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(54) Title: DEVICE BASED ON CELLULOSIC SUBSTRATE

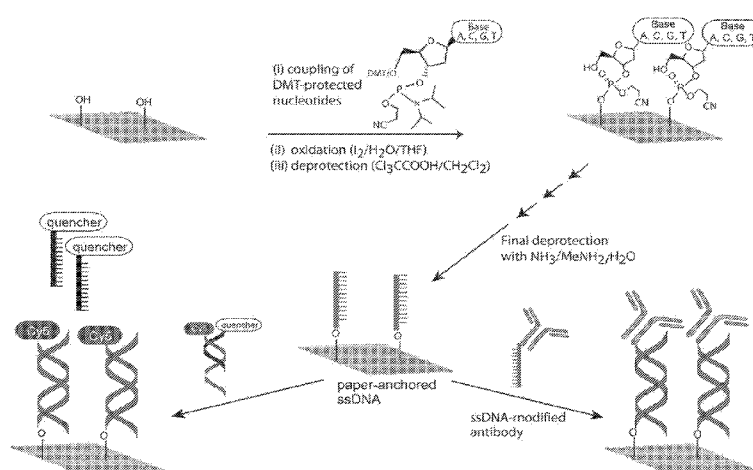


FIG. 1

(57) Abstract: A device is described, including: a cellulosic substrate comprising a plurality of hydroxyl groups; and one or more oligonucleotide covalently bonded to one or more of the hydroxyl groups. In certain embodiments, the oligonucleotide is DNA or RNA.

DEVICE BASED ON CELLULOSIC SUBSTRATE

Incorporation by Reference

[0001] All patents, patent applications and publications cited herein are hereby incorporated by reference in their entirety. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described herein.

Related Application

[0002] This application claims priority to U.S. Provisional Application No. 62/250,063, filed November 3, 2015, the contents of which are hereby incorporated by reference in its entirety.

Government Funding Clause

[0003] This invention was made with support from the United States government under Grant No. HDTRA1-14-C-0037 awarded by the Defense Threat Reduction Agency, Grant Nos. N66001-2-C4207 and N66001-14-2-4053 awarded by the Department of Defense/DARPA, and Grant No. R01GM065865 from National Institute of Health. The United States government has certain rights to this invention.

Field of the Invention

[0004] The present disclosure generally relates to the field of cellulosic-based devices.

Background

[0005] Microarrays are convenient tools for the multiplex analysis of several biological samples in clinical diagnostics. A microarray is a solid support bearing microscopic features that can detect specific target molecules and generate diagnostic data. The standard method of fabrication for microarrays is pin-spotting—a method in which a robotic system deposits small volumes of a solution containing a probe (usually DNA, RNA, antibody, or protein) onto a glass, silicon, or polymer-based substrate. Alternative methods include microstamping, inkjet printing, laser writing, or electrospray deposition, among others. These substrates can be derivatized with poly-L-lysine, polyamidoamine dendrimer, amino-terminated silanes, aldehydes, carboxylic acids, or other reactive groups that facilitate attachment. Existing methods for the fabrication of microarrays rely on complex equipment for processing, and

require a series of lengthy purification and functionalization steps; the substrates commonly used are neither flexible nor inexpensive, and are difficult to integrate in low-cost diagnostics systems intended for use in resource-limited settings.

[0006] Paper-based microfluidic systems (μ PADs) have emerged in recent years as a promising technology to address the growing need for simple, quantitative, point-of-care diagnostic devices capable of detecting different analytes from the same specimen in a single run. Paper is a useful substrate for the fabrication of microarrays through its high surface area (due to its high surface roughness and internal porosity) and high density of accessible hydroxyl functional groups. Paper is also inexpensive, flexible, easily shaped by cutting or folding, and disposable by incineration.

[0007] To carry out separate assays simultaneously with minimal cross talk on a micro paper-based analytical device (a μ PAD), distinct microzones may vary either in terms of access to stored reagents required for the detection of each target, or in their affinity for the target molecule. The first approach has received significant attention following the development of 3D μ PADs—systems which distribute the sample via vertical flow to independent test zones that store distinct reagents. The second approach has, so far, been largely ignored, probably due to the scarcity of methods available for assembling high quality microarrays on paper. To enable sensitive assays, these microarrays can be immobilized (preferably covalently, or, if noncovalent, with very low dissociation constants) and at high density on the surface of the substrate.

[0008] Thus, there is a growing need in clinical diagnostics for efficient, quick, parallel, and multiplex analysis of biomarkers from small biological samples.

Summary

[0009] Described herein are cellulosic substrate-based devices including one or more oligonucleotides bonded to the cellulosic substrate. The devices may include an array of the oligonucleotides. In certain embodiments, the oligonucleotides are bonded with the cellulosic substrate in high density per area of the cellulosic substrate.

[0010] Also described herein are methods for detecting fluorophore-linked DNA oligonucleotides, methods for assembling microarrays of DNA-conjugated antibodies/antigens on the cellulosic substrates, methods for detecting protein

antigens/antibodies. In certain embodiments, the device may include an array of two or more different types of oligonucleotides each capable of carrying out different detections, *e.g.*, detecting DNA and antibodies in the same device.

[0011] In one aspect, a device is described, including: a cellulosic substrate including at least one hydrophilic zone including a plurality of hydroxyl groups; and one or more first oligonucleotide each covalently bonded to the cellulosic substrate through one or more of the hydroxyl groups by a -O- linker.

[0012] In any of the preceding embodiments, the first oligonucleotide is DNA or RNA.

[0013] In any of the preceding embodiments, the first oligonucleotide is single strand DNA.

[0014] In any of the preceding embodiments, the device includes one or more second oligonucleotide different from the first oligonucleotide and are covalently bonded to the hydroxyl groups.

[0015] In any of the preceding embodiments, the first or second oligonucleotide is covalently bonded to the hydroxyl groups directly or through a linker.

[0016] In any of the preceding embodiments, the cellulosic substrate is paper.

[0017] In any of the preceding embodiments, the first oligonucleotide bonded to the hydroxyl groups of the cellulosic substrate has a density of more than about $0.1 \times 10^{14}/\text{cm}^2$, about $0.2 \times 10^{14}/\text{cm}^2$, about $0.5 \times 10^{14}/\text{cm}^2$, about $1.0 \times 10^{14}/\text{cm}^2$, about $2.0 \times 10^{14}/\text{cm}^2$, about $3.0 \times 10^{14}/\text{cm}^2$, about $4.0 \times 10^{14}/\text{cm}^2$, about $5.0 \times 10^{14}/\text{cm}^2$, about $6.0 \times 10^{14}/\text{cm}^2$, or about $10.0 \times 10^{14}/\text{cm}^2$.

[0018] In any of the preceding embodiments, the device includes an array of the first oligonucleotides.

[0019] In any of the preceding embodiments, the device includes includes an array of the first oligonucleotides and an array of the second oligonucleotides.

[0020] In any of the preceding embodiments, the device further includes one or more third oligonucleotides complementary to the first oligonucleotides and hybridized with the first oligonucleotides.

[0021] In any of the preceding embodiments, the device further includes one or more antibodies or antigens bonded to the first, second, or third oligonucleotides.

[0022] In any of the preceding embodiments, the device is a microfluidic analytical device and the cellulosic substrate further includes one or more hydrophilic channels in fluidic communication with the hydrophilic zone.

[0023] In any of the preceding embodiments, the cellulosic substrate includes one or more cellulosic layers and the hydrophilic zone and the hydrophilic channel are on the same or different cellulosic layers.

[0024] In any of the preceding embodiments, the cellulosic substrate further includes one or more sample deposition zone in fluidic communication with the hydrophilic zone.

[0025] In any of the preceding embodiments, the device further includes one or more hydrophobic areas defining the hydrophilic zone.

[0026] In any of the preceding embodiments, the device further includes one or more hydrophobic materials defining the hydrophilic zone.

[0027] In any of the preceding embodiments, the first or second oligonucleotide includes 2-1000 nucleotides.

[0028] In another aspect, a method of preparing the device of any of the embodiments described herein is disclosed, including:

providing the cellulosic substrate including at least one hydrophilic zone including a plurality of hydroxyl groups; and
covalently bonding one or more first oligonucleotide to one or more of the hydroxyl groups.

[0029] In any of the preceding embodiments, the bonding step is conducted by an automatic oligonucleotide synthesizer.

[0030] In yet another aspect, a method of preparing an antigen or antibody-bonded device is described, including:

providing the device of any of the embodiments described herein;

contacting the first oligonucleotide with a third oligonucleotide complementary to the first oligonucleotide and bonded with an antigen or antibody; and
hybridizing the first and third oligonucleotides.

[0031] In yet another aspect, a method of detecting a target antigen or target antibody is described, including:

providing the device of any of the embodiments described here;
providing a sample containing a target antigen or target antibody which is specific to the antibody or antigen, respectively, bonded to the first, second, or third oligonucleotides; and
forming a conjugate between the target antigen/antibody and the oligonucleotide-bonded antibody/antigen.

[0032] In any of the preceding embodiments, the target antigen or target antibody includes a florescent molecule.

[0033] In yet another aspect, a method of detecting a DNA is described, including:

providing the device of any of the embodiments described herein;
providing a sample containing a DNA including complementary first and second strand oligonucleotides, wherein at least one of the first and second strand oligonucleotides is complementary to the first oligonucleotide bonded to the device; and at least one of the first and second strand oligonucleotides is bonded with a fluorescent molecule;
contacting the first oligonucleotide with the sample to allow the first oligonucleotide to hybridize with one of the first and second strand oligonucleotides.

[0034] In yet another aspect, a method of detecting a first target and second target different from the first target is described, including:

providing the device of any of the embodiments described herein;
providing one or more samples containing the first and second targets;
wherein the first target is specific to the first oligonucleotide and the second target is specific to second oligonucleotide; and the first and second targets are each

independently selected from the group consisting of a DNA, an antigen, and antibody;
and

allowing the one or more samples to react with the device.

[0035] It is contemplated that any embodiment disclosed herein may be properly combined with any other embodiment disclosed herein. The combination of any two or more embodiments disclosed herein is expressly contemplated. It is contemplated that embodiments can be variously combined or separated without parting from the invention.

BRIEF DESCRIPTION OF DRAWINGS

[0036] The invention is described with reference to the following figures, which are presented for the purpose of illustration only and are not intended to be limiting.

[0037] Figure 1 is a schematic representation of DNA synthesis on paper and its applications to nucleic acid detection and formation of antibody and protein arrays, according to one or more embodiments described herein.

[0038] Figures 2A-2B show the synthesis of DNA oligonucleotides on paper, according to one or more embodiments described herein. Figure 2A shows that the absorbance at 470 nm, produced by the release of DMT carbocation after the coupling of a nucleotide, is used to monitor the yield of each nucleotide addition step. The sequence used in this study was 5'-CGATCCACTACAAGCTTGCCATCATGTCGATC-3'. Figure 2B shows a HPLC trace of DNA fragments cleaved from the paper by reducing the disulfide bridge (the sequence is 5'-CGATCCACTACAAGCTTTTS-STTTTTTTTTTTTTTT-3'). The arrow indicates the peak of the full-length oligonucleotide product. Other marked peaks indicate truncation oligonucleotide products.

[0039] Figures 3A-3C illustrates fluorescence-based detection of DNA oligonucleotides using strand displacement within paper-anchored ssDNA arrays, according to one or more embodiments described herein.

