Fabrication of substrate in the development of a DNA microarray for diagnostic applications

To be submitted as Major Project in partial fulfilment of the requirement for the degree of

MASTER OF TECHNOLOGY

IN

INDUSTRIAL BIOTECHNOLOGY

Submitted by

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DECLARATION

Certified that the project report entitled "**Fabrication of substrate in the development of a DNA microarray for diagnostic applications**" submitted by me is in partial fulfilment of the requirement for the award of the degree of Master of Technology in Industrial Biotechnology, Delhi Technological University. It is a record of original research work carried out by me under the supervision of **Dr. JaiGopal Sharma**, Department of Biotechnology, Delhi Technological University, Delhi and under the guidance of **Dr. Ravikrishnan Elangovan**, Indian Institute of Technology, New Delhi in collaboration with **Dr. Saroj Kumar**, Delhi Technological University, Delhi.

The matter embodied in this project report is original and has not been submitted for the award of any Degree/ Diploma.

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CERTIFICATE

This is to certify that the dissertation entitled: **"Fabrication of substrate in the development of a DNA microarray for diagnostic applications",** submitted by **SRISHTI MUNJAL (2K15/IBT/13)** in the partial fulfilment of the requirements for the reward of the degree of **Masters of Technology in INDUSTRIAL BIOTECHNOLOGY**, Delhi Technological University, is an authentic record of the work carried out under the supervision of **Dr. JaiGopal Sharma, Department of Biotechnology**.

The information and data enclosed in this dissertation is original and has not been submitted for the award of any other degree elsewhere.

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Contents

LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic Acid
SNP	Single Nucleotide Polymorphism
HT	High throughput
Oligo	Oligonucleotide
mRNA	Messenger Ribonucleic Acid
cDNA	Complementary DNA
PCR	Polymerase Chain Reaction
EST	Expressed Sequence Tag
PM	Perfect match
MM	Mismatch
CARD	Chemistry and Reagent Device
Th1 and Th2	T helper cells
MEMs	MicroElectroMechanical systems
PDMS	Poly dimethylsiloxane
GPS	3-glycidyloxypropyl) trimethoxysilane
APTES	(3- aminopropyl) triethoxysilane
APMES	Aminophenyltrimethoxysilane
MPTS	(3- mercaptopropyl) trimethoxysilane
SA	Succinic Anhydride
TNBS	2,4,6- trinitrobenzenesulfonic acid
DMSO	Dimethylsulfoxide
ss	single stranded
PNA	Peptide Nucleic Acid
EDC	(1- ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
NHS	N-hydroxy succinamide

BSA	Bovine Serum Albumin
PBS	Phosphate Buffer Saline
MES	Morpholino ethanesulfonic acid
SSC	saline sodium citrate
CCD	charge-coupled device
TEA	Triethylamine
THF	Tetrahydrofuran
ATR-FTIR	Attenuated Total Reflectance-Fourier Transform InfraRed

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ABSTRACT

High throughput techniques (HT) are the need of the hour as a large amount of data needs to be generated for diagnosis or treatment of a disease. DNA microarrays have been devised to provide with this task. But the problem is that the equipment is too bulky and expensive to be owned by small scale industries. This work aims to address the same issue and fabricate substrates to immobilize the probes specific for pathogens or biomarkers of a specific disease. The probes were covalently attached to the substrate, which would be chemically modified to be amenable for attachment, and then hybridized with the target sequence to know the correctness of the system. The efficiency of the system checked by fluorescence images, FTIR data, and surface graphs.

Keywords: High throughput, DNA microarray, biomarkers, FTIR

PART 1

INTRODUCTION

INTRODUCTION

DNA microarrays have been known and worked on for decades now. They are the most common tools to observe and diagnose diseases (1, 2), chromosomal abnormalities (3), SNPs (4) and mutations (5, 6). Being too expensive and bulky in nature, there is a need to manufacture and fabricate a portable DNA microarray that will be easy to use. Also, due to the diffusion limit, the process of hybridization in a conventional microarray is very time-consuming, this can be overcome by using microfluidic technology to enable flow through hybridization which is very efficient and can reduce the time period from 24 hrs to less than 15 minutes (7). This area has been extensively reviewed (8, 9), also with respect to merging with microfluidics (10, 11).

Glass slides have been used extensively for their properties like inertness, flatness, low fluorescence and low cost. Borosilicate glass and soda lime silicate are mostly flat and show low fluorescence. Flatness also ensures accurate immobilization and precise optical detection. The inertness of glass is another important aspect of choosing glass. It provides a platform for reactions that would not affect the ionic strength of buffer solutions used. Borosilicate glass has a much better resistance for leaching out alkalis as compared to soda lime silicate in acidic conditions and a still better resistance in hot water.

Contact angle is one important property to determine the wettability of a surface. It is defined by the angle made by liquid-solid interface and the vapour-liquid interface. As the angle increases, the hydrophobicity of the surface increases. A 0^{o} angle corresponds to a highly hydrophilic liquid which would completely wet the surface and at an angle greater than 150^{o} the liquid is said to be superhydrophobic that forms a spherical droplet.

Surface cleaning is the first and foremost step to remove all the organic and inorganic impurities from the substrate. The second step functionalization attaches a functional group to the substrate to attach a probe with complementary group. Silanization is one of the most widely used functionalization method used to create free primary amino groups. This is used even in the case wherein there is a need to create a carboxy moiety on the substrate.

Immobilization is the third step wherein the probe gets confined to the substrate physically, chemically or through affinity binding. Hybridization of the target to the probe is the final step of the protocol wherein ambient temperature for the probe and target is given.

OBJECTIVES

With the increasing need to determine the susceptibility of diseases in humans, there is an urgent need to develop systems that are cheaper, quicker and portable.

Modifying the surface chemistry of the substrate that is being used, helps attach the oligonucleotides that are needed to be detected.

The present study focuses on the modification of glass slides for the detection of cancer related oligonucleotide.

Specific aims

1. Carboxylation of surface
2. Characterization
3. Attachment of oligonucleotide probe, hybridization of target and labelled probe
4. Fluorescence detection

Part 2

LITERATURE REVIEW

REVIEW OF LITERATURE

Microarray

Prior to the human genome project, three platforms were observed to determine the events in a cell, proteomics- alterations in protein levels, transcriptomics- change in mRNA transcripts and metabolomics- variations in metabolites present in a cell. The former two fields have their own share of disadvantages ranging from varied protein production from different amino acids making the measurement of protein tedious in proteomics to metabolites having a range of chemical properties (12). Northern blotting technique was devised to determine RNA expression in order to characterize gene activity. But with this method, only one gene was probed per experiment, making it an extremely slow pace to characterize over 30,000 genes in the human genome. The 1990s saw the rise of the development of high throughput device called the microarray, wherein simultaneously many genes could be assessed (13, 14, 15). This design is allowing scientists to associate a disease with a gene activity at a very high pace (16). Miniaturized recognition sites consisting of binding reagents are attached which have the ability to differentiate complementary molecules. Two general types of microarrays exist: *in situ* synthesis and spotted arrays. The probe is synthesized on the substrate in the former whereas in the latter a synthesized strand is deposited on the substrate.

