

Serial No. 352,126

Filing Date 1 December 1994

Inventor Linda A. Chrisey
Walter J. Dressick
Jeffrey M. Calvert



NOTICE

The above identified patent application is available for licensing. Requests for information should be addressed to:

OFFICE OF NAVAL RESEARCH
DEPARTMENT OF THE NAVY
CODE OCCC3
ARLINGTON VA 22217-5660

| | |
|---------------------|-------------------------------------|
| Accession For | |
| NTIS CRA&I | <input checked="" type="checkbox"/> |
| DTIC TAB | <input type="checkbox"/> |
| Unannounced | <input type="checkbox"/> |
| Justification | |
| By | |
| Distribution / | |
| Availability Codes | |
| Dist | Avail and/or Special |
| A-1 | |

This document has been approved for public release and sale; its distribution is unlimited.

19950307 062

1
2
3 SELECTIVE ATTACHMENT OF NUCLEIC ACID MOLECULES TO PATTERNED SELF-
4 ASSEMBLED SURFACES
5

6 **Background of the Invention**

7 **1. Field of the Invention**

8 The present invention relates generally to the attachment of
9 nucleic acids to substrates and more particularly to the formation
10 of a pattern of nucleic acid molecules attached to a substrate.
11

12 **2. Description of the Background Art**

13 Biomolecules, preferably nucleic acids, have been immobilized
14 on a variety of solid surfaces, for a number of known applications,
15 including DNA and RNA oligomer synthesis; separation of desired
16 target nucleic acids from mixtures of nucleic acids including RNA;
17 conducting sequence-specific hybridizations to detect desired
18 genetic targets (DNA or RNA); creating affinity columns for mRNA
19 isolation; quantification and purification of PCR reactions;
20 characterization of nucleic acids by AFM and STM; and for sequence
21 determination of unknown DNAs, such as the human genome. A number
22 of methods have been employed to attach nucleic acids to solid
23 surfaces, but there is an increasing need to immobilize multiple
24 nucleic acids of unique or distinct sequences and which retain
25 their specific functions in a high resolution, spatially
26 controlled fashion on rugged, solid substrates.

27 The above applications have used a variety of substrates for

1 DNA immobilization, including polymeric membranes (nylon,
2 nitrocellulose), magnetic particles, mica, glass or silica, gold,
3 cellulose, and polystyrene.

4 Methods which have been employed for the attachment of
5 preformed synthetic or naturally occurring nucleic acids to solid
6 surfaces for the above mentioned applications and on the
7 aforementioned substrates have included: electrodeposition, UV
8 crosslinking, (nitrocellulose, nylon); electrostatic, covalent
9 linking, and exploitation of strong intermolecular ligand/receptor
10 binding as for enzyme- or protein-linked affinity methods.

11 Certain methods, such as the UV-crosslinking and some covalent
12 attachment methods, are considered non-specific, that is the
13 reactive group on the nucleic acid involved in attachment to the
14 surface cannot be well controlled, such as crosslinking of
15 nucleic acids to aminosilane films using glutaraldehyde (for
16 binding DNA to optical fibers) and UV-crosslinking methods for
17 attachment to nylon or nitrocellulose membranes.

18 While many of these attachment modalities may be appropriate
19 for the attachment of a single nucleic acid species to the desired
20 surface, most are inadequate for the attachment of multiple
21 species, especially at the high resolutions required for
22 applications involving very large numbers of DNA species. For
23 example, biotin-labeled DNA molecules of different sequences could
24 be separated as a collective group from a milieu of other molecules
25 using streptavidin-functionalized magnetic particles, but this

1 technique cannot resolve the individual DNA molecules of different
2 sequences, as would be required for applications involving multiple
3 hybridization events such as the simultaneous detection of many
4 genetic targets.

5 UV crosslinking of multiple nucleic acid species to a single
6 filter can be achieved, but at low resolution, as separation of
7 molecules of different sequence is accomplished by placement of
8 liquid portions of nucleic acids at the desired position on the
9 filter. This method is limited in the number of reactions that
10 could be accomplished on a given surface area, which is a
11 limitation for automation of applications on a micrometer scale.

12 Non-specific covalent linking methods, such as the reaction of
13 glutaraldehyde with amines, are inadequate because the
14 glutaraldehyde can react with different moieties on the nucleic
15 acid, may form polymeric materials, and diminish the number of
16 functional nucleic acid molecules on the surface. Prior art
17 covalent attachment methods meet the criterion of specific directed
18 immobilization of DNA through a selected chemical moiety on the DNA
19 to a specific moiety on the surface, but do not address the issue
20 of pattern or array formation using spatial control of the
21 immobilization process.

22 Before the present invention, no attempts were made to define
23 spatially resolved regions in which nucleic acids are selectively
24 bound from regions to which nucleic acids are excluded, other than
25 by placement of liquid aliquots (generally $>1 \mu\text{l}$) of nucleic acids

1 to the desired position on the filter, wafer, etc. To date, other
2 efforts to spatially separate individual nucleic acid species have
3 focused on the use of polystyrene, 96-well microtiter plates or
4 minidisks of glass for the purposes of sequence analysis or
5 hybridization reactions. The method whereby an epoxysilane was
6 used to modify a SiO₂ surface, and subsequently reacted with amino-
7 terminated DNA oligomers was useful for immobilization of multiple,
8 unique DNA species, but required the use of a fluid microjet
9 apparatus to deliver small volume aliquots to the surface. This
10 approach to spatially resolving discrete nucleic acid species on
11 the surface results in problems with cross-contamination between
12 closely adjacent areas which are meant to contain distinct
13 molecules, and also has an inherent limit to smallest feature size
14 achievable, dependent upon the fluid delivery system employed.
15 Additionally, the fluid microjet apparatus is expensive.

16 Methods have been described in the literature for the
17 spatially controlled modification of surfaces with peptides and
18 proteins, and also for synthetic nucleic acids. For example, DNA
19 oligomers have been synthesized, at the substrate surface, in a
20 stepwise fashion (i.e., one nucleic acid base at a time) using
21 photochemical activation of photosensitive protecting groups on
22 the DNA base monomers followed by condensation of subsequent
23 nucleic acid bases, capping and oxidation of the phosphorous from
24 P(III) to P(V). The specific conditions of the surface
25 modification chemistry and solid substrate utilized were not

1 described. The DNA oligomers are arranged in small-scale arrays
2 using initial photolithographic modification of the substrate
3 through a mask. This approach is quite limited in its ability to
4 fabricate arrays containing distinctly different or unrelated DNA
5 molecules. Such arrays are best prepared using standard solid
6 phase DNA oligomer synthesis and purification techniques on a large
7 scale, followed by immobilization of the pre-formed DNA molecules
8 in defined patterns on the surface. This method also does not
9 permit the immobilization of DNA species other than synthetic
10 molecules, such as isolated sequences of naturally-derived DNA.
11 The third drawback to this previous method is that each individual
12 with its component DNA array must be fabricated individually, that
13 is, the attachment of the final DNA sequence requires sequential
14 irradiation and condensation steps for each oligomer set, adding
15 numerous additional steps. For example, synthesis of a 20 base DNA
16 oligomer (a 20-mer) by this previous method requires eighty steps,
17 twenty photolysis steps and twenty nucleic acid condensations,
18 twenty oxidations of the phosphorous group and twenty cappings of
19 any unwanted hydroxy groups. This number of steps can seriously
20 compromise the quality and homogeneity of the final oligomer
21 product. If each step of the forty steps proceeds in 99% yield
22 --an optimistic value-- the overall yield of correctly synthesized
23 oligomers is only 67%. For a 95% average yield per step, the
24 overall yield of correctly synthesized oligomers would drop to 13%.
25 Clearly, the likelihood of using prior art methods to correctly

1 synthesize a usefully large number of identical nucleic acid
2 oligomers upon a single surface is small if not nil. This problem
3 also bears on the difficulties that the prior art has faced in
4 immobilizing hybridizable nucleic acid oligomers. The ability to
5 hybridize requires a minimum length of nucleic acid. Yet, with
6 each nucleic acid condensation step, the prior art methods provide
7 a significantly reduced yield of correctly synthesized nucleic acid
8 oligomer and increased potential for the generation of non-
9 hybridizable failure sequences.

10
11 **Summary of the Invention**
12

13 It is an object of the present invention to covalently or non-
14 covalently immobilize a controlled density of functional nucleic
15 acid molecules (hereinafter abbreviated as NAMs), particularly
16 nucleic acid oligomers, on a substrate under conditions that
17 maintain the specific function of the molecule.

18 It is another object of the present invention to provide a
19 method for preparing surfaces which contain at least one co-planar
20 patterned area to which NAMs may be immobilized, as well as at
21 least one area resistant to the covalent or non-covalent attachment
22 of NAMs, resulting in the formation of arrays or patterns of NAMs.

23 It is a further object of the present invention to
24 sequentially immobilize distinct NAMs in a well-defined pattern on
25 the surface of a substrate.

1 It is yet another object of the present invention to provide
2 sensors or biomaterials for a variety of biological, analytical,
3 electronic or optical materials uses.