[0040] Figure 4 illustrates the detection of fluorescent goat anti-rabbit IgG in a solution of goat serum, using a paper-anchored IgG microarray formed by the hybridization of ssDNA (sequence S2) synthesized directly on the surface of paper, with complementary ssDNA-conjugated IgG (black squares), according to one or more embodiments described herein.

[0041] Figures 5A and 5B show a device using paper-anchored ssDNA arrays for the detection of hCRP in a solution of human serum, according to one or more embodiments described herein. Figure 5A shows the schematic of the sandwich ELISA for hCRP and a calibration plot for fluorescence versus the concentration of hCRP. Figure 5B shows the images of the results using 16 to 100 pg mL⁻¹ concentrations of hCRP.

[0042] Figures 6A-6C show a device using paper-anchored ssDNA arrays for the multiplex detection of fluorescently-labeled nucleic acids and antibodies, according to one or more embodiments described herein. Figure 6A is a schematic of the process. Figure 6B shows the image of a device assembled using two paper-based arrays adjacent to each other, supported by vinyl plastic tape. Figure 6C shows the fluorescence intensity obtained using seven independent devices when adding a mixture of FQ and Ab, buffer only, FQ only, or AB only, to the device.

[0043] Figure 7 shows PAGE gel of non-denatured (left) and denatured (right) IgG-DNA complexes, according to one or more embodiments described herein. Arrows indicate the position of the IgG-DNA complexes (left, <160 kDa) and of the heavy (~50 kDa) and light chains (~25 kDa) derived from the IgG-DNA complexes (right).

[0044] Figure 8 shows the design and fabrication of paper-based devices, according to one or more embodiments described herein.

DETAILED DESCRIPTION

[0045] The foregoing and other features and advantages of various aspects of the invention(s) will be apparent from the following, more-particular description of various concepts and specific embodiments within the broader bounds of the invention(s). Various aspects of the subject matter introduced above and discussed in greater detail below may be implemented in any of numerous ways, as the subject matter is not limited to any particular manner of implementation. Examples of specific implementations and applications are provided primarily for illustrative purposes.

[0046] In one aspect, a device is described, including a cellulosic substrate having at least one hydrophilic zone including a plurality of hydroxyl groups; and one or more first oligonucleotide covalently bonded to one or more of the hydroxyl groups by forming a “-O-” linkage. As used herein and as understood by one of ordinary skill in the art, covalently

bonding oligonucleotide refers to the formation of a linkage of “-O-” between the cellulosic substrate and the oligonucleotide, *e.g.*, cellulosic substrate-O-oligonucleotide. Non-limiting examples of the first oligonucleotide include DNA and RNA. In certain embodiments, the first oligonucleotide is a single strand DNA, a double strand DNA, a messenger RNA, a transfer RNA. In certain embodiments, the first oligonucleotide is a siRNA, miRNA, long ncRNA, DNA or RNA aptamers. In certain specific embodiments, the first oligonucleotide is single strand DNA.

[0047] In some embodiments, the first or second oligonucleotide includes about 2-1000 nucleotides. In some embodiments, the first or second oligonucleotide includes about 5, 10, 15, 20, 25, 30, 35, 50, 100, or 200 nucleotides, or a number of nucleotides in any range bounded by any two values disclosed herein. In one specific embodiment, the first oligonucleotide includes 32 nucleotides.

[0048] In some embodiments, the device includes one or more second oligonucleotide different from the first oligonucleotide and are also covalently bonded to the hydroxyl groups. The first or second oligonucleotide may be covalently bonded to the hydroxyl groups directly or through a linker. The linker may be an alkyl chain (*e.g.*, $-(CH_2)_n-$, where n is an integer from 1 to 20), optionally substituted by one or more of halogen, NR_1R_2 , S, -S-S-, or OR_1 ; wherein each R_1 and R_2 are independently alkyl. Alternatively, the linker may be a disulfide linker, an oligosaccharide, a polypeptide, another oligonucleotide (*e.g.*, an oligonucleotide that is not sterically hindered) and/or other linker known in the art.

[0049] As used herein, the phrase “cellulosic substrate” includes cellulose and other cellulosic substrate known in the art. A cellulosic substrate includes articles of manufacture such as paper and cardboard that are made primarily of cellulose. It also includes modified cellulose, for example, where the hydroxyl groups of cellulose can be partially or fully reacted with various reagents to afford derivatives with useful properties such as nitrocellulose, cellulose esters and cellulose ethers. Non-limiting examples of the cellulosic substrate include paper, cellulose, cellulose derivatives, woven cellulosic materials, and non-woven cellulosic materials. Non-limiting examples of derivatives of cellulose include nitrocellulose or cellulose acetate.

[0050] In some embodiments, the cellulosic substrate is paper. Paper is inexpensive, widely available, readily patterned, thin, lightweight, and can be disposed of with minimal

environmental impact. Furthermore, a variety of grades of paper are available, permitting the selection of a paper substrate with the weight (*i.e.*, grammage), thickness and/or rigidity and surface characteristics (*i.e.*, chemical reactivity, hydrophobicity, and/or roughness) desired for the fabrication of a particular analytical device. Suitable papers include, but are not limited to, chromatography paper, card stock, filter paper, vellum paper, printing paper, wrapping paper, ledger paper, bank paper, bond paper, blotting paper, drawing paper, fish paper, paper towel, wax paper, photography paper, nitrocellulose, cellulose acetate, cellulosic paper, toilet paper, tissue paper, notebook paper, Kim Wipes, VWR Light-Duty Tissue Wipers, Technicloth Wipers, newspaper, any other paper that does not include binders, cloth, and porous polymer film. In general, any paper that is compatible with the disclosed bonding method may be used. In certain embodiments, the paper includes Whatman chromatography paper No. 1.

[0051] Applicants have surprisingly found that the first or second oligonucleotide can be bonded to the hydroxyl groups of the cellulosic substrate in high density per area of the cellulosic substrate. In certain embodiments, the first or second oligonucleotide bonded to the cellulosic substrate has a density of more than about $0.1 \times 10^{14}/\text{cm}^2$, about $0.2 \times 10^{14}/\text{cm}^2$, about $0.5 \times 10^{14}/\text{cm}^2$, about $1.0 \times 10^{14}/\text{cm}^2$, about $2.0 \times 10^{14}/\text{cm}^2$, about $3.0 \times 10^{14}/\text{cm}^2$, about $4.0 \times 10^{14}/\text{cm}^2$, about $5.0 \times 10^{14}/\text{cm}^2$, about $6.0 \times 10^{14}/\text{cm}^2$, or about $10.0 \times 10^{14}/\text{cm}^2$, or in a ranged bounded by any two values disclosed herein. Without wishing to be bound by any particular theory, it is believed that the high surface area, high roughness of the cellulosic substrate (*e.g.*, paper), and/or its porosity, increase the area accessible to reagents and allow for larger numbers of oligonucleotides to be attached per area. Additionally, compared with other substrate, cellulosic substrates also contain high density of available hydroxyl for bonding with the oligonucleotides. Thus, high density of bonded oligonucleotide per area of the cellulosic substrate can be achieved. In certain embodiments, the first or second oligonucleotide is bonded to the cellulosic substrate in a density 2, 5, 10, 15, 30, 50, 100, or 200 times (or in any range bounded by any two values disclosed herein) higher than the density of the oligonucleotide bonded to other types of substrates such as glass or polymer.

[0052] In some embodiments, the device as described herein includes an array of the first or second oligonucleotides. In some embodiments, the device as described herein includes an array of the first oligonucleotides and an array of the second oligonucleotides different from the first oligonucleotide. The arrays of the one or more types of oligonucleotides offer the

ability to conduct multiplex assays of the same or different kinds. In some embodiments, the arrays or the device described herein can be used for detecting fluorophore-linked DNA oligonucleotides, assembling microarrays of DNA-conjugated antibodies/antigens on the cellulosic substrates, and/or detecting protein antigens/antibodies. In certain embodiments, the device may include an array of two or more different types of oligonucleotides each capable of carrying out different detections, *e.g.*, detecting DNA and antibodies in the same device. Thus, in these exemplary devices, a first area of the device contains a first oligonucleotide while a second area of the device contains a second oligonucleotide different from the first oligonucleotides. As a result, a device as described herein may be used to detect two or more different types of biological molecules contained in the sample.

[0053] In some embodiments, the device as described herein utilizes the bonded first or second oligonucleotide as a structural anchor for attaching additional molecules to offer additional functions and/or structural features. In some embodiments, the device as described herein further includes one or more third oligonucleotides complementary to the first oligonucleotides and hybridized with the first oligonucleotides. One particular example is that the first oligonucleotide is a ssDNA which hybridizes with the complementary third oligonucleotide. Thus, in some embodiments, a device having a dsDNA bonded to the hydroxyl groups of the cellulosic substrate can be obtained.

[0054] In some embodiments, the first, second, or third oligonucleotide has antibodies or antigens bonded and the device may include one or more antibodies or antigens bonded to the cellulosic substrate. If the device includes an array of oligonucleotides, an array of the antibodies or antigens can be bonded to the cellulosic substrate. In some specific embodiments, the complementary third oligonucleotide has an antibody or antigen attached to it and upon its hybridization with the first oligonucleotide, the antibody or antigen can be attached to the cellulosic substrate. In some embodiments, a further antigen/antibody specific to the cellulosic substrate-bond antibody/antigen are bond to the cellulosic substrate through the specific antibody-antigen recognition. In some embodiments, further biologically important molecules such as proteins and enzymes can be attached to the cellulosic substrate as well.

[0055] In some embodiments, the device is a microfluidic analytical device and the cellulosic substrate further comprises one or more hydrophilic channels in fluidic communication with the hydrophilic zone. The hydrophilic channels or hydrophilic zone of the cellulosic

substrate may be formed by surrounding the hydrophilic zone or channel with hydrophobic barriers such as polymer (*e.g.*, photoresists). In certain embodiments, the cellulosic substrate comprises one or more cellulosic layers and the hydrophilic zone and the hydrophilic channel are on the same or different cellulosic layers. In certain embodiments, the cellulosic substrate further includes one or more sample deposition zone in fluidic communication with the hydrophilic zone. In an analytic assay, a sample containing a target molecule can be deposited in the sample deposition zone and through capillary action, the sample can flow to the hydrophilic zone, optionally through the hydrophilic channel, to undergo reactions or interactions with the first oligonucleotide or one or more biological molecule (*e.g.*, antigen, antibody, protein, or enzyme) bonded to the first oligonucleotide. More examples of the hydrophobic barriers and microfluidic devices can be found in PCT/US2007/081848, the entire content of which is incorporated by reference.

[0056] In some embodiments, a method of preparing the device described herein is disclosed, including: providing the cellulosic substrate comprising at least one hydrophilic zone comprising a plurality of hydroxyl groups; and covalently bonding one or more first oligonucleotide to one or more of the hydroxyl groups. In certain embodiments, the bonding step is conducted by an automatic oligonucleotide synthesizer or any other methods known in the art.

[0057] In some embodiments, a method of preparing an antigen or antibody-bonded device is described, including providing the device of any one of the embodiment described herein; contacting the first oligonucleotide with a third oligonucleotide complementary to the first oligonucleotide and bonded with an antigen or antibody; and hybridizing the first and third oligonucleotides.

