Title: METHOD FOR PREPARING SUBSTRATES HAVING IMMOBILIZED MOLECULES AND SUBSTRATES

Abstract: A method for the efficient immobilization of molecules onto substrate surfaces that employs an isocyanate compound to form a reactive isocyanate surface, nanoparticles onto surfaces as well as silylated molecules such as silylated oligonucleotides or proteins onto unmodified surfaces such as a glass surface is provided. Also provided are compounds, devices, and kits for modifying surfaces such as glass surfaces.
METHOD FOR PREPARING SUBSTRATES HAVING IMMOBILIZED MOLECULES AND SUBSTRATES

CROSS-REFERENCE

This application claims the benefit of U.S. Provisional application nos. 60/568,767 and 60/568,879, both filed on May 6, 2004 and is a continuation-in-part of U.S. serial no. 10/447,073, filed May 28, 2004 which claims the benefit of U.S. Provisional application No. 60/383,564, filed May 28, 2003, and is a continuation-in-part of U.S. serial no. 10/194,138, filed July 12, 2002 which claims the benefit of priority from U.S. Provisional application nos. 60/305,369, filed July 13, 2001 and 60/363,472, filed March 12, 2002, which are incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

Surface modification plays an important role in micro-array biomolecule detection technology for controlling backgrounds and spot morphology. Several modifications were developed using different type of commercially available silanes such as silyl amines, aldehydes, thiols etc. for immobilization of biomolecules such as oligonucleotides. After coating the surface with reactive silanes, the next challenge is immobilization of required biomolecules on the modified surface. The surface loadings always vary with different silanes and even same silane may not give reproducible results. Reproducibility of optimum surface loading has always been a great challenge in this field since surface loading dictates the performance of the assay. Even with simple linear molecules for immobilization, the optimum loading on the surface is difficult to achieve. Attaching DNA to a modified glass surface is a central step for many applications in DNA diagnostics industry including gene expression analysis. In general, DNA can be attached to a glass surface either through non-covalent, ionic interactions, or through multi-step processes or simple coupling reactions. Several methods have been reported in the literature using glass surface modified with different types of silylating agents. See, for instance, Nucleic Acids research, vol 22, 5456-5465 (1994); Nucleic Acids research, vol 24, 3040-3047 (1996); Nucleic Acids research, vol 24, 3031-3039 (1996); Nucleic Acids research, vol 27, 1970-1977 (1999); Angew. Chem. Int. Ed, 38, No.9, 1297 (1999); Analytical biochemistry 280, 143-150 (2000). All these reported methods involve

Indeed, many of the current immobilization methods suffer from one or more of a number of disadvantages. Some of these are, complex and expensive reaction schemes with low oligonucleotide loading yields, reactive unstable intermediates prone to side reactions and unfavorable hybridization kinetics of the immobilized oligonucleotide. The efficient immobilization of oligonucleotides or other molecules on glass surface in arrays requires a) simple reliable reactions giving reproducible loading for different batches, b) stable reaction intermediates, c) arrays with high loading and fast hybridization rates, d) high temperature stability, e) low cost, f) specific attachment at either the 5'- or 3'-end or at an internal nucleotide and g) low background noise.

One important development in DNA detection methods involves the use of gold nanoparticle probes modified with oligonucleotides to indicate the presence of a particular DNA. For instance, one such method is described in application number PCT/US00/17507, which is incorporated by reference herein in its entirety. Typically, oligonucleotides are attached to a nanoparticle that have sequences complementary to the nucleic acid to be detected. The nanoparticle conjugate formed by hybridization to the nucleic acid results in a detectable change, thereby indicating the presence of the targeted nucleic acid. Many methods of detecting nucleic acids utilize an array substrate, such as described in U.S. published application number 2004/0072231, which is incorporated herein by reference in its entirety. By employing a substrate, the detectable change can be amplified using silver staining techniques and the sensitivity of the assay is greatly increased.

In cases involving nanoparticle-labeled probes, particularly gold nanoparticle probes, for detection of target analytes on capture substrates, the detection of extremely low amounts of target analytes in a sample may be complicated by a relative high background signal due to non-specific binding of the nanoparticle-based detection probes onto substrate surfaces. Similarly, in cases involving relatively low concentrations of
target analyte, it would be desirable to confirm that the absence of nanoparticle-labeled
detection probes immobilized on the surface of substrates is either due to the absence of
the target analyte in a sample or due to defective substrate surface preparation.
Accordingly, a substrate and method of preparation which eliminates or substantially
reduces the level of background noise in nanoparticle-based detection systems would be
highly desirable. In addition, a method for direct immobilization of nanoparticles on a
substrate surface would be useful in several detection methods, including those described
above, such as a positive control to detect hybridization efficiency (and therefore quality)
of different batches of modified substrates and for detecting targets using surface plasmon
resonance (SPR) angle shift techniques with different sized DNA modified nanoparticle
probes.

The present invention represents a significant step in the direction of meeting or
approaching several of these objectives.

SUMMARY OF THE INVENTION

The present invention fulfills the need in the art for methods for the attachment of
molecules such as oligonucleotides or proteins onto substrates surfaces such as
unmodified glass surfaces or polymeric substrates without the need for laborious
synthetic steps, with increased surface loading densities, and with greater reproducibility
and which avoids the need for pre-surface modifications. Molecules such as DNA (either
labeled or unlabeled) can be silylated at either the 3' or 5' ends as discussed below and
the 3' or 5'-silylated DNA may then be covalently attached directly to a surface such as a
pre-cleaned glass surface (Scheme) for use in hybridization assays. Furthermore,
thorough the use of certain silylating reagents, it is now possible to further enhance
surface loading densities by using modified silylating agents having multiple molecules
attached thereto. Moreover, through the use of certain silylating reagents in combination
with spacer molecules such as polymers with free amino groups and crosslinker
molecules, it is possible to prepare substrates that are surprisingly suitable for use in
nanoparticle-based detection systems. The present invention thus provides novel methods
for attaching molecules onto a substrate, devices prepared by such methods, and
compositions. This method provides great advantages over the present technology in
terms of simplicity, cost, speed, safety, and reproducibility.
Thus, in one embodiment of the invention, a method is provided for making a substrate for use in target analyte detection. The method comprises: (a) providing a substrate having a surface; (b) contacting said surface with a isocyanate compound so as to provide a surface comprising free isocyanate groups, the isocyanate compound is a member selected from the group consisting of:

$$\text{Si(NCY)}_4;$$

$$(R_1)(R_2)(R_3)\text{Si-X-NCY}$$

$$\text{[([R_1](R_2)(R_3)\text{Si-X-Z-CYNH}])}_2\text{-Si (NCY)}_2$$

$$(R_1)(R_2)(R_3)\text{Si-X-Z-CYNH-Si (NCY)}_3$$

$i;$

$\text{vi; and}$

$\text{iv;}$

wherein $R_1$, $R_2$ and $R_3$ independently represents $C_1$-$C_6$ alkoxy, $C_1$-$C_6$ alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of $C_1$-$C_6$ alkyl and $C_1$-$C_6$ alkoxy; $X$ represents linear or branched $C_1$-$C_{20}$ alkyl or aryl substituted with one or more groups selected from the group consisting of $C_1$-$C_6$ alkyl and $C_1$-$C_6$ alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; $Y$ represents oxygen or sulfur; and $Z$ represents oxygen or NH, with the proviso that at least one of $R_1$, $R_2$, or $R_3$ represents $C_1$-$C_6$ alkoxy.

In another embodiment of the invention, a method is provided for making a substrate for use in target analyte detection. The method comprises: (a) providing a substrate having a surface; (b) contacting said surface with a isocyanate compound so as to provide a surface comprising free isocyanate groups, the isocyanate compound is a member selected from the group consisting of:

$$\text{Si(NCY)}_4;$$

$$(R_1)(R_2)(R_3)\text{Si-X-NCY}$$

$$\text{[([R_1](R_2)(R_3)\text{Si-X-Z-CYNH}])}_2\text{-Si (NCY)}_2$$

$$(R_1)(R_2)(R_3)\text{Si-X-Z-CYNH-Si (NCY)}_3$$

$i;$

$\text{vi; and}$

$\text{iv;}$

wherein $R_1$, $R_2$ and $R_3$ independently represents $C_1$-$C_6$ alkoxy, $C_1$-$C_6$ alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of $C_1$-$C_6$ alkyl and $C_1$-$C_6$ alkoxy; $X$ represents linear or branched $C_1$-$C_{20}$ alkyl or aryl substituted with one or more groups selected from the group consisting of $C_1$-$C_6$ alkyl and $C_1$-$C_6$ alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; $Y$ represents oxygen or sulfur; and $Z$ represents oxygen or NH, with the proviso that at least one of $R_1$, $R_3$, or $R_3$ represents $C_1$-$C_6$ alkoxy; (c) contacting said surface comprising free isocyanate groups with a spacer molecule so as to provide a surface comprising free
amino groups; and (d) contacting said surface comprising free amino groups with a linker molecule so as to provide a reactive surface having free reactive groups.

In one aspect of this embodiment, steps (c) and (d) may be repeated one or more times.

In another aspect of this embodiment, the method comprises after step (d): (c) contacting said reactive surface with at least one type of capture probe specific for the target analyte so as to provide a surface comprising immobilized capture probes; and (f) contacting said surface comprising immobilized capture probes with a capping agent so as to block residual unreacted free isocyanate groups on areas of the surface not having immobilized capture probes and produce a substrate having substantially low signal background due to non-specific nanoparticle binding relative to a surface not contacted with a capping agent.

In another aspect of this embodiment of the invention, the method further comprises: (i) contacting said reactive surface with at least one type of capture probe specific for the target analyte so as to provide a surface comprising immobilized capture probes; and (ii) contacting said surface comprising immobilized capture probes with a capping agent so as to block residual unreacted free isocyanate groups on areas of the surface not having immobilized capture probes and produce a substrate having substantially low signal background due to non-specific nanoparticle binding relative to a surface not contacted with a capping agent.

In another embodiment of the invention, a method is provided for making a substrate for use in target analyte detection. The method comprises: (a) providing a substrate having a surface; (b) contacting said surface with an isocyanate compound so as to provide a surface comprising free isocyanate groups, the isocyanate compound is a member selected from the group consisting of:

$$\text{Si(NCY)}_4;$$
$$\text{Si-X-NCY;}$$
$$[(\text{R}_1)(\text{R}_2)(\text{R}_3)\text{Si-X-Z-CYNH}]_{2-3}\text{Si(NCY)}_2$$
$$\text{Si-X-Z-CYNH-Si(NCY)}_3$$

wherein \( \text{R}_1, \text{R}_2 \) and \( \text{R}_3 \) independently represents \( \text{C}_1-\text{C}_6 \) alkoxo, \( \text{C}_1-\text{C}_6 \) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \( \text{C}_1-\text{C}_6 \) alkyl and \( \text{C}_1-\text{C}_6 \) alkoxy; \( \text{X} \) represents linear or branched \( \text{C}_1-\text{C}_{20} \) alkyl or aryl substituted with one or more groups selected from the group consisting of \( \text{C}_1-\text{C}_6 \) alkyl and \( \text{C}_1-\text{C}_6 \) alkoxy.
alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; Y represents oxygen or sulfur; and Z represents oxygen or NH, with the proviso that at least one of R₁, R₂, or R₃ represents C₁-C₆ alkoxy; (c) contacting the surface comprising free isocyanate groups with water so as to provide a surface comprising free amino groups; and (d) contacting said surface comprising free amino groups with a linker molecule so as to provide a reactive surface having free reactive groups.

In one aspect of this embodiment of the invention, the method further comprising, after step (d): (e) contacting said surface comprising free isocyanate groups with a spacer molecule so as to provide a surface comprising free amino groups; and (f) contacting said surface comprising free amino groups with a linker molecule so as to provide a reactive surface having free reactive groups.

In one aspect of this invention, steps (e) and (f) may be repeated one or more times.

In another aspect of this embodiment of the invention, the method further comprises: (i) contacting said reactive surface with at least one type of capture probe specific for the target analyte so as to provide a surface comprising immobilized capture probes; and (ii) contacting said surface comprising immobilized capture probes with a capping agent so as to block residual unreacted free isocyanate groups on areas of the surface not having immobilized capture probes and produce a substrate having substantially low signal background due to non-specific nanoparticle binding relative to a surface not contacted with a capping agent.

In yet another embodiment of the invention, a method for making a substrate for use in detection of a target analyte is provided. The method comprises: (a) providing a substrate having a surface; (b) contacting said surface with a isocyanate compound so as to provide a surface comprising free isocyanate groups; the isocyanate compound is a member selected from the group consisting of:

\[
\text{Si}(\text{NCY})_4; \\
(R_1)(R_2)(R_3)\text{Si-X-NCY} \quad \text{i}; \\
[(R_1)(R_2)(R_3)\text{Si-X-Z-CYNH}]_2\text{-Si (NCY)}_2 \quad \text{vi}; \quad \text{and} \\
(R_1)(R_2)(R_3)\text{Si-X-Z-CYNH-Si (NCY)}_3 \quad \text{iv};
\]

wherein R₁, R₂ and R₃ independently represents C₁-C₆ alkoxy, C₁-C₆ alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of C₁-C₆...
alkyl and C₁-C₆ alkoxy; X represents linear or branched C₁-C₃₀ alkyl or aryl substituted with one or more groups selected from the group consisting of C₁-C₆ alkyl and C₁-C₆ alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; Y represents oxygen or sulfur; and Z represents oxygen or NH, with the proviso that at least one of R₁, R₂, or R₃ represents C₁-C₆ alkoxy; (c) contacting said surface comprising free isocyanate groups with a spacer molecule so as to provide a surface comprising free amino groups; (d) contacting said surface comprising free amino groups with a linker molecule so as to provide a reactive surface having free reactive groups; (e) contacting said reactive surface with at least one type of capture probe specific for the target analyte so as to provide a surface comprising immobilized capture probes; and (f) contacting said surface comprising immobilized capture probes with a capping agent so as to block residual unreactive free isocyanate groups and produce a substrate having substantially low signal background due to non-specific nanoparticle binding relative to a surface not contacted with a capping agent.

In any of the above methods for making a substrate, the isocyanate compound may be selected from the group consisting of 2-Trimethoxysilane-6-triisocyanatosilancenureabenzene, 3-(triethoxysilyl) propylisocyanate, and tetraisocyanatosilane.

The spacer molecule can be any substance having a molecular structure that provides a plurality of functional groups. In one embodiment, the spacer molecule includes at least one first functional group that can react with free isocyanate groups previously attached to the surface; at least one second functional group for attachment to a cross-linker molecule which can then be subsequently attached to another spacer molecule or to a capture probe; and at least one optional third functional groups for providing a negative charge. Both the first and second functional groups are any suitable nucleophilic group that can react with a reactive functional group such as isocyanate. Examples of nucleophilic groups include –OH, -SH, -NH₂, and -NH₂. The optional third functional group includes a carboxylate group. Preferably, the first and second functional groups are free amino groups.

Spacer molecules may include many different types of polymers; preferably those incorporating multiple functional groups. Representative examples of these types of polymers include, without limitation, poly (dimmer acid-co-alkylpolyamine)-95, poly(dimmer acid-co-alkylpolyamine)-140, poly(allylamine), and poly(m-xylendiamine-
epichlorohydrin diamine terminated, and PAMAM dendrimer generation 0. Types of polymers also include, without limitation, carbohydrates and polysaccharides. A representative example includes neomycin. Other spacer molecules include low molecular weight compounds that provide the designated functionality; preferred examples include 3,3'-diaminobenzidene, and tris(2-aminoethyl)amine.

Any suitable capping reagent that deactivates reactive moieties may be used. Examples of capping reagents include amino acid, protein, carbohydrate, carboxylate, thiol, alcohol, and amine. A representative, but non-limiting, example includes glycine.

Representative, but non-limiting examples of isocyanate compound include phenylene 1,4-diisocyanate, tolylene-2,6-diisocyanate, tolylene-α,4-diisocyanate, and isophorone diisocyanate.

Non-limiting examples of linker molecules include ethylene glycolbis (succinimidyloxy succinate), disuccinimidyl suberate, 1,6-diisocyanato hexane, methylene bis-(4-cyclohexylisocyanate, glutaric dialdehyde, methylene-p-phenyl diisocyanate, and triethyl citrate.

Any suitable substrate may be used in the above methods. Preferably, the substrate includes at least one group that reacts with the isocyanate compound such as hydroxyl, amino, or carboxylate groups.

In still yet another embodiment of the invention, a substrate for use in target analyte detection is provided. The substrate comprises a surface modified by any of the above methods.

In still yet another embodiment of the invention, the substrate comprises a surface having a polymeric layer comprising free amino groups capable of binding said capture probes, and negatively charged ionic groups.

In another aspect of this invention, the substrate surface produces a background signal upon imaging using visual or fluorescent light having substantially reduced background signal relative to a substrate not having said polymeric layer.

In still yet another aspect of this invention, the substrate has a refractive index ranging from 1.400 to 1.900.

In another embodiment of the invention, a kit is provided for detecting target analytes. The kit comprises any of the above substrates and substrates prepared by the above methods.
In another embodiment of the invention, a method is provided for detecting one or more target analytes in a sample, the target analyte having at least two binding sites. The method comprises: (a) providing a substrate prepared by any one of the methods of the present invention, said substrate having at least one type of capture probes immobilized on a surface of the substrate, each type of capture probes specific for a target analyte; (b) providing at least one type of detection probe comprising a nanoparticle and a detector probe, the detector probe specific for a target analyte; (c) contacting the capture probes, the detection probes and the sample under conditions that are effective for the binding of the capture probes and detector probes to the specific target analyte to form an immobilized complex onto the surface of the substrate; (d) washing the surface of the substrate to remove unbound nanoparticles; and (e) observing for the presence or absence of the complex as an indicator of the presence or absence of the target molecule.

In another embodiment of the invention, a method is provided for immobilizing a nanoparticle onto a surface, said method comprising the steps of: (a) providing a substrate having a surface and a nanoparticle having oligonucleotides bound thereto, at least a portion of the oligonucleotides have a free amine group at an end not bound to the nanoparticle; (b) contacting the nanoparticle with an agent so as to form a reactive intermediate, said agent having a formula i:

\[(R_1)(R_2)(R_3)Si-X-NCY\]

wherein \(R_1\), \(R_2\) and \(R_3\) independently represents \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkyl and \(C_1-C_6\) alkoxy; \(X\) represents linear or branched \(C_1-C_{20}\) alkyl or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkyl and \(C_1-C_6\) alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; and \(Y\) represents oxygen or sulfur, with the proviso that at least one of \(R_1\), \(R_2\) or \(R_3\) represents \(C_1-C_6\) alkoxy; and (b) contacting the reactive intermediate with said surface so as to immobilized the molecule onto said surface.

In one aspect of this embodiment, the surface is a glass surface.

In another aspect of this embodiment, the surface has at least one group that reacts with the reactive intermediate. Representative examples of groups include hydroxyl, amino, or carboxylate group. A non-limiting example of agent includes 3-(isocyanatopropyl) triethoxysilane or 3-(isocyanatopropyl)dimethylmonoethoxysilane.
In another aspect of this embodiment, the oligonucleotides may be bound to the nanoparticle through a functional moiety such as a thiotic acid, alkyl thiol or disulfide group (e.g., epiandrostone disulfide).

In another embodiment of the invention, a method is provided for immobilizing a nanoparticle onto a surface. The method comprises the steps of: (a) providing a substrate having a surface comprising reactive moieties that reacts with amine groups and a nanoparticle having oligonucleotides bound thereto, at least a portion of the oligonucleotides have a amine group at an end not bound to the nanoparticle; and (b) contacting the reactive moieties with the nanoparticle so as to immobilized the nanoparticles onto said surface.

In one aspect of this embodiment, the surface is a glass surface.

In another aspect of this embodiment, the surface has at least one group that reacts with the reactive intermediate. Representative examples of groups include hydroxyl, amino, or carboxylate group. A non-limiting example of agent includes 3-(isocyanatopropyl) triethoxysilane or 3-(isocyanatopropyl)dimethylmonoethoxysilane.

In another aspect, the oligonucleotides may be bound to the nanoparticle through a functional moiety such as a thiotic acid, alkyl thiol or disulfide group (e.g., epiandrosterone disulfide).

In another aspect, the reactive moieties comprise isocyanates, anhydrides, acyl halides, or aldehydes.

In another embodiment of the invention, kits are provided for preparing modified substrates. The kits may include optional reagents for silylating molecules and optional substrates, buffers for carrying out assays including washing and binding steps.

In another embodiment of the invention, a method is provided for immobilizing a molecule onto a surface, said method comprising the steps of:

(a) contacting the molecule with an agent so as to form a reactive intermediate, said agent having a formula i:

\[(R_1)(R_2)(R_3)\text{Si-X-NCY} \quad \text{i} \]

wherein \(R_1\), \(R_2\) and \(R_3\) independently represent \(C_1\text{-}C_6\) alkoxy, \(C_1\text{-}C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1\text{-}C_6\) alkyl and \(C_1\text{-}C_6\) alkoxy; \(X\) represents linear or branched \(C_1\text{-}C_{20}\) alkyl or aryl substituted
with one or more groups selected from the group consisting of \( C_{1-6} \) alkyl and \( C_{1-6} \) alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; and \( Y \) represents oxygen or sulfur, with the proviso that at least one of \( R_1, R_2 \) or \( R_3 \) represents \( C_{1-6} \) alkoxy; and

(b) contacting the reactive intermediate with said surface so as to immobilize the molecule onto said surface.

In one aspect of this embodiment, a method is provided for immobilizing a molecule onto a glass surface.

In another embodiment of the invention, a method is provided for immobilizing a molecule onto a surface, said method comprising the steps of:

(a) contacting \( Si(NCY)_4 \) wherein \( Y \) represents oxygen or sulfur with an agent so as to form a first reactive intermediate, said agent having a formula ii:

\[
(R_1)(R_2)(R_3)Si-X-Z \quad \text{(ii)}
\]

wherein \( R_1, R_2 \) and \( R_3 \) independently represents \( C_{1-6} \) alkoxy, \( C_{1-6} \) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \( C_{1-6} \) alkyl and \( C_{1-6} \) alkoxy; \( X \) represents linear or branched \( C_{1-20} \) alkyl or aryl substituted with one or more groups selected from the group consisting of \( C_{1-6} \) alkyl and \( C_{1-6} \) alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; and \( Z \) represents a hydroxy or amino group, with the proviso that at least one of \( R_1, R_2 \) or \( R_3 \) represents \( C_{1-6} \) alkoxy;

(b) contacting the first reactive intermediate with a molecule so as to form a second reactive intermediate;

(c) contacting the second reactive intermediate with said surface so as to immobilized the molecule onto said surface. The method allows for the production of branched captured molecules structures such as branched oligonucleotides on a surface which is useful for enhancing detection of target analytes such as nucleic acids.

In one aspect of this embodiment of the invention, a method is provided for immobilizing a molecule onto a glass surface.

In another embodiment of the invention, a compound is provided having the formula iii:
(R₁)(R₂)(R₃)Si-X-NHCYL-M

wherein R₁, R₂ and R₃ independently represents C₁-C₆ alkoxy, C₁-C₆ alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of C₁-C₆ alkyl and C₁-C₆ alkoxy; X represents linear or branched C₁-C₂₀ alkyl or aryl substituted with one or more groups selected from the group consisting of C₁-C₆ alkyl and C₁-C₆ alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; Y represents oxygen or sulfur; L represents a linking group; and M represents a molecule, with the proviso that at least one of R₁, R₂, or R₃ represent C₁-C₆ alkoxy.

In another embodiment of the invention, a compound is provided having a formula iv:

(R₁)(R₂)(R₃)Si-X-Z-CYNH-Si(NCY)₃

wherein R₁, R₂ and R₃ independently represents C₁-C₆ alkoxy, C₁-C₆ alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of C₁-C₆ alkyl and C₁-C₆ alkoxy; X represents linear or branched C₁-C₂₀ alkyl or aryl substituted with one or more groups selected from the group consisting of C₁-C₆ alkyl and C₁-C₆ alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; Y represents oxygen or sulfur; and Z represents oxygen or NH, with the proviso that at least one of R₁, R₂, or R₃ represents C₁-C₆ alkoxy.

In another embodiment of the invention, a compound is provided having a formula v:

(R₁)(R₂)(R₃)Si-X-Z-CYNH-Si(NHCYL-M)₃

wherein R₁, R₂ and R₃ independently represents C₁-C₆ alkoxy, C₁-C₆ alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of C₁-C₆ alkyl and C₁-C₆ alkoxy; X represents linear or branched C₁-C₂₀ alkyl or aryl substituted with one or more groups selected from the group consisting of C₁-C₆ alkyl and C₁-C₆ alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; Y represents oxygen or sulfur; L represents a linking group; and Z
represents oxygen or NH₂ and M represents a molecule, with the proviso that at least one
of R₁, R₂, or R₃ represent C₁-C₆ alkoxy.

In another embodiment of the invention, a compound is provided having a
formula vi:

\[(R₁)(R₂)(R₃)Si-X-Z-CYNH)₂-Si(NCY)₂ \quad \text{vi} \]

wherein R₁, R₂ and R₃ independently represents C₁-C₆ alkoxy, C₁-C₆ alkyl, phenyl, or
aryl substituted with one or more groups selected from the group consisting of C₁-C₆
alkyl and C₁-C₆ alkoxy; X represents linear or branched C₁-C₂₀ alkyl or aryl substituted
with one or more groups selected from the group consisting of C₁-C₆ alkyl and C₁-C₆
alkoxy, optionally substituted with one or more heteroatoms comprising oxygen,
nitrogen, or sulfur; Y represents oxygen or sulfur; and Z represents oxygen or NH, with
the proviso that at least one of R₁, R₂, or R₃ represents C₁-C₆ alkoxy.

In another embodiment of the invention, a compound is provided having a
formula vii:

\[(R₁)(R₂)(R₃)Si-X-Z-CYNH)₂Si(NHCYL-M)₂ \quad \text{vii} \]

wherein R₁, R₂ and R₃ independently represents C₁-C₆ alkoxy, C₁-C₆ alkyl, phenyl, or
aryl substituted with one or more groups selected from the group consisting of C₁-C₆
alkyl and C₁-C₆ alkoxy; L represents a linking group; X represents linear or branched C₁-
C₂₀ alkyl or aryl substituted with one or more groups selected from the group consisting
of C₁-C₆ alkyl and C₁-C₆ alkoxy, optionally substituted with one or more heteroatoms
comprising oxygen, nitrogen, or sulfur; Y represents oxygen or sulfur; and Z represents
oxygen or NH; and M represents a molecule, with the proviso that at least one of R₁, R₂,
or R₃ represents C₁-C₆ alkoxy.

These and other embodiments of the invention will become apparent in light of
the detailed description below.
DESCRIPTION OF THE DRAWINGS

Figure 1 is a scheme that illustrates one embodiment of the invention. The scheme shows the modification of a molecule such as an oligonucleotide modified at either a 3'-amino or 5'-amino to produce a silylated DNA intermediate. This silylated intermediate is then spotted onto a surface of a substrate, e.g., glass substrate and washed.

Figure 2 illustrates spot morphology after spotting a substrate with a DMF solution containing a silylated DNA in water or DMF. Branching and spreading of the spot was observed with the aqueous solution.

Figure 3 illustrates spot morphology using a DMF solution containing a silylated DNA spotted on an overhydrated substrate. Branching of the spot was observed with the overhydrated substrate.

Figure 4 illustrates spot morphology with an aqueous solution containing no silylated DNA (blank control) and with silylated DNA (silyl).

Figure 5 is (a) a scheme that illustrates another embodiment of the invention. The scheme shows the coupling of a tetraisocyanatosilane with a 1-amino-4-triethoxysilylbenzene to form a first reactive intermediate 4. The reactive intermediate is then coupled to an oligonucleotide having a free 3' or 5'-amino group to silylated DNA intermediate as a second reactive intermediate containing three molecules bound thereto. This silylated intermediate is then spotted onto a surface of a substrate, e.g., glass substrate. In part (b), a scheme is provided that illustrates another embodiment of the invention. The scheme shows the coupling of a tetraisocyanatosilane with a 1-amino-4-triethoxysilylbenzene to form a first reactive intermediate 4. The reactive intermediate is then coupled to an oligonucleotide having a free 3' or 5'-amino group to silylated DNA intermediate as a second reactive intermediate containing two molecules bound thereto.