4 These and other objects have been achieved by the present
5 method for forming a pattern of nucleic acid molecules,
6 particularly nucleic acid oligomers, on a surface. A layer of
7 molecules bearing reactive functional groups, such as organosilane
8 reagents, are covalently bound to the surface of a substrate. If
9 desired, the layer may be further modified with a
10 heterobifunctional crosslinker that attaches to a reactive
11 functional group of the covalently bound molecule at one active
12 site and includes a second active site available for attachment to
13 a NAM or modified NAM. The covalently bound molecules and any
14 heterobifunctional cross-linker are selected so that the ability of
15 the surface to bind NAMs is modified by exposure of that surface to
16 suitable radiation (e.g., electrons, ions, x-rays and glow
17 discharge or other plasma), typically u.v. radiation. After the
18 surface is irradiated in a selected pattern, the surface is exposed
19 to the NAM to be attached. The NAM can be derived from synthetic
20 (man-made from nucleic acid monomers) or naturally occurring
21 sources.

22 The process may be repeated, permitting the attachment of a
23 plurality of NAMs, each in a distinct pattern.

24
25 **Brief Description of the Drawings**

1 A more complete appreciation of the invention will be readily
2 obtained by reference to the following Description of the Preferred
3 Embodiments and the accompanying drawings in which like numerals in
4 different figures represent the same structures or elements,
5 wherein:

6 Fig. 1 is a schematic flow chart for nucleic acid patterning
7 according to one embodiment of the present invention.

8 Fig. 2 is a schematic flow chart for nucleic acid patterning
9 according to another embodiment of the present invention.

10 Fig. 3 shows the binding of a DNA oligomer to an EDA-modified
11 silicon substrate.

12 Fig. 4 is a schematic flow chart illustrating two alternative
13 embodiments of the method of the present invention.

14 Fig. 5 is a schematic flow chart illustrating yet another
15 method of nucleic acid patterning according to the present
16 invention.

17 Fig. 6 shows the structure of the phosphorothioate DNA
18 backbone.

19
20 **Detailed Description of the Preferred Embodiment(s)**

21
22 Initially, an organosilane coating is covalently attached to
23 the hydroxylated surface of a substrate having exposed hydroxyl
24 groups, such as silicon dioxide, to form an organosilane film or
25 coating. This film or coating is typically monolayer or a bilayer.

1 The organosilane is selected to have at least one reactive site
2 that covalently binds to the hydroxylated surface of the substrate
3 and another reactive site that is incapable of binding either to
4 other organosilane molecules of the coating or to the substrate.

5 Thus, the organosilane molecules of the coating have a bound
6 reactive site and a free reactive site. This free reactive site
7 remains available for binding to a molecule distinct from both the
8 substrate and other organosilane molecules in the coating.

9 Specifically, the free reactive site of the organosilane may
10 directly bind to a modified or unmodified nucleic acid molecule
11 (see Fig. 1 and Fig. 2) or may indirectly bind to a modified or
12 unmodified nucleic acid molecule via a heterobifunctional
13 crosslinker (see Fig. 1, Fig 2 and Fig. 3). If the free reactive
14 site of the organosilane is directly bound to a nucleic acid
15 molecule, the binding is typically non-covalent (i.e., ionic
16 bonding, or, less often, hydrogen bonding). Advantageously, non-
17 covalent bonding of nucleic acid molecules generally disposes of
18 any need to modify the desired nucleic acid molecules, as it
19 typically may be performed using native nucleic acid molecules. On
20 the other hand, non-covalently bound systems are expected to be
21 less robust than covalently bound systems in the fabrication of
22 arrays of oligomers of nucleic acids.

23 As shown in Fig. 1, an aminosilane coating 10 is applied over
24 an SiO₂ substrate 12. The resulting structure 14 is irradiated
25 with ultraviolet light (at 193 nm, for example) through a mask 16

1 positioned over aminosilane coating 10. The u.v. irradiation
2 removes the portions of aminosilane coating 10 that were exposed
3 through mask 16. Upon removal of mask 16, aminosilane coating 10
4 remains on those regions protected by the mask and are the native
5 oxide of substrate 12 is exposed at any region 18 not protected by
6 mask 16. A nucleic acid oligomer, shown here as oligomeric DNA 19,
7 binds to the aminosilane coating 10, but not to the native oxide
8 18. This process forms a positive tone image in both the
9 aminosilane film and also in the DNA.

10 Fig. 2 shows the use of a positive-tone photoresist in the
11 method of the present invention. An aminosilane coating 10 is
12 applied over an SiO₂ substrate 12. A positive-tone photoresist 20
13 is then applied over the entire surface of aminosilane coating 10.
14 The resulting structure is irradiated with ultraviolet light
15 through a mask 22 positioned over the photoresist. Development and
16 washing removes the exposed portion of photoresist 20 and reveals
17 underlying aminosilane film 10. Oligomeric DNA 19 binds to the
18 exposed portions of aminosilane coating 10, but not to the
19 remaining portions of photoresist 20. In the schemes of both Fig.
20 1 and Fig. 2, a surface having patterns of exposed amino functional
21 groups is used for selective non-covalent attachment of DNA
22 oligomers.

23 Any heterobifunctional crosslinker used should have a
24 reactive site for bonding with the free reactive sites of the
25 organosilane molecules and a free reactive site for directly

1 bonding to the nucleic acid to be attached. For covalent bonding
2 of oligomeric nucleic acid molecules to a substrate surface, the
3 desired nucleic acid sequence is typically modified to contain a
4 functional group that is reactive with the free reactive site of
5 the selected heterobifunctional crosslinker (Fig. 3). Therefore,
6 the patterned amine surfaces of Figs. 1 and 2 may also be used for
7 the covalent attachment of NAM oligomers.

8 The exposed surface of the substrate, whether it is a native
9 organosilane coating or an organosilane coating that has been
10 reacted with a heterobifunctional crosslinker, may then be overlaid
11 with a layer of photoresist material. While the present invention
12 can accommodate the use of either a negative tone or a positive
13 tone photoresist, a positive tone photoresist is typically used.
14 A positive- tone photoresist is one in which the areas exposed to
15 irradiation become soluble in the developer, whereas areas which
16 are protected from irradiation by opaque areas of the mask remain
17 largely insoluble when treated with developer, i.e., the
18 photoresist blocks access to the underlying crosslinker layer with
19 its second reactive group. Conversely, a negative-tone photoresist
20 is soluble only in the unexposed regions. For either type of
21 photoresist, areas which remain coated with photoresist do not
22 become coated with NAMs, either via non-covalent or covalent
23 methods, i.e., these areas are largely resistant to NAM binding
24 (Fig. 2).

25 In any event, the assembly containing the organosilane coating

1 and any heterobifunctional crosslinker and/or photoresist is then
2 irradiated, either in a defined pattern or through a mask which
3 defines a pattern. The mask or irradiation pattern defines a
4 pattern of areas exposed to the irradiation and areas unexposed to
5 the irradiation on the substrate surface. Irradiation may be from
6 numerous types of exposure sources, including light (infrared,
7 visible, ultraviolet, x-ray), electron beams, ion beams and plasma.

8 Where no heterobifunctional crosslinker or photoresist is used
9 before the irradiation step, the organosilane molecules (such as
10 aminosilanes) in the irradiated portions of the organosilane
11 coating are photolytically transformed to destroy the amine
12 (nucleic acid binding) functional group and to produce a surface
13 that is not a good surface for binding NAMs. The free reactive
14 amine sites of the organosilane molecules in the unexposed portions
15 of the organosilane coating remain available for the subsequent
16 binding of NAMs, either directly or via heterobifunctional
17 crosslinkers.

18 Where no heterobifunctional crosslinker is used before the
19 irradiation step, but a positive-tone photoresist is employed, the
20 photoresist coating at first renders the free reactive sites of the
21 organosilane molecules unavailable for binding with NAMs. Upon
22 irradiation, the exposed areas of the photoresist become soluble
23 in the selected developer. Thus, these exposed areas of the
24 photoresist are removed during development. The removal of the
25 exposed areas of the positive photoresist renders the free reactive

1 sites of the underlying organosilane molecules available for
2 bonding to NAMs, either directly or via heterobifunctional
3 crosslinkers. If a negative-tone photoresist were used, the
4 unexposed regions would be soluble in and removable by the
5 developer, while the regions exposed to irradiation would become
6 insoluble in the developer and would remain after development.

7 The use of a heterobifunctional crosslinker simplifies the
8 covalent attachment of NAMs. A heterobifunctional crosslinker has
9 one first activated functional group reactive toward a specific
10 chemical moiety on the exposed portion of the silane molecules, and
11 a second activated functional group (which is not reactive toward
12 either the first functional group of the crosslinker, nor with the
13 free reactive group on the organosilane molecules) which will
14 preferentially react with a specific chemical moiety on the NAM to
15 effect a stable, covalent bond between the NAM and the hydroxylated
16 surface.

17 A photoresist may also be applied over the layer of
18 heterobifunctional crosslinker. If a positive tone photoresist is
19 applied, the portions of the photoresist exposed to irradiation
20 will become soluble in the selected developer and will thus be
21 removed during development. The unexposed regions remain insoluble
22 in the developer. Thus, the unexposed portions of the photoresist
23 remain and prevent the covalent and non-covalent binding of NAM's
24 to the second activated functional group of the heterobifunctional
25 crosslinker. If a negative tone photoresist is used, the exposed

1 portions of the photoresist are converted from a developer-soluble
2 state to a developer insoluble state. In that scenario, only the
3 unexposed portions of the photoresist will be removed during
4 subsequent development.