[0058] In other embodiments, a method of detecting a target antigen or target antibody, including: providing the device described herein including one or more antibodies or antigens bonded to the first, second, or third oligonucleotides; providing a sample containing a target antigen or target antibody which is specific to the antibody or antigen, respectively, bonded to the first, second, or third oligonucleotides; and forming a conjugate between the target antigen/antibody and the oligonucleotide-bonded antibody/antigen. To facilitate the detection, the target antigen or target antibody may have an attached florescent molecule and upon bonding, the florescent molecule may release a detectable florescent signal.

[0059] In still other embodiments, a method of detecting a DNA is described, including providing the device described herein; providing a sample containing a DNA containing complementary first and second strand oligonucleotides, wherein at least one of the first and second strand oligonucleotides is complementary to the first oligonucleotide bonded to the device; and at least one of the first and second strand oligonucleotides is bonded with a fluorescent molecule; contacting the first oligonucleotide with the sample to allow the first oligonucleotide to hybridize with one of the first and second strand oligonucleotides. Thus, in these embodiments, the first oligonucleotides may have an affinity to the first strand oligonucleotides which is higher than the affinity between the first and second strand oligonucleotides. As a result, the first oligonucleotide replaces one of the first and second strand oligonucleotides in the target molecule. A fluorescent molecule may be released to result in a detectable signal. On other embodiments, the target DNA may further include a quencher molecule preventing the fluorescent signal until the detection. In certain embodiments, one of the first and second strand oligonucleotides has the fluorescent moiety attached and the other has the quencher molecule attached. The binding of the first oligonucleotide with one of the first and second strand oligonucleotides separates the fluorescent molecule from the quencher molecule to result in a detectable fluorescent signal.

[0060] In still other embodiments, a method of detecting a first target and second targets different from the first target is described, including: providing the device described herein containing one or more second oligonucleotide different from the first oligonucleotide covalently bonded to the hydroxyl groups; providing one or more samples containing the first and second targets; wherein the first target is specific to the first oligonucleotide and the second target is specific to second oligonucleotide; and the first and second targets are each independently selected from the group consisting of a DNA, an antigen, and antibody; and allowing the one or more samples to react with the device. Non-limiting examples of the first and second targets include oligonucleotide, antigen, antibody, protein, and other enzymes.

[0061] In still other embodiments, a kit is described, including one or more of the device described in any of the embodiments disclosed herein. In certain embodiments, the kit includes one or more of the device described in any of the embodiments disclosed herein; and instructions for using the kit to conduct any of the detection method described herein. In certain embodiments, the kit includes one or more of the device described in any of the embodiments disclosed herein; and instructions for providing a sample containing a target

antigen or target antibody which is specific to the antibody or antigen, respectively, bonded to the first, second, or third oligonucleotides; and instructions for forming a conjugate between the target antigen/antibody and the oligonucleotide-bonded antibody/antigen. To facilitate the detection, the target antigen or target antibody may have an attached florescent molecule and upon bonding, the florescent molecule may release a detectable florescent signal.

[0062] In still other embodiments, the kit includes one or more of the device described in any of the embodiments disclosed herein; instructions for providing a sample containing a DNA containing complementary first and second strand oligonucleotides, wherein at least one of the first and second strand oligonucleotides is complementary to the first oligonucleotide bonded to the device; and at least one of the first and second strand oligonucleotides is bonded with a fluorescent molecule; and instructions for contacting the first oligonucleotide with the sample to allow the first oligonucleotide to hybridize with one of the first and second strand oligonucleotides. Thus, in these embodiments, the first oligonucleotides may have an affinity to the first strand oligonucleotides which is higher than the affinity between the first and second strand oligonucleotides.

[0063] In still other embodiments, the kit includes one or more of the device described herein containing one or more second oligonucleotide different from the first oligonucleotide covalently bonded to the hydroxyl groups; instructions for providing one or more samples containing the first and second targets; wherein the first target is specific to the first oligonucleotide and the second target is specific to second oligonucleotide; and the first and second targets are each independently selected from the group consisting of a DNA, an antigen, and antibody; and instructions for allowing the one or more samples to react with the device. Non-limiting examples of the first and second targets include oligonucleotide, antigen, antibody, protein, and other enzymes.

Experimental Section

[0064] In certain embodiments, described herein is a new procedure for assembling microarrays of ssDNA and proteins on paper. This method starts with the synthesis of DNA oligonucleotides covalently linked to paper, and proceeds to generate DNA arrays capable of simultaneously capturing DNA, DNA-conjugated protein antigens, and DNA-conjugated antibodies. The synthesis of ssDNA oligonucleotides on paper is convenient and effective, with 32% of the oligonucleotides cleaved and eluted from the paper substrate being full-

length by HPLC for a 32-mer. These ssDNA arrays can be used to detect fluorophore-linked DNA oligonucleotides in solution, and as the basis for DNA-directed assembly of microarrays of DNA-conjugated capture antibodies on paper, detect protein antigens by sandwich ELISAs. Paper-anchored ssDNA arrays with different sequences can be used to assemble paper-based devices capable of detecting DNA and antibodies in the same device, and enable simple microfluidic paper-based devices.

[0065] In certain embodiments, the efficient synthesis of DNA oligomers 32 nucleotides in length on the surface of paper and the fabrication of simple paper-based devices that integrate nucleic acid and protein microarrays are described. First, we demonstrate that the arrays of ssDNA can be used to detect fluorescently labeled DNA oligomers in solution. Next, we show that we can produce microarrays of proteins and antibodies that are two orders of magnitude more dense (based on projected footprint) than microarrays fabricated by pin-spotting. We transformed the paper-anchored ssDNA arrays into antibody microarrays via complementary-strand hybridization with ssDNA-conjugated antibodies, and show that microarrays of capture antibodies can be used to increase the sensitivity of paper devices. Last, we show that we can use paper-anchored ssDNA arrays with different sequences to assemble paper-based devices capable of detecting DNA and antibodies in the same device.

[0066] The technique we used to fabricate microarrays takes advantage of the ease with which the surface of paper can be modified to synthesize oligomers of single-stranded DNA (ssDNA) directly. The synthesis of DNA on unmodified paper eliminates potentially time-consuming and costly purification procedures, and simplifies downstream processing. To expand the potential impact of these arrays in low-cost, point-of-care clinical diagnostics, we transformed the DNA microarrays into protein microarrays in situ using a technique developed by Jiang and Heath for use on the surface of silicon, glass, PDMS and other synthetic polymers. *See, e.g.,* R. C. Bailey, G. A. Kwong, C. G. Radu, O. N. Witte, J. R. Heath, *J. Am. Chem. Soc.* 2007, 129, 1959; C. Boozer, J. Ladd, S. Chen, S. Jiang, *Anal. Chem.* 2006, 78, 1515. The technique uses an antibody that is chemically linked to ssDNA and is complementary to a surface-bound ssDNA; the antibody is immobilized on the surface via sequence-specific hybridization. DNA-directed immobilization reduces protein denaturation and enables greater orientational freedom of the antigen-binding sites than either covalent immobilization or non-specific adsorption, and yields a larger proportion of immobilized proteins (antibodies or antigens) that have unhindered binding domains. Other

advantages of DNA-directed immobilization include increased homogeneity and reproducibility, and the consumption of less amount of antibody per experiment.

Direct Synthesis of Single-stranded Oligonucleotides using Paper as the Solid Support.

[0067] The high density of hydroxyl groups on the surface of cellulose paper makes it a useful substrate for the chemistry of phosphoramidite synthesis of ssDNA. Whatman chromatography paper has a uniform structure and is free of coatings or binders that could interfere with the synthesis process. Figure 1 outlines the procedure used for the solid-phase synthesis of DNA carried out using paper as solid support. The post-synthesis deprotection is carried out in situ, and the process does not require purification steps.

[0068] Current methods for fabrication of DNA microarrays require synthesis of DNA on-bead, deprotection and cleavage from the bead, purification by HPLC, and spotting on substrate. The low cost of the paper support (\$0.0007/cm² Whatman Chr 1 chromatography paper), and the lack of pre- or post-synthesis steps required to activate the substrate or purify the products make the process simple and cost-effective. It is contemplated that the method described herein is compatible with processing on a large scale.

[0069] The high surface roughness of the paper, and its porosity, increase the area accessible to reagents and allow for larger numbers of oligonucleotides to be synthesized per area (calculated based on its planar projected footprint) than on a flat substrate (e.g. glass or polymer) with the same surface chemistry. On other substrates, increasing surface area (by applying acrylamide gels to glass slides, for example) to allow the immobilization of larger amounts of DNA resulted in greater signal intensities and an increased dynamic range. We took advantage of the high surface area of the paper by using a paper with a relatively high profile root mean square roughness parameter, RR.M.S. of $6.4 \pm 1.9 \mu\text{m}$, area root mean square roughness parameter SR.M.S., of $10.7 \pm 0.6 \mu\text{m}$, and the porosity, or the volume fraction of void, of ~68%. While the real surface area is usually proportional to the surface roughness, the real surface area of a rough surface is challenging to quantify; since the measurements used in fluorescent and colorimetric assays refer to intensity per two-dimensional area, we quantified the surface density of nucleotides in terms of nucleotides per projected (also referred to as plane, flat, or apparent) area of paper.

[0070] The terminal DMT protective group of the oligonucleotide, if not cleaved at the end of the synthesis, provided a useful way of characterizing the density of the oligonucleotide on

the apparent surface of paper (Figures 2A-2B). The cleavage of the terminal DMT under acidic conditions can be monitored at 495 nm by UV spectrometer. The surface density, ρ_{SP} (defined as the number of molecules of oligomer per cm^2 of projected area S_p) can be obtained from the UV absorbance at 495 nm, A_{495} , using Eq 1, where ϵ is the molar absorptivity of DMT, v is the volume of the solution in which the DMT is cleaved, d is the optical path length, and N_A is Avogadro's number.

$$\rho_{SP} = \frac{A_{495 \text{ nm}} \times v \times N_A}{\epsilon \times S_p \times d} \quad (1)$$

[0071] Using Eq. 1, for ϵ of DMT $7.2 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$, we estimated the density of oligonucleotides per projected area of paper to be $4.5 \pm 0.5 \times 10^{14} \text{ cm}^{-2}$. This value is two orders of magnitude higher than that of the current standards for DNA immobilization on 2-D substrates (for example, using pin spotting or adsorption on poly-l-lysine coated glass slides). With the exception of the first five coupling cycles, the UV absorbance at 495 nm of the DMT cleavage solution has plateaued, indicating that the phosphoramidite coupling and deprotection reactions occurred efficiently during the synthesis. The lower coupling efficiency observed in the first five coupling steps can be attributed to sluggish reactions of the DNA phosphoramidites to the less-accessible hydroxyl groups buried in the paper fibers. Since extending reaction time of the initial coupling rounds did not improve the coupling yields, we introduced spacer nucleotides of ten nucleotides at the 3' end of the directly synthesized oligonucleotide on paper and designed the probe-binding region to be located at the 5' end.