Figure 6 illustrates the results of detection of M13 capture sequences using a DNA array chip prepared as described in Example 1 (method no. 1). In plate no. 1, a non-complementary nanoparticle-labeled oligonucleotide probe was used. In plates nos. 2 and 3, a specific complementary nanoparticle-labeled oligonucleotide probe was used. As expected, the plates using the specific complementary probes showed detection events. See Example 3.

Figure 7 illustrates the results of detection of Factor V target sequence using a sandwich hybridization assay. A DNA array chip was prepared as described in Example
1 (method no. 1) using Factor V capture probe. The DNA chip performed as expected. See Example 4.

Figure 8 illustrates the results of detection of MTHFR target sequence using a DNA array chip prepared as described in Example 1 (method no. 1). The DNA chip performed as expected. Plate No. 1 shows that the detection probe does not hybridized above its melting temperature. Plate No. 2 showed detection of a 100mer MTHFR synthetic target. Plate No. 3 showed detection of a MTHFR PCR product. See Example 5. See Example 5.

Figure 9 illustrates the results of detection of Factor V target sequence using a DNA array chip prepared as described in Example 1 (method no. 1). The DNA chip performed as expected. No non-specific background noise was observed. See Example 6.

Figure 10 illustrates the results of detection of Factor V target sequence using a DNA array chip prepared as described in Example 1 (method no. 1). The DNA chip performed as expected. The probes reacted specifically to the target sequence and no cross-hybridization between the probes and targets was observed. See Example 7.

FIG 11: Shows the schematic diagram for making polymer coated slides and DNA printing on EGS linker slides.

FIG 12: Shows the chemistry model picture of polymer and EGS modifications on glass surface.

FIG 13: Shows the stepwise diagram of making polymer coated slides with the different polymers and linkers.

FIG 14: Shows the graphical diagram that compares a polymer coated slide and a dendrimer slide.

FIG 15: Shows arrayed CY3-oligonucleotides intensity comparison on polymer coated slide and dendrimer slide.

FIG 16a: Shows the polymer coated slide performance using positive control and factor V target.

FIG 16b: Shows the performance of tris(2-aminoethylene) modified slide performance using gold nanoparticle probes.

FIG 16c: Shows polymer-95 coated slide performance in an assay format using gold nanoparticle probes.

FIG 16d: Shows background after spreading gold nanoparticle probes on dendrimer slide and amplify with silver.
FIG 16e: Shows background after spreading gold nanoparticle probes on polymer-95 slide and amplify with silver.

FIG 16f: Shows the target detection on polymer-95 slide using different target concentrations in each well.

FIG 16g: Shows multiplex detection on polymer-95 modified slide.

FIG 16h: Shows positive control detection on polymer-95 slide.

FIG 16i: Shows positive control detection on commercial slide for comparison.

FIG 16j: Shows target detection on polymer-95 slide in top rows and controls in lower rows.

FIG 16k: Shows target detection on polymer-95 slide in wells 2, 3, 4, 5, 7, 8, 9 and 10. All wells have PCR DNA target; wells 1 and 6 do not have target.

FIG 16l: Shows target detection on polymer-95 slide using silver stained gold nanoparticles.

FIG 16m: Shows target detection on polymer-95 slide using silver stained gold nanoparticles.

FIG 16n: Shows target detection on polymer-95 slide using silver stained gold nanoparticles.

FIG 16o: Shows genomic target detection on polymer-95 slide. Signal was observed in wells 1, 7, 8, 9 clearly signals were observed.

FIG 17: Shows the immobilization of gold nanoparticles on aldehyde modified surface using silver amplification.

FIG 18: Shows the schematic diagram of attaching silyl linked gold nanoparticles to unmodified surface and silver amplification.

FIG 19: Shows attaching different types of silyl linked gold nanoparticles printed on an unmodified surface using silver amplification.

FIG 20: Shows the difference in signal intensity based on salt content in gold nanoparticle preparation.

Figure 21 shows schematically the chemical reactions involved in the production of a layer on top of a substrate surface. The first step of the scheme shows the attachment of the bifunctional disilyl moiety to the surface of the substrate which is labeled “A” on top of the surface. Once the surface is functionalized with “A” the available reactive isocyanate groups can subsequently be reacted with a nucleophile for printing of a biomolecule “P”, adding a spacer group “S” or hydrolysis to produce a primary amine on
the surface. Once the isocyanate groups are printed with a biomolecule "P", the surface is then blocked with a nucleophilic molecule "B". After the isocyanate groups of "A" are reacted with a multi-functional spacer group "S", such as a di or tetraamine, any residual isocyanate groups are capped "C" with a nucleophilic molecule. After capping, another multifunctional molecule such as a diisocyanate "DI" can be added to produce available reactive isocyanates on the surface. If the isocyanate is on the surface it is considered the linker group "L". The new surface "Substrate-A-L-DI" has multiple pathways again and can be printed, hydrolyzed or an additional linker added followed by a diisocyanate. After the last spacer "S" is added and reacted with a diisocyanate or other bifunctional electrophile, this produces the final surface having free isocyanates. This last bifunctional electrophile or mixture of bifunctional molecules to be added is a linker "L" which can then be used for printing of biomolecules. An isocyanate surface which has been hydrolyzed to produce the primary amine groups on the surface is now open for addition of a bifunctional electrophile "DI" and can then go through all the combinations. See Exemple 14.

Figure 22. shows the chemical structures for the spacer molecules.

Figure 23 shows the isocyanate and activate ester chemical structures.

Figure 24. shows the synthesis of 2-trimethoxy-6-(triisocyanatosilaneurea)benzene (4)

Figure 25. shows addition of the bifunctional disilyl 4 to the substrate surface to produce a coated substrate with free isocyanate groups on glass (5a) or plastic (5b).

Figure 26. shows the hydrolysis of the isocyanate groups on the surface to give primary amine groups which then is attached a linker group "L". The first example is with 1,6-hexamethylene diisocyanate to give a surface with free isocyanate groups, and the second example is with the triester of citric acid to give an activated ester group on the surface available for printing.

Figure 27. shows the addition of a spacer dendrimer followed by the addition of the linker 1,6-hexamethylene diisocyanate.

Figure 28. shows the addition of the spacer 3,3'-Diaminobenzidine.

Figure 29. continued from Figure 28. shows the capping of isocyanates after the addition of the spacer.
Figure 30. continued from Figure 29. shows the addition of diisocyanate, which could be arrayed or the process continued with the addition of another spacer.

Figure 31. continued from Figure 30. shows the addition of P95 polymer to free isocyanates on glass substrate.

Figure 32. continued from Figure 31. shows the addition of the linker group 4,4'-Dicyclohexylmethane diisocyanate which can be printed or an additional spacer added.

Figure 33. continued from Figure 29. shows the addition of the spacer 3,3'-Diaminobenzidine.

Figure 34. continued from Figure 31. shows the addition of the linker group 4,4'-Dicyclohexylmethane diisocyanate which can be printed or an additional spacer added.

Figure 35. continued from Figure 29. shows the addition of PoXyl polymer as a spacer.

Figure 36. continued from Figure 35. shows the addition of 4,4'-Dicyclohexylmethane diisocyanate as a linker which can be printed or an additional spacer added.

Figure 37. shows the addition of glycine to block any isocyanate on the surface after the substrate is arrayed with a biomolecule.

Figure 38. continued from Figure 25. shows the addition of the spacer P95 to the anchor group, followed by the addition of ethylene glycolbis(succinimidylsuccinate) as a linker.

Figure 39. shows image of glass substrate (10i) with 1,6-hexamethylene linker: Substrate 10i is arrayed with three columns and four rows in each of the three wells. Each of the three spots in any row has the same capture arrayed on the slide. The type of capture by row is wild type capture, mutant capture, negative control and positive control capture DNA sequences from top to bottom. Wells 1 and 3 show genomic wild type target with positive and wild type nanoparticle probes binding to both the positive and genomic captures. Well 2 shows nanoparticle probes without any genomic DNA, demonstrating that there is no nonspecific binding to the substrate.

Figure 40. shows image of glass substrate with triethyl citrate and 1,6-hexamethylene diisocyanate linker mixture (10p): Substrate 10p is arrayed with three columns and four rows in each of the five wells. Each of the three spots in any row has the same capture arrayed on the slide. The type of capture by row is positive control, wild type capture, mutant capture, and negative control capture DNA sequences from top
to bottom. Well 1 shows positive nanoparticle control probes, with no DNA target, binding to positive control captures. Wells 2 and 4 show PCR wild type target with wild type nanoparticle probes binding to the wild type captures. Well 3 shows mutant nanoparticle probes with mutant DNA target binding to the mutant captures only. The control, well 5 contained wild type and mutant nanoparticle probes with no DNA targets and demonstrate no non-specific binding of the nanoparticle probes.

Figure 41. shows image of glass substrate with triethyl citrate linker (10b): Substrate 10b is arrayed with three columns and four rows in each of the five wells. Each of the three spots in any row has the same capture arrayed on the slide. The type of capture by row is positive control, wild type capture, mutant capture, and negative control capture DNA sequences from top to bottom. Well 1 shows positive nanoparticle control probes, with no PCR target, binding to positive control captures. Well 2 shows PCR heterozygous DNA type target with wild type and mutant nanoparticle probes binding to both the wild type and mutant captures. Well 3 shows mutant nanoparticle probes with mutant DNA target binding to the mutant captures only. Well 4 shows wild type nanoparticle probes with wild type DNA target binding to the wild captures only. The control, well 5 contained wild type and mutant nanoparticle probes with no DNA targets and demonstrate no non-specific binding of probes.

Figure 42. shows glass substrate with Methylene diisocyanate linker (10n): Substrate 10n is arrayed with three columns and four rows in each of the five wells. Each of the three spots in any row has the same capture arrayed on the slide. The type of capture by row is positive control, mutant type capture, wild type capture, and negative control capture DNA sequences from top to bottom. Well 1 shows positive nanoparticle control probes, with no DNA target, binding to positive control captures. Wells 2 and 3 show genomic wild type DNA target with wild type nanoparticle probes binding to the wild type and mutant captures. Well 4 shows mutant type nanoparticle probes with mutant type DNA target binding to the mutant captures only. The control, well 5 contained wild type and mutant nanoparticle probes with no DNA targets and demonstrate no non-specific binding of probes.

Figure 43. shows glass substrate with triethyl citrate and methylenediphenyl diisocyanate linkers (10r): Substrate 10r is arrayed with three columns and four rows in each of the five wells. Each of the three spots in any row has the same capture arrayed on the slide. The type of capture by row is positive control, mutant type capture, wild type
capture, and negative control capture DNA sequences from top to bottom. Well 1 shows positive nanoparticle control probes, with no DNA target, binding to positive control captures. Well 2 shows PCR heterozygous type DNA target with wild type and mutant nanoparticle probes binding to both the wild type and mutant captures. Well 3 shows wild type nanoparticle probes with wild type DNA target binding to the wild type captures only. Well 4 shows mutant type nanoparticle probes with mutant type DNA target binding to the mutant captures only. The control, well 5 contained wild type and mutant nanoparticle probes with no DNA targets and demonstrate no non-specific binding of probes.

Figure 44. shows glass substrate with 4,4’-Dicyclohexyl methane diisocyanate linker (10m): Substrate 10m is arrayed with three columns and four rows in each of the five wells. Each of the three spots in any row has the same capture arrayed on the slide. The type of capture by row is positive control, wild type capture, mutant capture, and negative control capture DNA sequences from top to bottom. Well 1 shows positive nanoparticle control probes, with no DNA target, binding to positive control captures only. Wells 2 and 4 show PCR wild type target with wild type nanoparticle probes binding to the wild type captures only. Well 3 shows mutant nanoparticle probes with mutant DNA target binding to the mutant captures only. The control, well 5 contained wild type and mutant nanoparticle probes with no DNA targets and demonstrates no non-specific binding of the probes.

Figure 45. shows glass substrate with 1,6-Hexamethylene diisocyanate linker (10L): Substrate 10L is arrayed with three columns and four rows in each of the five wells. Each of the three spots in any row has the same capture arrayed on the slide. The type of capture by row is negative control, mutant type capture, wild type capture, and positive control capture DNA sequences from top to bottom. Well 1 shows positive nanoparticle control probes diluted 2 logs compared to positive control probe concentration in Figure 40. DNA target was not used and binding to the positive control captures was demonstrated. Wells 2, 3 and 4 show PCR wild type target with wild type nanoparticle probes binding to the wild type captures only. Well 4 contained the highest concentration of target with 3 and 2 each containing a log reduction in target respectively. The control, well 5 contained wild type and mutant nanoparticle probes with no DNA targets and demonstrates no non-specific binding of the probes.
Figure 46. shows glass substrate with methylenediphenyl diisocyanate linker. 
(10n): Substrate 10n is arrayed with three columns and four rows in each of the five wells. Each of the three spots in any row has the same capture arrayed on the slide. The type of capture by row is positive control, wild type capture, mutant capture, and negative control capture DNA sequences from top to bottom. Well 1 shows positive nanoparticle control probes, with no DNA target, binding to positive control captures only. Wells 2 and 4 show PCR wild type target with wild type nanoparticle probes binding to the wild type captures only. Well 3 shows mutant nanoparticle probes with mutant DNA target binding to the mutant captures only. The control, well 5 contained wild type and mutant nanoparticle probes with no DNA targets and demonstrates no non-specific binding of the probes.

Figure 47. shows two separate slides for a comparison of a Nanosphere plastic substrate (10h) with a commercially available glass substrate. The top slide (plastic) shows the wild type and mutant differentiation using formamide gradient in different wells on a HMDI surface. A similar experiment was conducted on the commercially available glass slide to compare with Nanosphere modified slide. The modified plastic slide demonstrated higher intensity and discrimination at 30% formamide concentration between wild type and mutant capture sequences and synthetic DNA targets. This is compared to the commercial slide at 40% formamide to obtain discrimination between wild type and mutant synthetic DNA targets. Assay conditions: To each well 100 μL of aliquot was prepared using hybridization buffer, wild type, mutant and control gold nanoparticle probes, and increasing concentrations of formamide. Assay was developed on modified slide following general assay conditions and imaged on Verigene® instrument.

Figure 48. shows the plastic substrate with a 1,6 hexamethylene diisocyanate linker on the surface (10K). Substrate 10K is arrayed with three columns and four rows in each of the five wells. Each of the three spots in any row has the same capture arrayed on the slide. The type of capture by row is mutant type capture, negative control, wild type capture, and positive control capture DNA sequences from top to bottom. Wells 1 and 5 show positive nanoparticle control probes, with no DNA target, binding to positive control captures only. Wells 2, 3 and 4 show genomic wild type target with wild type and positive control nanoparticle probes binding to the wild type and positive control captures only.
Figure 49. shows the plastic substrate with 4,4'-dicyclohexylmethane diisocyanate linker on the surface (10e). Substrate 10e is arrayed with three columns and four rows in each of the five wells. Each of the three spots in any row has the same capture arrayed on the slide. The type of capture by row is wild type -1, wild type -1, mutant -I, wild type - II, mutant -II and positive control captures. All the targets were hybridized to respective captures at room ambient temperature following regular assay protocol to check the capture presence on the modified slide. Wells-1,3,4,5 are showing all the genomic captures and well 2 is showing positive control capture.

Figure 50. shows the plastic substrate with 4,4'-dicyclohexylmethane diisocyanate linker on the surface (10e). Substrate 10e is arrayed with three columns and four rows in each of the five wells. Each of the three spots in any row has the same capture arrayed on the slide. The type of capture by row is mutant - I, wild type I, mutant - II, wild type - II, and positive control capture DNA sequences from top to bottom. Wells 1 and 2 were used for positive controls and 3, 4 and 5 were used for checking to differentiate genomic wild type target from mutant. In wells 3, 4 and 5 both positive control probe and gene specific probe were used to see the signal alignment.

Figure 51. shows the plastic substrate with 4,4'-dicyclohexylmethane diisocyanate linker on the surface (10e). The sample on the top was blocked with glycine after being arrayed with DNA captures, while the substrate on the bottom was not modified after arraying. Both samples tested with genomic DNA mutant and wild type. Each was arrayed and assayed and developed the same. The substrate (bottom) that was not block visually shows higher background and is approximately 2.5 to 3 times higher in signal response. Substrate 10e is arrayed with three columns and four rows in each of the five wells. Each of the three spots in any row has the same capture arrayed on the slide. The type of capture by row is wild type -1, wild type -1, mutant -1, wild type – II, mutant –II and positive control captures.

Figure 52. shows several different surfaces prepared on plastic. Surfaces contain 3,3’-diaminobenzidene (DAB) linker with, 1,6-hexamethylene diisocyanate (HMDI), 4,4'-methyleneedianiline diisocyanate (MDI), or triethyl citrate (T) linker on the surface. Substrates were arrayed with wild type (WT), mutant (MT), and positive control (PC) captures. Background (BGR) wells were developed without probes or DNA, and water control (WC), wells were exposed to water. Each slide was developed using wild type or mutant DNA targets at 1.9 nM (Signal 100), 190 pM (Signal 10) and 19 pM (Signal 1).
and the appropriate nanoparticle probes. After silver enhancement all slides were scanned on the Verigene® detector system. The signal response was plotted and compared across different modified substrates.

Figure 53. shows several different surfaces prepared on glass. Surfaces labeled “HMDI” are from the 10m preparation, “MDI” from the 10n preparation, “T” from the 10o preparation, “D-t” from the 10p preparation and “D” from the 10a preparation. Iterations are from different samples. Substrates were arrayed with wild type (WT), mutant (MT), and positive control (PC) captures. Background (BGR) wells were developed without nanoparticle probes or target DNA. Water control (WC) wells were exposed to water. Each slide was developed using wild type or mutant DNA targets at 1.9 nM (Signal 100), 190 pM (Signal 10) and 19 pM (Signal 1) and the appropriate nanoparticle probes. After silver enhancement all slides were scanned on the Verigene® detector system. The signal response was plotted and compared across different modified substrates.

Figure 54. shows the mean signal intensities when comparing glass and plastic substrates and also comparing different spacer groups on plastic. Slides were developed with positive, negative, mutant, and wild type DNA captures and developed utilizing nanoparticle probes as previously described herein. PoXyl was completed induplicate.

Figure 55. shows that glass (10h) and plastic (10m) substrates were arrayed uniformly with Cy3 capture probes.

Figure 56. shows a one step hybridization of a Cy5 probe to glass (10m) and plastic (10h) substrates with increasing probe concentrations. As the probe concentration increases so does the fluorescence response from the detector.
DESCRIPTION OF THE INVENTION

All patents, patent applications, and references cited herein are incorporated by reference in their entirety.

As defined herein, the term "molecule" refers to any desired substance, such as a desired specific binding member, that may be immobilized onto the surface of the substrate. The "specific binding member," as defined herein, means either member of a cognate binding pair. A "cognate binding pair," as defined herein, is any ligand-receptor combination that will specifically bind to one another, generally through non-covalent interactions such as ionic attractions, hydrogen bonding, Vanderwaals forces, hydrophobic interactions and the like. Exemplary cognate pairs and interactions are well known in the art and include, by way of example and not limitation: immunological interactions between an antibody or Fab fragment and its antigen, hapten or epitope; biochemical interactions between a protein (e.g. hormone or enzyme) and its receptor (for example, avidin or streptavidin and biotin), or between a carbohydrate and a lectin; chemical interactions, such as between a metal and a chelating agent; and nucleic acid base pairing between complementary nucleic acid strands; a peptide nucleic acid analog which forms a cognate binding pair with nucleic acids or other PNAs. Thus, a molecule may be a specific binding member selected from the group consisting of antigen and antibody-specific binding pairs, biotin and avidin binding pairs, carbohydrate and lectin bind pairs, complementary nucleotide sequences, complementary peptide sequences, effector and receptor molecules, enzyme cofactor and enzymes, and enzyme inhibitors and enzymes. Other specific binding members include, without limitation, DNA, RNA, polypeptide, antibody, antigen, carbohydrate, protein, peptide, amino acid, carbohydrate, hormone, steroid, vitamin, drug, virus, polysaccharides, lipids, lipopolysaccharides, glycoproteins, lipoproteins, nucleoproteins, oligonucleotides, antibodies, immunoglobulins, albumin, hemoglobin, coagulation factors, peptide and protein hormones, non-peptide hormones, interleukins, interferons, cytokines, peptides comprising a tumor-specific epitope, cells, cell-surface molecules, microorganisms, fragments, portions, components or products of microorganisms, small organic molecules, nucleic acids and oligonucleotides, metabolites of or antibodies to any of the above substances. Nucleic acids and oligonucleotides comprise genes, viral RNA and DNA, bacterial DNA, fungal DNA, mammalian DNA, cDNA, mRNA, RNA and DNA fragments, oligonucleotides, synthetic oligonucleotides, modified oligonucleotides,
single-stranded and double-stranded nucleic acids, natural and synthetic nucleic acids, and aptamers. Preparation of antibody and oligonucleotide specific binding members is well known in the art. Molecules may be immobilized onto substrates and serve as capture probes for target analytes. Molecules may also include a detection label such as a fluorophore or nanoparticle. The molecules (M) have at least one or more nucleophilic groups, e.g., amino, carboxylate, or hydroxyl, that are capable of linking or reacting with the silylating agents to form a reactive silylated molecule which is useful for modifying the surfaces of substrates. These nucleophilic groups are also capable of reacting with reactive moieties such as isocyanate groups and crosslinker molecules on the surfaces of substrates. These nucleophilic groups are either already on the molecules or are introduced by known chemical procedures.

The terms "label" or “detection label” refers to a detectable marker that may be detected by photonic, electronic, opto-electronic, magnetic, gravity, acoustic, enzymatic, or other physical or chemical means.

The phrase “spacer molecule” refers can be any substance having a molecular structure that provides a plurality of functional groups. In one embodiment, the spacer molecule includes at least one first functional group that can react with free isocyanate groups previously attached to the surface; at least one second functional group for attachment to a cross-linker molecule which can then be subsequently attached to another spacer molecule or to a capture probe; and at least one optional third functional groups for providing a negative charge. Both the first and second functional groups are any suitable nucleophilic group that can react with a reactive functional group such as isocyanate. Examples of nucleophilic groups include –OH, –SH, –NH₂, and –NH₃⁺. The optional third function group includes a carboxylate group. Preferably, the first and second functional groups are free amino groups. Spacer molecules may relate to substances such polymers, carbohydrates, antibiotics having a plurality of one or more types of nucleophilic groups, e.g., amino, carboxylate, or hydroxyl groups that are capable of linking or reacting with reactive moieties such as isocyanate groups on the surface of substrates or with linker molecules.

Spacer molecules may include many different types of polymers; preferably those incorporating multiple functional groups. Representative examples of these types of polymers include, without limitation, poly (dimmer acid-co-alkylpolyamine)-95, poly(dimmer acid-co-alkylpolyamine)-140, poly(allylamine), and poly(m-xylylendiamine-
epichlorohydrin diamine terminated, and PAMAM dendrimer generation 0. Types of
polymers also include, without limitation, carbohydrates and polysaccharides. A
representative example includes neomycin. Other spacer molecules include low
molecular weight compounds that provide the designated functionality; preferred
examples include 3,3'-diaminobenzidine, and tris(2-aminoethylamine). The term
"capping reagent" refers to a substance that deactivates reactive moieties that may be
present on regions of a substrate surface after printing or attachment of capture probes
onto the substrate surface. Just prior to capture probe attachment, a surface having free
amino groups may be treated with a bifunctional crosslinker molecule of which one
functional group reacts with the free amino groups. Another functional group of the
crosslinker molecule is available to bind directly or indirectly to the capture probe. The
presence of any remaining reactive functional groups after capture probe attachment is
generally undesirable as these moieties may react and bind target molecules or detection
labels and produce substantial background noise. Thus, any remaining unreacted reactive
moieties are generally deactivated prior to use of the substrate for target detection.
Examples of capping reagents include amino acid, protein, carbohydrate, carboxylate,
thiol, alcohol, and amine. A representative, but non-limiting, example includes glycine

The term "crosslinker molecules," "linker molecules," or "linker compound"
refers to a molecule that serves as a bridge or link between different substances or a
substance and a surface of a substrate. In one embodiment, the linker molecule is capable
of forming at least two covalent bonds such as a bond between a spacer molecule and a
free hydroxyl, amino, or carboxylate group on a substrate. Non-limiting examples of
linker molecules include ethylene glycolbis (succinimidylsuccinate), disuccinimidyl
suberate, 1,6-diisocyanatohexane, methylene bis-(4-cyclohexylisocyanate, glutaric
dialdehyde, methylene-p-phenyl diisocyanate, and triethyl citrate.

As defined herein, the term "substrate" refers any solid support suitable for
immobilizing oligonucleotides and other molecules are known in the art. These include
nylon, nitrocellulose, activated agarose, diazotized cellulose, latex particles, plastic,
polystyrene, glass and polymer coated surfaces. These solid supports are used in many
formats such as membranes, microtiter plates, beads, probes, dipsticks, optical fibers, etc.
Of particular interest as background to the present invention is the use of glass and nylon
surfaces in the preparation of DNA microarrays which have been described in recent
years (Ramsay, Nat. Biotechnol., 16: 40-4 (1998)). The journal Nature Genetics has
published a special supplement describing the utility and limitations of microarrays (Nat.Genet., 21(1): 1-60 (1999). Also of interest are optical substrates such as the ones described in U.S. Patent No. 6,807,352, which is incorporated by reference in its entirety. Typically the use of any solid support requires the presence of a nucleophilic group to react with the silylated molecules of the invention that contain a "reactive group" capable of reacting with the nucleophilic group. Suitable nucleophilic groups or moieties include hydroxyl, sulphydryl, and amino groups or any moiety that is capable of coupling with the silylated molecules of the invention. Chemical procedures to introduce the nucleophilic or the reactive groups onto solid support are known in the art, they include procedures to activate nylon (U.S. Pat. No. 5,514,785), glass (Rodgers et al., Anal. Biochem., 23-30 (1999)), agarose (Highsmith et al., J., Biotechniques 12: 418-23 (1992) and polystyrene (Gosh et al., Nuc. Acid Res., 15: 5353-5372 (1987)). The preferred substrate is glass.

The substrates may have surfaces that are porous or non-porous. As defined herein, the term "porous" means surface means that the surface permits diffusion to occur. The term "non-porous" surface means that the surface does not permit diffusion to occur.

The term "analyte," or "target analyte", as used herein, is the substance to be quantitated or detected in the test sample using substrates or devices prepared by the method of the present invention. The analyte can be any substance for which there exists a naturally occurring specific binding member (e.g., an antibody, polypeptide, DNA, RNA, cell, virus, etc.) or for which a specific binding member can be prepared, and the analyte can bind to one or more specific binding members in an assay.

In one embodiment of the invention, a method is provided for immobilizing a molecule onto a substrate surface, said method comprising the steps of contacting the molecule with an agent so as to form a reactive intermediate, said agent having a formula i:

\[(R_1)(R_2)(R_3)\text{Si-X-NCY} \quad i\]

wherein \(R_1, R_2\) and \(R_3\) independently represents \(C_1-C_8\) alkoxy, \(C_1-C_8\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkyl and \(C_1-C_6\) alkoxy; \(X\) represents linear or branched \(C_1-C_{20}\) alkyl or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkyl and \(C_1-C_6\) alkoxy.
alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; and Y represents oxygen or sulfur, with the proviso that at least one of R_1, R_2 or R_3 represents C_1-C_6 alkoxy; and contacting the reactive intermediate with said surface so as to immobilized the molecule onto said surface.