5 Any heterobifunctional crosslinker used may be attached either
6 before or after the substrate has been irradiated. If attached to
7 the surface before irradiation, only regions of the surface in
8 which the second activated functional group is exposed after
9 development will bind NAMS. If attached to the surface after
10 irradiation, the heterobifunctional crosslinker will attach only to
11 those regions of the substrate surface where the organosilane
12 molecules have exposed free reactive sites. Depending on the
13 nature of the radiation-induced transformation, free reactive sites
14 may be either created or destroyed in the exposed regions of the
15 substrate.

16 Fig. 4 further illustrates the method of the present
17 invention. In Fig. 4, silica substrate 40 has an aminosilane
18 coating 42 thereon, and is irradiated through mask 44. The
19 portions 46 of aminosilane coating 42 exposed through mask 44 are
20 removed by the irradiation, leaving a hydroxylated Si-OH surface.
21 Resulting structure 47 may then be contacted with negatively
22 charged DNA oligomer 48. The negatively charged DNA oligomer 48
23 electrostatically binds to the remaining portions of the
24 aminosilane coating, but not to the exposed regions.
25 Alternatively, resulting structure 47 can be backfilled with a

1 second organosilane that is attached in the exposed regions of the
2 surface. In the instance of Fig. 4, backfill is accomplished by
3 attaching commercially available linear perfluorinated alkylsilane
4 group 49 to portions 46. After backfilling, the resulting
5 structure is contacted with negatively charged DNA oligomer 48.
6 The negatively charged DNA oligomer 58 electrostatically binds to
7 the remaining portions of the aminosilane coating, but not to the
8 perfluoroalkyl groups.

9 Fig. 5 shows yet another alternative embodiment according to
10 the present invention. In Fig. 5, an SiO_2 substrate 100 is coated
11 with a coating of a silane 102 that is resistant to the binding of
12 nucleic acid oligomers. The resulting assembly is irradiated with
13 ultraviolet light through patterned mask 104. The ultraviolet
14 light selectively removes the non-DNA-binding silane from those
15 regions 106 exposed thereto through mask 102, leaving a
16 hydroxylated surface on SiO_2 substrate 100. When resulting
17 structure 108 is contacted with a solution of, for example, an
18 amino-terminated silane such as $(\text{CH}_3\text{O})_3\text{Si}-(\text{CH}_2)_3\text{NH}(\text{CH}_2)_2\text{NH}_2$ (N-(2-
19 aminoethyl)-3-aminopropyltrimethoxysilane, abbreviated as "EDA"),
20 the silane functionality reacts with the free hydroxyl groups in
21 regions 106. The amino terminus remains available for covalently
22 binding NAMS, such as DNA oligomers 110. Alternatively, the amino
23 terminus can be reacted with a heterobifunctional crosslinker that
24 binds NAMS.

25 In conjunction with the method of the present invention, one

1 may also employ known blocking agents to prevent non-specific
2 binding and/or absorption of NAMs, proteins, enzymes, conjugates or
3 small molecules used to detect the NAMs. Typical blocking agents
4 include proteins, carbohydrates, detergents and amino acids.

5 The substrates to be modified for use in the method and
6 product of the invention include materials which have or can be
7 modified to have surface hydroxyl groups which can react with
8 silanes. Suitable substrates are preferably inorganic materials,
9 including but not limited to silicon, glass, silica, diamond,
10 quartz, alumina, silicon nitride, platinum, gold, aluminum,
11 tungsten, titanium, various other metals and various other
12 ceramics. Alternatively, polymeric materials such as polyesters,
13 polyamides, polyimides, acrylics, polyethers, polysulfones,
14 fluoropolymers, etc. may be used as suitable organic substrates.
15 The substrate used may provided in any suitable form, such as
16 slides, wafers, fibers, beads, etc.

17 The nucleic acid-binding silanes useful for the invention can
18 bind to the substrate's hydroxyl groups, and include a wide variety
19 of silanes. Amino-terminated silanes are preferred for attachment
20 of NAMs, although thiol-terminated silanes may also be employed,
21 particularly in cases where the silane is to be reacted with a
22 thiol-reactive group on a heterobifunctional crosslinker. Other
23 silanes containing terminal functional groups such as olefins,
24 acetals, epoxy and benzylhalides are also useful from crosslinker
25 attachment. The hydroxyl-reactive terminus of the silane may be,

1 for example, trichloro, trimethoxy, triethoxy, monoethoxy,
2 chlorodimethyl or dimethylmethoxy. Typical silanes useful in the
3 present invention include amino-terminated silanes such as EDA,
4 trimethoxysilylpropyldiethylenetriamine (DETA), and
5 (aminoethylaminomethyl)-phenylethyltrimethoxysilane (PEDA),
6 aminopropyltriethoxysilane and aminobutyldimethylmethoxysilane..

7 Typically, the irradiation step is performed at a u.v.
8 wavelength, although other wavelengths may be used, especially
9 where differentiation between irradiated and non-irradiated regions
10 of the surface is accomplished with the aid of a photoresist. U.V.
11 radiation, when used, may be carried out using any number of UV
12 sources, including pulsed ArF excimer laser light (193 nm,
13 typically 0.1 - 10 mJ/cm²-pulse) and mercury lamps for typical
14 total exposures of 0.01-10 J/cm² of exposed surface area. Patterns
15 are typically generated by exposure through a lithographic mask
16 composed of UV opaque and UV transparent regions in contact with
17 the surface, but may also be formed using projection printing,
18 direct laser writing, or electron beam lithography.

19 The crosslinking agents bearing two different reactive
20 functional groups are known as heterobifunctional crosslinkers, and
21 the two functional groups are reactive toward different and
22 distinct chemical moieties, typically thiols, benzylhalides and
23 amines. Other functional groups are known and can be useful in the
24 method of the present invention. Functional groups reactive toward

1 amines include but are not limited to N-succinimide esters,
2 isothiocyanates, imidoesters and nitroaryl halides. Thiol reactive
3 groups include but are not limited to maleimides, iodoacetyl,
4 pyridyl disulfide, and other disulfides. A heterobifunctional
5 crosslinker found useful for the covalent attachment of a thiol-
6 modified synthetic DNA to an aminosilane-modified substrate was
7 succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB), which has an
8 amino reactive succinimide ester and a thiol reactive maleimide
9 group.

10 The photoresist to be coated onto the crosslinker-modified
11 silane layer, and later patterned using UV irradiation can include
12 numerous varieties, but must be sensitive to relatively low doses
13 of light, and must require developers that do not remove or react
14 with the crosslinker itself or the underlying silane or substrate.
15 The developers and strippers used to remove the resist must also
16 not interfere with the activity of the immobilized NAMs. A typical
17 photoresist used in this invention is S-1400-11™ (a
18 diazonaphthoquinone-type produced by Shipley Corp.; a positive-tone
19 resist), with the aqueous alkaline developer MF312 CD27™ (0.27M
20 tetramethylammonium hydroxide, produced by Shipley Corp.). An
21 example of a negative photoresist that is useful in the present
22 invention is the chemically amplified type, of which SAL601-ER7™
23 (Shipley), is an example.

24 The use of an overall negative-tone process, such as that
25 shown in Fig. 2, is particularly advantageous in the formation of

1 arrays or patterns composed of multiple, different NAMS (having
2 differing base sequence or function). In this scheme, the use of
3 a photoresist allows one to sequentially expose reactive areas of
4 the crosslinker layer by irradiating the photoresist in the desired
5 pattern(s). After fabrication of the array(s) or patterns is
6 completed, residual photoresist may be removed, without
7 compromising the functional performance of the NAMS.

8 The active agent to be attached to the modified surfaces can
9 include numerous biomolecules, but for the present invention are
10 chiefly nucleic acids molecules. Typically, these nucleic acid
11 molecules are natural or synthetic oligomers of DNA or RNA which
12 may be modified with a thiol or amino group in a specific location
13 in the oligomer (or by incorporating a ribose sugar which may then
14 be oxidized). Typically, these oligomers include from about 4 to
15 about 400 bases, and more typically from about 20 to about 150
16 bases. Even larger nucleic acid oligomers may be created using
17 these immobilized oligomers as primers for synthesis of amplified
18 nucleic acids or by incorporation of modified nucleotides during
19 the amplification process. The biomolecules to be attached by the
20 method of the present invention may be also be labeled, for
21 example, with fluorescent tags, enzymes, small antigens,
22 radioactive elements chemiluminescent tags, magnetic tags, metal
23 particles or other contrast agents. The preferred thiol-terminated
24 DNA oligomers are easily prepared using automated solid-phase
25 synthesis, preferably using phosphoramidite chemistry with a thiol-

1 modifier controlled pore glass support (i.e., the thiol group will
2 be at the 3'-terminus of the molecule), but may also be prepared
3 using a thiol-modifier phosphoramidite useful for incorporation of
4 the thiol at the 5'-terminus of the oligomer, or by incorporation
5 of sulfur at any number of desired positions (from 0-n, where n =
6 the number of nucleic acid bases in the specific oligomer) within
7 a given oligomer by using a sulfur containing oxidizer, such as
8 tetraethyldithiouram (TETD, Applied Biosystems, Inc) which forms
9 phosphorothioate linkages at the selected positions within the
10 backbone of the oligomer.