[0072] In order to confirm the identity of the full-length DNA oligonucleotide generated by synthesis on paper and measure the yield, we introduced a cleavable disulfide bond in the sequence. After completing the synthesis, the terminal DMT group was left on for use as a HPLC handle. The paper-anchored DNA oligonucleotide was cleaved by reduction of the disulfide group with dithiothreitol (DTT), and the eluted material was subjected to HPLC. The main HPLC peaks were analyzed by Electrospray Ionization Mass Spectrometry (ESI-MS). Mass spectroscopy confirmed that the HPLC peak eluting at 30.9 min (Figure 2B), with a mass of 6217.9 Da, was within 1.0 Da of the expected mass (with ^{13}C isotope correction), 6217.1 Da, of the full-length product after cleavage. The yield of the desired full-length oligonucleotide was estimated using absorbance data at 260 nm, as the ratio of the area of the peak corresponding to the full-length oligonucleotide (confirmed by mass spectrometry) and

the sum of the areas of the peaks in the chromatogram corresponding to truncation and full-length products. The ratio of the amount of full-length product to the total amount of oligonucleotides cleaved and eluted from paper substrate is 32 % according to HPLC.

Use of ssDNA Arrays to Detect Fluorescently-labeled Oligonucleotides.

[0073] To minimize the effects of non-specific adsorption, we used a competitive assay to detect a fluorescently-labeled target oligonucleotide, F. A Cy5 reporter was attached to the 5' end of the target. F is also complementary to the DNA oligomer S1 synthesized directly on the surface of the paper. A paper-anchored array of an oligomer S2 (see table 1 for full sequence), with a sequence non-complementary to F, was used as a negative control.

[0074] A short ssDNA probe, Q, was designed to be complementary to F and labeled with a dark quencher (Iowa Black RQ) at the 3' end. F and Q were mixed in a 1:9 ratio and allowed to hybridize by heating to 37 °C, and the solution was then allowed to cool to room temperature. When F and Q hybridize, the fluorophore (Cy5) on F is brought in close proximity to the quencher (Iowa Black RQ), and the hybridized product does not fluoresce.

[0075] The ssDNA anchored on the paper surface was designed to have a higher affinity for F than the probe Q. The assay is based on the competition between the DNA oligomer anchored on the paper microzone and Q for hybridization with F; blocking and washing steps are not required because a fluorescent signal is produced only as the ssDNA synthesized on paper displaces Q (from the FQ complex) to hybridize with F.

[0076] Figures 3A-3C shows the fluorescent signal recorded after solutions of the FQ complex in concentrations between 50 nM and 500 pM are added to the ssDNA arrays on the surface of paper and allowed to incubate at 37 °C for 30 min. We are able to detect concentrations of DNA oligomer as low as 500 pM using paper-anchored arrays of ssDNA strands complementary to F. The sequence S1 used for the DNA array (CA) is complementary to the target DNA oligomer F, while the sequence S2 used for a control array (NCA), is orthogonal to F. A solution FQ (non-fluorescent) was prepared by hybridizing fluorescently-labeled oligomer F and quencher-labeled oligomer Q, mixed in 1:9 molar ratio. The arrays are incubated with solutions corresponding to oligomer F at concentrations between 50 and 0.5 nM. A: Schematic of the process. B. Plot of fluorescence intensity as a function of the concentration of FQ. C. Bottom: Fluorescence image of the paper device,

showing, qualitatively, the dependence of the fluorescence intensity on the concentration of FQ. The error bars represent one standard deviation ($n=7$).

Characterization of Paper-based DNA-directed Antibody Arrays.

[0077] We formed microarrays by incubating solutions of ssDNA-conjugated rabbit IgG on disks (3mm diameter) of paper on the surface of which ssDNA with complementary sequence had been synthesized. The dsDNA (formed from the hybridization of the two ssDNA strands) anchored the IgG to the surface of the paper.

[0078] Disks of untreated paper were used as controls. The surface of the disks was blocked with a BSA solution in PBS, then washed with PBS, and hybridized with ssDNA-conjugated rabbit IgG (100 nM in PBS). Unbound conjugates were removed by washing three times with PBST buffer.

[0079] We used a fluorescently-labeled antibody (DL549 anti-rabbit IgG) as a model analyte to test the performance of the array. DL549 anti-rabbit IgG in ten-fold dilutions (1 pM to 1 nM) in a solution of goat serum (10% serum in PBS) was added to each paper-anchored IgG microarray disk and incubated for 30 min.

[0080] The mean fluorescence intensity of both test and control zones was measured; Figure 4 shows the calibration data in the form of the output fluorescent signal versus the concentration of DL549 anti-rabbit IgG in the sample. The LOD is $\sim 10 \text{ ng mL}^{-1}$ (or $\sim 67 \text{ pM}$) for the assay based on DNA arrays on paper. Paper anchored ssDNA arrays with a sequence (S1) noncomplementary ssDNA-conjugated IgG served as a control (blue triangles). Red circles depict an immunoassay performed on untreated paper, incubated with a solution of rabbit IgG, and then blocked with a solution of BSA. Calibration plot of the output signal of the immunoassay versus the concentration of goat anti-rabbit IgG in the sample ($N = 7$). The error bars represent one standard deviation. Dotted red arch indicate the use of a covalent linker to conjugate ssDNA to rabbit IgG.

ELISA Using Paper-based DNA-directed Antibody Arrays.

[0081] One goal behind developing the paper-anchored antibody array technique is to measure the levels of a clinically-relevant protein in biological fluids from humans, animals, and plants. To do so, we assembled devices using paper-anchored arrays of capture antibody

in microzones, and used these devices to quantify levels of hCRP spiked into diluted human serum using a sandwich ELISA assay. We formed these microarrays by incubating solutions of ssDNA-conjugated anti-hCRP antibody on disks of paper on the surface of which ssDNA with complementary sequence had been synthesized. The dsDNA (formed from the hybridization of the two ssDNA strands) anchored the anti-hCRP antibody to the surface of the paper.

[0082] Diluted human serum samples were spiked with recombinant hCRP at concentrations ranging from 16 pg mL⁻¹ to 1000 pg mL⁻¹ and applied to the microzones. We used a biotinylated anti-hCRP as detection antibody, and streptavidin Cy5 as a fluorescent probe (see the experimental section for details). Figures 5A-5B shows the calibration curve for concentration of hCRP vs. fluorescence intensity. The data were fit to Hill's equation with $R_2=0.993$. The LOD was 17 pg mL⁻¹ (~1 pM). Figure 5A shows the schematic of the sandwich ELISA for hCRP and a calibration plot for fluorescence versus the concentration of hCRP. The capture antibody (anti hCRP) is conjugated to a ssDNA strand complementary to the ssDNA strand synthesized on paper. The detection antibody is labeled with biotin (Biotin anti hCRP). Streptavidin Cy5 is used to quantify the concentration of hCRP. Figure 5B shows the images of the results using 16 to 100 pg mL⁻¹ concentrations of hCRP. C) Calibration plot for fluorescence versus the concentration of hCRP. Each datum is the mean of seven replicates (N=7), and the error bars represent the standard deviations of the measurements.

Multiplexed Assay for Detection of Fluorescently-labeled Nucleic Acids and Proteins.

[0083] In order to show that paper-anchored ssDNA arrays with different sequences can be used to assemble paper-based devices capable of detecting DNA and antibodies from a single sample, in the same device, we used a solution containing a mixture of two model targets, a fluorescently-labeled DNA oligomer, F, and a fluorescently-labeled antibody, anti-rabbit IgG antibody conjugated to DL549. Figures 6A-6B shows a schematic of the process.

[0084] To prepare a paper-based analytical device that incorporates both ssDNA and protein microarrays, we synthesized independently, on paper, ssDNA with orthogonal sequences S1 and S2 (see Table 1) and shaped the paper strips into half-disks with 3mm diameter by cutting. The array of ssDNA with sequence S1 was used without further modification to form the device. To form the protein microarray, we incubated the half disk with ssDNA with sequence S2 synthesized on its surface with a solution of complementary ssDNA-conjugated

rabbit IgG; the dsDNA formed from the hybridization of the two ssDNA strands anchored the IgG to the surface of the paper. We placed the two half-disks on which ssDNA and protein microarrays were formed adjacently, in order to form a single continuous paper microzone, and assembled a device as described in Figure 8. To prevent non-specific adsorption, the surface of the paper was blocked using a solution of BSA (1% in PBS, pH 7.6) for 30 min.

[0085] The fluorescently-labeled target oligomer F was pre-hybridized to the quencher-labeled oligomer Q, as described in the Supporting Information. A solution containing a mixture of FQ and anti-rabbit IgG antibody conjugated to DL 549 (Ab), both at 5 nM concentration, was added to each device, incubated for 30 min at 37° and allowed to cool to room temperature. The microzones were then washed three times with PBS, and imaged with a fluorescence scanner.

[0086] Figure 6B shows an image of a typical device. The same device is scanned in both the Cy5 (Ch1, top) and Cy3 (Ch2, middle) fluorescence channels, and the signal is overlaid in the bottom image. Figure 6C shows the average fluorescence data obtained using seven independent devices. In Figure 6C, normalized the average fluorescence of the microarray probe to the average fluorescence intensity of the adjacent control microarray probe (i.e. the ratio of the signal from a probe to the signal related to nonspecific binding or cross-hybridization, in the fluorescence channel and in the same device).

[0087] Thus, in some embodiments, a method for assembling microarrays of ssDNA and proteins on the surface of paper is described. The strategy is based on the synthesis of ssDNA directly on paper, with modified 2'-deoxynucleoside phosphoramidites sequentially coupled to a growing oligonucleotide chain that is anchored in the hydroxyl groups present on the surface of cellulose paper. This strategy of fabricating microarrays on paper is cost effective because the crude product of the synthesis is sufficiently pure to allow us to specifically differentiate the complementary DNA strand from other sequences with minimal non-specific interactions. This synthetic efficiency allows us to avoid distinct steps of DNA synthesis, purification, and immobilization; these steps are time consuming and underlie the majority of the production costs (as a reflection of solvents and reagents).

[0088] Through hybridization with complementary strands of DNA, these microarrays can capture fluorescent-labeled DNA, DNA-conjugated protein antigen, and DNA-conjugated antibodies. We demonstrate the use of these microarrays to perform a sensitive sandwich

ELISA to detect human CRP (LOD 13 ng mL⁻¹), and a multiplex assay capable of detecting DNA and antibodies in the same device. The versatility of this strategy offers new approaches to integration with simple microfluidic devices, and of expansion of the repertoire of analyses, and the sensitivity of the assays, that can be conducted using paper.

Materials

[0089] All the reagents needed for the synthesis of DNA in this study were purchased from Glen Research Co. Ltd, and used without further purification. Oligonucleotides cleaved from paper anchored DNA arrays were separated by reverse-phase high-pressure liquid chromatography (HPLC, Agilent 1200) using a C18 stationary phase (Eclipse-XDB C18, 5 μ m, 9.4 $\times$ 200 mm) and an acetonitrile/100 mM aqueous triethylammonium acetate gradient. UV spectrometry was measured on a Beckman Coulter DU800 spectrometer. LC-MS experiments were performed on an Alliance 2695 (Waters) HPLC system using a UPLC BEH C18 column (1.7 μ m, 2.1 $\times$ 50 mm) stationary phase and 6 mM aqueous triethylammonium bicarbonate/methanol mobile phase interfaced to a Q-ToF Micro massspectrometer (Waters).

