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Review Article

Surface Modification Chemistries of Materials Used in Diagnostic Platforms with Biomolecules

Mukesh Digambar Sonawane and Satish Balasaheb Nimse

Institute for Applied Chemistry and Department of Chemistry, Hallym University, Chuncheon 200-702, Republic of Korea

Correspondence should be addressed to Satish Balasaheb Nimse; satish_nimse@hallym.ac.kr

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Biomolecules including DNA, protein, and enzymes are of prime importance in biomedical field. There are several reports on the technologies for the detection of these biomolecules on various diagnostic platforms. It is important to note that the performance of the biosensor is highly dependent on the substrate material used and its meticulous modification for particular applications. Therefore, it is critical to understand the principles of a biosensor to identify the correct substrate material and its surface modification chemistry. The imperative surface modification for the attachment of biomolecules without losing their bioactivity is a key to sensitive detection. Therefore, finding of a modification method which gives minimum damage to the surface as well as biomolecule is highly inevitable. Different surface modification technologies are invented according to the type of a substrate used. Surface modification techniques of the materials used as platforms in the fabrication of biosensors are reviewed in this paper.

1. Introduction

In recent years, biosensors became one of the indispensable tools as point-of-care (PoC) diagnostics [1]. A biosensor is an analytical device which combines a biological component with a physicochemical detector, used for the detection of an analyte [2]. The general aim of the design of any biosensor is to allow the rapid, accurate, and convenient testing in the PoC settings where the patient is receiving care [3].

Advances in biosensor technologies have enabled developing the diagnostic biosensors which have high accuracy, high speed, and an ability of parallel screening of multiple analytes [4]. So far, numerous types of biosensors have been developed [5]. Classification of the biosensors can be rationalized by looking at the principle biomolecular interactions used in that particular biosensor. In general, most of the biosensors are majorly based on the antibody/antigen interactions [6], enzymatic interactions [7], DNA-DNA interactions [1, 8], cellular structures/cells [9], or biomimetic materials [10, 11].

Irrespective of a type of biosensor and a final detection step of an analyte either at the solid-liquid interface or in the solution phase involving nanoparticle (NP), the surface attachment of antibody, enzyme, DNA, or cell is inevitable. It is well known that the conformation of biomolecules such as antibodies and enzymes plays a crucial role in determining both efficiency and selectivity of these molecules for analytes [12]. Therefore, the performance of the biosensors greatly depends on the surface chemistry of the materials used and also the chemistries used in the conjugation of the components of biosensors such as antibodies and enzymes on the surface. The attachment chemistries used for the immobilization processes can alter the natural molecular environment of proteins, thus resulting in the loss of their activity indicated by a significant drop in the sensitivity and selectivity. Various surfaces used for the immobilization of biomolecules include silicon, glass (silicon dioxide), nitrocellulose, gold, silver, polystyrene, and graphene. In this critical review, we have highlighted the materials and respective chemistries used for the immobilization of biomolecules. Merits and demerits of some of the materials and their surface modification chemistries for the application in the selective and sensitive detection of analytes are discussed.

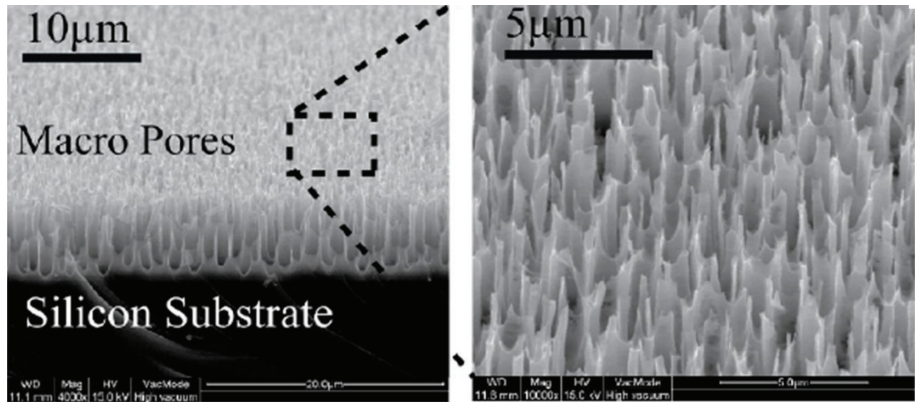


Figure 1: The macroporous surface of modified silicon (adapted from [13]).

2. Material and Techniques for Surface Modification

Modification of a surface is crucial to producing biomolecule detection platforms. In general, the functional groups present in biomolecules are allowed to react with the functional groups on the modified surfaces for their immobilization. Most common biomolecules immobilized on the surface for the fabrication of the diagnostic devices are DNA, proteins, and carbohydrates. DNA oligomers can be synthesized to have terminal amine and aldehyde groups. Proteins naturally contain amine, sulfhydryl, and carboxylic acid functional groups. Carbohydrates, in general, have hydroxyl functional groups and amine functional groups in case of glucosamine. Depending on these groups, surfaces of the substrate are modified for biomolecule attachment. The efficiency of the detection platform is strongly affected by the proper immobilization of biomolecules. Therefore, the materials and their surface modification chemistries are discussed in detail. The common surfaces used in the diagnostic devices are silicon, glass slide, glass membranes, carbon, nitrocellulose, polystyrene, silver, gold, and so forth.

2.1. Silicon. Silicon is the element of periodic table that rarely occurs in pure form. It occurs in a stable oxidized form. The typical application of silicon in electronic devices is as a semiconductor. Electrochemical detection of protein is well known because it is label-free and allows real-time detection. Physical adsorption is one of the choices for antibody immobilization as it does not need temperature and humidity controls [13]. The shorter time of immobilization makes assay faster compare to other methods [14]. Porous form of silicon surface makes it more efficient for the immobilization due to formation of pseudo-three-dimensional surface [15, 16]. The porous silicon (P-Si) surface shows high spot homogeneity, low internal fluorescence, little wetting ability, and less nonspecificity [17]. Depending on the pore size the P-Si has three categories, microporous (less than 10 nm), mesoporous (10–50 nm), and macroporous (larger than 50 nm). It has been reported that the macroporous silicon surface is highly suitable for antibody immobilization [13]. There are several reports on the techniques used for the fabrication of silicon surface.

2.1.1. Electrochemical Modification. The physisorption of biomolecules on the P-Si depends heavily on its micro- and nanomorphology controlled by the surface etching conditions and selection of silicon type. A great deal of work is reported on the fabrication of micro- and nanoporous silicon for antibody adsorption [18, 19]. Lee et al. selected a boron silicon wafer with a specific resistivity (~6–8 Ωcm) and placed it in the electrochemical cell.

As depicted in Figure 1, a self-supporting layer of P-Si is fabricated by growing an anodic oxide followed by its dissolution using an electropolishing current in a 15% hydrofluoric acid solution leading to the formation of pores. The macroporous P-Si is then cut into pieces and fitted in microtitre plate for deposition of capture antibodies (cAb) for sandwich immunoassay [20]. There are several reports on the applications of the P-Si surfaces for the electrochemical detection of various chemicals and bacteria [21, 22].

2.1.2. Covalent Modification. P-Si microparticles have high and unique reflectance properties. The antibodies which can target and capture specific antigens or cells can be covalently immobilized on the P-Si microparticles. The hydride-terminated surface of the P-Si microparticles can be passivated by hydrosilylation with dialkyne species. Guan and coworker used Cu(I)-catalyzed alkyne-azide cycloaddition (CuAAC) followed by succinimidyl activation reaction for coupling of the antibodies to P-Si. As shown in Figure 2, antibody modified P-Si microparticles were employed for the selective capture and detection of HeLa cells [23]. Similar CuAAC based surface modification for adhesion of cell surface has also been reported [24].

Other applications of P-Si surface include fabrication of protein microarray for PSA detection with the limit of detection (LOD) of 800 fg/mL. A successful covalent modification of P-Si has been reported by Rossi et al. for detection of MS2 virus with the LOD of 2×10^7 plaque-forming

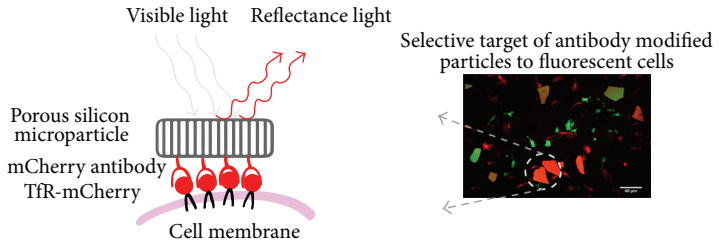


Figure 2: Covalent bonding of antibodies to P-Si microparticles for detection of cells (adapted from [23]).