11 The activity of the biomolecule must be maintained after
12 immobilization to the surface. For example, immobilized DNA or
13 RNA probes must retain their ability to hybridize to a
14 complementary DNA or RNA molecule in a sequence-specific manner, or
15 to function as primers for nucleic acid amplification techniques.

16 The process and products of this invention can be used in many
17 areas of the art. For example, DNA hybridization analysis to
18 detect or identify a genetic target such as a specific nucleic acid
19 sequence, microorganism, genetic disorder etc., using an
20 immobilized nucleic acid probe is well known in the art.
21 Similarly, the use of immobilized probes to perform sequence
22 determination of a nucleic acid of undetermined sequence using
23 sequential hybridization reactions to a large number of immobilized
24 probes has been reported. Likewise, the use of large populations
25 of different, non-immobilized nucleic acid molecules have been used

1 to identify small and macromolecular ligands that are capable of
2 sequence-specific binding with certain nucleic acid molecules.
3 Also, nucleic acid oligomers immobilized on surfaces may be used as
4 primers for the amplification of other nucleic acids molecules of
5 known or unknown sequence using standard thermal cycling techniques
6 and the addition of a second, unimmobilized appropriate primer.

7 In an application involving an immobilized DNA oligomer used
8 for capture and detection of a complementary (target) DNA molecule,
9 the complementary (target) sequence may be labeled with biotin, a
10 fluorescent dye or radioactivity (typically ³²P) using synthetic,
11 labeled primers, or direct incorporation of the label into the
12 molecule to be detected during an amplification step. For the
13 types of patterned arrays described in the present invention, a
14 significant advantage over the prior art is the capability to
15 immobilize large numbers of identical, oligomeric nucleic acid
16 probe molecules in a small surface area. Using the present
17 invention, immobilized nucleic acids confined to areas of 1 μM X 10
18 μM have been detected and resolved from adjacent nucleic acid
19 modified areas, and nucleic acids immobilized according to the
20 invention have been found suitable for hybridization with
21 complementary but not with mismatched nucleic acid molecules.

22 The specific conditions for each of the steps described for
23 the present invention, to obtain patterns or arrays of oligomeric
24 NAMs that may be non-covalently or covalently bound to the surface,
25 may vary greatly depending on the specific reagents and equipment

1 used. The crosslinker, photoresist and NAM should be chosen such
2 that none of these (or steps used to process or remove these) are
3 destructive to the underlying silane film, and the assembly of
4 steps are designed to promote maximum density of functional NAMs in
5 a designated area on the surface. The density of functional NAMs
6 in a designated area may be increased, for example, by using a
7 commercially available C3-C6 spacer between thiol and the NAM, by
8 varying the density of the organosilane film on the substrate, or
9 by designing the immobilized NAM so it lacks ability to bind itself
10 (i.e., not self-complementary), or by choosing a substrates with
11 greater surface area, such as glass or fused silica.

12 In the preferred usage, covalent immobilization of functional
13 nucleic acids on silicon substrates uses an amino-terminated
14 silane, and a heterobifunctional crosslinker to bind a high density
15 of synthetic, thiol-terminated DNA oligomer to a planar surface. It
16 has been determined using radiolabeled thiol-modified DNA oligomers
17 composed of 20 bases that densities of 45-75 pMoles/cm² are
18 achieved on silicon; depending on the exact base sequence of the
19 immobilized oligomer between 3 and 47% of the immobilized molecules
20 are capable of selective hybridization with a complementary DNA
21 oligomer.

22 Information that may assist in the practice of the present
23 invention can be found, for example, in Eigler (sic) et al., USP
24 5,077,210 and Ligler et al., United States Patent Application
25 07/691,491, filed April 26, 1991 and its parent application, USSN

1 07/182,123, all of which are incorporated by reference herein in
2 their entirety, for all purposes. Additionally, the attachment of
3 aminosilanes to a silica substrate is further discussed in Lowe et
4 al., USP 4,562,157, the entirety of which is incorporated herein by
5 reference for all purposes.

6
7 Having described the invention, the following examples are
8 given to illustrate specific applications of the invention
9 including the best mode now known to perform the invention. These
10 specific examples are not intended to limit the scope of the
11 invention described in this application.

12
13 **EXAMPLES**

14
15 **Example 1 - Selective, Non-Covalent Attachment of Phosphodiester**
16 **DNA Oligomers to Organosilane Surfaces**

17 Thin films of the organosilanes (SAMs) were formed on acid-
18 cleaned fused silica slides from N-(2-aminoethyl)-3-aminopropyltri-
19 methoxysilane (EDA), trimethoxysilylpropyldiethylenetriamine
20 (DETA), and the fluorinated silanes 13F (tridecafluoro-1,1,2,2-
21 tetrahydrooctyl-1-dimethylchlorosilane), and 13F-Cl₃,
22 (tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane). An
23 acid-cleaned slide was also tested. The methods used for acid
24 cleaning and SAM preparation are described in J.M. Calvert, W.J.
25 Dressick, C.S. Dulcey, J.H. Georger, D.A. Stenger, T.S. Koloski and

1 G.S. Calabrese, Polymers for Microelectronics, ACS Symposium
2 Series, V537, edited by C.D. Wilson, L.F. Thompson, and S. Tagawa,
3 American Chemical Society, Washington, DC, 1993, p210; and R.L.
4 Geer, D.A. Stenger, M.S. Chen, J.M. Calvert, R. Shashidar, Y.
5 Jeong, and P. Pershan, submitted to Langmuir. The slides were
6 immersed in a solution of radiolabelled (γ -32P) synthetic DNA
7 oligomer of sequence (GT)₁₀, which was prepared by automated DNA
8 synthesis using standard phosphoramidite chemistry on an Applied
9 Biosystems Model 394 synthesizer. The trityl-on oligomer was
10 purified (to >90% full length material) using C18 solid phase
11 extraction columns. Radiolabeling of the 5-OH group of the
12 oligomer was accomplished using γ -32P-ATP (DuPont NEN) and T4
13 polynucleotide kinase from Gibco-BRL following manufacturer's
14 protocol. The radiolabeled synthetic DNA was separated from free
15 radioactive γ -32P-ATP using NENSORB purification columns (DuPont-
16 NEN), following manufacturer's directions. The purified
17 radiolabeled DNA was diluted in high purity distilled and deionized
18 (>18m Ω) H₂O to a concentration of 3 μ M as determined by UV
19 absorbance at 260 nm, and extinction coefficients calculated using
20 Oligo 4.1 software (National Biosciences, Inc). The slides were
21 immersed for 17 hr at room temperature, with shaking, and were then
22 washed in three changes of fresh, high purity distilled and
23 deionized water, and dried on filter paper. The slides were placed
24 on autoradiographic film for 18 hrs. Following development of the
25 film, the slides were broken, placed in liquid scintillation vials

1 with 10 ml of Fisher brand Scintiverse IV scintillant, and
2 counted. Quantification of the radiolabeled DNA was accomplished
3 by determining the oligomer concentration using UV spectroscopy, as
4 well as the number of counts per minute (CPM) for an aliquot of
5 known volume, then converting this to a specific activity for the
6 oligomer (CPM/pMole). The results are given in tabular form below
7 (Table 1), and indicate that the aminosilane SAMs of EDA and DETA
8 permit attachment of the radiolabeled DNA, while the acid-cleaned
9 slide, the 13F and 13F-Cl₃ SAMs did not bind appreciable amounts of
10 DNA. These results were supported by observation of the
11 characteristic DNA absorbance peak at 260 nm in the UV spectra of
12 the EDA and DETA, but not on the acid-cleaned, 13F, or 13F-Cl₃-
13 treated slides.

14
15 DNA also bound to films prepared from (aminoethylaminomethyl)
16 phenethyl-trimethoxysilane (PEDA) on fused silica (1% PEDA in 1 mM
17 CH₃COOH in MeOH, 20 min at 25C, rinse twice in MeOH, then baked at
18 120C for 5 min), as demonstrated by the presence of the
19 characteristic DNA absorbance peak at 260 nm in the UV spectrum of
20 these slides.

21
22 Other organosilanes to which the DNA does not appear bind as
23 evidenced by the lack of an absorbance peak at 260 nm (DNA
24 absorbance maxima) include acid-cleaned fused silica slides treated
25 as follows: octadecyltrichlorosilane (1% in toluene, 5 min, 25C) or

1 chloromethylphenyltrichlorosilane (1% in toluene, 10 min, 25C) or
2 phenethyltrichlorosilane (1% in toluene for 6-7 min, 25C) or
3 mercaptomethyldimethylethoxysilane (2% in toluene for 60 min); all
4 were rinsed in two changes of fresh toluene, then baked on a hot
5 plate at 120C for 3 min, before treatment with 1 μ M synthetic DNA
6 oligomer [5'-(CAGT)₅-3'] in dI water for 1 hr at room temperature.
7 Slides were rinsed and dried as described above, then examined
8 using UV spectroscopy. No absorbance at 260 nm was detected for
9 any of these organosilane films, nor for acid-cleaned fused silica
10 only.