In practice, the molecule is contacted with the agent in solution. Generally, the molecule is dissolved in a solution and agent is added drop-wise to the molecule solution. Suitable, but non-limiting, examples of solvents used in preparing the solution include DMF, DMSO, ethanol and solvent mixtures such as DMSO/ethanol. The preferred solvent is ethanol. Water is preferably excluded from the reaction solvent because water may interfere with the efficient modification of the molecule. However, if water is necessary to increase solubility of the molecule in the solution, the amount of water generally ranges from about 0.1% to about 1%, usually no greater than 1%.

The amount of molecule to agent generally ranges from about 1 to about 1.5 typically from about 1 to about 1.1, preferably from about 1 to about 1 molar equivalents. The reaction may be performed in any suitable temperature. Generally, the temperature ranges between about 0 °C and about 40 °C, preferably from about 20 °C to about 25 °C. The reaction is stirred for a period of time until sufficient amount of molecule and agent reacts to form a reactive intermediate. The reactive intermediate has a structure defined by formula iii.

Thereafter, the reaction solution containing the reactive intermediate is then concentrated and dissolved in desired solvent to provide a spotting solution which is then applied to the surface of a substrate. The reactive intermediate is applied as a spotting solution. Any suitable solvent may be used to prepare the spotting solution. Suitable, but non-limiting, examples of solvents used in preparing the spotting solution include DMF, DMSO, and ethanol as well as any suitable solvent mixtures such as DMF/pyridine. Any suitable concentration of the spotting solution may be prepared, generally the concentration of the spotting solution is about 1 mM. Any suitable spotting technique may be used to produce spots. Representative techniques include, without limitation, manual spotting, ink-jet technology such as the ones described in U.S. Patent nos. 5,233,369 and 5,486,855; array pins or capillary tubes such as the ones described in U.S. patent nos. 5,567,294 and 5,527,673; microspotting robots (e.g., available from Cartesian); chipmaker micro-spotting device (e.g., as available from TeleChem Interational). Suitable spotting equipment and protocols are commercially available such
as the ArrayIt® chipmaker 3 spotting device. The spotting technique can be used to produce single spots or a plurality of spots in any suitable discrete pattern or array.

In the preferred embodiment, the agent is triethoxysilylisocyanate. The preferred molecule is a nucleic acid.

In another embodiment of the invention, a method is provided for immobilizing a molecule onto a substrate surface, said method comprising the steps of contacting Si(NCY)₄ with an agent so as to form a first reactive intermediate, said agent having a formula ii:

\[(R₁)(R₂)(R₃)Si-X-Z\]  

wherein R₁, R₂ and R₃ independently represents C₁-C₅ alkoxy, C₁-C₅ alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of C₁-C₅ alkyl and C₁-C₅ alkoxy; X represents linear or branched C₁-C₂₀ alkyl or aryl substituted with one or more groups selected from the group consisting of C₁-C₅ alkyl and C₁-C₅ alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; wherein Y represents oxygen or sulfur; and Z represents a hydroxy or amino group, with the proviso that at least one of R₁, R₂ or R₃ represents C₁-C₅ alkoxy; contacting the first reactive intermediate with a molecule so as to form a second reactive intermediate; and contacting the second reactive intermediate with said surface so as to immobilized the molecule onto said surface.

In this embodiment of the invention, the method provide for a modification of substrate surfaces with branched molecules so as to increase molecule loading on the substrate surface. These branched molecules behave like dendrimers to enhance sensitivity in assay performance. In practice, either Si(NCO)₄ or Si(NCS)₄ are reacted with a compound of formula ii to form a first reactive intermediate having the formula iv:

\[(R₁)(R₂)(R₃)Si-X-Z-CYNH-Si(NCY)₃\]  

wherein R₁, R₂ and R₃ independently represents C₁-C₅ alkoxy, C₁-C₅ alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of C₁-C₅ alkyl and C₁-C₅ alkoxy; X represents linear or branched C₁-C₂₀ alkyl or aryl substituted with one or more groups selected from the group consisting of C₁-C₅ alkyl and C₁-C₅
alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; Y represents oxygen or sulfur; and Z represents oxygen or NH, with the proviso that at least one of R₁, R₂, or R₃ represents C₁-C₆ alkoxy.

Generally, Si(NCO)₄ or Si(NCS)₄ is dissolved in a suitable dry solvent as described above. In practice, ethanol is the preferred solvent. The resulting ethanol solution is contained in a reaction flask and a solution of formula ii compound is added to the reaction flask. The formula ii solution may include any of the dried solvents described above. In practice, ethanol is the preferred solvent. The reaction temperature generally ranges from about 0 °C to about 40 °C, preferably about 22 °C. The reaction mixture is allowed to stir from about 1 min to about 60 min, usually about 5 min to about 10 min, until it reaches completion. The molar amount of Si(NCO)₄ or Si(NCS)₄ to formula ii compound generally ranges from about 3:1 to 1:1, preferably about 1:1.

Thereafter, the molecule is contacted with the first reactive intermediate to form a second reactive intermediate having the formula v:

\[(R₁)(R₂)(R₃)\text{Si-X-Z-CYNH-Si(NHCYCL-M)}₃ \quad v\]

wherein R₁, R₂ and R₃ independently represent C₁-C₆ alkoxy, C₁-C₆ alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of C₁-C₆ alkyl and C₁-C₆ alkoxy; X represents linear or branched C₁-C₂₀ alkyl or aryl substituted with one or more groups selected from the group consisting of C₁-C₆ alkyl and C₁-C₆ alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; L represents a linking group; Y represents oxygen or sulfur; and Z represents oxygen or NH; and M represents a molecule, with the proviso that at least one of R₁, R₂, or R₃ represent C₁-C₆ alkoxy. The linking group L may be a nucleophile that is naturally present or chemically added to the molecule such as an amino, sulhydryl group, hydroxy group, carboxylate group, or any suitable moiety. L may represent -NH₂, -S-, -O-, or -OOCC-. The molecule is contacted with the first reactive intermediate in solution. Generally, the molecule is dissolved in a solvent and added dropwise to the reaction flask containing the first reactive intermediate. The molecule is generally mixed in any suitable solvent as described above. The molar amount of molecule to first reactive intermediate generally ranges from about 1 to about 10 typically from about 1 to about 3,
preferably from about 1 to about 4. The reaction may be performed in any suitable temperature. Generally, the temperature ranges between about 0 °C and about 40 °C, preferably from about 20 °C to about 25 °C. The reaction is stirred for a period of time until sufficient amount of molecule and first reactive intermediate reacts to form a second reactive intermediate. Generally, an excess amount of molecule is used to react with the first reactive intermediate. In practice, typically at least 3 equivalents of molecule to 1 equivalent of first reactive intermediate is used.

Thereafter, the second reactive intermediate is then applied to the surface of a substrate using techniques described above.

In another aspect of this invention, if the ratio of Si(NCO)₄ or Si(NCS)₄ to formula ii compound is about 1:2 equiv./equiv., a first reactive intermediate is formed having the formula vi:

$$((R_1)(R_2)(R_3)Si-X-Z-CYNH)_2Si(NCY)_2$$  \[vi\]

wherein R₁, R₂ and R₃ independently represents C₁-C₆ alkoxy, C₁-C₆ alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of C₁-C₆ alkyl and C₁-C₆ alkoxy; X represents linear or branched C₁-C₂₀ alkyl or aryl substituted with one or more groups selected from the group consisting of C₁-C₆ alkyl and C₁-C₆ alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; Y represents oxygen or sulfur; and Z represents oxygen or NH, with the proviso that at least one of R₁, R₂, or R₃ represents C₁-C₆ alkoxy. Preferably, R₁, R₂ and R₃ represent methoxy, X represents phenyl, Y represents oxygen, and Z represents NH.

Thereafter, the molecule is contacted with the first reactive intermediate of formula vi as described above to produce a second reactive intermediate having the formula vii:

$$((R_1)(R_2)(R_3)Si-X-Z-CYNH)_2Si(NHCYL-M)_2$$  \[vii\]

wherein R₁, R₂ and R₃ independently represents C₁-C₆ alkoxy, C₁-C₆ alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of C₁-C₆ alkyl and C₁-C₆ alkoxy; L represents a linking group; X represents linear or
branched C₁⁻C₂₀ alkyl or aryl substituted with one or more groups selected from the group consisting of C₁⁻C₆ alkyl and C₁⁻C₆ alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; Y represents oxygen or sulfur; and Z represents oxygen or NH; and M represents a molecule, with the proviso that at least one of R₁, R₂, or R₃ represent C₁⁻C₆ alkoxy. The linking group L may be a nucleophile that is naturally present or chemically added to the molecule such as an amino, sulphydryl group, hydroxy group, carboxylate group, or any suitable moiety. L may represent –NH, –S–, –O–, or –OOC–. Generally, an excess amount of molecule is used to react with the first reactive intermediate. In practice, typically at least 3 equivalents of molecule to 1 equivalent of first reactive intermediate is used.

Therefore, the second reactive intermediate is then applied to the surface of a substrate using the techniques described above.

In another embodiment of the invention, a compound is provided having the formula iii:

\[(R₁)(R₂)(R₃)Si-X-NHCYL-M\]  

wherein R₁, R₂ and R₃ independently represents C₁⁻C₆ alkoxy, C₁⁻C₆ alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of C₁⁻C₆ alkyl and C₁⁻C₆ alkoxy; L represents a linking group; X represents linear or branched C₁⁻C₂₀ alkyl or aryl substituted with one or more groups selected from the group consisting of C₁⁻C₆ alkyl and C₁⁻C₆ alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; Y represents oxygen or sulfur; and M represents a molecule, with the proviso that at least one of R₁, R₂, or R₃ represent C₁⁻C₆ alkoxy. The linking group L may be a nucleophile that is naturally present or chemically added to the molecule such as an amino, sulphydryl group, hydroxy group, carboxylate group, or any suitable moiety. L may represent –NH, –S–, –O–, or –OOC–. In the preferred embodiment, R₁, R₂, and R₃ represent alkoxy, L represents –NH, X represents propyl, and Y represents O. The compound is useful for modifying substrate surfaces with a desired molecule.

In another embodiment of the invention, a compound is provided having a formula iv:
(R_1)(R_2)(R_3)Si-X-Z-CYNH-Si (NCY)_3

wherein R_1, R_2 and R_3 independently represents C_1-C_6 alkoxy, C_1-C_6 alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of C_1-C_6 alkyl and C_1-C_6 alkoxy; X represents linear or branched C_1-C_20 alkyl or aryl substituted with one or more groups selected from the group consisting of C_1-C_6 alkyl and C_1-C_6 alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; Y represents oxygen or sulfur; and Z represents oxygen or NH, with the proviso that at least one of R_1, R_2, or R_3 represents C_1-C_6 alkoxy. In the preferred embodiment, R_1, R_2, and R_3 represent ethoxy or methoxy, X represents benzyl, Y represents oxygen, and Z represents NH. The compound is useful for modifying molecules so that they can be attached to substrate surfaces.

In another embodiment of the invention, a compound is provided having a formula v:

(R_1)(R_2)(R_3)Si-X-Z-CYNH-Si (NHCYL-M)_3

wherein R_1, R_2 and R_3 independently represents C_1-C_6 alkoxy, C_1-C_6 alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of C_1-C_6 alkyl and C_1-C_6 alkoxy; L represents a linking group; X represents linear or branched C_1-C_20 alkyl or aryl substituted with one or more groups selected from the group consisting of C_1-C_6 alkyl and C_1-C_6 alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; Y represents oxygen or sulfur; and Z represents oxygen or NH; and M represents a molecule, with the proviso that at least one of R_1, R_2, or R_3 represents C_1-C_6 alkoxy. The linking group L may be a nucleophile that is naturally present or chemically added to the molecule such as an amino, sulfhydryl group, hydroxy group, carboxylate group, or any suitable moiety. L may represent -NH, -S-, -O-, or -OOC-. In the preferred embodiment, R_1, R_2, and R_3 represent methoxy or ethoxy, X represents 3- or 4- phenyl, Y represents oxygen, and Z represents NH. The compound is useful for modifying molecules so that they can be attached to substrate surfaces.

In another embodiment of the invention, a device is provided for the detection of target analytes in a sample. The device comprises a surface having an immobilized molecule as a specific binding member to the target analyte, wherein said surface is
prepared by any of the above methods. The preferred surface is a glass surface. The surface may have one or more different specific binding members attached thereto in an array to allow for the detection of different portions of a target analyte or multiple different types of target analytes.

In another embodiment of the invention, a kit is provided. The kit may comprise one or more containers containing any of the silylating agents mentioned above with an optional substrate, and a set of instructions.

The present invention is also directed to derivatizing substrate surfaces (e.g., glass, plastic, metal) have a variety of surface properties (i.e., porous and nonporous, impermeable and nonimpermeable) for use in nanoparticle-based detection of a target analyte. In particular, the substrate will typically have a surface comprising bound free hydroxyl, amino, or carboxylate groups, or a combination thereof. The substrate surface is then contacted with a disilyl compound having both silyl alkoxy and silyl isocyanate groups. Contacting the disilyl compound is such that the silyl alkoxy group preferentially contacts to the surface of the substrate so as to provide an anchor molecule having free isocyanate groups. The subsequent attachment of spacer molecules is made possible by reacting to the free isocyanate groups.

The substrate surface anchor group providing the free isocyanate groups can then be reacted with a spacer molecule; addition of a suitable spacer molecule provides free amino groups for attachment of an additional spacer molecule while also providing the desired surface characteristics. Suitable spacer molecules can be comprised of multiple free amino groups; some free amino groups react with the free isocyanate group provided by the anchor molecule whereas the other free amino groups provide a surface for attachment of an additional spacer molecule. A preferred spacer of this type is 3,3'-diaminobenzidine. Other suitable spacer molecules can generally be comprised of multifunctional groups, in particular, amino groups as well as functional groups providing a negatively charged moiety. Examples of suitable spacer molecules are polymers; examples of polymers include, but are not limited to, poly(dimmer acid-co-alkylyphamine)-95, poly(dimmer acid-co-alkylyphamine)-140, poly(allylamine), poly(m-xylendiamine-epichlorohydrin diamine terminated, tris(2-aminoethylamine), and PAMAM dendrimer generation 0. A preferred polymer is one which is also comprised of negatively charged functional groups, an example of which is a carboxylic acid. An example of a preferred polymer includes poly (dimmer acid-co-alkylpolyamine)-95 and
poly(dimer acicd-co-alkylpolyamine)-140. Other suitable spacers include carbohydrate polymers, and antibiotics such as neomycin.

Alternately, the anchor group providing free isocyanate groups can optionally be reacted with water without attaching a spacer molecule; the reaction with water is such that the free isocyanate groups undergo hydrolysis to produce free amino groups. Attachment of spacer molecules can then be carried out as described.

Linker molecules comprise functional groups capable of reacting with the free amino groups provided on the surface, and can be attached in any step where free amino groups are provided. According to the present invention, the linker molecules provide suitable functional groups (e.g. carboxylic acids, ester groups, and isocyanates) for attaching capture probes (i.e during arraying) that are specific for a target analyte. Preferred linker molecules include ethylene glycolbis (succinimidylsuccinate), disuccinimidyl suberate, 1,6-diisocyanatohexane, methylene bis-(4-cyclohexylisocyanate), glutaric dialdehyde, methylene-p-phenyl diisocyanate, and triethyl citrate.

A linker group can be attached directly to the hydrolyzed anchor group. Preferably, additional spacer molecules are attached to provide a substrate surface having the desired surface characteristics. Additional spacer molecules can be attached to any surface comprised of free amino groups by first reacting a diisocyanate compound to the free amino group surface to provide free isocyanate groups. Preferred diisocyanate compounds include phenylene 1,4-diisocyanate, tolylene-2,6-diisocyanate, tolylene-α,4-diisocyanate, and isophorone diisocyanate. The additional spacer molecule can then be attached via the reaction between the free isocyanate group on the surface and free amino group of the spacer molecule; attachment of any number of spacer molecules that result in an amenable surface according to this invention is contemplated, however, the preferred number of spacer molecules is between 2 and 7.

After the linker molecule is attached to the surface, the substrate is arrayed in discrete predetermined areas on the surface to attach a capture probe. More than one type of capture probes can be contacted with the surface; each type of capture probes is specific for a particular target analyte. A preferred capture probe is a nucleic acid.

A principle advantage of the method of the invention is that many types of amine-linked compounds can be coupled in a three-dimensional way to the polymer layer, thus maximizing availability for hybridizing target DNA and RNA biomolecules on the
surface for detection purposes. The immobilizing amine molecules do not directly contact the substrate surface but rather contacting the polymer coated on the surface of the substrate. The copolymer layer on the glass surface contains both amine and acid groups with amide bond linkage throughout the molecular chain. Without wishing to be bound by any particular theory, it is believed that the copolymer coating of the invention provides good results because the acid groups in the chain contribute to controlling the nonspecific binding of gold nanoparticle probes to the surface which in turn leads to minimization of background noise. Example 1 provides a general protocol for preparing polymer-coated substrates.

Any substrate can be used in the invention. Suitable substrates include transparent solid surfaces (e.g., glass, quartz, plastics and other polymers), opaque solid surface, and conducting solid surfaces. The substrate can be any shape or thickness, but generally will be flat and thin. Preferred are glass substrates, such as glass slides.

In one embodiment of the invention, a method is provided for making a substrate for use in target analyte detection. The method comprises: (a) providing a substrate having a surface; (b) contacting said surface with a isocyanate compound so as to provide a surface comprising free isocyanate groups, the isocyanate compound is a member selected from the group consisting of:

$$\text{Si(NCY)}_4;$$

$$\text{(R_1)(R_2)(R_3)Si-X-NCY i;}$$

$$\text{[(R_1)(R_2)(R_3)Si-X-Z-CYNH]}_2\text{-Si (NCY)_2 vi;}$$

$$\text{(R_1)(R_2)(R_3)Si-X-Z-CYNH-Si (NCY)_3 iv;}$$

wherein $R_1$, $R_2$ and $R_3$ independently represents $C_1-C_6$ alkoxy, $C_1-C_6$ alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of $C_1-C_6$ alkyl and $C_1-C_6$ alkoxy; $X$ represents linear or branched $C_1-C_{20}$ alkyl or aryl substituted with one or more groups selected from the group consisting of $C_1-C_6$ alkyl and $C_1-C_6$ alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; $Y$ represents oxygen or sulfur; and $Z$ represents oxygen or NH, with the proviso that at least one of $R_1$, $R_2$, or $R_3$ represents $C_1-C_6$ alkoxy. In another embodiment of the invention, a method is provided for making a substrate for use in target analyte detection. The method comprises: (a) providing a substrate having a surface; (b) contacting said surface with a isocyanate compound so as to provide a surface.
comprising free isocyanate groups, the isocyanate compound is a member selected from the group consisting of:

\[
\text{Si(NCY)}_4; \\
(R_1)(R_2)(R_3)\text{Si-X-NCY} \quad \text{i;}
\]

\[
[(R_1)(R_2)(R_3)\text{Si-X-Z-CYNH}_2\text{-NCY}]_2 - \text{Si (NCY)}_2 \quad \text{vi; and}
\]

\[
(R_1)(R_2)(R_3)\text{Si-X-Z-CYNH-Si (NCY)}_3 \quad \text{iv;}
\]

wherein \( R_1, R_2 \) and \( R_3 \) independently represents \( C_1-C_6 \) alkoxy, \( C_1-C_6 \) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \( C_1-C_6 \) alkoxy and \( C_1-C_6 \) alkoxy; \( X \) represents linear or branched \( C_1-C_{20} \) alkyl or aryl substituted with one or more groups selected from the group consisting of \( C_1-C_6 \) alkyl and \( C_1-C_6 \) alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; \( Y \) represents oxygen or sulfur; and \( Z \) represents oxygen or \( \text{NH} \), with the proviso that at least one of \( R_1, R_2, \) or \( R_3 \) represents \( C_1-C_6 \) alkoxy; (c) contacting said surface comprising free isocyanate groups with a spacer molecule so as to provide a surface comprising free amino groups; and (d) contacting said surface comprising free amino groups with a linker molecule so as to provide a reactive surface having free reactive groups.

In one aspect of this embodiment, steps (c) and (d) may be repeated one or more times.

In another aspect of this embodiment, the method comprises after step (d):

(e) contacting said reactive surface with at least one type of capture probe specific for the target analyte so as to provide a surface comprising immobilized capture probes; and (f) contacting said surface comprising immobilized capture probes with a capping agent so as to block residual unreacted free isocyanate groups on areas of the surface not having immobilized capture probes and produce a substrate having substantially low signal background due to non-specific nanoparticle binding relative to a surface not contacted with a capping agent.

In another aspect of this embodiment of the invention, the method further comprises: (i) contacting said reactive surface with at least one type of capture probe specific for the target analyte so as to provide a surface comprising immobilized capture probes; and (ii) contacting said surface comprising immobilized capture probes with a capping agent so as to block residual unreacted free isocyanate groups on areas of the surface not having immobilized capture probes and produce a substrate having
substantially low signal background due to non-specific nanoparticle binding relative to a surface not contacted with a capping agent.

In another embodiment of the invention, a method is provided for making a substrate for use in target analyte detection. The method comprises: (a) providing a substrate having a surface; (b) contacting said surface with a isocyanate compound so as to provide a surface comprising free isocyanate groups, the isocyanate compound is a member selected from the group consisting of:

\[ \text{Si(NCY)}_4; \]
\[ (R_1)(R_2)(R_3)\text{Si-X-NCY} \]
\[ [(R_1)(R_2)(R_3)\text{Si-X-Z-CYNH}]_2\text{Si (NCY)}_2 \]
\[ (R_1)(R_2)(R_3)\text{Si-X-Z-CYNH-Si (NCY)}_3 \]

wherein R1, R2 and R3 independently represents C1-C6 alkoxy, C1-C6 alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of C1-C6 alkyl and C1-C6 alkoxy; X represents linear or branched C1-C20 alkyl or aryl substituted with one or more groups selected from the group consisting of C1-C6 alkyl and C1-C6 alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; Y represents oxygen or sulfur; and Z represents oxygen or NH, with the proviso that at least one of R1, R2, or R3 represents C1-C6 alkoxy; (c) contacting the surface comprising free isocyanate groups with water so as to provide a surface comprising free amino groups; and (d) contacting said surface comprising free amino groups with a linker molecule so as to provide a reactive surface having free reactive groups.

In one aspect of this embodiment of the invention, the method further comprising, after step (d): (e) contacting said surface comprising free isocyanate groups with a spacer molecule so as to provide a surface comprising free amino groups; and (f) contacting said surface comprising free amino groups with a linker molecule so as to provide a reactive surface having free reactive groups.

In one aspect of this invention, steps (e) and (f) may be repeated one or more times.

In another aspect of this embodiment of the invention, the method further comprises: (i) contacting said reactive surface with at least one type of capture probe specific for the target analyte so as to provide a surface comprising immobilized capture probes; and (ii) contacting said surface comprising immobilized capture probes
with a capping agent so as to block residual unreacted free isocyanate groups on areas of the surface not having immobilized capture probes and produce a substrate having substantially low signal background due to non-specific nanoparticle binding relative to a surface not contacted with a capping agent.

In yet another embodiment of the invention, a method for making a substrate for use in detection of a target analyte is provided. The method comprises: (a) providing a substrate having a surface; (b) contacting said surface with a isocyanate compound so as to provide a surface comprising free isocyanate groups, the isocyanate compound is a member selected from the group consisting of:

\[
\text{Si(NCY)}_4; \\
(R_1)(R_2)(R_3)\text{Si-X-NCY} \quad \text{i;}
\]

\[
[(R_1)(R_2)(R_3)\text{Si-X-Z-CYNH}]_2\text{Si (NCY)}_2 \quad \text{vi; and}
\]

\[
(R_1)(R_2)(R_3)\text{Si-X-Z-CYNH-Si (NCY)}_3 \quad \text{iv;}
\]

wherein \(R_1, R_2, \) and \(R_3\) independently represents \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkyl and \(C_1-C_6\) alkoxy; \(X\) represents linear or branched \(C_1-C_{20}\) alkyl or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkyl and \(C_1-C_6\) alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; \(Y\) represents oxygen or sulfur; and \(Z\) represents oxygen or NH, with the proviso that at least one of \(R_1, R_2, \) or \(R_3\) represents \(C_1-C_6\) alkoxy; (c) contacting said surface comprising free isocyanate groups with a spacer molecule so as to provide a surface comprising free amino groups; (d) contacting said surface comprising free amino groups with a linker molecule so as to provide a reactive surface having free reactive groups; (e) contacting said reactive surface with at least one type of capture probe specific for the target analyte so as to provide a surface comprising immobilized capture probes; and (f) contacting said surface comprising immobilized capture probes with a capping agent so as to block residual unreactive free isocyanate groups and produce a substrate having substantially low signal background due to non-specific nanoparticle binding relative to a surface not contacted with a capping agent.

In any of the above methods for making a substrate, the isocyanate compound may be is selected from the group consisting of 2-Trimethoxysilane-6-triisocyanatosilanceneureabenzene, 3-(triethoxysilyl) propylisocyanate, and tetraisocyanatosilane.
The spacer molecule can be any substance having a molecular structure that provides a plurality of functional groups. In one embodiment, the spacer molecule includes at least one first functional group that can react with free isocyanate groups previously attached to the surface; at least one second functional group for attachment to a cross-linker molecule which can then be subsequently attached to another spacer molecule or to a capture probe; and at least one optional third functional groups for providing a negative charge. Both the first and second functional groups are any suitable nucleophilic group that can react with a reactive functional group such as isocyanate. Examples of nucleophilic groups include –OH, –SH, –NH2, and –NH2. The optional third function group includes a carboxylate group. Preferably, the first and second functional groups are free amino groups.

Spacer molecules may include many different types of polymers; preferably those incorporating multiple functional groups. Representative examples of these types of polymers include, without limitation, poly (dimmer acid-co-alkylpolyamine)-95, poly(dimmer acid-co-alkylpolyamine)-140, poly(allylamine), and poly(m-xylendiamine-epichlorohydrin diamine terminated, and PAMAM dendrimer generation 0. Types of polymers also include, without limitation, carbohydrates and polysaccharides. A representative example includes neomycin. Other spacer molecules include low molecular weight compounds that provide the designated functionality; preferred examples include 3,3'-diaminobenzidine, and tris(2-aminoethylamine).

Any suitable capping reagent that deactivates reactive moieties may be used. Examples of capping reagents include amino acid, protein, carbohydrate, carboxylate, thiol, alcohol, and amine. A representative, but non-limiting, example includes glycine.

Representative, but non-limiting examples of isocyanate compound include phenylene 1,4-diisocyanate, tolylene-2,6-diisocyanate, tolylene-α,4-diisocyanate, and isophorone diisocyanate.

Non-limiting examples of linker molecules include ethylene glycolbis (succinimidylsuccinate), disuccinimidyl suberate, 1,6-diisocyanatohexane, methylene bis-(4-cyclohexylisocyanate, glutaric dialdehyde, methylene-p-phenyl diisocyanate, and triethyl citrate.

Any suitable substrate may be used in the above methods. Preferably, the substrate surface (e.g., glass, plastic, metal) includes at least one group that reacts with the
disilyl compounds of the present invention, such as hydroxyl, amino, or carboxylate groups, or any combination thereof.

Substrate materials amenable for nanoparticle-based detection methods encompass a variety of relevant surface properties. Thus, in one aspect of the present invention, the substrate material has a refractive index in the range of 1.400 to 1.900. In another aspect of the invention, the substrate material provides a light transmittance, that is, the amount of light which passes through the substrate without being either absorbed or being reflected by the surface, either by way of light passing through substrate material parallel to the horizontal axis, or light passing through the substrate material perpendicular to the horizontal axis, of greater than 80%.

In yet another aspect of the invention, the substrate surface modified produces a background signal upon imaging using visual or fluorescent light having substantially reduced background signal relative to a substrate not having said polymeric layer. The substrate surface prior to attaching capture probes has a preferred water contact angle in the range of 25 – 75 degrees.

In yet another embodiment of the invention, the substrate comprises a surface having a polymeric layer comprising negatively charged ionic groups and free isocyanate groups capable of binding said capture probes.

In still yet another embodiment of the invention, a substrate for use in target analyte detection is provided. The substrate comprises a surface modified by any of the above methods.