[0090] Chromatography paper (Whatman #1 Chr) was purchased from GE Healthcare (NJ, USA). General Purpose Vinyl Tape (764 Black, 5.0 mil) was purchased from 3M (St. Paul, MN). Polystyrene microtiter plates (UltraCruz ELISA Plate, high binding, 96 well, Flat bottom) were purchased from Santa Cruz Biotech (Dallas, TX, USA). Rabbit IgG, Streptavidin-Cy5, bovine serum albumin (BSA) solution (10 % m/m in DPBS), human serum, and phosphate buffered saline (PBS) pH 7.6 (25 °C) were purchased from Sigma Aldrich (St Louis, MO, USA). Tablets of Tris Buffered Saline Buffer (TBS) were purchased from Utech Products Inc (Schenectady, NY). Goat anti-rabbit IgG antibody labeled with DyLight™ 549 (DL549 anti-rabbit IgG) was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Mouse anti-human CRP (capture antibody, Part 842676), biotinylated mouse anti-human CRP (detection antibody, Part 842677), recombinant human CRP (Part 842678) were purchased from R&D Systems (Minneapolis, MN).

[0091] Absorbance and fluorescence measurements were performed using a microtiter plate reader (model SpectraMax M2, Molecular Devices, Vienna, VA, USA). Fluorescence measurements were performed using a Typhoon FLA 9000 scanner (GE Healthcare, Wilmington, MA, USA).

[0092] The DNA sequences (from 5' to 3') used in this study are summarized in Table 1:

Table 1: DNA sequences in one or more embodiments described herein.

Name	Sequence	Bases	Properties
S1	CGATCCACTACAAGCTTGCC ATCATGTTCGATC	32	Synthesized on Paper
S2	GTGAAGTGGCAGTATCTTTT TTTTTTTTTTTTTTTT	35	Synthesized on Paper
CS1	GGCAAGCTTG TAGTGGATCG /3ThioMC3-D/	20	Complementary to S1, thiolated, for conjugation with hCRP
CS2	GATACTGCCACTTCAC/3Thio MC3-D/	16	Complementary to S2, thiolated, for conjugation with rabbit IgG
F	GGCAAGCTTG TAGTGGAT CG/3Cy5Sp/	20	Complementary to S1, 3' Cy5 modification
Q	/5IAbRQ/CGATCCACTACA	12	Complementary to F, 5' Iowa Black Quencher modification

[0093] All the purified oligonucleotides (F, Q, CS1, CS2) used in this work were purchased from Integrated DNA Technologies, Inc. (Coralville, IA).

[0094] We conjugated proteins (rabbit IgG and mouse anti-human CRP) with thiolated DNA oligomers (IDT DNA, Inc) using sulfosuccinimidyl 4-[p-maleimidophenyl]butyrate (Sulfo-SMPB) from Pierce Biotechnology (Rockford, IL, USA). All chemicals and reagents were used as received without further purification.

Direct DNA synthesis on paper

[0095] A commercial filter paper (Whatman Chromatography paper No. 1) was cut into ~2 cm × 2 cm squares for convenient handling, scrolled, and inserted into DNA synthesis columns, and subjected to DNA synthesis using a standard 1 μmole DNA synthesis protocol

on a PerSeptive Biosystems Expedite DNA synthesizer (model 8909). After synthesis, the paper was removed from the synthesis column and the DNA was deprotected with AMA (1:1 v/v of 30% aqueous ammonium hydroxide: 40% aqueous methylamine) at 65 °C. The paper was rinsed with methanol and water, and then dried. Figure 1 shows the process used for DNA synthesis on paper.

Post-synthesis Characterization of Paper-anchored DNA Arrays

[0096] The deprotection of DMT before each coupling step provide a means to assess the success of each coupling step in generating the full-length oligonucleotide on paper support. DNA was synthesized on paper in DMT-on mode (leaving the last DMT protective group on the 5' terminus of the oligonucleotide) and was treated with a 100 µL of 3 % trichloroacetic acid in dichloromethane for 10 minutes, and the UV absorbance (at 495 nm), A₄₉₅, of the resulting solution was measured.

Yield of Oligonucleotides in Paper-anchored DNA Synthesis

[0097] The target sequence for the DNA synthesized on paper in this assay was 5'-DMT-CGATCCACTACAAGCTTTTS-STTTTTTTTTTTTTT-3'; after cleavage with dithiothreitol (DTT), the full-length product forms an oligonucleotide with sequence 5'-DMT-CGATCCACTACAAGCTTTT-SH -3'. The full length and truncation products generated after cleavage oligonucleotides that can be analyzed in the eluent by HPLC and LC-MS.

[0098] We used HPLC to determine the yield of the synthesis process, calculated based on the integration of the analytical HPLC signals that correspond to oligonucleotides. The HPLC yield was defined as the ratio of the integration of the peak corresponding to the full-length product (confirmed by LC-MS), divided by the sum of all peak integrations combined.

Fabrication of simple paper-based devices

[0099] In certain embodiments, paper-based devices were fabricated using a similar strategy from strips of paper with DNA-anchored arrays using tape (General Purpose Vinyl Tape, 764 Black, 5.0 mil, from 3M (St. Paul, MN)). as a support (Figure 8). We cut paper into disks with a 3-mm diameter using a biopsy punch. We trimmed tape into ~1 cm × 1 cm squares and bored a 2-mm diameter hole at the center using a biopsy punch. The paper was sandwiched between the two layers of tape such that the disk-shaped paper was placed over the holes.

Formation of dsDNA arrays

[0100] Oligomers F and Q (sequences in Table 1) were purchased from IDT DNA Inc and used as received, without further purification. We prepared stock solutions (100 μ M) by dissolving the oligomers in water.

[0101] A working solution containing 50 nM of F and 450 nM of Q was prepared in TBS buffer (25mM Tris, 140mM NaCl, and 3 mM KCl, pH 7.6) from AMESCO. The hybridization product FQ was prepared by heating the mixture to 37 °C in a water bath and allowing it to cool to room temperature. The working solution was further diluted in TBS to a series of concentrations of FQ between 25 nM and 0.5 nM.

[0102] Microzones in a paper-based device were prepared by cutting strips of paper with anchored DNA arrays into disks with a 3-mm diameter, using a biopsy punch. The device was assembled as described in the previous section (see Figure 8). Blocking was not necessary because the hybridization product FQ does not fluoresce. A solution of FQ (10 μ L) was added to each microzone of a paper-based device, and was allowed to incubate at 37 °C for 30 min in a humidity-controlled chamber. The device was allowed to cool to room temperature after this step. The fluorescence produced after the DNA anchored on paper displaces Q to hybridize to F was recorded using a fluorescence scanner.

Formation of antibody-oligonucleotide conjugates

[0103] Antibody-ssDNA conjugates were synthesized by chemically linking thiol-terminated ssDNA (purchased from IDT DNA, Inc) with a protein (rabbit IgG or mouse anti hCRP2). A water-soluble crosslinker, sulfosuccinimidyl 4-[p-maleimidophenyl]butyrate (Sulfo-SMPB), containing an NHS-ester and a maleimide reactive group connected by a spacer, was added in 20-fold molar excess (1 mM in PBS, pH 7.6) to an equal volume of a 50- μ M solution of the protein (in PBS, pH 7.6). The reaction mixture was then incubated for an hour with mixing at 1000 rpm at room temperature, using a vortex mixer (Benchmark Scientific, Inc), according to the manufacturer's instructions. The derivatized protein were desalted by Nap-10 size exclusion columns (GE Healthcare) and diluted in PBS, pH 7.6, to 500 μ L. Thiolated ssDNA (25 μ M) was added at a 1:1 molar ratio and the mixture was incubated for an hour with mixing at 1000 rpm at room temperature. Unreacted oligomers were removed by ultrafiltration with a 100000 MW cut-off membrane (EMT Millipore).

[0104] The volume of the solution containing the protein-ssDNA conjugate was adjusted to 100 μ L (10mg/mL concentration, measured using a Thermo Scientific NanoDrop Spectrophotometer based on absorption at 280 nm[2]) and stored in 5- μ L aliquots at -20°C. A small portion of the antibody-ssDNA conjugate was subjected to denaturing PAGE. A shift in the position of the bands corresponding to IgG heavy and light chains to higher molecular weights confirmed the conjugation (Figure 7).

Formation of DNA-directed protein arrays

[0105] The strip of paper-anchored ssDNA was cut into disks with a 3-mm diameter using a biopsy punch. The surface of the disks was blocked with 50 μ L of a 1% wt/vol solution of BSA in PBS, pH 7.6, for 30 min. The disks were washed with PBS, and then incubated with 50 μ L of a solution of DNA-protein conjugates (100 nM in PBS) at 37° C for 30 min, then at room temperature (23 ± 3 °C) for 30 min. Unbound conjugate was removed by washing three times with 50- μ L volumes of a PBST buffer (0.05% Tween in PBS, pH 7.6).

Characterization of rabbit IgG arrays on paper

[0106] The rabbit IgG array was incubated with a 50- μ L volume of a solution of a fluorescently-labeled anti-IgG antibody, DL549 anti-rabbit IgG (monoclonal goat anti-rabbit IgG antibody conjugated with a proprietary florescent dye, DyLight549) containing 10% vol/vol goat serum for 30 min. Unbound antibody was removed by washing with a total volume of 100 μ L of PBST buffer (0.05% Tween in PBS, pH 7.6). The disks were placed in a 96-well black plate with clear bottom (purchased from Corning) and the fluorescence intensity was scanned with excitation and emission wavelengths of 544 nm and 590 nm, respectively, in a microtiter plate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA).

Sandwich ELISA for detection of hCRP

[0107] Mouse anti-human CRP (capture antibody, Part 842676), biotinylated mouse anti-human CRP (detection antibody, Part 842677), recombinant human CRP (Part 842678) were part kit (Part DCP00) of a purchased from R&D Systems (Minneapolis, MN). DNA-directed arrays of capture antibody (Mouse anti-human CRP) were formed on the surface of paper and subsequently used to fabricate paper-based devices as described before (see Figure 8).

[0108] The devices were suspended in air, using an empty pipette-tip box, to prevent the vertical flow of reagent solutions away from the test zones. The solutions of recombinant human C-reactive protein (hCRP) were prepared in a 10 % v/v solution of human serum in PBST (0.05% Tween in PBS, pH 7.6). The microzones were incubated with 10 μ L per well of solutions of human serum spiked with concentrations of hCRP between 16 and 1000 pg mL⁻¹, for 20 min. The microzones were then washed three times with 20 μ L PBST and incubated with 10 μ L of biotinylated mouse anti-human CRP detection antibody (250 ng mL⁻¹ in PBS, pH 7.6). After incubation for 20 min, each microzone was washed three times with 20 μ L PBST. Detection was completed by adding 10 μ L of 100 ng mL⁻¹ Streptavidin-Cy5 in PBS buffer, pH 7.6 to each microzone; unbound Streptavidin-Cy5 was removed by washing three times with 20- μ L volumes of PBST.

Immunoassays on untreated hydrophilic paper

[0109] Paper-based devices were assembled as described in Figure 8. We used non-specific adsorption to immobilize rabbit IgG on untreated (used as received from the manufacturer) paper, by adding 5 μ L of a solution of rabbit IgG in PBS, pH 7.6, to the test zone, and allowed it to dry for 10 min at room temperature. We blocked microzones by adding 5 μ L of a 1% wt/vol solution of BSA in PBS, pH 7.6, and allowing it to dry for 10 min. A volume (5 μ L) of a solution containing the DL549 anti-rabbit IgG in a solution of PBS with 10% vol/vol goat serum was added to each zone and allowed to incubate for 5 min. Each microzone was washed three times with 10 μ L of PBS. Each microzone was scanned, and the fluorescence intensity was measured using ImageJ.