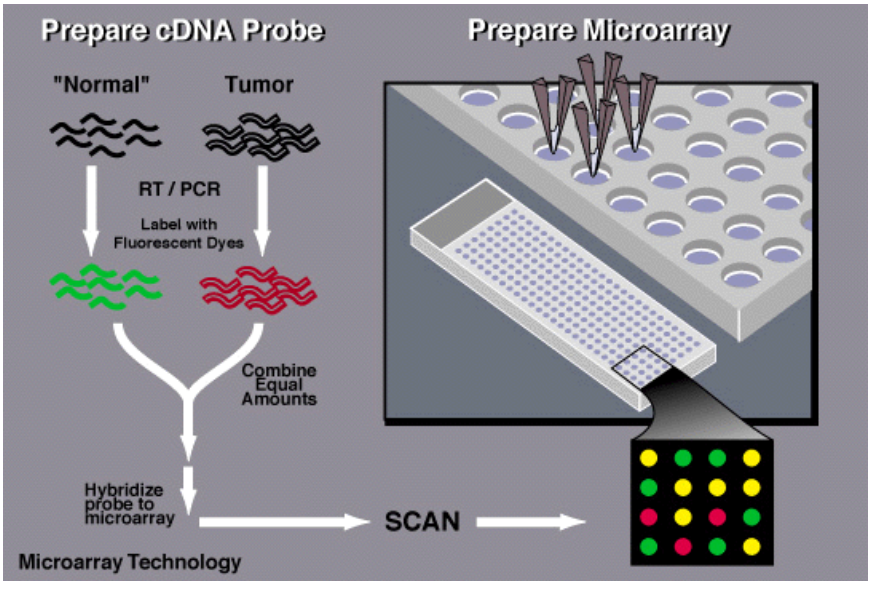

Figure1: Microarray overview (courtesy: National Human Genome Research Institute, U.S)

Types of microarrays

There exist two main types of DNA microarrays, namely, spotted and oligonucleotide microarrays.

Spotted DNA arrays:

This type supports attachment of single stranded DNA or a cDNA prepared as a result of reverse transcriptase PCR reaction (17). Up to 20,000 spots can be put on a slide having replicates, control sets and test. Each spot will have an oligonucleotide expressing a gene or Expressed Sequence Tag (EST). The test and control would be labeled by different fluorescent dyes so as to get a quantitative ratio between the two.

Oligonucleotide arrays:

The advantage that this type enjoys as compared to spotted is that oligonucleotides can be *in situ* synthesized bypassing the need for attachment to the substrate. But the length of the fragment is restricted to 50-70 expressing different fragments of the same gene, giving absolute results. Recently there have been customized microarrays available, providing perfect match (PM) sequences as well as some mismatches (MM), with a single base change to check for the efficiency of hybridization. Some widely used methods are Affymetrix CARD and Rheonix CARD (12, 18, 19, 20). This method allows researchers to check for allelic variation also and variants formed due to alternate splicing.

Table1: Commercially available automated microarray platforms (modified from (72))

Company	Product name	Number of genes	Reporter	Application
Affymetrix Inc.	Gene Chip	10000-260 000 oligos	Fluorescent	Expression profiling, polymorphism analysis
Rheonix, Inc., Ithaca, NY	Rheonix CARD	27 or 40 targets	Optical and fluorescent	Pathogen identification
Brax. Cambridge, UK		1000 oligos	Mass Spectrometry	Novel gene identification
BD Clontech	Atlas nylon array Atlas glass array	1176 cDNAs 1081 cDNAs	Radioisotope Fluorescent	Expression profiling
Hyseq Inc.	HyChip™	Up to 8000 oligos	Radioisotope or fluorescent	Large scale sequencing
Incyte Pharmaceuticals Inc.	GEM	1000-10000 oligo/PCR fragments	Radioisotope or florescent	Expression profiling polymorphism analysis
Molecular Dynamics Inc. Nanogen	Storm® FlorImager®	Co. 10000 cDnNAs	Fluorescent	Expression profiling and novel gene identification
Nanogen	Semiconductor microchip	Eventally 10000 oligos	Fluorescent	Short tandem repeat identification
Protogene Laboratories		Upto 8000 oligos	Fluorescent	Expression profiling and polymorphism analysis
Sequenom	MassArray Spectro Chip	250 per chip	Mass spectrometry	Novel gene identification
Stratagene	Discovery-3 microarray	4000 cDNAs	Fluorescent	Expression profiling and novel gene identification
German Cancer Institute,		1000 PNA prototype	Fluorescent/mass spectrometry	Expression profiling and diagnostics

Switch from commercial to in-house

Research institutions are now carrying out experiments in the lab itself as opposed to visiting array specific locations. This has been made possible by the emergence of industries that provide the essentials of the experiment. From buffers to probes, individual products as well as kits, everything is available commercially. This reduces the set-up time but time and effort in establishing and optimization of protocols are still required. (72)

Advantages

A large number of sample analysis simultaneously has saved a lot of time and resources. This high amount of data generated also allows for building of molecular interactions in the cell, when considering mRNA microarrays, beneficial for faster determination of gene expression, thereby helping in disease identification.

Microarrays also turn out to be highly sensitive when the difference of even a single base is needed which are difficult with other methods (7). As opposed to the conventional need of knowledge for the genes and pathways involved in host and pathogen genomes, microarrays surpass this. Previously unknown pathways can also be elucidated with the help of microarrays, also providing knowledge about levels of gene expression (9).

Applications

Diagnosis of disease

Of the many applications of microarrays, the most important are diagnosis of diseases. Arrays can provide unknown data on alterations in gene expression in various diseases, the most sought after being cancer. These studies could then lead to new therapeutic advances thus helping in treating the disease (21). Genetic profiling is another use seen for microarrays. This could be done for tumor subtypes (22), as observed by Alizadeh AA *et al* the different stages of B-cell lymphoma (23). Predisposition to a specific disease could also be elucidated, making it a very important detection assay which could prove beneficial for the susceptible population (24). Organism identification is another advantage offered by microarrays. Sequence variants of the genes can be made and checked for pathogen polymorphism. Enteric pathogens were determined by Chizhikov *et al* by observing the antigenic determinants with the help of gene sequences of pathogens, namely *E. Coli*, *Shigella spp.* and *Salmonella spp* (25). Another group Wang *et al* developed a microarray to determine 140 viruses with the

hybridization pattern being the characteristic feature (26). microarrays are proving to be indispensable for studying microbial populations.

Microorganism evolution

Conserved genes over evolution give an idea of how divergent have species become. This can be studied using microarrays. Hakenbeck *et al* compared 20 *pneumococcal spp.* from over 2000 genes from a reference strain (27). Dobrindt *et al* used cDNA microarrays to prove that gene deletion and acquisition via bacteriophage contributed to evolutionary process (28).

Virulence

Gene determination for observing proteins acting as adhesions, toxins, tissue damage has been done following global analysis. Merrell *et al* used competitive hybridization to compare pathogen's gene expression profiles (29). Mamoun *et al* studied parasite development. They used expressed sequence tags isolated at five stages of development of *Plasmodium falciparum* (30).

Immunology studies

Studying the immune cells at the differentiation, maturation and activation helps determine the gene expression at these specific stages. The first line of defense provided by the innate immune system has gained importance recently to know the genetic alterations involved. Naour *et al* determined the expression of 255 genes during the differentiation process of dendritic cells (31). The second line of defence specific for the pathogens has also been enough elucidated. Rogge *et al* determined the genes differentiating T helper cells Th1 and Th2 (32).

Difference in immune response due to diversity of organisms

A study done by Huang *et al* observed the differences in dendritic cell reaction in host cell to different pathogen exposure (33). These included a bacterium, a fungi and a virus. Microarray studies can also show the pathways involved in immune reactions. The gene expression for cytokines and chemokines was also increased. Avalos *et al* analyzed the response of the host for an intracellular pathogen *T. gondii*, for which an increase in gene expression was observed (34). Blader *et al* observed an upregulation of gene expression of enzymes involved in glycolysis and cholesterol biosynthesis (35). Okabe *et al* observed a difference in the expression of hepatocellular tumors borne from hepatitis B or hepatitis C (36).