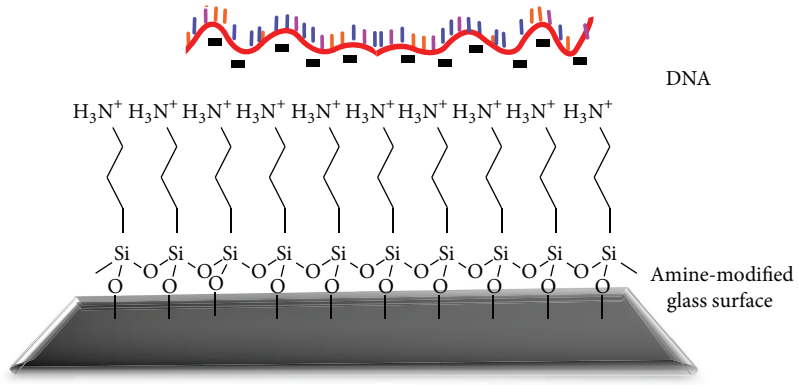


Figure 3: Physisorption of DNA on the amine-modified glass surface.

units per mL (pfu/mL) [25]. As explained earlier, the strict fabrication protocol is needed for accurate size of pores. Change in the concentrations of reagents and change in current and time affect the reproducibility of the result. Other covalent ethylene glycol modified silicon surfaces for specific adsorption of protein through photochemical reaction [26] and silicon covalent surface modification for agarose cross-linking in form of catheter have been reported for infection inhibition and omental wrapping [27].

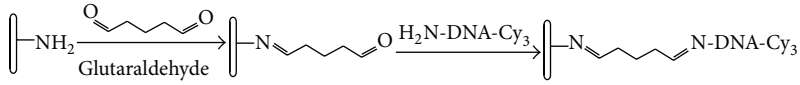
2.2. Glass (SiO_2). A glass is made up of a silicon dioxide (SiO_2). The oxide layer protects the silicon from chemical degradation and reactions. Silicon dioxide is abundant in nature and thermally stable due to a large number of silicon and oxygen bonds. Glass substrates are easily available and simple to handle and have high mechanical stability. Glass surfaces are widely used for the immobilization of DNA, proteins, and other low molecular weight biomolecules [28]. In diagnostic field, glass surface plays a crucial role as microarray platform for detection of various pathogenic DNA as well as biomarker protein [29, 30].

2.2.1. Physisorption Based Modification. Physical adsorption or physisorption is one of the simplest techniques used for the biomolecule immobilization on glass surfaces. As shown in Figure 3, the negatively charged phosphate backbone on DNA forms an ionic interaction with the positively charged surface of amine-modified glass surface through charge interactions [38].

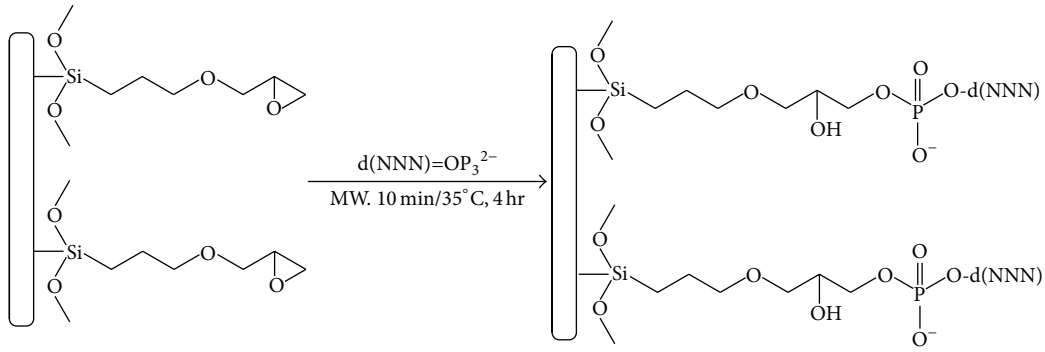
Lemeshko et al. reported that the aminosilanization of the glass surface by treatment with 3-(aminopropyl) trimethoxysilane (APTMS) affords the positively charged surface. Unfortunately, the immobilization of DNAs with this method does not result in the reproducible DNA detection platform. Because the multiple interaction of negatively charged DNA backbone with the cationic surface orients it parallel to the glass surface, thus its availability for hybridization with the complimentary DNA (cDNA) is significantly low.

The random orientation of the immobilized DNAs is also related to the nonspecific hybridization and low reproducibility. The change in pH of solution and temperature significantly affects the performance of the platform based on the method of physisorption.

2.2.2. Covalent Modification. Due to various disadvantages of physisorption methods, the covalent surface modification strategy has been widely used. Covalent bond is stronger and more stable than electrostatic interaction at solution-surface interfaces [40, 41]. The glass surfaces and be modified with the different functional groups suitable for making covalent bonds with the biomolecules to be immobilized. In general, the biomolecules have amine, carboxylic acid, and sulfhydryl functional groups. These functional groups can be used for the immobilization of biomolecules on the surface. There are



Scheme 1: Aldehyde modification of glass slide.



Scheme 2: Covalent attachment of phosphorylated DNA.

various reports on the glass surface modification for the covalent attachment of biomolecules.

(1) Aldehyde Modification. For the aldehyde modification of the glass surface, the silanol groups on the surface are converted to an amine by different methods using amine modification reagents such as (3-Aminopropyl)triethoxysilane (APTES) [42]. Fixe et al. reported the aldehyde modification using glutaraldehyde. As shown in Scheme 1, the amine-modified surface is then treated with glutaraldehyde (2.5% v/v) in 0.1 M PBS for 2 h at room temperature. The slide is then washed with water and dried. The solution containing amine-modified DNAs is spotted on the slide and incubated in humidified chamber for up to 24 h to afford the DNA microarray. However, it is important to note that the unreacted aldehyde functional groups on the glass surface should be blocked by reacting them with the sodium borohydride ($NaBH_4$) [43].

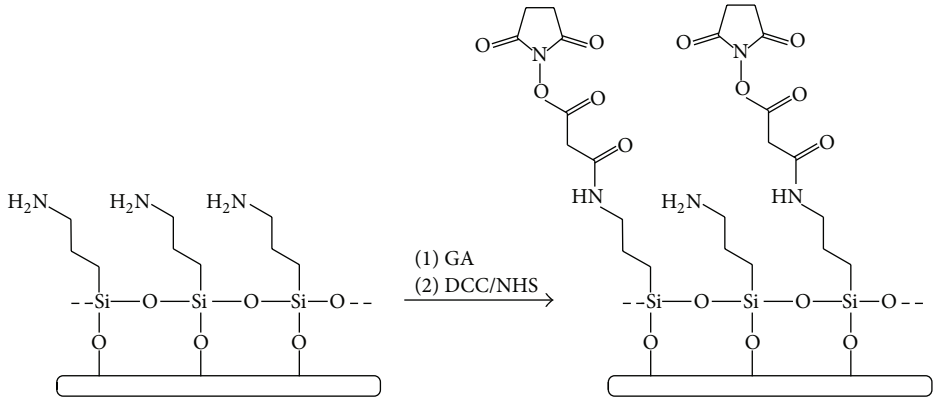
Similar to the DNA immobilization, the cAb can also be immobilized on the aldehyde-modified glass surfaces to afford protein microarrays. However, the cAbs on the protein microarrays obtained by using aldehyde chemistry have a lower binding affinity as well as reduced specificity for the target antigens [44]. Another method of aldehyde modification of ester functionalized slide can be done by reduction to alcohol followed by controlled oxidation of alcohol to aldehyde using pyridinium chlorochromate (PCC) [45]. Apart from the glass chips surface, the glass bead surface is also modified to produce a surface with aldehyde functions using APTES and glutaraldehyde reagents [46].

(2) Epoxy Modification. Apart from silanization, epoxylation is a commonly used approach for glass surface modification. The epoxy functional group on the epoxy-modified glass surfaces are allowed to react with the amine-containing biomolecules for their immobilization [47]. Thus, the amine-modified DNAs and proteins with their native amine groups can be immobilized by this method on the glass surfaces for the generation of DNA microarray and protein microarray, respectively. As shown in Scheme 2, the epoxy functionalized surfaces can be used for the covalent attachment of phosphorylated DNAs.

The advantage of this method is that the 3′ phosphate group can make a covalent bond with epoxy groups. This allows immobilization of larger fragments of DNA or PCR products on the surface. As explained by Mahajan et al., the epoxylation of glass surface is carried out by keeping the glass in a 2% solution glycidoxypropyltrimethoxysilane (GOPTS) in toluene for 4-5 h at 50°C followed by washing and drying. After epoxylation of glass surface, the probe immobilization is done by spotting the 3′ phosphate DNA on the surface and microwave treatment for 10 min in a buffer of pH 10. After the DNA immobilization, excess epoxy groups are masked by using capping buffer of pH 9 containing 0.1 M Tris with 50 mM ethanolamine, for 15 min at 50°C [48].

One of the disadvantages of this approach is the need for high pH at which glass surface starts to degrade leading to the inconsistent results [49]. Furthermore, the high pH also damaged the native three-dimensional structure of proteins resulting in decreased sensitivity and increased nonspecific interactions.

