting property of the product is having the array on the bottom of the tube.

Detection of label free genetically modified organism (GMO) target DNA sequences was also achieved with a sandwich hybridization based array platform (Cansiz *et al.*, 2012). This customly based system does not require specialized printers and scanners and offers an economical target detection platform for multiplex GMO target sequences in plant originated samples.

Meanwhile, the development of nucleic acid based arrays that capable of scoring large numbers of single nucleotide polymorphisms (SNPs) made great progress, and became widely used. SNP arrays are also used for direct to consumer (DTC) genetic profiles. They do valid and useful results and have been extensively practised, providing a large market for these SNP arrays which are now the dominant species in the DNA array world (Gunderson *et al.*, 2006); (Jordan, 2012).

1.3 Single Nucleotide Polymorphisms and Detection Platforms

Changes in the sequence of particular genes cause many genetic diseases which might be also malignant and the most abundant form of genetic variation is the single nucleotide polymorphisms (SNPs) (Antonarakis *et al.*, 1985); (Landegren *et al.*, 1988a); (Wabuyele *et al.*, 2003). At the vast majority (some 99%) of genomic sites, every human carries the same base residue on both chromosomal homologs. The remaining codes much of the diversity among humans, including differences in disease susceptibility. Major part of these DNA variants are compromised of "single nucleotide polymorphisms (SNPs)", "genome positions at which there are two distinct nucleotide residues (alleles) that each appears in a significant portion of the human population". They occur approximately once in every 300-1000 nucleotides (Cutler *et al.*, 2001); (Dong *et al.*, 2001); (Hacia *et al.*, 1999); (Schafer and Hawkins, 1998). In literature, there is now detailed information about SNP frequency within specific genes and the dispersal patterns of SNPs across the human genome (Cargill *et al.*, 1999); (Halushka *et al.*, 1999).

Most common single gene disorders in humans are "Hemoglobinopathy" and "thalassemia". About 7% of the world population carries a globin gene mutation, and in the vast majority of cases it is inherited as an autosomal recessive trait. Hemoglobins are the proteins containing iron-protoporphyrin IX (heme) as a prosthetic group. In adult humans, the most common hemoglobin type is a tetramer (which contains 4 subunit proteins) called hemoglobin A, consisting of two α and two β subunits non-covalently bound, each made of 141 and 146 amino acid residues, respectively. This is denoted as α2β2 and encoded by the α and β globin gene loci. Inherited abnormalities of the hemoglobin tetramer may be itemized into two categories: those that are characterized by structural anomalies of the hemoglobin chains, and others that result from an array of molecular defects that either reduce or completely restrict the synthesis of one or more of the polypeptide chains of the hemoglobin molecule. The term 'hemoglobinopathy' refers to the former disorders, whereas the latter defines the term 'thalassemia (Schafer and Hawkins, 1998); (Watson, 1954). To date, over 1,200 different mutant alleles have been characterized at the molecular level and each country has

its own mutational spectrum of Hb variants and thalassemia mutations. In Turkey being located in Mediterranean region, the most frequently observed mutation is IVS–1–110 (G>A). Around Denizli district in Aegean Region, the rate of β thalassemia carriers with abnormal hemoglobins is 3,5% according to the records of Department of Public Health, Denizli. The incidence of β thalassemia is 2,1% in our country and this rate differs according to the regions. In our country a prevention program for hemoglobinopathies is carried out in 33 cities by the Ministry of Health of Turkish Republic. The basic aim of this program is the determination of possible risks fort the couples who are planning marriage in the premarital period and help the born of healhy individual by prenatal diagnosis in pregnancy period (Akar *et al.*, 1987); (Atalay *et al.*, 2005); (Hardison *et al.*, 2001); (Patrinos *et al.*, 2004); (Tadmouri and Basak, 2001); (Weatherall and Clegg, 2008). In this context, it is clearly seen that new, accelarated, cheap and contemporary molecular methods like specific array platforms are needed to be introduced into practice for hemoglobinopathies and other SNPs in the country.

Many new technologies for SNP genotyping have been developed that can be classified as: (a) allele-specific hybridisation, for example, TaqMan™ assay (Livak, 1999) and molecular beacons assay (Tyagi and Kramer, 1996; Tyagi *et al.*, 1998), oligonucleotide microarrays (Wang *et al.*, 1998; Cargill *et al.*, 1999; Hacia *et al.*, 1999); (b) allele-specific nucleotide incorporation, like primer extension (Pastinen, 2000), and pyrosequencing (Nyren *et al.*, 1993; Alderborn et al., 2000); (c) allele-specific oligonucleotide ligation (OLA, ligase chain reaction (Barany, 1991), padlock probes (Nilsson et al., 1997), and rolling circle amplification (Banér et al., 1998; Lizardi et al., 1998) and (d) allele-specific invasive cleavage invader assay (Hall et al., 2000) and the still widely used traditional method restriction site analysis (Kwok, 2001); (Schwonbeck *et al.*, 2004).

Nowadays, important genotype-phenotype relationships are being unraveled and personalized medicine is becoming a medical reality. Many SNPs precondition the individuals to have a certain disease or trait or react to a drug in a different way, thus they are highly useful in diagnostics and drug development. Their

analysis is also used for agriculture, food testing, identity testing, pathogen identification, drug discovery and development, pharmacogenomics and even nutrigenomics researches (Liboredo and Pena, 2014); (Carlson, 2008). There are a variety of studies in literature for microarray or nucleic acid chip based detection methods. There have been also many prototypes on development and products on the market that some of them will be presented here.

Journal of Analytical Chemistry published a study in 2009 which presents an integrated system that can detect α thalassemia from saliva samples in a rapid way. Genes can be specifically amplified by the on-chip PCR module and immediately detected using the optical detection module (Lien *et al.*, 2009). GenFlex Tag Array, which is a product of Affymetrix Company, was also developed to be used in performing mutation analysis at B-globin gene. The GenFlexTM Tag Array enables the interrogation of up to 2000 nucleic acid reaction products. Through collaboration with ASPER Biotech (Tartu, Estonia), Papasavva *et al.* (2006) developed a microarray system called "thalassochip." It contains all the common and frequent mutations and polymorphisms of the Mediterranean region. Another product named as globin strip assay which could be used for diagnosis of α and β thalassemia, was developed and marketed by ViennaLab Diagnostics (Austria). The Globin StripAssay is based on reverse hybridization of biotinylated PCR products to a parallel array of allele-specific oligonucleotides immobilized on membrane test strips. Hybridization-triggered fluorescence strategy for label-free and multiplex miRNA detection on graphically encoded silica suspension array was also developed by Jiang *et al.* (2014).

Metal nanoparticles are also introduced into SNP detection platforms both for specificity and signalling approaches. Bao *et al.* (2005) published a microarray based method that allows multiplex SNP genotyping in total human genomic DNA without the need for target amplification.

Oligonucleotide ligation assays (OLA) are rapid, specific and sensitive reactions for the detection of known point mutations and they are based on the covalent joining of two adjacent oligonucleotide probes by a DNA ligase when they are

hybridized to a complementary DNA template. (Figure 1.13). These characteristics allow non-stringent ligation conditions, which can be used to type multiple nucleotide substitutions in a single assay (Landegren *et al.*, 1988b); (Tobe *et al.*, 1996).

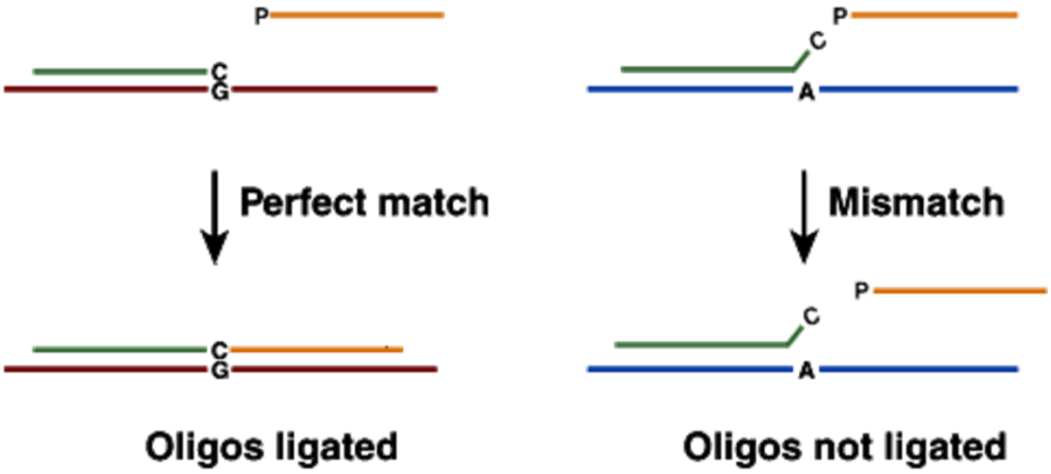

Figure 1. 13 Principle of oligonucleotide ligation assay. Template DNA corresponding to the wild-type (WT) allele is denatured to form a single-stranded target DNA. Allele-specific and common probes are hybridized to the target DNA. The WT allele-specific and the common probes are ligated to each other by DNA ligase enzyme since the template DNA is complementary to the WT allele-specific probe (perfect match, left). No ligation occurs between the mutant allele-specific and the common probe, since there is a mismatch between the template DNA and the mutant allele-specific probe (mismatch, right)

Beginning of the development of several analytical methods utilizing DNA ligases, for example, OLA and ligase chain reaction (LCR) was started in 1988, ligate short single-stranded DNA probes as a means to detect sequence variants (Alves and Carr, 1988; Landegren *et al.*, 1988). In 1990, thermostable DNA ligase became available commercially and enabled the temperature cycling of the ligation reaction (Barany, 1991). Furthermore, the breakthrough in OLA began in 1990 when PCR was coupled to the assay prior to ligation reaction (Nickerson *et al.*, 1990). These improvements made this assay more useful for nucleic acid based diagnostics of inherited diseases in the clinical laboratories. Nowadays, for the detection of a wide variety of relevant mutations, the method is used in numerous clinical and research laboratories worldwide (ROMPPANEN, 2005). Deng *et al.* (2004) developed a solid phase platform that the capture probes were immobilized on glass slides, and signal probes having biotin modifications for further enzyme based visualization. Ligation reactions between probes in the

presence of a complementary target were carried out on chips in the presence of T4 DNA ligase and the products were directly visualized on chips through enzyme-linked assay. Zhang *et al*. (2008) described an on-chip oligonucleotide ligation approach that arrayed a series of functionalized beads in a single microfluidic channel for detection of low-abundant point mutations in p53 gene.

1.4 Aim of the Study

In this study, the main aim is to develop a sandwich hybridization based detection platform for single nucleotide polymorphisms (SNPs). In the frame of this purpose, different approaches and platforms were used to improve the single nucleotide mismatch discrimination ability and to investigate the applicability of various materials and environments for the system.

During the time period; custom modification of different surfaces, nucleic acid probe designs under the criteria of mismatch detection and their immobilization, optimisation of the sandwich hybridization conditions, application of different signal methods to the platform, integration of OLA method and nanoparticle based controlled release approaches to the systems are performed.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

In this study all chemicals were chosen in the analytical grade category and purchased from Sigma-Aldrich (USA), Merck (Germany) or AppliChem (Germany) unless otherwise specified. All solutions were prepared with ultrapure water which has 18.2 MΩcm resistance.

2.1.2 Buffers and solutions

Preparations and composition of buffers and solutions were given in Appendix A.

2.1.3 Oligonucleotides used in the study

All oligonucleotides were purchased from Integrated DNA Technologies (IDT) with standard desalting or HPLC purification and used without further purification. They were resuspended in nuclease free water up to defined stock concentrations and stored at -20°C. Sequences and their modification are given in tables under the related chapters.

2.1.4 Crosslinker and disulfide bond reducing agent

Cross-linkers were purchased from the company Pierce (IL, USA). Sulfo-EMCS (N-[ε-maleimidocaproyloxy]sulfosuccinimide ester) (MW: 410.33 g/mol) with a 9.4 Å spacer arm length and $SM(PEG)_2$ (succinimidyl-[(N-maleimidopropionamido)-diethyleneglycol]ester) (MW: 425.39 g/mol) with a 17.6 Å spacer arm length were used as heterobifunctional crosslinkers. Reducing disulfide bond modifications on oligonucleotides ends' was achieved with TCEP (Tris(2-carboxyethyl)phosphine) and free thiol groups were formed.

2.1.5 Support Materials

Standard sized Poly-L-lysine (PLL) coated microscope slides were purchased from the company Menzel Gläser (Braunschweig, Germany). Standard sized, uncoated microscope slides were bought from the company Marienfield GmbH & Co. (Lauda-Königshofen, Germany) for silanization experiments. Glass capillary tubes with an interior diameter of 1,1 mm were purchased from Paul Marienfeld GmbH & Co. Mesostructured MCM-41 hexagonal silica particles with 4.5-4.8 nm unit cell sizes were also used in the study. Their pore volume and pore sizes are 0.98 cm^3/g and 2.1-2.7 nm, respectively. HF180 coded Hi-Flow ™ Plus nitrocellulose membrane (Millipore) was used in the study which has 180 ± 45 (sec/4cm) capillary flow times. They are backed by the producer on 6 cm x 30 cm (± 0.05 cm) standard sized backing cards. (Standard backing material: 250 μm (± 50 μm), white polystyrene). They were cutted in 3 mm wide as for the preparation of seperate strips. Porous matrices that are used for the sample application pad and absorbent pad were purchased as cellulosic fiber materials under the SureWick®name (Millipore).

2.1.6 Coating Materials

Epoxy silane (3-glycidoxylpropyltrimethoxysilane) and amine silane (3-aminopropyltrimethoxysilane) coating materials were purchased from Sigma Aldrich and Fluka, respectively.

2.1.7 Fluorescein and Quantum Dots

Qdot® 565 ITK™ Streptavidin Conjugate Kit from Invitrogen provides the Qdot® streptavidin conjugate. A single isomer derivative of fluorescein, 6-FAM (6-carboxyfluorescein), is attached to 3' end of oligos by manufacturer upon request.

2.1.8 Horseradish Peroxidase and 3,3',5,5'-Tetramethylbenzidine

Horseradish Peroxidase and 3,3',5,5'-Tetramethylbenzidine (TMB) were purchased from Pierce Biotechnology.

2.2 Experimental Strategy

This study comprises four main parts; construction of lab-made glass platforms, single nucleotide polymorphism detection strategies based on sandwich hybridization, oligonucleotide ligation reaction mediated sandwich hybridization and silica nanoparticle supported lateral flow strip platforms (Figure 2.1).

The classical structure of a DNA microarray platform is fundamentally based on a solid, inert, impermeable support and a coating that links the inorganic glass to biomolecular probes suited for hybridization. The composition and morphology of the solid support and the choice of surface chemistry affect several critical requirements for a succesful array platform. In the context of these issues, first part of this study is designed to acquire a customly designed functionalized surface for the specialized needs of the system.

Sandwich hybridization system offers a number of advantages when compared to the direct hybridization. First of all, labeling is not required for the target molecule which provides flexibility in the signal development strategy thus a signal probe can be functionalized via a variety of molecules like fluorescent, biotin, nanoparticles, etc. Furthermore, as two hybridization events must occur to generate a signal, sandwich hybridization is stated to be more specific than direct hybridization assays. Second part of the study takes the advantage of sandwich

hybridization format in order to detect single nucleotide polymorphisms in target sequences.

Detailed examinations for surface functionalization chemistry, substantial issues on immobilization of probes and oligonucleotide-surface interactions were done in both of these two parts.

In the third part of the study, to develop a relatively more effective platform for discriminating single nucleotide mismatches, different solid support systems and enzymatic reactions were used as alternative approaches. The platform explained in this part inside glass capillary tubes, can detect single nucleotide polymorphisms through the oligonucleotide ligation assay still in sandwich hybridization format. Signalling mechanism is based on the HRP catalyzed oxidation reaction of TMB and coloured products can be detected with unaided eye.

Outcomes of the first three parts structure the last part of the study which mainly uses surface functionalization chemistries, surface-probe interactions and HRP/H_2O_2/TMB reaction system for signalling. Distinctively, silica nanoparticles and nitrocellulose membrane based lateral flow platforms are used in a combined way for controlled release of TMB substrate; dependent on the full complementarity of probes. HRP catalyzed oxidation reaction of 3,3',5,5'-Tetramethylbenzidine (TMB) also constitutes the signalling mechanism in which the blue coloured products can be observed via unaided eye.

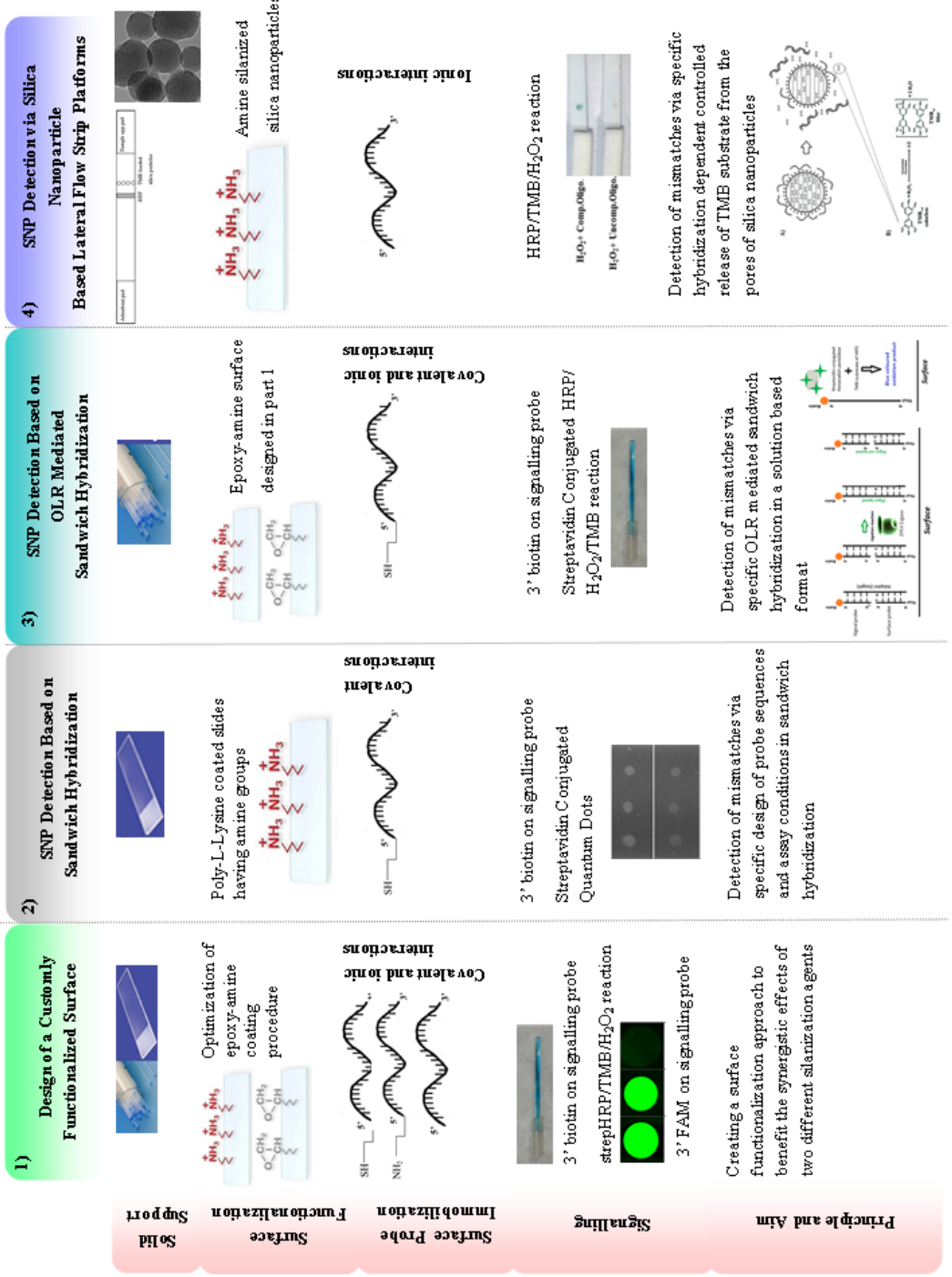

Figure 2. 1 Overview for experimental strategy. 1) Design of a customly functionalized surface, 2) SNP detection based on sandwich hybridization, 3) SNP detection based on OLR mediated Sandwich hybridization, 4) SNP detection via silica nanoparticle based lateral flow strip platfroms

2.3 Methods

2.3.1 Construction of Lab-Made Glass Platforms

Two types of glass platforms were used in the study as "glass capillary tubes" and "glass microscope slides". Glass capillary tubes with an interior diameter of 1.1 mm and commercially sold 76 x 26 x 1 mm sized uncoated glass microscope slides were used in this study. Surface cleaning and functionalization were done based on a previous method of (Chiu *et al.*, 2003) with some modifications.

2.3.1.1 Cleaning the Glass Slides and Glass Capillary Tubes

Glass capillary tubes were cleaned by soaking into 10 % (w/v) Sodium hydroxide (NaOH) solution in 60 % (v/v) ethanol for at least 3 hours with gentle shaking at room temperature, rinsed with ultrapure water 5 times with shaking. Approximately 30 capillaries can be cleaned in this way by using a single 50 mL plastic tubes. Following the washing steps capillary tubes were put in large centrifuge tubes and then dried by spinning at 1000 g for 2 min. Glass microscope slides were also cleaned in a similar way but using staining jars according to Hellendahl which have 75 mL liquid holding capacity. Slides were dried with Slide Spinner at 4800 rpm, for 15 seconds.

2.3.1.2 Epoxy Amine Coating of the Glass Slides and Glass Capillary Tubes

The coating solution was made by stirring a mixture of between 0.2 and 1 % epoxy (3-glycidyloxypropyltrimethoxysilane, GPS) and amine (3-aminopropyltrimethoxysilane- APS) at 4°C for 30 min in ultrapure water. In order to find proper epoxy and amine silanes' concentrations and their combinations, different parameters were designed in trials as shown below (Table 2.1). In these trials uncoated glass surfaces were used as negative control elements. Additionally, ultrapure water or absolute acetone were also compared by using seperately as solvents, and different concentration ratios for silanes and epoxy resin material were also tried in different experiments (Table 2.3). Glass

slides were then immersed in and capillary tubes were filled with coating solution for 5 minutes before being spun dry, and then were put in an oven at 80°C for 20 hours.

Table 2. 1 Combination ratios of epoxy and amine materials in coating solution

Coating material	**Epoxy (3-glycidyloxypropyl trimethoxysilane)**	**Amine (N,N-diethyl 3- aminopropyl trimethoxysilane)**
Percent Value (v/v) in solvent	0.2 1 0.2 0	0.2 0.2 0 0

2.3.1.3 Determination of Probe Immobilization Efficiency of Epoxy-Amine (EA) Coated Surface

Different parameters were designed in trials as shown in Table 2.1 above in order to choose proper epoxy and amine silanes' concentrations and/or their combinations. A 1:1 ratio as 0.2 % epoxy and 0.2 % amine solution was chosen and used for the following experiments.

In order to observe the performance of newly created surface in terms of binding reactions between epoxy amine (EA) surface and different end modifications on oligonucleotides three different probes were used in immobilization studies (Table 2.2). Thiol modified and unmodified probes were designed specifically to hybridize a part of Cauliflower Mosaic Virus (CaMV) 35S promoter. Amino

modified probe is an aptamer sequence having affinity for thrombin (Bock *et al.*, 1992).

Table 2. 2 Oligonucleotides used during probe immobilization optimization steps

	5’	3’	Sequence
End Modification	Amine (NH_2)	FAM	5’/5AmMC6T/ATAGGTTGGTGTGGGTTGG/6-FAM/-3’
	Thiol (SH)	FAM	5’/5ThioMC6D/AAAAAAAAAGCATCTTCAACGATGGCCT TTCCTTT/6-FAM/-3’
	None	FAM	5’TCGCAATGATGGCATTTGTAGGAGC/6-FAM/-3’

Related to the different chemical reactivities of different end modifications towards EA coated surface, independent immobilization mixes were prepared for each of the three probes. Final concentration of 10 µM for probe with a 5’ thiol group, heterobifunctional cross linker Sulfo EMCS and TCEP with final concentrations of 2 mM and 0.1 mM, respectively were mixed in 10 mM (pH 7.2) phosphate buffer (PBS). Reduction of disulfide bonds needed a 30 min incubation at room temperature. 10 µM of probe with a 5’ amine group and 5’ unmodified probe were prepared in 150 mM (pH 8.5) sodium phosphate buffer without crosslinker or S-S bond breaker TCEP. Prepared immobilization solutions were applied on individual glass slides with a volume of 0.5 µL for each spot with a micropipette. Incubation for spotted slides took 4 hours at room temperature in a petri dish containing dH_2O at the base. At the end of incubation, slides were put in jars according to Hellendahl which had been filled with 150 mM Sodium Phosphate buffer (pH 8.5) including 0.1 % or 0.5 % SDS, in order to

optimize washing stringency (Table 2.3). Slides were washed with 130 rpm agitation during 10 minutes. After completing the main washing step, washing solution was discarded and jars filled with ultrapure water and following 3 times wash-offs were performed with 130 rpm agitation during 15 minutes. Slides were then dried with slide spinner.

Table 2.3 Solvent type and post immobillization washing regimes with a function of coating material combination

Washing Conditions	**Coating combination**	**Solvent type**
10 minutes washes with phosphate buffer including 0.1 % SDS and 3X15 minutes washes with ultrapure H_2O	0.2 % epoxy resin 0.2 % amine	acetone
	0.2 % epoxy % 0.2 amine	acetone
	0.2 % epoxy 0.2 % amine	ultrapure H_2O
	uncoated	uncoated
10 minutes washes with phosphate buffer including 0.5 % SDS and 3X15 minutes washes with ultrapure H_2O	0.2 % epoxy resin 0.2 % amine	acetone
	0.2 % epoxy % 0.2 amine	acetone
	0.2 % epoxy 0.2 % amine	ultrapure H_2O
	uncoated	uncoated

Sandwich hybridization application was also tried on newly created surfaces after determining the proper coating and post immobilization washing ways. In these experiments, besides EA coated surfaces commercially sold PLL coated glass microscope slides were also used. Surface probes were thiol modified and signal probes were FAM modified. 5'thiol-3'FAM modified probes were used as positive control parameters. Sandwich structure is made up of; surface-stabilized probe which is semi-complementary to the target DNA strand, an adaptor (target) that will be stabilized to the surface by surface probe, and a signal probe which is

hybridizable to a part of the adaptor where surface probe is not. Sequences are given below (Table 2.4).

Zeiss LSM 510 confocal laser scanning microscope (Jena, Germany) was used for scanning the slides using a low magnification (4X, NA 0.1) objective at 1024x1024 resolution and 12-bit dynamic range.

Table 2.4 Probe and target sequences used in the sandwich assay

Probe Type	Sequence
Surface probe	5'/5ThioMC6D/AAAAAAAAAGCATCTTCAACGATGGCCT TTCCTTT-3'
Signal probe	5'TCGCAATGATGGCATTTGTAGGAGC/6-FAM/-3'
Adapter (target)	5'GCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGC CATCGTTGAAGATGC 3'
Positive control	5'/5ThioMC6D/AAAAAAAAAGCATCTTCAACGATGGCCT TTCCTTT/6-FAM/-3'

2.3.2 Single Nucleotide Polymorphism Detection based on Sandwich Hybridization

2.3.2.1 Platform and Surface Construction

Every point in the DNA array has a sandwich structure, as shown in Figure 2.2, made up of; surface-stabilized probe which is semi-complementary to the target

DNA strand, an adaptor (target) that will be stabilized to the surface by surface probe, and a signal probe which is complementary to a part of the adaptor where surface probe is not.

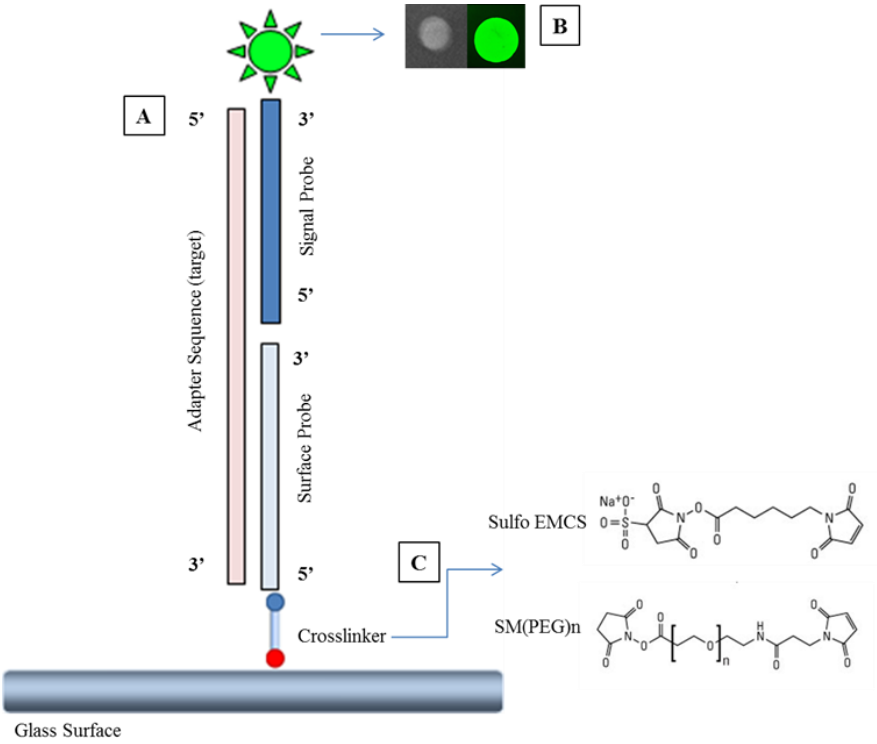

Figure 2. 2 Schematic drawing of the platform. A: Sandwich platform has a surface attached oligonucleotide (surface probe, 25 mer) which is designed to partly hybridize to an adapter sequence (Adapter, 52mer) which in turn is detected by hybridization to a second labeled probe (Signal probe, 26mer), B: typical spot morphology acquired with the UV illuminator and confocal laser scanning microscope, C: Heterobifunctional crosslinkers used in surface activation.

Discriminating oligonucleotide base for the mutated oligonucleotide base on adapter molecule was placed on surface or signal probe. As a support material standard sized (75x25x1 mm) sized PLL coated microscope slides were used.

2.3.2.2 Heterobifunctional Cross-linker and Oligonucleotides

Heterobifunctional cross linker $SM(PEG)_2$ (succinimidyl-[(N-maleimidopropionamido)-diethyleneglycol]ester) (MW: 425.39) with a 17.6 A° spacer arm length was used to activate the PLL coated glass surface in a final 2 mM concentration (Figure 2.1). It was suspended in Dimethyl sulfoxide (DMSO) and stored (-20) °C as aliquots. Oligonucleotides used in the study were given in Tables 2.5 and 2.6.