Device using paper-anchored ssDNA arrays for the multiplex detection of fluorescently-labeled nucleic acids and antibodies.

[0110] DNA with orthogonal sequences S1 and S2 (see Table 1) was synthesized independently on the surfaces of paper. The strips of paper were cut into disks with a 3-mm diameter using a biopsy punch, then into half-disks using a blade. For the paper with anchored DNA of sequence S2, the surface of the half-disks was blocked with 25 μ L of a 1% wt/vol solution of BSA in PBS, pH 7.6, for 30 min, and the half-disks were washed with PBS, and then incubated with 25 μ L of a solution of DNA-rabbit IgG conjugates (100 nM in PBS) at 37° C for 30 min, then at room temperature (23 $\pm$ 3 °C) for 30 min. Unbound

conjugate was removed by washing three times with 25- μ L volumes of a PBST buffer (0.05% Tween in PBS, pH 7.6).

[0111] Paper-based devices were assembled as described in Figure 8, except that the two half-disks of paper (one half with ssDNA with sequence S1, and another with the DNA-anchored rabbit IgG array described before) were placed in close proximity to form a single microzone. An solution containing 5 nM of FQ (prepared by hybridizing oligonucleotides F and Q in a 1:9 ratio, as described before) and 5 nM of DL549 anti-rabbit IgG in TBS buffer containing 10% vol/vol goat serum, was added to the microzone, incubated for 30 min at 37° and allowed to cool to room temperature. The microzones were then washed three times with PBS, and imaged with a fluorescence scanner. We normalized the average fluorescence of the microarray probe to the average fluorescence intensity of the adjacent control microarray probe (*i.e.*, the ratio of the signal from a probe to the signal related to nonspecific binding or cross-hybridization, in the fluorescence channel and in the same device).

Immunoassays in standard polystyrene plates

[0112] The wells of a standard polystyrene 96-well plate (Corning) were incubated with a 50- μ L volume of a solution of rabbit IgG (10 μ g/mL, in PBS pH 7.4) for 2 hr at room temperature. The wells were washed three times using 100- μ L volumes of PBST buffer (0.05% Tween in PBS, pH 7.6), and blocked using 50 μ L of a 1% wt/vol solution of BSA in PBS, pH 7.6, for 60 min. The wells were washed three times using 100- μ L volumes of PBST buffer, then were incubated with a 50- μ L volume of a solution of DL549 anti-rabbit IgG (monoclonal goat anti-rabbit IgG antibody, conjugated with DyLight549, from Jackson ImmunoResearch, Inc.) containing 10% vol/vol goat serum, for 60 min. The wells were washed five times using 100- μ L volumes of a PBST buffer, and the fluorescence intensity was determined at 530 nm in a microtiter plate reader.

[0113] While for purposes of illustration a preferred embodiments of this invention has been shown and described, other forms thereof will become apparent to those skilled in the art upon reference to this disclosure and, therefore, it should be understood that any such departures from the specific embodiment shown and described are intended to fall within the spirit and scope of this invention.

[0114] Unless otherwise defined, used or characterized herein, terms that are used herein (including technical and scientific terms) are to be interpreted as having a meaning that is

consistent with their accepted meaning in the context of the relevant art and are not to be interpreted in an idealized or overly formal sense unless expressly so defined herein.

[0115] Although the terms, first, second, third, etc., may be used herein to describe various elements, these elements are not to be limited by these terms. These terms are simply used to distinguish one element from another. Thus, a first element, discussed below, could be termed a second element without departing from the teachings of the exemplary embodiments. Spatially relative terms, such as “above,” “below,” “left,” “right,” “in front,” “behind,” and the like, may be used herein for ease of description to describe the relationship of one element to another element, as illustrated in the figures. It will be understood that the spatially relative terms, as well as the illustrated configurations, are intended to encompass different orientations of the apparatus in use or operation in addition to the orientations described herein and depicted in the figures. For example, if the apparatus in the figures is turned over, elements described as “below” or “beneath” other elements or features would then be oriented “above” the other elements or features. Thus, the exemplary term, “above,” may encompass both an orientation of above and below. The apparatus may be otherwise oriented (e.g., rotated 90 degrees or at other orientations) and the spatially relative descriptors used herein interpreted accordingly. Further still, in this disclosure, when an element is referred to as being “linked to,” “on,” “connected to,” “coupled to,” “in contact with,” etc., another element, it may be directly linked to, on, connected to, coupled to, or in contact with the other element or intervening elements may be present unless otherwise specified.

[0116] The terminology used herein is for the purpose of describing particular embodiments and is not intended to be limiting of exemplary embodiments. As used herein, singular forms, such as “a” and “an,” are intended to include the plural forms as well, unless the context indicates otherwise. Additionally, the terms, “includes,” “including,” “comprises” and “comprising,” specify the presence of the stated elements or steps but do not preclude the presence or addition of one or more other elements or steps.

Claims

What is claimed:

1. A device comprising:
 - a cellulosic substrate comprising at least one hydrophilic zone comprising a plurality of hydroxyl groups; and
 - one or more first oligonucleotide each covalently bonded to the cellulosic substrate through one or more of the hydroxyl groups by a -O- linker.
2. The device of claim 1, wherein the first oligonucleotide is DNA or RNA.
3. The device of claim 1 or 2, wherein the first oligonucleotide is single strand DNA.
4. The device of any one of the preceding claims, wherein the device comprises one or more second oligonucleotide different from the first oligonucleotide and are covalently bonded to the hydroxyl groups.
5. The device of any one of the preceding claims, wherein the first or second oligonucleotide is covalently bonded to the hydroxyl groups directly or through a linker.
6. The device of any one of the preceding claims, wherein the cellulosic substrate is paper.
7. The device of any one of the preceding claims, wherein the first oligonucleotide bonded to the hydroxyl groups of the cellulosic substrate has a density of more than about $0.1 \times 10^{14}/\text{cm}^2$, about $0.2 \times 10^{14}/\text{cm}^2$, about $0.5 \times 10^{14}/\text{cm}^2$, about $1.0 \times 10^{14}/\text{cm}^2$, about $2.0 \times 10^{14}/\text{cm}^2$, about $3.0 \times 10^{14}/\text{cm}^2$, about $4.0 \times 10^{14}/\text{cm}^2$, about $5.0 \times 10^{14}/\text{cm}^2$, about $6.0 \times 10^{14}/\text{cm}^2$, or about $10.0 \times 10^{14}/\text{cm}^2$.
8. The device of any one of the preceding claims, comprising an array of the first oligonucleotides.
9. The device of claim 4, wherein the device comprises an array of the first oligonucleotides and an array of the second oligonucleotides.
10. The device of any one of the preceding claims, further comprising one or more third oligonucleotides complementary to the first oligonucleotides and hybridized with the first oligonucleotides.
11. The device of any one of the preceding claims, further comprising one or more antibodies or antigens bonded to the first, second, or third oligonucleotides.

12. The device of any one of the preceding claims, wherein the device is a microfluidic analytical device and the cellulosic substrate further comprises one or more hydrophilic channels in fluidic communication with the hydrophilic zone.
13. The device of claim 12, wherein the cellulosic substrate comprises one or more cellulosic layers and the hydrophilic zone and the hydrophilic channel are on the same or different cellulosic layers.
14. The device of claim 12, wherein the cellulosic substrate further comprises one or more sample deposition zone in fluidic communication with the hydrophilic zone.
15. The device of claim 12, further comprising one or more hydrophobic areas defining the hydrophilic zone.
16. The device of claim 12, further comprising one or more hydrophobic materials defining the hydrophilic zone.
17. The device of any one of the preceding claims, wherein the first or second oligonucleotide comprises 2-1000 nucleotides.
18. A method of preparing the device of any of the preceding claims, comprising:
 - providing the cellulosic substrate comprising at least one hydrophilic zone comprising a plurality of hydroxyl groups; and
 - covalently bonding one or more first oligonucleotide to one or more of the hydroxyl groups.
19. The method of claim 18, wherein the bonding step is conducted by an automatic oligonucleotide synthesizer.
20. A method of preparing an antigen or antibody-bonded device, comprising:
 - providing the device of claim 1;
 - contacting the first oligonucleotide with a third oligonucleotide complementary to the first oligonucleotide and bonded with an antigen or antibody; and
 - hybridizing the first and third oligonucleotides.
21. A method of detecting a target antigen or target antibody, comprising:
 - providing the device of claim 11;
 - providing a sample containing a target antigen or target antibody which is specific to the antibody or antigen, respectively, bonded to the first, second, or third oligonucleotides; and

forming a conjugate between the target antigen/antibody and the oligonucleotide-bonded antibody/antigen.

22. The method of claim 21, wherein the target antigen or target antibody comprises a florescent molecule.

23. A method of detecting a DNA, comprising:

providing the device of claim 1;

providing a sample containing a DNA comprising complementary first and second strand oligonucleotides, wherein at least one of the first and second strand oligonucleotides is complementary to the first oligonucleotide bonded to the device; and at least one of the first and second strand oligonucleotides is bonded with a fluorescent molecule;

contacting the first oligonucleotide with the sample to allow the first oligonucleotide to hybridize with one of the first and second strand oligonucleotides.

24. A method of detecting a first target and second target different from the first target, comprising:

providing the device of claim 4;

providing one or more samples containing the first and second targets;

wherein the first target is specific to the first oligonucleotide and the second target is specific to second oligonucleotide; and the first and second targets are each independently selected from the group consisting of a DNA, an antigen, and antibody; and

allowing the one or more samples to react with the device.