(3) Carboxylate Modification. The amine-modified DNAs are commonly used because of the simple chemistry involved in their conjugation with fluorescence molecules with carbonyl functional groups. Same chemistry can be attributed to immobilizing the amine-modified DNAs on the carboxylic acid modified glass surface. The coupling between carboxylic acid and amine is direct without any linker in between them. The 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) is a commonly used reagent for amide coupling reactions. Other common coupling reagents are N-hydroxysuccinimide (NHS), hydroxybenzotriazole (HOBt), and N,N,N′,N′-Tetramethyl-O-(benzotriazol-1-yl)uronium



Scheme 3: Surface modification with activated carboxylic acid.

tetrafluoroborate (TBTU). The free amino functional groups in lysine containing proteins are targeted for the immobilization of proteins on the glass surface through amide coupling reactions. The polyamidoamine (PAMAM) dendrimer is used as a linker for immobilization of the DNAs on the glass surfaces [50, 51]. PAMAM was initially invented in the 1980s to increase the amino groups on the outer surface of sphere [52]. Its application is now extended for DNA immobilization. Firstly, the silylation is performed using 95 : 3 : 2 v/v solution of ethanol, water, and APTES by treating the glass slides for 2 h.

As depicted in Scheme 3, Benters et al. treated the silylated surface overnight with the solution of glutaric anhydride (GA) in DMF. Slides are then washed with DMF multiple times to remove the unreacted reagents. Carboxyl groups are activated by using activating reagent such as 1 mM solution of NHS or dicyclohexylcarbodiimide (DCC) in DMF for 1 h followed by washing with DMF. For the covalent attachment of dendrimer, 100 μL of 10% PAMAM in methanol is allowed to react with the activated carboxyl functional groups on the surface for 12 h at room temperature followed by washing with methanol to remove the excess dendrimer. Before the immobilization of DNAs on the surface, these dendrimers are activated by using GA/NHS as described above [53].

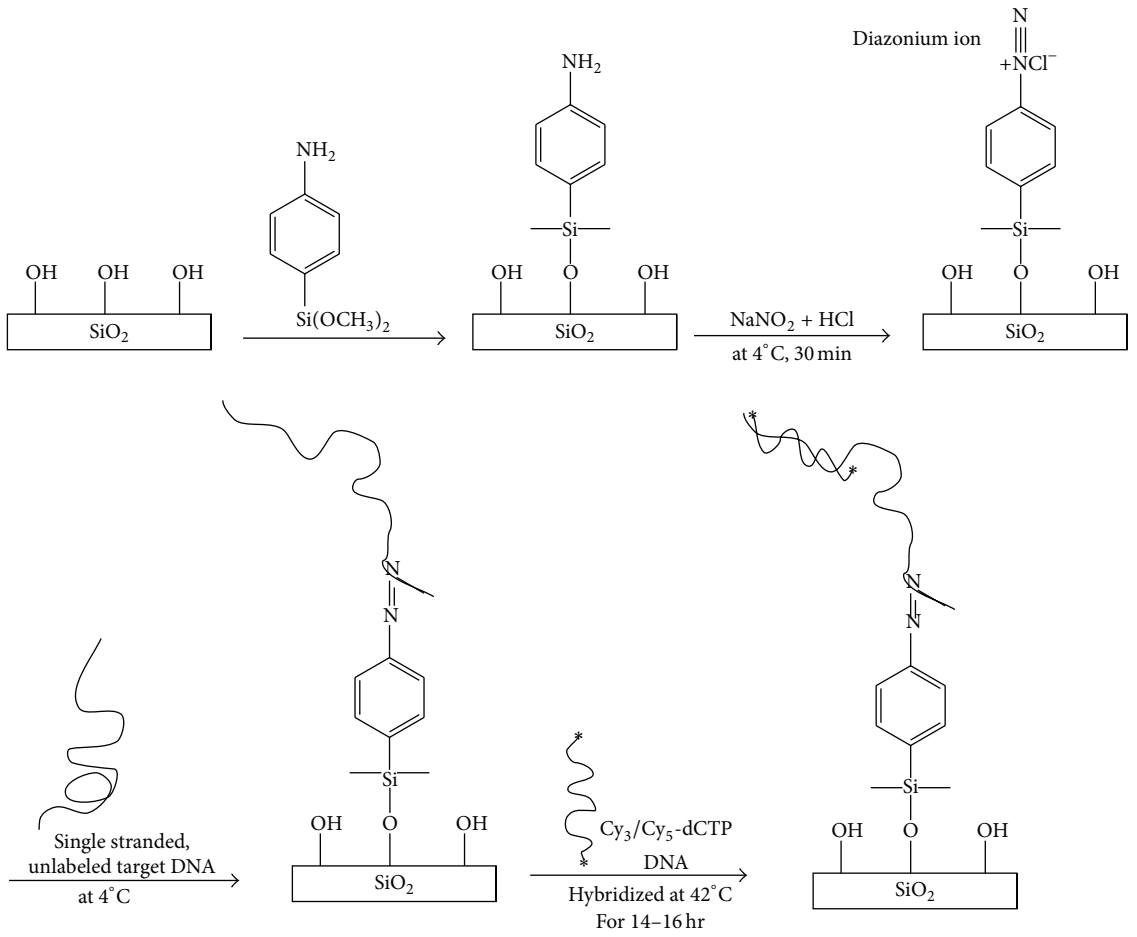
(4) Diazotization. Diazo, one of the stable bonds in organic chemistry, makes its use imperative in the surface modification process. For diazotization, the amine-modified surface is needed. Commonly, a cleaned glass using piranha solution for 30 min followed by deionized (DI) water is used [54]. The cleaned glass surface is then allowed to react with the (4-aminophenyl) trimethoxysilane in ethanol solution for 30 min. After silanization, the amine-modified surface is treated with the sodium nitrite ($NaNO_2$) to generate diazobenzyl surface as depicted in Scheme 4. The diazotization reaction is done at 4°C using solution of 40 mL water, 80 mL HCl (400 mM), and 3.2 mL $NaNO_2$ (200 mM) as reported by Alwine et al. [55]. After the reaction, surface is washed with ice-cold sodium acetate buffer, deionized water, and ethanol. Then, the solution containing the probe DNAs is spotted on the ice-cold diazotized surface and air dried for 1-2 h. Dolan et al. mentioned that the unreacted diazo groups on the surface are blocked by reaction with the glycine or UV-cross-linked followed by baking for two hours at 80°C [56]. The immobilized DNAs can be allowed to hybridize with the PCR products for the detection and discrimination of pathogens.

(5) Surface Modification with Supramolecules. In the last decade, tremendous work is done on the various applications of supramolecules, in particular, calixarenes [31, 57, 58]. The calix[4]crown-5 derivatives are well known for their ability to capture the cationic substrates including metal cations and ammonium ions [59]. The cavities of calixarenes are well known to capture the amino groups of proteins and form stable complexes with them. According to the report, Lee et al. exploited the ability of calixarenes to form complexes with proteins to fabricate the protein microarrays on glass surfaces as depicted in Figure 4 [60, 61]. For the fabrication of the protein microarrays, the calix[4]crown-5 derivatives containing aldehyde functional groups are reacted with the amine functional groups on the amine-modified glass chip to generate a monolayer.

Then, the protein in PBS solution, with 30% glycerol is spotted on calix[4]crown-5 derivative modified glass surface and incubated at 37°C for 3 h. Then, the chip is washed with the 10 mM PBS solution containing 0.5% Tween 20 for 10 min at room temperature and dried under a stream of N_2 gas. The unreacted calix[4]crown-5 derivatives, the free amine groups on the glass surface, are blocked by immersing the chip into the 3% BSA in PBS solution for 1 h at room temperature.

Similar to protein microarrays [62], Nimse et al. reported the DNA microarrays based on the 9G technology, which uses the interactions of the monolayer of calix[4]arene derivatives on the glass surface and the DNAs appended to the nine consecutive guanines (9G) are also reported [32]. The aminocalix[4]arene (AMCA) derivative with aldehyde functional groups is allowed to react with the amine-modified glass surface to generate the AMCA slides. As depicted in Figure 5, the determination of contact angle of water on the AMCA modified glass surface indicates that the AMCA molecules make the glass surface hydrophobic.

Once the AMCA immobilization is done, the solution containing DNA probes appended to 9G is spotted on the



Scheme 4: Covalent immobilization of probe DNA on a glass surface by diazotization and subsequent hybridization.