Substrate

One of the initial steps of the experiment is the choice of substrate. This would depend on the functional group that is to be attached directly to the surface and the type of probe to be immobilized. It could range from silicon to glass to gold and the coating could vary from polymers like polylysine to polyacryl-hydrazide (8). Silicon and glass have been in great demand due to their successful integration in the field of microelectronics and microelectromechanical systems (MEMs). Glass has been extensively used due to its properties like chemical inertness, flatness, low fluorescence, and low cost. Borosilicate glass and soda lime silicate are mostly flat and show low fluorescence. Flatness also ensures accurate immobilization and precise optical detection. The inertness of glass is another important aspect of choosing glass. It provides a platform for reactions that would not affect the ionic strength of buffer solutions used. Borosilicate glass has a much better resistance for leaching out alkalis as compared to soda lime silicate in acidic conditions and a still better resistance in hot water. Polymers are the new age materials that have started being used due to their inexpensiveness and simple manufacturing processes (37) allowing the fabrication of one time use devices. Soft polymers like poly (dimethylsiloxane) have also gained attention in the past decade. Major advantages include easy integration with outside components and stability at a varied range of temperatures. It finds efficient utilization in microfluidics for designing devices such as to identify axonal communication among different hippocampal subregion networks (38) or devising a triboelectric-piezoelectric hybrid nanogenerator (39).

Contact angle

Wettability is a topic that has received a lot of interest in industrial processes like lubrication, oil recovery and spray quenching (40, 41, 42). Superhydrophobic surfaces are being used in a number of applications including self-cleaning, nanofluidics and electrowetting (43, 44). The surface wetness of a liquid is determined by the contact angle, i.e. the angle formed by the solid-liquid and liquid-vapour interface. Liquids making angles less than 90° on the surface favors wetting and spreading over the surface whereas with angles greater than 90° tend to decrease the surface contact to form a droplet. Liquids forming 0° angle is termed to completely wet the surface whereas a liquid is said to be superhydrophobic when it forms an angle greater than 150° (45).

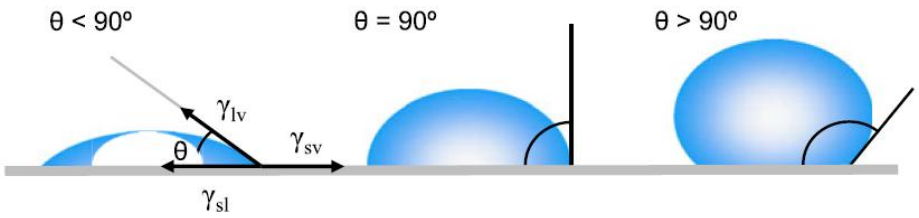

Figure 2: Different contact angles formed by liquid on a surface (45)

Surface cleaning

It is considered as the most crucial step, for any impurity could result in non- specific binding. It is very important to remove all types of contaminants be it metallic, oil-soluble or water-soluble. A method is optimized that uses methanol and concentrated hydrochloric acid to overcome problems like hazardous nature of constituents such as that of Piranha. By the subsequent addition of concentrated sulphuric acid, all organic and inorganic impurities get removed.

Functionalization

Functionalization involves attaching a functional group to the slide onto which the complementary group of the probe will attach. The most commonly used functionalization molecules are alkoxysilanes. Three hydrolysable alkoxy groups are present with a central silicon atom bonded with a terminal functional group.

An amine functionalization is needed when a carboxyl group needs to be linked to the glass surface as the latter is a highly tedious protocol (46). The commonly used silanes include (3-glycidyloxypropyl) trimethoxysilane (3- GPS) (47, 78), (3- aminopropyl) triethoxysilane (49), aminophenyltrimethoxysilane (50), (3- mercaptopropyl) trimethoxysilane (3-MPTS) (51), and haloacetamidosilanes (52). Mono-, di- or trialkoxysilanes have been widely used. Silanols and alcohols are liberated in the first step involving hydrolysis of the alkoxy group. Condensation with the substrate then forms siloxane linkages. The surface hydroxyl groups influence the reactivity of the surface with the silanol groups. Kallury *et al* has shown that out of the three conformations of the reaction product of APTES and glass or metal oxide substrate, the preferred one is where there are two sites are attached and the third one is free (53). Moon *et al* have observed that silanes consisting of three alkoxy groups and one alkyl group give rise to multilayer and non-uniform structures on silica as contrary to silanes containing two alkoxy groups and two alkyl groups which give rise to defined single layer

deposition (54). The functionalization reaction can occur in both organic and polar solvents. In case of polar solvents, acidic (55) and basic (56) conditions have observed to enhance polymerization in the solution itself rather than reacting with the surface. Special attention needs to be given to the reaction basics depending on a number of factors like temperature, solvent, concentration, possibility of side-reactions, by-products, etc.

Carboxyl groups can be generated on the surface by using either silanes with carboxy groups (57) or polymers (58). Due to side product formation, the attachment between amine-bound surface and carboxy-bound probe was not possible (59). To overcome this, the carboxy moiety was bound to an aromatic group (60). This amine group can then be reacted with molecules like polyacrylic acid or succinic anhydride (61, 62) to convert into a carboxyl moiety. Kralj S *et al* showed that when functionalizing 3-(2-aminoethylamino) propylmethyldimethoxysilane (APMS) with SA, only the terminal primary amine reacts with SA and not the secondary amines due to steric hindrances (63).

To quantify the amount of silanization on the glass surface, 2,4,6- trinitrobenzenesulfonic acid (TNBS)/Igepal/sodium tetraborate solution is used. An orange coloured compound is obtained giving an absorbance at 335-345 nm. The disadvantage of this method is that it gives the quantitative estimation for small molecular amines only because the reaction time is small and the by-product of TNBS does not interfere with the analysis. But with matrices having a large number of amine groups, analysis in aqueous media yields substantial amount of picric acid. Hence the reaction is done in DMSO. To check for carboxylate- immobilized slides, ethylenediamine is used followed by TNBS/Igepal/sodium tetraborate solution (64).

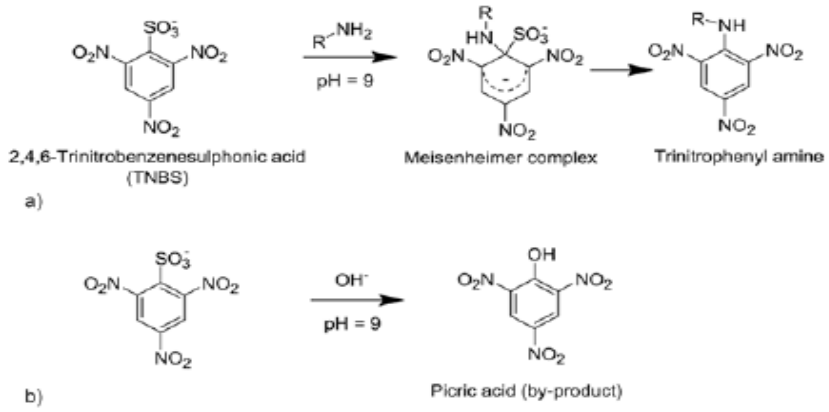

Figure 3: Reaction of 2, 4, 6-trinitrobenzenesulphonic acid (TNBS) with (a) primary amines and (b) hydroxyl ions (64)

Immobilization

Immobilization involves attachment of the synthesized probe with the substrate. The probe is custom-synthesized with a functional group complementary to that on the surface. Various methods for attachment exist including physical, chemical and affinity-based. There exists both pros and cons of all the aforementioned techniques but the best results have been shown with chemical bonding due to the attachment being strong, reproducible and unaffected by physical parameters (65). Physical adsorption does not require any modification of the probe. Ionic interactions are the most common attractions that occur between, for example, negatively charged DNA backbone and positively charged surface. Cai H. *et al* and Xu C. *et al* immobilized ssDNA on a glassy carbon electrode (66, 67). This type of attachment results in non-uniform immobilization of probes with random orientations. Also, these attractions can be disrupted by changes in pH or temperature of buffers. The highly specific streptavidin-biotin interaction offers another way of attachment (68). A two-step attachment method, wherein biotin is first attached to the surface using a crosslinker is followed by addition of streptavidin. Biotin has high affinity for both avidin and streptavidin but latter is preferred for having isoelectric point equal to five, avoiding non-specific interactions (69). The problem with this highly specific binding is that the binding capacity of streptavidin deteriorates with time. The sensitivity and specificity of these interactions are also doubted due to the instability of immobilized streptavidin and non specific interactions. This method turns out to be very expensive due to the multiple steps involved (70). A relatively new technology that overcomes the problem of closely spaced probes is nanocones. These are cone shaped dendrons that establish uniform spacing (71).