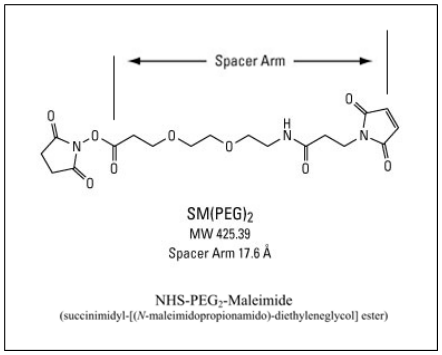

Figure 2. 3 Molecular structure of heterobifunctional cross linker $SM(PEG)_2$

Table 2. 5 Oligonucleotides used in the SNP discrimination via sandwich hybridization, discriminator is surface probe

Type	Sequence *Discriminator: Surface Probe*
Wild type Adapter (target)	5'TCTTGGGTTTCTGATAGGCACTGACTCTCTCTGCCTATTG GTCTATTTTCCC-3'
Mutant Adapter (target)	5'TCTTGGGTTTCTGATAGGCACTGACTCTCTCTGCCTATT**A** GTCTATTTTCCC-3'
Signal Probe	5'-AGTCAGTGCCTATCAGAAACCCAAGA/3Bio/-3'
Mutant Surface Probe with sp9	5'/5ThioMC6D//iSp9/GGGAAAATAGAC**T**AATAGGCAGAGA-3'
Mutant Surface Probe with polyT's	5'/5ThioMC6D/TTTTTTTTTTTTTTTGGGAAAATAGAC**T**AATA GGCAGAGA-3'
Mutant Surface Probe (A) with sp9	5'/5ThioMC6D//iSp9/GGGAAAATAGAC**A**AATAGGCAGAGA-3'

Table 2. 6 Oligonucleotides used in the SNP discrimination via sandwich hybridization, discriminator is signal probe

Type	Sequence *Discriminator: Signal Probe*
Wild type Adapter (target)	5'TCTCTGCCTATTGGTCTATTTTCCCACCCTTAGGCTGCTGGTGG TCTACCCT-3'
Mutant Adapter (target)	5'TCTCTGCCTATTAGTCTATTTTCCCACCCTTAGGCTGCTGGTGG TCTACCCT-3'
Surface Probe with sp9	5'-/5ThioMC6-D//iSp9/AGGGTAGACCACCAGCAGCCTAAGGG-3'
Mutant Signal Probe	5'-GGGAAAATAGACTAATAGGCAGAGA/3Bio/-3'
Mutant Signal Probe (A)	5'-GGGAAAATAGACAAATAGGCAGAGA/3Bio/-3'
Positive Control Probe	5'/5ThioMC6D//iSp9/GGGAAAATAGACCAATAGGCAGAGA/3Bio/3'

2.3.2.3 Surface Probe Immobilization

In the present study, it is possible to perform reduction of disulfide bonds, activation of Poly-L-Lysine coated glass surface and immobilization of the capture probe at one step in immobilization solution. Final concentration of 10 µM for surface probe with a 5' thiol group, heterobifunctional cross linker $SM(PEG)_2$ and TCEP with final concentrations of 2 mM and 0.1 mM, respectively were mixed in 10 mM PBS. Reduction of disulfide bonds needed a 30 min incubation at room temperature. Following the incubation, the immobilization solution was applied on the glass slides as 0.5 µL for each spot. Incubation for spotted slides took 90 minutes at 30°C in a petri dish containing

dH_2O. At the end of incubation, slides were put in jars according to Hellendahl which had been filled with 5X Saline-Sodium Citrate (SSC) buffer containing 0.1% Sodium Dodecyl Sulfate (SDS) and washed with 130 rpm agitation during 15 minutes. After completing the main washing step, washing solution was discarded and jars filled with ultrapure water and following 3 times wash-offs were performed with 130 rpm agitation during 15 minutes. Slides were then dried with slide spinner at 4800 rpm in 15 seconds without any splashing.

2.3.2.4 Surface Blocking

To perform surface blocking, dried slides were put in a jar according to Hellendahl which had been filled with 2 % Bovine Serum Albumin (BSA) in 10 mM PBS at pH 7.2. Following 2 hours incubation period at room temperature, blocking solution was discarded and slides were washed two times with 0.1X SSC (pH 7.0) for 15 seconds. Two additional rinsing steps were also done with ultrapure water. All washing steps were performed with 130 rpm agitation at room temperature. After washing, slides were dried with a spinner

2.3.2.5 Sandwich Hybridization

Hybridization solution containing 20 µM adapter and 20 µm signal probe in 0.6X saline sodium citrate (SSC), 0.5% SDS and 2% BSA was prepared and kept at specified hybridization temperature till application. Then, it was applied to the slide and covered with a cover slip. Slides were then incubated at a specified temperature for 5 hours in foil covered humidifying chambers in a dark incubator. Hybridization temperatures were tried as 50°C, 55°C and 60°C. After incubation period slides were washed for 5 minutes under 130 rpm agitation with 1X SSC containing 0.1% SDS and 0.1X SSC, respectively. Water rinsing steps were done for 3 times each for 1 minute with 130 rpm agitation, too. All post hybridization washing solutions were preheated to specified hybridization temperature. Slides were then dried with slide spinner for 15 seconds and stored in foil-covered dry jar until signal molecule application.

2.3.2.6 Quantum Dot Application

Qdot® 565 ITK™ Streptavidin Conjugate Kit from Invitrogen provides the Qdot® streptavidin conjugate as 250 µL of a 2 µM solution. 1.5 uL of 2 uM QD and 148.5 uL invitrogen incubation buffer were prepared to have a final 0.02 uM concentration and applied on to spots of each slide. After application, a coverslip was used to spread the QD solution uniformly throughout the spots. Slides were placed in humidifying chamber and incubated at 30 °C for 1 hour. Following the incubation period slides were washed for 5 minutes under 130 rpm agitation with 1X SSC and 0.1X SSC, respectively. Water rinsing steps were done for 3 times each for 1 minute with 130 rpm agitation, too. Slides were then dried with slide spinner for 15 seconds and stored in foil-covered dry jar until UV imaging.

2.3.2.7 UV Imaging and Intensity Analysis

Slides were placed under UV illuminating instrument. In this study, Vilber-Lourmart Infinity 1000 was used as a UV light source and images were taken in JPEG format. Experiments were conducted in duplicates. Net fluorescence intensity values were calculated by subtracting background intensity from spot intensity using Image J (Abràmoff *et al.*, 2004) software. Figure 2.4 summarizes the scanning and analysis process.

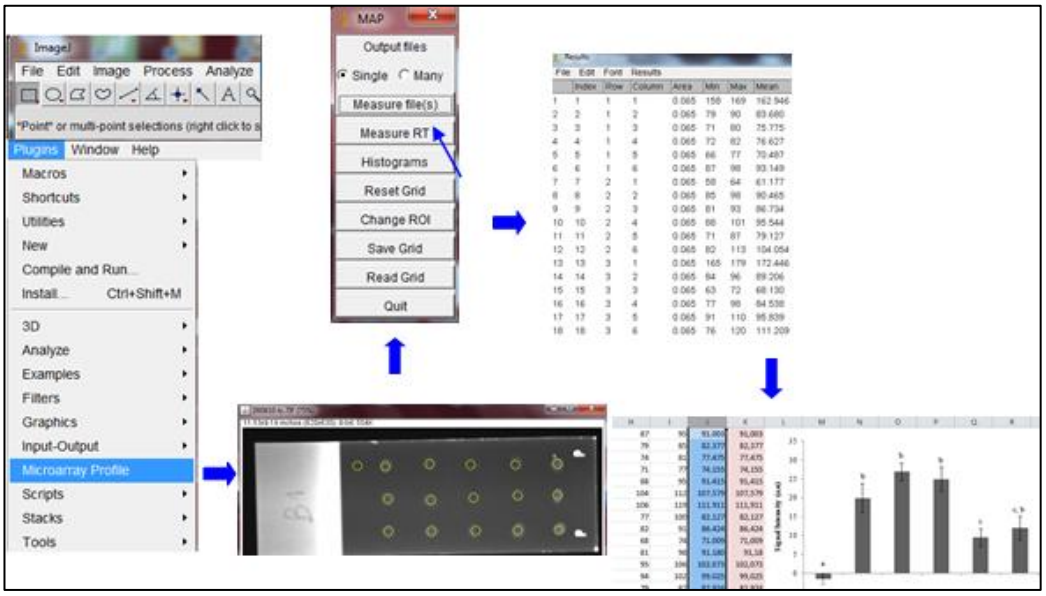

Figure 2. 4 Schematic presentation of scanning and intensity analysis of the spots.

2.3.2.8 Statistical Analysis

Minitab statistical software program was used for calculation of the mean values and standard error of means (SEM) of replicates in terms of color intensity. The variance in mean values of different treatments was also evaluated in one way analysis of variance (ANOVA) at 95 % confidence interval.

2.3.3 Single Nucleotide Polymorphism Detection based on Oligonucleotide Ligation Reaction Mediated Sandwich Hybridization in Glass Capillary Tubes

In this study, the system for SNP detection employs an on-surface approach including glass capillary tubes, that can detect single nucleotide polymorphisms (SNPs) through the Oligonucleotide Ligation Reaction (OLR).

2.3.3.1 Glass Capillary Platform

Glass capillary tubes with an interior diameter of 1,1 mm were purchased from Paul Marienfeld GmbH & Co. Surface cleaning and functionalization were done based on a previous method of Chiu *et al.* (2003) with some modifications in which the method was also described in Section 2.3.1. Oligonucleotides used in the study were given in Table 2.7 below.

Table 2. 7 Oligonucleotides used in the Ligation Reaction Mediated Sandwich Hybridization

Name	Type	Sequence (5'-3')
SNP1.Slig.Adp_N	**Adapter (target)**	TAGGCACTGACTCTCTCTGCCTATTGGTCTATTTTCCCACCCTTAGGCTG
SNP1.Slig.Adp_M	**Adapter (target)**	TAGGCACTGACTCTCTCTGCCTATT**A**GTCTATTTTCCCACCCTTAGGCTG
SNP1.Slig.P1	**Signal Probe**	/5Phos/AATAGGCAGAGAGAGTCAGTGCCTA/3Bio/
SNP1.Slig.P2_FM	**Surface Probe**	/5ThioMC6-D//iSp9/CAGCCTAAGGGTGGGAAAATAGAC**T**
Positive control		/5ThioMC6-D//iSp9/GGGAAAATAGACCAATAGGCAGAGA/3Bio/

2.3.3.2 Surface Probe Immobilization and Blocking

Reduction of disulfide bonds of surface probe , activation of epoxy-amine coated walls of capillaries and immobilization of the surface probe was performed in immobilization solution in which the method was also described in Section 2.3.2.3. Instead, the immobilization solution is prepared in larger volumes to fully cover the inside pool of the capillaries. A standard volume to fill a capillary tube

is 80 µL and an example table for preparation of immobilization solution is given in Table 2.8 below.

Table 2. 8 Standard surface probe immobilization mixture for capillaries

Ingredients	Stock Concentration	Amount	Final Concentration
5' Thiolated Surface Probe	100 uM	9 µl	10 uM
PBS Buffer	10 mM	66 µl	7.3 mM
$SM(PEG)_2$ in DMSO	30 mM	6 µl	2 mM
TCEP	1 mM	9 µl	0.1 mM

After filling the capillaries with immobilization solution they were incubated for 1.5 h at 30°C. An automated syringe pump is used in washing steps to ensure a controlled volume and ease of usage. To be washed capillary tubes were connected each other with plastic tubing parts. (Figure 2.5).

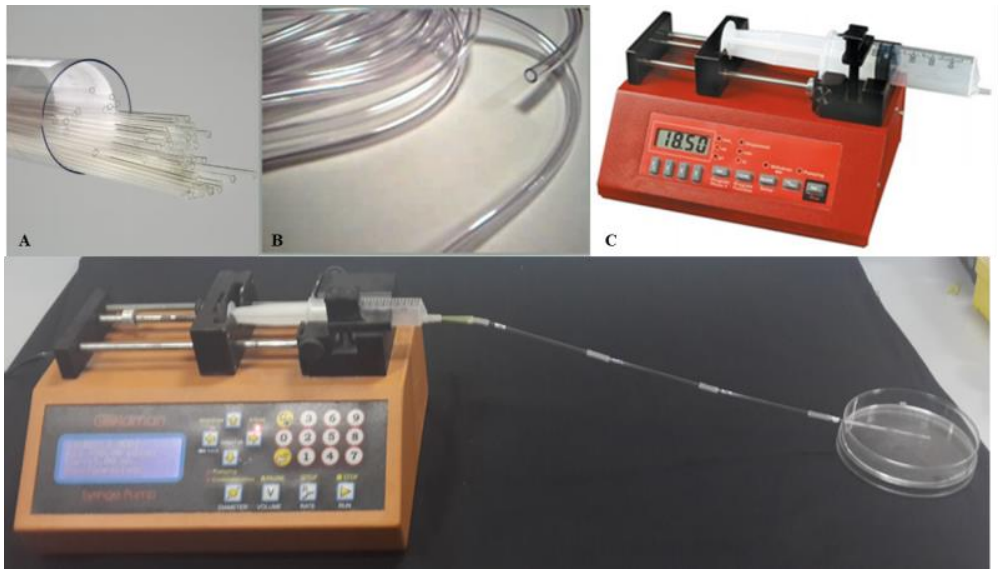

Figure 2. 5 Physical components of the washing platform. A) Glass capillary tubes with an interior diameter of 1,1 mm, B) Plasting tubing material to connect the capillaries each other during filling and washing steps via syringe pump,C) automated syringe pump. Assembly of the components are also given in the image.

Capillaries were washed with the defined solution with 5 mL volume of a syringe which is a proper volume to wash 6 capillaries. Volume in the syringe or size of the syringe can be changed according to the number of capillaries. After placing the syringe and connected capillaries on to the syringe pump platform, steps were started with 50 uL/sec speed until the immobilization solution completely leave the capillaries, then continue with 10 uL/sec rate. Rinsing steps with ultrapure water for 3 runs with a 10 uL/sec rate followed the main washing process. Drying was performed by placing the capillaries in centrifuge tubes and spinning at 1000 g for 2 minutes. Blocking was done by filling the capillaries with 2% BSA (in 10 mM PBS) and incubating for 1 h at room temperature. Capillaries were washed with 0.1X SSC via pipetting 5-6 times for each cap at the end of 1 h of blocking and dried as described above.

2.3.3.3 Oligonucleotide Ligation Reaction and Post Reaction Washing Steps

Sample reaction mixture is given in Table 2.9. Variables are; probe concentrations, addition of BSA, PEG, DMSO and the related reaction mixtures are given in Tables 2.10, 2.11, 2.12, 2.13 and 2.14. Mixtures are prepared in 80 µL volumes to fill the capillaries, and reaction period takes 30 minutes at 45° C in a temperature controlled incubator. Post reaction washing regimes for each of the reaction tables are given in text under related tables.

Table 2. 9 Sample reaction mixture for oligonucleotide ligation reaction between surface and signal probes

Ingredients	Stock Concentration	Final Concentration
Adapter (target)	100 uM	5 µM
Signal Probe	100 mM	5 µM
Taq DNA Ligase	40U/µL	20U
Taq DNA Ligase Buffer	10 X	1 X
BSA	10%	2%
dH_2O		

Post reaction washes after the first reaction in which the mixture is given in Table 2.8 is performed via a controlled syringe pump. 6M urea in 1X PBS is used for the denaturation step of the first group. Capillaries were filled with this solution and then washed with 0.1% SDS containing 1X SSC solution, 0.1 X SSC solution and 3 times ultrapure water with syringe pump under a 50 uL/sn rate. For the second group, glyoxal reaction mixture (DMSO 50%, Glyoxal 20%, 1X PBS 20%, Glycerol 10%) is applied denaturation step of the first group. Capillaries were filled with this solution and then washed with 0.1% SDS containing 1X SSC solution, 0.1 X SSC solution and 3 times ultrapure water with syringe pump

under a 50 uL/sn rate. Drying of the capillaries was performed after washing steps by placing the capillaries in centrifuge tubes and spinning at 1000 g for 2 minutes.

Table 2. 10 Reaction control mixture without Taq DNA Ligase, for oligonucleotide ligation reaction between surface and signal probes

Ingredients	Stock Concentration	Final Concentration
Adapter (target)	100 uM	5 µM
Signal Probe	100 mM	5 µM
Taq DNA Ligase Buffer	10 X	1 X
BSA	10%	2%
dH_2O		

Table 2. 11 Standard reaction mixture with Taq DNA Ligase, for oligonucleotide ligation reaction between surface and signal probes

Ingredients	Stock Concentration	Final Concentration
Adapter (target)	100 uM	5 µM
Signal Probe	100 mM	5 µM
Taq DNA Ligase	40U/µL	20U
Taq DNA Ligase Buffer	10 X	1 X
BSA	10%	2%
dH_2O		

For the reactions that the components given in Tables 2.10 and 2.11, denaturation was done by heat application. Following the reaction, capillaries were incubated in a waterbath at 95°C for 15 minutes and then washed with then washed with 0.1% SDS containing 1X SSC solution, 0.1 X SSC solution and 3 times ultrapure water with syringe pump under a 50 uL/sn rate. All solutions were also preheated

to 95°C. Drying of the capillaries was performed after washing steps by placing the capillaries in centrifuge tubes and spinning at 1000 g for 2 minutes.

Table 2. 12 Reaction mixture for oligonucleotide ligation reaction between surface and signal probes, including DMSO and uncomplementary adapter with the same molarity of complementary adapter (5 µM)for competitive hybridization

Ingredients	**Stock Concentration**	**Final Concentration**
Adapter (target)	100 uM	5 µM
Uncomplementary adapter	100 uM	5 µM
Signal Probe	100 mM	10 µM
Taq DNA Ligase	40U/µL	20U
Taq DNA Ligase Buffer	10 X	1 X
DMSO	10%	3%
dH_2O		

Table 2. 13 Reaction mixture for oligonucleotide ligation reaction between surface and signal probes including DMSO and uncomplementary adapter with the same molarity of complementary adapter (10 μM) and signal probe for competitive hybridization

Ingredients	Stock Concentration	Final Concentration
Adapter (target)	100 uM	10 μM
Uncomplementary adapter	100 uM	10 μM
Signal Probe	100 mM	10 μM
Taq DNA Ligase	40U/μL	20U
Taq DNA Ligase Buffer	10 X	1 X
DMSO	10%	3%
dH_2O		

Table 2. 14 Reaction mixture for oligonucleotide ligation reaction between surface and signal probes including DMSO and uncomplementary adapter with the same molarity of complementary adapter and signal probe for competitive hybridization. Mixture also contains 1% PEG8000.

Ingredients	Stock Concentration	Final Concentration
Adapter (target)	100 uM	10 μM
Uncomplementary adapter	100 uM	10 μM
Signal Probe	100 mM	10 μM
Taq DNA Ligase	40U/μL	20U
Taq DNA Ligase Buffer	10 X	1 X
DMSO	10%	3%
PEG8000	10%	1%
dH_2O		

For the reactions that the components given in Tables 2.10, 2.11, 2.12, 2.13 and 2.14 denaturation was done by heat application. Following the reaction, capillaries were incubated in a waterbath at 95°C for 15 minutes and then washed with then washed with 0.1% SDS containing 1X SSC solution, 0.1 X SSC solution and 3 times ultrapure water with syringe pump under a 50 uL/sn rate. All solutions were also preheated to 95°C. Drying of the capillaries was performed after washing steps by placing the capillaries in centrifuge tubes and spinning at 1000 g for 2 minutes.

2.3.3.4 Horseradish Peroxidase and 3,3',5,5'-Tetramethylbenzidine (TMB) Reaction

Streptavidin conjugated Horseradish Peroxidase (HRP-Sav) was diluted as 1:10.000 ratio in 1% Skim Milk in 1X PBS (pH 7.2) and applied into capillaries. After 1 hour incubation at 37°C capillaries were washed with 2X PBS and 0.05 % Tween-20 pH 7.0 for 5 runs with a rate of 50 ul/sn via syringe pump. Rinsing steps were done 3 times with a rate of 50 ul/sn with ultrapure water and after all capillaries were dried by placing the capillaries in centrifuge tubes and spinning at 1000 g for 2 minutes. Commercially sold TMB solution applied into capillaries and 10 minutes incubation at dark were done for blue product evaluation.

2.3.3.5 Photography and Success Rate Calculation

Beside photographic results, success rates were also calculated for each test parameter. Success rate looks at a data sample for either successes or failures, which may also be called as “yes” or “no” expressions. Among six replicates from the finally optimized experimental conditions, successes are divided by the total number of attempts to find success rate and expressed as percentage. Image J software was used for colour intensity analysis and results coming from test parameters were poroportioned with respect to their own positive control values.

2.3.3.5 Statistical Analysis

Minitab statistical software program was used for calculation of the mean values and standard error of means (SEM) of replicates in terms of blue color intensity. The variance in mean values of different treatments was also evaluated in one way analysis of variance (ANOVA) at 95 % confidence interval.

2.3.4 Single Nucleotide Polymorphism Detection Based on Silica Nanoparticle Based Lateral Flow Strip Platforms

2.3.4.1 Optimization of HRP-TMB reaction constituents

Oxidation of the benzidine derivative, TMB, by horseradish peroxidase in the presence of H_2O_2 was firstly catalyzed in solution phase to determine the proper stochiometry to get the visual results of blue colour formation. First of all stock solution were prepared for reaction constitutents. TMB weighted and dissolved in DMSO with a required concentration. Hydrogen peroxide was commercially available in market as 35 % stock in water which also equals to 11.42 M H_2O_2. Horseradish peroxidase was purchased as powder, weighted and dissolved in PBS solution as 1 mg/mL as a stock concentration. Preliminary studies were conducted with different concentrations of TMB, H_2O_2 and HRP in which the parameters are given in Table 2.15

Table 2. 15 HRP-TMB reaction constituents with various molar ratio combinations.

TMB Concentrations (mM)					
4	2	1	0,5	0,25	0,125
1M H_2O_2					
1 µg HRP			**0.1 µg HRP**		
H_2O_2 Concentrations (M)					
3,6	1,8	0,9	0,45	0,225	0,1125
1mM TMB					
0.1 µg HRP					

2.3.4.2 Preparation of TMB loaded silica nanoparticles

Mesostructured MCM-41 hexagonal silica nanoparticles with 4.5-4.8 nm unit cell sizes were used in the study. Their pore volume and pore sizes are 0.98 cm^3/g and 2.1-2.7 nm, respectively. 3,5,3,5 tetramethylbenzidine was dissolved in DMSO with a final 0.4 M concentration. Different concentrations of TMB were prepared with dilution from the main stock as; 0.2 M, 0.1 M, 0.05 M, 0.025 M. Silica nanoparticles were weighted as 10 mg and mixed with 2000 µl 1X PBS. Various concentrations of TMB were added as 20 µl to each seperate tube. Silica nanoparticles are incubated with TMB in an eppendorf tube overnight. Tubes containing silica and TMB is centrifuged at 14000 rpm for 5 min following the incubation period and supernatant was discarded. Pellet was resuspended in 1000 µl 1X PBS and homogenically aliquoted to seperate tubes as 100 µl. Aliquotes were again centrifuged at 14000 rpm for 5 min and supernatants were discarded. If the present pellets were not to be used immediately, then they were let to have an overnight airdry process in room temperature and afterwards storage at 4°C. Before proceeding to silanization phase, some optimization experiments were done with TMB loaded silica nanoparticles.

Optimization studies were initiated with in-solution trials. First of all, pellets and supernatants coming from the centrifugation of 100 µl aliquotes and one of the 100 µl aliquote of itself were proned to an oxidation reaction which is catalyzed by HRP in the presence of H_2O_2 and colour formation degrees were photographed. In the other trial, pellets coming from silica nanoparticles, dopped with various concentrations of TMB, were placed in a similar reaction. After photographing, samples were centrifuged, supernatants were discarded and pellet was resuspended in 10 µl PBS. Following this, OD values were measured both for pellets and supernatants via Nanodrop spectrophotometer.

2.3.4.3 Preparation of Lateral Flow test strips

Test strips in this study are composed of three main parts as nitrocellulose membrane, sample application pad and absorbent pad as diagrammed below in Figure 2.6.

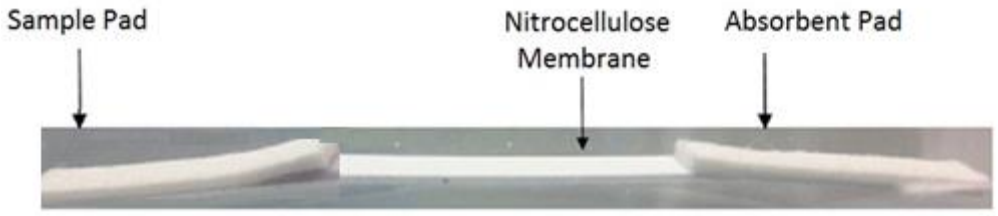

Figure 2. 6 Components of the customized lateral flow nitrocellulose membrane test strip

HF180 coded Hi-Flow ™ Plus nitrocellulose membranes (Millipore) were used in the study which have 180 ± 45 (sec/4cm) capillary flow times. They are backed by the producer on 6 cm x 30 cm (± 0.05 cm) standard sized backing cards. These membrane sticked cards are cut into 4 mm wide. Sticky coverings was removed for backing the sample application and absorbent pads. Porous matrices that are

used for the sample application pad and absorbent pad were purchased as cellulosic fiber materials under the SureWick®name (Millipore). Sample application pad (17 mm x 4 mm), absorbent pad (17 mm x 4 mm), and nitrocellulose membrane (60 mm x 4 mm) were prepared separately and then mounted together. Materials were the same for sample application and absorbent part and they were not proned to any chemical or physical application before using.

2.3.4.4 Application of TMB loaded silica nanoparticles on nitrocellulose membrane

0.4, 0.2, 0.1, 0.5 and 0.25 M TMB were added to air dried silica particles and they were suspended in 50 μL PBS and centrifuged at 14000 rpm for 5 minutes and supernatants were discarded. Pellets were finally resuspended seperately in 5, 10, and 20 μL PBS and applied on nitrocellulose membrane cards.

2.3.4.5 Running silica nanoparticle based lateral flow strip assay

Suspensions and HRP were applied on nitrocellulose membrane cards in two different orders called as “strip1” design and “strip2” design in order to understand and optimize the flow behaviours of the assay (Figure 2.7). In “strip1” design silica suspension was applied on card with a location of 6 mm below the sample application pad, and let to dry for 10 minutes at dark. HRP was applied 4 mm below the silica suspension and let to dry for 10 minutes, too. In “strip2” design HRP was applied on card with a location of 6 mm below the sample application pad, and let to dry for 10 minutes at dark. Silica suspension was applied 4 mm below the silica suspension and let to dry for 10 minutes, too. After completing the process on membrane cards, 3.5 % H_2O_2 was sent from sample application pad and waited until it reaches to absorbent pad.

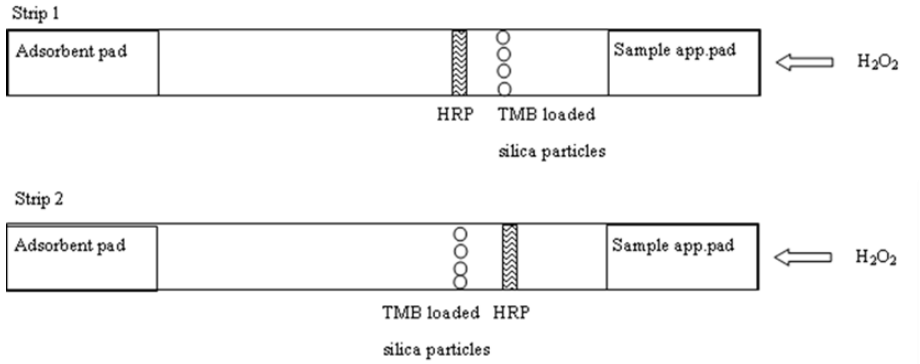

Figure 2. 7 Design of nitrocellulose membrane strips in terms of the position of TMB and HRP lines according to sample application pad.

2.3.4.6 Silanization of the silica nanoparticle surface

Silanization phase were proceeded with air dried silica nanoparticles containing 0.2 M TMB which were stored at 4°C. Silanization mixture containing; 50 µL amino-silane, 10 µL 0.2 M TMB in DMSO and 950 µL ethanol ph 5.5 with acetic acid were added on pellets. Prior to centrifugation at 14000 rpm for 5 minutes and discarding supernatants, pellets were incubated with silanization mixture for 3 hours by mixing at room temperature. Final pellets were resuspended in PBS and homogenically aliquoted as 100 µL. Aliquotes were centrifuged again and if the pellets were not to be used immediately, then they were let to have an overnight airdry process in room temperature and afterwards storage at 4°C.

2.3.4.7 Oligonucleotide binding to the surface

An oligonucleotide binding mixture containing 100 µM oligonucleotide and 0.2 M TMB in PBS were added in 21 µL volume to the pellets coming from 100 µL aliquotes (final probe concentration: 47.6 µM for each aliquote). Tubes were let to air dry overnight following an 30 minutes room temperature mixing incubation. For control experiments, same mixture lack of oligonucleotide was applied to another tube of pellet.

2.3.4.8 Application of TMB loaded silica nanoparticles on nitrocellulose membrane

Air dried silica particles including 0.2 M TMB and binded oligonucleotides on surface were suspended in 50 µL PBS and centrifuged at 14000 rpm for 5 minutes and supernatants were discarded. Pellets were finally resuspended in 10 µL PBS applied on nitrocellulose membrane cards.

2.3.4.9 Running silica nanoparticle based lateral flow strip assay

Suspension were applied on nitrocellulose membrane cards with a location of 6 mm below the sample application pad, and let to dry for 10 minutes at dark. HRP was applied 3 mm below the silica suspension and let to dry for 10 minutes, too. After completing the process on membrane cards, complementary and uncomplementary oligonucleotide was suspended in 3.5 % H_2O_2 with final concentrations of 100 µM. Oligonucleotide-H_2O_2 mixture were sent from sample application pad and waited until it reaches to absorbent pad.

2.3.4.10 Detection of SNP Mutated complementary oligonucleotides on assay

Detection of SNP mutated oligonucleotides were also done with the same assay procedure. Oligonucleotides having one, two and three mismatches to the oligonucleotide on the surface were applied through the application pad in H_2O_2.

2.3.4.11 Microscopy and photography

Images of the strips were photographed with a camera and also stereomicroscope images were examined.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Construction of a Custom Designed Functionalized Surface

Nature of the immobilization surface is a key component in array platforms. Composition and morphology of the solid substrate material and the surface chemistry, which is a kind of bridge between support and immobilized molecules, are the main constituents of an array platform.

3.1.1 Determination of Solid Support and Its Preparation

Solid supports for the platforms in this study were chosen as the inside cavity surface of ‘glass capillary tubes’ and ‘glass microscope slides’ for the current and following three experimental parts. In the last experimental part, solid supports other than glass were used and they will be introduced in relevant sections. Glass microscope slides were the first supports during the invention period of DNA microarrays mainly because of their availability. However, its permanence throughout this area is also attributable to its intrinsic properties, including low fluorescence, excellent flatness, chemical inertness, and low cost (Conzone and Pantano, 2004) . That being said glass is chemically inert, it is possible to activate its surface by silanization and several modifications.

Preparing a clean surface is a prerequisite for generating a microarray slide. Standard glass microscope slides may possibly contain some environmental contaminants simply like dust on the surface which need to be excessively cleaned before coating processes. Otherwise irregularities may arise on surface coating

and they might affect all the upstream processes. Removing glass contaminants can be achieved by many methods but generally classified as acid, alkali or physical cleaning approaches. Alkaline solutions are readily available to most labs and their disposal is less of an issue than with acidic effluents. In this study, 10 % (w/v) Sodium hydroxide (NaOH) solution in 60 % (v/v) ethanol was used as the cleaning solution. This type of alkaline glass cleaning works by etching and incubation time is dependent on the level of glass contamination. Alkaline cleaning application lasted for 3 hours in this study which is a three times longer period than the generally accepted duration that is sufficient to remove most contaminants. In literature, overnight incubation have been also published, and giving convenient results (Erdogan *et al.*, 2001). Thereby, it might be concluded that prolonging the alkaline cleaning period is also not a miscue to achieve an efficient coating. Alcohol presence in the solution helps removing the adsorbed organics from the surface, thus increasing the efficiency of the alkaline cleaning application (Aboytes *et al.*, 2003). Cleaning solution in this study was also prepared by dissolving sodium hydroxide pellets in 60 % (v/v) ethanol. Consecutive water rinsing steps also followed the alkaline process to halt the etching of glass and cleaning was finalized by a spin dry process.

3.1.2 Surface Coating Agent Choice for the Glass Supports

For specific detection, realized by a base-pair interaction with targets, the probes must be placed on the surface in an addressable structure (Heise and Bier, 2005). “Ionic interaction” and “covalent immobilization” are the two main strategies to place the molecules on a prepared support. Ionic interaction strategy does not require any nucleic acid modification and thus it is generally recognized as the simplest method for immobilization and occurs between the negatively charged groups present on the oligonucleotide probe and positive charges covering the surface (Sassolas *et al.*, 2008). These kind of interactions can cause some major drawbacks depending on the scope of the experiment as insufficient exposure of functional domains to target, largely due to a many of unpredictable orientations that the immobilized oligonucleotide can adopt upon binding to the glass surface.

On the other hand, covalent immobilization process results in more proper orientation of oligonucleotides which enhance their exposure, binding and hybridization with complementary molecules (Du *et al.*, 2005). In this chapter, covalent immobilization is chosen as the attachment strategy which requires the functionalization of the surface and modification of the nucleic acid surface probe.