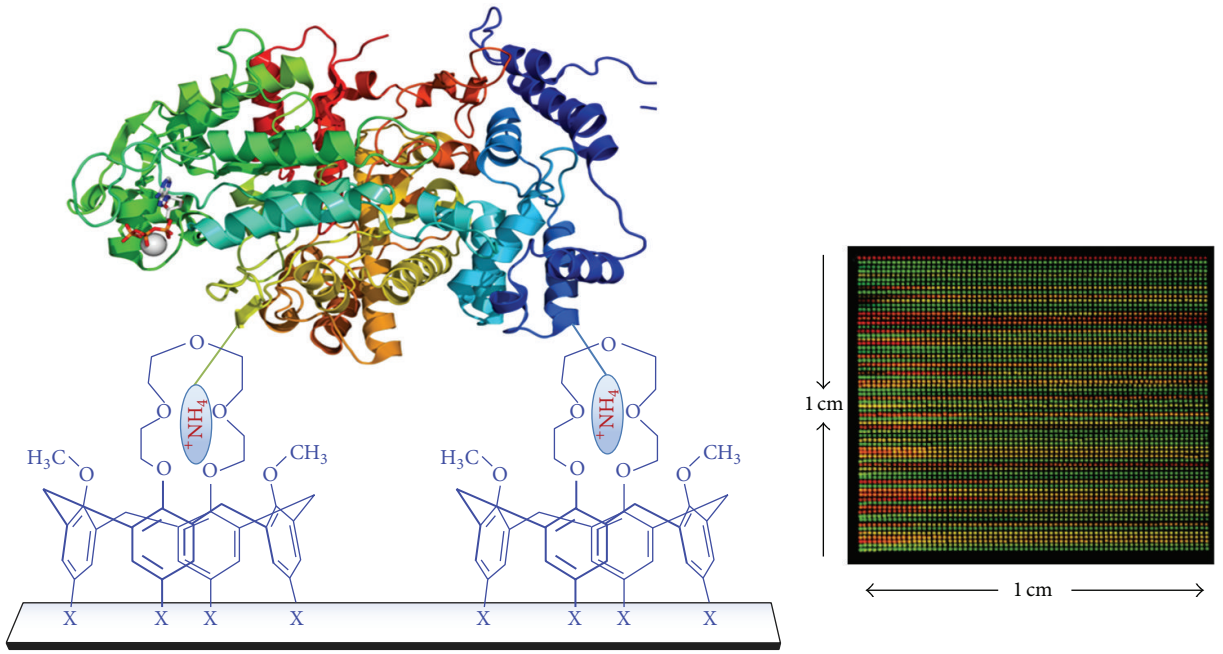
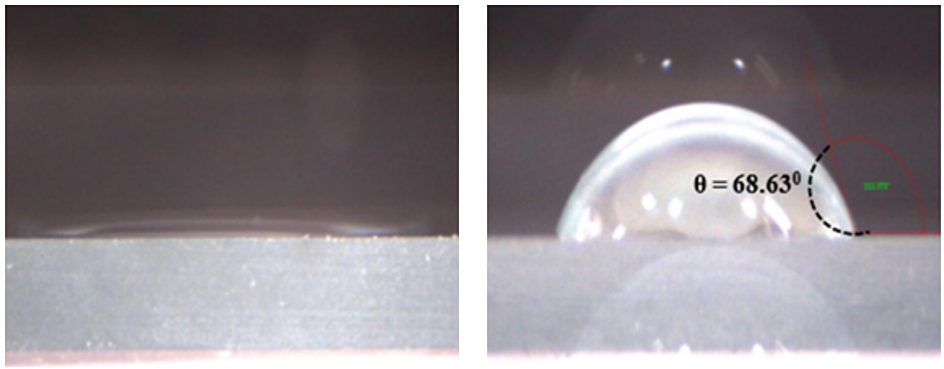


Figure 4: Interaction of calix[4]crown-5 derivative with proteins for the fabrication of protein microarray (adapted from [31]).



(a) (b)

Figure 5: Contact angle comparison: (a) bare glass and (b) AMCA modified glass slide (adapted from [32]).

surface by using microarray. After incubation at room temperature for 4 h, the slides are washed to remove excess DNAs and dried. The free AMCA molecules on the surface are then blocked with the 4x SSC solution containing BSA and 0.1% SDS. Finally, the slides are dried to obtain 9G DNAChips [63, 64].

2.3. Carbon. In recent years, a nanoparticle-based structure like nanotubes and nanowires has got tremendous attention in biomedical and diagnostic fields [65, 66]. Before 1991, sp^3 hybridized form of carbon (diamond), sp^2 hybridized forms like graphite, and C60 fullerene were the only known forms of carbon with potential applications [67]. In 1991, allotropes like carbon nanotubes (CNT) were first reported and successfully synthesized by Iijima with multiwall faces (MWCNT) [68].

Due to thermal, electrical, and magnetically unique properties, CNT became an important material in the development of biosensor platforms. Commonly reported method for preparation of CNT includes chemical vapour deposition [69, 70], arc discharge [71], and laser ablation [72].

Apart from CNT, other carbon-based nanostructures including carbon dots, carbon fibers, and PDMS also attracted researchers due to their diverse properties for biomolecule conjugation [73, 74]. The major advantage of carbon nanomaterial is their large surface area which allows maximum space for biomolecule attachment.

Immobilization of protein, carbohydrate, and nucleic acid on the CNT surfaces allows various biological applications [75, 76]. The most important part in biomolecule conjugation is the surface functionalization of carbon-based material. The general techniques for carbon surface modification are (physisorption) noncovalent and covalent modifications. There are several reports on the different techniques of surface modification of carbon-based surfaces. Due to the vastness of the research on carbon-based material, only few methods are described in this paper.

2.3.1. Physisorption. The hydrophobic character of CNT sidewalls and the strong π-π interactions between the individual tubes causes them to aggregate and reduce their solubility in common solvents, which is considered as a problem for their biological applications. However, the same hydrophobic character of the CNT surface is exploited for fabrication of biosensors by immobilizing biomolecules onto the CNT surfaces through hydrophobic-hydrophobic interactions [77–79].

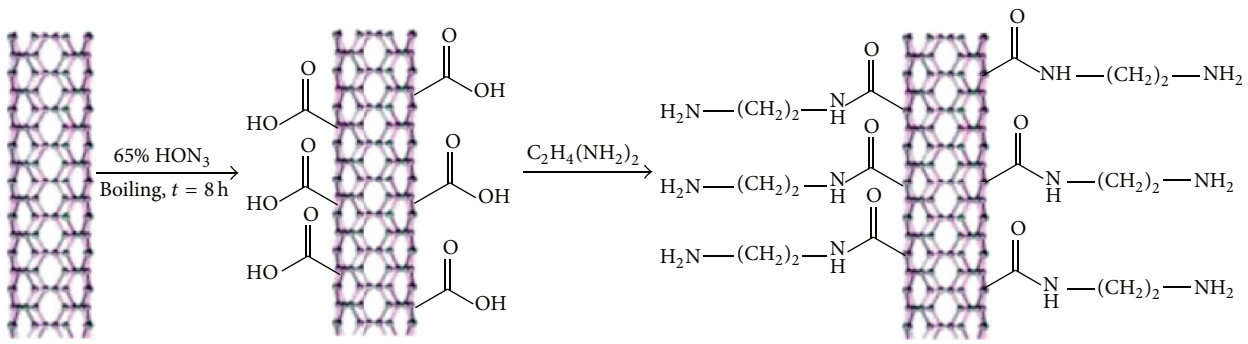
The advantage of noncovalent interaction is a limited distortion of the three-dimensional structure of a protein or enzyme to be immobilized. The hydrophobic amino acids in proteins and enzymes develop the π-π interactions with the hydrophobic part of CNT, which make them adsorbed on the surface of CNT [80–83]. As mentioned earlier, the platforms modified by noncovalent interactions suffer from the typical drawbacks of nonspecific interactions resulting in the decreased specificity and sensitivity of a biosensor.

To eliminate the problem of nonspecific interactions of target proteins with the sensor surface, the novel approach of polyethylene oxide (PEO) polymer coating is adopted by many researchers. The PEO is well known for its protein repelling ability [84]. Bomboi et al. reported the use of Tween 20 which is composed of three PEO branches and P103, another molecule with hydrophobic polypropylene oxide units, to modify the surface of CNT before adsorption of proteins on it. The surface of Tween 20 and P103 modified CNT becomes slightly hydrophilic compared with bare CNT, which results in the decrease in its interaction with the hydrophobic amino acid chains of biomarker proteins [85].

As depicted in Figure 6, for the selective immobilization of streptavidin on the CNT surface, Chen et al. used mixed noncovalent and covalent approach, which allows the highly specific immobilization of streptavidin and blocks the nonspecific binding of other proteins like BSA [33].

Conjugation of a fluorescent molecule on the CNT surface is an immerging technology for highly sensitive detection of biomolecules. Nakayama-Ratchford et al. reported the immobilization of the fluorescent molecules like fluorescein on the surface of CNT through π-π interactions [86]. There are several reports on the applications of CNT surfaces modified with the fluorescent molecules through noncovalent interactions [87–90].

2.3.2. Chemical Modification. To prevent the nonspecific binding and to avoid the leaching of biomolecules from the



Scheme 5: Amine modification of CNT.

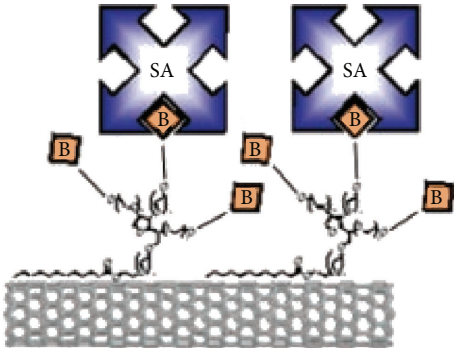


Figure 6: Streptavidin recognition by a nanotube coated with biotinylated Tween (adapted from [33]).

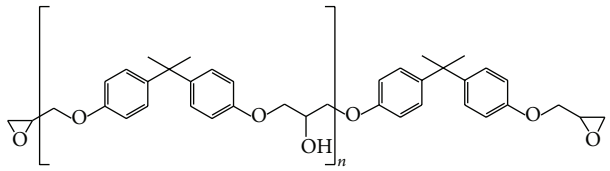


Figure 7: Structure of Epon resin 828.