11
12
13 **Table 1. Binding of Radiolabeled Oligomer IV (3 μ M) to SAMs on**
14 **Fused Silica.**

| | H ⁺ - cleaned slide | EDA | DETA | 13F | 13F-Cl ₃ |
|-------------------------------|--------------------------------------|-------------------|--------------------|----------------|---------------------|
| CPM | 5630 | 85,155 \pm | 86,370 \pm | 11,417 \pm | 5882 \pm |
| Bound | | 7373 | 19,419 | 1212 | 518 |
| pMoles DNA/in ² | 5.8 | 87.9 \pm 7.6 | 89.1 \pm 20.0 | 11.8 \pm 1.3 | 6.1 \pm 0.5 |

1 **Example 2 - Colorimetric Detection of a Biotin-Labeled Oligomer**
2 **Attached to DETA SAM**

3 A DETA SAM was formed on acid-cleaned fused silica as
4 described (Calvert et. al., Polymers for Microelectronics). A DNA
5 oligomer of the sequence: 5' -biotin-ACTG-ACTG-ACTG-ACTG-ACTG-3' was
6 prepared using standard phosphoramidite chemistry. The biotin
7 group was incorporated using either a commercially available
8 biotin-phosphoramidite (to place the biotin at the 5' terminus of
9 the oligomer) or 1 μ M biotin modifier controlled pore glass
10 synthesis column (to place the biotin group at the 3'-terminus of
11 the oligomer, both from Glen Research, Sterling, VA), following the
12 manufacturer's instructions. The labeled oligomer was purified
13 before further use using a C18 solid phase extraction column.
14 Aliquots (5 μ l of a 1 μ M solution in high purity, distilled and
15 deionized water) of the biotin-labeled DNA oligomer of sequence
16 (ACTG)₅, were placed on the surface of the DETA SAM-modified fused
17 silica slide. At the end of 15 min, the droplets were washed away
18 by immersion in three changes of water. The slide was then placed
19 in 20 ml of blocking solution A [to prevent non-specific adsorption
20 of protein; 1% purified casein, 0.1% sarkosyl, 0.02% SDS in 5X SSC
21 (0.75M sodium citrate-sodium chloride buffer)] for 15 min at 24°C.
22 Streptavidin-horseradish peroxidase (SA-HRP) was added to a final
23 dilution of 1:500, and the slide incubated for 15 min at 24°C. The
24 slide was then immersed twice in PBS-0.05% Tween 80, and once in
25 PBS (1 - 5 min each time), and finally placed in a solution

1 containing the HRP substrate tetramethylbenzidine (TMB). TMB
2 produces a blue colored precipitable product when it reacts with
3 peroxidase. Blue circles corresponding to the location of the 5 μ l
4 aliquots of DNA solution placed on the fused silica slide were
5 observed following this treatment. The surrounding areas on the
6 fused silica slide remained transparent and did not turn blue,
7 indicating that the biotin-labeled DNA oligomer was confined to
8 regions on the DETA SAM-modified slide where the aliquots were
9 originally placed, and that no non-specific binding of the SA-HRP
10 to these other areas of the slide occurred. Had non-specific
11 binding of the SA-HRP occurred on the slide, areas of the slide not
12 treated with the biotin-labeled DNA oligomer would have also turned
13 blue.

14
15 **Example 3 - Selective Hybridization of a DNA Oligomer Attached**
16 **by Electrostatic Binding to an Aminosilane Film on**
17 **Fused Silica Slides**

18 Small aliquots (5 μ l of 1 μ M solution in water) of a DNA
19 oligomer of sequence 5'-(ACTG)₅-3' (I) were placed on DETA SAMs on
20 fused silica slides (prepared as described in Example 1), then
21 rinsed off in two changes of PBS-0.05% Tween, and one time in PBS
22 (5 min per treatment). The slides were placed in blocking buffer
23 A (see Example 2) for 15 min, then two slides were immersed in a 1
24 μ M solution of a complementary oligomer [II, 5'-(CAGT)₅-3'] and two
25 were placed in a 1 μ M solution of a mismatched oligomer [III, 5'-

1 (CAAT)₅-3'], for 1 hr at room temperature. The slides were rinsed
2 briefly in two changes of 2X SSC (0.3M sodium citrate-sodium
3 chloride buffer, pH 7.0), then 1 slide of each pair was placed in
4 0.1X SSC (0.015M sodium citrate-sodium chloride, pH 7.0) at 23°C,
5 and the other slide in 0.1X SSC at 30°C. [The annealing
6 temperature of the perfectly matched I/II pair is estimated at 32°C
7 in 0.1X SSC using algorithms employed by Oligo 4.1 software]. The
8 slides were left for 15 minutes, then all slides were exposed to
9 1:500 dilution of streptavidin-horseradish peroxidase in blocking
10 buffer A for 15 min at room temperature. The slides were rinsed
11 twice in PBS, then placed in a solution of the HRP substrate, TMB.
12 (see example 2, above). Blue circles were observed on the slides
13 on which aliquots of oligomer I had been placed, then treated with
14 the complementary, biotin-labeled oligomer II, however, no reaction
15 was seen with the mismatched I/III pair. These results indicate
16 that a DNA oligomer immobilized via electrostatic means on an
17 organosilane SAM is able to form duplexes selectively with its
18 complementary partner, but will reject hybridization with a
19 mismatched oligomer.

20
21 **Example 4 - Demonstration of Electrostatic Binding of DNA**
22 **Oligomer to Aminosilane Film on Fused Silica**

23 DETA SAMs were formed on fused silica slides as described in
24 Example 1. Slides were immersed in 1 μM water solution of DNA
25 oligomer 5'-(ACTG)₅-3' for 1 hr at 25C, then rinsed in 3 changes of

1 dI water. UV spectra were taken of the dried slides. Duplicate
2 slides were then placed in solutions containing: high purity dI
3 water only; 50 mM Na-phosphate, pH 7.6; 50 mM Na-phosphate, 250 mM
4 NaCl, pH 7.6; or 50 mM Na-phosphate, 1M NaCl pH 7.6 for 1, 2, 4 and
5 21 hr at 25C. The UV absorbance at 260 nm (DNA maxima) was
6 compared for all slides. At high salt (50 mM Na-phosphate, pH 7.6
7 with either 250 mM NaCl or 1 M NaCl, after 2 hrs 80% of the DNA has
8 desorbed from the DETA surface. For 50 mM Na-phosphate, 1M NaCl pH
9 7.6, after 21 hr, less than 10% of the adsorbed DNA remains. These
10 results indicate that electrostatic interactions between the
11 negatively charged phosphate groups of DNA and positively charged
12 amino groups of the aminosilane SAMs are responsible for DNA
13 deposition from solution onto these SAMs.

14
15 **Example 5 - Selective, Electrostatic Binding of Double Stranded**
16 **Herring Sperm DNA on Aminosilane Films on Fused**
17 **Silica Slides**

18 Acid cleaned and DETA-treated fused silica slides were treated
19 with a 100 $\mu\text{g/ml}$ solution of herring sperm DNA in water for 30 min
20 at 25C. Slides were rinsed 3 X in dI water and dried under a
21 stream of filtered N_2 . UV spectra were taken of the slides before
22 and after treatment. Acid cleaned slides did not bind double-
23 stranded DNA, while the DETA SAM modified slides showed the
24 characteristic DNA absorbance peak at 260 nm ($A_{260} = 0.0071$, vs -
25 0.0004 for acid-cleaned slide).

1 **Example 6 - Non-Covalent Binding of P(S) DNA on Aminosilane**
2 **Films on Fused Silica Slides**

3 The phosphorothioate oligomer [P(S)], a congener of the native
4 phosphodiester DNA molecule in which the negatively charged
5 internucleotidic phosphate oxygen is replaced by a negatively
6 charged sulfur atom, is prepared using standard phosphoramidite
7 chemistry on the automated DNA synthesizer using a commercially
8 available thiolating/oxidizing reagent, tetraethylthiouram (TETD)
9 instead of the customary I₂-based oxidizer. Purification and
10 quantification of the product DNA oligomer are performed as in
11 Examples 1 and 2. The structure of the phosphorothioate DNA
12 congener is given in Fig. 6.

13 Fused silica slides were treated with PEDA (as described in
14 Example 1), then immersed in a 1 μM solution of phosphorothioate
15 DNA oligomer [5'-(ACTG)₅-3', P(S)] in dI water for 1 hr at 25C.
16 The slides were rinsed 3 times in dI water, dried under filtered
17 N₂, then examined using UV spectroscopy. The slides which were
18 treated with the P(S) oligomer yielded the characteristic DNA
19 absorbance peak at 260 nm, while the untreated slide did not
20 display this peak.

21 **Example 7 - Photochemical Modification of a PEDA Film to Form**
22 **DNA Patterns**

23 PEDA films were prepared on fused silica slides as described
24 in Example 1. Advancing contact angle measurements determined
25 using 5-10 μl water droplets on a Zisman-type goniometer yielded

1 values of 60 +/-2 degrees.

2 Following formation of PEDA SAM on fused silica, a slide was
3 clamped to a chrome-on-quartz lithographic mask and exposed to
4 monochromatic 193 nm deep UV laser pulses using a Questek Ar-F
5 excimer laser. Total exposures of 350 mJ/cm² (0.3 mJ/pulse) were
6 employed. The smallest features on the mask employed were 100 μM
7 x 100 μM pads. PEDA slides patterned in this way were subsequently
8 immersed in a 1 μM solution of biotinylated oligomer 5'-(ACTG)₅-3'
9 for 30 min at room temperature, then the slide was blocked for 15
10 min and labeled DNA detected using SA-HRP as described in Example
11 2, above. Pattern features were successfully observed using
12 bright-field and differential contrast microscopy at 50X
13 magnification.