Probes can be proteins, DNA, RNA or aptamers (others). A special interest has been generated in microRNAs with reviews. PCR amplified products, cDNA have popularly been used but the process is time consuming and costly. PNAs (Peptide Nucleic Acids) are now being used as they allow more spots than cDNAs but require the need to be sequenced (72).

This modification is possible due to bioconjugation involving cross-linking reagents that have reactive groups to bind other molecules functionalized with groups like carboxy-, amino-, sulfo- etc. Some of the widely used linkers are aldehydes (glutaraldehyde, formaldehyde) (73-75), carbodiimides (1- ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC)) (76-78), N-hydroxy succinimide. Primary target of these linkers is amine, which they accomplish through acylation or alkylation. EDC in conjunction with NHS activate carboxyl

groups to form amide bonds with amines (79, 80). An intermediate o-acylisourea is formed when carbodiimide reacts with carboxyls in an acidic pH. But the intermediate is unstable in aqueous solutions; therefore, stabilization is needed for effective coupling to amines. The widely used method is addition of NHS (81, 82). Surpassing the disadvantage of the above-mentioned linkers requiring functional groups on targets for conjugation, a new class of photolinkers are gaining popularity. These photolinkers are able to activate inert targets also giving it an edge over the conventional techniques (83). They have found uses in surface modifications (84, 85), organic synthesis (86) among others.

A spacer molecule is incorporated to avoid steric hindrance and allow proper binding. This spacer could be a string of nucleotides bound together (87) or carbon chains of varying lengths. The probes need to be spotted on the activated glass slides with the help of a spotter that would transfer a definite volume of probe to attach with the surface (8).

Pre hybridization blocking is an important step to inhibit any non-specific binding on the slide. This is done by using Bovine Serum Albumin (BSA) (88) or casein.

Hybridization

The hybridization process needs to be fastened to reduce the time period through a flow through mechanism (7) instead of the conventional diffusion process. This mechanism is operable when the whole protocol is miniaturized from isolation of target DNA to hybridization.

Part 3

MATERIALS AND METHODS

MATERIALS AND METHODS

1. REAGENTS

Materials:

Table 2: List of reagents and their sources

SOURCE	CHEMICALS
TCI India	(Amino propyl)-triethoxy silane
Sisco Research Laboratories Pvt. Ltd.	EDC, triethylamine, tetrahydrofuran, N-morpholino ethanesulfonic acid, sodium chloride, potassium chloride, sodium phosphate dibasic, potassium dihydrogen orthophosphate
Blue Star	Cover glass
Spectrochem	Succinic Anhydride, N-hydroxy succinamide
Fischer scientific	Hydrochloric acid, sulfuric acid, methanol, toluene, ethanol
Integrated DNA technologies primers	DNA/RNA probes

2. BUFFERS

Buffer helps to maintain a constant pH. Any change in pH caused by acid or base is regulated by the buffer. Different buffers are used for different reactions.

1. Phosphate Buffer Saline (PBS) (10X) (1L) (pH=7)

The most common and widely used being PBS. This is an ideal buffer for making DNA solutions.

In 800mL of distilled water, dissolve-

- 8gm of Sodium Chloride (NaCl)
- 0.2gm of Potassium Chloride (KCl)
- 1.44gm of Sodium phosphate dibasic (Na_2HPO_4)
- 0.24gm of Potassium dihydrogen orthophosphate (KH_2PO_4)
- Add water to make 1L

2. **Morpholino ethanesulfonic acid Buffer (MES) (0.1M) (pH=4.5)**

MES is used as an ideal buffer when performing the activation of carboxyl groups in order to react with amines.

In 800mL of distilled water, dissolve 20.32 gm of MES hydrate buffer salt. NaOH was used to balance the pH. Make up the left volume with distilled water.

3. **Saline sodium citrate Buffer (SSC) (20X) (1L) (pH=7)**

This is a buffer used for washing. The high content of salt and detergent SDS makes it ideal for removing unbound molecules.

In 800mL of distilled water, dissolve-

- 175.3 gm sodium chloride (NaCl)
- 88.2 gm sodium citrate
- 1% Sodium Dodecyl Sulphate (SDS)
- Adjust the pH to 7.0
- Make up the left volume with distilled water

3. EQUIPMENTS

1. **Heating plate**

A device used for maintaining temperatures around 300°C. It was useful in this work during cleaning phase to remove any leftover sulphuric acid from the substrate.

Figure 4: Heating plate (Environmental and Industrial Biotechnology Lab, DTU)

2. **Oven**

An instrument used for maintaining temperature of over 200°C. This equipment was of great importance in this experiment for surface silanization. APTES needed to be cured for efficient coating.

Figure 5: Hot air oven (RTSE) (Environmental and Industrial Biotechnology Lab, DTU)

3. **Centrifuge**

A device utilizing centrifugal force to sediment down particles present in a solution according to their Svedberg unit. This equipment was used in this particular experiment to dry the slides after each treatment.

Figure 6: Centrifuge (5810R, Eppendorf) (Environmental and Industrial Biotechnology Lab, DTU)

4. **Fluorescent microscope**

A microscope that is capable of sensing fluorescing molecules. It utilizes light in the visible range and on the basis of absorption and emission range

of the molecules, the charge-coupled device (CCD) senses the movement and captures it.

Figure 7: Fluorescence microscope (Olympus IX71) (Molecular Motor Lab, IIT, Delhi)

5. **FTIR-ATR Spectroscopy (Fourier Transform InfraRed- Attenuated Total Reflection)**

A highly advanced technique used to observe the chemical structure of a compound. It gives various peaks for the groups present in a compound. IR spectra is obtained on a single beam Perkin Elmer (Spectrum BX Series, USA) with the following scan parameters- scan range: 4400-650 cm^{-1}.

Figure 8: FTIR-ATR instrument (USIC, Delhi University)

PROTOCOLS

Surface cleaning

1. Glass slides were soaked in 1:1 ratio of Methanol (MeOH) and Hydrochloric acid (HCl) for 30 minutes.
2. They were then rinsed with MilliQ in a water bath sonicator for 15 minutes.
3. Concentrated sulphuric acid (H_2SO_4) was then added to the slides and incubated for 30 min.
4. They were then rinsed with MilliQ in a water bath sonicator for 15 min.
5. The slides were placed in the slide holder and then placed in boiling water, first equilibrated with the hot water itself for 30-45 minutes.
6. Rinsing with MilliQ and centrifugation at 1000 rpm for 10 minutes was performed.
7. The slides were then kept in petri plates sealed with parafilm.

Functionalization

1. 5% APTES (v/v) in toluene was incubated in plastic slide holders for 20 minutes at 70°C.
2. The slides were then rinsed with toluene, curated for 1hour at 110°C and centrifuged.
3. The slides were then incubated at room temperature in a solution made up of 5 mg/mL Succinic Anhydride (SA) and 5% TriEthylAmine (TEA) dissolved in TetraHydroFuran (THF) for 2 hr.
4. The slides were rinsed with THF, ethanol and MilliQ followed by centrifugation.