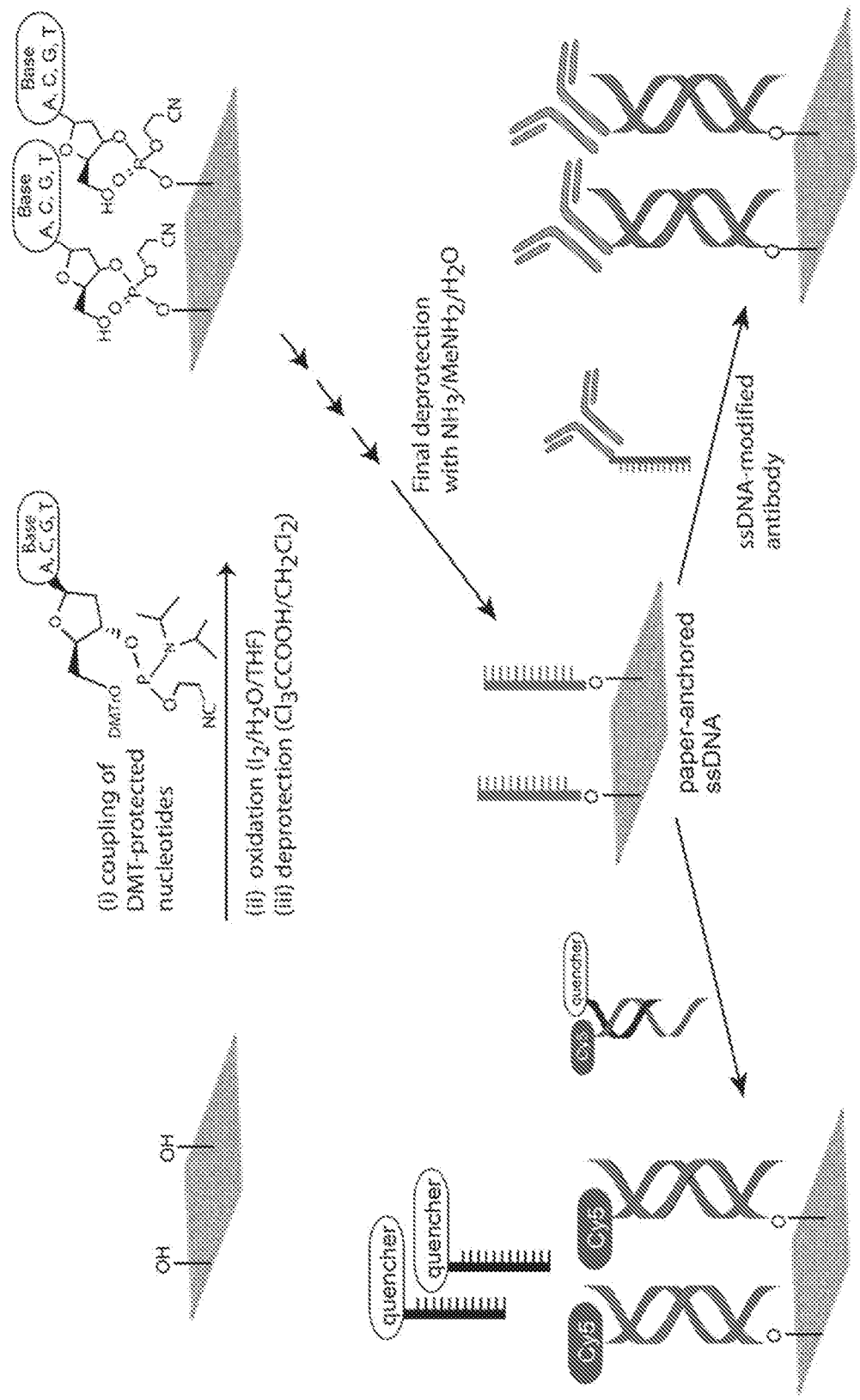


FIG. 1

FIG. 2A

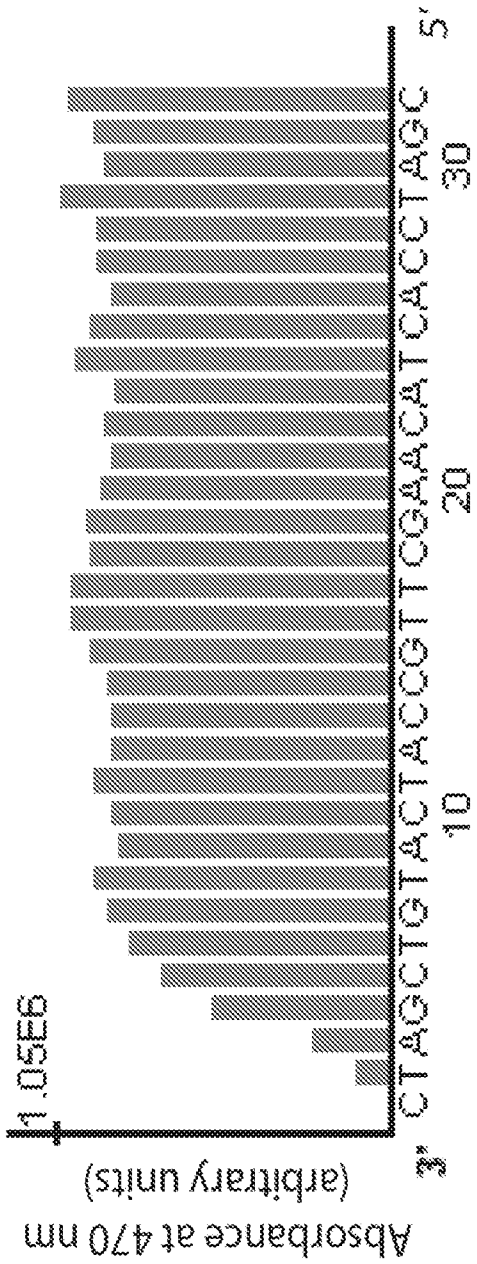
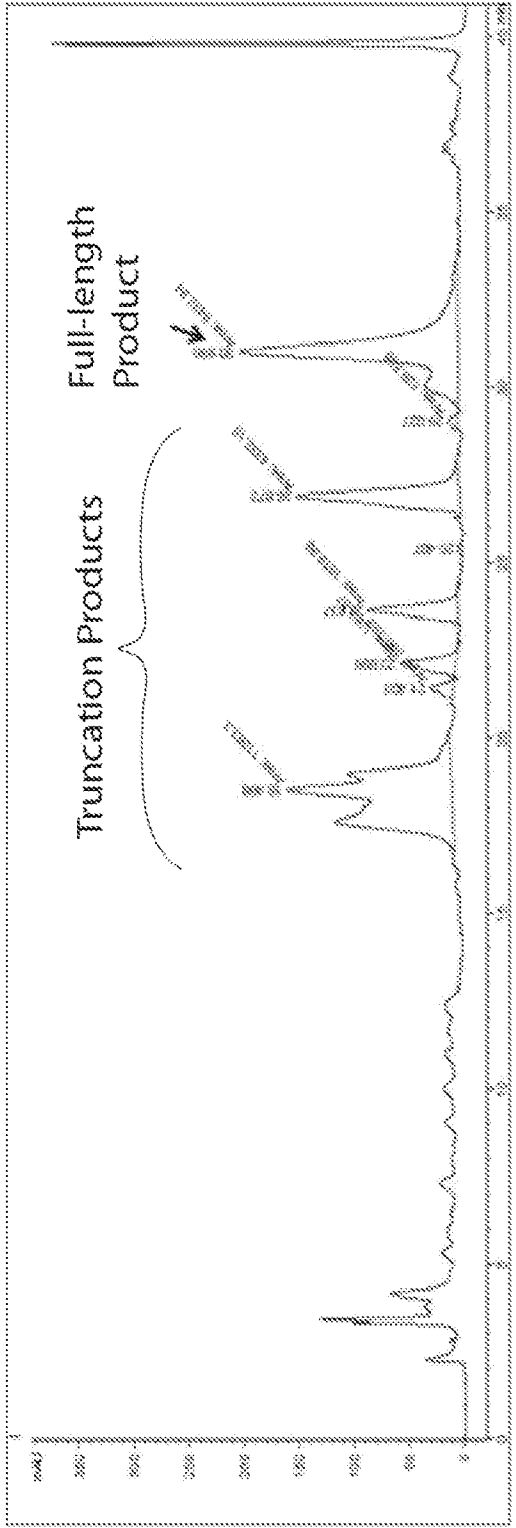