14
15
16 **Example 8 - Patterned Non-Covalent Immobilization of Synthetic**
17 **DNA on Negative Photoresist-Coated Aminosilane**
18 **Films on Fused Silica Slides**

19 Fused silica slides were treated with DETA as described above
20 (Example 1) to form films. DETA slides were then spin-coated with
21 negative photoresist SAL601-ER7™ (Shipley) for 30 seconds at 7K
22 rpm, then baked at 90C for 30 min. A lithographic mask was placed
23 on top of the photoresist-treated slide, then the assembly was
24 exposed to a 254 nm UV lamp for 3 seconds (17.4 mJ, 5.8 mW/cm²).
25 The slide was developed by baking at 115C for 5 min, then treatment

1 in MF312 CD27 developer for 6 minutes. The slides were rinsed with
2 dI water, and dried under filtered N₂. A 1 μM solution of 5'-
3 biotin-(ACTG)₅-3' in dI water was pipetted onto the exposed and
4 developed surface of the DETA/SAL601-E17™-treated slide, and
5 allowed to sit for 15 min at 25°C. The slide was rinsed in three
6 changes of dI water, then immersed in blocking buffer A (see
7 Example 2, above) for 15 min at 25°C. The slide was rinsed three
8 times in 0.1M Tris-0.015M NaCl, pH 7.5, then treated with 1:500
9 dilution of SA-HRP (see Example 2) in blocking buffer A for 15 min
10 at 25°C. After rinsing slides three times in 0.1M Tris-0.015M
11 NaCl, pH 7.5, they were immersed in the HRP substrate TMB,
12 whereupon blue color appeared on the slide as a negative tone image
13 (blue appears where the chrome regions on mask protected the
14 underlying photoresist/DETA; regions which were irradiated are not
15 removed by the developer). Differential interference contrast
16 microscopy was used to visualize the smallest feature sizes (100
17 μM² pads).

18
19 **Example 9 - Covalent Immobilization Using Aminosilane Films**
20 **Modified with a Heterobifunctional Crosslinker, and**
21 **Thiol-Modified DNA Oligomers**

22 Thiol-modified DNA oligomers were prepared using standard
23 phosphoramidite chemistry and an automated DNA synthesizer with the
24 following substitutions: a commercially available, 1 μM 3'-thiol
25 C3 S-S modifier DNA synthesis column (Glen Research) was used

Docket No.: N.C. 76,081
Inventor's Name: Chrisey et al.

PATENT APPLICATION

1 instead of the usual DNA base (A, C, T or G) synthesis column, and
2 a 0.02M I₂ oxidizer was used instead of the usual 0.1M I₂ oxidizer.
3 Purification and quantification of the product DNA oligomer was
4 performed as described in Examples 1 and 2. A concentrated
5 solution of the protected disulfide form of the oligomer [5'-
6 (ACTG)₅-S-S-3'] was prepared in water, then treated with a solution
7 of dithiothreitol (DTT) in phosphate buffer (final concentrations:
8 30 nMoles DNA oligomer 0.04M DTT, 0.17M Na-phosphate, pH 8.0) for
9 15 -20 hr at 25C to reduce the disulfide oligomer to its free thiol
10 form [5'-(ACTG)₅-SH-3']. Immediately before addition to SMPB
11 crosslinker-modified DETA or EDA substrates (see below),
12 extraction of the solution (typically 1 ml volume) with 3
13 equivalent portions of ethyl acetate (to remove excess DTT), is
14 performed to yield the free thiol form of the oligomer. This form
15 of the oligomer will be referred to as 5'-Biotin-(ACTG)₅-SH-3'
16 henceforth.

17 EDA and DETA films were prepared on fused silica slides as
18 described in Example 1. A 1 mM solution of the heterobifunctional
19 crosslinker SMPB {succinimidyl 4-(p-maleimidophenyl)butyrate} was
20 prepared by dissolution in 100 μl DMSO, then mixture with 30 ml of
21 20% DMSO, 80% MeOH. EDA or DETA slides were treated with the SMPB
22 solution in glass jars for 2 hr at 25°C. Slides were rinsed three
23 times in fresh MeOH, and dried under a stream of filtered N₂.
24 Slides were then transferred to a fresh glass jar along with 30 ml
25 degassed buffer (10 mM HEPES, 5 mM EDTA, pH 6.6) to which

1 approximately 30 nMoles of the free thiol form of the DNA oligomer
2 has been added, then placed into a Ar-purged glovebag. The
3 reaction is conducted under a positive pressure of Ar (to avoid
4 oxidation of the thiol-modified DNA oligomer) for 2 hr at 25C, then
5 slides are rinsed once in fresh HEPES buffer and twice in dI water,
6 the dried under a stream of filtered N₂.

7
8 To remove non-covalently bound DNA from the slides, slides were
9 treated for 24 hr with 50 mM Na-phosphate, 1M NaCl, pH 7.6 at 25C,
10 then rinsed three times with dI water, and dried under N₂ (see
11 Example 4).

12
13 Reactions were followed by UV spectroscopy, after each succeeding
14 layer was deposited on the slide: DETA or EDA, then SMPB, then
15 total DNA, then covalently bound DNA remaining after treatment
16 under high salt concentrations to remove any electrostatically-
17 bound DNA. EDA and DETA films show no absorbance above 210 nm; the
18 SMPB film shows a small absorbance at approximately 220 nm (due to
19 the phenyl group), while the total DNA peak is clearly observed at
20 260 nm, and is reduced by approximately 30% following treatment
21 under high salt conditions.

1 on-glass mask, then rinsed briefly in 1 M NaCl. Following this
2 rinse, the slides were treated with deprotected 5'-biotin-(ACTG)₅-
3 SH-3' DNA as described in Example 9 for EDA and DETA films. The
4 slides were treated with 50 mM Na-phosphate, 1M NaCl, pH 7.6 for 24
5 hr to remove electrostatically (non-specifically) bound DNA, then
6 were blocked using Buffer A. The biotin-labeled DNA was visualized
7 using a enzyme-linked colorimetric assay as described in Example 5.
8 Patterns were visible using either brightfield or differential
9 interference contrast (DIC) microscopy at 50X magnification.

10
11 **Example 12 - DNA Patterns Fabricated Using UV Lamp**
12 **Irradiation of Positive Photoresist-Coated**
13 **Aminosilane Films on Fused Silica Substrates**

14 DETA films were prepared on fused silica slides as described
15 in Example 1, then treated with a 1 mM SMPB solution as described
16 in Example 9. S-1400-11 positive-tone photoresist (Shipley Corp.)
17 was spin-coated onto the DETA/SMPB surfaces (5K rpm) and then baked
18 at 90C for 30 min. Slides were exposed through a chrome-on glass
19 mask using a 254 nm UV lamp (40 - 50 mJ/cm² total exposure, 8 - 10
20 sec), then developed using MF312 CD27TM developer (Shipley Corp) for
21 50 sec. With the positive tone resist, features that are not
22 protected by the chrome areas of the mask dissolve away upon
23 development, exposing the underlying silane/crosslinker layers.
24 The slides are treated under an Ar atmosphere with a 1 μM solution
25 of deprotected 5'-biotin-(ACTG)₅-SH-3' DNA oligomers as described

1 in Example 9, then slides are rinsed with fresh HEPES buffer and dI
2 water and dried under N₂. Visualization of DNA patterns using the
3 colorimetric method described in Example 5 was accomplished without
4 further treatment of the slides. Patterns were also visible using
5 the colorimetric detection scheme following treatment of the slides
6 for 24 hr in 50 mM Na-phosphate, 1 M NaCl to remove
7 electrostatically (i.e., non-specifically) bound DNA from the
8 surface.

9
10 **Example 13 - Demonstration of Retention of Covalently**
11 **Immobilized DNA on Aminosilane-Crosslinker**
12 **Surfaces Following Treatment with Photoresist**
13 **Developer MF312 CD27**

14 DETA films were prepared on fused silica slides (see Example
15 1), and treated with the SMPB crosslinker as described in Example
16 9. Slides were then treated with : 1) MF312 CD27 developer (50
17 sec) followed by the deprotected 5'-Biotin-(ACTG)₅-SH-3' DNA
18 oligomer (see Example 9), or 2) the deprotected 5'-Biotin-(ACTG)₅-
19 SH-3' DNA oligomer (see Example 9), then the MF312 CD27 developer
20 for 50 sec. In each case, DNA was visualized following detection
21 using colorimetric methods described in Example 5. These results
22 suggest that both the aminosilane-crosslinker linkage and the
23 crosslinker-DNA-biotin linkages may be stable to the harsh
24 developing (pH >13) conditions require for the photoresist.