Activation

1. Pre-conditioning in 100 mM MES, 150 mM NaCl, pH 5 (MES buffer) for 15 min.
2. Incubation in 4 mM EDC, 10 mM NHS in MES for 30 min.
3. Washing with SSC was done followed by milliQ.
4. Centrifugation was done.

Immobilization and hybridization

The DNA and RNA probes taken had sequences:

DNA Probe: 5'-H_2N-AAA AAA AAA AAA AAA BCD EFG HIJ KL-3'

DNA Label: 5'-ABC DEF GHI JK-Fluorescein-3'

RNA Target: 5'-UAB CDE FGH IJK LMN OPQ RST U-3', a microRNA specific for cancer.

The protocol followed:

1. The probe was added at a concentration of 200 nM, 20 µL in slides and incubated for 2 hr under moisture environment.
2. The slides were washed with SSC followed by centrifugation.
3. Blocking with 1% BSA in PBS was done followed by centrifugation.
4. Target was added and incubated for 15 min and rinsed with SSC and water in ultrasonicator waterbath followed by centrifugation.
5. Label was added for 15 min and incubated and rinsed with SSC and water in ultrasonicator waterbath.

Characterization

To qualitatively determine the active process taking place, three controls were taken

- Control 1: Probe and Label added
- Control 2: Target and Label added
- Control 3: only Label added

Another characterization technique undertaken was FTIR-ATR spectroscopy. This was used to determine the chemical nature of the slide. Spectra recorded at room temperature in the range of 4000-650 cm^{-1}. The concentrations used in the protocol were not sensitive enough for the device where the silicon dioxide bonds of the slide surpassed the modifications.

Detection

The slides were viewed under Olympus IX71 with a 60X magnification lens. All the images related work was done with the help of imagej software. Surface graphs and integrated density were determined by the software.

PART 4

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

Surface cleaning

The slides were thoroughly cleaned in MeOH and HCL solution for 30 minutes. Following which the slides were thoroughly rinsed with milliQ. This is done to remove any organic or inorganic impurities that may be present on the slides. Hydroxylation of the slides was done by sulphuric acid converting silicon dioxide to silanol.

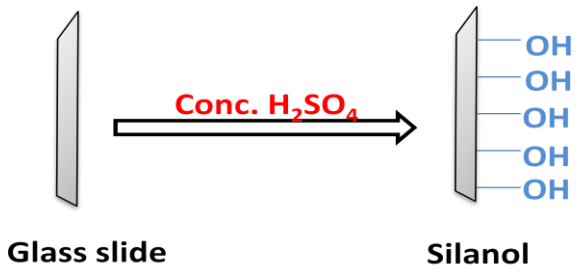

Figure 9: Cleaning and hydroxylation of slides with sulphuric acid was done.

Functionalization

Silanization of cleaned slides was done by incubating them with APTES at 110°C.

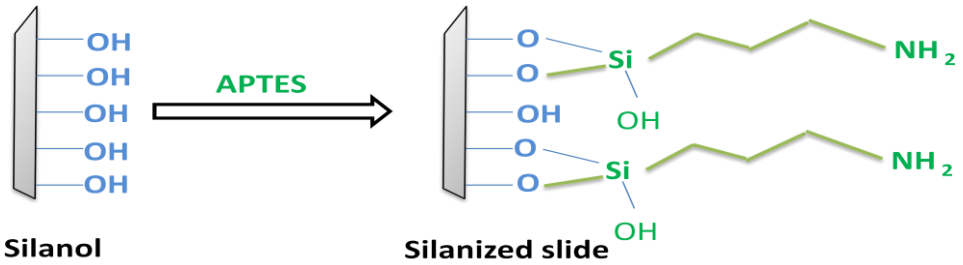

Figure 10: Silanization of slides on reaction with APTES.

APTES treated slides were then reacted with Succinic anhydride to introduce carboxyl groups.

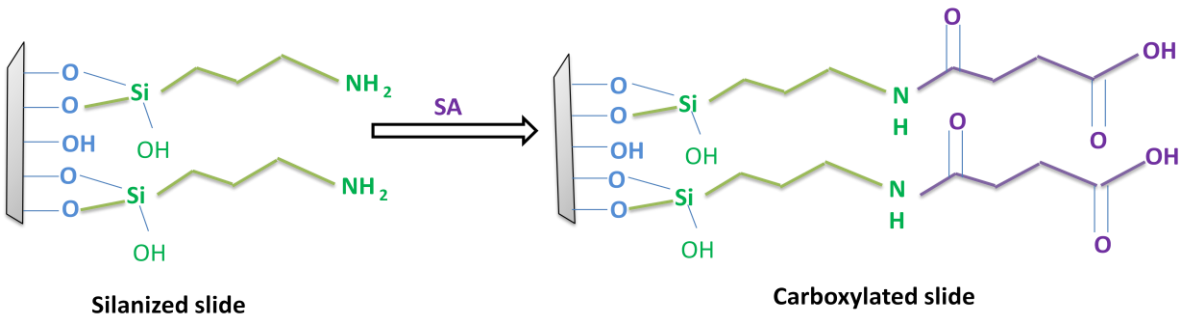

Figure 11: Introduction of carboxyl group to silanized slides.

Activation

Activation of carboxyl group introduced slides was done with EDC and NHS.

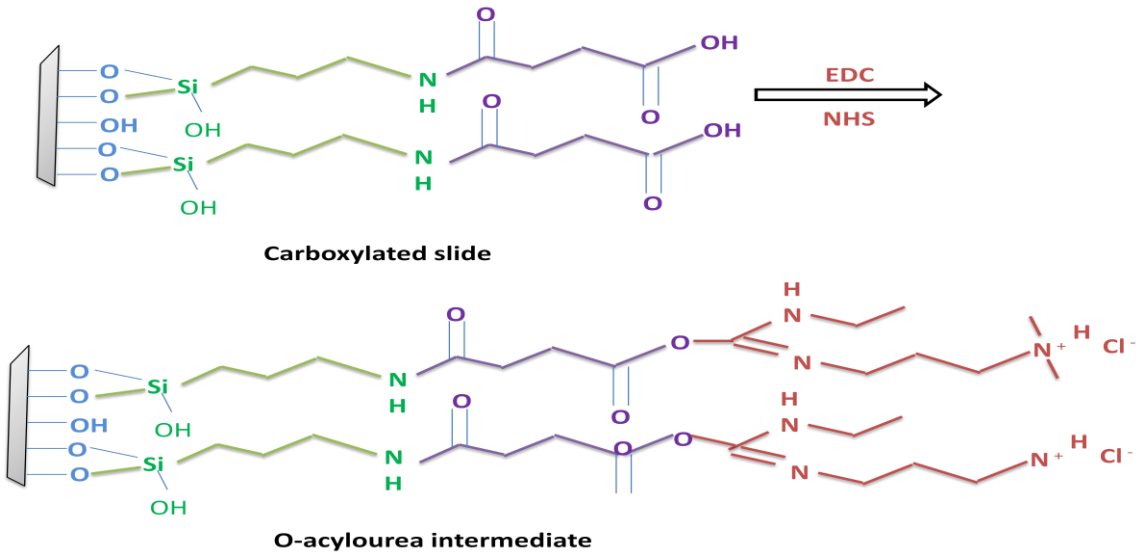

Figure 12: Activation of carboxylated slide with EDC and NHS for covalent immobilization of aminated probe.

Immobilization

Immobilization of DNA on activated glass slides involved the reaction between carboxylic group of slide and amine group of DNA forming an amide bond.