There have been various materials to modify glass surfaces for immobilization of DNA (oligonucleotides and/or cDNA). Poly-L-lysine(PLL), N,N-Diethyl-3-aminopropyltrimethoxysilane(APS),3-Glycidyloxypropyl)trimethoxysilane (GPS) and aldehyde or carboxylic acid are the most common ones. Covalent immobilization of GPS and APS to the silicon bearing hydroxide functional groups on the surface of glass can be counted as an advantage over PLL coatings. Moreover, silanized surfaces offer a more consistent surface than PLL, with lower background and higher signal intensities (Benters *et al.*, 2002). Glass surface covalently accepts silanes via silylethers, leaving the alkylfunctional group available for further manipulation. As a result, silanized surface can be used directly to bind nucleic acids or be further reacted with a variety cross-linkers having homo- or heterobifunctional properties to introduce a variety of surface chemistries. (Sorribas *et al.*, 2001); (Aboytes *et al.*, 2003) but there have been also oligonucleotide immobilization ways on PLL coated slides via crosslinkers in a similar way like the silanized ones. On the other hand, PLL coatings generally have budget prices than any other silane coatings but lack of longer shelf lives.

Surface coating agent choice depends on the scope of the experiment, availability of the material, shelf life of the constructed platform and cost. Throughout the thesis both coatings “Poly-L-Lysine and Epoxy-Amine” were used depending on the necessities of the designed experiments and details are given in related sections. A custom made coating which will be also used in different parts of the theses, is discussed in the current chapter.

Although the general strategy is coating the surface with only one type of material, it is also possible to make a custom combination depending on the

scope. In this study, to build a superior surface for enhanced probe immobilization, combination of epoxy and amine silanes were used in order to have more than one kind of functional interaction between the nucleic acids and the glass surface. They both covalently bond to clean glass via the SiOH groups of the glass surface. Custom modifications for the specific nature of our platform were done on a previous method by Chiu *et al* (2003).

In order to understand the nature of customly created platform in terms of immobilization dynamics, first of all one needs to examine the components of this synergistic platform seperately. In the case of epoxy silane, it is known that it allows amine-terminated DNA to be covalently bind to the surface via an amine-initiated nucleophilic ring opening reaction that guides for a covalent bond formation between the epoxy groups and the amine terminated DNA (Gheorghe and Guiseppi-Elie, 2003) (Taylor *et al.*, 2003). Unmodified nucleic acids can also bind to an epoxysilane surface via exocyclic amino groups of the bases (Figure 3. 1).

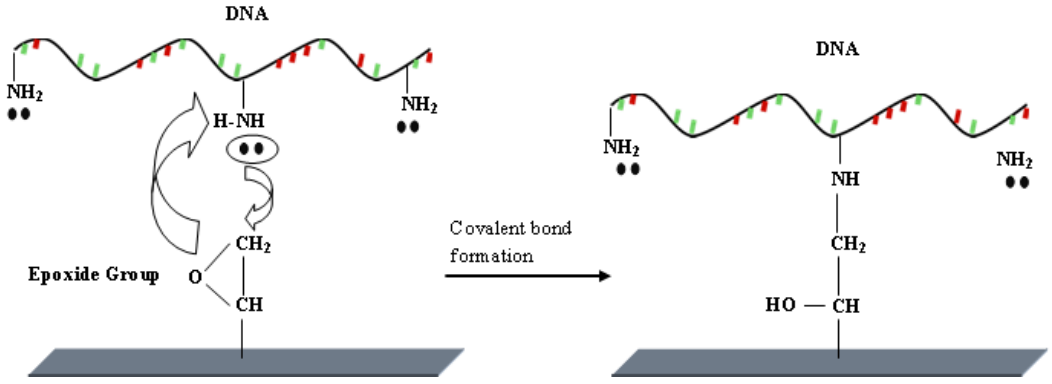

Figure 3. 1 Schematic drawing of a DNA molecule coupling to epoxy functionalized glass surface via amine-initiated nucleophilic ring opening reaction that guides for a covalent bond formation between the epoxy and the terminal or internal amine groups of DNA.

On the other side, a surface treated with amino silane interacts via ionic interactions with DNA, with the negatively charged phosphate backbone of DNA and amine functions being positively charged (Figure 1.3). In this study; rather than primary amine, tertiary amine was used. Tertiary amine molecules have low reactivity towards the epoxy group upon their mixing together before coating on the slide surface. Besides, the tertiary amine group provides a permanent positive charge that is not affected even by high pH, as is the primary amine group (Chiu *et al.*, 2003).

In order to find proper epoxy and amine silanes' concentrations and their combinations, different parameters were designed in trials as shown in Table 2.1. In these trials uncoated glass surfaces were used as negative control elements. Performance of newly created surface in terms of binding reactions was investigated with three different probes as one with no modification and the others are 5' thiol and 5' amine modified. Additionally, all of them had 3' end FAM modification for signalling (Table 2.2). TCEP reduction of S-S bonds of thiol modified probe and cross linking via Sulfo EMCS were also done.

Although the spot morphologies failed to cover the standards in this first trial, it is clear to have greater signal intensities when compared to background with 0.2 % (v/v) epoxy and 0.2 % (v/v) amine combination for all the three probe types. As a result, first trials pointed the 0.2 % (v/v) epoxy and 0.2 % (v/v) amine combination as the proper choice to be hold for the following experiments (Figure 3.1). Besides, almost the same signal and background intensities with uncoated surface (negative control) and 1 % epoxy - 0.2 % amine and 0.2% epoxy parameters directed the study towards trying different solvents and post immobilization washing conditions.

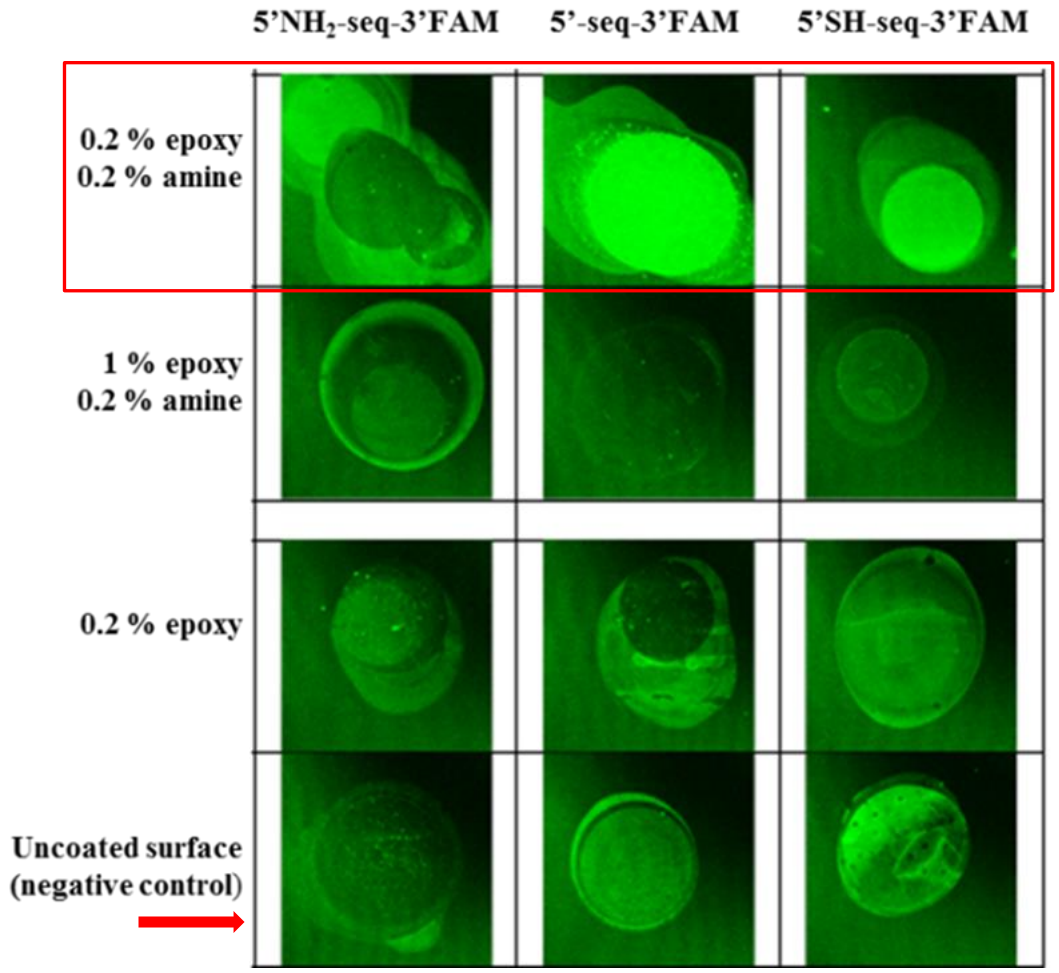

Figure 3. 2 Confocal laser scanning results of surface coating with different concentration combinations of epoxy and amine silanes. Each row shows the results from the surfaces coated with the combinations indicated far-left. Results given in the same columns represents the immobilization of 5' amine group modified, unmodified and 5' thiol modified oligonucleotide surface probes, respectively. In common, all probes are modified with FAM groups on 3' ends. Uncoated surface represents for negative control.

3.1.3 Determination of Solvent Type and Post Immobilization Washing Conditions on Surface Probe Binding Characteristics

Silanization reactions can be done in various ways like in aqueous solution, totally in organic solvent, organic solutions containing a small amount of water, and even

in the vapor phase. Room temperature or elevated temperature conditions can be also used (Hermanson, 2013). Post immobilization washing scavenges the weakly attached probe molecules and buffer components that can create background noise. Washing away the weakly or unspecifically attached probes also reveals the actual immobilization efficiency of the surface.

Effective and proper concentration ratios for coating were determined as the combination of 0.2 % epoxy and 0.2 % amine in the previous trials. Determining proper solvent type and slide washing conditions were done after preparing this coating combination in different solvents and trying different washing applications after oligonucleotide probe binding on the surface. Besides, epoxy resin material that was already available on hand were added to trials to see if it was a better alternative to present epoxy coating material. Different parameters were designed in trials as shown in Table 2.3. In these trials uncoated glass surfaces were used as negative control elements. In this part of the study, silanization mixes were prepared seperately both in aqueous solution and in acetone as organic solvent. Results of the scans are shown in Figure 3.2. and Figure 3.3 below.

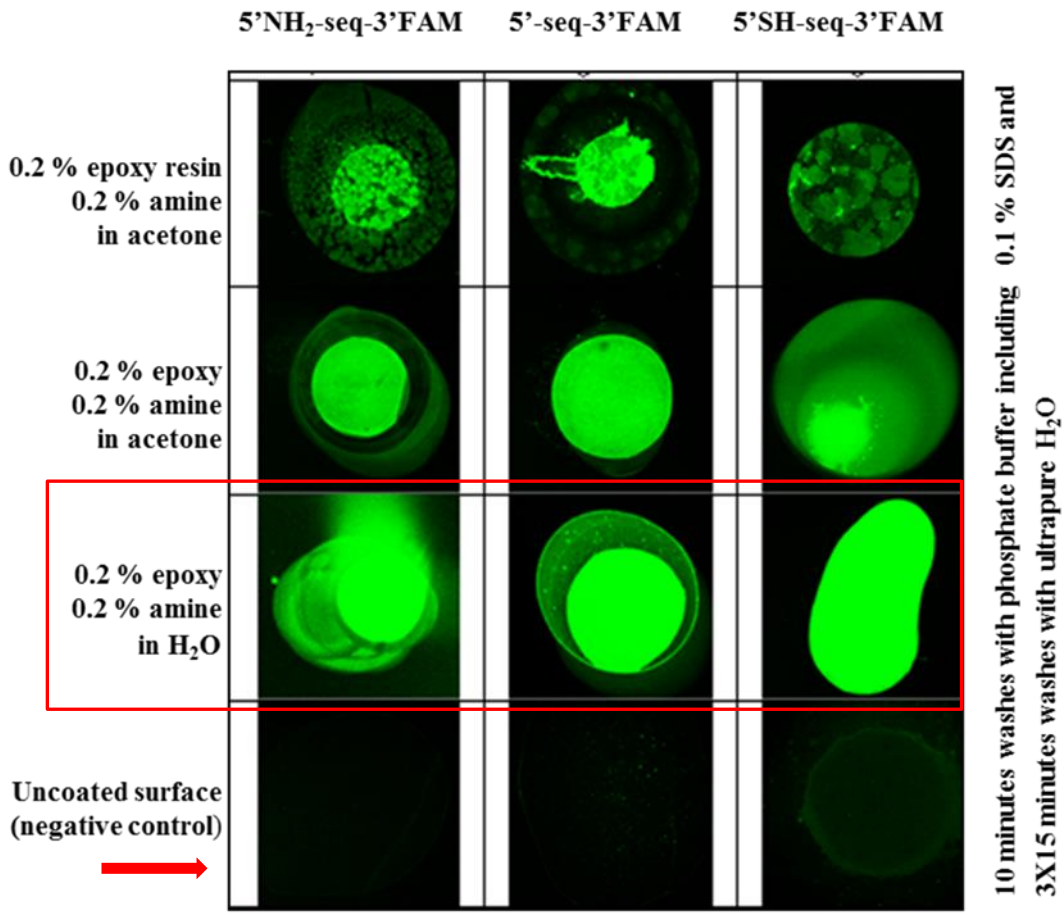

Figure 3. 3 Confocal laser scanning results of surface coating with different solvents. Post hybridization washing conditions were performed with phosphate buffer including 0.1 % SDS during 10 minutes and 3X15 minutes washes with ultrapure H_2O. Each row shows the results from the surfaces coated with the solvents indicated far-left. Results given in the same columns represents the immobilization of 5' amine group modified, unmodified and 5' thiol modified oligonucleotide surface probes, respectively. In common, all probes are modified with FAM groups on 3' ends. Uncoated surface represents for negative control.

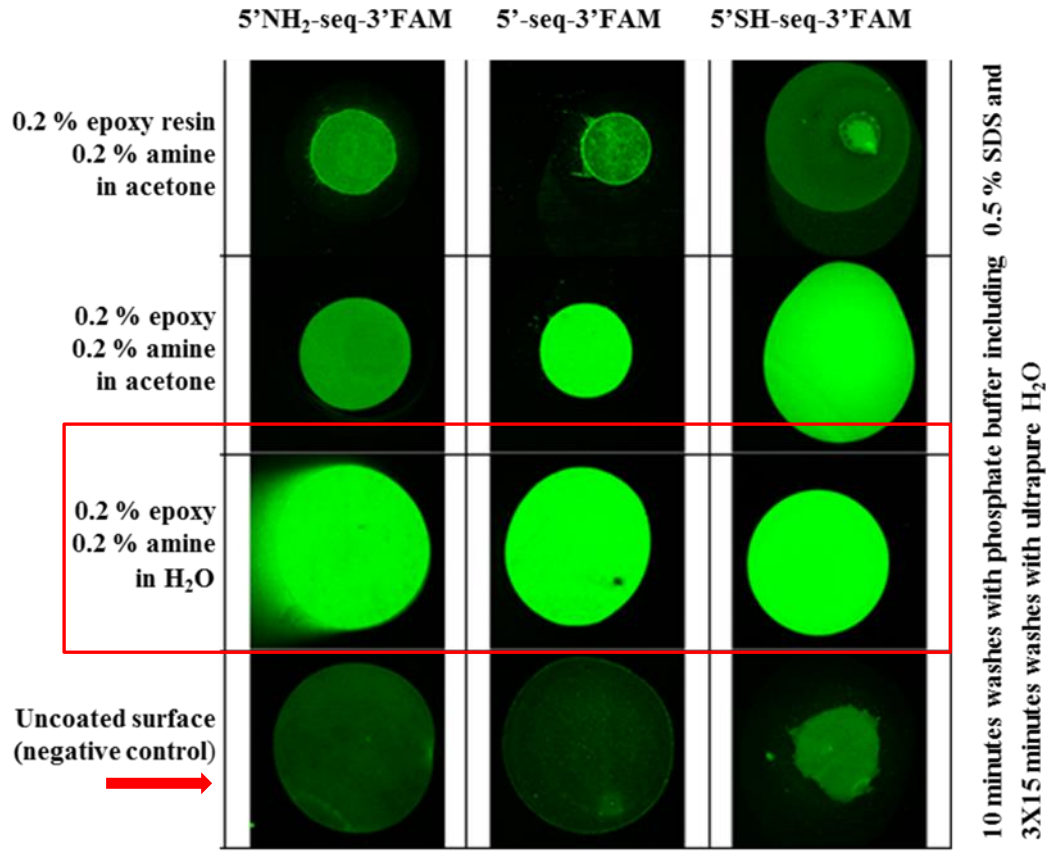

Figure 3. 4 Confocal laser scanning results of surface coating with different solvents. Post hybridization washing conditions were performed with phosphate buffer including 0.5 % SDS during 10 minutes and 3X15 minutes washes with ultrapure H_2O. Each row shows the results from the surfaces coated with the solvents indicated far-left. Results given in the same columns represents the immobilization of 5' amine group modified, unmodified and 5' thiol modified oligonucleotide surface probes, respectively. In common, all probes are modified with FAM groups on 3' ends. Uncoated surface represents for negative control.

It is clear from the scan images that epoxy resin material could not be succesful to construct a smooth coating layer. Instead, deposition of material is seen in clusters building mounds and collapses which can cause nonspecific binding of probes on the surface. Actually it is a form of crystallization of material related with the baking period for coating and also incubation period for immobillization. It is also known that temperature intervals of as little as 20 to 30°C are the most common cause of crystallization. Visually this also looks like salt crystallization effect on

microarrays related with buffers. Although it is compensated partially in more harsh washing conditions, the parameter was removed in further experiments.

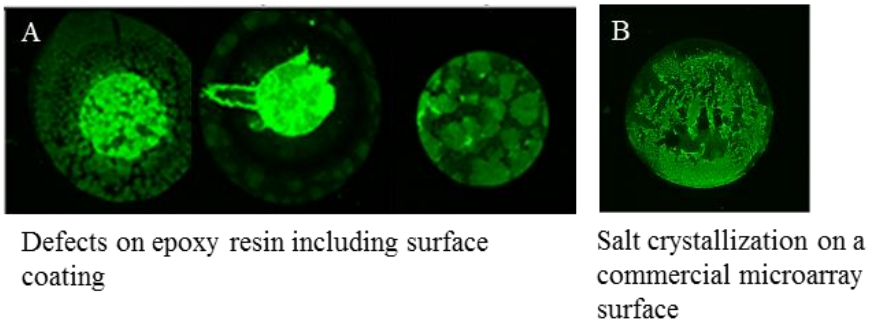

Figure 3. 5 Comparison of defects on epoxy resin containing surface coating (A) with salt crystallization effect on a commercial aminosilanized microarray substrate (B). Image of parameter B is taken from Ressine *et al.* (2007)

Using acetone as organic solvent for silanization did not show significant difference from the aqueous reaction in terms of signal intensities of unmodified and thiol modified probes while the amine terminated probes showed better signal intensities on silanized surfaces with aqueous reaction. Although spot morphologies do not satisfy the expectations in Figures 3.2 and 3.3, it can be held accountable from pipetting process that better morphologies are gained in following experiments for the same parameters. In the following replicate experiments, epoxy resin material, which had an inefficient surface performance, was eliminated from the trials. Other conditions were replicated and both washing steps and solvent type were verified (Figures 3.5 and 3.6).

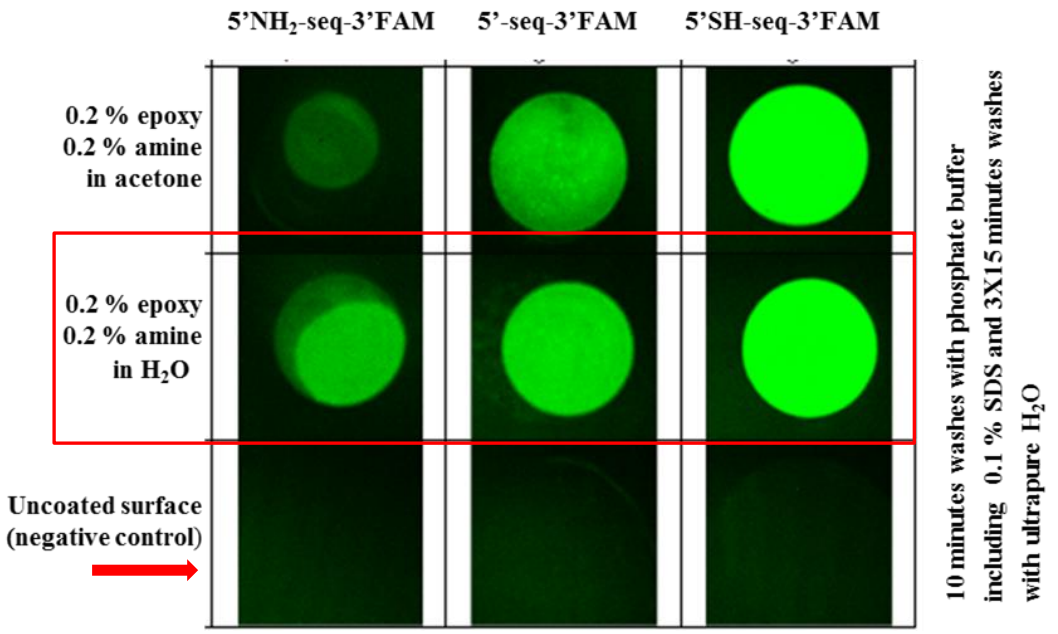

Figure 3. 6 Confocal laser scanning results of 0.2 % epoxy – 0.2 % amine coating combination with different solvents. Post hybridization washing conditions were performed with phosphate buffer including 0.1 % SDS during 10 minutes and 3X15 minutes washes with ultrapure H_2O. Each row shows the results from the surfaces coated with the solvents indicated far-left. Results given in the same columns represents the immobilization of 5' amine group modified, unmodified and 5' thiol modified oligonucleotide surface probes, respectively. In common, all probes are modified with FAM groups on 3' ends. Uncoated surface represents for negative control.

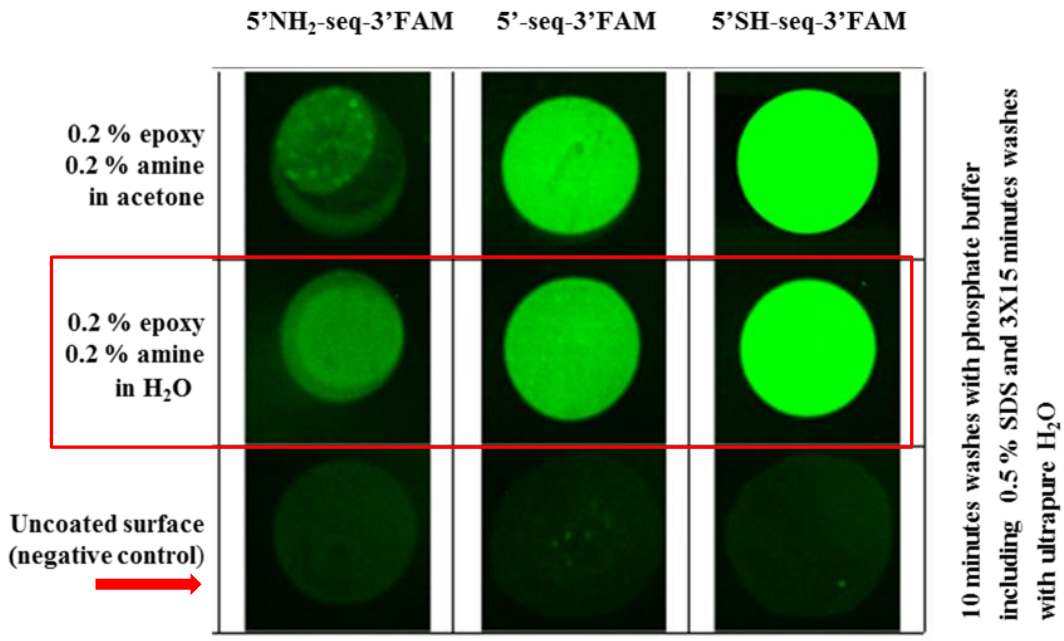

Figure 3. 7 Confocal laser scanning results of 0.2 % epoxy – 0.2 % amine coating combination with different solvents. Post hybridization washing conditions were performed with phosphate buffer including 0.5 % SDS during 10 minutes and 3X15 minutes washes with ultrapure H_2O. Each row shows the results from the surfaces coated with the solvents indicated far-left. Results given in the same columns represents the immobilization of 5' amine group modified, unmodified and 5' thiol modified oligonucleotide surface probes, respectively. In common, all probes are modified with FAM groups on 3' ends. Uncoated surface represents for negative control.

It was observed from the trials that the ultrapure water is the proper solvent for epoxy-amine coating combination as dissolving the coating material in acetone does not cause any significant enhancement on signals. The purpose of washing the surface following the immobilization is to remove any labeled targets that have not attached specifically. Determination of washing regime mainly based on the relatively higher signal intensities on negative control slides, which correspond to background noise, under 0.5 % SDS containing phosphate buffer washing conditions when compared with that of containing 0.1 % SDS. Washing slides after surface probe binding with phosphate buffer including 0.1 % SDS seems more effective to eliminate unspecific binding of probes on array surface.

As a result, for unmodified probes we can propose that negatively charged phosphate backbone of the DNA interacts with the positively charged amine groups on the surface. Epoxy groups can also form a covalent binding with the amine groups on the bases of the oligonucleotide probe which are already approximated via ionic interactions. Amine and thiol terminated probes are also covering all these ways except some additional specific reactions. Amine functionalized probes have an extra covalent linkage with epoxy groups through its 5' amine modification, and thiolated ones have also one with amine groups one surface via Sulfo EMCS heterobifunctional cross linker. As also expressed by Chiu *et al.* (2003) presence of each interaction simultaneously with the other probably enhances the effect of the other to create the synergistic effect observed. As a consequence of these expressions, relatively brighter signals gained with the thiol modified probes might be attributed to the usage of all interactions explained above. Thiolated probes have ionic interaction with amine groups through their negatively charged phosphate backbone, covalent conjugation with amine groups via cross linker and covalent conjugation of nitrogenous bases with epoxy groups while the amine modified or unmodified probes only use one or two of these interactions.

3.1.4 Sandwich Hybridization Applications on Modified Surfaces

In the second part of the study, sandwich hybridization was done on epoxy-amine coated glass slide surfaces (Figure 3.8). Ultrapure water solved 0.2 % epoxy - % 0.2 amine combination was used to coat the surfaces. Following the surface probe binding and/or post sandwich hybridization on coated array, 10 minutes wash was done with phosphate buffer including 0.1 % SDS and 3 times 15 minutes washes with ultrapure H_2O. In these experiments, besides epoxy-amine coated surfaces commercially sold poly-l-lysine coated glass microscope slides were also used for comparison. In trial, surface probes were thiol modified and signal probes were FAM modified (Table 2.4). 5'-thiol-3'FAM modified probes were also used as positive control parameters.

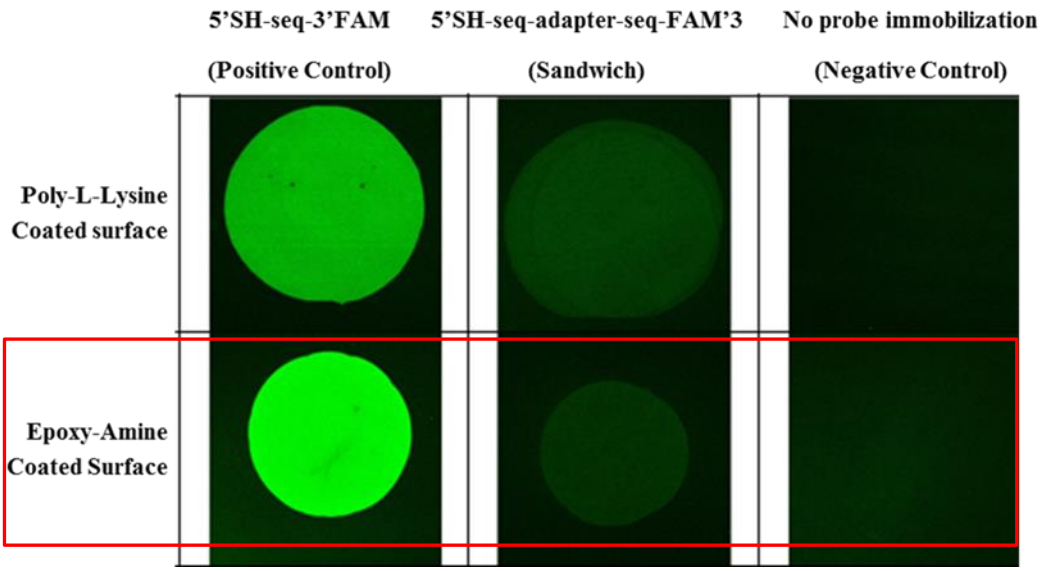

Figure 3. 8 Confocal laser scanning results of sandwich hybridization assay on PLL and EA coated surfaces. Each row shows the results from the surfaces coated with the material indicated far-left. Results given in the same columns represents the positive control probe, sandwich hybridization and negative control, respectively. In common, surface probes are modified with thiol at 5 ' ends and signal probes have FAM groups on 3' ends.

Signal intensites belong to positive control probes are significantly higher than sandwich hypridization and negative control parameters for both Poly L Lysine (PLL) and epoxy-amine (EA) coated surfaces. Differences in signal intensity and spot diameter are clearly seen between PLL and EA coated surfaces for positive control probe. Sandwich hybridization based application on both surfaces does not differ in terms of signal intensity however spot diameter parameter also gives a variation like in the positive control probe application. These variation in spot diameter can be attributed to the variation in surface texture and hydrophobicity related with different coatings. Relatively lower intensities coming from sandwich hybridization might be caused by the washing steps each of which was applied after immobilization and hybridization.

Observing the succeeding results coming from newly created glass microscope slide surface led us to think for different support materials or topographies

innovated with this coating application. Previously determined assay conditions were used for coating the glass capillary tubes which can have a future usage in fluidic based array platforms. Uncoated, bare glass capillary tubes, and commercially sold PLL coated ones were used as control parameters (Figure 3.9). Positive control probes and signal probes in sandwich hybridization have 3' biotin modification. Following the immobilization, hybridization and washing steps, streptavidin conjugated Horseradish Peroxidase (HRP-Sav) was applied into environment. After the incubation period, commercially sold TMB solution applied into capillaries for blue coloured product evaluation. Details of the reaction will be described and discussed in relevant sections. As a result, newly created EA surface gave positive signals both for positive control probe and sandwich hybridization parameters, which indicates the utility of the new material in future experiments.

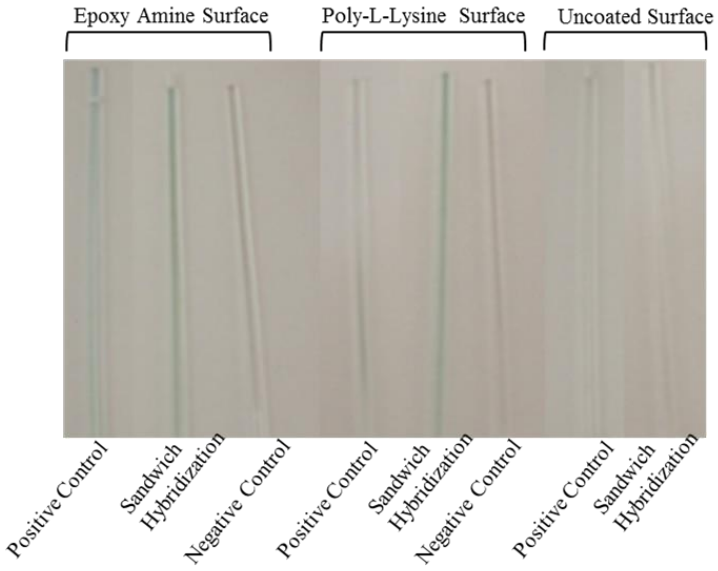

Figure 3. 9 Photography of sandwich hybridization assay on EA, PLL and uncoated surfaces. Results given as the positive control probe, sandwich hybridization and negative control, respectively. In common, surface probes are modified with thiol at 5 ' ends and signal probes have biotin modification on 3' ends.