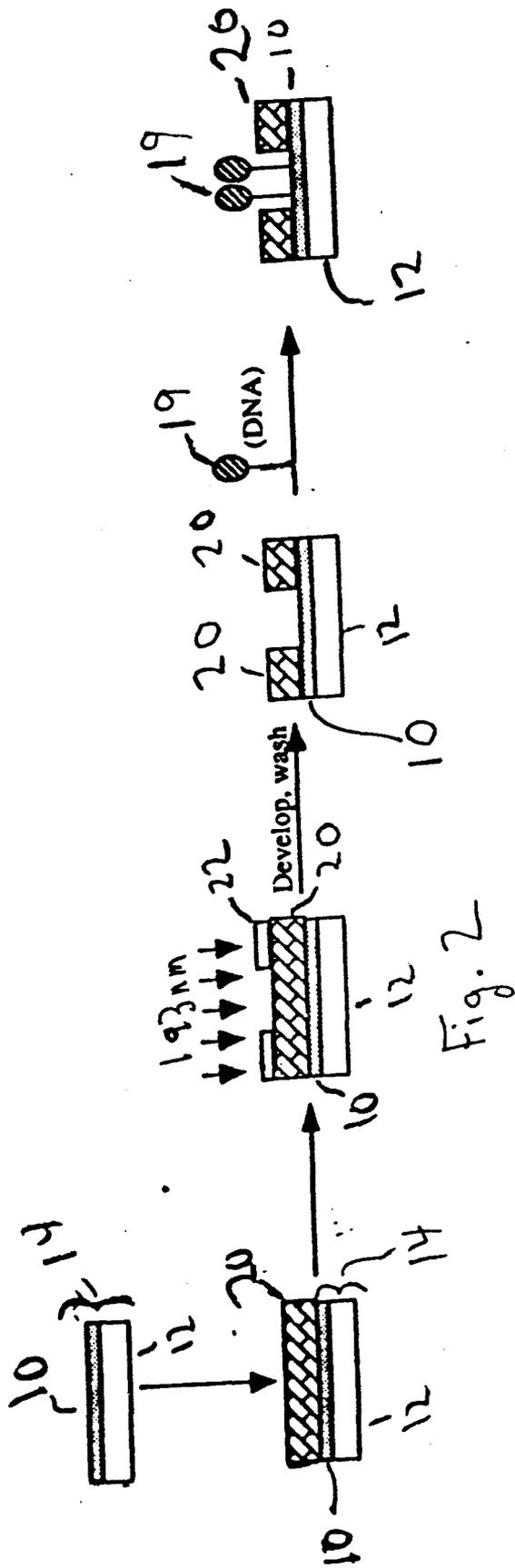
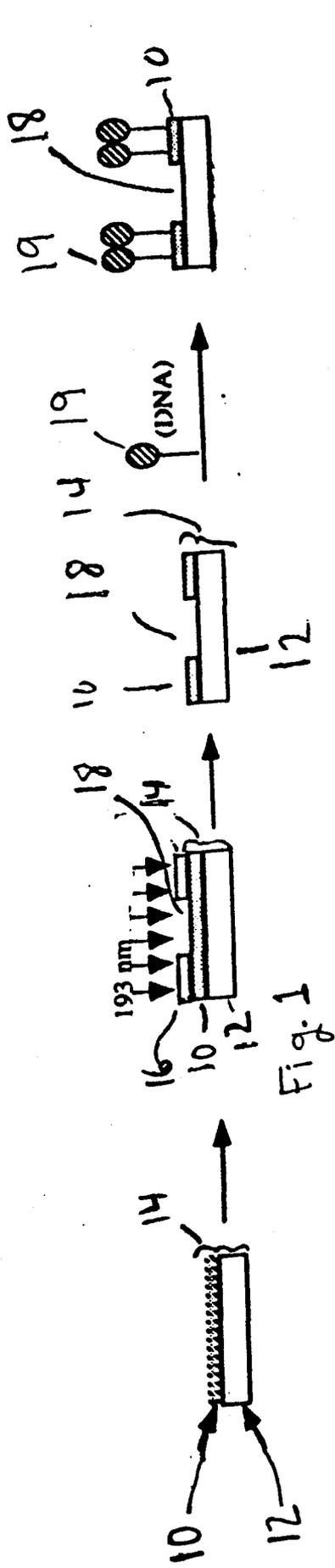
1 Obviously, many modifications and variations of the present
2 invention are possible in light of the above teachings. For
3 example, although the present specification has described the
4 invention with particular reference to organosilanes as examples of
5 nucleic acid-binding coating molecules, it should be understood
6 that other molecules may be used for the nucleic acid-binding
7 substrate coating. That is, any coating molecules having one
8 functional group that reacts with the surface and another
9 transformable functional group that does not bind to the surface or
10 other coating molecules, but whose ability to directly or
11 indirectly bind nucleic acid molecules is reversed by irradiation
12 (i.e., irradiation destroys or creates the ability to covalently or
13 non-covalently bind, either directly or indirectly, nucleic acid
14 molecules), may be used for the substrate coating in place of the
15 nucleic acid-binding organosilane molecules. It is therefore to be
16 understood that, _____, the
17 invention may be practiced otherwise than as specifically
18 described.

Docket No.: N.C. 76,081
Inventor's Name: Chrisey et al.

PATENT APPLICATION

ABSTRACT

Patterns of pre-formed hybridizable nucleic acid oligomers are formed upon a substrate. The substrate is coated with molecules, such as aminosilanes, whose reactivity with nucleic acid molecules can be transformed by irradiation. The coated substrate exposed to patterned irradiation then contacted with pre-formed nucleic acid oligomers. The binding of the preformed nucleic acid oligomers to the coating molecules may be covalent or non-covalent (for example, ionic bonding or hydrogen bonding). If desired, a heterobifunctional crosslinker may be employed, before or after irradiation, with the coating to promote covalent binding of the nucleic acid oligomers to the coating molecules. Also, the irradiation step may be performed with the assistance of a positive-tone or negative-tone photoresist.