In another embodiment of the invention, a kit is provided for detecting target analytes. The kit comprises any of the above substrates and substrates prepared by the above methods.

In another embodiment of the invention, a method is provided for detecting one or more target analytes in a sample, the target analyte having at least two binding sites. The method comprises: (a) providing a substrate prepared by any of the methods of the invention, said substrate having at least one type of capture probes immobilized on a surface of the substrate, each type of capture probes specific for a target analyte; (b) providing at least one type of detection probe comprising a nanoparticle and a detector probe, the detector probe specific for a target analyte; (c) contacting the capture probes, the detection probes and the sample under conditions that are effective for the binding of the capture probes and detector probes to the specific target analyte to form an
immobilized complex onto the surface of the substrate; (d) washing the surface of the substrate to remove unbound nanoparticles; and (e) observing for the presence or absence of the complex as an indicator of the presence or absence of the target molecule.

The present invention is also directed to a method for immobilizing nanoparticles on a substrate surface. 3' amine linked oligonucleotides may be synthesized. The 5' end of the 3' amine linked oligonucleotides are attached to nanoparticles, for instance via sulfide linkers. Techniques for functionalizing oligonucleotides with sulfide groups and attachment to nanoparticles are described for instance in published U.S. patent application numbers 2003/0143598A1 and 2002/0155442A1, each of which is incorporated herein by reference in its entirety. A preferred sulfide linker for linking the oligonucleotide to the nanoparticle is an epiandrosterone linker. The oligonucleotide is additionally modified on the 3' end to form a 3' end modified oligonucleotide, which is then contacted to the nanoparticle surface. The nanoparticles with the modified oligonucleotides attached thereto are then contacted with an aldehyde modified substrate surface, resulting in immobilization of the nanoparticles on the substrate surface.

In another embodiment, the method of the invention comprises synthesizing 3' silyl functionalized oligonucleotides to form a 3' end modified oligonucleotide. The modified oligonucleotides are attached to the surface of the nanoparticles through sulfide linkers on the oligonucleotide. The nanoparticles with the 3' silyl linked oligonucleotides attached thereto are then contacted with an aldehyde modified substrate surface, resulting in immobilization of the nanoparticles on the substrate surface.

The immobilization of nanoparticles on substrate surfaces as described herein is useful in several detection techniques. For example the immobilization method of the invention is useful for analyzing silver amplification reagents on a glass surface. For instance, gold nanoparticles immobilized on glass surfaces by the methods of the invention can be used as a positive control in silver amplification based DNA detection assay techniques. Alternatively, a DNA probe can be directly hybridized to the DNA capture strand which is coupled to the glass surface as a positive control. This hybridization method varies from batch to batch of modified glass slides and signal is dependent on hybridization efficiency.

In addition, using the method of the invention one can avoid the capture-probe hybridization procedure in assays that utilize a capture probe for immobilizing a nanoparticle on the substrate surface. Generally, in such assays, a positive control is
provided by hybridizing an oligonucleotide on a nanoparticle to a complementary oligonucleotide on the substrate surface. The invention eliminates this hybridization step, because the nanoparticle is directly immobilized on the substrate surface.

Further, the direct immobilization of nanoparticles according to the invention is also highly useful in detecting DNA targets using surface plasmon resonance (SPR) angle shift technique with different sizes of DNA modified nanoparticle probes. When DNA target is hybridized to the immobilized nanoparticle linked capture strand and DNA modified nanoparticle detection probe in a sandwich assay format, SPR angle shift can be measured using spectroscopy techniques. This provides a method for detecting target DNA using SPR spectroscopy in the presence of large size DNA linked nanoparticle probes. Even single nuclear polymorphs (SNPs) can be detected using this nanoparticle linked capture–nanoparticle linked probe method.

Any substrate whose surface can be modified to provide a surface comprised of aldehyde groups can be used in the invention. Suitable substrates include transparent solid surfaces (e.g., glass, quartz, plastics and other polymers), opaque solid surface, and conducting solid surfaces. The substrate can be any shape or thickness, but generally will be flat and thin. Preferred are glass substrates, such as glass slides.

Thus, in one embodiment of the invention, a method is provided for immobilizing a nanoparticle onto a surface, said method comprising the steps of: (a) providing a substrate having a surface and a nanoparticle having oligonucleotides bound thereto, at least a portion of the oligonucleotides have a free amine group at an end not bound to the nanoparticle; (b) contacting the nanoparticle with an agent so as to form a reactive intermediate, said agent having a formula i:

\[(R_1)(R_2)(R_3)Si-X-NCY\]

wherein \(R_1\), \(R_2\) and \(R_3\) independently represent \(C_1\)-\(C_6\) alkoxy, \(C_1\)-\(C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1\)-\(C_6\) alkyl and \(C_1\)-\(C_6\) alkoxy; \(X\) represents linear or branched \(C_1\)-\(C_{20}\) alkyl or aryl substituted with one or more groups selected from the group consisting of \(C_1\)-\(C_6\) alkyl and \(C_1\)-\(C_6\) alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; and \(Y\) represents oxygen or sulfur, with the proviso that at least one of \(R_1\), \(R_2\) or \(R_3\) represents \(C_1\)-\(C_6\) alkoxy; and (b) contacting the reactive intermediate with said surface so as to immobilized the molecule onto said surface.
Nanoparticles useful in the practice of the invention include metal (e.g., gold, silver, copper and platinum), semiconductor (e.g., CdSe, CdS, and CdS or CdSe coated with ZnS) and magnetic (e.g., ferromagnetite) colloidal materials. Other nanoparticles useful in the practice of the invention include ZnS, ZnO, TiO₂, AgI, AgBr, HgI₂, PbS, PbSe, ZnTe, CdTe, In₂S₃, In₂Se₃, Cd₃P₂, Cd₃As₂, InAs, and GaAs. The size of the nanoparticles is preferably from about 5 nm to about 150 nm (mean diameter), more preferably from about 5 to about 50 nm, most preferably from about 10 to about 30 nm. The nanoparticles may also be rods. Other nanoparticles useful in the invention include silica and polymer (e.g. latex) nanoparticles.


Suitable nanoparticles are also commercially available from, e.g., Ted Pella, Inc. (gold), Amersham Corporation (gold), Nanoprobes, Inc. (gold), and Quantum Dot Inc. (core-shell semiconductor particles such as CdSe/ZnS).

The nanoparticles, the oligonucleotides or both are functionalized in order to attach the oligonucleotides to the nanoparticles. Such methods are known in the art. For instance, oligonucleotides functionalized with alkanethiols at their 3'-termini or 5'-termini readily attach to gold nanoparticles. See Whitesides, *Proceedings of the Robert A. Welch*

In one aspect of this embodiment, the surface is a glass surface.

In another aspect of this embodiment, the surface has at least one group that reacts with the reactive intermediate. Representative examples of groups include hydroxyl,
amino, or carboxylate group. A non-limiting example of agent includes 3-(isocyanatopropyl) triethoxysilane or 3-(isocyanatopropyl)dimethylmonoethoxysilane.

In another aspect of this embodiment, the oligonucleotides may be bound to the nanoparticle through a functional moiety such as a thiotic acid, alkyl thiol or disulfide group (e.g., epiandrosterone disulfide).

In another embodiment of the invention, a method is provided for immobilizing a nanoparticle onto a surface. The method comprises the steps of: (a) providing a substrate having a surface comprising reactive moieties that reacts with amine groups and a nanoparticle having oligonucleotides bound thereto, at least a portion of the oligonucleotides have a amine group at an end not bound to the nanoparticle; and (b) contacting the reactive moieties with the nanoparticle so as to immobilized the nanoparticles onto said surface.

In one aspect of this embodiment, the surface is a glass surface.

In another aspect of this embodiment, the surface has at least one group that reacts with the reactive intermediate. Representative examples of groups include hydroxyl, amino, or carboxylate group. A non-limiting example of agent includes 3-(isocyanatopropyl) triethoxysilane or 3-(isocyanatopropyl)dimethylmonoethoxysilane.

In another aspect, the oligonucleotides may be bound to the nanoparticle through a functional moiety such as a thiotic acid, alkyl thiol or disulfide group (e.g., epiandrosterone disulfide).

In another aspect, the reactive moieties comprise isocyanates, anhydrides, acyl halides, or aldehydes.

In another embodiment of the invention, kits are provided for preparing modified substrates. The kits may include optional reagents for silyating molecules and optional substrates, buffers for carrying out assays including washing and binding steps.
EXAMPLES

The invention is demonstrated further by the following illustrative examples. The examples are offered by way of illustration and are not intended to limit the invention in any manner. In these examples all percentages are by weight if for solids and by volume if for liquids, and all temperatures are in degrees Celsius unless otherwise noted.

Example 1: Preparation of DNA array chips

This Example provides a general procedure for the covalent attachment of a molecule, e.g., 3’ or 5’-silylated DNA, directly to surfaces such as pre-cleaned glass surface via single silylated molecule or dendritic silylated molecule procedure.

(a) Method No. 1

As shown in Figure 1, a method is shown for attaching a 3’-amino or 5’-amino DNA molecule to a pre-cleaned glass surface. 3’-Amine linked DNA is synthesized by following standard protocol for DNA synthesis on DNA synthesizer. The 3’ amine modified DNA synthesized on the solid support was attached through succinyl linker to the solid support. After synthesis, DNA attached to the solid support was released by using aqueous ammonia, resulting in the generation of a DNA strand containing a free amine at the 3’-end. The crude material was purified on HPLC, using triethyl ammonium acetate (TEAA) buffer and acetonitrile. The dimethoxytrityl (DMT) group was removed on the column itself using triflouroacetic acid.

After purification, 1 equivalents of 3’-amine linked DNA was subsequently treated with 1.2 equivalents of triethoxysilyl isocyanate (GELEST, Morrisville, PA, USA) for 1-3h in 10% DMSO in ethanol at room temperature. Traces of water that remained in the DNA following evaporation did not effect the reaction. After 3h, the reaction mixture was evaporated to dryness and spotted directly on pre-cleaned glass surface using an arrayer (Affymetrix, GMS 417 arrayer with 500 micron pins for spotting). Typically, 1mM silylated DNA was used to array a glass surface and the arrayed substrate is then kept in the chamber for 4h-5h. Thereafter, the slides were incubated in nanopure water for 10 minutes to remove the unbound DNA, washed with ethanol, and dried in the dessicator. After drying, these plates were tested with target DNA samples.

In a preliminary study using linear silyl oligonucleotides prepared by the above procedure to spot a glass surface, it was observed that spotting in DMSO or DMF
medisurprisingly controlled spot branching or diffusion. See Figure 2. The spot morphology was clean and discrete. If the substrate was overhydrated in the dessicator chamber prepared by filing a portion of a chamber with water and storing the glass slides on a rack above the water level overnight, the slides become overhydrated. Undesirable branching of the spot was observed on overhydrated slides, even when DMSO or DMF solvent is used. See Figure 3. When water was used as the sole solvent for spotting, the resultant spots were branched out and spread to other spots. See Figure 4. Without being bound to any theory of operation, an aqueous spotting solution and/or the presence of water in a overhydrated substrate results in the polymerization of silyl oligonucleotides and thus interfered with the modification of the surface with the desired molecule. Thus, dried polar aprotic solvents such as DMF, DMSO and dried polar solvents like ethanol, isopropanol and mixture of solvents like DMF/Pyridine were found to be suitable solvents for arraying the silyl modified oligonucleotides. The presence of water (>1%) in the spotting solution or over hydration of slides results in spot branching after arraying. Spot branching is undesirable because it may lead to false positive results in binding studies.

(b) Method No. 2

As shown in Figure 5, a method is shown for attaching multiple 5' or 3' amino DNA molecules to a glass surface. To 1 equivalent of silyl amine in dry acetonitrile, 1.2 equivalents of tetrasiocyanate is added dropwise and the reaction mixture is stirred at room temperature for 10 minutes to form compound 3. 5' or 3'-amine linked oligonucleotide is synthesized and deprotected using aqueous ammonia conditions by conventional procedures. After HPLC purification, 5' or 3'-amine free oligonucleotide is treated with compound 3 in a 1:10 DMSO/ethanol (v/v) mixture. After 10 minutes, the modified oligonucleotides are evaporated under vacuum and spotted on unmodified glass surface in DMSO or DMF media.

Example 2: Detection of Factor V target sequence using a DNA array chip

This Example illustrates that DNA plates prepared as described in Example 1 are useful for sandwich hybridization assays for detection of nucleic acid targets.

(a) Gold Colloid preparation:
Gold colloids (13 nm diameter) were prepared by reduction of HAuCl₄ with citrate as described in Frens, *Nature Phys. Sci.*, **241**, 20 (1973) and Grabar, *Anal. Chem.*, **67**, 735 (1995). Briefly, all glassware was cleaned in aqua regia (3 parts HCl, 1 part HNO₃), rinsed with Nanopure H₂O, then oven dried prior to use. HAuCl₄ and sodium citrate were purchased from Aldrich Chemical Company. Aqueous HAuCl₄ (1 mM, 500 mL) was brought to reflux while stirring. Then, 38.8 mM sodium citrate (50 mL) was added quickly. The solution color changed from pale yellow to burgundy, and refluxing was continued for 15 min. After cooling to room temperature, the red solution was filtered through a Micron Separations Inc. 1 micron filter. Au colloids were characterized by UV-Vis spectroscopy using a Hewlett Packard 8452A diode array spectrophotometer and by Transmission Electron Microscopy (TEM) using a Hitachi 8100 transmission electron microscope. Gold particles with diameters of 13 nm will produce a visible color change when aggregated with target and probe oligonucleotide sequences in the 10-35 nucleotide range.

(b) **Synthesis Of Oligonucleotides:**

Oligonucleotides were synthesized on a 1 micromole scale using a Milligene Expedite DNA synthesizer in single column mode using phosphoramidite chemistry. Eckstein, F. (ed.) *Oligonucleotides and Analogues: A Practical Approach* (IRL Press, Oxford, 1991). All solutions were purchased from Milligene (DNA synthesis grade). Average coupling efficiency varied from 98 to 99.8%, and the final dimethoxytrityl (DMT) protecting group was cleaved from the oligonucleotides to do final epiandrosterone coupling on the synthesizer itself. Capture strands were synthesized with DMT on procedure and purified on HPLC system.

(c) **Purification of oligonucleotides**

Reverse phase HPLC was performed with using Agilent 1100 series system equipped with Tosch Biosep Amberchrom MD-G CG-300S column(10x118mm, 35μm particle size) using 0.03 M Et₃NH⁺ OAc⁻ buffer (TEAA), pH 7, with a 1%/min. gradient of 95% CH₃CN/5% TEAA. The flow rate was 1 mL/min. with UV detection at 260 nm. The final DMT attached was deprotected on HPLC column itself using 1-3 % trifluoro acetic acid and TEAA buffer. After collection and evaporation of the buffer contained the DMT cleaved oligonucleotides, was then evaporated to near dryness. The amount of
oligonucleotide was determined by absorbance at 260 nm, and final purity assessed by reverse phase HPLC.

The same protocol was used for epiandrosterone linked-oligonucleotides for probe preparation and no DMT removal needed.

(d) Attachment Of Oligonucleotides To Gold Nanoparticles

Probes used in the Example: (3'-act tta aca ata g-a2o-Epi-5' and 3'-t taa cac tgc c-a20-Epi-5') (SEQ ID NO:1) was attached in the following fashion. These probes were designed for M13 target sequence detection.

A 1 mL solution of the gold colloids (15nM) in water was mixed with excess (3.68 :M) 5’-epiandrosterone linked-oligonucleotide (33 and 31 bases in length) in water, and the mixture was allowed to stand for 12-24 hours at room temperature. Then, 100 µL of a 0.1 M sodium hydrogen phosphate buffer, pH 7.0, and 100 µL of 1.0 M NaCl were premixed and added. After 10 minutes, 10 µL of 1% aqueous NaN₃ were added, and the mixture was allowed to stand for an additional 20 hours then increased the salt concentration to 0.3. After standing 4h at 0.3 M NaCl again increased to 1M Nacl and kept further 16h. This "aging" step was designed to increase the surface coverage by the epi disulfide linked-oligonucleotides and to displace oligonucleotide bases from the gold surface. Somewhat cleaner, better defined red spots in subsequent assays were obtained if the solution was frozen in a dry-ice bath after the 40-hour incubation and then thawed at room temperature. Either way, the solution was next centrifuged at 14,000 rpm in an Eppendorf Centrifuge 5414 for about 15 minutes to give a very pale pink supernatant containing most of the oligonucleotide (as indicated by the absorbance at 260 nm) along with 7-10% of the colloidal gold (as indicated by the absorbance at 520 nm), and a compact, dark, gelatinous residue at the bottom of the tube. The supernatant was removed, and the residue was resuspended in about 200 µL of buffer (10 mM phosphate, 0.1 M NaCl) and recentrifuged. After removal of the supernatant solution, the residue was taken up in 1.0 mL of buffer (10 mM phosphate, 0.1 M NaCl) and 10 µL of a 1% aqueous solution of NaN₃. Dissolution was assisted by drawing the solution into, and expelling it from, a pipette several times. The resulting red master solution was stable (i.e., remained red and did not aggregate) on standing for months at room temperature, on
spotting on silica thin-layer chromatography (TLC) plates, and on addition to 2 M NaCl, 10 mM MgCl₂, or solutions containing high concentrations of salmon sperm DNA.

For examples 2-5 we prepared different set of Factor V probes using an aqueous solution of 17nM (150 µL) Au colloids, as described above, was mixed with 3.75 µM (46 µL) 5’-epiandrosterone-9α-tatctccetgce (SEQ ID NO:2), and allowed to stand for 24 hours at room temperature in 1 ml Eppendorf capped vials. A second solution of colloids was reacted with 3.75 µM (46 µL) 5’-epiandrosterone-9α-attctctgct-3’ (SEQ ID NO:3). Note that these oligonucleotides are non-complementary. The residue was dissolved using the same procedure described above and the resulting solution was stored in a glass bottle until further use.

(e) Hybridization conditions

Stock buffer solution: For the hybridization buffer, the following stock solution was used: 3.0 NaCl, 0.3 M Na-Citrate, 10mM MgCl₂, 4.0 mM NaH₂P0₄ and 0.005% SDS.

Hybridization assay was performed using diluted buffer (0.78M NaCl, 70 mM sodium citrate, 2.64 mM MgCl₂, 1.1mM sodium phosphate, 0.01%) from the stock buffer solution by adding 0.5% of Tween. In a typical experiment procedure, target and probe were mixed with the hybridization buffer and heated the mixture at 95°C for 5 minutes. After cooling to room temperature aliquots were transferred on to the glass substrate and placed in humidity chamber for hybridization (Different assays were done at different temperature conditions since each probe has a different melting temperature). After hybridization, plates were washed with two different wash buffers and spin dried. Plates dried were treated with silver amplification solutions (silverA+silverB) (silver amplification kit available from SIGMA, St.Louis, MO 63178, catalog no: S 5020 and S 5145) and the data was collected from the amplified plates using an imaging system for data collection described in (Nanosphere, Inc. assignee) U.S. Patent application no. 10/210,959 and PCT/US02/24604, both filed August 2, 2002, which are incorporated by reference in their entirety.

(f) Target Sequence used

This Factor V target sequence was used in examples 2-6 for detection. M13 probes were used in example 1 for direct probe targeting to capture strand test the plates
and no target detection was performed here. But from example 2-5 Factor V target detection was done in presence of Factor V probes and M13 probes. Here M13 probes served as controls. In plate no: 5 different combination of assay were performed on one plate including Factor V wild type and mismatch detection. Each well in plate no: 6 was clearly defined with target and probes used.

Factor V wild type sequence:
5’gacatcgcttcctggctaatagctactactctaactcttgacagcagatccctgacagcaggaagatactataattggttaaagctg3’ (SEQ ID NO:4)

Probe sequence:
Probe FV (13D): 5’-Epi-a20-tatcctgac 3’ (SEQ ID NO: 5)
Probe FV (26D): 5’-Epi-a20-attccattgac 3’ (SEQ ID NO: 6)

Capture Strand Sequence For factor V target detection:
5’-tcc tga tga aga tta gac att ctc gtc- NH-CO-NH- Si-(OEt)3-3’ (SEQ ID NO:7)

Stock buffer solution: For the hybridization buffer, the following stock solution was used: 3.0 NaCl, 0.3 M Na-Citrate, 10mM MgCl2, 4.0 mM NaH2PO4 and 0.005% SDS.

Example 3: Detection of M13 target sequence using DNA array chip
In this Example, probe was targeted directly to the capture strand and a detection assay was performed. Plates Nos. 1-3 were prepared as described in Example 1 (method no. 1). In Plates 2 & 3, probes (Figure 6) were clearly hybridized to the capture strand within 45 minutes. The gold colloid nanoparticles hybridized to the capture were clearly visible before silver amplification. In plate no 1 (Figure 6), a different probe was used and the assay was developed to show the specificity. After silver stain development, signals were not shown on the glass surface even after silver amplification. This experiment established the specificity of the DNA chip prepared in accordance with the invention.

M13 Capture sequence:
5’-tga aat tgt c- NH-CO-NH-- Si-(OEt)3-3’ (SEQ ID NO: 8)
Probe used on plates Nos. 2-3 plates:
3'-act tta aca ata g-a20-Epi-5' (SEQ ID NO: 9)

On plate no.1, a detection probe 3'-t taa cac tcg c-a20-Epi-5' (SEQ ID NO:10) was used which was non-complementary to the capture strand for sequence specificity testing (no signals). This clearly showed the specificity of the both capture strand sequence and the probe. In both cases, 6nM probe was used in diluted buffer conditions. In a typical experimental procedure, 30μl of the diluted buffer (1.3M NaCl, 130mM sodium citrate, 4.38mM MgCl2, 1.82mM sodium phosphate, 0.003% SDS) and 20μl of probe (10nM) was flooded on the arrayed glass chip and allowed to hybridize for 1.5h at room temperature. The final concentration of probe was 4nM and buffer concentration was 0.78M NaCl, 70 mM sodium citrate, 2.64mM MgCl2, 1.1mM sodium phosphate, 0.002% SDS. Thereafter, the chip was washed with 0.75 M sodium chloride, 75mM citrate and 0.05% Tween buffer and then washed again with 0.5M sodium nitrate buffer. Then plates were treated with silver amplification solutions silver A+ SilverB (1mL+1mL = total 2mL) for 4 minutes and washed with nanopure water. Finally, the plates were exposed to the imaging system for data collection as discussed above.

**Example 4: Detection of Factor V target sequence using a DNA array chip**

In this Example, two different silanized capture strands were spotted directly on the plate and detected. The plate was prepared as described in Example 1 (method no. 1). The middle row always carried the positive control capture with other capture on top and bottom rows. Here, wild type, mutant and heterozygous samples were used for the detection. All samples were showed signals in the proper place using the above mentioned assay conditions. See Figure 7.

a) Positive controls capture sequence:
5'-tga aat tgt tat c- NH-CO-NH- Si-(OEt)3-3’ (SEQ ID NO:10)
 Probe used was for positive control:
3’-act tta aca ata g-a20-Epi-5’ (SEQ ID NO:11)

b) Probes used for target detection are:
Probe FV 13D (probe for wild type target): 5’-Epi-a20-tatcctgcc 3’ (SEQ ID NO:12)

Probe FV 26D (probe for mutant target): 5’-Epi-a20-attcctgcc13’ (SEQ ID NO:13)

Capture Strand Sequence For factor V target detection:
5’-tcc tga tga aga tta gac att ctc gtc- NH-CO-NH-- Si-(OEt)3-3’

Factor V wild type target sequence:
5’gacatcgctttgctagagacttcttaactgtagacagatccccctggacaggaagtaacaggtatgtttgccttgaagtaaccttcag 3’ (SEQ ID NO: 13)

Mutant Factor V target sequence:
gtagactactttcatactgtagacagatccccctggacaggaagtaacaggtatgtttgccttgaagtaaccttcag-3’
(SEQ ID NO:14)

Heterozygous: 50% of wild type and 50% of mutant target.
Well 1: Heterozygous- Probe 26D was used
Well 2: Heterozygous- Probe 13D was used
Well 3: Control- with probe 26D, only positive control should show up
Well 4: Control- with probe13D, only positive control should show up
Well 5: Mutant - target with mutant probe 26D + positive control probe
Well 6: Mutant target –with wild type probe 13D + positive control
Well 7: Heterozygous- with probe 26D
Well 8: Heterozygous- with probe 13D
Well 9: Wild type target - with mutant probe 26D
Well 10: Wild type target- with wild type probe13D

Example 5: Detection of MTHFR target sequence on a DNA array plate

In this Example, an MTHFR 100mer synthetic target and 208 base pair PCR product (10nM ~50nM) was used in the detection assay. The plates were prepared as described in Example 1 (method no. 1). Alternative wells were used as controls using M13 target and MTHFR 18mer probe and did not show even traces of silver, following
silver signal amplification. As shown in plate no. 1 (Figure 8), an experiment was performed at 70°C to show that probe does not hybridize above melting temperature (MTHFR target and 18mer probe). The results show probe specificity and that at high temperature, the probes are not binding nonspecifically to the silyl oligo-attached substrate.

100mer synthetic target:
5'-aag cac tgt aag gag aag gtg tct gcg gga gcc gat tgt ttc tgt gat acg ctt ttc cgc tgt aag gca tgc acc ga-3' (SEQ ID NO: 14)

18mer probe sequence used on all three plates:
3'-ctg tgt aag aag gcg ttt-A20-Epi-5' (SEQ ID NO: 15)

PCR product: 208 base pair

5'ccttgagcaaggctggagccagcctctctctgtcaggtctcatcccatgtgtgctggagttcccccaagcaccaccccgaagcaagggagcttgagagctggactgaagcgacagtgggaggggagcgaggatttcatcatacgcaacgctttttttgagctgacaaggagaaggcatgcagcatggagcagtgggactctgtgccccctgtgccccccgatctttctccacaggtggaggggggacagcagggccagcataactcctctccaccccactctcacg (SEQ ID NO:16)

Experimental conditions:

In a typical experimental procedure (on plate no:2), to 30μl of the diluted buffer (1.3M NaCl, 130mM sodium citrate, 4.38mM MgCl2, 1.82mM sodium phosphate, 0.003% SDS), 10μl of 18mer probe (10nM) and 2 μl of 100mer synthetic target (10 μM) 8μl of water were mixed and flooded on the arrayed glass chip and allowed to hybridize for 1.5h at room temperature. The final concentration of probe was 2nM and target concentration was 400pM and buffer concentration was 0.78M NaCl, 70 mM sodium citrate, 2.64 mM MgCl2, 1.1mM sodium phosphate, 0.01%). After that washed with 0.75 M sodium chloride, 75mM citrate and 0.05% Tween buffer and then washed again with 0.5M sodium nitrate buffer. After that plates were treated with silver A+ SilverB (1mL+1mL = total 2mL) (silver amplification kit available from SIGMA, St.Louis, MO 63178, catalog no: S 5020 and S 5145) for 4 minutes and washed with nanopure water.

Finally plates were exposed to imaging system for data collection as discussed above. In
Example 3 on plate no:2, wells no: 2 1,4, 5, 8 are controls and controls made up with M13 synthetic target and MTHFR 18mer probe (5'-'tat gct tcc ggc tgc tgc tat gtt gtt gaa att tga cgg ata aca att tca-3'). (SEQ ID NO: 17)

As mentioned earlier, the experiment on plate no.1 (Figure 8) was performed at 70 °C to show that above melting temperature probe 18mer probe did not bind to the capture probe.

Plate no.3 (Figure 8) was generated following the same experimental procedure and using the same probes. 10 μl (2nM-10nM) of MTHFR PCR product was used as target. Plate no.3 wells 2, 3, 6 and 7 are the controls with Factor V 99mer mutant target and MTHFR 18mer probe.