3.2 Single Nucleotide Polymorphism Detection Based on Sandwich Hybridization

In this study, every point in the DNA array has a sandwich structure instead of positive and negative control parameters. The structure as shown in Figure 3.10, made up of; surface immobilized probe (surface probe) which is semi-complementary to the target (adapter) strand, an adapter (target) that will be stabilized to the support by surface probe, and a signal probe which is complementary to a part of the adapter where surface probe is not. Discriminating oligonucleotide base for the mutated point on adapter molecule was placed on surface probe or signal probe.

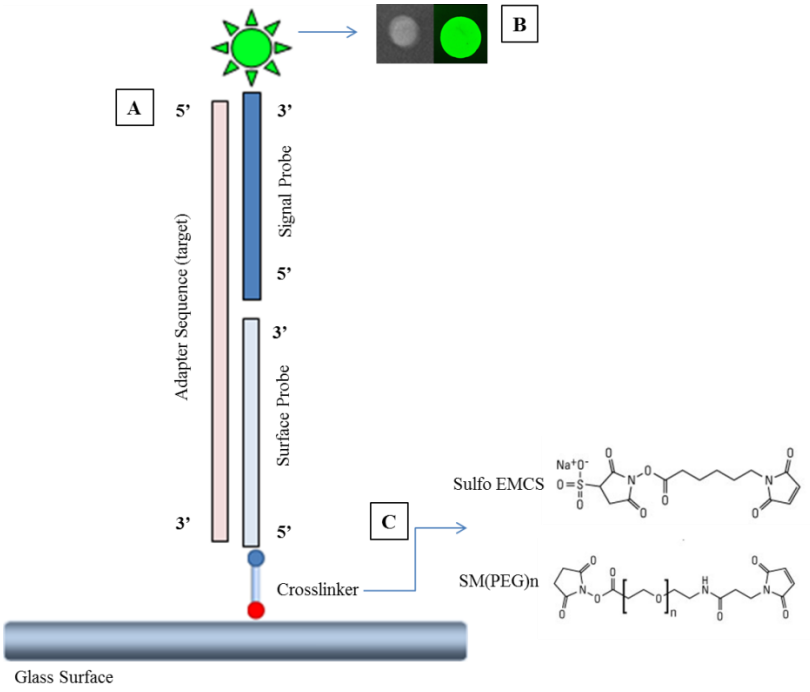

Figure 3. 10 Schematic representation of the platform. A: Sandwich platform has a surface attached oligonucleotide (surface probe, 25 mer) which is designed to partly hybridize to an adapter sequence (Adapter, 52mer) which in turn is detected by hybridization to a second labeled probe (Signal probe, 26mer), B: typical spot morphology acquired with the UV illuminator and confocal laser scanning microscope, C: Heterobifunctional crosslinkers used in surface activation.

3.2.1 Design of Oligonucleotide Probe Sequences and Their Functionalizations

In this part of the study, oligonucleotide probes and targets were designed to discriminate a single nucleotide change (G>A) on β-globin gene which causes β-IVS-I-110 type of beta thalassemia. Firstly, 52 base pair length wild type and mutated target sequences were selected from β-globin gene. Wild type and mutated target sequences were unmodified, and only differ in one nucleotide at the 40^{th} base position. Target sequence is needed to be complemented by surface and signal probes in a half and half manner in sandwich hybridization format and mismatch should be covered by one of the probes at the center or close to the center. Sequences for probes are given in Tables 2.5 and 2.6.

Throughout the study discriminatory probe (surface or signal probe) is complementary to the mutated form of the target. Mutated target differs from the wild type only in one nucleotide at the 40^{th} base position as already mentioned above. In this context, higher signal intensities are expected from the "mutant surface probe-mutant adapter-signal probe" or "surface probe-mutant adapter-mutant signal probe" sandwich hybridization formats. Besides, "mutant surface probe (A)" and "mutant signal probe (A)" named probes are also added to study as different control perspectives. SNP described here is a Guanine to Adenine change and mutant probes has a Thymine complementary base for mutated target while mutant(A) probes have an Adenine base which is not complementary to both the wild type and mutated target (Tables 2.5 and 2.6).

It is known that duplex instability is important for mismatch discrimination and is related to both position and type of the mismatch between target and probe. According to literature; internal mismatches, at the center or very close the center; have more destabilizing effects on hybridization stability when compared to the ones at ultimate or penultimate positions (Naiser *et al.*, 2008); (Özkumur *et al.*, 2010); (Peyret *et al.*, 1999); (Piao *et al.*, 2008). All these guidelines were taken into consideration during designing process and target area was chosen among different candidates. It is also a general consideration for a sequence to

have a GC content between 40% and 60% as the nonspecific hybridization issues may increase for GC ratios outside of this range (Keller and Manak, 1993). In the current design, GC ratios are 46.2 % and 44.2 % for wild type and mutant adapter sequences, respectively. ΔG values for possible hairpin and self dimer structures are between the range of -0.96 and -4.59 kcal.mole^{-1} and their T_m (°C) values lower than the hybridization temperatures in the assay format.

Following the determination of target region, surface and signal probes were designed as complementary ones. Signal probes, in common, have 3' end biotin modification which will be further conjugated to streptavidin functionalized quantum dots.

Surface probes that will be immobilized on PLL surface have 5' end thiol modifications for covalent attachment. As stated previously, covalent immobilization process of oligonucleotide probes on a support material results in more proper orientation of oligonucleotides which enhance their exposure, binding and hybridization with complementary molecules (Du *et al.*, 2005). Besides, covalent immobilization requires modification of oligonucleotide at its 5' or 3' end and so thus the surface, such that each partner represents an active pair of functional groups. The coupling reaction between the modified nucleic acid and the activated surface can be carried out directly or via a crosslinker moiety (Tjong *et al.*, 2014). Zammatteo *et al* (2000) and Chrisey *et al* (1996) have both stressed the requirement for efficient covalent coupling chemistries like carbodiimide-mediated coupling, aldehyde activation and heterobifunctional crosslinkers' applications that can provide stability and consistency to DNA microarrays.

Crosslinkers covalently bind two unequal chemical groups which otherwise would remain unreactive for each other, and they act as physical spacers which provide greater accessibility and/or freedom to each of the conjugated molecules (Chrisey *et al.*, 1996a); (Chrisey *et al.*, 1996b). Heterobifunctional cross linkers contain two different reactive entities that can conjugate two different functional targets. As an example, one part of a crosslinker may contain an amine-reactive group, while the other part may have a sulfhydryl-reactive group. By this way,

crosslinking reaction is directed towards the selected parts of target molecules, thus getting better control over the conjugation process (Hermanson, 2013). SM(PEG)n reagents, which has been also used in this study, are heterobifunctional crosslinkers with N-hydroxysuccinimide (NHS) ester and maleimide groups that allow covalent conjugation of amine and sulfhydryl containing molecules. They also contain soluble polyethylene glycol (PEG) spacer arms which improve water solubility of reagent and conjugate, reduce the potential for aggregation of the conjugate, and increases flexibility of the crosslink (ThermoScientific, 2014). In our study, 5' thiol modified surface probes were immobilized to PLL coated surface via $SM(PEG)_2$, a heterobifunctional crosslinker molecule which contains two PEG spacer arm molecules as indicated with subscript.

Conjugation chemistry of the reaction can be mainly described as follows; N-hydroxysuccinimde (NHS) ester end of the crosslinker react with primary amines on the surface at pH 7-9 to form amide bonds, while maleimide end react with sulfhydryl groups specifically on the surface probes at pH 6.5-7.5 to form stable thioether bonds. Therefore, pH 7.2-7.5 environment is needed for conjugation experiments involving this type of heterobifunctional crosslinker (Hermanson, 2013); (ThermoScientific, 2014). In our study, we also performed the reaction for NHS ester and maleimide groups simultaneously in pH 7.2 PBS buffer.

Attaching one end of the surface probe to a solid support induces some constraints that needed to be considered while developing that kind of platforms as the hybridisation of the oligonucleotides to target nucleic acids should be as free as possible from interference from the solid support. Steric hindrance (or steric interference) which prevents the target in solution to make close approach/hybridize to the immobilized probe (Shchepinov *et al.*, 1997), is one of the main constraints which is needed to be taken into account. Steric hindrance is a general term that covers multiple factors which affect hybridization reaction between immobilized probe and target in a negative manner. It involves both physical constraints involving less accessibility at surface and electrostatic and physiochemical effects occurring between nucleic acids and surface (Poulsen *et*

al., 2008). It is also proposed that when an immobilised molecule is spatially removed from the solid support by a spacer molecule of any kind, the closer it is to the solution state and the more likely it is to react freely with dissolved molecules (Shchepinov *et al.*, 1997). When considered from the aspect of physical constraints, surface immobilized probes without any spacer molecule are just about within reach of each other on the surface. Besides, ends of the probes closest to the surface are less accessible than the ends furthest away and tethered molecules may crowd each other. On the other hand, oligonucleotides on long spacers are better able to extend away from their neighbours and from the surface to allow interaction with the target (Southern *et al.*, 1999). It can be concluded that spacers help to overcome this kind of steric interference which is a main constraint during development of hybridization platforms. Spacer molecules can be chosen as poly nucleotide addings like poly T's OR Poly A's at the end of the sequence, or other chemical forms like polyethyleneglycol (PEG) based spacers.

In order to determine the spacer molecule type throughout the study, we have tried to immobilize thiolated surface probes having poly T or PEG spacers. PEG spacers (sp9) (MW: 212.14 g/mol) and thymine nucleotides (15 mer) (MW: 4501 g/mol) are located between the sequence and disulfide group on the probes (Table 2.5). Images of the platform upon UV light illumination are given below (Figure 3.10).

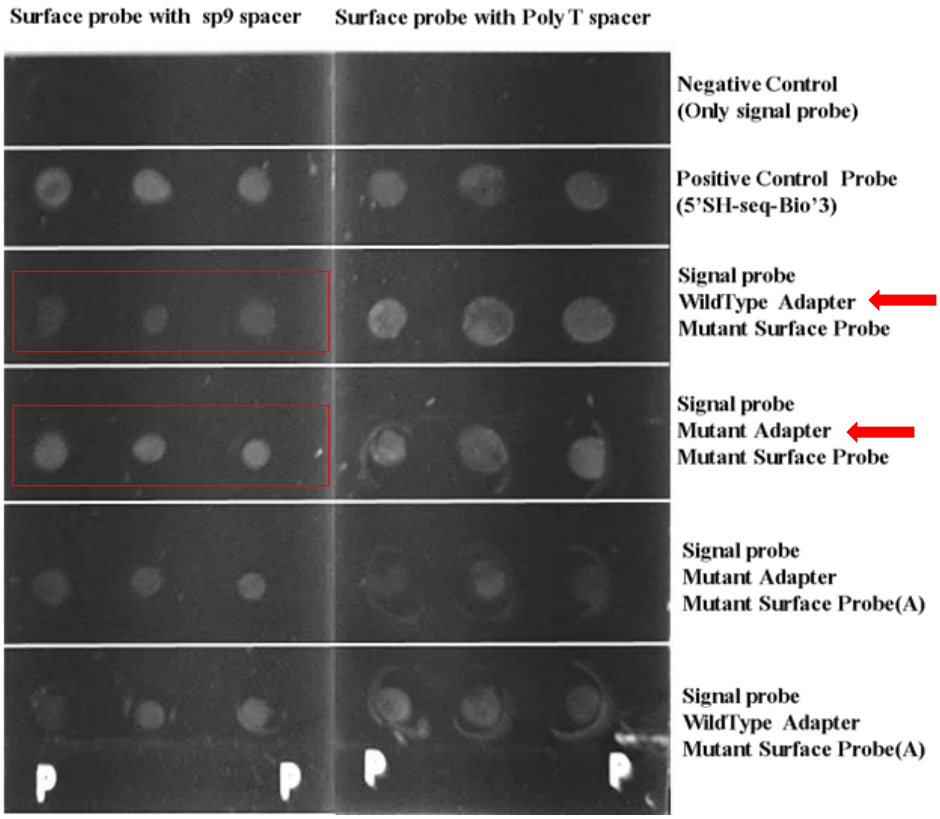

Figure 3. 11 Images of platform upon UV light immobilization with surface probes having poly T or sp9 spacers. Each row shows the results from the application of negative/positive control probes or sandwich hybridization that each of which is explained on the right side of the image.

According to the image of the platform seen above, spot morphologies are differing according to the spacer molecule type. Spots achieved from sp9 spaced surface probes are prominently smaller diameters than that of poly T spaced probes. Besides, it is observed that outer contours are smooth and less unspecific spreads are present for sp9 parameter. Sp9 is a triethylene glycol chain generally used for 5' or internal modifications of oligonucleotide probes (Figure 3.11). It is roughly 20 Å in length. On the other hand, fifteen base long thymine spacer can be roughly estimated as 75 Å long, although the length of a single stranded oligonucleotide depends on the confirmation which can vary with respect to its immediate physical and chemical environment (Smith *et al.*, 1996).

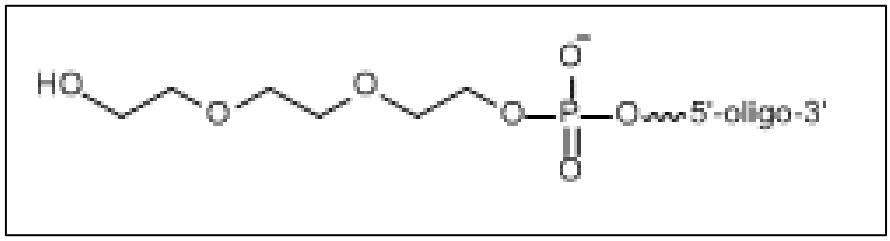

Figure 3. 12 Structure of a Spacer 9 (PEG); 5' Modification (Biosearch Technologies, 2014)

It should be also noted that the thymidine nucleotides can also bind to PLL coated surface via electrostatic interactions in an unordered way, in addition to thiol group binding. Relatively larger spot diameters in poly T spaced probes might be also caused by this additional and unspecific attraction to the surface. To our knowledge, there has not been any specific studies in literature comparing polynucleotide and chemical spacers' effects on surface immobilization of oligonucleotide probes in glass support based array studies. However, Hurst *et al*. (2006) investigated the effects of spacer composition that influence DNA coverage on gold nanoparticles. They used adenine (A), thymine (T), and PEG spacers and proposed that the tendency of the A and T spacers as DNA bases to interact with the gold will cause the DNA to partially lie on the gold surface.

Having more uniform and approximated spots is especially valid for sandwich hybridization formats where the spacer molecules are required to overcome steric interferences for getting better hybridization rates. It is also clearly seen in the quantitative results summarized in Figure 3.13 and Figure 3.14 that the platform prepared with sp9 spaced surface probes has better discrimination ability for mutated targets. Intensity values for sandwich hybridizations with wild type and mutated targets were compared with the control lines of their own in each slide which were prepared with sp9 and poly T spaced surface probes seperately. Signal intensity gained from the sp9 spaced mutant surface probe is statistically same with the positive control values. On the other hand, there is a statistically significant decrease in signal intensity in wild type adapter sandwich due to one

mismatch when compared to positive control and mutant adapter sandwich. Smooth spot contours and specific signal intensities for mismatch discrimination on sp9 slides may be attributed to higher probe flexibility and consequent increase in specific target-probe hybridization. For this reason, sp9 modification was selected as the standard spacer for the developing platform.

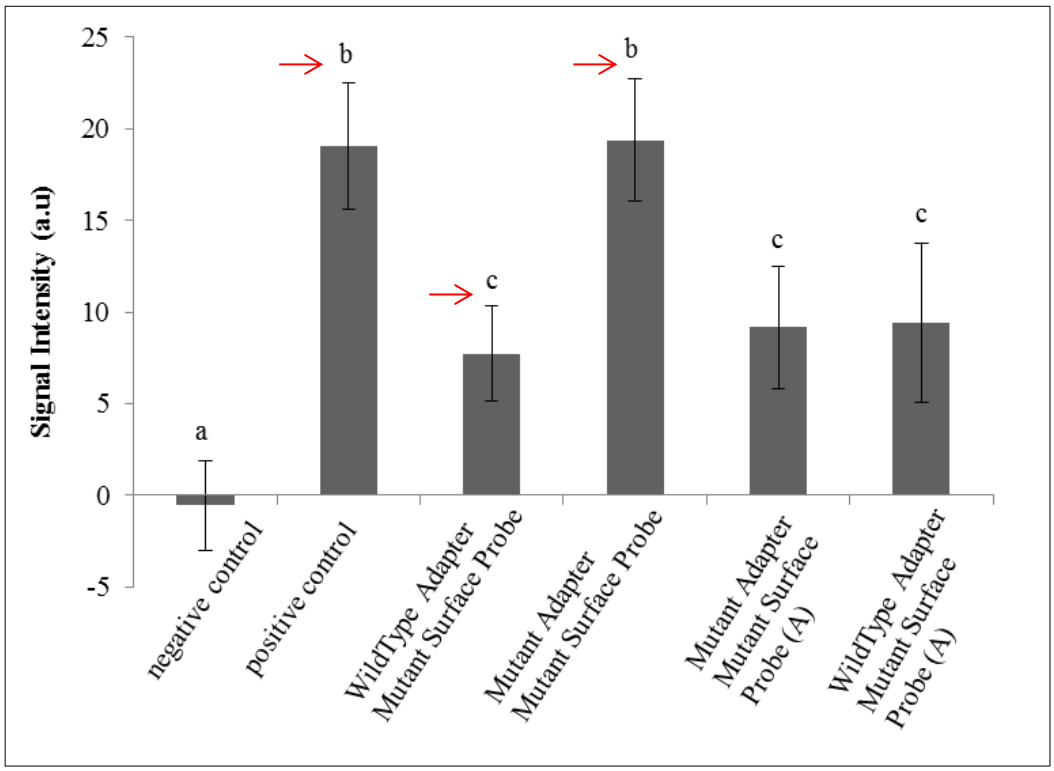

Figure 3. 13 Signal intensity values for the slides having sp9 spaced surface probes. Negative and positive control parameters were also indicated. The height of the histograms represents the average fluorescence pixel intensity of all spots. Error bars represent standard error of mean calculated from 6 spots for each parameter. Different letters indicate significant difference at $p < 0.05$.

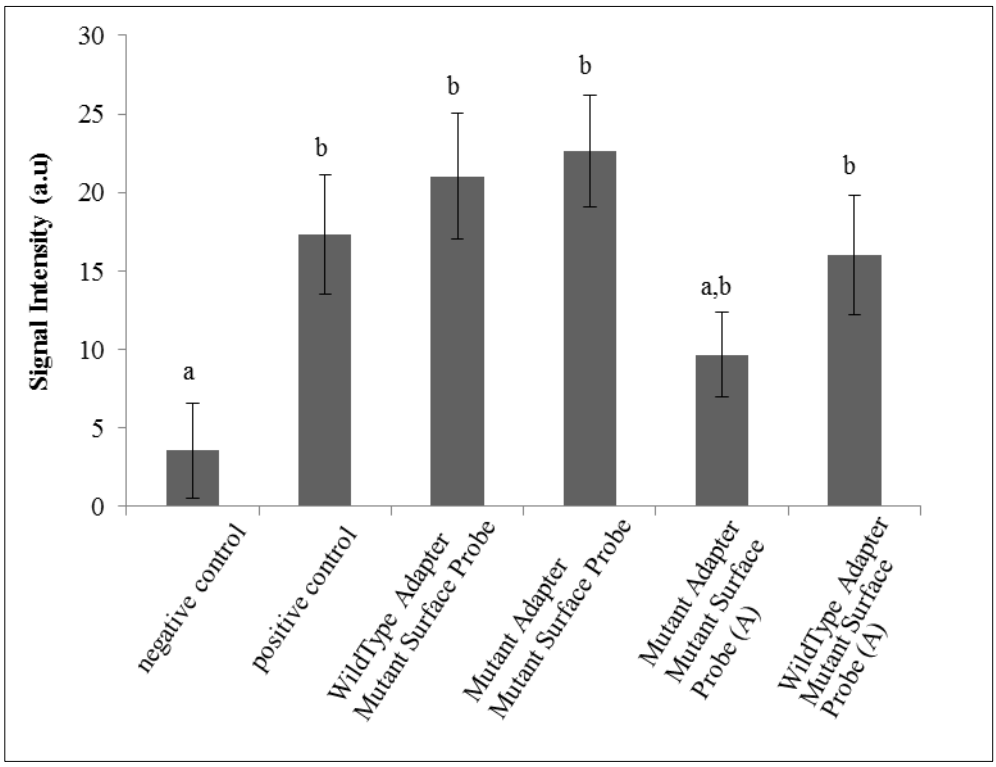

Figure 3. 14 Signal intensity values for the slides having poly T spaced surface probes. Negative and positive control parameters were also indicated. The height of the histograms represents the average fluorescence pixel intensity of all spots. Error bars represent standard error of mean calculated from 6 spots for each parameter. Different letters indicate significant difference at $p < 0.05$.

3.2.2 Optimization of Sandwich Hybridization Conditions

Hybridization efficiency strongly depends on surface probe density in both the efficiency of duplex formation and the kinetics of target capture. Increment in the surface probe density also increases the amount of target to be captured. However, exceeding the optimal densities may reduce hybridization efficiencies via steric interferences (Peterson *et al.*, 2001). Besides, probe concentration can also affect hybridization efficiency as high probe densities effectively cluster large number of negatively charged DNA together in the spot which will cause electrostatic repulsion of negatively charged DNA probe and target. In this part of the study, surface probe densities were kept constant at 10 μM. Determination of the

concentration is based on the different optimization studies for sandwich hybridization based platforms which were carried out in our laboratory previously (Cansız, 2010); (Gül, 2010). Prior to crosslinking and consequent surface immobilization disulfide groups at the 5‘ end of the probe need to be reduced to thiol form.

Tris(2-carboxyethyl)phosphine (TCEP) was used as a reducing agent for disulfide bonds. TCEP reduces disulfide bonds as effectively as dithiothreitol (DTT). However DTT have to be removed before certain sulfhydryl-reactive cross-linking and. this removal process may include methods like column purification or ethylacetate removal. Those methods are time consuming and not completely effective to remove all traces of DTT which will further negatively effect conjugation reaction. On the other hand, unlike DTT, TCEP does not have to be removed before cross-linking reaction. Besides, compared to DTT, TCEP is more stable, more effective, and able to reduce disulfide bonds at lower pHs. It was also reported that a 1:1 ratio of TCEP to disulfides required nearly one hour to complete the reduction (Mery *et al.*, 1993). In our study, reduction of disulfide bonds of surface probe , activation of PLL coated surface with $SMPEG_2$ crosslinker and immobilization of the surface probe was performed one easy step in pH 7.2 PBS immobilization solution in 1.5 hour.

Sandwich hybridization simply requires two adjacent, non overlapping probes; a surface immobilized and a signal probe which are complementary to a defined target molecule. Design of the probe sequences were explained in section 3.2.1, which are mainly influenced by the target region. Concentration of signal probe and target were kept as 20 μM for each which have been also previously examined in our laboratory.

Sandwich hybridization construct has three different sequence components so thus the optimization of hybridization parameters is more complex than two probe system platforms. Hybridization temperature is the most considerable factor while developing those kind of platforms.

Stringency of hybridization conditions are determined by the factors that affect

the stability of hybrids. Since hybridization occurs most readily at 25°C below the melting temperature (T_m) of the hybrids, the calculation of T_m is a necessary first step. As the temperature is elevated towards the T_m value, hybridization stringency also increases. It is also known that the estimation of T_m is more complex in hybridization systems employing more than one probe, just like in the sandwich hybridization and conditions need to be selected that are a compromise between the requirements of each probe. For oligonucleotide probes, hybridization temperature nearly equals 5°C-10°C below the Tm. (Keller and Manak, 1993).

We manually calculated the T_m values according to the formula (Sambrook *et al.*, 2001) below and also with a computer based application (OligoAnalyzer® 3.1, IDT). Formula is for the oligonucleotides 14 bases and longer up to 60–70 nucleotides.

Tm = 81.5 + 16.6(log10[Na+]) + 0.41(%G + C) – (600/N), where N is the chain length.

Table 3. 1 Melting temperature data for oligonucleotide probes. (Discriminator is surface probe and signal probe is common)

		Discriminator: Surface Probe
Probe Type	**Calculated T_m with formula (°C)**	**OligoAnalyzer® 3.1 (°C)**
Signal Probe	60.07	64.2
Mutant Surface Probe	56.41	58.6
Mutant Surface Probe (A)	56.41	59.3

According to the computated T_m values for probes, we have decided to apply 50°C, 55°C and 60°C hybridization temperatures. Mismatch discrimination requires more stringent environment than regular sandwich hybridization assays so thus the temperature was not kept below 50°C. Visual images of the platform gained via UV illumination is given in Figure 3.15. It is obvious that there is a decrease in signal intensity of probes related with the increase in hybridization temperature. Difference can be also seen for standard positive control probes in graphical expresssion (Figure 3.16)

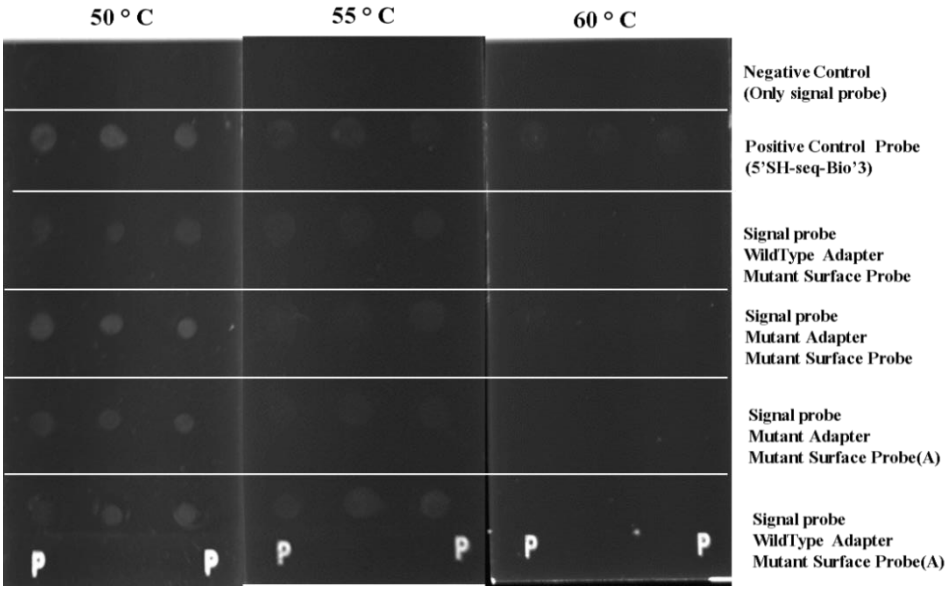

Figure 3. 15 Images of platform upon UV light immobilization with surface probes sp9 spacers at 50°C, 55°C and 60°C hybridization temperature Each row shows the results from the application of negative/positive control probes or sandwich hybridization that each of which is explained on the right side of the image.

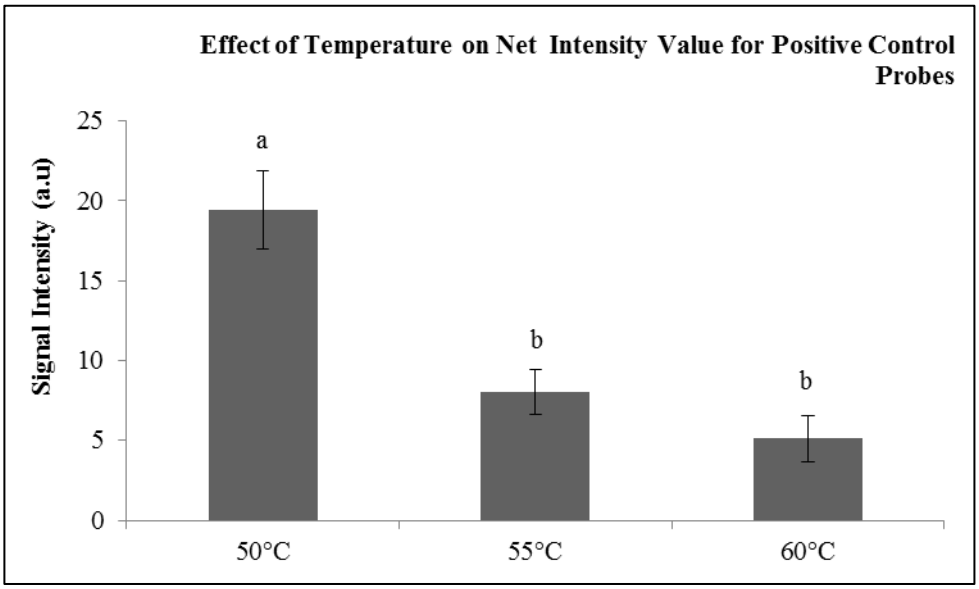

Figure 3. 16 Effect of temperature on net intensity values of positive control probes. The height of the histograms represents the average fluorescence pixel intensity of all spots. Error bars represent standard error of mean calculated from 6 spots for each parameter. Different letters indicate significant difference at $p < 0.05$.

Comparison were done by referencing only the positive control probes in order to see the effect of temperature on immobilization and signalling mechanism independent from hybridization conditions. Differences in signal intensity of positive control probes can be attributed to a kind of vapourization related defect during additional hybridization times at elevated temperatures after their process had finished on surface. Additional post hybridization washing steps may also decrease the signal up to some point. These two parameters for positive control probes should also propose some clues about the assay. These may be the fundamental factors that can affect the basal signal.

In terms of mismatch discrimination, sandwich hybridization parameters with their negative and positive controls are also quantified and results are diagrammed in Figures 3.17, 3.18 and 3.19 for 50°C, 55°C and 60°C, respectively.

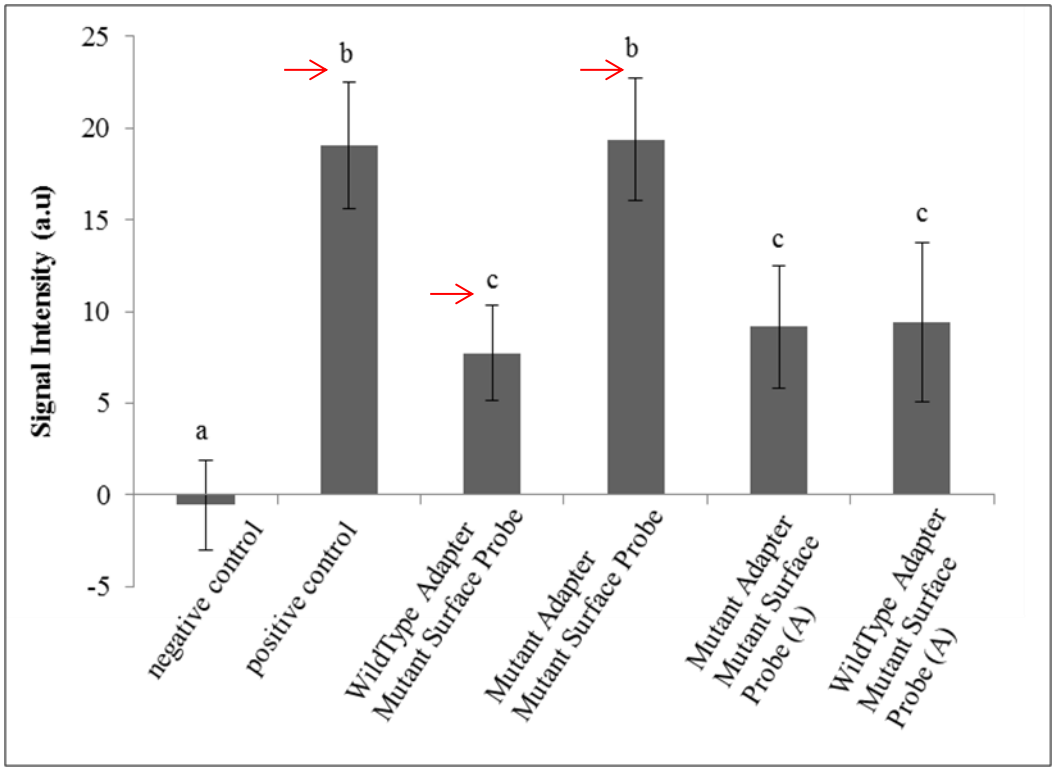

Figure 3. 17 Signal intensity values for the slides having sp 9 spaced surface probes at 50°C hybridization temperature. Negative and positive control parameters were also indicated. The height of the histograms represents the average fluorescence pixel intensity of all spots. Error bars represent standard error of mean calculated from 6 spots for each parameter. Different letters indicate significant difference at $p < 0.05$. (Figure is previously given as 3.12)

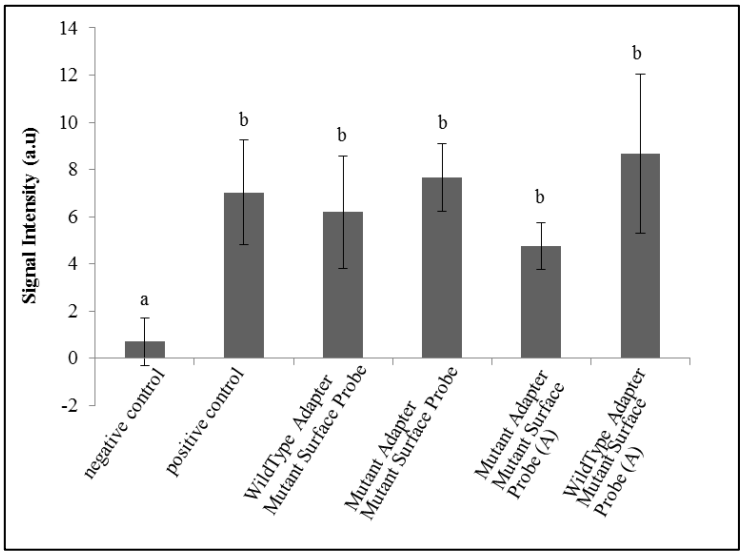

Figure 3. 18 Signal intensity values for the slides having sp 9 spaced surface probes at 55°C hybridization temperature. Negative and positive control parameters were also indicated. The height of the histograms represents the average fluorescence pixel intensity of all spots. Error bars represent standard error of mean calculated from 6 spots for each parameter. Different letters indicate significant difference at $p < 0.05$.