CNT surface, a covalent modification technique is reported. There are two types of covalent modifications, namely (i) indirect method, for example, carboxylation of CNT surface, and (ii) direct method, for example, fluorination of CNT surface where surface carbons change from sp^2 to sp^3 hybridization. Esterification or amidation and sidewall covalent attachment are other approaches for covalent attachment [91, 92].

(1) Carboxyl Modification. The modification of the sensor surfaces with the carboxyl groups is a general and highly used surface modification method. Once the surface is modified with the carboxyl groups, it can be converted to a variety of functional groups through reaction with appropriate reagents. Furthermore, the carboxylated surfaces can be directly conjugated with proteins or DNA through their amine functional groups using coupling reagents. A mixture of sulphuric acid and nitric acid is a commonly used reagent for the carboxylation of CNT surface.

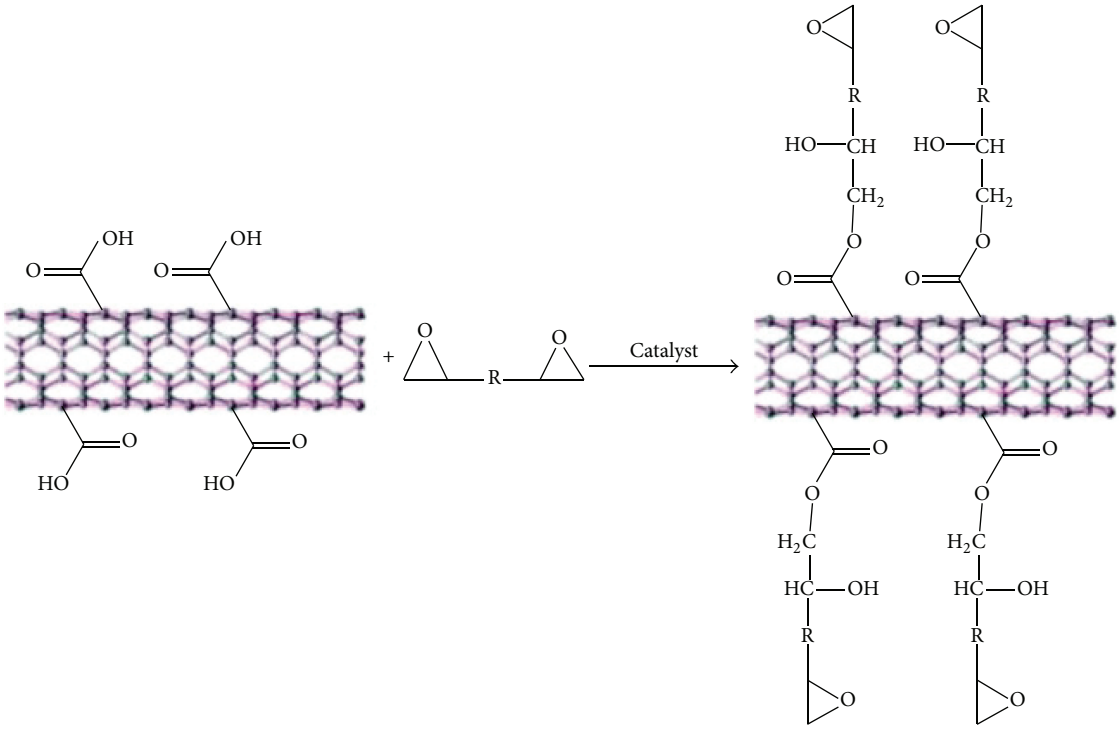
Marshall et al. reported the method for carboxyl modification of CNT [93]. According to this method, a 2 mg sample of SWCNT is added to the 75% mixture of concentrated sulphuric acid and nitric acid (1 mL). The mixture is allowed to sonicate in sonicator at 20°C. The reaction mixture is then diluted to 250 mL by using deionized water. The carboxylated CNTs are then filtered through polytetrafluoroethylene filter (PTFE) of 0.45 μm size. Finally, the collected nanotubes are washed with water at acidic pH followed by ethanol and dried in a vacuum desiccator.

(2) Amine Modification. Amide coupling is the most commonly used conjugation technique in biomolecule attachment. Either the amine or acid part of DNA or protein can be used for conjugation using amide coupling reaction. For the conjugation of DNA to CNT through its phosphate group, amine modification is needed.

Tam et al. reported the DNA sensor based on the amine modification of MWCNT as depicted in Scheme 5 [94]. The MWCNT were boiled in the 15 M nitric acid for 12 h to generate the carboxylated MWCNT. The purified carboxylated MWCNT were reacted with ethylenediamine to obtain the amine-modified MWCNT. The amine-modified MWCNT were conjugated to DNA by using EDC coupling reaction.

Other reports for amine modification of CNT include the use of nitric acid and sulphuric acid mixture for carboxylation of the surface followed by ethylenediamine reaction [95]. There are several reports on the covalent conjugation of fluorescent molecules to the CNT surfaces [96–98].

(3) Epoxy Modification. Epoxide group is a highly reactive functional group used in many substitution reactions as an electrophile. Eitan et al. reported the covalent attachment of epoxide-terminated molecules to carbon nanotubes by reaction between epoxide rings and carboxylic acid groups that are initially formed on the nanotube surface [99]. For epoxidation of CNT, the surface is first carboxylated by dispersing the CNT in 75% solution of sulphuric acid in nitric acid for 3 h. The purified carboxylated CNT were kept in the acetone with sonication for 1 h. Then, the solution containing the carboxylated CNT is mixed with the solution of Epon resin 828 (Figure 7) in acetone to allow the reaction between Epon resin 828 and carboxyl groups on the CNT surface as depicted in Scheme 6.



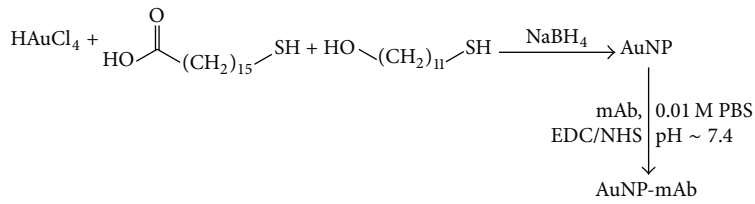
Scheme 6: Epoxy modification of CNT.

The mixture of solutions is sonicated for 1 h followed by stirring and heating at 70°C. The reaction was quenched by addition of appropriate amount of potassium hydroxide. Then, the epoxilated CNT were purified and a number of epoxy groups on the CNT surface were determined by using thermogravimetric analysis.

2.4. Nitrocellulose. A PoC diagnosis system is always expected to deliver final results in few minutes. Though the microarrays based on the glass and gold surfaces are useful for detection of multiple analytes at a time, they need longer time for final data interpretation. Microfluidic systems based on paper or nitrocellulose membranes are emerging surfaces for detection of bacteria, viruses, and proteins. A paper is advantageous due to its hydrophilic nature which allows the aqueous solutions to penetrate without the need of any external force [100]. The essential constituent of paper is cellulose. The cellulose has hydroxyl functional groups which can be modified and changed according to surface properties needed for a particular detection device [101]. Due to low cost and high availability, paper is preferred for large scale production. Moreover, the devices required for the tests are portable and disposable [102–105]. The very first example of paper based detection is glucose detection in urine [106] and pregnancy test kit [107]. There are different types of paper like Whatman® filter paper, glossy paper [108], membrane, and so forth. Whatman filter is used because of its high penetration and flow rate [109–112], but in some cases, it does not have the necessary characteristic for surface modification. Membranes, on the other hand, are planner sheets and provide larger surface area for immobilization of biomolecules such as DNA and protein. The nitrocellulose (NC) membrane is broadly used for immobilization of DNA [113], protein [114], and enzymes [115] due to its hydrophobic surface.

2.4.1. Physical Adsorption. Single-stranded DNAs are irreversibly bound to the surface of the NC membrane with noncovalent interactions. The primary interactions are hydrophobic and electrostatic between the positively charged surface of the membrane and negatively charged DNAs. The nitrocellulose offers very high binding capacity for DNAs and proteins. Fabrication of NC membranes can be done by different methods including the photolithography. The wax patterning method is fast, cheap, and easy to process and does not need organic solvents. The NC membranes with the pore size of 0.45 μm do not need pretreatment for penetration. The fabrication process includes mainly printing and baking steps which can be finished in 10 min. As shown in Figure 8, firstly, the wax is printed on the surface of NC membrane and baked at 125°C for 5 min in the oven. The melting of the wax on membrane makes it more hydrophobic and affords surface suitable for the immunoassay. For immunoassays, Lu et al. coated the cAbs on the surface, and the free area is blocked with the 1% BSA.

During baking, melted wax passes through the membrane. The 121° contact angle of backside membrane assures the melting of wax thoroughly. The efficiency of wax treated membrane is compared with the untreated membrane by immobilizing fluorescence labeled goat antihuman IgG. The immobilization on wax printed surface is more uniform than the untreated membrane. Apart from this, a large volume of liquid can be used because ring effect of protein on the



Scheme 7: Immobilization on antibodies on AuNP.