FIG. 2B



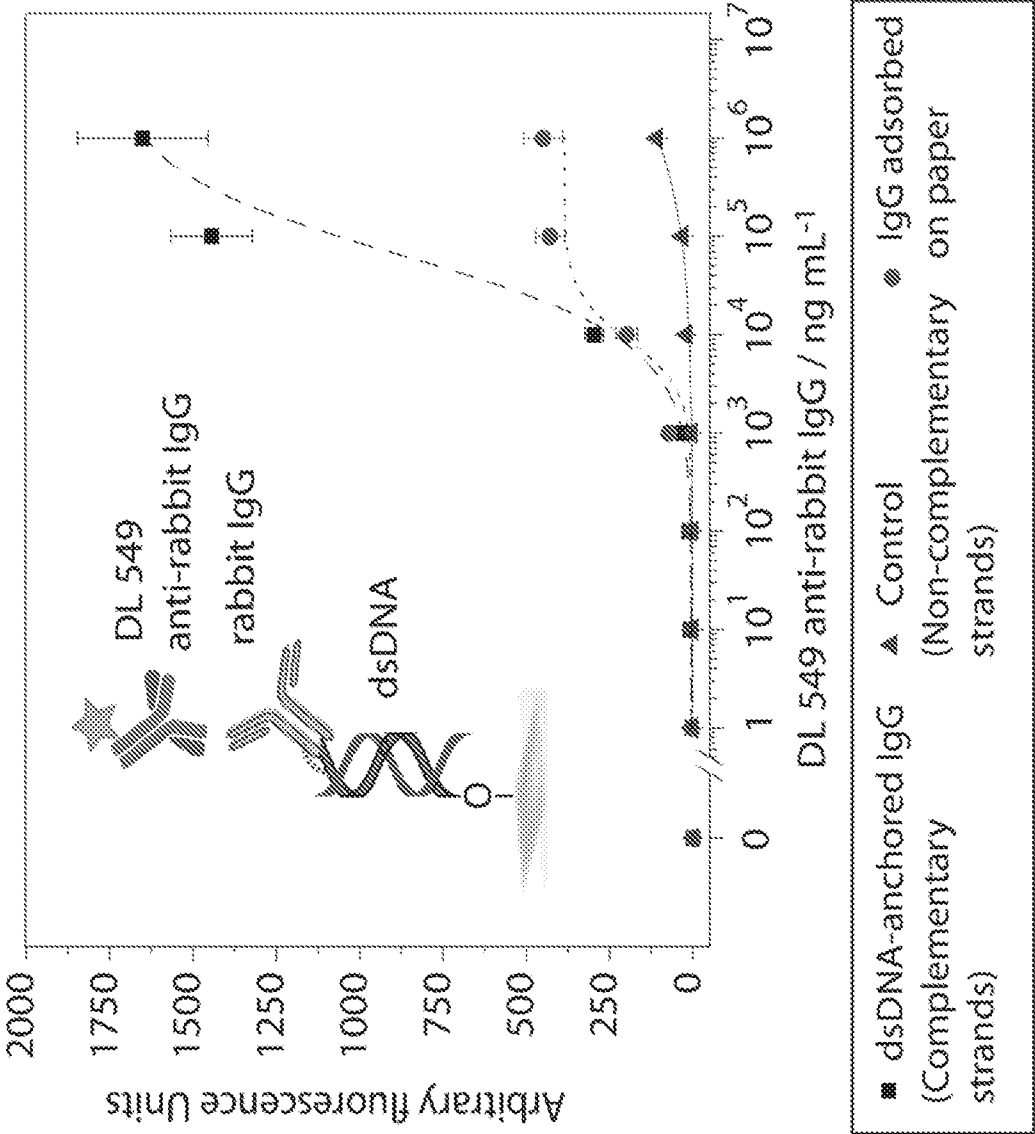


FIG. 4

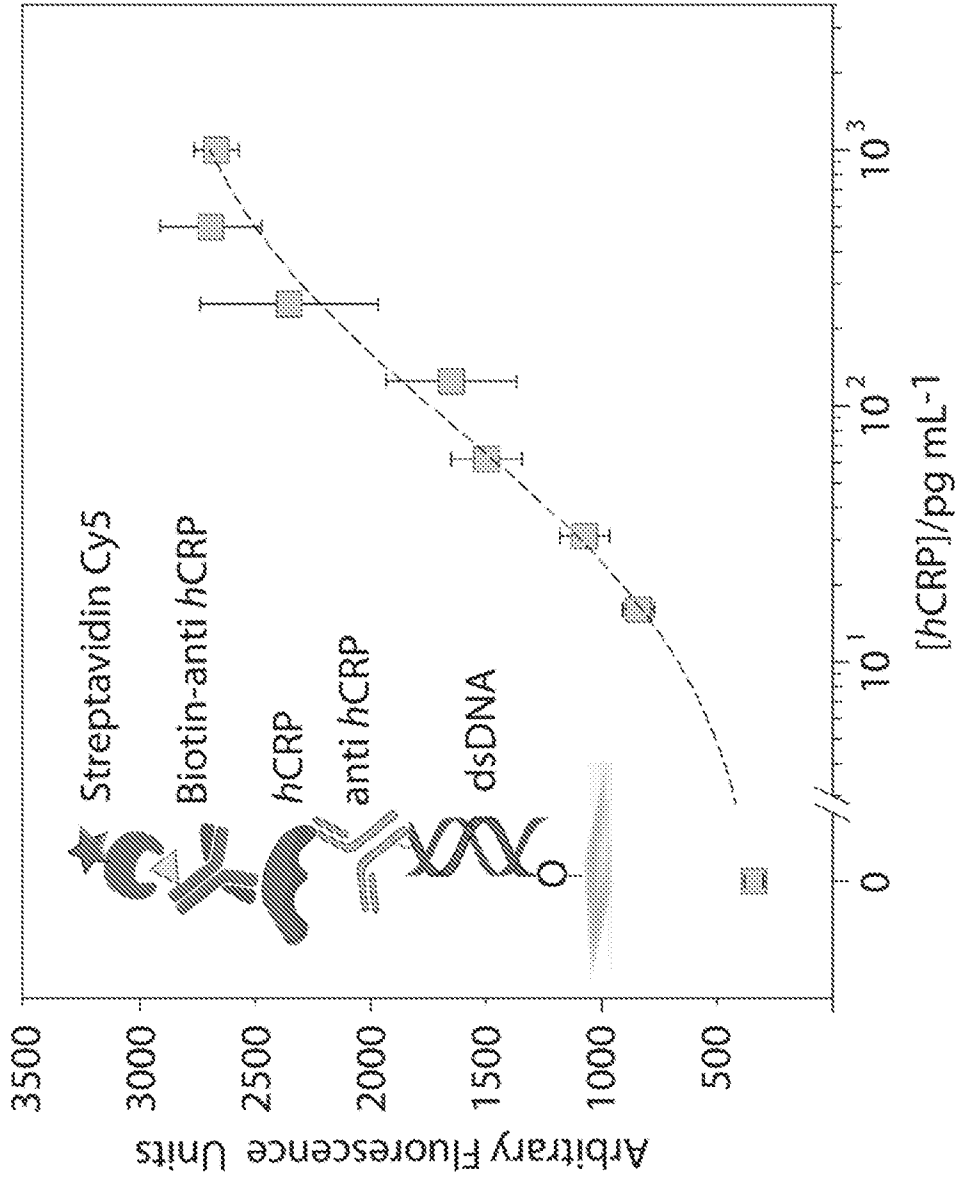


FIG.
5A

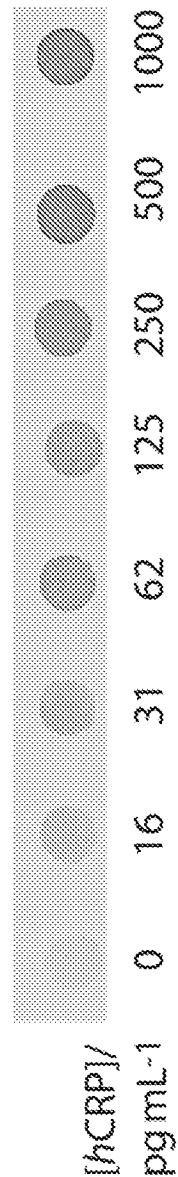


FIG.
5B

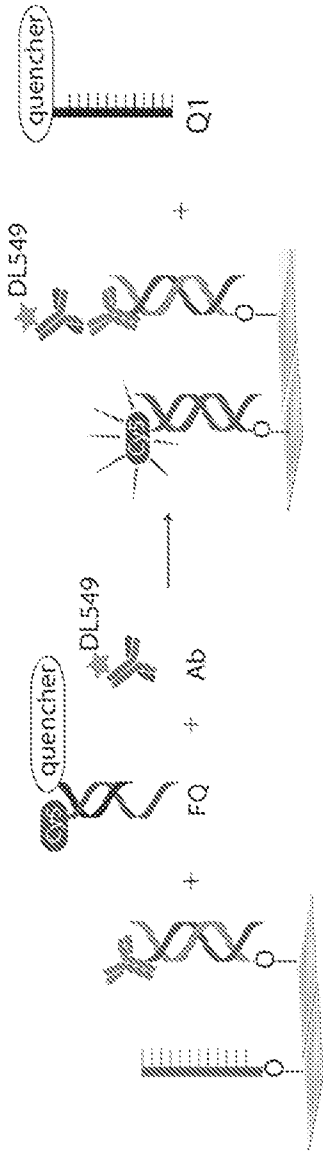


FIG.
6A

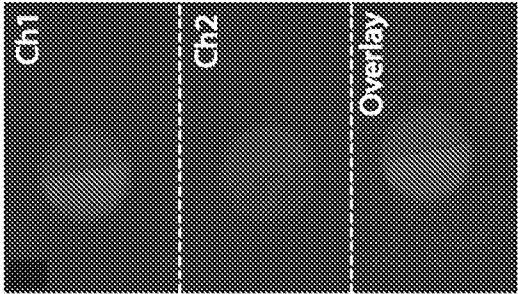


FIG.
6B

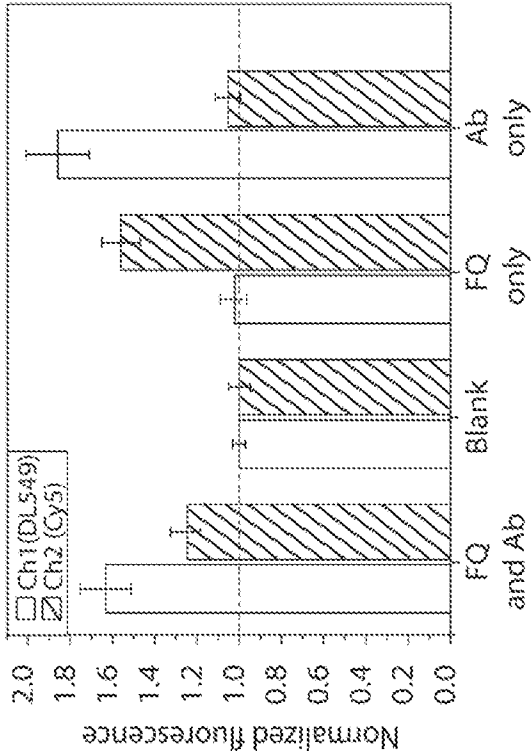


FIG.
6C

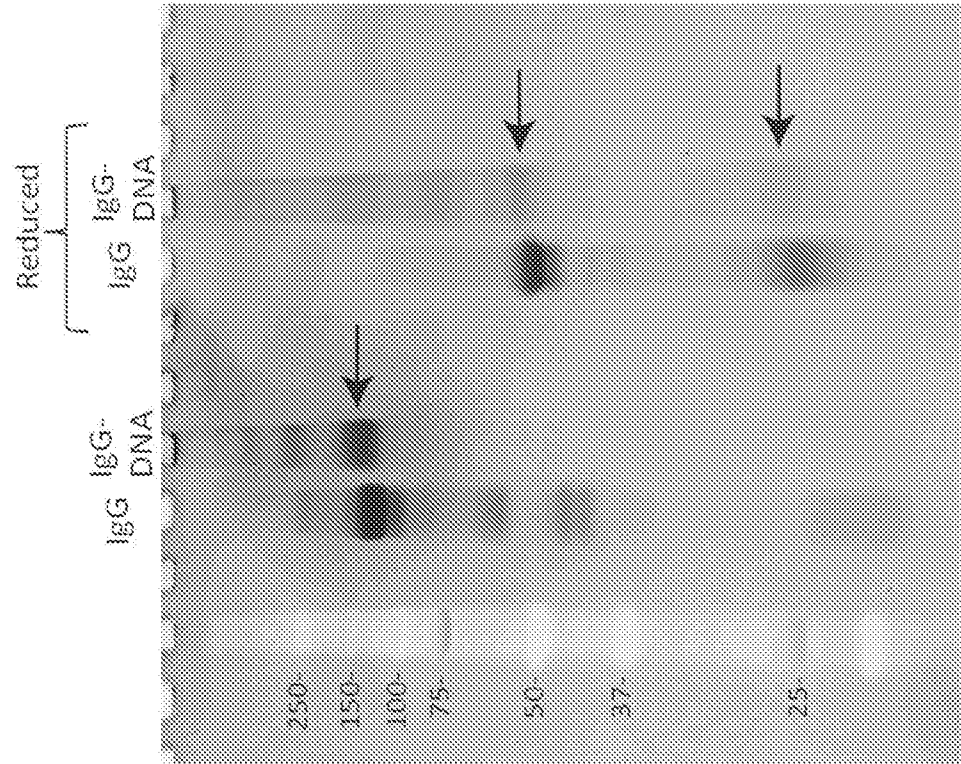


FIG. 7

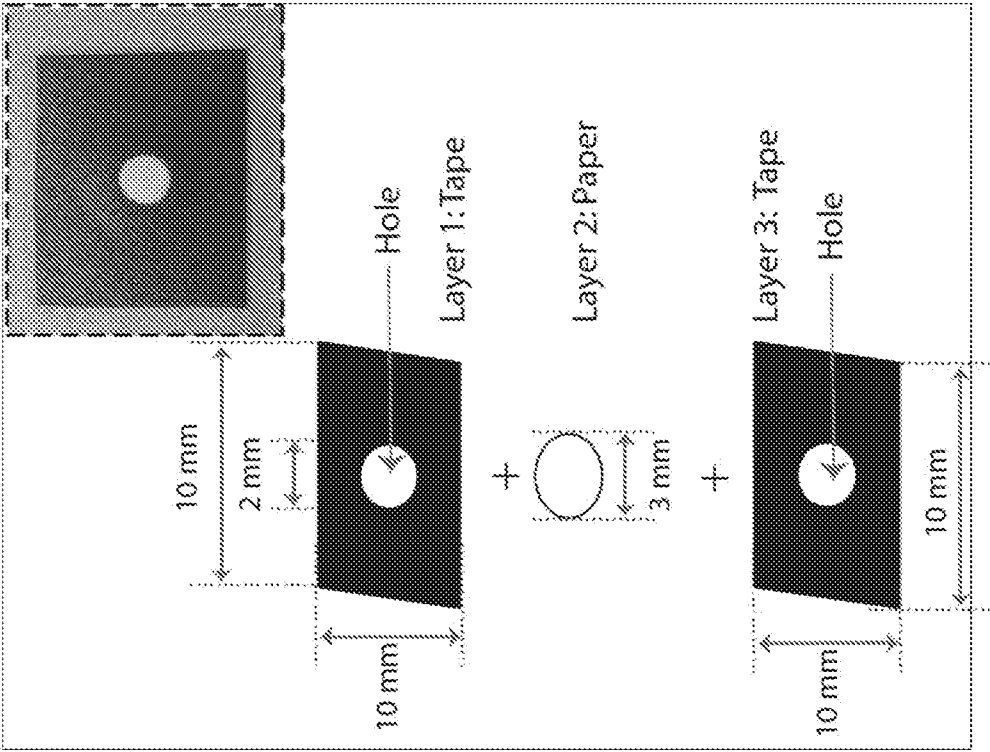


FIG. 8