Factor V 99mer Mutant Factor V target had the following sequence:
5'gtaggactctcatactgtaagagcgacagttccctggacagtaaggaataacagttttttgtctgtaggtgtaacccctcag-3')
(SEQ ID NO: 18)

Example 6: Detection of Factor V target sequence on DNA array plate
In this Example and in the following Example 7, the same capture strands were arrayed on the plate. The purpose of this experiment was to find out the difference in intensity of the spots after silver development when same oligomer was spotted on the slide at different places. Positive control was spotted in the middle of two Factor V 4G oligomer captures on the slide. The results are shown in Figure 9.

Capture strand sequence for Factor V target detection was:
5' tcc tga tga tga ttg att cac ttc-NH-CO-NH- Si-(OEt)3-3' (SEQ ID NO:19)

Positive capture control capture spotted was (M13):
5' tga aat tgt tat c-NH-CO-NH-- Si-(OEt)3-3' (SEQ ID NO:20)

The target sequence used was wild type Factor V 99base pair single strand DNA having the following sequence:
gtaggactactcctcaatctgtagagagaagaggatccctggagagaggtgattttttgtctgtagttacccctcag-3')
(SEQ ID NO:21)
Mutant Factor V target had the following sequence:
\[\text{gtaggactacttcaactgtgaagagacagatccctggacaggttaaggaatacaggtatattttgtccttgaagtaacctttcag-3'}\] (SEQ ID NO:22)

and probes used had the following sequence:
probe FV 13D: 5'-Epi-a20-ttacctgc 3' (SEQ ID NO:23),
probe FV 26D: 5'-Epi-a20-attacctgccc 3' (SEQ ID NO:24).

Capture Strand Sequence for factor V target detection:
\[5'-\text{tcc tga tga aga tta gac att ctc gtc-} \text{NH-CO-NH--Si-(OEt)}_3-3'\] (SEQ ID NO:25)

Positive control sequence: 5'-tga aat tgt tat c-NH\textsubscript{2}-3' (SEQ ID NO: 26)
and probe used for positive control was: 3'-act tta atc atc g-a20-Epi-5' (SEQ ID NO: 27)

In a typical experimental procedure, to 25\textmu l of the diluted buffer (1.3M NaCl, 130mM sodium citrate, 4.38mM MgCl\textsubscript{2}, 1.82mM sodium phosphate, 0.003% SDS), 10\textmu l of probe (10nM) and 10 \textmu l of PCR target (15-50nM) and 5 \textmu l of positive control probe (10nM) were mixed and flooded on the arrayed glass chip and allowed to hybridize for 1.5h at room temperature. The final concentration of probe was 2nM, and buffer concentration was 0.78M NaCl, 70 mM sodium citrate, 2.64 mM MgCl\textsubscript{2}, 1.1mM sodium phosphate, 0.01%). that the plates was then washed with 0.75 Sodium chloride, 75mM citrate and 0.05% tween buffer and then washed again with 0.5M Sodium Nitrate buffer. The plates were treated with silver A+ SilverB (1mL+1mL = total 2mL) for 4 minutes and washed with nanopure water. Finally, the plates were exposed to the imaging system described above for data collection. Both positive control probe and target reacted probe were mixed and the assay was run to show the selectivity of the probe. The wells were identified as follows:

Wells 1, 6, 8 and 9 have only positive control probe with target and buffer.

Wells 2, 5 had both positive control probe and target probe with targets and buffer.
Wells 4, 7 and 10 have only target probe with target and buffer and here positive control probe and target were absent.

Well 3 did not have any target and positive control probe but it had target probe and buffer.

These results (Figure 9) show that probes were specific to target detection and no non-specific background noise was observed when target was absent.

**Example 7: Detection of Factor V target sequence**

In this Example, all capture strands pattern is the same as described in Example no.6. Moreover, the same experimental conditions and concentrations described in Example 6 were used to perform the assay at 52°C. Wild type and mutant targets were given in the example 6. The results are shown in Figure 10. The wells are identified as follows:

Well 1: Positive control probe directly probing to the capture strand in the same buffer conditions mentioned in example 4.

Well 2: Factor V Probe 5'-Epi-a20-attcttgccct-3' (26D) (SEQ ID NO: 27) and Factor V 99base pair mutant target, positive control probe and buffer.

Well 3: Factor V Probe 5'-Epi-a20-attcttgccct-3' (26D) (SEQ ID NO: 28) and Factor V 99base pair mutant target and hybridization buffer.

Well 4: Probe 13D and Factor V mutant PCR target, positive control and hybridization buffer.

Well 5: Probe 13D and Factor V mutant PCR target, and hybridization buffer.

Well 6: Control (MTHFR target and Probe 13D and hybridization buffer).
Well 7: Wild type Factor V target, probe (26D), positive control probe and hybridization buffer,

Well 8: Wild type Factor V target and probe (26D), and hybridization buffer.

Well 9: Wild type Factor V target, probe 13(D), positive control probe and hybridization buffer.

Well 10: Wild type Factor V target, probe 13(D), and hybridization buffer.

Probe FV 13D: 5'-Epi-a<sub>20</sub>-tacctctgcc-3' (SEQ ID NO: 29)

Probe FV 26D: 5'-Epi-a<sub>20</sub>-attccctgct-3' (SEQ ID NO: 30)

These results (Figure 10) show that probes were reacted specifically to the target and there is no cross hybridization between probes and targets were observed when probes were mixed with different targets.

**Example 8: Protocol for preparing polymer-coated substrates**

A general protocol for preparing polymer-coated substrates and for printing amine modified DNA on the polymer coated surface is as follows.

Amine modified DNA is made from gene synthesizer using standard protocols and purified on HPLC. Oligonucleotides are printed using pH 8.5 300mM phosphate buffer using GMS Affymatrix<sup>®</sup> arrayer. Figure 11 is a schematic showing a representative method for preparing a substrate for detecting target analytes involving modifying a substrate with an isocyanate compound (2-trimethoxysilane-6-(triisocyanatosilaneurea)benzene) to form a surface having isocyanate groups, contacting the surface having the isocyanate groups with a spacer molecule (poly(dimmer acid-co-alkylpolyamine)-95) having a plurality of amino groups to form a surface having free amino groups, contacting the surface having free amino groups with a linker molecule (EGS) to form a surface having reactive moieties. The resulting surface can be used to attach capture probes such as nucleic acids molecules.
Materials used:

Glass slides: Gold seal products, catalogue no: 3011
Fisher Scientific, Catalogue no: 12-544-1
Silanes: 3-(Triethoxysilyl)propylisocyanate, Sigma-Aldrich, Catalogue no: 41336-4.
m-aminophenyltrimethoxysilane, Gelest, catalogue no:SIA0599.0
Tetraisocyanatosilane, Gelest, catalogue no: SIT125.0
Polymers: Poly(dimmer acid-co-alkylpolyamine)-140, Sigma-Aldrich,
Catalogue no: 191043
Poly(dimmer acid-co-alkylpolyamine)-90, Sigma-Aldrich,
Catalogue no: 191019
Neomycin, Sigma-Aldrich, Catalogue no: N1142
Poly(allylamine), Sigma-Aldrich, Catalogue no: 479144
Poly(m-xylylenediamine-epichlorohydrine), diamine terminated,
Sigma-Aldrich, Catalogue no: 456888
Tris(2-aminoethylamine), Sigma-Aldrich, Catalogue no: 22563-0
Panam Dendrimer Generation7, Sigma-Aldrich, Cat no: 53672-5
Linkers: Ethyleneglycol-bis-(succinimidyl-succinate) EGS, Pierce, cat no:
21565
Disuccinimidyl Suberate,(DSS), Pierce, cat no:21555
1,6 Diisocyanatohexane, Sigma-Aldrich, Catalogue no: D 12470-2
Glutaric Dialdehyde, Sigma-Aldrich, Catalogue no: 34085-5

Cleaning Slides: All the slides were first soaked in NaOH (5% in water) for 30
minutes at room temperature and washed with water to obtain the pH of 7. Then they
were soaked in 5%-HCl for 30 minutes at room temperature and again washed one time
with water. Finally, the slides were treated with 3% H2O2 in 5%-HCl for 3 hours at room
temperature, washed with water three times (till pH=7) and with Ethyl Alcohol three
times, and dried by spinning. Afterward, the air-dried slides were cured in the oven
overnight at 120 OC

Step1: Synthesis of 2-Trimethoxysilane-6-(triisocyanatosilaneurea) benzene:
1.96 (0.01M) of Tetraisocyanatosilane and 2.13g (0.01M) of m-Aminophenyltrimethoxysilane were mixed and stirred for 20 minutes at room temperature. 200 mL of Ethyl alcohol was added to the mixture and stirred additional 60 minutes at room temperature (see Figure 11).

**Step 2: Silanation:**

Twenty five slides were treated with solution 4.09g of 2-Trimethoxysilane-6-(triisocyanatosilaneurea) benzene (0.01M) (1) in 200mL solution of Ethanol for 2 hours at room temperature under slight agitation. After 2h of reaction, slides were removed from the bath and washed three times with Ethanol and used for the next step without further drying (see Fig. 11).

**Step 3: polymer coating:**

1.5 g of poly(dimer acid-co-polyamine) - 95 (2) (purchased from Aldrich Chemicals, St. Louis, MO, USA) was added to the pyridine-dichloromethane mixture (150mL; 5: 1) and stirred for 1.5 hours at room temperature. Silylated slides were placed in this solution and kept at room temperature for 4.5 hours. Then slides were washed in the following order (see Fig. 11)

- two times with pyridine (for 5 min)
- two times with dichloromethane (for 5 min each time)
- three times with ethanol (for 5 min each time).

**Step 4: Cross linker addition on the surface:**

**EGS-Cross linker**

a). Slides prepared in Step 3 were treated with 20mM solution of (0.456g in 50ml of DMSO) ethylene glycol-bis-succinimidylsuccinate (3-EGS) for 4 hours at room temperature. The treated slides were then washed with ethanol: DMSO mixture (9:1) once, three times with ethanol, then dried. Figure 11 illustrates the chemistry of the branched silane coating: Branched silane was used for the surface coating to improve the sensitivity of target detection. In fact, this branched silane helped in reproducibility of target detection and to some extent in improving sensitivity. Figure 12 is a model picture of polymer coating on glass surface showing the polymer positioned on the glass surface angularly which is optimal for DNA printing.

**Diisocyanate cross linker**

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b). Slides prepared in Step 3 were treated with 26mM solution of (0.436g in 100ml DMSO) of 1,6-Diisocyanatohexane (3-C6) for 4 hours at room temperature. The treated slides were then washed once with ethanol: DMSO (9:1) mixture, three times with ethanol and dried. After linker addition, 3' amine modified DNA was arrayed and kept in the humid chamber for 12h. the arrayed slide was washed with water prior to assay development, then dried.

Example 9: **Cy3 Oligonucleotide spotting on polymer coated slides and dendrimer modified slides and average intensity comparison:**

Various polymers and linkers were evaluated as surface coatings to produce branched surfaces. Figure 13 provides a schematic diagram for representative polymers and linkers and order of use. Among all these polymers, poly(dimer acid co-alkyl poly amine)-95 gave good results in detecting target DNA using gold nanoparticles probes and gave reproducible results with minimum background noise. Amine rich compounds like dendrimers, Tris (2-amino ethylamine), poly (allylamine) coated surfaces gave little higher backgrounds compared to Poly (dimer acid co-alkyl poly amine)-95 coated surface using gold nanoparticle probes. Without being bound by any theory of operation, it is believed that the gold nanoparticles are binding to unreacted amines on the surface. High backgrounds were observed using CY3 labeled oligonucleotides printing on amine-rich dendrimer surface.

For all polymer surface modifications, the procedures described in Example 8, including slide cleaning and surface modification, were followed. All the slides were cleaned prior to the dendrimer coating using cleaning procedure from page 2. The slides were treated with 5% 3-isocyanatopropyltriethoxysilane in anhydrous ethanol and kept at room temperature for 1h, washed with ethanol three times, and dried in dessicator under vacuum. The dried slides were then treated with Panam Starbust® G7 dendrimer (0.15% final concentration) in DMSO and kept for 5h at room temperature. The treated slides washed with DMSO and ethanol (2 times) and dried in dessicator under vacuum. Finally, dendrimer-linked slides were cross-linked with 100mM EGS linker in DMSO for 6h and washed with DMSO, ethanol successively and dried in dessicator under vacuum. After overnight drying in a dessicator, the slides were used for DNA printing.
In this example, CY3-linked oligonucleotide were printed on the polymer-coated surface and washed after 6h with water. CY3 intensity from oligonucleotide coupled to the surface was measured using fluorescent scanner. The results are shown in Figure 14. This Figure shows the average spot intensity of amine linked CY3 linked oligonucleotide coupled to the modified surface. Different types of slides after CY3 oligonucleotide spotting are shown in Figures 15 (a) for 3E and 3D and Figure 16. Figure 15(a) and (b) illustrate polymer-coated slides after CY3-linked oligonucleotide spotting. Figure 16 illustrates dendrimer modified slides after CY3-linked oligonucleotide spotting.

Comparing polymer-coated slides (Figure 15(a) and (b)) with the dendrimer slides (Figure 16), the dendrimer slides produced higher background noise when employed in gold nanoparticle-based assay and CY3 attachment studies. Without being bound by any theory of operation, it is believed that this may be due to the excess amine groups on the surface creating a positively-charged environment that absorbs negatively-charged molecules like DNA gold probes.

The protocol used in this Experiment is as follows:

1. Oligo Solution prepared –FV43H+Cy3 (75nmole+60pmole)
2. Total 960 spots per slide were spotted using Cartesian arrayer
3. The slides were hydrated for 18 hours in a chamber
4. The slides were then dried and scanned, also washed and scanned, then analyzed
5. An oligo mix was prepared using the below amounts of DNA:
   a. FV43H = GGCGAGGAATA-(peg)3-NH2 (75nmoles) +
   b. CY3 oligo = Cy3-TCATCATCA-(Spacer18)-NH2 (60 pmoles)
6. Solution is dispensed across the substrate, the substrate was hydrated which allows the oligo mix to bind to the substrate
7. Substrates were then dried and washed with 0.2% SDS followed by MilliQ water
8. Substrates were then scanned using GenePix 4100A Scanner at 400 PMT, 40um resolution
9. The scanned images are saved and gridded using GenePix Pro 4 software.
10. Data was gathered and organized in MS Excel
11. Higher signal intensity is consistent with more Cy3 tags being present and therefore more binding capacity
The assay results do not indicate about how much DNA was hybridized and/or how proficient the substrate was in limiting background after silver stain. All the slides made following the above protocol were shown in Figures 15(a) and (b) and Figure 16 and a comparison of their intensity is shown in Figure 14. This test shows that amine-linked oligonucleotides can be covalently coupled to the modified surface all over the slide.

**Example 10: Evaluation of Polymer coated substrates**

This Example illustrates detection of PCR products using the polymer-coated substrates of the invention. A one-step hybridization assay procedure based on nanoparticle-based probes was used.

**PCR Product Detection:** Slides 1, 2 & 3 PCR amplified duplex (80nM) were used for detection. The results are shown in Figures 16(a)-(c) respectively.

**FV99 PCR product**

CTGAAAGGGTTACTTTCAAGGACAAAAATACCTGTATTCCTCGCCTGTCAGGATCTGC
TCTTACAGATTGAACTGATGTCTATTAGCCCAGAGCGATGTC

**Captures:**

Wild type Factor V capture: FV43H - 5' - GGC GAG GAA TA- (spacer 18)$_3$-NH$_2$ - 3'

Mutant Factor V capture: FV44H - 5' - AGG CAA GGA AT- (spacer 18)$_3$-NH$_2$ - 3'

Positive control capture: PHA2H - 5' - TGA AAT TGT TAT C- (spacer 18)$_3$-NH$_2$ - 3'

Factor V probe: FV45Q - 5' Epi -AAA AAA AAA AAA AAA - (Spacer 18)$_1$ - CT TCT AAT CTG TAA GAG CAG 3'

Positive control probe: PHA1D - 5' Epi -AAA AAA AAA AAA AAA AAA AAG ATA ACA ATT TCA-3'

Nanoparticle loading was performed by standard protocol. Typically, 5'-epi disulfide (epi = epiandrosterone) oligonucleotide was loaded on citrate modified gold nanoparticles and kept at room temperature for 24h in the dark (4µM of modified oligonucleotide loaded per 1mL). Then salt addition was started and increased to 0.5 M in 6h of time and kept in the salt conditions total 40h at room temperature in the dark. After 40h oligonucleotide-linked gold nanoparticles were filtered and centrifuged using plastic
tubes. The nanoparticle conjugates were then washed with water and resuspended in 0.1
M NaCl, 10nM phosphate, 0.01% azide pH: 7 buffer.

**Experimental procedure for slides 1, 2 & 3:** All the slides were washed with
0.2% SDS and water just prior to the experiment and dried by spinning at the room
temperature. Aliquots for the assay were prepared using Factor V probe and positive
control probe and place in the wells. Typically, 35ul of hybridization buffer (2X SSC, 0.2
tween), 5ul of water, 5 ul of target, 10 ul of colloid were mixed for each well and heated
at 97°C and cooled at room temperature for 3 minutes. Then aliquots were transferred to
the respective wells on the slide using pipette. For positive controls we used water in
place of target since it is probe-capture hybridization. After 120 minutes hybridization at
40 °C, slides were washed with 5M NaNO₃ and amplified with a commercial silver
amplification solution for 4 minutes at room temperature. Then washed with water twice
and dried by spinning the slide. Slide was imaged on VERIGENE (Nanosphere Imaging
System) and transferred the images to the word file.

**Slide no 1:** Xylene polymer coated slide used for DNA detection. The slide was
prepared in accordance with Example 8. Wells 1 & 3 are used for positive controls and 2
& 4 used for wild type target. See Figure 16(a).

**Slide no 2:** PCR duplex detection on Tris-(2, amino ethylene) coated slide. The
slide was prepared in accordance with Example 1. As shown in the picture this amine rich
slide gave backgrounds with gold nanoparticle probes. See Figure 16(b)

**Slide no 3:** PCR wild type duplex detection on polymer-95 coated surface. The
slide was prepared as described in Example 8. Wells 1, 4, 5, 9 &10 used for probe
controls. Wells 2, 3, 7 & 8 used for wild type factor V target and well 6 used for +ve
control probe. See Figure 16(c).

From the above three slides and as shown in Figures 16(a)-(c), copolymer (dimer
acid-co-alkyl polyamine)-95 slides worked better in terms of background and sensitivity.
However, slide no: 2 which is coated with tris (2, amino ethylene) slide gave higher
background using gold nanoparticle probe in the assay. This result tells us that amine rich
surfaces are not suitable for gold nanoparticles assay for direct use and may be blocking
is necessary.

**Slide no 4:** Dendrimer-linked slide: Dendrimer slide and polymer -95 coated were
compared using gold nanoparticles. See Figures 16(d) and (e). The dendrimer slide was
Dendrimer slide showed higher backgrounds compared to polymer coated slide in our experiment.

**Experimental procedure for slide 4**: Both the slides were washed with 0.2% SDS and water just prior to the experiment. After washing, all the slides were dried by spinning at room temperature. 700ul of hybridization buffer (2X SSC, 0.2 tween), 100ul of water, 200ul of colloid were mixed and layered on the slide using pipette. After 60 minutes at 40°C, slides were washed with 5M NaNO₃ and amplified with a commercial silver amplification solution for 4 minutes at room temperature. The silver-treated slides were washed with water twice and dried by spinning and imaged on VERIGENE.

**Slide no 5**: In this experiment PCR target was diluted in different concentrations and used for detection on polymer -95 coated slides. See Figure 16(f).

**Experimental procedure**: The modified slide was washed with 0.2% SDS and water just prior to the experiment. After washing, all the slides were dried by spinning at room temperature. In each well different concentration of target was used to check the sensitivity (180nM, 1.8nM, 180pM & 18pM respectively). Typically, 35ul of hybridization buffer (2X SSC, 0.2 tween), 5ul of water, 5 ul of target, 10 ul of colloid were mixed for each well and heated at 97°C and cooled at room temperature for 3 minutes. Then aliquots were transferred to the respective wells on the slide using pipette. After 120 minutes hybridization at 40°C, slides were washed with 5M NaNO₃ and amplified with silver for 4 minutes at room temperature. Then washed with water twice and dried by spinning the slide. Slide was imaged on VERIGENE (Nanosphere Imaging System) and transferred the images to word file. From the images, it was observed that 18pm target could be detected using polymer (dimer acid co alkyl amine)-95-coated slides.

**Slide no 6**: This slide shows the detection of wild type, mutant and heterozygous targets detection simultaneously on polymer -95 coated slides. See Figure 16(g).

**Experimental Procedure for slide no. 6**: The modified slide was washed with 0.2% SDS and water just prior to the experiment. After washing, all the slides were dried by spinning at room temperature. Different types of samples such as wild type, mutant and heterozygous targets were used in different wells to check the specificity. Typically, 35ul of hybridization buffer (2X SSC, 0.2 Tween), 5ul of water, 5 ul of target, 10 ul of colloid were mixed for each well, heated at 97°C, then cooled at room temperature for 3 minutes. Aliquots were then transferred to the respective wells on the slide using pipette.
For positive controls, water was used in place of target since it is probe-capture hybridization. After 120 minutes, hybridization at 40°C, the slides were washed with 5M NaNO₃ and amplified with a commercial silver amplification solution for 4 minutes at room temperature. Then washed with water twice and dried by spinning the slide. Slide was imaged on VERIGENE (Nanosphere Imaging System) and transferred the images to word file.

**Slide no 7:** A polymer -95 slide was compared with a commercial Codelink® slide (Amersham Corp.) using direct capture-probe hybridization assay. The instant polymer-95 coated slide of the invention performed nearly the same like the Codelink® slide, based on the results of this experiment. See Figure 16(h).

**Experimental Procedure:** In this experiment positive control probe directly hybridized to the capture strand to compare polymer coated slide with codelink slide. To 35ul of hybridization buffer (2X SSC, 0.2 tween), 15ul of water, 5 ul of colloid were mixed and added to each well. The slide kept under humidity chamber for 1h and washed with 5M NaNO₃ solution at room temperature and amplified with silver solution for four minutes. Both polymer coated slide (a) and codelink (b) slide gave similar result in terms hybridization.

The entire slides showed from 1 to 7 were developed using positive controls and Factor V PCR targets. From the results shown in Figures 16(a)-(i), it was concluded that the polymer -95 coated substrate was comparable with the commercially available Codelink® slides. However, amine-rich polymer coated slides had higher backgrounds (slide 2 (Figure 16(b)) and 4a) (Figure 16(d)) in gold nanoparticle-based assay. In conclusion, amine-coated surfaces were not as good for gold nanoparticle probe-based assays and blocking steps were need to obtain better results.

After completing PCR product detection, detection of genomic targets Factor V and Factor II were attempted in a one-step assay format on polymer -95 coated slides. All the results using genomic target are shown below.

**Captures used:**

- **FV genomic WT** - 5’- TGG ACA GGC GAG GAA TAC AGG TAT -NH₂ - 3’
- **FV genomic Mut** - 5’- CTG GAC AGG CAA GGA ATA CAG GTA TT -NH₂ - 3’

**Detection Probe used**
FV Epi Pro 46 - 5' epi- CCA CAG AAA ATG ATG CCC AGT GCT TAA CAA GAC CAT ACT ACA GTG A 3'

Experimental Procedure for slides G1 to G3: A modified slide was washed with 0.2% SDS and water just prior to the experiment. After washing, all the slides were dried by spinning at room temperature. Typically, 35ul of hybridization buffer (2X SSC, 0.2 tween), 5ul of formamide, 5 ml of genomic target (4 mg per ml), 2 ml of magnesium chloride (24.5mM) 10 ml of colloid were mixed for each well and heated at 97°C and cooled at room temperature for 5 minutes. All the controls prepared using water instead of target DNA. Then, aliquots were transferred to the respective wells on the slide using pipette. After 120 minutes hybridization at 40 °C, the slides were washed with 5M NaNO₃ and amplified with a commercial silver amplification solution for 4 minutes at room temperature. The treated slides were then washed with water twice and dried by spinning the slide. Slides were imaged on VERIGENE (Nanosphere Imaging System) and the images were transferred into MS Word files. Where “T” is marked in that well target was used and where “C” is marked there control used.

Slide G1: Genomic DNA Factor V (5ug /ul sample) detection and total 20ug of the sample was used in the assay. Assay was performed at 40 °C and both mutant and wild type capture showed the signals because both wild type and mutant hybrids have above 40 °C melting point. See Figure 16(j).

Slide G 2: Genomic DNA Factor V (5ug /ul sample) detection and total 20ug of the sample was used in the assay. Assay was performed at 40 °C and both mutant and wild type capture were shoed up at 40 °C. See Figure 16(k).

Slide G 3: Two times diluted genomic DNA sample detection (2.5 ug/ul sample) and total 10 ug of the genomic DNA was used for each well. See Figure 16(L).

Slide G4: Genomic DNA Factor V (5ug /ul sample) detection and total 20ug of the sample was used in the assay. Assay was performed at 47 °C and at higher. See Figure 16(m) temperature target bound very weakly to mutant capture as shown in the picture.

Experimental Procedure for slides G4 to G5: Modified slide was washed with 0.2% SDS and water just prior to the experiment. After washing, all the slides were dried by spinning at room temperature. Typically, 35ul of hybridization buffer (2X SSC, 0.2 tween), 5ul of formamide, 5 ul of factor V genomic target (4 ug per ml), 2ul of
magnesium chloride (24.5 mM) 10 ul of colloid were mixed for each well and heated at 97°C and cooled at room temperature for 5 minutes. All the controls prepared using water instead of target DNA. Then aliquots were transferred to the respective wells on the slide using pipette. After 120 minutes hybridization at 47 °C, slides were washed with 5M NaNO₃ and amplified with a commercial silver amplification solution for 4 minutes at room temperature. Then washed with water twice and dried by spinning the slide. Slide was imaged on VERIGENE (Nanosphere Imaging System) and transferred the images to word file. Where “T” is marked in that well target was used and where “C” is marked there control used. Here we could differentiate wild type capture to mutant capture at 47 °C.

**Slide G5**: Genomic DNA Factor V (5ug /ml sample) detection and total 20 ug of the sample was used in the assay. Assay was performed at 47 °C and here mutant did not show up and wild type capture showed the signal. See Figure 16(n).

**Slide G6**: Genomic DNA Factor II (5ug /ml sample) detection and total 20 ug of the sample was used in the assay. Genomic DNA Factor II (5ug /ml sample). See Figure 16(o).

**Experimental Procedure for slide G6**: Modified slide was washed with 0.2% SDS and water just prior to the experiment. After washing, all the slides were dried by spinning at room temperature. Typically, 35ul of hybridization buffer (2X SSC, 0.2 tween), 5ul of formamide, 5 ml of genomic target (4 ug per ul), 2 ul of magnesium chloride (24.5mM) 10 ul of factor II colloid were mixed for each well and heated at 97°C and cooled at room temperature for 5 minutes. Then aliquots were transferred to the respective wells on the slide using pipette. After 120 minutes hybridization at 40 °C, slides were washed with 5M NaNO₃ and amplified with silver for 4 minutes at room temperature. Then washed with water twice and dried by spinning the slide. Slide was imaged on VERIGENE (Nanosphere Imaging System) and transferred the images to word file. Where “T” is marked in that well target was used and where “C” is marked there control used. All the controls prepared using water instead of target DNA. The results are shown in Figures 16(j) to (o).

In conclusion, we demonstrated both Factor V PCR and genomic sample target detection on newly developed copolymer-95 coated surface using oligonucleotides modified gold nanoparticle probes. This surface process is simple and economic to prepare in a commercially required environment.