5'-ACTGACTGACTGACTGACTG-3'-SH

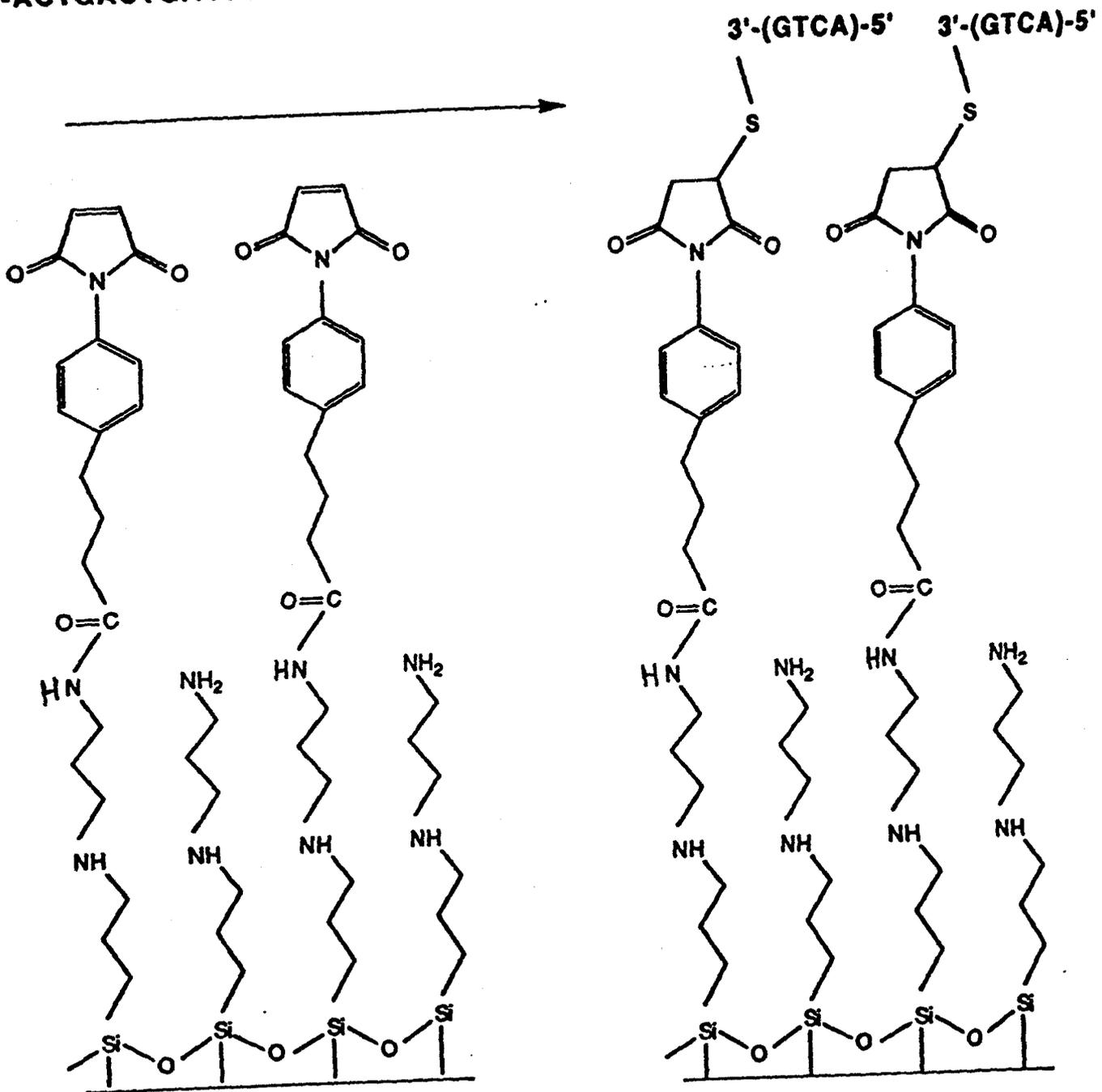


Fig. 3

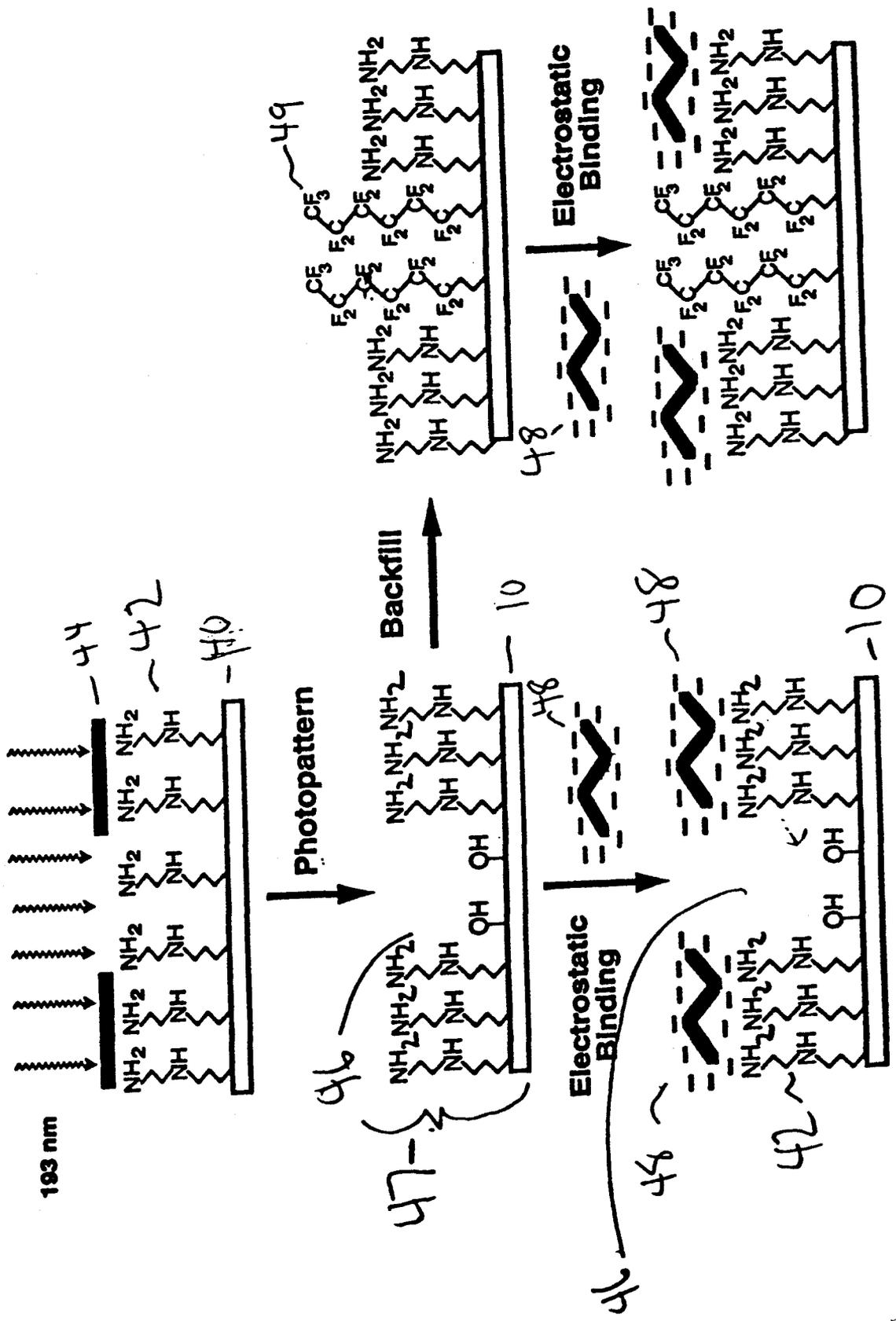


Fig. 4

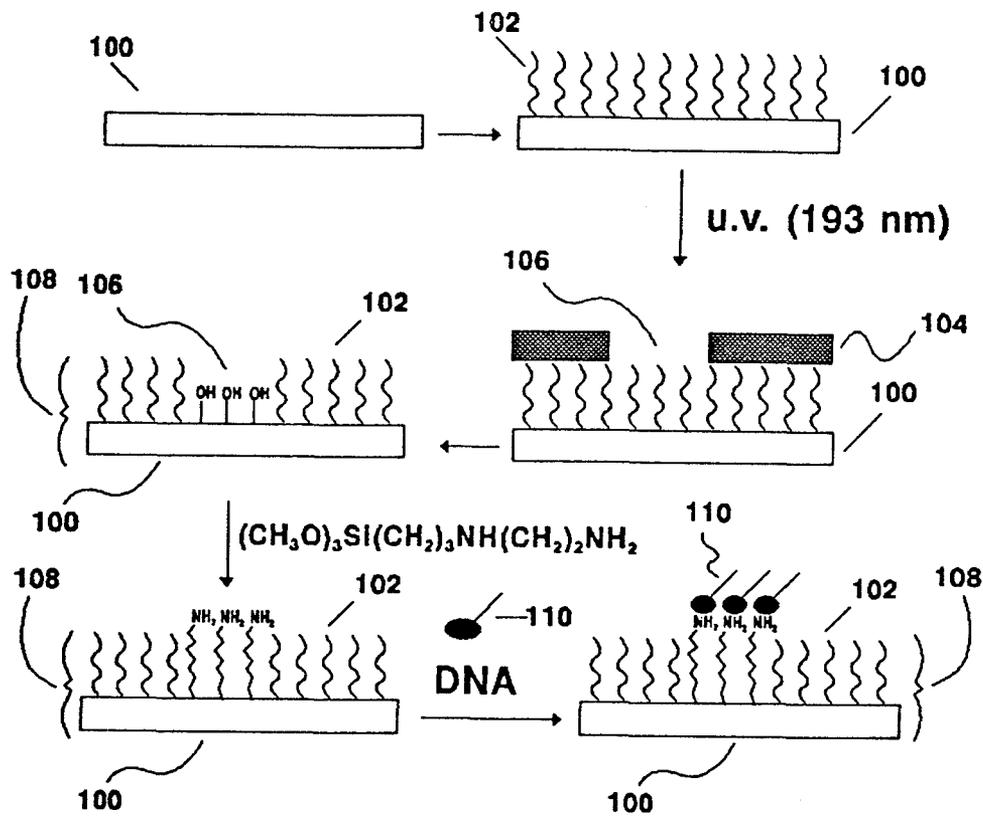


Fig. 5

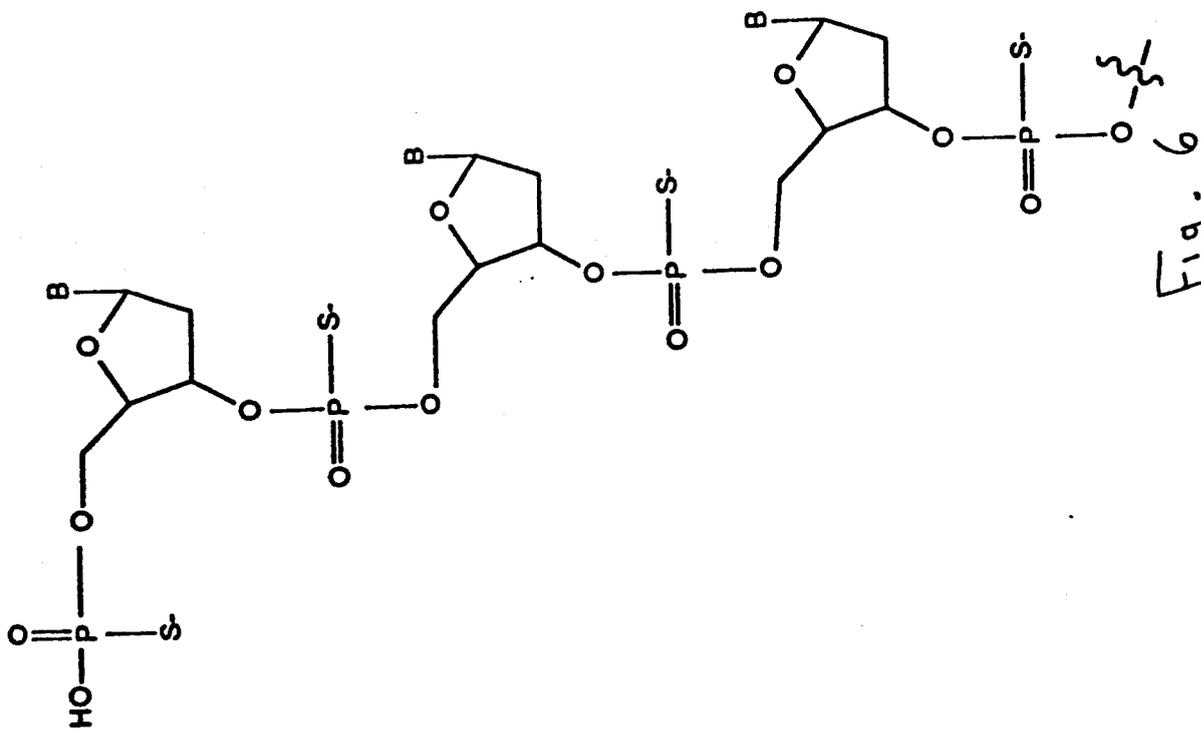


Fig. 6