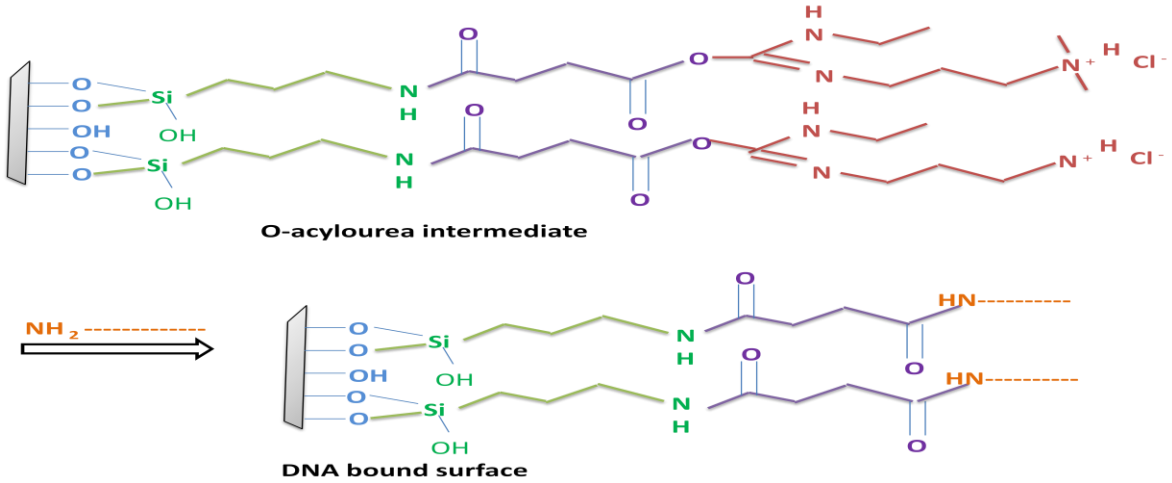

Figure 13: Reaction mechanism of covalent immobilization of DNA probe on activated substrate.

Characterization

The characterization of substrate was done by ATR-FTIR and the functional group and their peaks were observed as shown in figure 14. The characteristic peak of siloxane bond was observed in the range of 800-900 cm^{-1}. After slide was treated with APTES, a new peak was observed at 3200-3400 cm^{-1} confirming appearance of primary amine. The carbon-nitrogen bond in APTES can be seen at 1080-1360 cm^{-1}. After treatment with Succinic Anhydride, a new peak was observed at 1710-1780 cm^{-1} corresponding to a carboxyl group.

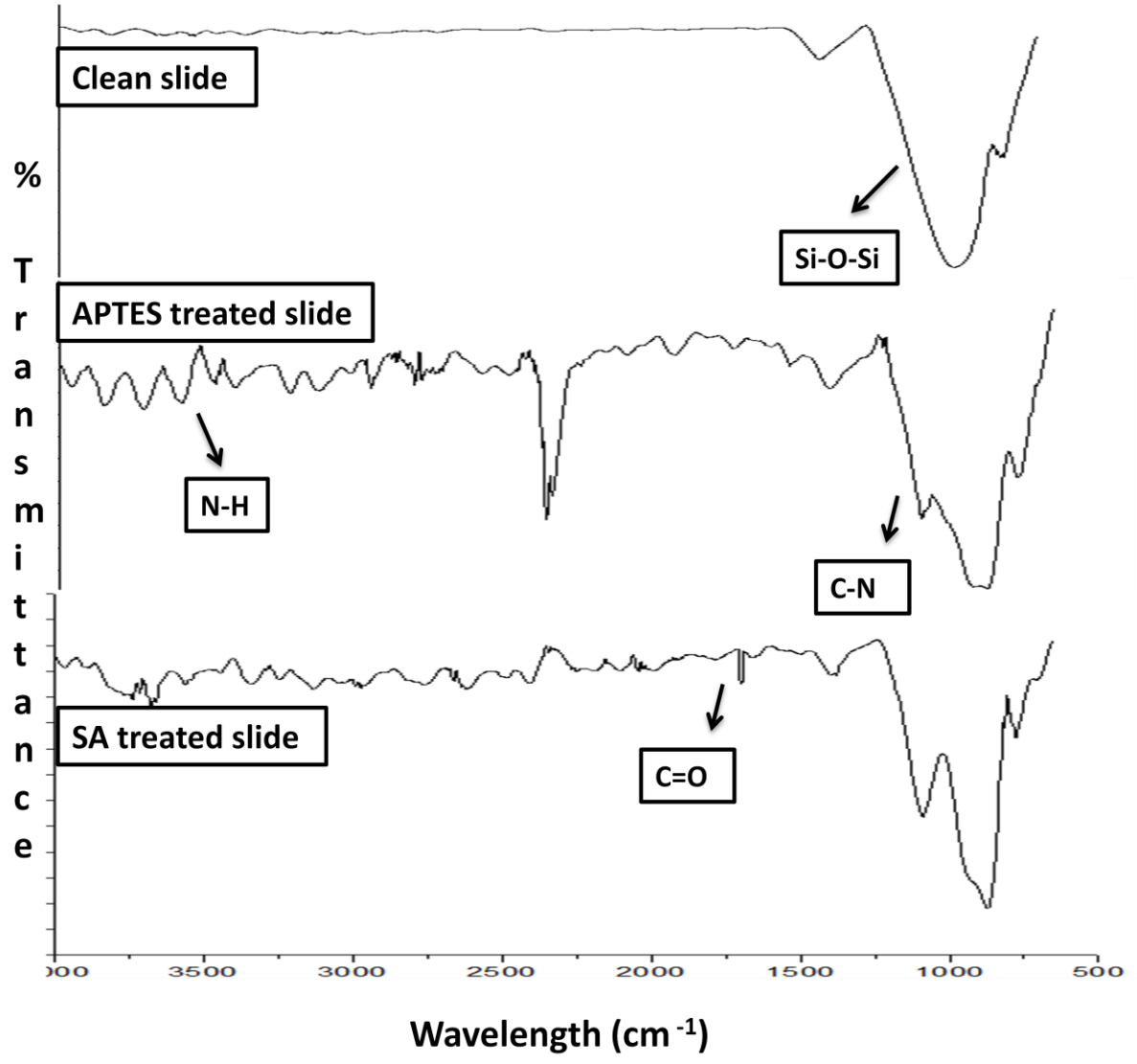

Figure 14: ATR-FTIR spectra of clean slide, APTES treated slide and SA treated slide.

Detection

The slides were then viewed under the microscope and observed for controls (clean slide, slide with only label, slide with probe and label and slide with target and label) and samples

at different concentrations of DNA probe added, i.e. 60 nM, 80 nM, 100 nM and 200 nM. It was observed that the best results were seen with 200 nM. The fluorescent images and the surface graphs are seen in figures 15, 16 and 17. The controls showed almost negligible fluorescence indicating very less or no binding of the probe to the surface.