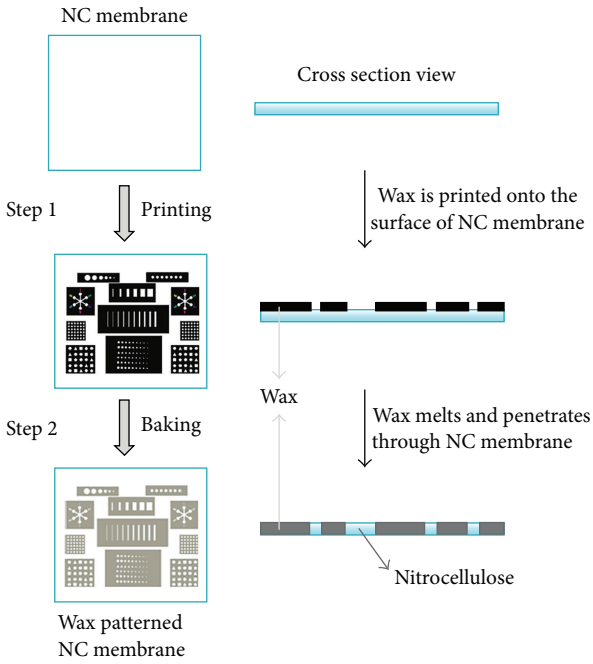


Figure 8: Wax printing on nitrocellulose membranes (adapted from [34]).

untreated membrane is avoided. Wax printing method generates 100 μm microchannel, which removes the micrometer size contaminants and makes the wax printed membranes suitable for the applications in microfluidic assays. Apart from immobilization, wax printing has application in dot immunoassay and sample purifications [34].

Due to advantages of NC membrane for high binding capacity and good flow of solution, it is also used on the chip surfaces. The glass slide can be coated with the 12 μm layer of NC to afford a platform with the intrinsic properties of NC. The solution of cAbs in PBS buffer containing 10% glycerol is then spotted on the modified surface. After spotting, the slides are incubated for one hour. After incubation, slides are washed with 0.1% Tween 20 in PBS buffer and dried to generate the platform for detection of biomarker proteins [116].

Apart from routine microarray applications, NC membrane is also used for the detection of circulating tumor cells (CTC) as reported by Zhang et al. The binding of CTCs by the cAbs immobilized on the NC membrane is detected by Surface Enhanced Raman Scattering (SERS), as depicted in Figure 9.

Usually, the antibodies specific for cancer cells are used as a capture substrate and immobilized on the surface. The NC membrane is cut into 1 cm × 1 cm pieces. Then, these pieces are immersed in the ethanol and then tiled on the PMMA wafer. After washing, the membrane is activated by using PBS buffer containing antibodies at 37°C for 30 min. During this process, the cAbs are avidly immobilized on the NC membrane. The 1% BSA solution is loaded to block the free surface area to avoid any background signals due to nonspecific interactions. Finally, the membranes are washed with PBS buffer and stored at 4°C. The gold nanoparticle and Raman probes are used to detect the CTCs by SERS [35].

2.5. Gold. Application of nanoparticles is a most focused area in the field of inorganic chemistry and it has also attracted imaginations of many biologists too. Inorganic nanoparticles including gold, silver, and ferrous oxides have found many important applications in cell signaling, drug delivery, and colorimetric and fluorescence-based detections. Gold nanoparticles (AuNP) functionalized with proteins, DNAs, or Raman probes are well known in the field of biosensors and nanobiotechnology for their applications in the molecular diagnostics [117–119], protein detection [120–122], gene regulation [123], and cell imaging [124].

2.5.1. Covalent Modification

(1) Thiol Based Modification. AuNPs are commonly used in the electrochemical and optical detection systems. The conjugation with biomolecule does not change the optical properties of AuNP [125]. Di Pasqua et al. reported thiol modification for the detection of *E. coli* O157:H7, *E. coli* specific antibodies were conjugated with the AuNPs [126]. As shown in Scheme 7, the 10 nm AuNPs are carboxylated and then coupled to *E. coli* specific monoclonal antibody (mAb) through amide coupling.

For the generation of AuNPs, to the solution containing 410 mg of $HAuCl_4 \cdot 3H_2O$ in 6 mL of water, ethanoic solutions of 11-mercapto-1-undecanol and 16-mercaptohexadecanoic acid are added. Upon addition of $NaBH_4$ to this mixture at 0°C, the solution turns brown. The product AuNP capped with alkanethiol pendant alcohol and carboxylic acid functional groups precipitate upon three hours of stirring. The precipitated particles are then thoroughly washed with ethanol and an ethanolic solution containing a small amount of 1 M HCl. The AuNPs are then dried under vacuum for ten hours. For the conjugation of mABs with the AuNPs, the carboxylic acid functional groups on the AuNPs are

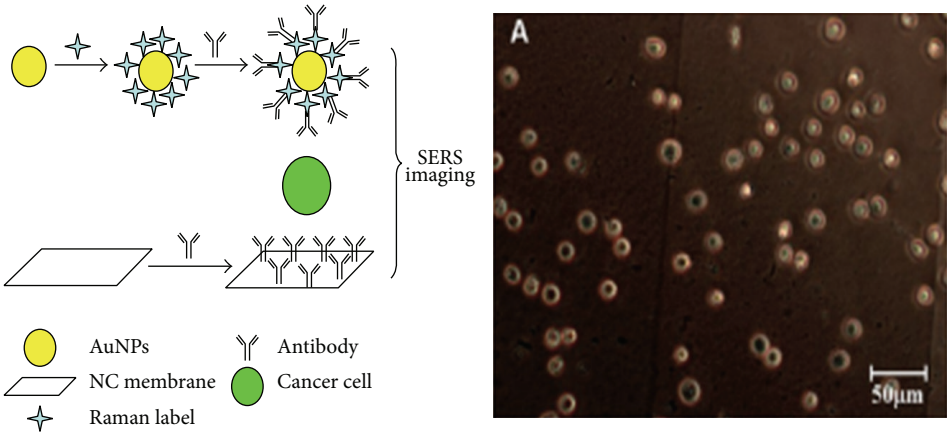
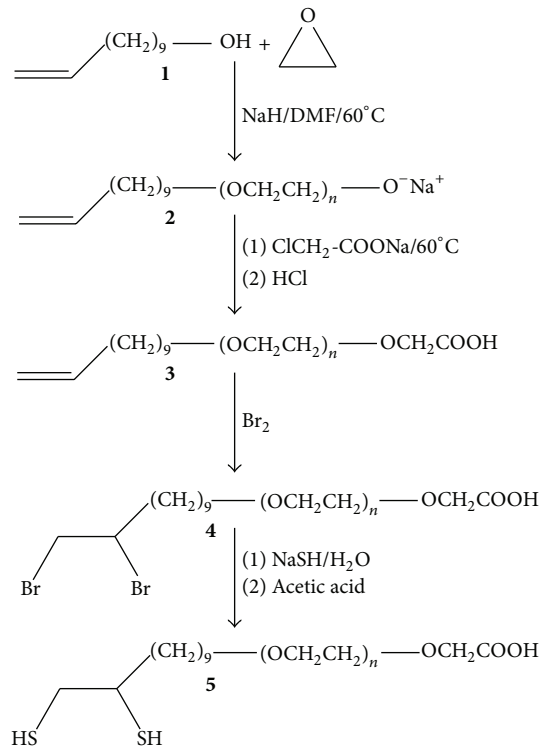


Figure 9: Detection of circulating tumor cells on NC membranes containing immobilized cAbs (adapted from [35]).

activated by reacting the AuNPs with 1-ethyl-3-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) in the PBS buffer. The mABs are then added to this solution, and the suspension is stirred at room temperature for 30 min. The mAB-conjugated AuNPs are then collected by centrifugation and washing with PBS and ethanol. The mAB-conjugated AuNPs successfully bind to *E. coli* when incubated in PBS at pH 7.4. In other reports, the AuNPs were found very effective in the detection of cancerous pancreatic cells.