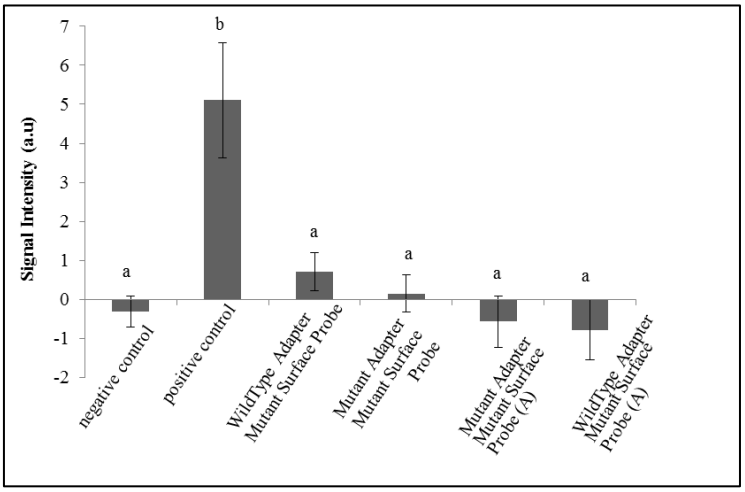

Figure 3. 19 Signal intensity values for the slides having sp 9 spaced surface probes at 60°C hybridization temperature. Negative and positive control parameters were also indicated. The height of the histograms represents the average fluorescence pixel intensity of all spots. Error bars represent standard error of mean calculated from 6 spots for each parameter. Different letters indicate significant difference at $p < 0.05$.

Both visual images and graphics indicate the higher mismatch discrimination efficiency for 50°C hybridization temperature. Under this temperature, surface probes which are complementary to the mutated adapter sequence can hybridize efficiently and give significantly higher intensities than they give with the wild type target. Besides, another type of a surface probe "mutant surface probe (A)", which also has a one mismatch for the adapter but different from "mutant surface probe" (T>A), give significantly lower intensities. Signal intensity gained from the mutated target discrimination is also similar with the intensity gained from positive control probes. In all three temperature trials, positive and negative controls have affirmative results which shows the reliability of the platform. Results of mismatch detection under 55°C and 60°C are not favourable as there is no observed visually and statistically difference for wild type and mutated targets.

Up to this point of experiments in this section,discriminating probe was the one which had been immobilized on surface. Another approach is also tried in which the discriminator is the signal probe. Mismatched base is localized at the center of signal probe, and sequence of adapters and probes are also changed. T_m calculations for new probes are given in Table 3.2.

Table 3. 2 Melting temperature data for oligonucleotide probes. (Discriminator is signal probe and surface probe is common)

		Discriminator: Signal Probe
Probe Type	**Calculated T_m with formula (°C)**	**OligoAnalyzer® 3.1 (°C)**
Surface Probe	66.35	69.7
Mutant Signal Probe	56.5	58.4
Mutant Signal Probe (A)	56.5	59.3

Hybridization temperatures for this approach were 50°C and 55°C, and 60° C was eliminated from the trials. Visual results and quantitative graphics are given below in Figures 3.20, 3.21 and 3.22

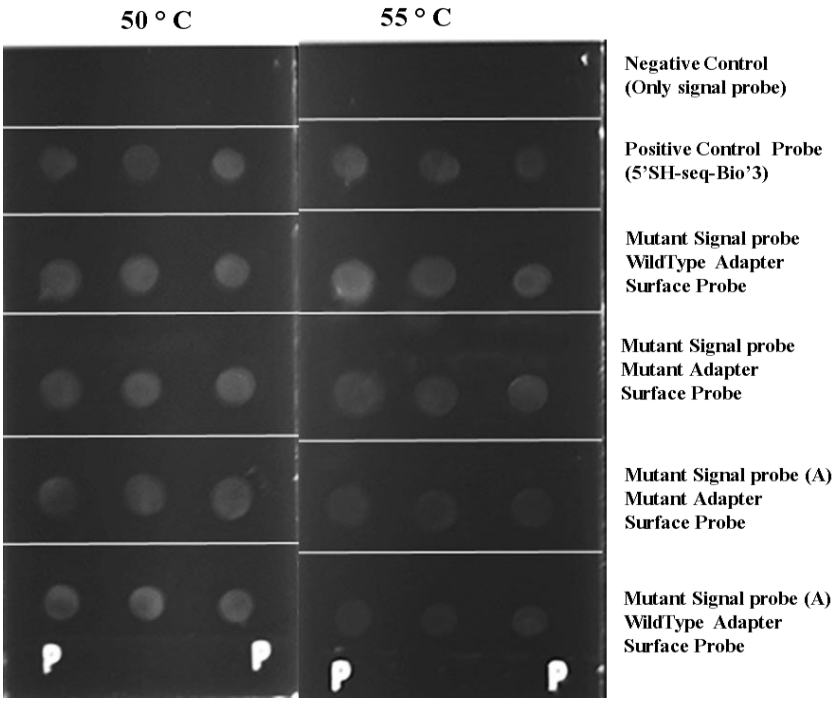

Figure 3. 20 Images of platform upon UV light immobilization at 50°C and 55°C hybridization temperature while the signal probe is mismatch discriminator. Each row shows the results from the application of negative/positive control probes or sandwich hybridization that each of which is explained on the right side of the image.

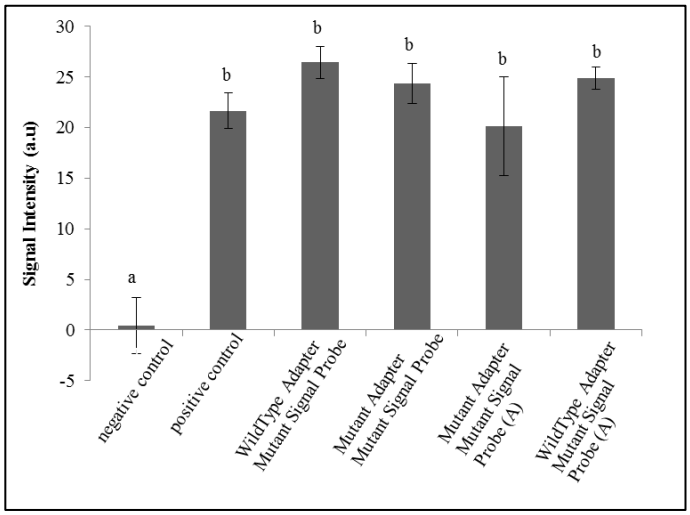

Figure 3. 21 Signal intensity values for the slides having sp 9 spaced surface probes at 50°C hybridization temperature. Negative and positive control parameters were also indicated. The height of the histograms represents the average fluorescence pixel intensity of all spots. Error bars represent standard error of mean calculated from 6 spots for each parameter. Different letters indicate significant difference at $p < 0.05$.

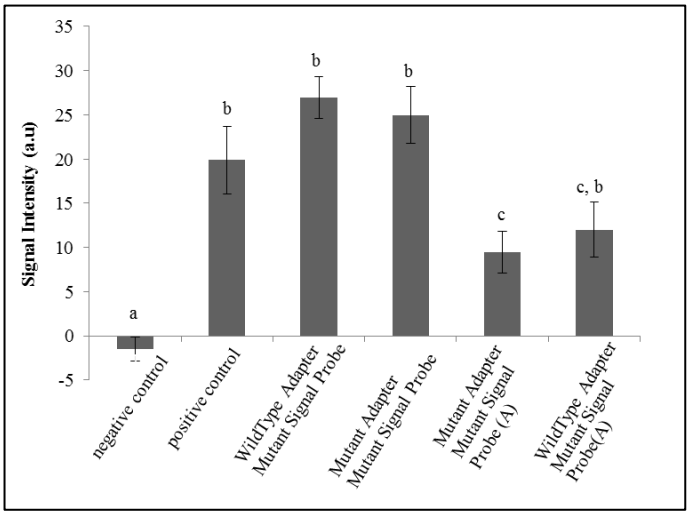

Figure 3. 22 Signal intensity values for the slides having sp 9 spaced surface probes at 55°C hybridization temperature. Negative and positive control parameters were also indicated. The height of the histograms represents the average fluorescence pixel intensity of all spots. Error bars represent standard error of mean calculated from 6 spots for each parameter. Different letters indicate significant difference at $p < 0.05$.

Both of the temperatures do not give affirmative results for mismatch detection when the discriminating nucleotide is on the signal probe. When we compare the two parts of the study in terms of location of the mismatch nucleotide on probes in sandwich format, it can be concluded that the selectivity of the binding increases when the discriminating oligonucleotide locates on the surface probe.

When mismatch nucleotide locates on signal probe, adapter binds to support via the surface probe bridge in any way. In this condition when the nucleotides of signal probe, other than the mismatched one, binds to adapter; it might be impossible to prevent unspecific events by washing regimes. Instead one nucleotide in signal probe, all the others are complementary with the adapter, even many of them removed remaining ones can cause signal as the probe is already carrying the signalling component. Increasing the stringency of washing regimes above the optimized point may also cause the displacement of specific events and decrease in specific signals. On the other hand, when the surface probe contains the mismatched oligonucleotide, these can be all overcomed by preventing the location of adapter on surface thus precluding the signal probes settlement on test from the beginning.

It was also observed that the selectivity of the binding increases when the surface probe attached to the support with a PEG spacer rather than a polynucleotide spacer. Besides, ideal hybridization temperature for sandwich formation is observed as 6°C to 8°C lower than the T_m of the discriminating probe and 10°C-14°C lower than the signal probe. Combination of these physical constraints together with locating the mismatch on surface probe constitutes the optimized and chosen conditions for our platform.

3.3 Single Nucleotide Polymorphism Detection based on Oligonucleotide Ligation Reaction Mediated Sandwich Hybridization in Glass Capillary Tubes

In this section, the system for SNP detection employs an on-surface approach with glass capillary tubes, that can detect single nucleotide polymorphisms (SNPs) through the oligonucleotide ligation reaction mediated sandwich hybridization.

In order to develop a relatively more effective platform for discriminating single nucleotide mismatches, we went towards alternative approaches using a different solid support and enzymatic reactions. The platform explained in this part employs a partially solution based approach inside glass capillary tubes, that can detect single nucleotide polymorphisms (SNPs) through the Oligonucleotide Ligation Assay. Oligonucleotide hybridization rates on surface based approaches are significantly lower than that of solution based models. One common explanation is the fact that the probes are more likely to react freely with other molecules and their complements when they are all in solution state. Volume inside the glass capillary tubes provides a good environment for hybridization process to be conducted in aqueous phase. Oligonucleotide ligation reaction is the other selective speciality of this platform. According to this, if surface and signal probe oligonucleotides are both perfectly hybridised to their adjacent targets they can be ligated during the ligation reaction and signals can be observed.

DNA ligases catalyze the synthesis of a phosphodiester bond between directly adjacent 3' hydroxyl and 5' phosphoryl groups of DNA segments. The enzymes act only when the DNA segments are perfectly hybridized to a complementary DNA sequence. Even a single base pair mismatch between two strands decreases significantly the efficiency of this enzyme, thus preventing the ligation (Wu and Wallace, 1989). This part of the study is mainly based on this phenomenon while creating mismatch discriminating platforms. Generalization of the assay is given in Figure 3.23.

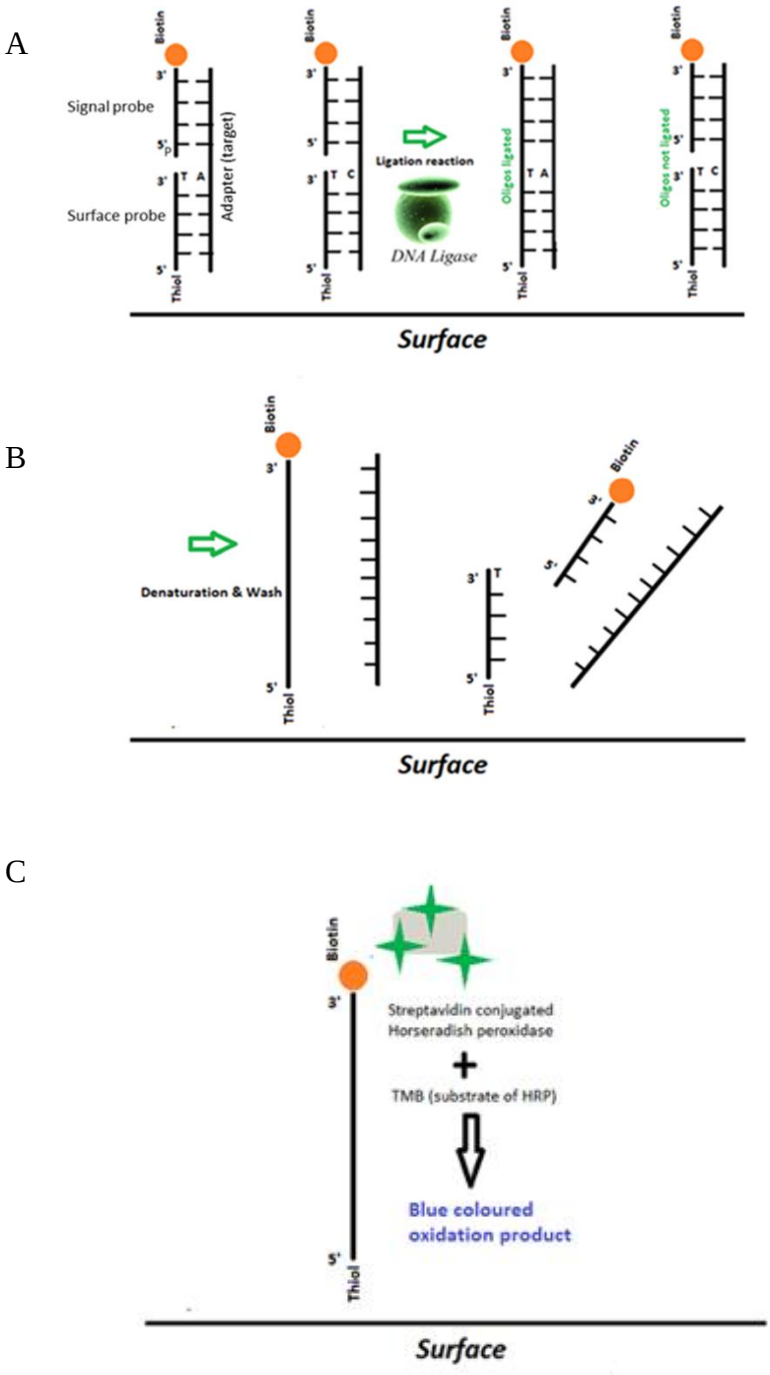

Figure 3. 23 Schematic view of the oligonucleotide ligation mediated SNP detection. A) DNA Ligase mediated ligation reaction of between oligonucleotide probes having one mismatch base or full complementarity to the adapter sequence. B) ligated probes stay on surface after denaturation and washing steps while the unligated signal probe (having mismatch) washed away. C) Surface bound new ligated probe (surface probe+signal probe) interacts with streptavidin conjugated signalling molecules through its biotin modification on 3' end.

In the study, signal probes have 3’ biotin modifications that will bind them to the streptavidin conjugated Horseradish Peroxidase (Strep-HRP). Following surface probe immobilization and ligation reaction steps, Strep-HRP will be applied into capillaries in order to functionalize the ligated probes. Sequence data for the oligonucleotides used in the study is given in Table 2.7.

HRP converts hydrogen peroxide to water, obtaining the two hydrogen atoms it needs for this from a “donor” molecule “3,3’,5,5’-tetramethylbenzidine” (TMB) . Thus, at the same time that hydrogen peroxide is being reduced, TMB is oxidized. In its reduced form TMB is colorless while it is blue in oxidized form. The enzymatic reaction constructing the signalling mechanism of the developing platform is given below in Figure 3.24.

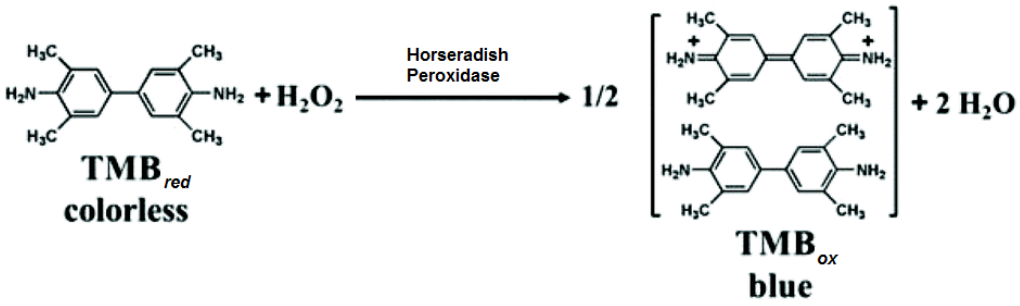

Figure 3. 24 Reaction view for HRP catalyzed oxidation reaction of TMB by H_2O_2

3.3.1 Surface Probe Immobilization and Blocking

Reduction of disulfide bonds of surface probe, activation of epoxy-amine coated walls of capillaries and immobilization of the surface probe was performed at one step in immobilization solution in which the method was also described in Section 2.3.3.2. Differing from the surface based systems in Section 3.2, the

immobilization solution is prepared in larger volumes to fully cover the inside pool of the capillaries. A standard volume to fill a capillary tube is 80 μL and an example table for preparation of immobilization solution is given in Table 2.8. Concentration of the probe having tethered to the inner wall surface of epoxy amine coated capilllary tube is kept constant as 10 μM. General steps during surface probe immobilization is similar with 3.2. except the larger volumes and washing instruments. An automated syringe pump is used in washing steps to ensure a controlled volume and ease of usage. Blocking was done by filling the capillaries with 2% BSA (in 10 mM PBS) and incubating for 1 hour at room temperature. Immobilization of 5' thiol functionalized oligonucleotide surface probes were successfully achieved on epoxy-amine coated glass capillaries. A common probe called as positive control added to each batch of experiments, which has a 5' thiol and 3' biotin modification. Sample reaction mixture is given in Table 2.8. Visual signals from positive control capillaries prove the efficiency of surface immobilization of probes with thiol modification (Figure 3.25)

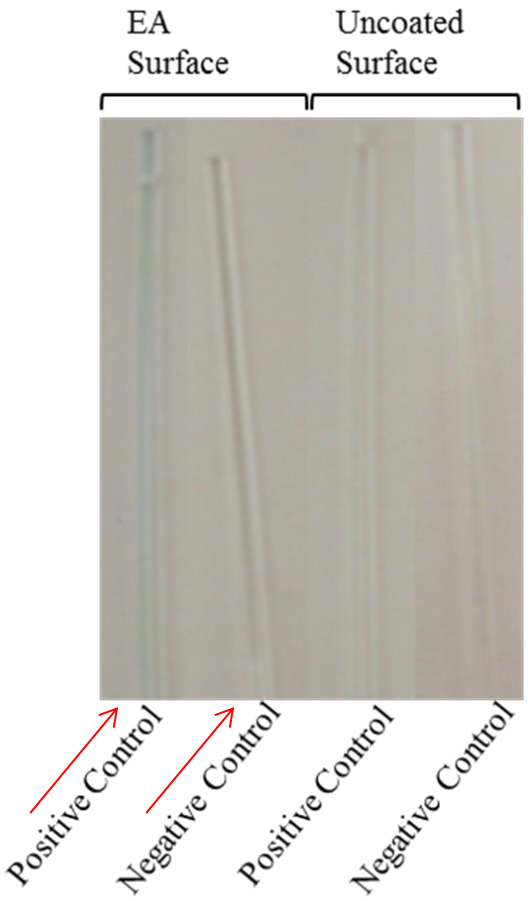

Figure 3. 25 Visual signal observation from HRP-TMB reaction on EA coated and uncoated surfaces. Positive control includes 5' thiol and 3' biotin functionalized probes on surface while the negative control parameter does not contain any oligonucleotide probe.

3.3.2 Oligonucleotide Ligation Reaction and Post Reaction Washing Regimes

Oligonucleotide Ligation Reaction which can discriminate single nucleotide polymorphisms (SNPs) through the reaction creates the main outline of this approach inside glass capillary tubes. Variables are; probe concentrations, concentrations of BSA, PEG, DMSO and/or Taq DNA ligase

Sample reaction mix is shown in Table 2.9. In order to optimize reaction conditions for better and specific signal acquirement, some variables were examined in the reaction and post reaction conditions. Mismatched nucleotide in mutated target is placed in the middle of the sequence thus encountering the 3' end of surface probe. It has been already discussed that the optimal specificity is achieved when the mismatched nucleotide is positioned at the center of the probe sequence of interest. As a result, hybridization of surface and signal probes to the complementary segments of target oligonucleotide is possible even in the presence of a mismatch nucleotide. However, even surface and signal probes get hybridized to target, in the presence of a mismatched nucleotide on surface probe, ligation reaction will not proceed to ligate these two probes. On the other side, in the case of ligation reaction, signal probe will be ligated to surface bound probe and thus it will stay on surface support.

Under standard reaction conditions given in Table 2.9, different post reaction washing regimes were applied in order to get rid of unligated probes and concurrent nonspecific visual signals. Urea and glyoxal mixture were used in seperate sets before commonly applied SDS containing SSC buffer and ultrapure water rinsing steps (Section 2.3.3.3).

As basically said, nucleic acids form structures stabilized by hydrogen bonds between nitrogenous bases. Double helical DNA molecules, when subjecte to extreme pH values, heated near 100° C or proned to some chemical agents, undergo significant changes in their physical properties. During the process, covalent bonds remain intact while the weak hydrogen bonds break, seperating the complementary strands. This physical change is known as denaturation (Rastogi, 2006). Glyoxal (ethanedial) reacts with nucleic acids, nucleotides, and their component bases and introduces an additional ring onto guanosine residues, thus sterically hindering the formation of G-C base pairs and consequently the renaturation of native structure (Hutton and Wetmur, 1973); (Nakaya *et al.*, 1968). Urea is known as a chaotropic denaturant, which unravel the secondary and double stranded structures by disrupting the hydrogen bonding between nitrogenous bases in DNA structure.

Visual signals acquired from the capillaries treated with urea and glyoxal in post reaction washes are given in Figure 3.26.

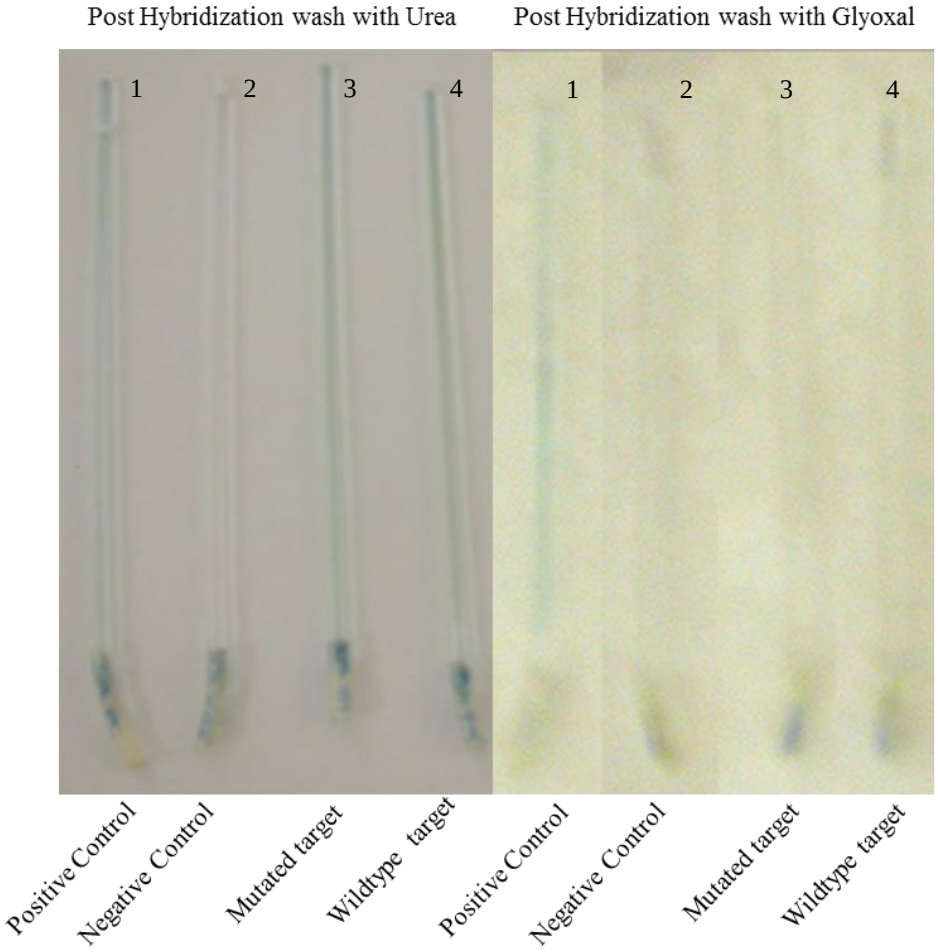

Figure 3. 26 Results of post reaction washes with urea and glyoxal, respectively. With Urea ;1) positive control, 2) negative control (only signal probe on surface) 3) lig.react. with mutated target (adapter), 4) lig.react with wildtype target (adapter); With Glyoxal ;1) positive control, 2) negative control (only signal probe on surface) 3) lig.react. with mutated target (adapter), 4) lig.react with wildtype target (adapter);

For both of the applications, positive control probes give visual signals by colour formation resulting from HRP-TMB reaction, and at the same time no signals were gathered from negative control parameter. This results again proofs the succesful immobilization of thiolated probes on EA surface and specific signal gaining related to the presence of biotinylated signal probe.

On the other hand, mismatched target could not be discriminated from the wildtype in urea application. This may possibly resulted from the insufficient denaturation of double stranded structure. Denaturation is expected to be concluded only with the presence of ligated signal and surface probes together as one on the surface. Glyoxal treatment results in totally negative visual signals for both mismatched and wildtype targets. Positive control probe immobilized capillaries are saved till the HRP-TMB reaction steps and does not prone to ligation reaction mixes and post reaction washing steps thus gained affirmative results from positive control probes only indicates the succesful immobilization and HRP-TMB reaction. In this context, lack of signal in glyoxal treated capillaries when compared with urea trated ones may be attributed to glyoxal application. Post reaction washes might be insufficient to get rid of all the glyoxal remainings and this might be resulted with the damage or inhibition of HRP activity.

Experiencing those insignificant results directed the study towards changing post hybridization washing regimes and reaction components. Visual results are totally given in Figures in Appendix B for optimization experiments and experimental steps are also explained in the following pages.

Heat denaturation was applied in spite of chemical denaturation application before washing steps in order to get rid of unspecific visual signals. Following the reaction, capillaries were incubated in a waterbath at 95°C for 15 minutes and then washed with solutions preheated to 95°C (Section 2.3.3.3). In order to clarify that the acquired visual signals are resulted from the ligation reaction, same experimental steps were performed without Taq DNA ligase (Tables 2.10 and 2.11), (Figure 1, Appendix B).

Positive control probes on EA coated surfaces are verifying the surface efficiency for immobilization via giving visual signals, and it is clearly independent from ligation reaction as well as the presence of Taq DNA ligase. It is also clearly seen that the positive control probe applied on an uncoated surface proofs the efficiency of our custom made surface functionalization by giving no visual signals. Negative controls, only having biotinylated signal probes on surface give weak signals when compared to other test and positive control parameters. It was also discussed previously that the unmodified oligonucleotides can also bind to newly created EA surface by their nitrogenous bases. Signal differences between positive and negative control parameters also explains the efficiency of thiol group conjugation to EA surface thus it is not affected by washing steps, while the non-covalent electrostatic interactions between nitrogenous bases and EA surface is partially removed by washing applications. Relatively week signal from no target control parameter may also be explained in the same way with the above. On the contrary, using uncomplementary target results in no visual signals in both of the applications. Positive and negative results coming from enzyme catalyzed and no enzyme application respectively for wildtype and mutated target also clearly shows that the acquired visual signals are resulted from the ligation reaction**.** Although there is not any significant discriminatory signals on the platform for mismatched target, it is obvious that the ligation reaction is succesfully proceeded.

In order to clarify and overcome the unspecific signals from the wildtype target, which may be attributed to nonspecific interaction between surface and signal probes, uncomplementary target oligonucleotide were also added to the ligation reaction mixture to cause a kind of competitive hybridization event. Reduction in binding sites due to hybridization of multiple targets but more efficiently steric crowding is theorized to adjust specific reaction events. Signal probe: uncomplementary target: wildtype target molar ratios are adjusted as 1:1:1 and 1:2:2. Besides, BSA was also excluded and 3% DMSO was added to the reaction mixture (Tables 2.12 and 2.13) Results gained as visual signals are given in Figure 2 in Appendix B. Relatively lower visual signals from “surfaceprobe-

(wt.target+uncomp.target)*2–signalprobe" sandwich parameter may be attributed to the specific hybridization rate adjusting effect of competitive hybridization. On the other hand, unspecific signal is still present for no target control parameter, similar with the previous experiments.

Polyethylene glycol (PEG) polymer has been used to enhance blunt end ligation efficiency and specificity in many studies. Xiao *et al*. (2007) also the investigated the effects of other additives on oligonucleotide ligation assay efficiency and specificity. The results showed that PEG was proved to be the best additive among them in which the highest yield was obtained with a relatively improved specificity. PEG molecules create macromolecular crowding which enhances ligation efficiency and specificity. It is also called as "volume excluder" and the crowded solutions caused by are more adequate model for intracellular conditions and so thus the enzyme activities than assays in dilute solutions (Fulton, 1982). In order to benefit from the enhancer property of PEG, we also added PEG8000 with a final concentration of 1% into ligation reaction mixtures (Table 2.13). Duplicate trials were done (Figures 3 and 4, Appendix B) for no target control parameter and wildtype target, and totally clear results were gained which may proof the efficiency of PEG on the specificity of the reaction.

Until this point of capillary experiments 1-Step™ Turbo TMB-ELISA (Pierce Biotechnology) substrate solution was used for HRP catalyzed reaction. In order to have brighter signals, another substrate solution; 1-Step™ Ultra TMB ELISA was also used which yields more sensitivity among the TMB substrates. First results are compromising when the wild type target containing capillaries give slightly lighter blue colour than that of mutated target (Figure 5, Appendix B). However, it is not sufficient for such a platform that needs to be visually read without any doubts. In order to reduce the unspecific visual signals, blocking step was performed in a more stringent way. 1% Tween 20, which is a polysorbate surfactant, is added to the blocking process to more effectively block the remaining available spaces to prevent the unspecific binding of signal probes (Figure 6, Appendix B).