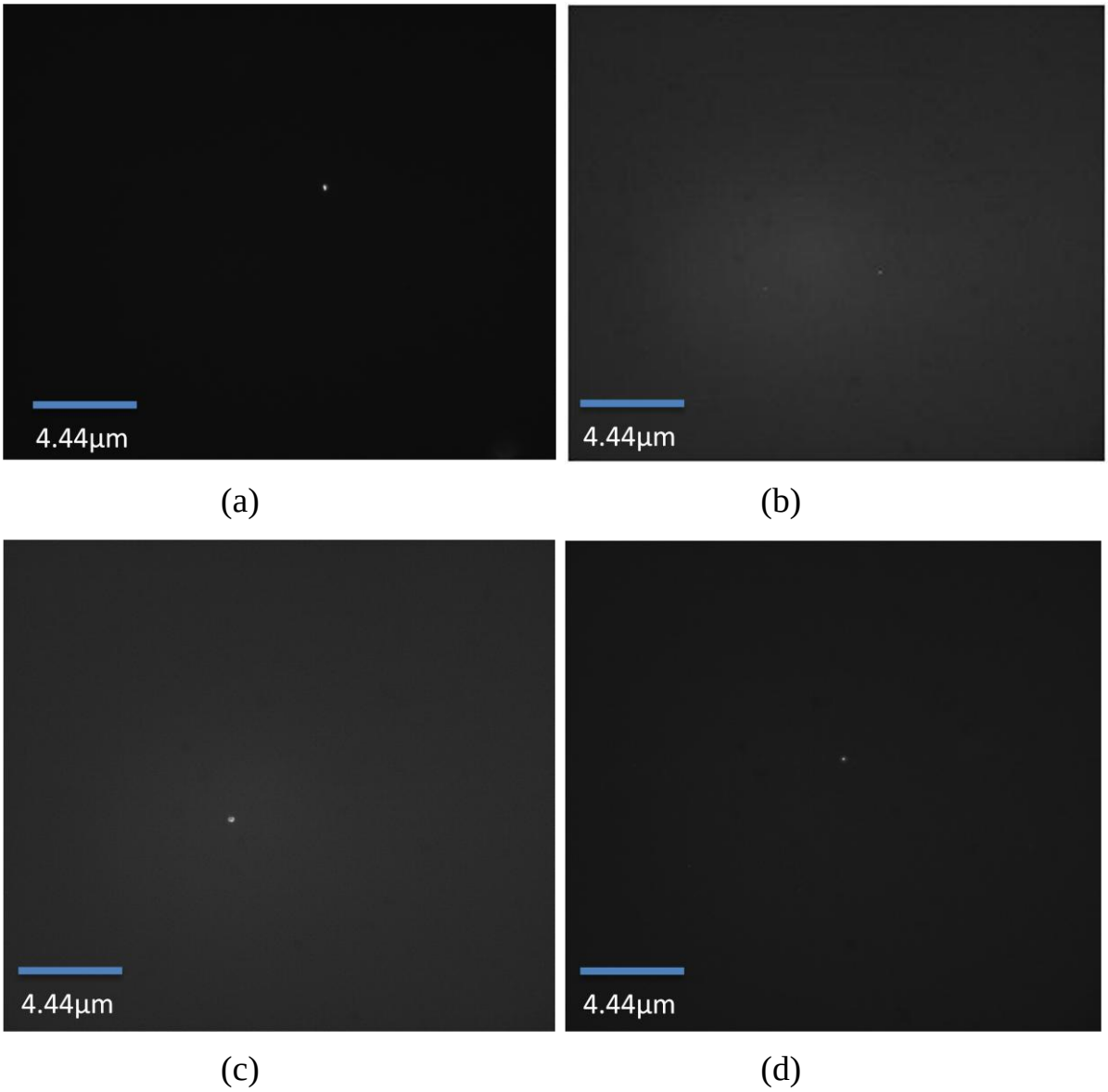

Figure 15: Fluorescent images for (a) clean slide, (b) activated slide with only label, (c) activated slide with probe and label and (d) activated slide with taget and label.

The increasing concentrations show an increasing trend in the intensity of fluorescence. This intensity was also measured at the brightest signals obtained in the images. Ten best readings were taken and their mean calculated, giving the average integrated density.

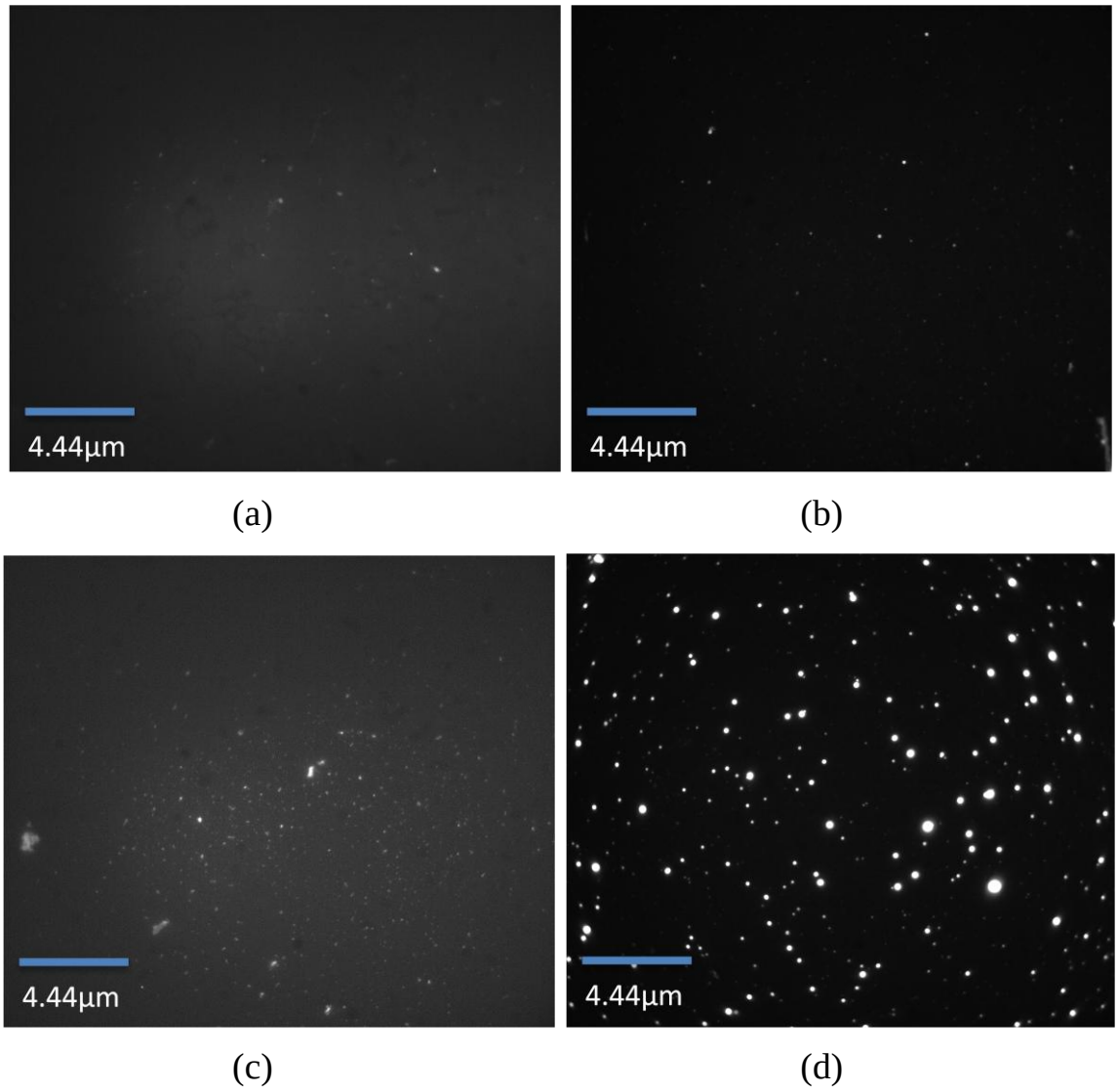

Figure 16: Fluorescent images were observed after addition of (a) 60 nM, (b) 80 nM, (c) 100 nM and (d) 200 nM concentrations of fluorescent labelled probe.

The surface graphs were obtained from the corresponding concentration images with the help of imagej software. These graphs give the peaks of the signals obtained. This is essential in cases where the signals are too light to be observed.