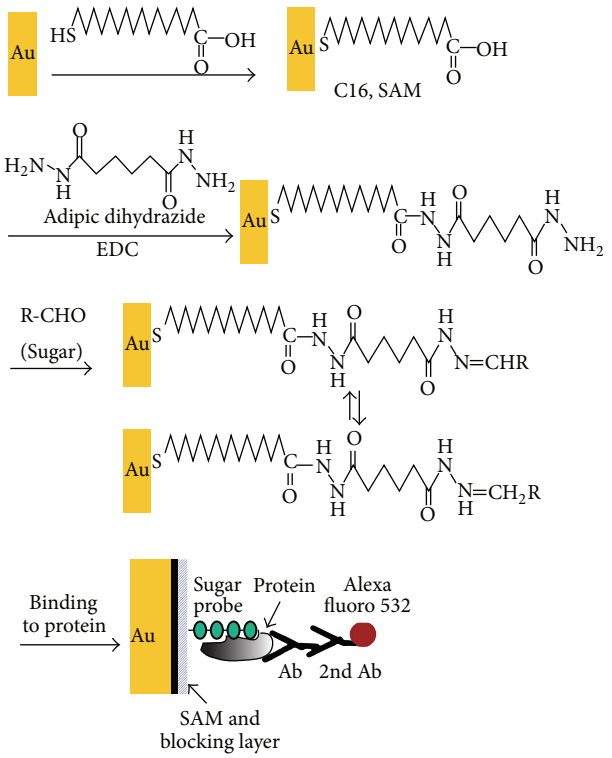
The AuNPs are known to suffer from the problem of aggregation upon long-term storage. Therefore, to solve this issue, the AuNPs are conjugated with the polyethylene glycol (PEG) to prevent their aggregation [127, 128]. The conjugation of PEG with the AuNPs makes the later hydrophilic, thus preventing agglomeration induced by hydrophobic interactions. A PEG with the heterobifunctional groups (dithiol at one end and a carboxyl at another end) is coated on the AuNP. The dithiol group allows stable anchoring heterobifunctional PEG ligands on the surface of AuNP, and the terminal carboxyl group is used for coupling with antibodies. The carboxy-terminated bifunctional PEG linker is synthesized by following a method reported by Eck et al. which is depicted in Scheme 8. The anionic polymerization of compound **1** with an excess of ethylene oxide allows synthesizing compound **2**. The treatment of compound **2** with chloroacetic acid at 60°C allows generating compound **3**. The reaction of an excess of the elemental bromine in dichloromethane at 0°C for four hours in the dark with compound **3** produces dibromide compound **4**. Compound **5** is obtained by reaction with compound **4** in water with the excess of sodium hydrosulfide hydrate.

The synthesized bifunctional PEG linker is then conjugated with the citrate-stabilized gold nanoparticles by simple ligand exchange. The citrate-stabilized gold nanoparticles are known to aggregate in the buffer containing salts. Therefore, the ligand exchange from citrate to PEG should be done in pure water. The PEGylated AuNPs are conjugated to monoclonal F19 antibodies for the detection of human pancreatic carcinoma through EDC/NHS coupling reaction [129].



Scheme 8: Synthesis of bifunctional PEG linker using conjugation with AuNP.

(2) Hydrazide Modification. According to the report by Zhi et al., the hydrazide-derivatized self-assembled monolayer on a gold surface is used for the efficient and selective anchoring of oligosaccharide [39]. The generated oligosaccharide microarrays allow the fluorescence-based detection of target proteins. The use of gold as a substrate for the fluorescence-based detection suffers from the drawbacks including fluorescence quenching and nonspecific surface adsorption of proteins. However, the use of ω-thiolated fatty acid (C_{16}) self-assembled monolayer between the gold surface and hydrazide



Scheme 9: Application of hydrazide-derivatized SAM on a gold surface for the fabrication of oligosaccharide microarray (adapted from [39]).

groups minimizes the quenching effect. Moreover, the effective blocking of the surface with the poly(ethylene glycol) aldehyde and BSA reduces the nonspecific adsorption of protein on the surface.

For the fabrication of the reported oligosaccharide microarrays, the gold substrate is prepared by following a method depicted in Scheme 9. The self-assembled monolayer (SAM) of 16-mercaptohexadecanoic acid (MHDA) is obtained by soaking the gold coated slides in the isobutyl alcohol for 2 days. The slides are then washed in ethanol to remove excess reagents and are dried under the nitrogen stream. The carboxyl functional groups on the SAM are converted to the hydrazide groups by incubation of the SAM covered slides in the dimethyl sulfoxide solution containing 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and adipic dihydrazide. The ethanol-washed and dried slides are then used for the oligosaccharide printing to obtain oligosaccharide microarrays.

The generated oligosaccharide microarray platform allows interrogation of carbohydrate-protein interactions in a high-throughput manner.

2.6. Silver. Similar to AuNPs, silver nanoparticles (AgNP) are also used for the immobilization of proteins and enzymes. The surface of AgNP allows adsorption of proteins. Hence, it can be utilized as a host matrix for various biomolecules. Silver is a suitable substrate for the immobilization of whole cell or the isolated enzymes [130, 131].

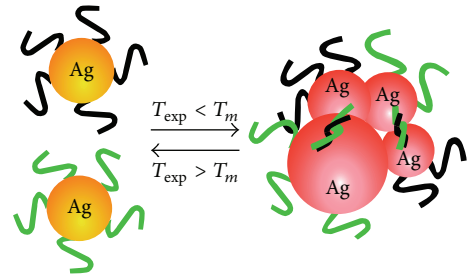


Figure 10: AgNPs-oligonucleotide conjugates (adapted from [36]).

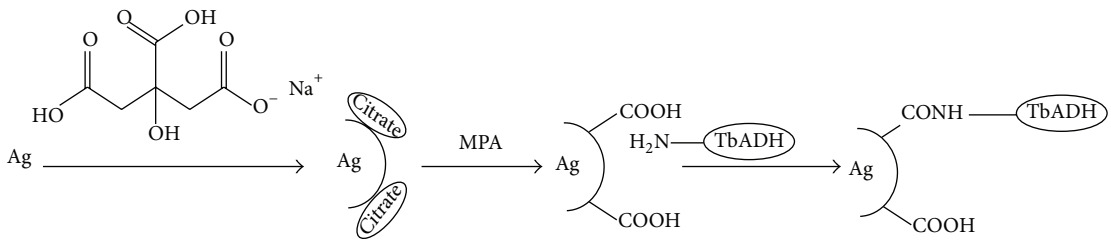
2.6.1. Electrostatic Modification. The electrostatic interactions allow the physical adsorption of biomolecules on the surface of AgNPs. Shen et al. reported that the AgNPs are synthesized by reduction of silver nitrate in the presence of sodium citrate. During the reduction process, the solution is sonicated for 15 min to obtain the yellow silver colloid which is then kept at 4°C for several days, away from light. Then, isolated nanoparticles can be used for physisorption of proteins at a pH higher than their isoelectric point [132].

2.6.2. Covalent Modification. As depicted in Scheme 10, the AgNPs prepared by the citrate reduction method and stabilized with citrate [133] can be functionalized with carboxyl groups upon treatment with 3-mercaptopropanoic acid (MPA). As reported by the Petkova et al., for the immobilization of alcohol dehydrogenase from *Thermoanaerobium brockii* (TbADH), the carboxyl-modified AgNPs were treated with the solution containing TbADH under continuous stirring for 7 h at 26°C, a research by [134]. The AgNPs modified with the TbADH were successfully used for the biotransformation studies.

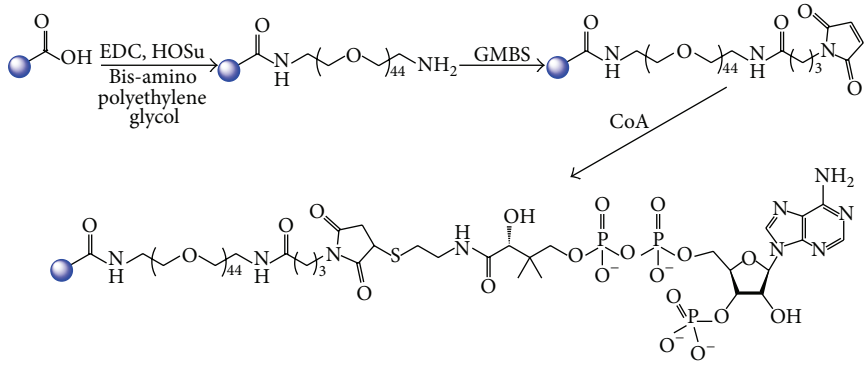
The application of a bifunctional linker for the modification of AgNPs is reported [135, 136]. The bifunctional linker, 11-mercaptoundecanoic acid, was used with 1-octanethiol in 1 : 1 ratio to generate the mixed SAM on the surface of AgNP. The IgG antibodies are then conjugated with the mixed SAM modified AgNPs. It is reported that, upon covalent conjugation of biomolecules with AgNPs, their stability and activity is maintained [137].

According to another report on the covalent modification, AgNPs-oligonucleotide conjugates are prepared by using DNA and triple cyclic disulfide moieties [36]. As depicted in Figure 10, the two types of AgNPs-oligonucleotide conjugates with complementary DNA sequences undergo DNA hybridization driven coagulation, resulting in the color change.

As depicted in Figure 11, the AgNP-DNA conjugates were prepared by treatment of AgNPs with the cyclic disulfide modified oligonucleotide. The AgNP and oligonucleotides were allowed to react with each other with 1 : 4 ratio in the presence of 1% sodium dodecyl sulfate and NaCl. The overnight incubation of solution results in the AgNP-DNA conjugates which are collected by centrifugation and washed to remove the excess DNAs.



Scheme 10: Covalent conjugation of enzymes on the surface of carboxyl-modified AgNPs.



Scheme 11: Covalent conjugation of coenzyme A to polystyrene nanoparticles.

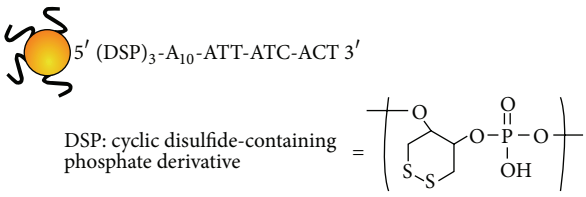


Figure 11: Conjugation of AgNPs with oligonucleotides by using DSP as a linker molecule (adapted from [36]).