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Example 11: Synthesis of 3' amine modified DNA linked gold nanoparticles (Fig. 17)

3'- amine linked oligonucleotide was synthesized on expedite gene synthesizer using 3'-amine support from Glen Research. At the end of the synthesis, 5’ end epiandrostosterone ("epi") disulfide phosphoramidite was attached to the 3'-amine oligonucleotide without using the detritylation step. After completing the synthesis the solid oligonucleotide linked support was dried and placed in ammonia solution at 55 °C overnight. After 18 hours of deprotection time, the ammonia was removed from the solution using a nitrogen flow into the tube. Then it was filtered and purified on HPLC using reverse phase column running under pH 7 phosphate buffer conditions. After purification, the 3’-amine and 5’- epi disulfide linked oligonucleotide was quantified by UV-Vis spectroscopy. The 3’-amine and 5’-epi disulfide oligonucleotide was loaded on citrate modified gold nanoparticles and kept at room temperature for 24 hours in the dark (4μM of modified oligonucleotide loaded per 1mL). See J. Am. Chem. Soc. 120, 1959-1964; Bioconjugate Chemistry, Vol 11, Number 2, P 289-291 which are incorporated by reference in its entirety. Then salt addition was started and increased to 0.5 M in 6 hours time and kept under salt conditions for a total of 40 hours at room temperature in the dark. After 40 hours the 3’ amine linked gold nanoparticles were filtered and centrifuged using plastic tubes. The sample was then washed with water and resuspended in 0.1 M NaCl, 10mM phosphate, 0.01% azide pH 7 buffer. The resulting DNA modified gold nanoparticle probes were stored in 0.1M NaCl, 10mM Phosphate and 0.01% sodium azide pH 7 buffer.

These 3’-amine modified DNA linked gold nanoparticles were then spotted on an aldehyde modified glass surface, at different concentrations for immobilization, using a Cartesian arrayer (see Example 13). After spotting slides were allowed to sit in the chamber for 8-12 hours and washed with water and amplified with silver to see the amplified signals of immobilized gold nanoparticles (Fig. 17).

Example 12: Synthesis of 3'-silyl linked gold nanoparticles preparation: (Fig 18)

3'- amine linked oligonucleotide was synthesized on an expedite gene synthesizer using 3'-amine support material obtained from Glen Research. At the end of the synthesis, 5’ end epi disulfide phosphoramidite was attached to the 3’-amine
oligonucleotide without using the detritylation step. After completing the synthesis, the solid oligonucleotide linked support was dried and put in ammonia solution at 55 °C overnight. After 18 hours of deprotection time all the ammonia was removed from the solution using nitrogen flow into the tube. It was then filtered and purified on HPLC using reverse phase column running under pH 7 phosphate buffer conditions. After purification, the 3'-amine and 5'-epi androsterone disulfide linked oligonucleotide was quantified using UV spectroscopy. The purified “3’amine-5’epianadrosterone” oligonucleotide was treated with 3-isocyanato propyl triethoxysilane in EtOH/DMSO mixture at room temperature to provide the 3’-silyl linked and 5’-epi disulfide oligonucleotide. After 1 hour, the reaction mixture was evaporated to dryness.

3’-silyl linked and 5’-epi disulfide oligonucleotide was loaded on citrate modified gold nanoparticles and kept at room temperature for 24 hours in the dark (4 μM of modified oligonucleotide loaded per 1mL). Salt addition was initiated and increased to 0.5 M in 6 hours of time and kept in the salt conditions total 40 hours at room temperature in the dark. The 3’ amine linked gold nanoparticles were then filtered and centrifuged using plastic tubes. The sample was then washed with water and resuspended in 0.1 M NaCl, 10 mM phosphate, 0.01% azide pH 7 buffer.

Example 13: Procedure for printing 3’ amine linked gold nanoparticles and 3’-silyl linked gold nanoparticles on glass slides:

3’-silyl linked DNA modified gold nanoparticles and 3’-amine modified gold nanoparticles (DSPs) were arrayed on slides as part of a two component system. Component A contained 2x the final concentration of DSPs in probe storage buffer (100 mM Sodium Chloride in 10mM Sodium Phosphate pH 7). Component B contained 2x Telechem Micro spotting Plus Buffer (Cat. I.D.: MSP, TeleChem International). Glass substrate slides used for arraying were NoAb Hydrogel Aldehyde Activated Slides obtained from Noab BioDiscoveries (Cat. number UAS0005HA).

Slides were arrayed using a Cartesian ProSys 4510-8SQ arrayer. The room temperature during the arraying process was between 20 and 25 °C, and the humidity inside the arrayer while the slides were being spotted was between 25% and 40% relative humidity. One hour after completion of the arraying run, the slides were incubated in a sealed chamber at greater than 85% relative humidity for 8-18 hours. The slides were
vacuum desiccated for at least one hour (but less than 24 hours), washed twice for two minutes with 0.2% sodium dodecyl sulfate (DNase, RNase, Protease free and 0.2um filtered) and washed twice for two minutes with MilliQ water. The slides were centrifuged to dryness (approx. 1 minute) and stored in a dessicator (relative humidity < 30%) until use.

The silver development process followed was as follows: Prior to enhancement Sigma silver solutions A and B were transferred from 4 °C to a 25 °C water bath and equilibrated for at least 20 minutes. Equal volumes of Sigma A and B were mixed, and used to develop signal for between 2-7 minutes. The reaction was stopped by adding 5% acetic acid, rinsed in acetic acid wash (x3), and slides finally rinsed with MilliQ water (x4). Slides were spun-dried for 45 seconds and scanned on an ArrayWorx E Biochip Reader.

The scan settings for the Arrayworx were: Channel: 4, Exposure time: 0.2 seconds, Sensitivity: Hi Dynamic Range and Resolution: 13.0080 um (Figures 19 and 20). In Fig. 19, four dilutions for each prep were spotted starting at 3.5 nM. The TriEthoxy seems to give a much larger signal and a dose response. No carry-over was observed. The spots to the right were analyzed and will be presented as data. The spots to the left were not analyzed.

In Fig. 20, 0.8 M means probe was loaded in 0.8M salt conditions and 0.5 M means probe was loaded in 0.5 M salt conditions. Triethoxy means triethoxy silyl linked gold nanoparticles and monoethoxy means dimethylmonoethoxy silyl liked gold nanoparticles. Above shows the average of all data from 8 slides. No error bars are shown because the variability from slide to slide (in signal) is very large. However, the same trend was observed. A dose response was observed in all conditions however, the triethoxy had significantly higher signal.

**Example 14: Preparation of additional substrates and evaluation**

In this Example, several additional substrates were prepared and evaluated. The reagents used and conditions are described below.

**Cleaning of Glass Slides (1a):**
Glass slides were washed and prepared by immersion in four separate solutions at ambient temperature followed by rinsing with water three times after each wash. First, the slides were in a 5% w/v solution of sodium hydroxide in water for 30 minutes. The slides were then washed in a 5% v/v hydrochloric acid solution for 30 minutes, followed by a 3% hydrogen peroxide:5% v/v hydrochloric acid solution (H₂O₂:HCl) for 30 minutes. After rinsing with water the slides were rinsed with ethanol, three times, dried by spinning using a slide centrifuge. After air drying, the slides were placed in an oven overnight at 120 °C.

Cleaning of Plastic Slides (1b):
Plastic slides were washed and prepared by immersion in four separate solutions at ambient temperature followed by rinsing with water three times after each wash. First, the slides were washed in an ethanol solution for 30 minutes, followed by a wash in a 5% w/v solution of sodium bicarbonate in water for 30 minutes. The slides were then washed in a 5% v/v hydrochloric acid solution for 30 minutes, followed by a 3% hydrogen peroxide:5% v/v hydrochloric acid solution (H₂O₂:HCl) for 30 minutes. After rinsing with water the slides were rinsed with ethanol, three times, dried by spinning using a slide centrifuge. After air drying, the slides were placed in a dessicator. Plastic is polycarbonate (Lexan®, Plexiglass®), polyphenol (Backlite®) and polycyclonornborne.

Preparation of 2-Trimethoxysilane-6-(trisocyanatosilaneurea) benzene (4):
Tetraisocyanatosilane (2) (6.6692 g, 0.034 mol) and m-aminophenyltrimethoxysilane (3) (7.2954 g, 0.0342 mol) were mixed and stirred for 20 minutes at room temperature. To the solution was added dry ethanol (850 mL) and was stirred an additional 45 minutes at room temperature to produce (4). 4 was left as a solution and added to the slides.

Silanation of Glass Slides (5a) with 4:
Glass slides (25) were treated with a 250 mL of 0.04 M (4) {((2-Trimethoxysilane-6-trisocyanatosilaneurea) benzene} for 2 hours at ambient temperature with slight agitation. The slides were then removed, washed with ethanol three times and dried by spinning using a slide centrifuge to produce (5a). The substrate (5a) was further dried overnight in a desiccator under vacuum.
Silanation of Plastic Slides (5b) with 4:
Plastic slides (65) in two containers were treated with a 300 mL of 0.04 M (4) \{(2-
Trimethoxysilane-6-trisocyanatosilaneurea) benzene\} each, for 2 hours at ambient
temperature with slight agitation. The slides were then removed, washed with ethanol
three times and dried by spinning using a slide centrifuge to produce (5b). The substrate
(5b) was further dried overnight in a desiccator under vacuum.

Reaction of 5a (glass) and 5b (plastic) with Diaminobenzidine (DAB) to give 6a
(glass) and 6b (plastic):
3,3'-Diaminobenzidine tetrahydrochloride dihydrate (8.9 g, 0.0225 mol) and triethyl
amine (11.84 g, 0.117 mol) (TEA) were added to 834 mL of ethanol and stirred for one
hour at room temperature and filtered. Twenty five silylated glass slides were placed in
250 mL of solution DAB at ambient temperature for 3 hours and then washed three times
with ethanol for 5 minutes followed drying via spinning using the slide centrifuge to
produce 6a. Sixty five plastic slides in two containers were treated with 300 mL and 250
mL each of a solution diaminobenzined ( DAB) for 3 hours at ambient conditions. The
plastic slides were washed with ethanol (3X) for five minutes each followed drying via
spinning using the slide centrifuge to produce 6b.

Reaction of 5b (plastic) with Water:Ethanol to produce 6c (plastic):
Slides (5b) were treated with 130 mL of water-ethanol mixture (1:1) under slight
agitation for 2 hours at ambient temperature. The slides were removed and washed with
ethanol (2 X 100 mL) and dried in a desiccator at ambient temperature under vacuum to
produce 6c.

Capping of residual isocyanate groups of 6a (glass) and 6b (plastic) with glyine to
produce 7a (glass) and 7b (plastic):
Glycine (6.38 g, 0.085 mol) was added to 850 mL of water and stirred for 15 minutes at
ambient temperature to make 0.1 M glycine solution. Twenty five glass slides (6a) and
plastic slides (6b) were placed in the 0.1 M Glycine solution at ambient temperature with
slight agitation for two hours. The slides were washed with water (2X) for five minutes,
followed with ethanol (3X) for five minutes each time and dried by spinning using a slide centrifuge to produce 7a (Glass) and 7b (plastic).

**Reaction of 7a (glass) and 7b (plastic) with tolylene-2,6-diisocyanate to produce 8a (glass) and 8b (plastic):**

To 0.2624 g (0.00146 mol) of tolylene-2,6-diisocyanate, 850 mL of dry hexane was added and stirred for 15 minutes under ambient conditions. Twenty four glass slides (7a) were placed in 250 mL of solution of tolylene diisocyanate and 64 plastic (7b) in two separate containers of 300 mL and 250 mL respectively, and were kept at ambient temperature for 4 hours. The slides were washed with hexane (3X) and dried by spinning using a slide centrifuge to produce 8a (glass) and 8b (plastic). Note: tolylene-2,6-diisocyanate was replaced with 1,4-phenylene diisocyanate with a similar process.

**Reaction of 7a (glass) with 1,4-Phenylene Diisocyanate to produce 8c (glass).**

1, 4-Phenylene diisocyanate, (0.095 g, 0.000594M) was added to 250 mL of dimethylformamide (DMF) (0.0024 mol/L solution) and stirred for 15 minutes at room temperature. Twenty-five glass slides (7a) were placed in the diisocyanate solution and kept at room temperature for 3 hours. After completion, slides were washed one time with DMF and three times with ethanol (for 5 min each time) and dried by spinning using a slide centrifuge to produce 8c.

**Reaction of 8a (glass) with Poly (dimer acid-co-polyamine)-95 (P95) to produce 9a (glass):**

Poly (dimer acid-co-polyamine)-95 (3.0 grams) was added to the mixture of 225 mL of pyridine and 35 mL of dichloromethane and stirred for 1.5 hours at ambient temperature. Twenty-four glass slides (8a) were treated with polymer solution for 4 hours under slight agitation at ambient temperature. The slides were then washed with pyridine for 5 minutes (2 x 250 mL), dichloromethane for 5 minutes (1 x 250mL), ethanol for 5 minutes (1 x 250 mL), di-isopropylethylamine:ethanol:water solution (5:127:127 mL ratio) for 2h followed with ethanol for 5 minutes (3 x 250 mL). Slides were dried by spinning using a slide centrifuge to produce 9a.
Reaction of 8b (plastic) with 3,3’-Diaminobenzidine Tetrahydrochloride Dihydrate (DAB) to produce 9b (plastic):
3,3’-Diaminobenzidine tetrahydrochloride dihydrate, (1.07 g, 0.0027mol), and triethyl amine (TEA) (1.4207 g, 0.014 mol) were added to 270 mL of ethanol and stirred for 1 hour at ambient temperature and filtered. Fifteen plastic slides (8b) were placed in solution and kept at temperature for 4 hours under slight agitation. The slides were then washed three times with ethanol (for 5 min each time) and dried by spinning using the slide centrifuge to produce 9b.

Reaction of 8b (plastic) with PAMAM Dendrimer Generation 0 to produce 9c (plastic):
A 20% methanol solution of PAMAM (0.58 mL) was added to 150 mL of ethanol, stirred for 30 minutes at ambient temperature. Five slides were placed in this solution and were treated for 4 hours under slight agitation. Then slides were washed three times with ethanol (for 5 min each time) and dried by spinning using the slide centrifuge to produce 9c.

Reaction of 8b (plastic) with Poly(m-xylylendiamine-epichlorohydrine), diamine terminated (PoXyl) to produce 9d (plastic):
PoXyl, (4.19 g, 0.00624 mol) was added to 520 mL of ethanol, stirred for 20 minutes at room temperature. Forty four plastic slides were placed in this solution. Slides were treated for 4 hours under slight agitation. Then slides were washed three times with ethanol (for 5 min each time) and dried by spinning using the slide centrifuge to produce 9d. Dried in vacuum overnight.

Reaction of 5b (plastic) with DAB-Am-8, Polypropyleneimine Octaamine Dendrimer, Generation 2.0 to produce 9e (plastic):
Polypropyleneimine Octaamine (0.60 g, 0.000775 M, MW = 773.31 g/mole) was added to 125 mL of ethanol and stirred for 30 minutes at ambient temperature. Eight silylated slides (5b) were reacted with the solution polyamine solution at ambient temperature for 2 hours. The slides were washed three times with ethanol (3 x 100 mL, for 5 min each time) and dried by spinning using the slide centrifuge.
Reaction of 8c (glass) with Poly (dimmer acid-co-polyamine)- 95 (P95) to produce 9f (glass):
Poly (dimmer acid-co-polyamine)- 95 (3.0 g) was added to the mixture of 225 mL of pyridine and 35 mL of Dichloromethane and stirred for 1.5 hours at ambient temperature. Twenty-five slides (8c) were placed in the P95 solution for 3 hours. Then slides were washed with pyridine (2X, 5 minutes each), dichloromethane (5 minutes), ethanol (5 minutes), a solution of triethylamine:ethanol (5 mL:225 mL) for 2 hours, and a final rinse with ethanol (3X, 5 minutes each). The slides were dried by spinning using a slide centrifuge to produce 9f.

Reaction of 5a (glass) with Poly (dimmer acid-co-polyamine)- 95 (P95) to produce 9g (glass):
Poly (dimer acid-co-polyamine) - 95 (1.5 g) was added to 150 mL pyridine-dichloromethane mixture (5: 1) and stirred for 1.5 hours at ambient temperature. Slides (5a) were placed in the P95 solution and kept at room temperature for 4.5 hours. Then slides were washed with pyridine (5 minutes each), dichloromethane (2X 5 minutes), ethanol (3X, 5 minutes). The slides were dried by spinning using a slide centrifuge to produce 9g.

Reaction of 9a (glass) with Hexamethylene diisocyanate (DCH) to produce 10a (glass):
Hexamethylene diisocyanate (DCH), 22.891 grams (0.1361 mol) was added to 208 mL of hexane and stirred for 15 minutes at ambient conditions. Fifteen slides (9a) were treated with DCH solution for 5 hours under slight agitation. The slides were washed with hexane (2 x 250 mL) and ethanol (2 x 250 mL). Slides were dried by spinning using a slide centrifuge and stored in a dessicator to produce 10a.

Reaction of 9a (glass) with Triethyl Citrate to produce 10b (glass):
Triethyl citrate (4.421 grams, 0.016 mol) and 3.48 mL of diisopropylethylamine (DIPEA) were added to 228 mL of ethanol and stirred for 15 minutes at room temperature. Fourteen glass slides 9a were treated with citrate solution for 5 hours under
slight agitation. The slides were washed with ethanol (3 x 250 mL) and dried by spinning using a slide centrifuge.

**Reaction of 9a (glass), 9b (plastic), 9c (plastic), 9d (plastic) with 4,4’-Dicyclohexylmethane diisocyanate (HMDI) to produce 10e (glass), 10f (plastic), 10g (plastic), 10h (plastic) respectively.**

A solution of 4,4’-Dicyclohexylmethane diisocyanate (HMDI) was prepared for 62 plastic and 23 glass slides from a combination of plastic and glass from 9a - 9d. HMDI (77.64 g, 0.296 mol) and 1050 mL of Hexane (0.26mol/L) were combined and stirred for 15 minutes at room temperature. Sixty two plastic slides in two containers were treated with 300 mL and 250 mL of HMDI solution and twenty three glass slides in a separate container were treated with 250 mL of HMDI solution at ambient temperature for 6 hours. The slides were washed three times with hexane, ethanol once and dried by spinning on the slide centrifuge to produce 10e (glass), 10f, 10h, 10g (plastic).

**Reaction of 6c (plastic) with 1,6-Hexamethylene diisocyanate (DCH) to produce 10i (plastic):**

1,6-Hexamethylene diisocyanate (13.08 g) and 75ml of dry ethanol were mixed together at ambient temperature. Five slides after (6c) were placed into vertical slide staining dish and treated with DCH solution for 4 hours under slight agitation. The slides were washed with ethanol (3 x 50 mL) and dried by spinning.

**Reaction of 6c (plastic) with Triethyl Citrate to produce 10j (plastic):**

Triethyl citrate (27.6 g) and triethyl amine(3.03 g) were added to 75 mL of ethanol and stirred for 15 minutes. Five slides from (6c) were treated with citrate solution for 24 hrs at ambient temperature under slight agitation. The slides were washed three times with ethanol (3 x 50 mL) and dried by spinning using the slide centrifuge.

**Reaction of 9e (plastic) with Hexamethylene diisocyanate (DCH) to produce 10k (plastic):**

1,6-Hexamethylene diisocyanate (DCH) (10.01 g, 0.060 M) was added to 120 mL of dry ethanol and mixed thoroughly at ambient temperature. Eight slides (9e) were treated with DCH solution for 5 hours at ambient temperature under agitation. The slides were
washed three times with ethanol (3x100mL) and dried by spinning using the slide centrifuge.

Reaction of 9f (glass) with 1, 6-Hexamethylene Diisocyanate to produce 10L (glass):
Four slides (9f) were placed into Vertical Slide Staining Dish containing 10 mL of a 0.5 M solution of (10.1 g in 9.9 mL of ethanol) 1, 6-hexamethylene diisocyanate in 110mL of EtOH. Slides were treated diisocyanate for 5 hours at ambient temperature. Slides were removed, washed three times with ethanol and dried by spinning using a slide centrifuge to give 10L.

Reaction of 9f (glass) with 4,4'-Dicyclohexylmethane diisocyanate (HMDI) to produce 10m (glass):
Four slides (9f) were placed in Vertical Slide Staining Dish containing 70 mL of 0.50 M solution of (9.18 g/ 8.6 mL (0.035 Mol) 4,4'-Dicyclohexylmethane diisocyanate (HMDI) in 61 mL of dimethylformamide (DMF) for 5 hours at ambient temperature. Slides were removed, washed three times with DMF and dried by spinning using a slide centrifuge to give 10m.

Reaction of 9f (glass) with 4,4'-Methylene diphenyl diisocyanate (MDI) to produce 10n (glass):
Four slides (9f) were placed in Vertical Slide Staining Dish containing 70 mL of 0.50 M solution of (8.76 g, 0.035 Mol) 4,4'-Methylene diphenyl diisocyanate (MDI) in 70 mL of dimethylformamide (DMF) for 5 hours at ambient temperature. Slides were removed, washed three times with DMF and dried by spinning using a slide centrifuge to give 10n.

Reaction of 9f (glass) with to produce 10o (glass)
Four slides (9f) were placed in Vertical Slide Staining Dish containing 120 mL of 0.50 M solution of (16.58 g, 0.006 Mol) triethyl citrate (T) in 101 mL of ethanol and (3.33 g, 0.034 M) of triethyl amine (TEA). Slides were kept at room temperature for 24 hours and washed three times with ethanol and dried by spinning using a slide centrifuge to give 10o.
Reaction of 9f (glass) with triethyl citrate and 1,6-Hexamethylenediisocyanate to produce 10p (glass):
Three slides (9f) were placed in Vertical Slide Staining Dish containing a solution of triethyl citrate (7.425 g, 0.027 mol), 1,6-Hexamethylenediisocyanate (1.35 g, 0.008 mol) and triethylamine (0.97 g, 0.010 mol) in in 61 mL of ethanol for 5 hours at ambient temperature. Slides were removed, washed three times with ethanol and dried by spinning using a slide centrifuge to give 10p.

Reaction of 9f (glass) with triethyl citrate and 4,4'-Dicyclohexylmethane diisocyanate (HMDI) to produce 10q (glass):
Three slides (9f) were placed in Vertical Slide Staining Dish containing a solution of triethyl citrate (7.425 g, 0.027 mol), 4,4'-Dicyclohexylmethane diisocyanate (HMDI) (2.10 g, 0.008 mol) and triethylamine (0.97 g, 0.010 mol) in in 60 mL of dimethylformamide (DMF) for 5 hours at ambient temperature. Slides were removed, washed three times with DMF and dried by spinning using a slide centrifuge to give 10q.

Reaction of 9f (glass) with Triethyl Citrate and 4,4'-Methylene diphenyl diisocyanate (MDI) to produce 10r (glass):
Three slides (9f) were placed in Vertical Slide Staining Dish containing a solution of triethyl citrate (7.425 g, 0.027 mol), 4,4'-Methylene diphenyl diisocyanate (MDI) (2.0 g, 0.008 Mol) and triethylamine (0.97 g, 0.010 mol) in in 62 mL of dimethylformamide (DMF) for 5 hours at ambient temperature. Slides were removed, washed three times with DMF and dried by spinning using a slide centrifuge to give 10r.

Reaction of 9f (glass) with ethylene glycol-bis-succinimidylsuccinate (3-EGS) to produce 10s (glass):
Slides 9f were treated with a 20 mM solution of (0.456g in 50 mL of DMSO) ethylene glycol-bis-succinimidylsuccinate (3-EGS) in DMSO for 4 hours at room temperature. The treated slides were then washed with ethanol: DMSO mixture (9:1) once, three times with ethanol, then dried.

Reaction of 9f (glass) with 1,6 Hexamethylenediisocyanate to produce 10t (glass):
Slides 9f were treated with a 26 mM solution of (0.436g in 100 mL DMSO) of 1,6-Hexamethylene diisocyanate for 4 hours at ambient temperature. The treated slides were then washed once with ethanol: DMSO (9:1) mixture, three times with ethanol and dried.

**Arraying of Slides:**
Amine modified oligonucleotide captures were arrayed using a pin style or non-contact arraying system. The capture oligonucleotides were dissolved in aqueous solutions to prepare a range of concentrations (40 mM to 400 mM) with (0.001% to a 0.1% concentration) sodium dodecylsulfate (SDS), 300 mM phosphate buffer at pH = 7.2, and/or formamide. Several different types of capture oligonucleotide sequences were prepared with either 5’ or 3’ modified amino sequences ranging from 10 to 100 bases in length. The oligonucleotides may also have 3’-amino modified linkers attached to the end.

**Blocking of Arrayed Slides:**
Ten slides were placed in a solution of 0.525 g of glycine in 70 mL of water and kept at ambient conditions under slight agitation for 2 hours. Slides were washed with SDS (0.2%) solution and three times with water. Slides were dried for 2 minutes on the slide centrifuge.

**Typical hybridization Conditions for Modified Slides – Single Step Method:**
Arrayed slides were washed with 0.2% SDS prior to the assay development. A reaction volume of 50 μL was prepared per well using hybridization buffer (10 x SSC; 0.1% tween, formamide concentration of 18-70%). Nanoparticle probes (1 nM) and genomic target concentrations of 1μg/1μl were used in the hybridization mixture. The hybridization mixture was heated at 97 °C for 5 minutes and cooled at ambient temperature for 5 minutes, then transferred to the wells. Slides were kept at 40 °C – 41 °C for hybridization under humid conditions and then washed with buffer (0.5 M NaNO₃, 0.01 % tween). The nanoparticles on the surface were then exposed to silver enhancement reagents (Sigma-Aldrich) for 5 minutes and imaged with Verigene® detector (Nanosphere) for data analysis. The development of nanoparticle probes with oligonucleotides is described elsewhere. (Techniques for functionalizing oligonucleotides with sulfide groups and attachment to nanoparticles are described for
instance in published U.S. patent application numbers 2003/0143598A1 and
2002/0155442A1, each of which is incorporated herein by reference in its entirety. A
preferred sulfide linker for linking the oligonucleotide to the nanoparticle is an
epiandrostosterone linker. See PCT/US01/01190, filed January 12, 2001, which is
incorporated by reference in its entirety.)

Each well is marked with appropriate letter and T, C, +ve C, M and Het.
T = Genomic wild type DNA target was used with specific probe and hybridization
buffer. No positive control probe and no other target were used.
C = Genomic probes mixed with hybridization buffer were used without any target or
positive control probe.
+ve C = Positive control probe in hybridization buffer was used without any target or any
other probes.
M = Genomic mutant type target was used in these wells with hybridization buffer and
specific probe. No positive control probe or other target were used.
Het = Genomic heterozygous type target was used in these wells with hybridization
buffer and specific probe. No positive control probe or other target were used.

Each well is marked with appropriate letter and T = genomic target used in these wells, C
= Control, no target, no +Ve control probe, +VeC = Positive control probe, no target, M=
mutant target used and Het = heterozygous target was used.

Typical hybridization Conditions for Modified Slides – Dual Step Method:
Step 1: Arrayed slides were washed with 0.2% SDS just prior to the assay development.
A total of 50 µL of reaction volume was prepared per well using hybridization buffer (10
x SSC; 0.1% tween, formamide concentration of 18-70%), genomic DNA target
concentrations varied from $10^2$ to $10^7$ copies per µL. Hybridization mixture was denatured
at 95 °C for 4 min and cooled at ambient temperature for 3 minutes then transferred to the
respective wells. Slides were incubated at 40 °C for 2h for hybridization under humid
conditions.
Step 2: After target hybridization, the slides were washed with NaNO₃, and tween and
spin dried. The probe solution was mixed with the hybridization mixture and was added
to each well and incubated at 40°C under humid conditions for 30-120 minutes. Slides
were washed with wash buffer and treated with silver solutions A and B for five minutes and imaged with Verigene® instrument for data analysis. The development of nanoparticle probes with oligonucleotides is described elsewhere (Techniques for functionalizing oligonucleotides with sulfide groups and attachment to nanoparticles are described for instance in published U.S. patent application numbers 2003/0143598A1 and 2002/0155442A1, each of which is incorporated herein by reference in its entirety. A preferred sulfide linker for linking the oligonucleotide to the nanoparticle is an epiandrosterone linker. See PCT/US01/01190, filed January 12, 2001, which is incorporated by reference in its entirety).

Each well is marked with appropriate letter and T, C, +ve C, M and Het.

T = Genomic wild type DNA target was used with specific probe and hybridization buffer. No positive control probe and no other target were used.

C = Genomic probes mixed with hybridization buffer were used without any target or positive control probe.