However, visual results are not covering the expectations from the platform. In addition to the new blocking way, post HRP application washing regimes also applied in a more stringent way. 0.05 % Tween 20 including 2x PBS wash buffer is replaced with 1 % Tween 20 including 2x PBS wash buffer in the new experiment. In this way, detection of mutated target can easily observed by colour formation (Figure 3.27). Optimization steps up to this point brought the platform to an affirmative level for mismatch discrimination. Although all six replicates does not give smooth colour formation patterns through the surface, differences in visualization can be easily distinguished.

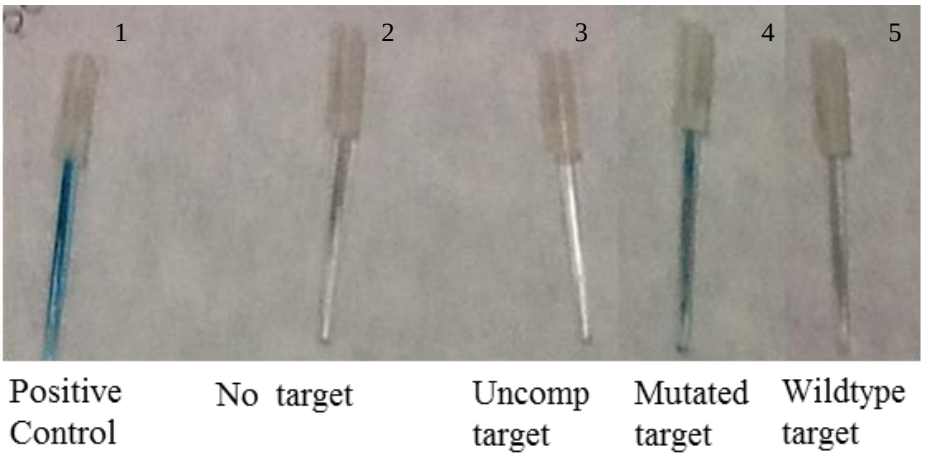

Figure 3. 27 Results of the reaction with 1-Step™ Ultra TMB ELISA substrate solution and Tween 20 including blocking and post HRP washing steps. 1) positive control, 2) lig.react. without target (adapter), 3) lig.react. with uncomplementarytarget(adapter),4) "surface probe-(mut.target+uncomp.target)*2-signal probe", 5)"surfaceprobe-(wt.target+uncomp.target)*2–signalprobe"

Beside photographic results, success rates were also calculated for each test parameter. Success rate looks at a data sample for either successes or failures, which may also be called as "yes" or "no" expressions. Among six replicates from the finally optimized experimental conditions, success rate to discriminate

mismatched target from the other parameters such as complementary target, random uncomplimentary target and without target controls is 71.5 %. Blue colour intensity of the capillaries from each parameter were also compared with their positive controls in their relevant sets. A graphical description is given below in Figure 3.28.

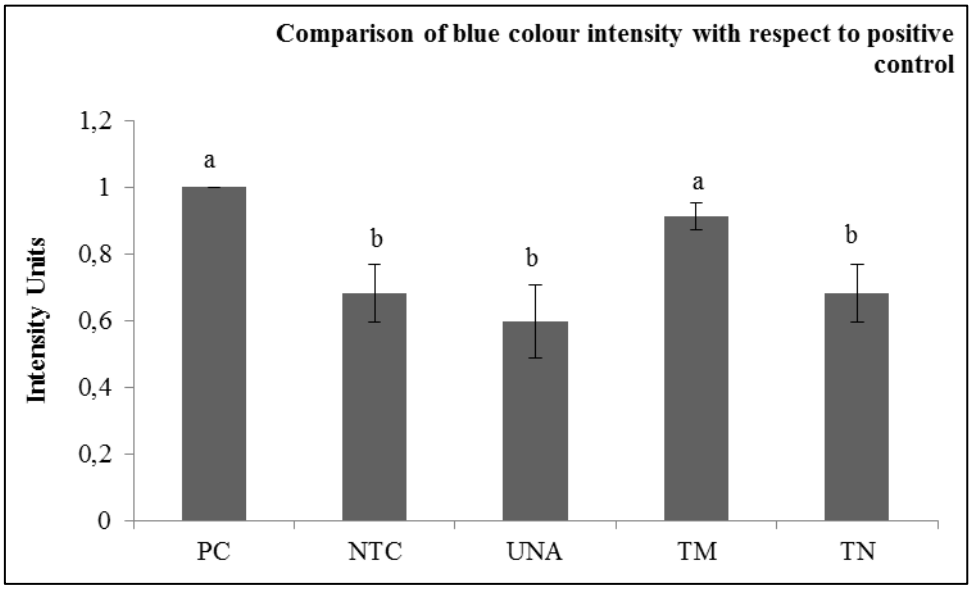

Figure 3. 28 Blue colour intensity of differen parameters on the platform with respect to their positive controls. PC: positive control, NTC: lig.react. without target (adapter), UNA: lig.react. with uncomplementarytarget(adapter), TM: lig.react. with mutated target, TN: lig.react. with wildtype target

Main bottleneck of the platform is denaturating the hybrid constituted by the unligated probe pair and the target (adapter) oligonucleotide. Under ideal conditions, ligation reaction occurs between the surface probe and signal probe if there is a complementary target. In this way, signal probe remains united with the surface probe and thus stays on support giving signals. Non-specific signals from wildtype target, uncomplimentary target and no target controls are possibly

resulting from the unspecific binding of signal probe on surface and ineffective denaturation after sandwich hybridization.

Effective immobilization capacity of epoxy-amine surface might be one of the disadvantages in this platform for causing nonspecific ionic interactions between signal probe and the surface. Another surface coating having switchable interactions with nitrogenous bases of probes and so thus the end modifications can be chosen. Aldehyde silanization might be an option in this condition. Schiff base formation between aldehydes and amines occurs readily in aqueous solutions, especially at elevated pH. This is a pH dependent process and the linkage, however, is not stable unless reduced by a reducing agent to secondary or tertiary amine bonds. Therefore, binding interactions between nitrogenous bases of probes and the surface can be prevented by adjusting the pH and not using reducing agents. In this way, surface binding reaction will only occur between thiol modified surface probe and aldehyde silanized support via a heterobifunctional cross linker. As an example, 3,3′-N-[ε-Maleimidocaproic acid] hydrazide, trifluoroacetic acid salt (EMCH) is such a crosslinker that conjugates sulfhydryls to carbonyls (aldehyde or ketones). On the other hand, ineffective denaturation conditions after sandwich hybridization to get rid of unligated signal probes and target regions might be enhanced. There might be especially a heated syringe pump system to perform heat denaturation and consequent washing steps simultaneously and more effectively.

3.4 Single Nucleotide Polymorphism Detection via Silica Nanoparticle Based Lateral Flow Strip Platforms

In this section, the system for SNP detection employs an on-surface approach together with MCM-41 mesostructured silica nanoparticles and lateral flow platform. Signalling mechanism in this developing platform is also based on the HRP catalyzed oxidation reaction of TMB. Detailed image describing the system is given in Figure 3.29.

MCM-41 mesostructured silica nanoparticles were filled with TMB substrate through their pores. Following TMB embedding incubation period, silica surface is modified with amine groups via 3-aminopropyltrimethoxysilane silanization material. This amine modification gives positively charged structure to the surface to have an ionic interaction with negatively charged phosphate backbone of the oligonucleotide probe. By this way, it is aimed to close the pore openings via theoretically horizontal position of probes on the surface. In literature, similar approaches with oligonucleotides, aptamers, proteins or any other molecules are generally referred as "molecular gate", "molecular lock" or "molecular valve" systems. These structures can respond to an external stimuli by opening or closing. Stimulus can be physical, chemical, or biological such as redox activation, pH, temperature or the presence of a specific enzyme, antibody or DNA. Their response constitutes a controlled release for the entrapped cargo molecules. In our platform, the oligonucleotide probes capping the pores can be opened via the presence of a complementary oligonucleotide probe. It is expected to arise from a dislocation reaction in the presence of a complementary strand. Surface bound probes and complementary target probes will hybridize, dislocate or change the orientation and thus the pores will be uncapped and entrapped TMB molecules will be released. On the contrary, pores are expected to stay closed if an uncomplementary target is applied because of the relative impossibility of hybridization between two probes and thus the dislocation from the pore openings. Under these conditions TMB will be retained inside the silica nanoparticles' pores. As a result, depending on the specific hybridization event, controlled release of TMB occurs.

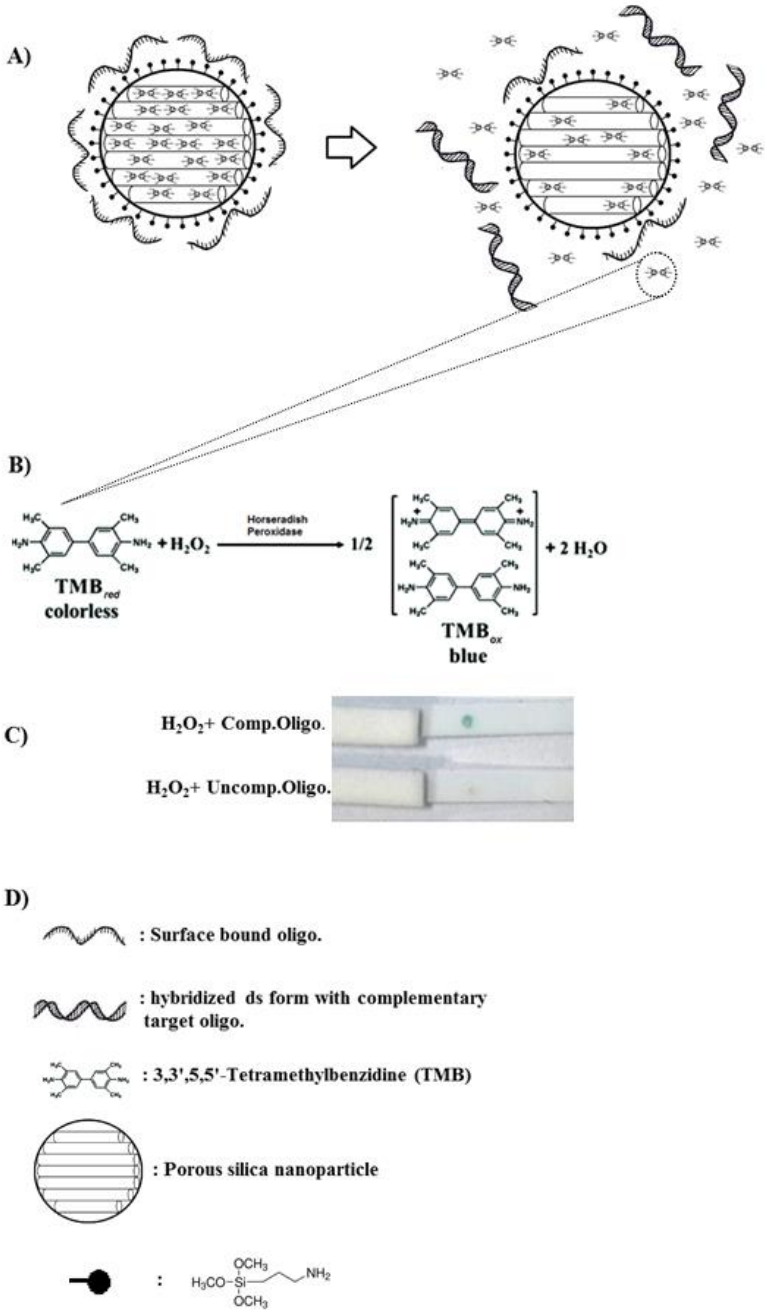

Figure 3. 29 Graphical description of the developing platform. A) TMB is embedded through the pores of silica particles, surface is silanized with amino groups and oligonucleotide probes bind to surface via ionic interactions and capped the pores. In the presence of a target complementary strand, hybridization and related displacement of the probes from the surface occurs, TMB is released through pores. B) HRP catalyzed oxidation of released TMB with H_2O_2 yields a visual blue product. C) Specific reaction on lateral flow strips with nitrocellulose membrane applied processed silica particles and target+H_2O_2 application through sample pad. D) Detailed description of test components (Figure is inspired from Climent *et al.* (2010))

In the platform, released TMB molecules are subjected to a redox reaction catalyzed by horseradish peroxidase with the presence of H_2O_2. TMB is oxidized while the H_2O_2 is reduced to H_2O and oxidized TMB gives a blue colour formation. Consequently, when the complementary target oligonucleotide applied silica particles are reacted with HRP/H_2O_2 system, blue product visualization is expected. On the contrary, if the target is not fully complementary, there would not be a hybridization event and thus the pores would remain capped without releasing TMB out. In conclusion, under the presence of uncomplementary target oligonucleotide, blue product visualization is not expected while the presence of complementary target oligonucleotide gives visualized blue coloured read-outs.

3.4.1 Optimization of HRP-TMB reaction constituents

HRP can catalyze the oxidation of TMB in the presence of H_2O_2 and there may be several TMB derivatives in the reaction system, including intermediates (e.g., cationic free radical, charge transfer complex, and di-imine) (Figure 3.30).

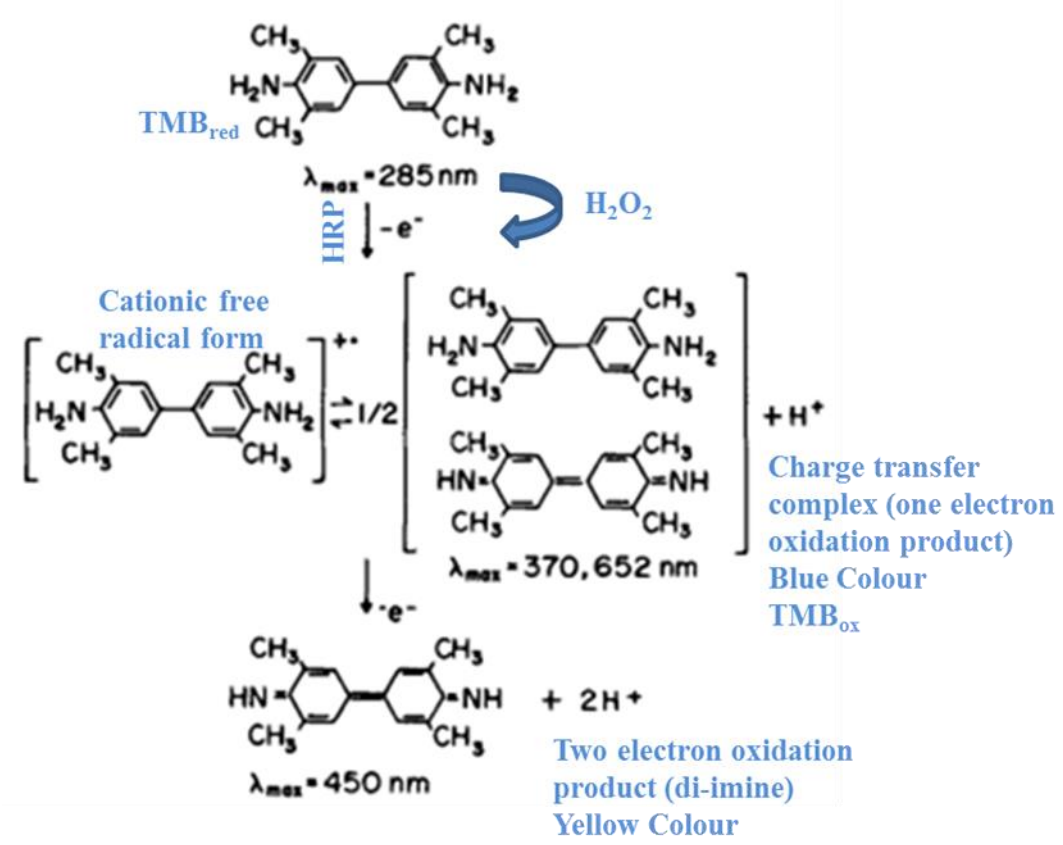

Figure 3. 30 Route for the HRP/H_2O_2/TMB system. Modified from Josephy *et al.* (1982)

The charge-transfer complex exhibits a blue color, with absorbance peaks at 652 and 370 nm and exist in rapid equilibrium with the radical cation structure. If sufficient acid exists (e.g. excess H_2O_2 or strong acidic condition), the blue product will be further oxidized to a yellow diimine, which is stable at acidic condition and has the maximal absorption wavelength at 450 nm while displaying a yellow color (Figure 3.30) (Gao *et al.*, 2011); (Josephy *et al.*, 1982); (Li *et al.*, 2009). HRP/TMB/H_2O_2 catalytic sytem is chosen as the signalling system for our developing platforms as the coloured products are susceptive to unaided eyes, and also allows for spectroscopy.

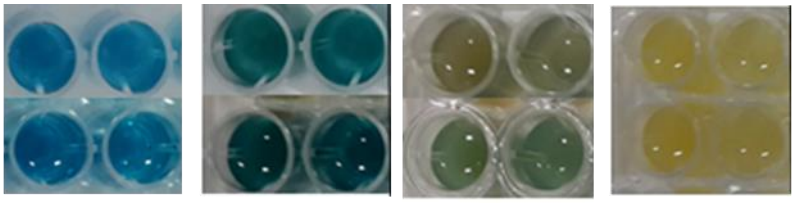

Figure 3. 31 Coloured products of the oxidation of TMB by horseradish peroxidase (HRP)/ H_2O_2 system

In this study, oxidation of the benzidine derivative, TMB, by horseradish peroxidase in the presence of H_2O_2 was firstly catalyzed in solution base to determine the proper stochiometry to get the visual results of blue colour formation. It is explained that HRP-mediated catalysis follows a ping-pong mechanism in which the enzyme first reacts with H_2O_2 to form an enzyme-oxygen free radical and then the free radical reacts with TMB. Therefore, concentrations of both TMB and H_2O_2 will affect the reaction kinetics (Gajhede et al., 1997); (Gaoet al., 2007) (Gao et al., 2011).

Commercially sold TMB substrate already including H_2O_2, is generally used in most of the studies in literature that the current enzymatic reaction is required for signalling. In this developing platform, TMB and H_2O_2 will participate into the system with silica nanoparticles and on nitrocellulose membrane through the sample application pad, respectively. Therefore, we could not use a premixed solution and need to prepare the unique components of the assay seperately. We firstly choose a stable H_2O_2 concentration of 1M (3.5% w/v), and observe the reaction under different TMB concentrations with a two different amount of HRP (Table 2.14).

H_2O_2 content is higher than usual to shorten the waiting time for the appearance of color and to enhance the blue colour formation in next experiments on nitrocellulose membranes seen by the unaided eye. This kind of molar adjustment

for TMB and H_2O_2, related with the aim of the experiment, was also done by Zhu *et al.* (2010). They also used the HRP-TMB reaction for a DNAzyme related thrombin detection on nitrocellulose membrane supported dot blot assay. Spectroscopic results for the blue product formation with different TMB concentrations under a stable H_2O_2 content is given below in Figure 3.32.

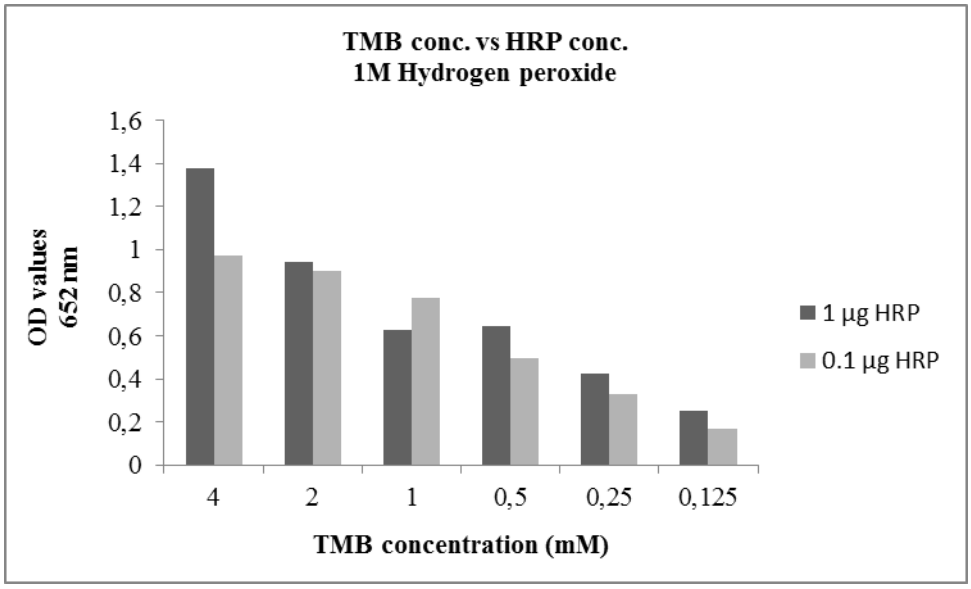

Figure 3. 32 Spectroscopic measurement of the reaction with various TMB concentrations and enzyme contents at 652 nm.

Figure 3.32 demonstrates the formation of blue product under stable 1 M H_2O_2 content but different TMB and enzyme contents. Measurements were done at the wavelength of 652 nm which is the absorbance peak region of blue coloured oxidation product. 1 µg enzyme content gives dramatic decreases in blue product formation with less TMB concentrations while it is more mild with lower enzyme content of 0.1 µg. All this points can be concluded that the enzyme content affects the reaction rate. Josephy *et al* (1982) also used various HRP concentrations with fixed TMB and H_2O_2 content. Formation of the blue product was measured as a

function of time following addition of enzyme by recording absorbance at 700 nm and it was observed that in each case, the final absorbance was the same. Thus, they also proposed that enzyme affects the rate of the reaction, but not the concentrations of the products formed.

In order to see the effect of different H_2O_2 amounts on the reaction we also fixed TMB content as 1 mM and HRP as 0.1 µg, which are the least amounts to gain affirmative results. Figure 3.33 demonstrates the formation of blue product under stable 1 mM TMB and 0.1 µg enzyme but different H_2O_2 contents. OD values are observed to be decrease proportionally with H_2O_2 concentrations.

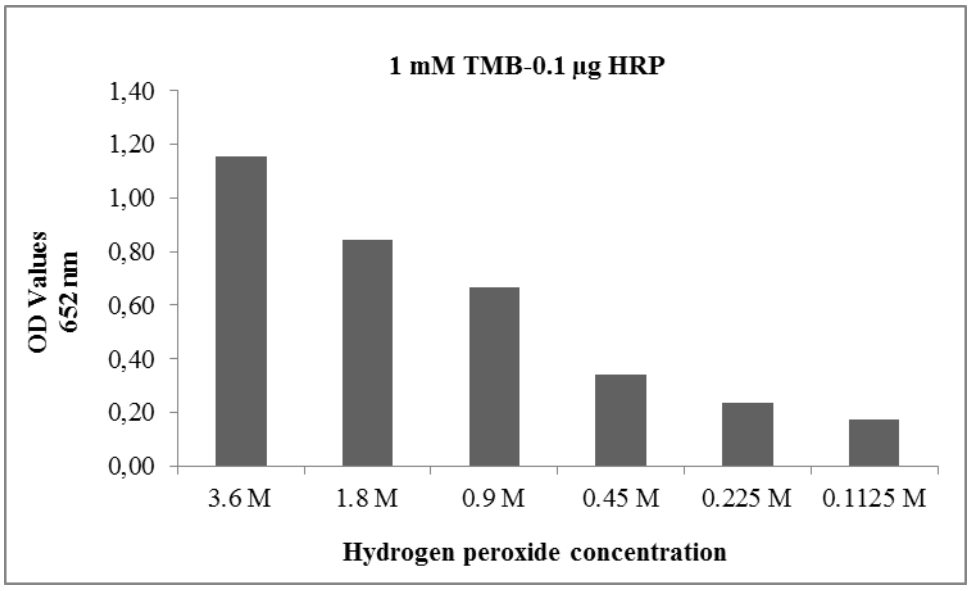

Figure 3. 33 Spectroscopic measurement of the reaction with various H_2O_2 concentrations and enzyme contents at 652 nm.

3.4.2 Preparation of TMB loaded silica nanoparticles

In molecular-gate-based platforms, cargo molecules which are desired to be selectively released are loaded into a nanoporous container. A gating molecule which can open or close it according to the presence of an external physical, chemical or biological stimulus, caps each pore on the nanoporous material. Redox activation, competitive binding, environmental factors like pH, temperature, and light, or a biological stimuli factor like enzymes, antibody, and DNA, can trigger various nanovalve systems (Klajn *et al.*, 2010); (Özalp *et al.*, 2014); (Yang, 2011). Mesoporous silica nanoparticles (MSNs) are used in many biomedical applications which are based on molecular gate or also called nano gate or nano valved systems for targeted drug delivery, targeting cancer cells for identification and treatment and also for biosensing. They have many excellent characteristics, such as good biocompatibility, rigidity, chemical stability, optical transparency, high surface areas, large pore volumes, uniform and tunable pore sizes. Controllable surface functionalization of MSNs can be carried out with a variety of organic functionalities, such as thiols, amines and carboxylic acid groups (He and Shi, 2011); (Rosenholm *et al.*, 2010); (Yang, 2011).

In this study, mesostructured MCM-41 hexagonal silica particles (Figure 3.34) with pore volume and pore sizes of 0.98 cm^3/g and 2.1-2.7 nm, respectively. There should be a good match between pore size and the molecule to be loaded.

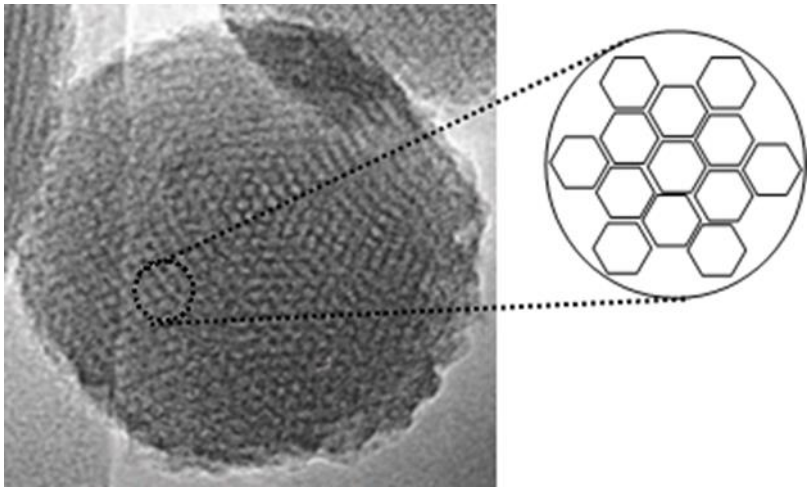

Figure 3. 34 TEM image of a MCM-41 hexagonal mesostructured silica nanoparticle (Trewyn *et al.*, 2007), and a closer view for pore structure.

3.4.3 Optimization of Reaction Parameters in Silica Nanoparticle Based Environment

Different concentrations of TMB were prepared with dilution from the main stock as; 0.4 M, 0.2 M, 0.1 M, 0.05 M and 0.025 M. in DMSO. TMB was loaded into the pores of bare MSN's with incubation periods. Before proceeding to silanization phase, some optimization experiments were done with TMB loaded silica nanoparticles.

3.4.3.1 In solution trials

Pellets coming from the MSN's TMB loading suspension solutions,which were assumed to include TMB embedded particles, were resuspended in H_2O_2 and HRP mixture. Pellets were colorless before addition of mixture and the colour formations after adding reaction components are seen in Figure 3.35. Final assumed concentrations of TMB in 1 mL PBS environment for the given concentrations are; 4 mM, 2 mM, 1 mM, 0.5 mM and 0.25mM, respectively.

Besides, H_2O_2 and HRP contents were kept constant as 1.142 M and 1 µg, respectively.

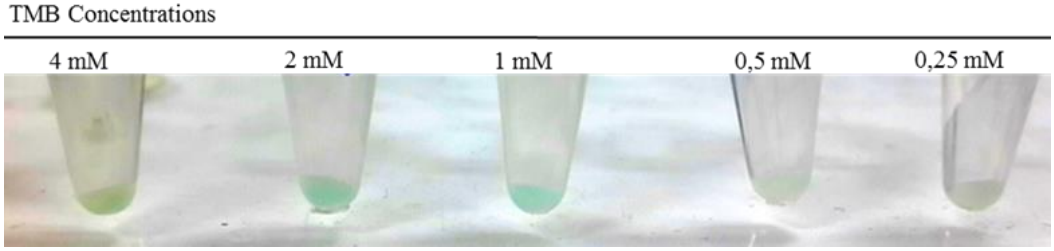

Figure 3. 35 Visualization of HRP catalyzed oxidation of TMB previously embedded in silica nanoparticles

Following the visualization of colour formations, samples were centrifuged, supernatants were collected seperately and pellets were resuspended in a lower volume of H_2O_2. OD_{652} and OD_{370} values which are specific absorbance peak regions for blue coloured oxidation product, were measured for both the resuspended pellets and collected supernatants. OD_{652} and OD_{370} values were about to zero for supernatants (data not shown) while the same values gave different results in pellets related with the concentration of TMB (Figures 3.36 and 3.37).

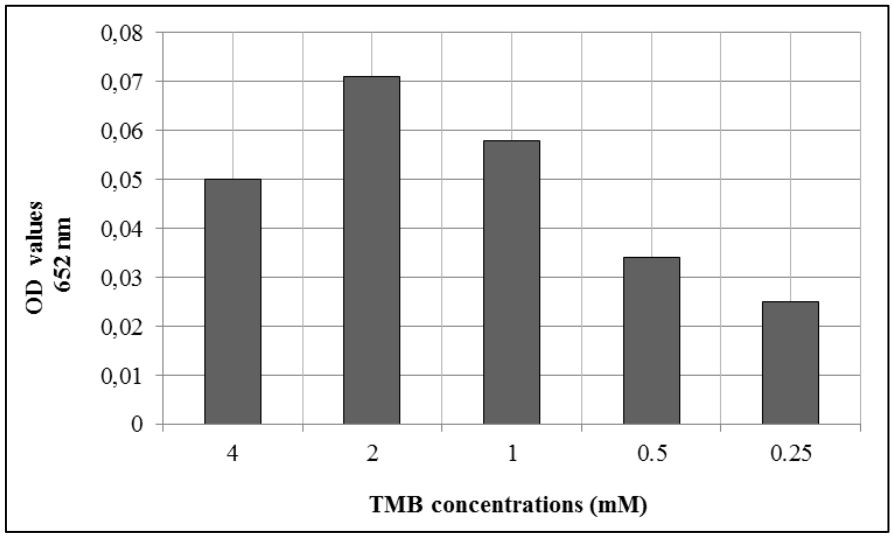

Figure 3. 36 Spectroscopic measurement of the reaction with the pellets of silica nanoparticle embedded TMB molecules of various concentrations at 652 nm wavelength.

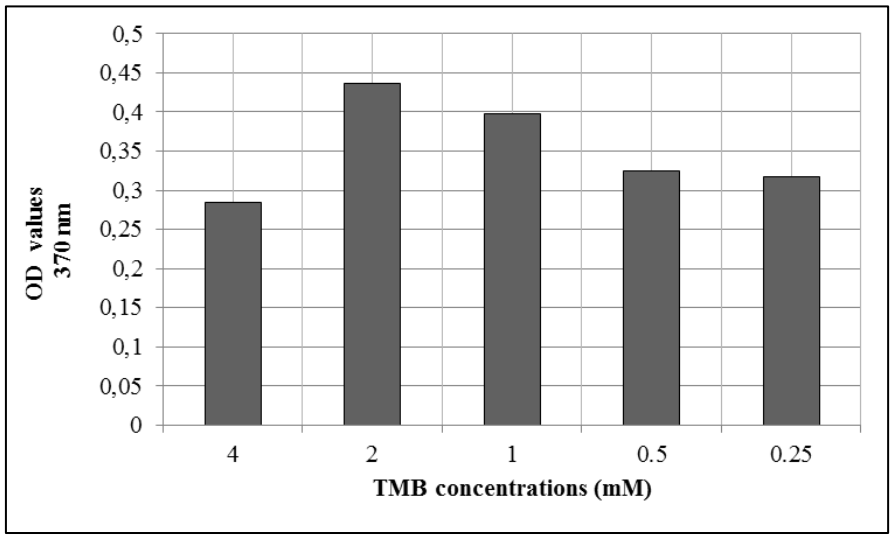

Figure 3. 37 Spectroscopic measurement of the reaction with the pellets of silica nanoparticle embedded TMB molecules of various concentrations at 370 nm wavelength.