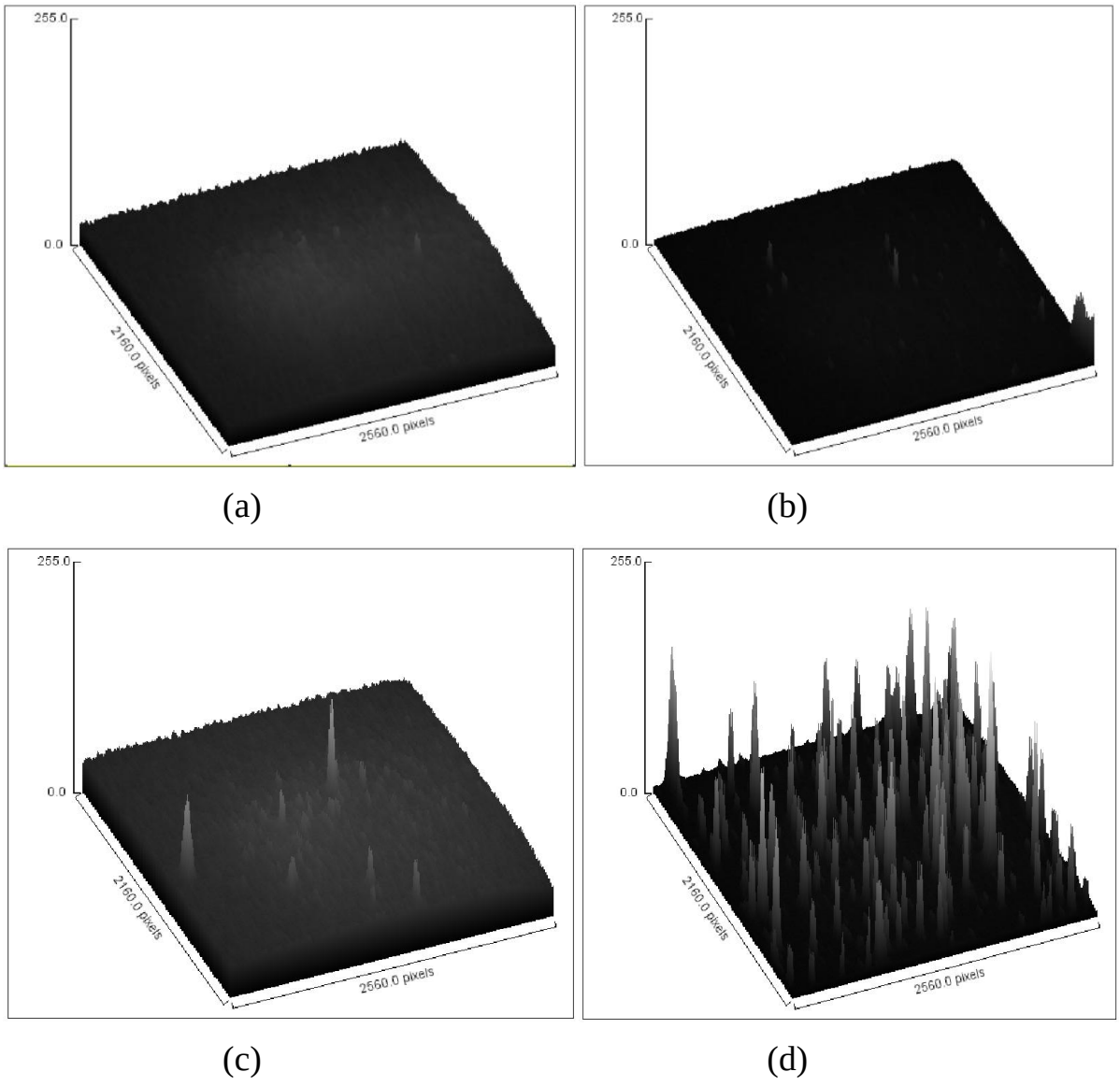

Figure 17: Surface graphs for (a) 60 nM, (b) 80 nM, (c) 100 nM and (d) 200 nM concentrations of probe.

A comparative graph for the four concentrations was also made to observe the trend. And there is an increasing graph seen corresponding to the optimized immobilization and hybridization seen at 200 nM.

Table 3: The corresponding Integrated densities of the different DNA concentrations taken.

DNA Concentration (nM)	Integrated density (px)
60	32985
80	67215
100	105485
200	458539

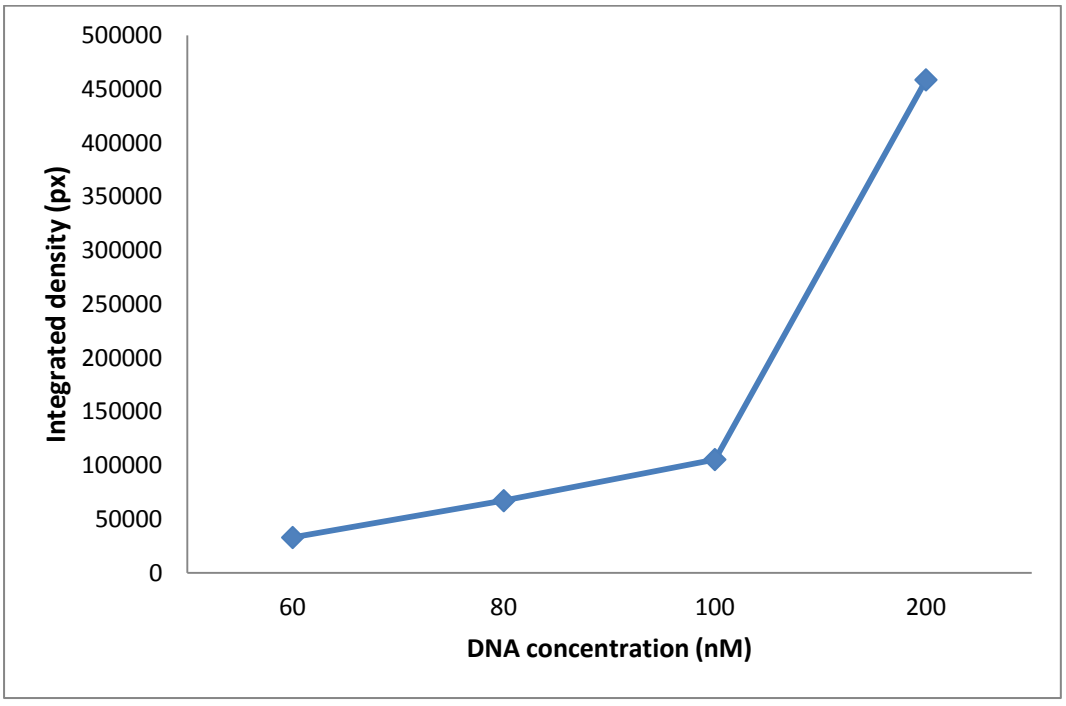

Figure 18: The relationship between DNA concentration and corresponding integrated densities.

PART 5

SUMMARY

SUMMARY

DNA microarray has become an indispensable tool in the modern diagnostic arena. But it has the limitation of being very expensive that could only be bought by the giants of the industry. This work is a step towards that improvement- the production of a portable, not too expensive microarray system. Fabrication of the chip containing probe DNA which would serve as the identification sequence for the pathogen to be identified is sought. Surface modification of a cheap, easily available substrate like glass, provides with a binding region for the probes. Attaching a fluorescent molecule with the probe gives the opportunity of an optical detection system. Controls lacking the probe immobilization or target were seen to give negligible intensity signal confirming the prior methodology. Optimization of the DNA probe concentration was done to check for the highest intensity. Surface graphs were also studied. Further work needs to be done in creating an array of probes on the same substrate and creating a full-fledged microarray device.

PART 6

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