+ve C = Positive control probe in hybridization buffer was used without any target or any other probes.

M = Genomic mutant type target was used in these wells with hybridization buffer and specific probe. No positive control probe or other target were used.

Het = Genomic heterozygous type target was used in these wells with hybridization buffer and specific probe. No positive control probe or other target were used.

Reagent List:
NaOH – Sodium Hydroxide; Pellets; Fisher Scientific
HCl – Hydrochloric Acid, 35-38%; Fisher Scientific
H₂O₂ – Hydrogen Peroxide, 30%; Fisher Scientific
EtOH – Ethyl Alcohol, 200 proof; ACS/USP Grade; Pharmco, Inc.
NaHCO₃ – Sodium Bicarbonate; Fisher Scientific
Si(NCO)₄ – Tetraisocyanatosilane; Gelest, Inc.
m-Aminophenyltrimetoxy silane, Gelest, Inc.
DAB – 3,3’-Diaminobenzidine tetrahydrochloride dihydrate
TEA – Triethylamine; Fisher Scientific
GLY – Glycine, Sigma-Aldrich
TDIC – Tolyene-2,6-diisocyanate, Aldrich
Hexn – Hexane, Fisher Scientific
PDIC – 1,4-Phenylene diisocyanate, Aldrich
P95 – Poly(dimer acid-co-polyamine)-95, Sigma-Aldrich
Pyridine – Sigma-Aldrich
DCM – Dichloromethane, Fisher Scientific
DiPEA – Diisopropylethylamine, Aldrich
PAMAM Dendrimer Generation 0, Sigma-Aldrich
PoXyl – Poly(m-xylidendiamine-epichlorohydrine), diamine terminated, Aldrich
DAB-Am-8 – Polypropyleneimine Octaamine Dendrimer, Generation 2.0, Aldrich
DCH – Hexamethylenediisocyanate, Sigma-Aldrich
T – Triethyl Citrate, Sigma-Aldrich
HMDI – 4,4′-Dicyclohexylmethane diisocyanate, Aldrich
MDI – 4,4′-Methylene-diphenyl diisocyanate, Aldrich
3-EGS – Ethylene glycol-bis-succinimidylsuccinate, Pierce
DMSO – Dimethyl sulfoxide, Aldrich
SDS – Sodium Dodecyl Sulfate, 20% solution, Fisher Scientific
Formamide, Sigma-Aldrich, Fisher Scientific
20xSSC Buffer, Invitrogen, Fisher Scientific
Tween 20 – Polyoxyethylene sorbitan monolaurate, Sigma-Aldrich
NaNO₃ – Sodium Nitrate, Sigma-Aldrich
Silver enhancer solution A, Sigma-Aldrich
Silver enhancer solution B, Sigma-Aldrich

EQUIPMENT AND MATERIALS

Timer refers to Fisher Scientific, Traceable Timer, Cat. # 06-662-55, Calibr. Due 06/05, #320808935

Centrifuge refers to Telechem Int’l, Microarray High-Speed Centrifuge, Cat. # MHC110V

Termix Stirrer refers to Fisher Scientific, Cat. # 14-493-120S, Model 120S

Hg-Thermometer refers to Fisher Scientific, Cat. # 103606, -10 °C - +350 °C, 1 °C, Ser. #2121

Filter paper, Whatman Int, Ltd., Cat. # 1202-320

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References:


7. (a) *Nucleic Acids research*, vol. 28, No.13 E71 (2000);  
   (b) Huber et al. WO 01/46214, published June 28, 2001  
   (c) Huber et al. WO 01/46213, published June 28, 2001  
   (d) Huber et al. WO 01/46464, published June 28, 2001


WHAT IS CLAIMED:

1. A method for immobilizing a molecule onto a surface, said method comprising the steps of:
   (a) contacting the molecule with an agent so as to form a reactive intermediate, said agent having a formula i:

   \[(R_1)(R_2)(R_3)\text{Si-X-NCY} \quad \text{i}\]

   wherein \(R_1\), \(R_2\) and \(R_3\) independently represents \(C_1\text{-}C_6\) alkoxy, \(C_1\text{-}C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1\text{-}C_6\) alkyl and \(C_1\text{-}C_6\) alkoxy; \(X\) represents linear or branched \(C_1\text{-}C_{20}\) alkyl or aryl substituted with one or more groups selected from the group consisting of \(C_1\text{-}C_6\) alkyl and \(C_1\text{-}C_6\) alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; and \(Y\) represents oxygen or sulfur, with the proviso that at least one of \(R_1\), \(R_2\) or \(R_3\) represents \(C_1\text{-}C_6\) alkoxy; and
   (b) contacting the reactive intermediate with said surface so as to immobilize the molecule onto said surface.

2. The method of claim 1 wherein the surface is a glass surface.

3. The method of claim 1 wherein the surface has at least one group that reacts with the reactive intermediate.

4. The method of claim 3 wherein the group comprises a hydroxyl or amino group.

5. The method of claim 1 wherein the agent is 3-(isocyanatopropyl)triethoxysilane.

6. The method of claim 1 wherein the molecule comprises a probe.
7. The method of claim 6, wherein the probe comprises a protein, a peptide, a nucleic acid, a peptide nucleic acid, an amino acid, a linked nucleic acid, a nucleoside triphosphate, a carbohydrate, a lipid, a lipid bound protein, an aptamer, a virus, a cell fragment, or a whole cell.

8. The method of claim 7, wherein the lipid bound protein comprises a G-protein coupled receptor.

9. The method of claim 6, wherein the probe comprises an antibody, an antigen, a receptor, or a ligand.

10. A method for immobilizing a molecule onto a glass surface comprising the steps of:
    (a) contacting the molecule with an agent so as to form a reactive intermediate, said agent having a formula i:

    \[(R_1)(R_2)(R_3)Si-X-NCY\]

    wherein \(R_1, R_2\) and \(R_3\) independently represents \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkyl and \(C_1-C_6\) alkoxy; \(X\) represents linear or branched \(C_1-C_{20}\) alkyl or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkyl and \(C_1-C_6\) alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; and \(Y\) represents oxygen or sulfur, with the proviso that at least one of \(R_1, R_2\) or \(R_3\) represents \(C_1-C_6\) alkoxy; and

    (b) contacting the reactive intermediate with the glass surface so as to immobilized the molecule onto said surface.

11. The method of claim 10, wherein the molecule is a probe.

12. The method of claim 11, wherein the probe comprises a protein, a peptide, a nucleic acid, a peptide nucleic acid, an amino acid, a linked nucleic acid, a nucleoside
triposphosphate, a carbohydrate, a lipid, a lipid bound protein, an aptamer, a virus, a cell fragment, or a whole cell.

13. The method of claim 12, wherein the lipid bound protein comprises a G-protein coupled receptor.

14. The method of claim 11, wherein the probe comprises an antibody, an antigen, a receptor, or a ligand.

15. A method for immobilizing a molecule onto a surface, said method comprising the steps of:

(a) contacting Si(NCY)$_4$ wherein Y represents oxygen or sulfur with an agent so as to form a first reactive intermediate, said agent having a formula ii:

$$(R_1)(R_2)(R_3)Si-X-Z$$

wherein $R_1$, $R_2$ and $R_3$ independently represents C$_{1-6}$ alkoxy, C$_{1-6}$ alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of C$_{1-6}$ alkyl and C$_{1-6}$ alkoxy; X represents linear or branched C$_{1-20}$ alkyl or aryl substituted with one or more groups selected from the group consisting of C$_{1-6}$ alkyl and C$_{1-6}$ alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; and Z represents a hydroxy or amino group, with the proviso that at least one of $R_1$, $R_2$ or $R_3$ represents C$_{1-6}$ alkoxy;

(b) contacting the first reactive intermediate with a molecule so as to form a second reactive intermediate;

(c) contacting the second reactive intermediate with said surface so as to immobilized the molecule onto said surface.

16. The method of claim 15 wherein the surface is a glass surface.

17. The method of claim 15 wherein the surface has at least one group that reacts with the reactive intermediate.
18. The method of claim 17 wherein the group comprises a hydroxyl or amino group.

19. The method of claim 15 wherein the agent is 4-aminophenyl trimethoxysilane, 4-aminophenyl triethoxysilane, 3-aminophenyltrimethoxysilane, 3-aminophenyltriethoxysilane, or mixtures thereof.

20. The method of claim 19 wherein the agent and tetraisocyanatosilane is present at a 4:1 molar ratio.

21. The method of claim 15 wherein the molecule and the first reactive intermediate is present in a 3:1 molar ratio.

22. The method of claim 15 wherein the first reactive intermediate has a formula iv:

\[(R_1)(R_2)(R_3)\text{Si-X-Z-CYNH-Si (NCY)}_3 \text{ iv}\]

wherein \(R_1, R_2,\) and \(R_3\) independently represents \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkyl and \(C_1-C_6\) alkoxy; \(X\) represents linear or branched \(C_1-C_{20}\) alkyl or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkyl and \(C_1-C_6\) alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; \(Y\) represents oxygen or sulfur; and \(Z\) represents oxygen or NH, with the proviso that at least one of \(R_1, R_2,\) or \(R_3\) represents \(C_1-C_6\) alkoxy.

23. The method of claim 22 wherein \(R_1, R_2,\) and \(R_3\) represents methoxy, \(X\) represents phenyl, \(Y\) represents oxygen, and \(Z\) represents NH.

24. The method of claim 22 wherein the second reactive intermediate has a formula v:

\[(R_1)(R_2)(R_3)\text{Si-X-Z-CYNH-Si (NHCYL-M)}_3 \text{ v}\]

wherein \(R_1, R_2,\) and \(R_3\) independently represents \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substitutes
alkyl and C_1-C_6 alkoxy; L represents a linking group; X represents linear or branched C_1-C_{20} alkyl or aryl substituted with one or more groups selected from the group consisting of C_1-C_6 alkyl and C_1-C_6 alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; Y represents oxygen or sulfur; and Z represents oxygen or NH; and M represents a molecule, with the proviso that at least one of R_1, R_2, or R_3 represents C_1-C_6 alkoxy.

25. The method of claim 24 wherein L represents a nucleophilic group from the molecule.

26. The method of claim 25 wherein the nucleophilic group comprises –NH, –S–, –O–, or –OOC–.

27. The method of claim 15 wherein the first reactive intermediate has a formula vi:

\[(R_1)(R_2)(R_3)Si-X-Z-CY\text{NH}_{2}Si\text{ (NCY)}_{2}\]  

vi

wherein R_1, R_2 and R_3 independently represents C_1-C_6 alkoxy, C_1-C_6 alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of C_1-C_6 alkyl and C_1-C_6 alkoxy; X represents linear or branched C_1-C_{20} alkyl or aryl substituted with one or more groups selected from the group consisting of C_1-C_6 alkyl and C_1-C_6 alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; Y represents oxygen or sulfur; and Z represents oxygen or NH, with the proviso that at least one of R_1, R_2, or R_3 represents C_1-C_6 alkoxy.

28. The method of claim 27 wherein R_1, R_2 and R_3 represents methoxy, X represents phenyl, Y represents oxygen, and Z represents NH.

29. The method of claim 27 wherein the second reactive intermediate has a formula vii:

\[(R_1)(R_2)(R_3)Si-X-Z-CYNH]_{2}Si[NHCYL-M]_{2}\]  

vii
wherein R1, R2 and R3 independently represents C1-C₆ alkoxy, C1-C₆ alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of C1-C₆ alkyl and C1-C₆ alkoxy; L represents a linking group; X represents linear or branched C1-C₂₀ alkyl or aryl substituted with one or more groups selected from the group consisting of C1-C₆ alkyl and C1-C₆ alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; Y represents oxygen or sulfur; and Z represents oxygen or NH; and M represents a molecule, with the proviso that at least one of R1, R2, or R3 represent C1-C₆ alkoxy.

30. The method of claim 29 wherein L represents a nucleophilic group from the molecule.

31. The method of claim 30 wherein the nucleophilic group comprises -NH, -S-, -O-, or -OOC-.

32. The method of claim 15 wherein the molecule comprises a probe.

33. The method of claim 32, wherein the probe comprises a protein, a peptide, a nucleic acid, a peptide nucleic acid, an amino acid, a linked nucleic acid, a nucleoside triphosphate, a carbohydrate, a lipid, a lipid bound protein, an aptamer, a virus, a cell fragment, or a whole cell.

34. The method of claim 33, wherein the lipid bound protein comprises a G-protein coupled receptor.

35. The method of claim 32, wherein the probe comprises an antibody, an antigen, a receptor, or a ligand.

36. A method for immobilizing a molecule onto a glass surface, said method comprising the steps of:

(a) contacting Si(NCY)₄ wherein Y represents oxygen or sulfur with an agent so as to form a first reactive intermediate, said agent having a formula ii:
(R_1)(R_2)(R_3)Si-X-Z

wherein R_1, R_2 and R_3 independently represents C_1-C_6 alkoxy, C_1-C_6 alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of C_1-C_6 alkyl and C_1-C_6 alkoxy; X represents linear or branched C_1-C_{20} alkyl or aryl substituted with one or more groups selected from the group consisting of C_1-C_6 alkyl and C_1-C_6 alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, and sulfur; and Z represents a hydroxy or amino group, with the provision that at least one R_1, R_2, or R_3 represents C_1-C_6 alkoxy;

(b) contacting the first reactive intermediate with a molecule so as to form a second reactive intermediate;

(c) contacting the second reactive intermediate with said surface so as to immobilized the molecule onto said surface.

37. The method of claim 36 wherein the surface is a glass surface.

38. The method of claim 36 wherein the surface has at least one group that reacts with the reactive intermediate.

39. The method of claim 38 wherein the group comprises a hydroxyl or amino group.

40. The method of claim 36 wherein the agent is 4-aminophenyl trimethoxysilane, 4-aminophenyl trimethoxysilane, 3-aminophenyltriethoxysilane, 3-aminophenyltriethoxysilane, or mixture thereof.

41. The method of claim 36 wherein the agent and tetraisocyanatosilane is present at a 1:1 molar ratio.

42. The method of claim 36 wherein the molecule and the first reactive intermediate is present in a 3:1 molar ratio.
43. The method of claim 36 wherein the first reactive intermediate has a formula iv:

\[(R_1)(R_2)(R_3)Si-X-Z-CYNH-Si(NCY)_3\]  iv

wherein \(R_1\), \(R_2\) and \(R_3\) independently represents \(C_1\)-\(C_6\) alkoxy, \(C_1\)-\(C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1\)-\(C_6\) alkyl and \(C_1\)-\(C_6\) alkoxy; \(X\) represents linear or branched \(C_1\)-\(C_{20}\) alkyl or aryl substituted with one or more groups selected from the group consisting of \(C_1\)-\(C_6\) alkyl and \(C_1\)-\(C_6\) alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; \(Y\) represents oxygen or sulfur; and \(Z\) represents oxygen or NH, with the proviso that at least one of \(R_1\), \(R_2\), or \(R_3\) represents \(C_1\)-\(C_6\) alkoxy.

44. The method of claim 43 wherein \(R_1\), \(R_2\) and \(R_3\) represents methoxy, \(X\) represents phenyl, \(Y\) represents oxygen, and \(Z\) represents NH.

45. The method of claim 43 wherein the second reactive intermediate has a formula v:

\[(R_1)(R_2)(R_3)Si-X-Z-CYNH-Si(NHCYL-M)_3\]  v

wherein \(R_1\), \(R_2\) and \(R_3\) independently represents \(C_1\)-\(C_6\) alkoxy, \(C_1\)-\(C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1\)-\(C_6\) alkyl and \(C_1\)-\(C_6\) alkoxy; \(L\) represents a linking group; \(X\) represents linear or branched \(C_1\)-\(C_{20}\) alkyl or aryl substituted with one or more groups selected from the group consisting of \(C_1\)-\(C_6\) alkyl and \(C_1\)-\(C_6\) alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; \(Y\) represents oxygen or sulfur; and \(Z\) represents oxygen or NH; and \(M\) represents a molecule, with the proviso that at least one of \(R_1\), \(R_2\), or \(R_3\) represents \(C_1\)-\(C_6\) alkoxy.

46. The method of claim 45 wherein \(L\) represents a nucleophilic group from the molecule.
47. The method of claim 46 wherein the nucleophilic group comprises \(-\text{NH}, -\text{S}, -\text{O},\) or \(-\text{OOC}\).

48. The method of claim 36 wherein the first reactive intermediate has a formula vi:

\[
[(R_1)(R_2)(R_3)\text{Si-X-Z-CYNH}_2\text{Si (NCY})_2 \quad \text{vi}
\]

wherein \(R_1, R_2\) and \(R_3\) independently represents \(\text{C}_1\-\text{C}_6\) alkoxy, \(\text{C}_1\-\text{C}_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(\text{C}_1\-\text{C}_6\) alkyl and \(\text{C}_1\-\text{C}_6\) alkoxy; \(X\) represents linear or branched \(\text{C}_1\-\text{C}_{20}\) alkyl or aryl substituted with one or more groups selected from the group consisting of \(\text{C}_1\-\text{C}_6\) alkyl and \(\text{C}_1\-\text{C}_6\) alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; \(Y\) represents oxygen or sulfur; and \(Z\) represents oxygen or NH, with the proviso that at least one of \(R_1, R_2,\) or \(R_3\) represents \(\text{C}_1\-\text{C}_6\) alkoxy.

49. The method of claim 48 wherein \(R_1, R_2\) and \(R_3\) represents methoxy, \(X\) represents phenyl, \(Y\) represents oxygen, and \(Z\) represents NH.

50. The method of claim 48 wherein the second reactive intermediate has a formula vii:

\[
[(R_1)(R_2)(R_3)\text{Si-X-Z-CYNH}_2\text{Si (NHCYL-M}_2 \quad \text{vii}
\]

wherein \(R_1, R_2\) and \(R_3\) independently represents \(\text{C}_1\-\text{C}_6\) alkoxy, \(\text{C}_1\-\text{C}_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(\text{C}_1\-\text{C}_6\) alkyl and \(\text{C}_1\-\text{C}_6\) alkoxy; \(L\) represents a linking group; \(X\) represents linear or branched \(\text{C}_1\-\text{C}_{20}\) alkyl or aryl substituted with one or more groups selected from the group consisting of \(\text{C}_1\-\text{C}_6\) alkyl and \(\text{C}_1\-\text{C}_6\) alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; \(Y\) represents oxygen or sulfur; and \(Z\) represents oxygen or NH; and \(M\) represents a molecule, with the proviso that at least one of \(R_1, R_2,\) or \(R_3\) represents \(\text{C}_1\-\text{C}_6\) alkoxy.
51. The method of claim 50 wherein L represents a nucleophilic group from the molecule.

52. The method of claim 51 wherein the nucleophilic group comprises -NH, -S-, -O-, or -OOC-.

53. The method of claim 36 wherein the molecule comprises a probe.

54. The method of claim 53, wherein the probe comprises a protein, a peptide, a nucleic acid, a peptide nucleic acid, an amino acid, a linked nucleic acid, a nucleoside triphosphate, a carbohydrate, a lipid, a lipid bound protein, an aptamer, a virus, a cell fragment, or a whole cell.

55. The method of claim 54, wherein the lipid bound protein comprises a G-protein coupled receptor.

56. The method of claim 36, wherein the probe comprises an antibody, an antigen, a receptor, or a ligand.

57. A device comprising a surface with an immobilized molecule, wherein said surface is prepared by the method of any one of claims 1, 10, 15, or 36.

58. The device of claim 57 wherein the surface is a glass surface.

59. The device of claim 57, wherein the molecule is a probe.

60. The device of claim 59, wherein the probe comprises a protein, a peptide, a nucleic acid, a peptide nucleic acid, a linked nucleic acid, an amino acid, a nucleoside triphosphate, a carbohydrate, a lipid, a lipid bound protein, an aptamer, a virus, a cell fragment, or a whole cell.

61. The device of claim 60, wherein the lipid bound protein comprises a G-protein coupled receptor.
62. The device of claim 59, wherein the probe comprises an antibody, an antigen, a receptor, or a ligand.

63. The device of claim 62, wherein the probe has been derivatized to contain one or more amino and/or hydroxyl groups.

64. A compound having the formula iii:

\[(R_1)(R_2)(R_3)Si-X-NHCYL-M\] iii

wherein \(R_1\), \(R_2\) and \(R_3\) independently represents \(C_1-C_6\) alkoxy, \(C_1-C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkyl and \(C_1-C_6\) alkoxy; \(L\) represents a linking group; \(X\) represents linear or branched \(C_1-C_{20}\) alkyl or aryl substituted with one or more groups selected from the group consisting of \(C_1-C_6\) alkyl and \(C_1-C_6\) alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; \(Y\) represents oxygen or sulfur; and \(M\) represents a molecule, with the proviso that at least one of \(R_1\), \(R_2\), or \(R_3\) represents \(C_1-C_6\) alkoxy.

65. The compound of claim 64 wherein \(L\) represents a nucleophilic group from the molecule.

66. The compound of claim 65 wherein the nucleophilic group comprises –NH, –S–, –O–, or –OOC–.

67. The compound of claim 64, wherein the molecule comprises a protein, a peptide, a nucleic acid, a peptide nucleic acid, an amino acid, a linked nucleic acid, a nucleoside triphosphate, a carbohydrate, a lipid, a lipid bound protein, an aptamer, a virus, a cell fragment, or a whole cell.

68. The compound of claim 67, wherein the lipid bound protein comprises a G-protein coupled receptor.
69. The compound of claim 64, wherein the molecule comprises an antibody, an antigen, a receptor, or a ligand.

70. A compound having a formula iv:

$$\text{(R}_1\text{)(R}_2\text{)(R}_3\text{)}\text{-Si-X-Z-CYNH-Si (NCY)}_3$$

wherein $R_1$, $R_2$ and $R_3$ independently represents $C_1$-$C_6$ alkoxy, $C_1$-$C_6$ alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of $C_1$-$C_6$ alkyl and $C_1$-$C_6$ alkoxy; $X$ represents linear or branched $C_1$-$C_{20}$ alkyl or aryl substituted with one or more groups selected from the group consisting of $C_1$-$C_6$ alkyl and $C_1$-$C_6$ alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; $Y$ represents oxygen or sulfur; and $Z$ represents oxygen or NH, with the proviso that at least one of $R_1$, $R_2$, or $R_3$ represents $C_1$-$C_6$ alkoxy.

71. The compound of claim 70 wherein $R_1$, $R_2$ and $R_3$ represents methoxy, $X$ represents phenyl, $Y$ represents oxygen, and $Z$ represents NH.

72. A compound having a formula v:

$$\text{(R}_1\text{)(R}_2\text{)(R}_3\text{)}\text{-Si-X-Z-CYNH-Si (NHCYL-M)}_3$$

wherein $R_1$, $R_2$ and $R_3$ independently represents $C_1$-$C_6$ alkoxy, $C_1$-$C_6$ alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of $C_1$-$C_6$ alkyl and $C_1$-$C_6$ alkoxy; $L$ represents a linking group; $X$ represents linear or branched $C_1$-$C_{20}$ alkyl or aryl substituted with one or more groups selected from the group consisting of $C_1$-$C_6$ alkyl and $C_1$-$C_6$ alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; $Y$ represents oxygen or sulfur; and $Z$ represents oxygen or NH; and $M$ represents a molecule, with the proviso that at least one of $R_1$, $R_2$, or $R_3$ represents $C_1$-$C_6$ alkoxy.

73. The compound of claim 72 wherein $L$ represents a nucleophilic group from the molecule.
74. The compound of claim 73 wherein the nucleophilic group comprises –NH, –S-, –O-, or –OOC-.

75. The compound of claim 72, wherein the molecule comprises a protein, a peptide, a nucleic acid, a peptide nucleic acid, an amino acid, a linked nucleic acid, a nucleoside triphosphate, a carbohydrate, a lipid, a lipid bound protein, an aptamer, a virus, a cell fragment, or a whole cell.

76. The compound of claim 72, wherein the lipid bound protein comprises a G-protein coupled receptor.

77. The compound of claim 72, wherein the molecule comprises an antibody, an antigen, a receptor, or a ligand.

78. A compound having a formula vi:

\[(R_1)(R_2)(R_3)\text{Si-X-Z-CYNH}_2\text{Si (NCY)}_2\]

vi

wherein R₁, R₂ and R₃ independently represents C₁-C₆ alkoxy, C₁-C₆ alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of C₁-C₆ alkyl and C₁-C₆ alkoxy; X represents linear or branched C₁-C₂₀ alkyl or aryl substituted with one or more groups selected from the group consisting of C₁-C₆ alkyl and C₁-C₆ alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; Y represents oxygen or sulfur; and Z represents oxygen or NH, with the proviso that at least one of R₁, R₂, or R₃ represents C₁-C₆ alkoxy.

79. The compound of claim 78 wherein R₁, R₂ and R₃ represents methoxy, X represents phenyl, Y represents oxygen, and Z represents NH.

80. A compound having a formula vii:

\[\{(R_1)(R_2)(R_3)\text{Si-X-Z-CYNH}_2\text{Si (NHCYL-M)}_2\]

vii
wherein R₁, R₂ and R₃ independently represents C₁-C₆ alkoxy, C₁-C₆ alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of C₁-C₆ alkyl and C₁-C₆ alkoxy; L represents a linking group; X represents linear or branched C₁-C₂₀ alkyl or aryl substituted with one or more groups selected from the group consisting of C₁-C₆ alkyl and C₁-C₆ alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; Y represents oxygen or sulfur; and Z represents oxygen or NH; and M represents a molecule, with the proviso that at least one of R₁, R₂, or R₃ represents C₁-C₆ alkoxy.

81. The compound of claim 80 wherein L represents a nucleophilic group from the molecule.

82. The compound of claim 81 wherein the nucleophilic group comprises –NH, -S-, -O-, or -OOC-.

83. A kit comprising:

   a container comprising a compound of formula i:

   \[(R₁)(R₂)(R₃)Si-X-NCY\]  

wherein R₁, R₂ and R₃ independently represents C₁-C₆ alkoxy, C₁-C₆ alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of C₁-C₆ alkyl and C₁-C₆ alkoxy; X represents linear or branched C₁-C₂₀ alkyl or aryl substituted with one or more groups selected from the group consisting of C₁-C₆ alkyl and C₁-C₆ alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; and Y represents oxygen or sulfur, with the proviso that at least one of R₁, R₂ or R₃ represents C₁-C₆ alkoxy; and

   optional substrate.

84. A kit comprising:
a first container comprising Si(NCY)\textsubscript{4} wherein Y represents oxygen or sulfur;

a second container comprising an agent having a formula ii:

\[(R_1)(R_2)(R_3)\text{Si-X-Z} \quad \text{ii}\]

wherein \(R_1\), \(R_2\) and \(R_3\) independently represent \(C_1\text{-}C_6\) alkoxy, \(C_1\text{-}C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1\text{-}C_6\) alkyl and \(C_1\text{-}C_6\) alkoxy; \(X\) represents linear or branched \(C_1\text{-}C_{20}\) alkyl or aryl substituted with one or more groups selected from the group consisting of \(C_1\text{-}C_6\) alkyl and \(C_1\text{-}C_6\) alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; and \(Z\) represents a hydroxy or amino group, with the proviso that at least one of \(R_1\), \(R_2\) or \(R_3\) represents \(C_1\text{-}C_6\) alkoxy; and

an optional substrate.

85. A kit comprising:

a container comprising a compound having the formula iv:

\[(R_1)(R_2)(R_3)\text{Si-X-Z-CYNH-Si (NCY)}_3 \quad \text{iv}\]

wherein \(R_1\), \(R_2\) and \(R_3\) independently represent \(C_1\text{-}C_6\) alkoxy, \(C_1\text{-}C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1\text{-}C_6\) alkyl and \(C_1\text{-}C_6\) alkoxy; \(X\) represents linear or branched \(C_1\text{-}C_{20}\) alkyl or aryl substituted with one or more groups selected from the group consisting of \(C_1\text{-}C_6\) alkyl and \(C_1\text{-}C_6\) alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; \(Y\) represents oxygen or sulfur; and \(Z\) represents oxygen or NH, with the proviso that at least one of \(R_1\), \(R_2\), or \(R_3\) represents \(C_1\text{-}C_6\) alkoxy; and

an optional substrate.