Visual results and corresponding graphics of absorbance values of silica pellet solutions at 370 and 652 nm, are in consistency in terms of blue product formation as it was already mentioned that the blue oxidation product of the reaction gives peaks at 370 and 652 nm. It can be also commented that the absence of blue product in supernatants after reaction, but presence in pellet measurements, corresponds to the location of the reaction. In solution state, TMB seems to diffuse from pores in order to participate in reaction but not completely spreading in the solution. Instead, it relatively stays on the surface or localize orbitary.

The UV chromophore of TMB in its original unreacted form gives a peak at 285 nm. The values both for the pellet and supernatant samples are given in Figures 3.38 and 3.39. According to this; even there have been any unreacted TMB molecules, they still localize on the silica particles rather than spreading into the solution. In supernant, only samples from 2 and 4 mM concentrations gave some absorbance but they are nearly hundred times lower than that of the contents in pellet sample. In summary, these results are also in correspondence with the idea discussed above about the location of TMB molecules.

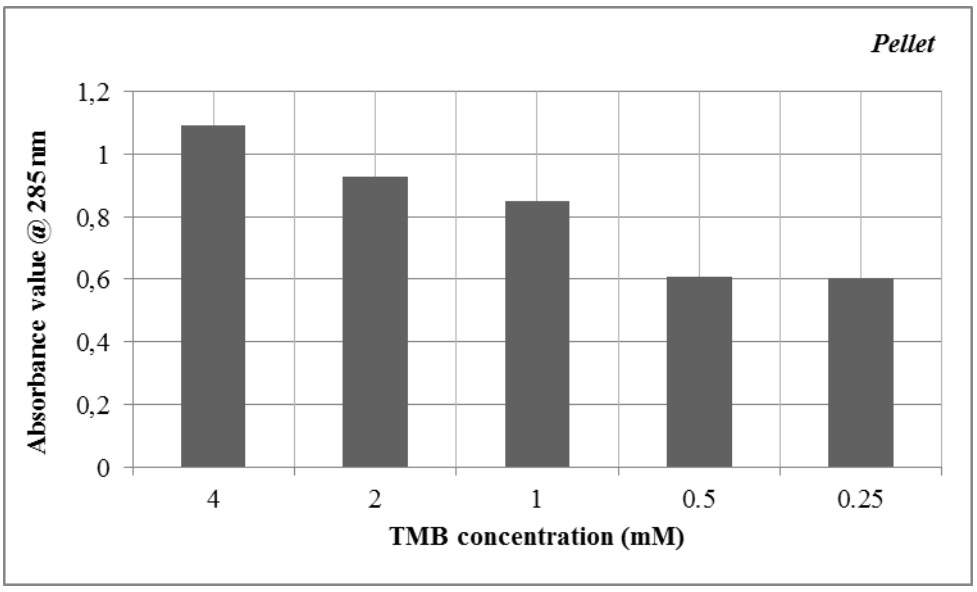

Figure 3. 38 Spectroscopic measurement for the unreacted TMB molecules of various concentrations in pellets at 285 nm wavelength.

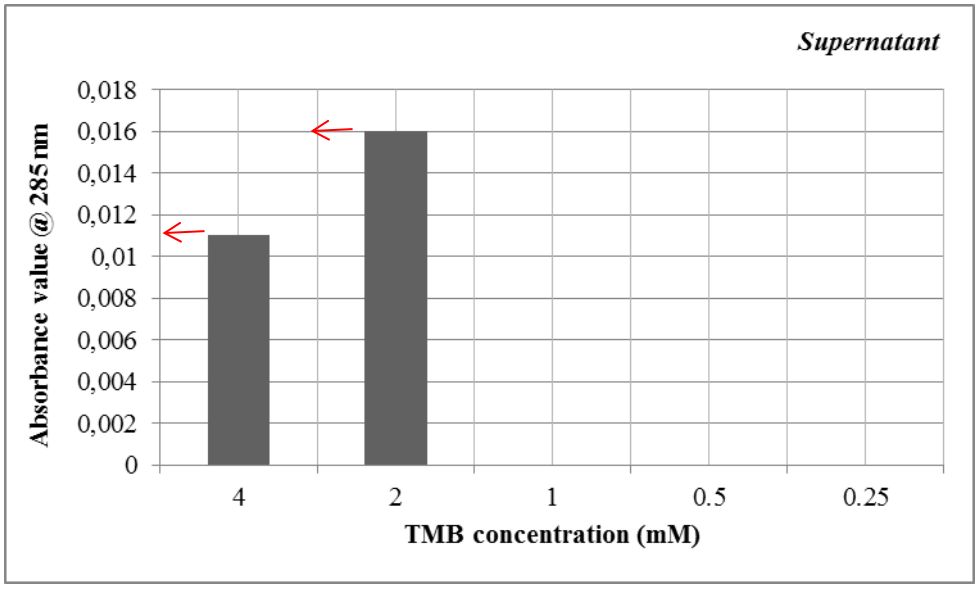

Figure 3. 39 Spectroscopic measurement for the unreacted TMB molecules of various concentrations in supernatants at 285 nm wavelength (Zero absorbance for 1, 0.5 and 0.25 mM concentrations)

3.4.3.2 On strip trials

Assay was also tried on HF180 nitrocellulose membrane strips for 4 mM, 2 mM and 1 mM final concentration of TMB loaded silica particles. This concentration interval was chosen on the basis of in solution trials which have the most promising ones to enhance the blue product visualization.

TMB embedded silica nanoparticles' pellets were dissolved in 5 µL of PBS and applied on strip as 1 µL. HRP (1mg/ml) was also applied as 1 µL. 50 µL of H_2O_2 was sent from the sample application pad. Only difference between strip 1 and strip 2 design is the position of TMB and HRP lines according to sample application pad (Figure 3.40).

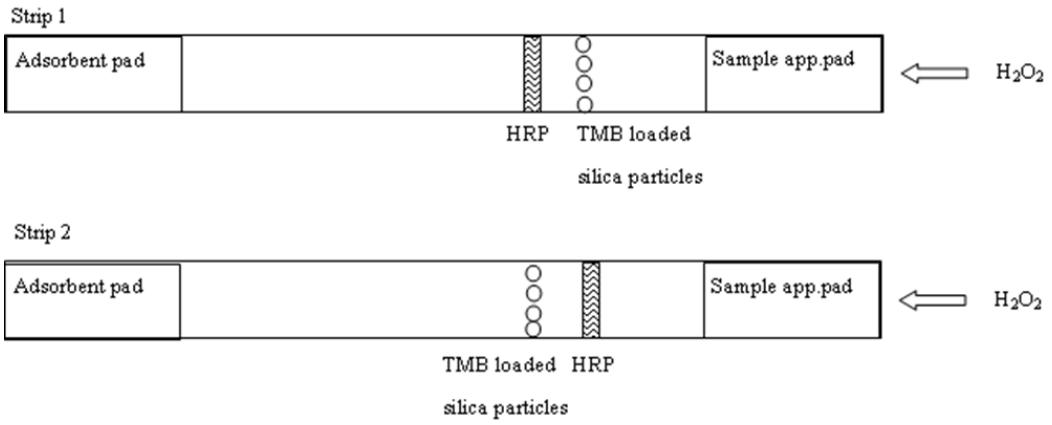

Figure 3. 40 Design of nitrocellulose membrane strips in terms of the position of TMB and HRP lines according to sample application pad

3.4.3.2.1 Preparation of Lateral Flow test strips

Lateral flow test strips used in this study were fabricated by assembling three components on an overlapping sequence. HF180 coded Hi-Flow ™ Plus nitrocellulose membranes (Millipore) were used which have 180 ± 45 (sec/4cm) capillary flow times. Porous matrices that are used for both the sample application pad and absorbent pad were purchased as cellulosic fiber materials and they were not proned to any chemical or physical application before using. Differing from the classical lateral flow test platforms, we did not use a conjugate pad element. Instead, we used the membrane platform as a controlled environment for the reaction to take place. Besides, sample and absorbent pads were used to control the flow of H_2O_2 through the reaction.

3.4.3.2.2 Running silica nanoparticle based lateral flow strip assay

TMB loaded silica nanoparticle suspensions and HRP were applied on nitrocellulose membrane cards in two different orders called as “strip1” design and “strip2” design in order to understand and optimize the flow behaviours of the assay. Designs are shown in Figure 3.40. After completing the process on

membrane cards, 3.5 % H_2O_2 was sent from sample application pad and waited until it reaches to absorbent pad. All of the strip 1 designs gave no colour formation while strip 2's could be visualized (Figure 3.41).

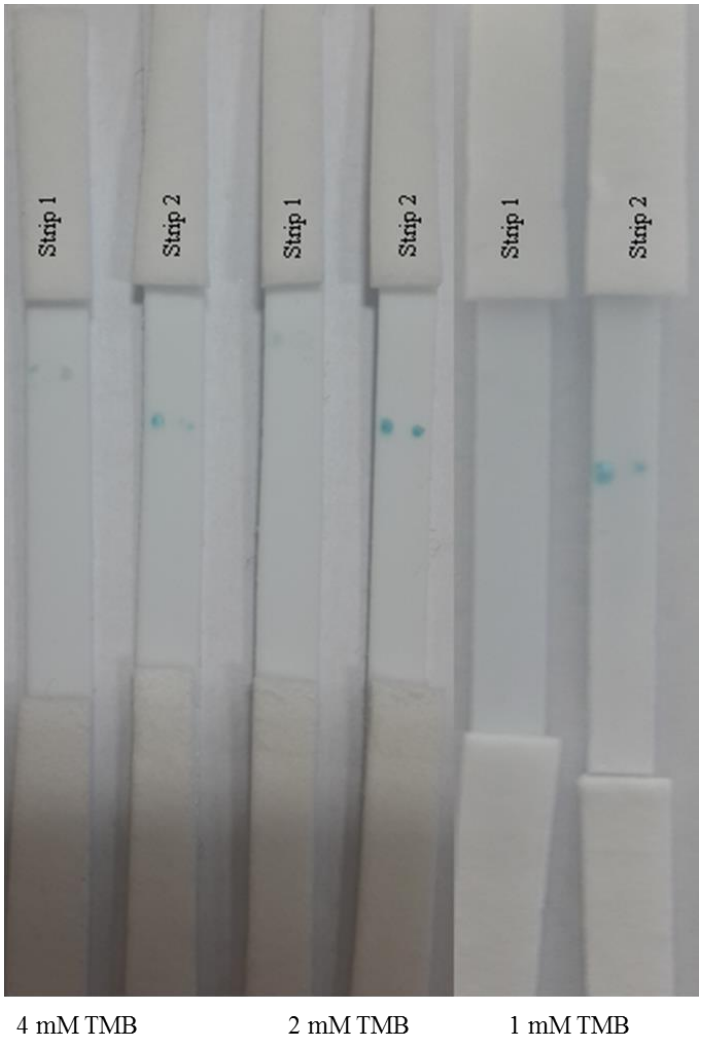

Figure 3. 41 Visualization of HRP catalyzed oxidation of TMB, previously embedded in silica nanoparticles, on nitrocellulose membrane based lateral flow strips. (Strip1 and strip2 designs in terms of the position of TMB and HRP lines according to sample application pad was previously described in Figure 3.40)

These trials are also in correspondence with the results in solution state that the TMB seems to diffuse from pores in order to participate in reaction but not completely spreading. Instead, it relatively stays on the surfaces or localize orbitary. Blue product formation can be only visualized when TMB loaded particles are below the HRP loading point, while the H_2O_2 and HRP mixture flows through them.

In the first trial in which the results are shown in Figure 3.41 silica nanoparticle suspensions were prepared with a final volume of 5 µL and applied as 1 µL on membranes. We were also curious about the concentration of silica particles for strip 2 design which gave visual results. In order to see the effect of dilution on pellets, both 10 µL and 20 µL suspensions were prepared and applied on membranes according to strip 2 design. Final TMB concentrations were also varied as 4 mM, 2 mM, 1 mM, 0.5 mM and 0.25 mM (Figure 3.42)

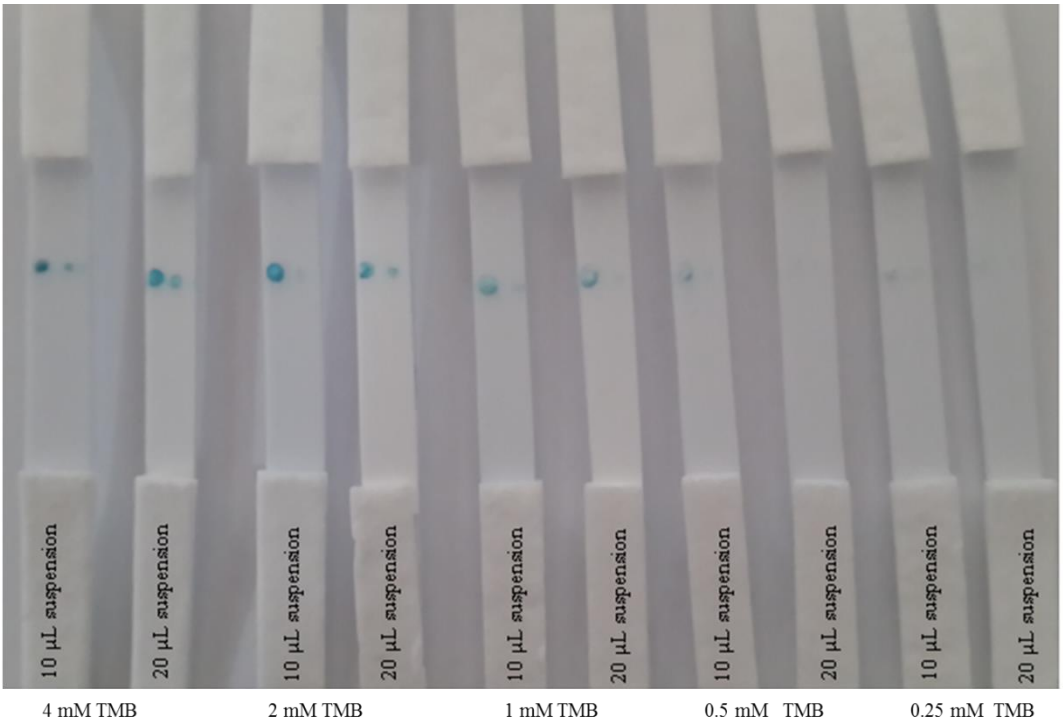

Figure 3. 42 Visualization of HRP catalyzed oxidation of TMB previously embedded in silica nanoparticles on "strip2" designed lateral flow platforms. Suspensions were prepared in 10 µL and 20 µL volumes for each TMB concentration.

According to the visual results in Figure 3.41 and 3.42, intensity of the blue colour decreases related with the concentration of TMB but suspension volume does not seem to have a dramatic effect on it. There is also not dramatically different change in colour when the results of 10 and 20 µL suspensions for 1 mM TMB are compared with that of 5 µL in Figure 3.41. We choosed 10 µL suspension for the batch of TMB embedded particles which are coming from a starting amount of 5 mg bare silica nanoparticles, in order to have better handling capacity and more homogenized application on membrane than 5 µL suspensions.

3.4.4 Oligonucleotide Loading on TMB Embedded Silica Nanoparticle Surface

In order to construct a molecular-gate-based platform with TMB embedded silica nanoparticles in combination with lateral flow systems, we decided to use oligonucleotides as capping molecules. As the main goal of this thesis is to develop SNP diagnostic platforms, this gate-based system is also directed to include oligonucleotide elements.

Shortly, oligonucleotides were used as capping molecules for the pores of silica nanoparticles which were loaded with TMB. These caps can be selectively opened in the presence of a specific complementary oligonucleotide, and let the TMB substrate out. Visual results are gained after adding the reaction components of H_2O_2 and HRP to the system.

3.5.4.1 Determination of Surface Functionalization Chemistry and Oligonucleotide Binding to the Surface

Silica nanoparticles are first loaded with TMB substrate and then their surface is functionalized through silanization. 3-aminopropyltrimethoxysilane (APS) is chosen as the silanization material. APS gives positively charged structure to the surface to have an ionic interaction with negatively charged phosphate backbone of oligonucleotide. By this way, it is aimed to have closed pores via theoretically horizontal position of probes on the surface. Because of the fact that oligonucleotides are expected to cover the pore openings, they do not need to have end modifications to covalently bind to surface in a defined, theoretically vertical position. In summary, oligonucleotide probes does not have any end modifications and adsorp to the amino modified surface by ionic interactions.

3.4.4.2 Running silica nanoparticle based lateral flow strip assay for Oligonucleotide Target Detection

Air dried silica particles including final 2 mM TMB, which previously showed better visualization and binded oligonucleotides on its surface, were suspended and applied on nitrocellulose membrane cards. HRP was also applied according to the strip 1 and strip 2 design. After completing the process on membrane cards, complementary and uncomplementary oligonucleotides was suspended in 3.5 % H_2O_2 with final concentrations of 100 μM. Oligonucleotide-H_2O_2 mixture were sent from sample application pad and waited until it reaches to absorbent pad.

As also shown in Figure 3.43, surprisingly strip 2 design did not give specific results in order to discriminate the presence of hybridization between silica surface bound probe and its complementary oligonucleotide. On the other hand, strip 1 design gave a visual signal in 15 minutes with the presence of complementary oligonucleotide. Its relatively smaller surface area can be attributed to the unhomogenized application of particles on surface (Figure 3.43 and Figure 3.44).

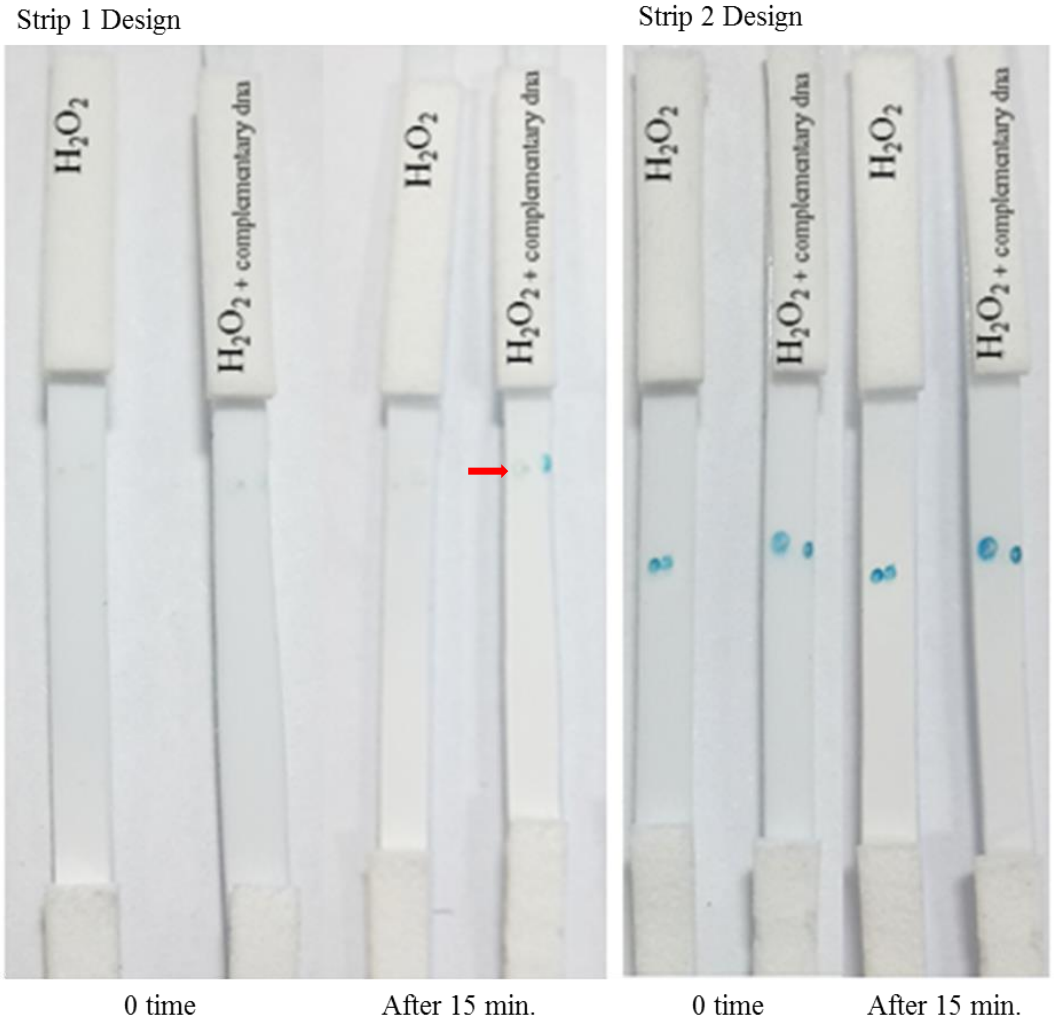

Figure 3. 43 Visualization of HRP catalyzed oxidation of TMB previously embedded in silica nanoparticles on nitrocellulose membrane based lateral flow strips on "0" time and after 15 minutes. Reaction is dependent on the hybridization of surface immobilized olignucleotide with a complementary probe. Strip1 and strip2 designs in terms of the position of TMB and HRP lines according to sample application pad was previously described in Figure 3.43. H_2O_2 marked ones are the controls for H_2O_2+complementary DNA test.

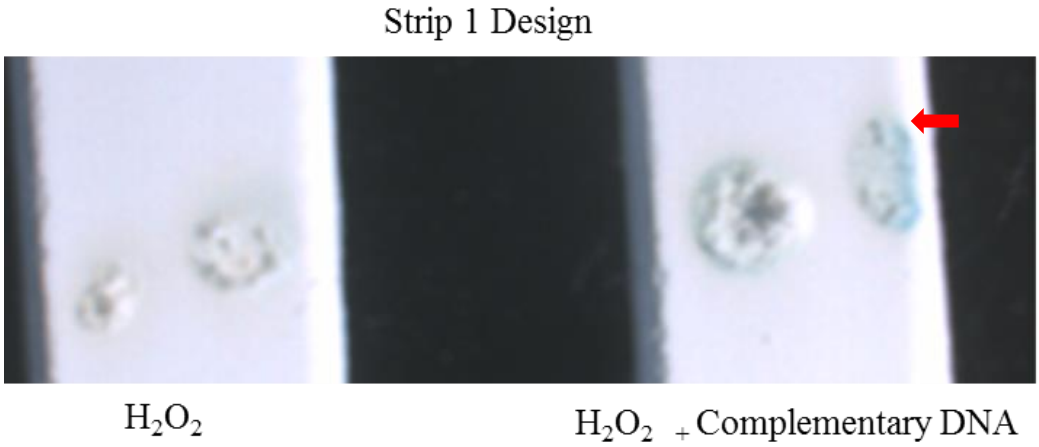

Figure 3. 44 Closer view with a microscope image for the "strip 1" design presented above in Figure 3.42, after 15 minutes. Reaction is dependent on the hybridization of surface immobilized olignucleotide with a complementary probe. Strip1 and strip2 designs in terms of the position of TMB and HRP lines according to sample application pad was previously described in Figure 3.43. H_2O_2 marked one stands for the control of H2O2+complementary DNA test.

Results from another replicate is given in Figures 3.45 and Figures 3.46, as the latest is the stereomicroscope image of the strips in the first figure. There are four different control parameters which were explained below the figures. Visual signals were only get from the test strip which were also proofed with other replicates.

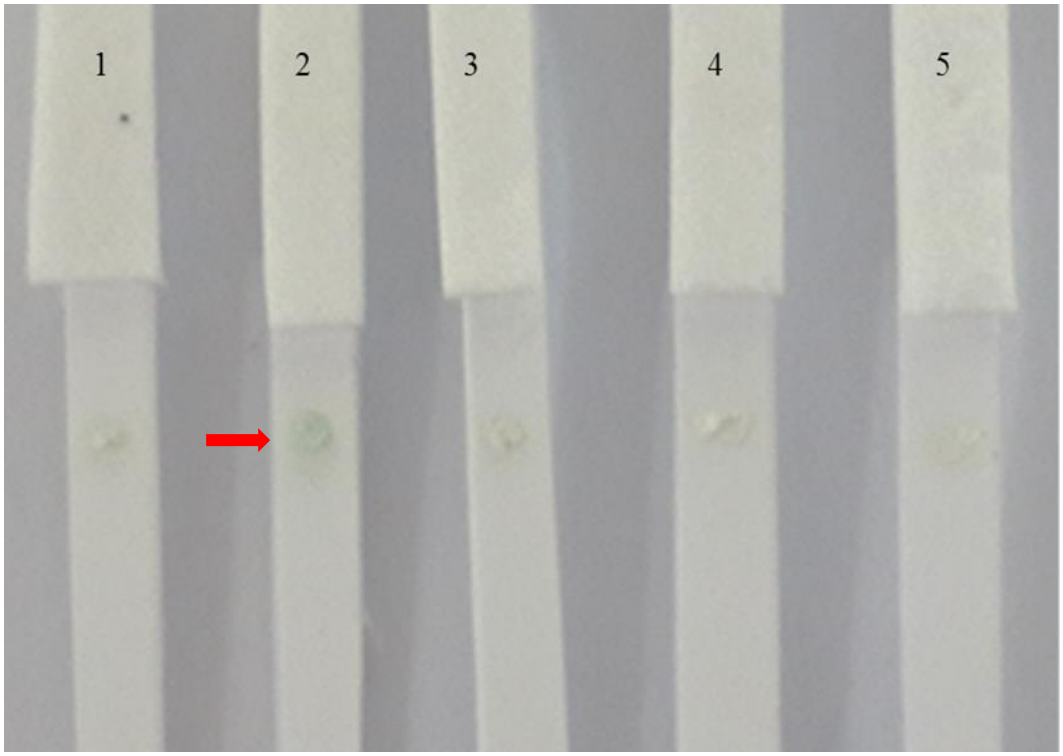

Figure 3. 45 Visualization of HRP catalyzed oxidation of TMB previously embedded in silica nanoparticles on nitrocellulose membrane based lateral flow strips. Reaction is dependent on the hybridization of surface immobilized oligonucleotide with a complementary probe, and design of "strip1" is used which was previously described in Figure 3.43. 1) surface oligonucleotide immobilized TMB embedded silica particles on strip, H_2O_2 delivery through sample pad, 2) surface oligonucleotide immobilized TMB embedded silica particles on strip, H_2O_2+complementary probe delivery through sample pad, 3) surface oligonucleotide immobilized TMB embedded silica particles on strip, H_2O_2 +uncomplementary probe delivery through sample pad, 4) TMB embedded silica particles without surface oligonucleotide on strip, H_2O_2 +uncomplementary probe delivery through sample pad, 5) TMB embedded silica particles without surface oligonucleotide on strip, H_2O_2 delivery through sample pad

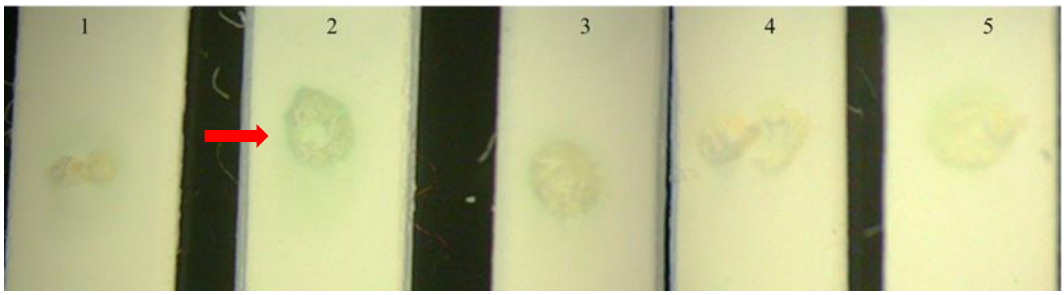

Figure 3. 46 Closer view with a microscope image for the Figure 3.44. 1) surface oligonucleotide immobilized TMB embedded silica particles on strip, H_2O_2 delivery through sample pad, 2) surface oligonucleotide immobilized TMB embedded silica particles on strip, H_2O_2+complementary probe delivery through sample pad, 3) surface oligonucleotide immobilized TMB embedded silica particles on strip, H_2O_2 +uncomplementary probe delivery through sample pad, 4) TMB embedded silica particles without surface oligonucleotide on strip, H_2O_2 +uncomplementary probe delivery through sample pad, 5) TMB embedded silica particles without surface oligonucleotide on strip, H_2O_2 delivery through sample pad.

These trials are also in correspondence with the results in solution state and first on strip trials that the little amount of TMB spreads through but mostly localizes near surface.

In strip 2 design HRP and H_2O_2 flow over particles and cause the reaction to take place. All of the TMB molecules participate in reaction as both diffused ones and unspecifically surface bound ones. As a result we can not observe any specific reaction depending on the presence of target molecule.

On the other hand, specific results are observed in the strip 1 design under the presence of complementary oligonucleotide. Although HRP is below the particles and can not reach to the particles in complete amount in this concept, because of the flow direction, some contrary comments are given in literature. Lu *et al.* (2009) and Carrilho *et al.* (2009) used wax patterning method in order to prevent cross-talk between near by samples and spreading of immobilized molecules. Nitrocellulose membrane also has a lateral and vertical wicking rate which may cause spreading of the material after application. It is also claimed that

combination of lateral and vertical wicking can cause a weak capture line (Jones, 1999). Although spreading and cross talking are seem to be problematic issues in classical nitrocellulose membrane platforms, they might be counted as advantageous clues in this developing platform to gain specific read outs.

In summary, an explanation of the process on the strip can be offered like the following; as a result of gate opening through oligonucleotide hybridization TMB molecules slightly diffuse from the pores and a minor amount of HRP reaches to the zone and reaction takes place. Under this selectivity, there is not any unspecific reaction with surface sticked TMB molecules but only with the diffused ones.

3.5.5 Detection of Mismatched Complementary Oligonucleotides

Promising results from the complementary probe discriminating applications in the experiments above directed the study towards mismatched probe detection. Experimental steps were similar as the previous ones, except the newly designed probes.

3.5.5.1 Design of Oligonucleotide Probe Sequences for Mismatch Discrimination

Bacillus sp. specific probes used for the assay were designed based on the published sequences of *sap* gene encoding S-layer protein (GenBank accession number. Z36946).

Duplex instability is important for mismatch discrimination and is related to both position and type of the mismatch between target and probe. According to literature; internal mismatches, at the center or very close the center; have more destabilizing effects on hybridization stability when compared to the ones at ultimate or penultimate positions (Naiser *et al.*, 2008); (Özkumur *et al.*, 2010); (Peyret *et al.*, 1999); (Piao *et al.*, 2008). As indicated above, equally important other point for effective mismatch discrimination is the type of nucleotide pairs

which will constitute the mismatch context . Piao *et al.* (2008) showed the hybridization stability trend affected by single mismatches as; G:T≈G:G>G:A>A:A≈T:T>A:C≈TC>C:C. Peyret *et al* (1999) also observed the trends in stabilities of like with like base mismatches at 37°C and published as; G:G > T :T ≈ A:A > C:C. Boulard et al (1997) also observed that C:C mismatches have a low stacking propensity and are stabilized by only one hydrogen bond with NMR and molecular dynamics analysis. Consequently, it has been seen that different studies rate C:C mismatches as the least stable of the four like with like base mismatches and other four different base mismatches. In the light of this information we introduced the C-C mismatch in the middle of the sequence in case of the single mismatch probes to maximize duplex instability (Table 3.3).

It was reported that the destabilizing effect of the second mismatch depends on many factors such as the type and location of the mismatches and the type of the neighbouring base (Ke and Wartell, 1993); (Werntges *et al.*, 1986). Piao *et al* (2008) observed that a second mismatch shifts the melting curve leftward further, indicating a decrease of melting temperature when they compared the melting curves of perfect match and 1-mismatch with second mismatch. According to this a cooperative melting feature when two MMs are present in a duplex is shown for 9 different double mismatch combinations. A:C and C:C double mismatch combination causes the melting temperature decrease more than other combinations. It has been reported previously that when two MMs are separated by ten nucleotides, the duplex is more unstable (Guo *et al.*, 1997). It was interpreted mainly on the basis of the helical structure. B-form DNA contains about ten basepairs per helical turn, and two MMs separated by ten nucleotides become the nearest neighbors across the major grove of the helix, showing a cooperative influence on the helical stability. We have designed the two mismatch probe according to these information from related literature with A:C and C:C double mismatch which are seperated from each other by ten nucleotides (Table 3.3). One more mismatch also introduced into sequence as the

third mismatch. Basically the third mismatched base is put just right hand side of one of the other mismatch near to the 5-end side (Table 3.3).