2.7. Polystyrene. Polystyrene is an aromatic polymer obtained by polymerization of styrene monomers [138]. The polystyrene-based NPs are widely used in the molecular diagnostics for the detection of biomarker proteins and genomic DNAs. Polystyrene can be employed as a coating on a variety of nanoparticles as its functional groups can be easily modified or conjugated with other molecules. The other application of polystyrene is in the fabrication of fluorescence beads (FBs). The fluorescence molecules are loaded into the polystyrene shell to produce FBs, which are then used in various sensing applications. The polystyrene is also used to coat the magnetic NPs so they can be conjugated with other biomolecules such as DNA and Proteins. The applications of polystyrene include but are not limited to the detection of *E. coli* [139], the detection of cardiac Troponin T (cTnT), the detection of Herpes simplex virus, and many more.

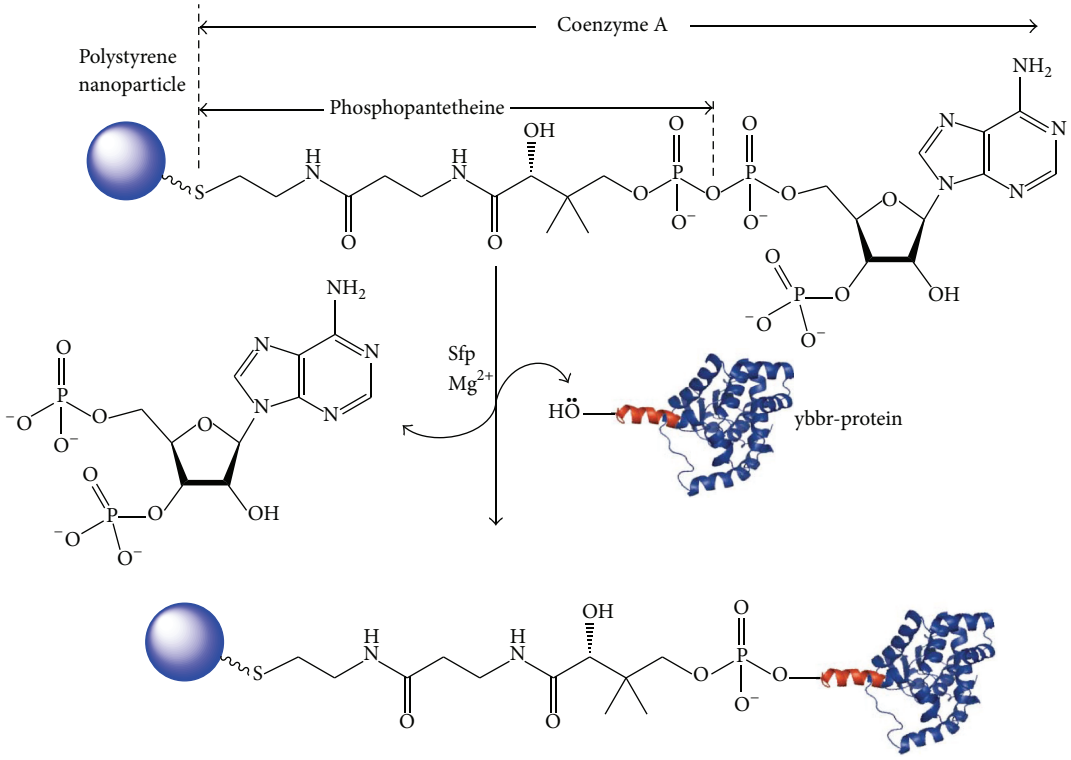
2.7.1. Covalent Modification. Covalent modification through carboxyl group is well known for modification of biomolecule. Most of the polystyrene-based nanoparticles bear large numbers of pendant carboxyl which increases the loading amount of biomolecule.

Enzyme catalyzed covalent immobilization of the proteins bearing a small 12-mer "ybbR" tag onto the polystyrene nanoparticles (PSNP) is recently reported by Wong et al. [140]. As depicted in Scheme 11, the carboxyl-PSNPs were reacted with the bis-amino-polyethylene glycol in the presence of HOSu and EDC to generate the amino-pegylated PSNPs. These PSNPs are then reacted with the g-maleimidobutyric acid succinimidyl (GMBS) to obtain maleimide-functionalized PSNPs. The maleimide-functionalized PSNPs upon chemoligation with coenzyme A (CoA) produce the CoA-derivatized PSNPs.

As depicted in Scheme 12, the protein of interest bearing ybbR-tag is immobilized on the CoA-derivatized PSNPs. The *phosphopantetheinyl transferase* (Sfp) catalyzed the reaction between the serine residue of the ybbR-tag and the phosphopantetheine moiety of CoA with the simultaneous loss of 3′,5′-adenine diphosphate results in the attachment of proteins onto the surface of PSNPs. It is reported that the enzymes immobilized by this method retain their activity.

There are several reports on the use of fluoromicrobeads for the detection of biomarkers. Primarily the fluoromicrobeads are made up of carboxylated polystyrene as a coating material entrapping the fluorescent molecules. According to the recent report by Song et al. [37], the fluoromicrobeads were used for the detection of the cTnT on the protein microarray as depicted in Figure 12.

The carboxylate modified fluoromicrobeads were conjugated with the anti cTnI mAb through EDC/NHS-mediated



Scheme 12: Protein conjugation to polystyrene NP.

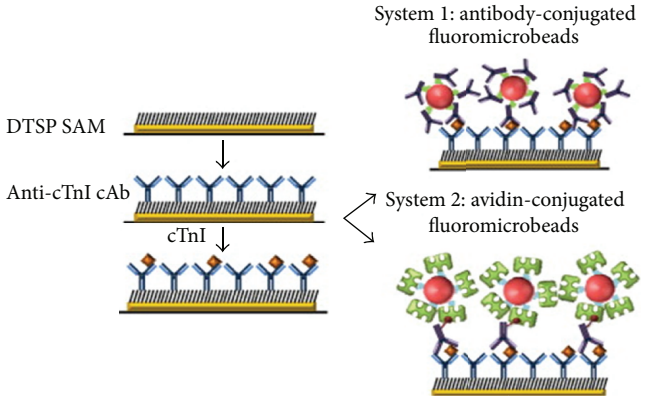


Figure 12: Application of the polystyrene-coated fluoromicrobeads for the detection of cTnI on the protein microarray (adapted from [37]).

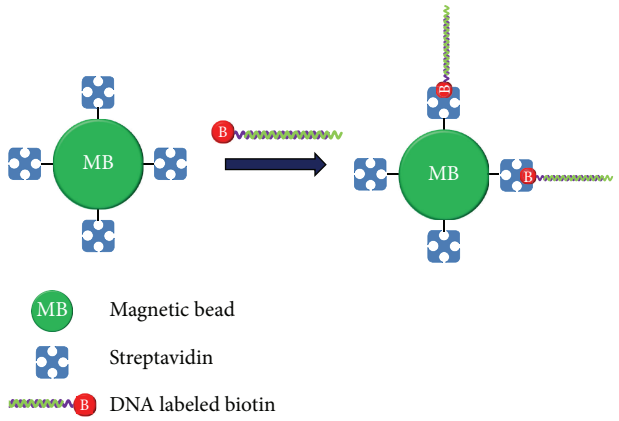


Figure 13: DNA attachment to the magnetic beads.

coupling reaction. The use of fluoromicrobeads allowed detecting the cTnI in plasma samples with a range of 0.1–100 ng/mL.

2.7.2. Affinity-Based Modification. The affinity interactions are used for the attachment of oligonucleotides with the fluoromicrobeads or polystyrene-coated magnetic beads. To generate the oligonucleotide conjugated fluoromicrobeads or magnetic beads, respective polystyrene-coated beads are first conjugated with the streptavidin using the EDC/NHS coupling reaction as reported by Zhang et al. [141]. As depicted in Figure 13, the streptavidin modified beads are then allowed to incubate with the biotinylated oligonucleotides. Due to the affinity of biotin for streptavidin, the biotinylated oligonucleotides can be readily immobilized on the streptavidin coated beads.

The binding affinity of streptavidin and biotin in solution is up to 2.5×10^{13} $(\text{mol/L})^{-1}$ [142]. This interaction is about a million times stronger than the antigen and antibody interaction. The antibodies directly immobilized onto the surface lose 90% of their biological activity [143]. However, the biotinylated antibodies immobilized on the streptavidin coated surface can retain their bioactivity [144].

3. Conclusions

Surface modification techniques of the materials used as platforms in the fabrication of biosensors are reviewed in this paper. There are several reports on materials and various methods for altering their physicochemical properties. Hence, one of these methods can be chosen according to the targeted use and needed physiochemical properties. It is important to note that the performance of the biosensor is highly dependent on the substrate material used and its meticulous modification for particular applications. Therefore, it is critical to understand the principles of a biosensor to identify the correct substrate material and its surface modification chemistry. Furthermore, it is important to look at the latest advances in the materials and techniques used for the fabrication of a biosensor before the processing.

Competing Interests

All authors declare that there is no conflict of interests.

Acknowledgments

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