86. A kit comprising:

a container comprising a compound having the formula vi:

\[
[(R_1)(R_2)(R_3)\text{Si-X-Z-CYNH}_{2}\text{-Si (NCY)}]_2 \quad \text{vi}
\]
wherein \( R_1, R_2 \) and \( R_3 \) independently represents \( \text{C}_1-\text{C}_6 \) alkoxy, \( \text{C}_1-\text{C}_6 \) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \( \text{C}_1-\text{C}_6 \) alkyl and \( \text{C}_1-\text{C}_6 \) alkoxy; \( X \) represents linear or branched \( \text{C}_1-\text{C}_{20} \) alkyl or aryl substituted with one or more groups selected from the group consisting of \( \text{C}_1-\text{C}_6 \) alkyl and \( \text{C}_1-\text{C}_6 \) alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; \( Y \) represents oxygen or sulfur; and \( Z \) represents oxygen or \( \text{NH} \), with the proviso that at least one of \( R_1, R_2, \) or \( R_3 \) represents \( \text{C}_1-\text{C}_6 \) alkoxy; and

an optional substrate.

87. The kit of claim 86 wherein \( R_1, R_2 \) and \( R_3 \) represents methoxy, \( X \) represents phenyl, \( Y \) represents oxygen, and \( Z \) represents \( \text{NH} \).

88. A kit comprising a compound having a formula vii:

\[
[(R_1)(R_2)(R_3)\text{Si-X-Z-CYNH}]/_2\text{Si (NHCYL-M)}_2 \quad \text{vii}
\]

wherein \( R_1, R_2 \) and \( R_3 \) independently represents \( \text{C}_1-\text{C}_6 \) alkoxy, \( \text{C}_1-\text{C}_6 \) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \( \text{C}_1-\text{C}_6 \) alkyl and \( \text{C}_1-\text{C}_6 \) alkoxy; \( L \) represents a linking group; \( X \) represents linear or branched \( \text{C}_1-\text{C}_{20} \) alkyl or aryl substituted with one or more groups selected from the group consisting of \( \text{C}_1-\text{C}_6 \) alkyl and \( \text{C}_1-\text{C}_6 \) alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; \( Y \) represents oxygen or sulfur; and \( Z \) represents oxygen or \( \text{NH} \); and \( M \) represents a molecule, with the proviso that at least one of \( R_1, R_2, \) or \( R_3 \) represents \( \text{C}_1-\text{C}_6 \) alkoxy.

89. The compound of claim 88 wherein \( L \) represents a nucleophilic group from the molecule.

90. The compound of claim 89 wherein the nucleophilic group comprises – \( \text{NH}, \text{-S-}, \text{-O-}, \) or \( \text{-OOC-} \).

91. A method for making a substrate for use in target analyte detection, said method comprising:
(a) providing a substrate having a surface;

(b) contacting said surface with an isocyanate compound so as to provide a surface comprising free isocyanate groups, the isocyanate compound is a member selected from the group consisting of:

\[
\text{Si(NCY)}_4; \\
(R_1)(R_2)(R_3)\text{Si-X-NCY} \quad \text{i;} \\
[(R_1)(R_2)(R_3)\text{Si-X-Z-CYNH}]_2\text{Si (NCY)}_2 \quad \text{vi; and} \\
(R_1)(R_2)(R_3)\text{Si-X-Z-CYNH-Si (NCY)}_3 \quad \text{iv;} \\
\]

wherein \( R_1, R_2 \) and \( R_3 \) independently represents \( C_1-C_6 \) alkoxy, \( C_1-C_6 \) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \( C_1-C_6 \) alkyl and \( C_1-C_6 \) alkoxy; \( X \) represents linear or branched \( C_1-C_{20} \) alkyl or aryl substituted with one or more groups selected from the group consisting of \( C_1-C_6 \) alkyl and \( C_1-C_6 \) alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; \( Y \) represents oxygen or sulfur; and \( Z \) represents oxygen or NH, with the proviso that at least one of \( R_1, R_2, \) or \( R_3 \) represents \( C_1-C_6 \) alkoxy.

92. The method of claim 91, further comprising, after step (b):

(c) contacting said surface comprising free isocyanate groups with a spacer molecule so as to provide a surface comprising free amino groups; and

(d) contacting said surface comprising free amino groups with a linker molecule so as to provide a reactive surface having free reactive groups.

93. The method of claim 92, further comprising repeating steps (c) and (d) one or more times.

94. The method of claims 92 or 93, further comprising, after step (d):

(e) contacting said reactive surface with at least one type of capture probe specific for the target analyte so as to provide a surface comprising immobilized capture probes; and

(e) contacting said surface comprising immobilized capture probes with a capping agent so as to block residual unreacted free isocyanate groups on areas of the surface not having immobilized capture probes and produce a substrate having
substantially low signal background due to non-specific nanoparticle binding relative to a surface not contacted with a capping agent.

95. The method of claim 91, further comprising:
   (c) contacting the surface comprising free isocyanate groups with water so as to provide a surface comprising free amino groups; and
   (d) contacting said surface comprising free amino groups with a linker molecule so as to provide a reactive surface having free reactive groups.

96. The method of claim 95, further comprising, after step (d):
   (e) contacting said surface comprising free isocyanate groups with a spacer molecule so as to provide a surface comprising free amino groups; and
   (f) contacting said surface comprising free amino groups with a linker molecule so as to provide a reactive surface having free reactive groups.

97. The method of claim 96, further comprising repeating steps (e) and (f) one or more times.

98. The method of claims 95, 96 or 97, further comprising:
   (i) contacting said reactive surface with at least one type of capture probe specific for the target analyte so as to provide a surface comprising immobilized capture probes; and
   (ii) contacting said surface comprising immobilized capture probes with a capping agent so as to block residual unreacted free isocyanate groups on areas of the surface not having immobilized capture probes and produce a substrate having substantially low signal background due to non-specific nanoparticle binding relative to a surface not contacted with a capping agent.

99. The method of claim 91 wherein the disilylisocyanate compound is selected from the group consisting of 2-Trimethoxysilane-6-triisocyanatosilanceureabenzene, 3-(triethoxysilyl) propylisocyanate, and tetraisocyanatosilane.
100. The method of claim 91 wherein the spacer molecule has at least two functional groups that can react with a isocyanate group.

101. The method of claim 100 wherein the spacer molecule is polymer, a carbohydrate, or antibiotic.

102. The method of claim 100, wherein the spacer molecule is a member selected from the group consisting of poly (dimmer acid-co-alkylpolyamine)-95, poly(dimmer acid-co-alkylpolyamine)-140, poly(allylamine), poly(m-xylendiamine-epichlorohydrin diamine terminated, tris(2-aminoethylamine), and PAMAM dendrimer generation 0, neomycin, and 3,3’-diaminobenzidine.

103. The method of any one of claims 94 or 98 wherein the capping reagent is a member selected from the group consisting of amino acid, protein, carbohydrate, carboxylate, thiol, alcohol, and amine.

104. The method of claim 103 wherein the capping reagent is glycine.

105. The method of claim 91 wherein the isocyanate compound is a member selected from the group consisting of phenylene 1,4-diisocyanate, tolylene-2,6-diisocyanate, tolylene-α,4-diisocyanate, and isophorone diisocyanate.

106. The method of claim 91 wherein the linker molecule is selected from the group consisting of ethylene glycolbis (succinimidylsuccinate), disuccinimidyl suberate, 1,6-diisocyanatohexane, methylene bis-(4-cyclohexylisocyanate, glutaric dialdehyde, methylene-p-phenyl diisocyanate, and triethyl citrate.

107. The method of any one of claims 94 or 98, wherein the capture probe is a nucleic acid.

108. The method of any one of claims 94 or 98 wherein more than one type of capture probes are contacted with the surface having second reactive moieties, each type of capture probes is specific for a particular target analyte.
109. The method of claim 94 or 98 wherein the capture probes are arrayed in discrete predetermined areas on the surface of the substrate.

110. A method for making a substrate for use in detection of a target analyte, said method comprising:

(a) providing a substrate having a surface;

(b) contacting said surface with a isocyanate compound so as to provide a surface comprising free isocyanate groups, the isocyanate compound is a member selected from the group consisting of:

$$\text{Si(NCY)}_4;$$

$$(R_1)(R_2)(R_3)\text{Si-X-NCY}$$

$$[(R_1)(R_2)(R_3)\text{Si-X-Z-CYNH}]_2-Si\text{ (NCY)}_2$$

$$[(R_1)(R_2)(R_3)\text{Si-X-Z-CYNH-Si (NCY)}]_3$$

wherein $R_1$, $R_2$ and $R_3$ independently represents $C_1-C_6$ alkoxy, $C_1-C_6$ alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of $C_1-C_6$ alkyl and $C_1-C_6$ alkoxy; $X$ represents linear or branched $C_1-C_{20}$ alkyl or aryl substituted with one or more groups selected from the group consisting of $C_1-C_6$ alkyl and $C_1-C_6$ alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; $Y$ represents oxygen or sulfur; and $Z$ represents oxygen or NH, with the proviso that at least one of $R_1$, $R_2$, or $R_3$ represents $C_1-C_6$ alkoxy;

(c) contacting said surface comprising free isocyanate groups with a spacer molecule so as to provide a surface comprising free amino groups;

(d) contacting said surface comprising free amino groups with a linker molecule so as to provide a reactive surface having free reactive groups;

(e) contacting said reactive surface with at least one type of capture probe specific for the target analyte so as to provide a surface comprising immobilized capture probes; and

(f) contacting said surface comprising immobilized capture probes with a capping agent so as to block residual unreactive free isocyanate groups and produce a substrate having substantially low signal background due to non-specific nanoparticle binding relative to a surface not contacted with a capping agent.
111. The method of any one of clams 91 or 110 wherein the substrate has at least one group that reacts with the isocyanate compound.

112. The method of claim 111 wherein the group comprises hydroxyl, amino, or carboxylate.

113. A substrate for use in detection of one or more target analytes, said substrate comprising a surface with an attached capture probe is prepared by the method of any of claims 94, 98, or 110.

114. A substrate comprising a surface having a polymeric layer comprising free amino groups capable of binding said capture probes, and negatively charged ionic groups.

115. The substrate of claim 114, wherein said surface produces a background signal upon imaging using visual or fluorescent light having substantially reduced background signal relative to a substrate not having said polymeric layer.

116. The substrate of claim 113, wherein said substrate has a water contact angle ranging from about 25 to 75 degrees.

117. The substrate of claim 116, wherein the substrate has a refractive index ranging from about 1.400 to 1.900.

118. A kit for detecting one or more target analytes comprising the substrate of any one of claims 22, 23, 25, or 25.

119. A method for detecting one or more target analytes in a sample, the target analyte having at least two binding sites, comprising:

(a) providing a substrate of any one of claims 91 or 110, said substrate having at least one type of capture probes immobilized on a surface of the substrate, each type of capture probes specific for a target analyte;
(b) providing at least one type of detection probe comprising a nanoparticle and a detector probe, the detector probe specific for a target analyte;
(c) contacting the capture probes, the detection probes and the sample under conditions that are effective for the binding of the capture probes and detector probes to the specific target analyte to form an immobilized complex onto the surface of the substrate;
(d) washing the surface of the substrate to remove unbound nanoparticles; and
(e) observing for the presence or absence of the complex as an indicator of the presence or absence of the target molecule.

120. A method for immobilizing a nanoparticle onto a surface, said method comprising the steps of:
(a) providing a substrate having a surface and a nanoparticle having oligonucleotides bound thereto, at least a portion of the oligonucleotides have a free amine group at an end not bound to the nanoparticle;
(b) contacting the nanoparticle with an agent so as to form a reactive intermediate, said agent having a formula i:

\[(R_1)(R_2)(R_3)\text{-Si-X-NCY} \quad \text{i}\]

wherein \(R_1\), \(R_2\) and \(R_3\) independently represents \(C_1\text{-}C_6\) alkoxy, \(C_1\text{-}C_6\) alkyl, phenyl, or aryl substituted with one or more groups selected from the group consisting of \(C_1\text{-}C_5\) alkyl and \(C_1\text{-}C_6\) alkoxy; \(X\) represents linear or branched \(C_1\text{-}C_{20}\) alkyl or aryl substituted with one or more groups selected from the group consisting of \(C_1\text{-}C_5\) alkyl and \(C_1\text{-}C_6\) alkoxy, optionally substituted with one or more heteroatoms comprising oxygen, nitrogen, or sulfur; and \(Y\) represents oxygen or sulfur, with the proviso that at least one of \(R_1\), \(R_2\) or \(R_3\) represents \(C_1\text{-}C_6\) alkoxy; and
(b) contacting the reactive intermediate with said surface so as to immobilized the molecule onto said surface.

121. The method of claim 120 wherein the surface is a glass surface.
122. The method of claim 120 wherein the surface has at least one group that reacts with the reactive intermediate.

123. The method of claim 122 wherein the group comprises a hydroxyl, amino, or carboxylate group.

124. The method of claim 120 wherein the agent comprises 3-(isocyanatopropyl) triethoxysilane or 3-(isocyanatopropyl)dimethylmonoethoxysilane.

125. The method of claim 120 wherein the oligonucleotides are bound to the nanoparticle through a functional moiety.

126. The method of claim 125 wherein the functional moiety comprises a thiotic acid, alkyl thiol or disulfide group.

127. The method of claim 126 wherein the disulfide group is an epiandrosterone disulfide.

128. A method for immobilizing a nanoparticle onto a surface, said method comprising the steps of:
   (a) providing a substrate having a surface comprising reactive moieties that reacts with amine groups and a nanoparticle having oligonucleotides bound thereto, at least a portion of the oligonucleotides have a amine group at an end not bound to the nanoparticle; and
   (b) contacting the reactive moieties with the nanoparticle so as to immobilized the nanoparticles onto said surface.

129. The method of claim 128 wherein the surface is a glass surface.

130. The method of claim 128 wherein the reactive moieties comprise isocyanates, anhydrides, acyl halides, or aldehydes.
131. The method of claim 128 wherein the oligonucleotides are bound to the nanoparticle through a functional moiety.

132. The method of claim 131 wherein the functional moiety comprises a thiotic acid, alkyl thiol, or disulfide group.

133. The method of claim 132 wherein the disulfide group is an epiandrosterone disulfide.

134. A substrate prepared by the methods of any one of claims 91 or 110.

135. A kit comprising a substrate of claim 132.
**Figure 1**

\[ \text{NH}_2 + \text{EtO-Si-NH-} \rightarrow 1:10 \text{DMSO-ethanol} \]

\[ \text{Room temp., 1-3h} \]

\[ \text{NH-CO-NH-Si-} \]

silylated DNA

\[ \text{silylated DNA} \rightarrow \text{RT} \]

\[ \text{... .... ...} \]

1. Wash with water for 10 minutes
2. Wash with ethanol and dry

Ready for testing with target DNA and probes
The only time "branching" was seen with the DMF buffer was the case above where it seems that the area around the spots is overhydrated.
Figure 5(a)

1. H₂N

2. OCN\_\text{Si}N\text{NCO}

3. OCN\_\text{Si}NH\text{CO-NH}\_\text{Si}OCH₃

4. Oligonucleotide-\text{NH}_₂

Coupling

5. Oligonucleotide-\text{HN-Oc-HN-SiNH-CO-NH-SiOCH₃}

Oligonucleotide-\text{HN-Oc-HN-NH-CO-NH-Oligonucleotide}

branched oligonucleotide

Print silyl oligonucleotide on the glass surface.
Figure 5(b)

\[
\begin{align*}
\text{H}_2\text{N} & \text{Si} \text{OCH}_3 \\
\text{OCH}_3 & \\
\text{OCN}_2 & \\
\text{NCO} & \\
\text{OCN}_3 & \\
\text{NCO} & \\
\hline
\text{H}_3\text{CO} & \text{HN-OC-HN-Si-NH-CO-NH} \\
\text{HN-OC-HN} & \text{Si-NH-CO-NH-Oligonucleotide} \\
\text{Oligonucleotide} & \text{HN-OC-HN} \\
\text{HN-OC-HN} & \text{Si-NH-CO-NH-Oligonucleotide} \\
\text{Oligonucleotide} & \text{HN-OC-HN} \\
\text{HN-OC-HN} & \text{Si-NH-CO-NH-Oligonucleotide} \\
\text{Oligonucleotide} & \\
\hline
\end{align*}
\]

3 Oligonucleotide-NH$_2$
Coupling

4

5 branched oligonucleotide

Print silyl oligonucleotide on the glass surface.
FIGURE 6

Plate Nos. 1 (first plate), 2 (middle plate), and 3 (last plate)
Figure 8

Hybridization did at 70 ° (Plate1)

Mthfr synthetic 100mer target (Plate2)

Mthfr PCR Product (Plate3)
Figure 10
**FIGURE 11A**

Scheme (second step)

2-Trimethoxysilane-6-(trisocyanatosilaneurea)benzene

**FIGURE 11B**

Scheme (third step)

Poly (dimer acid-co-alkylpolyamine)-95
FIGURE 11C

S C H E M E

(F o u r t h s t e p)

[Chemical structure diagram]

+ 3

3-EGS

(EGS) Ethylene glycolbis(succinimidylsuccinate)

FIGURE 11D
Schematic diagram for making slides

1. Cleaning slides
   - Poly(dimethylacrylamide)/polyamine@40
   - Poly(dimethylacrylamide)/polyamine@85
   - Nonylphenol
   - Poly(ethyleneimine)
   - Poly(methylenediamine)pentaether-ethyleneimine, diamine terminated

2. Stabilization of slides
   - Poly(dimethylacrylamide)/polyamine@45
   - Poly(dimethylacrylamide)/polyamine@85 TAMA
   - Tita(2-aminooctane) TAMA

3. Linkers
   - Poly(methylenediamine)pentaether-ethyleneimine, diamine terminated
   - Poly(methylenediamine)pentaether-ethyleneimine, triamine terminated
   - Quats, diethyleneglycol

FIGURE 13
Figure 5: Cy3-linked oligonucleotides printing on polymer (dimmer acid co-alkyl poly amine)-95 coated slide 3E) used EGS cross linker and 3D) used diisocyanate cross linker.
FIGURE 16A

Slide 1.

Each well:

- OOOOOO — Positive control capture
- OOOOOO — Positive Control capture
- OOOOOO — Wild type Factor

Here in well 1 & 3 only contained positive control probe with hybridization buffer and we see only positive control signals. Wells 2 & 4 contained factor V wild type PCR target and probe with buffer and we see factor V target signals.

FIGURE 16B

Slide no 2:

Each Well:

- OOO — Factor V mutant capture
- OOO — Positive Control capture
- OOO — Factor V Wild type capture

Unfortunately, this Tris (2-aminoethylene) modified surface gave backgrounds with nanoparticle probes. This confirms unreacted amines are binding to the gold nanoparticles in this case.
FIGURE 16C

Slide 3

Each well:  
OOO —— Positive control capture  
OOO —— wild type capture  
OOO —— mutant capture

Wells 1, 4, 5, 9, 10 were used Factor V probe with buffer but no target therefore no signals were observed. Well 6 used with positive control probe with buffer and positive control signals were observed. Wells 2, 3, 7 & 8 were used with FactorV probe + target and buffer and wild type target signals were observed.

FIGURE 16D

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td>10</td>
</tr>
</tbody>
</table>

+Ve control  Wt  Wt  cont  cont

FIGURE 16E

Slide 4. Here nanoparticles were spread on two slides for comparison. Nanoparticles are binding amine rich dendrimer slides and giving backgrounds after silver amplification.

4a) Dendrimer slide  4b) polymer -95 modified slide
FIGURE 16F

Slide 5.
Each well:

- OOO ---- wild type factor V capture
- OOO ---- buffer
- OOO ---- mutant factor V capture
- OOO ---- positive control

Here 1 & 5 contained 8pM PCR target with probe and buffer
2 & 6 contained 80pM PCR target with probe and buffer
3 & 7 contained 0.8nM PCR target with probe and buffer
4 & 8 contained 80nM PCR target with probe and buffer

Since it is wild type target detection, only wild type signals are showed up in the assay (top row)

![Image of a microarray with dilutions and wild type signals]

FIGURE 16G

Slide 6

Each Well:

- OOO ---- wild type factor V capture
- OOO ---- mutant factor V capture
- OOO ---- wild type capture
- OOO ---- positive control

Well 1: Factor V prone with buffer no signal
Well 2: 80nM Factor V target
Well 3: 80nM Mutant target
Well 4: 80nM Heterzygous target
Well 5: 0.8nM (100 times diluted) wild type target

![Image of a microarray with dilutions and wild type signals]
FIGURE 16H
Slide 7
Each well:

OOO ---- wild type factor V capture
OOO ---- mutant factor V capture
OOO ---- positive control

FIGURE 16I
All 10 wells in both slides contained only positive control probe and buffer. Both slides showed positive control signals in the right place.

1  2  3  4  5  

6  7  8  9  10

a) Nanosphere polymer coated slide  b) Commercial Codelink Slide

Slide G1
Each Well:

OOO ---- mutant genomic factor V capture
OOO ---- Buffer
OOO ---- Buffer
OOO ---- Wild type genomic factor V capture

Wells 1, 2, 3, 4 have target with probe and buffer. Wells 5, 6, 7, 8 and 10 have only factor V probe and buffer and no signals were expected in these wells.

1  2  3  4  5 

5  6  7  8  10
FIGURE 16K

Slide G2.

Each well:

- OOO ---- mutant genomic factor V capture
- OOO ---- Buffer
- OOO ---- Buffer
- OOO ---- Wild type genomic factor V capture

Wells 3, 4, 6, 8, 9 & 10 have target with probe and buffer. Wells 1, 2, 6 & 7 have only factor V probe and buffer and no signals were expected in these wells.

FIGURE 16L

Slide G3

Each Well:

- OOO ---- mutant genomic factor V capture
- OOO ---- Buffer
- OOO ---- Buffer
- OOO ---- Wild type genomic factor V capture

Wells 2, 3, 4, 5, 7, 8, 9 & 10 have target with probe and buffer. Wells 1 & 6 have only factor V probe and buffer and no signals were expected in these wells.
FIGURE 16M

Slide G4

Each well:

OOO ---- mutant genomic factor V capture
OOO ---- Buffer
OOO ---- Buffer
OOO ---- Wild type genomic factor V capture

Wells 2, 3, 4, 5, 7, 8, 9 & 10 have target with probe and buffer. Wells 1 & 6 have only factor V probe and buffer and no signals were expected in these wells.

FIGURE 16N

Slide G5

Each well:

OOO ---- mutant genomic factor V capture
OOO ---- Buffer
OOO ---- Buffer
OOO ---- Wild type genomic factor V capture

Wells 2, 3, 4, 5, 7, 8, 9 & 10 have target with probe and buffer. Wells 1 & 6 have only factor V probe and buffer and no signals were expected in these wells.
FIGURE 160

Slide G6

Each well:

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>OOOO</td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

Column 1: mutant, 2 buffer, 3 buffer and 4 wild type capture.
3'-Amine linked gold nanoparticles printing on aldehyde modified glass slide

3'-NH$_2$-ATCG-ATCG-ATCG-ATCG-Epi-5'

Citrate modified gold colloid

1

3'-amine modified gold colloid

2

3'-amine modified gold colloid spotting

3

After attaching amine modified gold nanoparticles to the aldehyde slide

4

Silver amplification

After silver amplification spots on the aldehyde modified glass slide.
FIGURE 18

3′-Silyl linked gold nanoparticles printing on unmodified glass slide

3′-monoethoxy Silyl modified gold colloid → Printing mono ethoxy silyly linked particles on unmodified glass surface → Silver amplification → After silver amplification

3′-Triethoxy Silyl modified gold colloid → Printing triethoxy silyl linked particles on a unmodified glass surface → Silver amplification → After silver amplification
**Figure 22:**

**Spacer Structures**

- **P95 Polymer**
- **PoXyl Polymer**
- **Neomycin**
- **3,3'-Diaminobenzidine**
- **PAMAM**
- **Polypropylenimine octamine**
Figure 23: Isocyanates and Activated esters used for Attachment of Spacers and the Linkers

(OCN)_3Si-NHCONH-\(\text{Si(OMe)}_3\)  2-Trimethoxysilane-6-(trisocyanatosilaneurea)benzene

\[\text{OCON} \quad \text{CH}_2 \quad \text{NCO} \]

\[\text{H}_3\text{C} \quad \text{N=N=O} \]

\[\text{O=CN} \quad \text{N=N=O} \]

\[\text{O=CN} \quad \text{N=N=O} \]

\(\text{NCO-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-NCO}\)  1,6 Hexamethylene diisocyanate (DCH)

\[\text{N-O-C-CH}_2\text{-CH}_2\text{-C-O-CH}_2\text{-CH}_2\text{-O-C-CH}_2\text{-CH}_2\text{-C-O-N} \]

(EGS) Ethylene glycolbis(succinimidylsuccinate)

\[\text{CH}_3 \quad \text{O} \quad \text{CH}_3 \quad \text{O} \quad \text{CH}_3 \quad \text{O} \quad \text{CH}_3 \]

Triethyl Citrate (T)
Figure 41:

<table>
<thead>
<tr>
<th>Well</th>
<th>1</th>
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<th>3</th>
<th>4</th>
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</tr>
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<tbody>
<tr>
<td>Probe</td>
<td>+Ve1</td>
<td>Het</td>
<td>Mut</td>
<td>Wt</td>
<td>C</td>
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<td>Wells</td>
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</tr>
<tr>
<td>+Ve-Control</td>
<td>WT-Capture</td>
<td>Mut-Capture</td>
<td>-Ve-Control</td>
<td></td>
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</tr>
<tr>
<td>Well</td>
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<td>------</td>
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<tr>
<td>Probe</td>
<td>+Ve 1</td>
<td>Wt</td>
<td>Mut</td>
<td>Wt</td>
<td>C</td>
</tr>
</tbody>
</table>

+Ve-Control
WT-Capture
MtT-Capture
-Ve-Control
Figure 47:
Nanopore plastic slide with formamide gradient:

Commercially available Glass slide with formamide gradient:

Formamide %

Wells

0 9 20 30 40

1 2 3 4 5
<table>
<thead>
<tr>
<th>Wells</th>
<th>Target</th>
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</thead>
<tbody>
<tr>
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<tr>
<td>3</td>
<td>+Ve and Wt</td>
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<tr>
<td>4</td>
<td>+Ve and Wt</td>
</tr>
<tr>
<td>5</td>
<td>+Ve and Wt</td>
</tr>
</tbody>
</table>

**Figure 48:**

- Mut-capture
- -Ve - capture
- Wt - Capture
- +Ve Control
Figure 50:
Figure 51:

Substrate Surface Blocked with Glycine

<table>
<thead>
<tr>
<th>Well</th>
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<th>2</th>
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<th>4</th>
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<td>Wt</td>
<td>Wt</td>
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<tr>
<td>MuT-Capture 1</td>
<td>WT-Capture 1</td>
<td>MuT-Capture 2</td>
<td>WT-Capture 2</td>
<td>+Ve-Control</td>
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</table>

Native Substrate Surface - No Blocking

<table>
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<tr>
<th>Well</th>
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<th>3</th>
<th>4</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Probe</td>
<td>+Ve 1</td>
<td>Wt</td>
<td>Mut</td>
<td>Wt</td>
<td>Wt</td>
</tr>
<tr>
<td>MuT-Capture 1</td>
<td>WT-Capture 1</td>
<td>MuT-Capture 2</td>
<td>WT-Capture 2</td>
<td>+Ve-Control</td>
<td></td>
</tr>
</tbody>
</table>
Glass (10m) arrayed across
The substrate in multiple rows
And columns with a Cy3 capture
To obtain uniform arraying

Plastic (10h) arrayed across
The substrate in multiple rows
And columns with a Cy3 capture
To obtain uniform arraying
Figure 56

Signal for Plastic and Glass Substrates after a Hybridization to a Cy5-probe

Average signal-background (pol, pm3a)

Probe Concentration (pM)

0 500 1000 1500 2000 2500 3000

10h

10m