Table 3. 3 Oligonucleotide probe designs for mismatch discrimination in nitrocellulose membrane supported silica nanoparticle system

Probe Name	***Sequence (5'-3')***
Target (T)	AGACTGACAAGCAGTTCGGTACAGAAGCAGCAAA
Complementary Probe (CP)	TTTGCTGCTTCTGTACCGAACTGCTTGTCAGTCT
Uncomplementary Probe (UP)	GAAAATAGAAAATTTTTCCTAAACGAAAAGGTTA
Mismatch 1 (MM1)	TTTGCTGCTTCTGTACCCAACTGCTTGTCAGTCT
Mismatch 2 (MM2)	TTTGCTGCTTCCGTACCGAACTCCTTGTCAGTCT
Mismatch 3 (MM3)	TTTGCTGCTTCCCTACCGAACTCCTTGTCAGTCT

Visualized results from the photography and microscopic images of strips are given in Figures 3.47 and 3.48. First, second and third strips in each figure correspond to the no sample control, complementary probe (CP) and uncomplementary probe (UP), respectively. Results are gained as the same with the results of section 3.4.4.2. Besides, strips 4, 5 and 6 corresponds to the complementary probes having one, two and three mismatched bases, respectively. CP and one mismatch probe gave visual product formation, as the MM1's is lower than that of CP. On the other hand UP, MM2 and MM3 did not give visual product which might be attributed to the increment in specificity as the similarity between sequences decreases.

Figure 3. 47 Visualization of HRP catalyzed oxidation of TMB previously embedded in silica nanoparticles on nitrocellulose membrane based lateral flow strips. Reaction is dependent on the hybridization of surface immobilized oligonucleotide with a complementary probe, and design of "strip1" is used which was previously described in Figure 3.43. 1) surface oligonucleotide immobilized TMB embedded silica particles on strip, H_2O_2 delivery through sample pad, 2) surface oligonucleotide immobilized TMB embedded silica particles on strip, H_2O_2+complementary probe delivery through sample pad, 3) surface oligonucleotide immobilized TMB embedded silica particles on strip, H_2O_2 +uncomplementary probe delivery through sample pad, 4) surface oligonucleotide immobilized TMB embedded silica particles on strip H_2O_2+MM1 probe delivery through sample pad, 5) surface oligonucleotide immobilized TMB embedded silica particles on strip H_2O_2+MM2 probe delivery through sample pad, TMB embedded silica particles without surface oligonucleotide on strip, 6) surface oligonucleotide immobilized TMB embedded silica particles on strip H_2O_2+MM3 probe delivery through sample pad, 7) TMB embedded silica particles without surface oligonucleotide on strip, H_2O_2 delivery through sample pad.

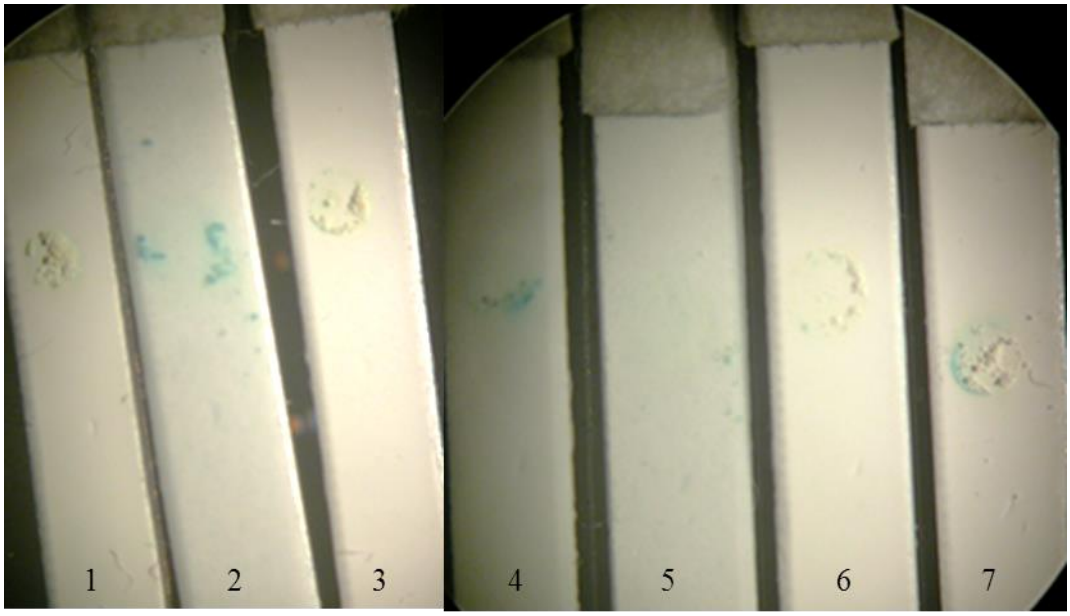

Figure 3. 48 Closer view with a microscope image for the Figure 3.46. Visualization of HRP catalyzed oxidation of TMB previously embedded in silica nanoparticles on nitrocellulose membrane based lateral flow strips. Reaction is dependent on the hybridization of surface immobilized olignucleotide with a complementary probe, and design of "strip1" is used which was previously described in Figure 3.43. 1) surface oligonucleotide immobilized TMB embedded silica particles on strip, H_2O_2 delivery through sample pad, 2) surface oligonucleotide immobilized TMB embedded silica particles on strip, H_2O_2+complementary probe delivery through sample pad, 3) surface oligonucleotide immobilized TMB embedded silica particles on strip, H_2O_2 +uncomplementary probe delivery through sample pad, 4) surface oligonucleotide immobilized TMB embedded silica particles on strip H_2O_2+MM1 probe delivery through sample pad, 5) surface oligonucleotide immobilized TMB embedded silica particles on strip H_2O_2+MM2 probe delivery through sample pad, TMB embedded silica particles without surface oligonucleotide on strip, 6) surface oligonucleotide immobilized TMB embedded silica particles on strip H_2O_2+MM3 probe delivery through sample pad, 7) TMB embedded silica particles without surface oligonucleotide on strip, H_2O_2 delivery through sample pad.

Another result from the replicates is also given in Figure 3.49, and better mismatch discrimination ability of the platform can be observed.

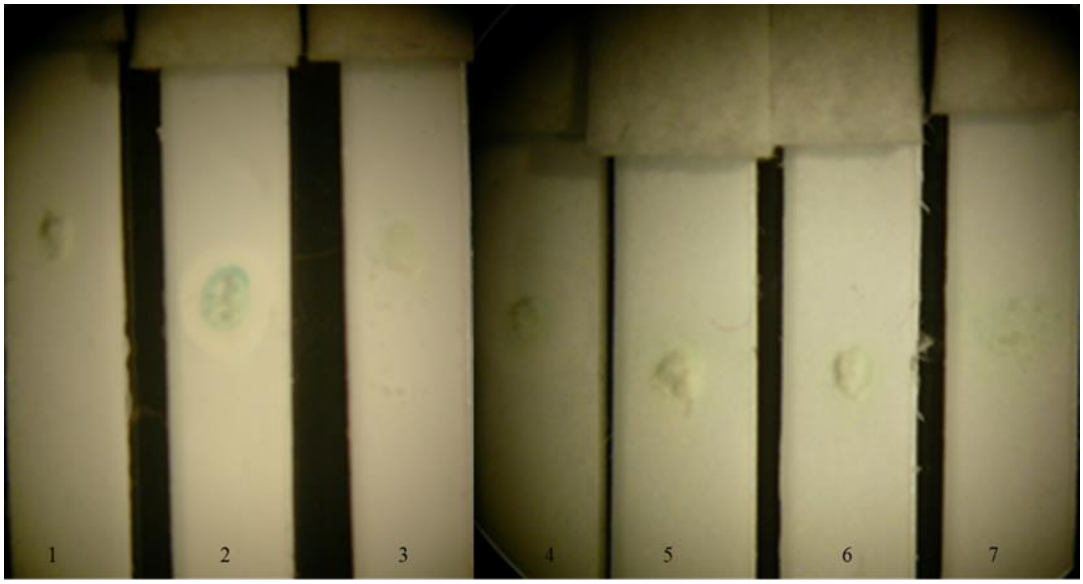

Figure 3. 49 Visualization of HRP catalyzed oxidation of TMB previously embedded in silica nanoparticles on nitrocellulose membrane based lateral flow strips. 1) surface oligonucleotide immobilized TMB embedded silica particles on strip, H_2O_2 delivery through sample pad, 2) surface oligonucleotide immobilized TMB embedded silica particles on strip, H_2O_2+complementary probe delivery through sample pad, 3) surface oligonucleotide immobilized TMB embedded silica particles on strip, H_2O_2+uncomplementary probe delivery through sample pad, 4) surface oligonucleotide immobilized TMB embedded silica particles on strip H_2O_2+MM1 probe delivery through sample pad, 5) surface oligonucleotide immobilized TMB embedded silica particles on strip H_2O_2+MM2 probe delivery through sample pad, TMB embedded silica particles without surface oligonucleotide on strip, 6) surface oligonucleotide immobilized TMB embedded silica particles on strip H_2O_2+MM3 probe delivery through sample pad, 7) TMB embedded silica particles without surface oligonucleotide on strip, H_2O_2 delivery through sample pad.

Last strips include silica nanoparticles embedded with TMB into pores which are not capped by oligonucleotides. They also give very light colour formation which is also expected because of the uncapped pores.

Beside photographic results, success rates were also calculated for each test parameter. Success rate looks at a data sample for either successes or failures, which may also called as “yes” or “no” expressions. Among four replicates, success rates are 100% for full complementary probe, uncomplementary probe,

MM2 and MM3 probes. On the other hand, strips for one mismatch (MM1) discrimination from the full complementary probe has 75% success rate.

Some superior properties of mesoporous silica nanoparticles (MSNs) such as biocompatibility, chemical stability, large pore volumes, uniform and tunable pore sizes, controllable surface functionalization, and resistance to microbial attack have made silica matrices highly attractive as the structural basis for various nanotechnological applications such as adsorption, catalysis, sensing, and separation. In addition, they can effectively protect loaded cargo molecules which makes them promising nanocarriers and ideal scaffolds for targeted drug/gene delivery systems and biosensors for intracellular controlled release and imaging applications (Descalzo *et al.*, 2006); (He *et al.*, 2009); (Rosenholm *et al.*, 2010); (Rosenholm *et al.*, 2009); (Yang, 2011)**.** There have been many studies in literature using these material for sensing and especially for controlled drug delivery applications.

However, to our knowledge, there has not been any study for SNP discrimination using this superior properties of silica nanoparticles. In our platform, we used oligonucleotide probes as capping molecules for controlled detection of complementarities or mismatches with the target strand. One study from Climent *et al.* (2010) used oligonucleotides as gating molecules. Their opening protocol occurs by a displacement reaction in the presence of a target complementary strand which will result in hybridization of the two oligonucleotides, the uncapping of the pores, release of the entrapped fluorescein cargo and consequent fluorescence detection. Differing from our platform, they only showed the capping efficiency of oligonucleotides and related controlled release. On the other hand, we showed the sensitive discrimination ability of the system for oligonucleotide complementarity, even for a one mismatch, in addition to proofing all these capabilities. Our platform is also supported by nitrocellulose membrane based lateral flow test strips which can scale the study up to point of care diagnostic systems.

The system developing here can be used for various diagnostic and detection aims. Determination of a presence of specific gene transformation in a sample can be monitored with our platform to detect if the sample product originates from a genetically modified organism (GMO) or not. Platform can be also used to detect the presence of a specific microorganism from food, feed, environmental samples like water and soil or a sample from a patient. More importantly it has been shown with this study that the detection of single nucleotide polymorphisms (SNP) is possible via the developing platform.

SNPs, the most common genetic variations between human beings, is a key enabler when the concept of personalized medicine is to be realized. Many SNPs precondition the individuals to have a certain disease or trait or react to a drug in a different way, thus they are highly useful in diagnostics and drug development. Their analysis is also used for agriculture, food testing, identity testing, pathogen identification, drug discovery and development, pharmacogenomics and even nutrigenomics researches (Carlson, 2008). Our silica nanoparticle based platform can be used for all these areas for the detection of specific traits. Complex nature of the test during construction process enables the usage of the completed test to be a user-friendly, basic, sensitive and specific. It will be also possible to use the platform both in laboratory and field or point-of-care environments.

CHAPTER 4

CONCLUSIONS

This study aims to develop different single nucleotide polymorphism (SNP) detection platforms that can be applied to point of care applications

Prior to the construction of the platform, construction of a custom designed functionalized surface is made with 3-aminopropyltrimethoxysilane (APS) and 3-Glycidyloxypropyltrimethoxysilane (GPS) groups. Effective combination of two groups is determined as 0.2% epoxy and 0.2% amine in aqueos environment. Post immobilization washing conditions are also optimized for the specific surface in order to gain best signals and less background noises. The coating system works effectively both on glass microscope slides and glass capillary tubes.

SNP detection firstly studied on Poly L Lysine (PLL) coated glass slides with a classical sandwich hybridization system. Format of the system differs according to the mismatched oligonucleotide location and different hybridization temperature regimes. Under optimized conditions of using sp9 spaced discriminatory surface probes and 50°C hybridization temperature, system can efficiently detect SNPs at single nucleotide level.

Another platform for SNP detection was examined on glass capillary tubes in which the surfaces are modified with the epoxy-amine combination coating. The platform can detect single nucleotide polymorphisms (SNPs) at single nucleotide level, through the Oligonucleotide Ligation Reaction with an efficiency of 71% under optimized conditions.

Last part of this study is a molecular gate based design to detect SNPs. Silica nanoparticles are used as scaffolds to include chromogen TMB molecules inside their pores and detector oligonucleotide probes on their surface by capping the pores. Processed particles and horseradish peroxidase (HRP) are applied on a nitrocellulose membrane based lateral flow strip surface. Pore opening occurs by a displacement reaction in the presence of a target complementary strand. This results in hybridization of the two oligonucleotides, the uncapping of the pores, and release of the entrapped TMB molecules. Released TMB molecules, related with the specific hybridization event, are easily oxidized by a reaction catalyzed by HRP with the presence of H_2O_2 in the environment and yield a blue coloured product for detection. The optimized detection platform works with 100% efficiency to discriminate the complementary and two or three mismatch sequences. Detection of one mismatch target was performed with 75% success rate which can be counted as a promising result for this novel platform.

In this study, the developed platforms can be reckoned as rapid, accurate and cost-effective systems that can be utilized in point of care applications for personalized medicine. Besides, specific detection events for pathogenic organisms or environmental samples might also be possible fields of application. It is also aimed to make it possible to use the platform both in laboratory and field or point-of-care environments.

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APPENDIX A

PREPARATION OF BUFFERS AND SOLUTIONS

100mM (10X) Phosphate Buffered Saline

(1370 mM NaCl, 100 mM Phosphate, 27 mM KCl)

80 g NaCl, 2 g KCl, 14.4 g Na 2 HPO 4 , 2.4 g KH_2PO_4 were dissolved in 1L of ultrapure H_2O and pH was adjusted to 7.2. Sterilization was performed by autoclaving and the buffer was stored at room temperature.

5X Saline-Sodium Citrate Buffer

(75 mM sodium citrate, 750 mM sodium chloride, pH 7.0)

22.06 g Sodium Citrate, 43.8 g Sodium Chloride were dissolved in 1L of distilled H_2O and pH was adjusted to 7.0. Sterilization was performed by autoclaving and the buffer was stored at room temperature.

10% SDS

10 g SDS was dissolved in 100mL of distilled H_2O.Sterilization was performed with filter sterilization. (Filter size: 0.4 μm) and the solution stored at room temperature.

2% BSA in 10 mM PBS

2 g BSA was dissolved in 100mL of 10mM PBS Buffer and filter sterilized (Filter size: 0.4 μm). The solution was stored at +4°C.

1 mM TCEP

0.00286 g of TCEP was dissolved in 1 mL distilled H_2O. A dilution was carried out by mixing 100 µL of 10 mM TCEP with 900 µL dH_2O and 40 aliquotes x 25 µL was stored at (-20)°C for maximum 2 months.

10 % Sodium Hydroxide (NaOH) in 60 % Ethanol

60 % Ethanol was prepared by adding 40 ml distilled H_2O to 60 ml absolute ethanol. 10 gr of NaOH was dissolved in 60 % ethanol and completed to 100 mL.

APPENDIX B

VISUAL DATA of OPTIMIZATION STEPS in SECTION 3.3.2

(Oligonucleotide Ligation Reaction and Post Reaction Washing Regimes)

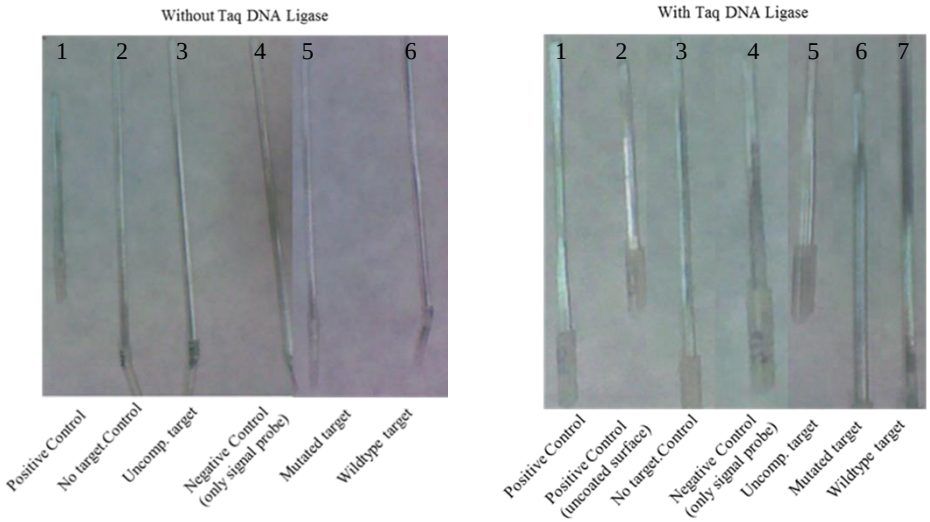

Figure B. 1 Results of post reaction (with/without Taq DNA ligase) application with heat denaturation. Without Taq DNA ligase;1) positive control, 2) lig.react. without target (adapter), 3) lig.react. with uncomplementary target (adapter), 4) negative control (only signal probe on surface), 5)lig. React. with mutated target (adapter), 6) lig.react with wildtype target (adapter); With Taq DNA ligase;1) positive control, 2) positive control probe on uncoated surface, 3) lig.react. without target (adapter), 4) lig.react. with uncomplementary target (adapter), 5) negative control (only signal probe on surface), 6)lig. React. with mutated target (adapter), 7) lig.react with wildtype target (adapter)

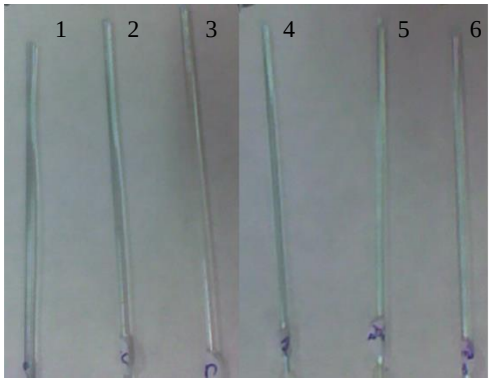

Figure B. 2 Results of competitive hybridization experiment. 1) positive control, 2) lig.react. without target (adapter), 3) lig.react. with uncomplementary target (adapter), 4) "surfaceprobe-wt.target–signalprobe",5)"surfaceprobe-(wt.target+uncomp.target)–signalprobe",6) "surfaceprobe-(wt.target+uncomp.target)*2–signalprobe"

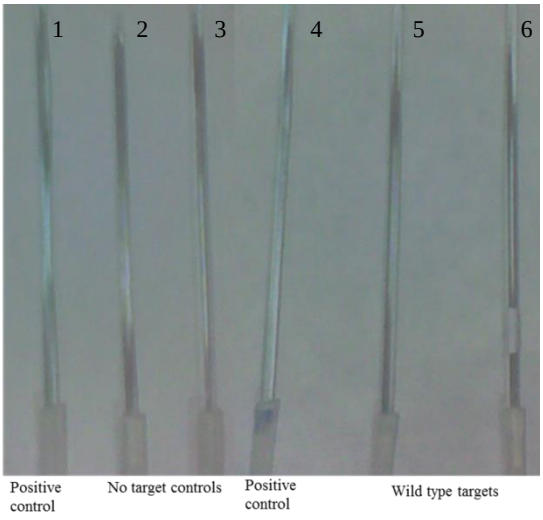

Figure B. 3 Results of competitive hybridization experiment with PEG addition into reaction mixture. 1) positive control, 2),3) lig.react. without target (adapter), 3) positive control, 4) "surfaceprobe-(wt.target+uncomp.target)*2–signalprobe"

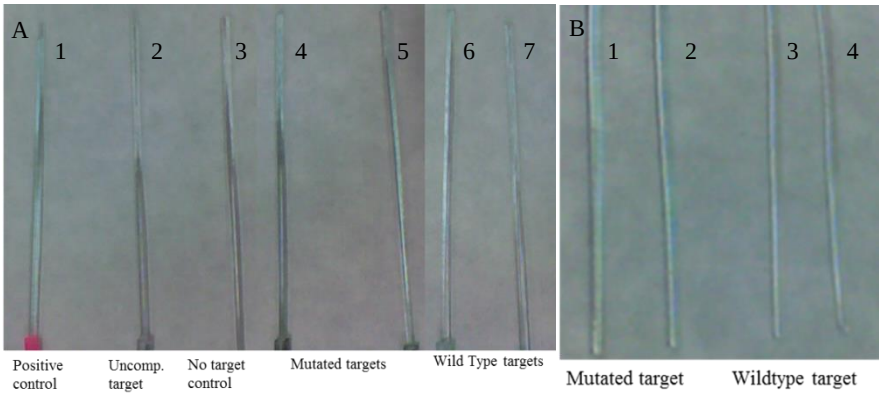

Figure B. 4 Results of competitive hybridization experiment with PEG addition in reaction mixture (A), 1) positive control, 2) lig.react. with uncomplementary target (adapter),3) lig.react. without target (adapter), 4) "surfaceprobe-(mut.target+uncomp.target)*2–signalprobe" 5) "surfaceprobe-(wt.target+uncomp.target)*2–signalprobe" ; B) Close view comparison of mutated and wild type targets.

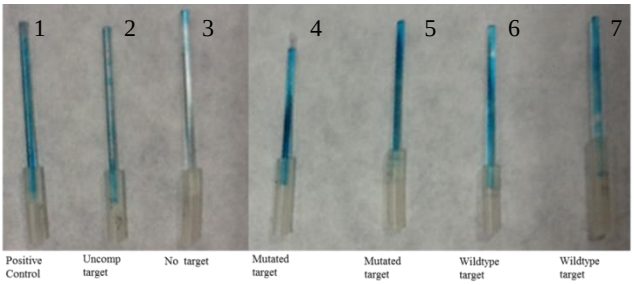

Figure B. 5 Results of the reaction with 1-Step™ Ultra TMB ELISA substrate solution. 1) positive control, 2) lig.react. with uncomplementary target (adapter),3) lig.react. without target (adapter), 4),5) "surfaceprobe-(mut.target+uncomp.target)*2–signalprobe" 6),7) "surfaceprobe-(wt.target+uncomp.target)*2–signalprobe"

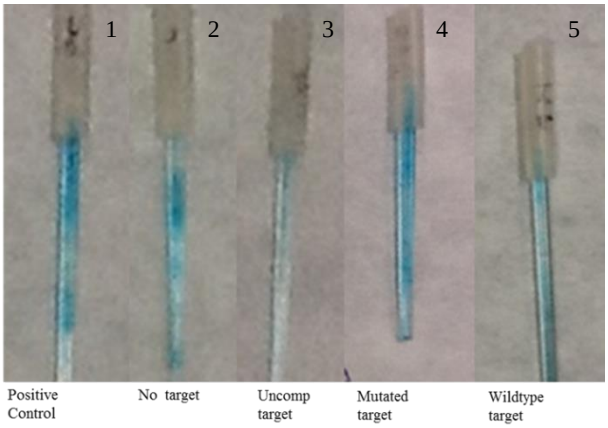

Figure B. 6 Results of the reaction with 1-Step™ Ultra TMB ELISA substrate solution and Tween 20 including blocking step. 1) positive control, 2) lig.react. without target (adapter), 3)lig.react. with uncomplementary target (adapter), 4) "surfaceprobe-(mut.target+uncomp.target)*2–signalprobe" 5) "surfaceprobe-(wt.target+uncomp.target)*2–signalprobe"

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2. Yılmaz R, **Akça O**, Baloğlu MC, Öz MT, Öktem HA, Yücel M (2012) Optimization of yeast (Saccharomyces cerevisiae) RNA isolation method for real-time quantitative PCR and microarray analysis. Afr. J. Biotechnol. 11(5): 1046-1053.

3. Celikkol Akcay U, **Ercan O**, Kavas M, Yildiz L, Yilmaz C, Oktem HA, Yucel M (2010) Drought-induced oxidative damage and antioxidant responses in peanut (Arachis hypogeal L.) seedlings. Plant Growth Regul. 61:21-28

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1. The original chapter: Mark D. Curtis (2008) Chapter 7:Recombinant DNA, vector design and construction. In: Stewart CN (Ed.) Plant Biotechnology and Genetics: Principles, Techniques and Applications. John Wiley & Sons, Inc. New Jersey, U.S.A.

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2. The original chapter: John Finer and Taniya Dhillon (2008) Chapter 11: Production of transgenic plants. In: Stewart CN (Ed.) Plant Biotechnology and Genetics: Principles, Techniques and Applications. John Wiley & Sons, Inc. New Jersey, U.S.A.

Translation: Kavas M, Kalemtaş G, **Ercan O,** Battal A, Gürel E, Öktem HA, Yücel M (2011) published by Nobel Akademik Yayıncılık Tic. Ltd. Şti. Ankara Turkey.

Thesis

"Effect of Drought and Salt Stresses on Antioxidant Defense System and Physiology of Lentil (Lens culinaris M.) Seedlings" . M.Sc. Thesis, Middle East Technical University, Graduate School of Natural and Applied Sciences, Biology, Supervisor: Prof. Dr. Hüseyin Avni Öktem, February 2008.

Presentations at International Conferences:

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Yılmaz R, **Ercan O**, Baloğlu MC, Öktem HA, Yücel M "Optimisation of Yeast RNA Isolation Method for LightCycler qPCR and Affymetrix GeneChip Analysis" BIOTECH METU 2009, International Symposium on Biotechnology: Developments and Trends, Middle East Technical University, Ankara, Turkey, September 2009

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Yılmaz R, Baloğlu MC, **Ercan O**, Öktem HA, Yücel M "Farklı Sıcaklık Stresleri Altında Ekmek Mayasında (*Saccharomyces cerevisiae*) Gen İfade Profillerinin Mikroarray Yöntemi ile İncelenmesi" 16th National Biotechnology Congress, December 2009, Antalya.

Ercan O, Baloğlu MC, Kavas M**,** Gökçay D**,** Köse FS, Öktem HA, Yücel M "Kuraklık Stresinin Kavun (*Cucumis melo* L.) Antioksidatif Savunma Sistemi Üzerindeki Etkisi" 16th National Biotechnology Congress, December 2009, Antalya.

Ercan O, Akcay UÇ, Kavas M**,** Peksel B, Eroğlu**,** Öktem HA, Yücel M "Tuz stresinin yerfıstığı (*Arachis hypogea* L.) antioksidatif savunma sistemi üzerindeki etkisi" 16th National Biotechnology Congress, December 2009, Antalya

Akcay UÇ, **Ercan O,** Kavas M, Yıldız L, Yılmaz Ç, Öktem HA, Yücel M "Kuraklık stresinin yerfıstığı (Arachis hypogea L.) antioksidatif savunma sistemi uzerindeki etkisi" 21st National Biochemistry Congress, October 2009, İstanbul

Ercan O, Yücel M, Öktem HA "Effect of drought and salt stresses on antioxidant defense system and physiology of lentil seedlings." 15th National Biotechnology Congress, Antalya, 2007.

Kavas M, **Ercan O**, Öktem HA, Yücel M "Optimisation of various parameters of microprojectile bombardment with immature wheat inflorescences" 15th National Biotechnology Congress, Antalya, 2007

Projects:

ODTÜ-BAP-ÖYP BAP-08-11-DPT 2002 K120510, Development of Microarray Chip Platforms for Diagnostic Purposes

BAP-08-11-2011-01 KBRN Uygulamaları için yeni nesil algılama sistemlerinin geliştirilmesi

ODTÜ-BAP-ÖYP BAP-08-11-DPT 2002 K120510, Effect of Drought and Salt Stresses on Antioxidant Defense System and Physiology of Lentil (Lens culinaris M.) Seedlings

BOREN, BOREN-2009-Ç0217. Bor stresi altında buğday bitkisinin gen ifade profillerinin incelenmesi ve Tarımborun bu strese tepki üzerine etkilerinin moleküler seviyelerde belirlenmesi. Öktem HA, Yücel M, Eyidoğan F, Öz MT, **Ercan O**, Kayhan C.

International Meetings, Workshops and Courses Attended:

- Workshop on Biomimetic and Bioanalytical Systems (PhD Symposium on Complex Enzymes for Biosensor Applications), November 2012, Luckenwalde/ Germany
- Potsdam Days in Bioanalysis (3rd Public Status Seminar "Lab in a Hankie" and 4th Potsdam Colloquium on Bioanalysis), November 2012, Potsdam, Germany,
- 8th Workshop, Molecular Interactions from Molecule to Product Innovation, September 2012, Berlin, Germany
- Biomimetic Functional Materials, Status Seminar of "Biohybride Funktionssysteme", May 2012, Potsdam, Germany
- Symposium on Bioactive Particles and Coatings, May 2012, Potsdam, Germany
- 1st German-Turkish Workshop on NanoBio and Biosensing Technologies, June 2011, Ankara, Turkey
- International Academy of Nanomedicine 2nd World Congress, October 2010, Antalya, Turkey
- 2nd International Molecular Methods in Food Microbiology Symposium Series; 2010, Şanlıurfa
- 1st Microarray Training and Data Analysis Course, Kasım 2008, Middle East Technical University Central Laboratory Molecular Biology and Biotechnology R & D Center/Ankara
- Nanomat International Workshop on Nanobiotechnology and Genome Technologies, November 2007, Antalya, Turkey

- NANO-TRII Nanoscience and Nanotechnology Congress, May 2006, Ankara, Turkey

Meetings and Courses Organized:

- 1st National Biotechnology Student Summit, Organizers: Öktem HA, Çökmüş C, Eyidoğan F, Tuncer AS, **Ercan O**, Baloğlu C, Öz MT, METU, Culture and Convention Center, Ankara, Turkey, September 23 – 24, 2011.
- 3rd Microarray Training and Data Analysis Course, Organizer: Yücel M, Instructors: Yılmaz R, Öz MT, **Ercan O**, Baloğlu MC, METU, Central Laboratory, Molecular Biology and Biotechnology R&D Center, Ankara, Turkey, December 20 – 25, 2010.
- 2nd Microarray Training and Data Analysis Course, Organizer: Yücel M, Instructors: Yılmaz R, **Ercan O**, Baloğlu MC, METU, Central Laboratory, Molecular Biology and Biotechnology R&D Center, Ankara, Turkey, 29-30 June and 1 July, 2009
- 16th National Biotechnology Congress, Antalya, Turkey,2009
- 15th National Biotechnology Congress, Antalya, Turkey,2009

Membership:

Turkish Biotechnology Association –Member of the Executive Board

Scholarships, Certificates and Awards:

Visiting Scientist, March 2012–March 2013, Fraunhofer-Institut für Biomedizinische Technik (IBMT), scholarship provided by Faculty Development Program (ÖYP) of METU, Ankara Turkey.

Publication Award, METU, 2010

Publication Award, TUBİTAK, 2010

Travel Grant from The Federation of European Societies of Plant Biology for FESPB 2008 Congress August 2008, Tampere, Finland

Computer Skills:

Microsoft Office Applications (Word, Excel, PowerPoint, Publisher), MINITAB, and Internet Applications

Languages:

Turkish: native speaker, English: fluent, German: beginner A2