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(54) Title: MICROFLUIDIC BIOSENSOR AND METHODS OF USE

(57) Abstract: The present invention relates to a method for detecting or quantifying an analyte in a test sample. This involves providing at least one test mixture where the test mixture includes a test sample, potentially containing analyte, a capture conjugate, and a marker conjugate. The capture conjugate includes a capture support and a first binding material, where the first binding material is selected to bind with a portion of the analyte. The marker conjugate includes a particle, a marker, and a second binding material, where the second binding material is selected to bind with a portion of the analyte other than the portion of the analyte for which the first binding material is selected. Reaction is permitted to occur in the test mixture between analyte present in the test sample and the first and second binding materials, thereby forming a product complex comprising analyte present in the test sample, the capture conjugate, and the marker conjugate. The reacted test mixture is exposed to a capture device having non-specific affinity for the capture support, whereby product complex present in the reacted test mixture is immobilized from the reacted test mixture. The presence or amount of the marker from the immobilized product complex is detected using a detection assembly. The presence or amount of the marker from the immobilized product complex is correlated with the presence or amount, respectively, of the analyte in the test sample. A device for carrying out this method and similar methods with other assay formats are also disclosed.

MICROFLUIDIC BIOSENSOR AND METHODS OF USE

[0001] This application claims the benefit of U.S. Provisional Patent Application Serial No. 60/549,729, filed March 3, 2004.

[0002] The subject matter of this application was made with support from the United States Government under The National Institutes of Health, Grant No. 1 R01 HD37109-01A1. The U.S. Government may have certain rights.

FIELD OF THE INVENTION

[0003] The present invention is directed to a microfluidic biosensor and to methods of using it.

BACKGROUND OF THE INVENTION

[0004] Molecular biology based technologies, such as the polymerase chain reaction (PCR), for detection of pathogenic microorganisms are slowly replacing culture based detection methods (Kow et al., *Journal of Medical Entomology* 378(4):475-479 (2001); Laue et al., *Journal of Clinical Microbiology* 37(8):2543-2547 (1999); and Killen et al., *Journal of Virol. Methods* 41(2):135-146 (1993)). While molecular methods tend to be more sensitive, specific, and faster than culture based methods, they are also limited by expensive equipment requirements (Baeumner et al., *Analytical Chemistry* 74:1442-1448 (2002)). Scientists are overcoming this limitation by miniaturizing molecular assays into a microfluidic format (Mondesire et al., *IVD Magazine* 9-14 (2000); Yu et al., *Micro Total Analysis Systems Conference*, Enschede, Netherlands 545-548 (2000); Kopp et al., *Science* 280:1046-1048 (1998); and Manz et al., *Journal of Chromatography* 593:253-258 (1992)). Microfluidics is the enabling technology base for the development of miniature devices that move, mix, control, and react fluid volumes in the micron range. Microfluidics offer obvious advantages in the reduced consumption of reagents; faster and more sensitive reactions due to enhanced effects of processes such as diffusion and mass transport; increased throughput through parallel processing; and reduced expenses in terms of power and reagent consumption. Most importantly, fabrication of microfluidic devices is inexpensive and allows the integration of several modules to automate analytical processes (Duffy et al., *Analytical Chemistry* 70:4974-

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4984 (1998); Jingdong et al., *Analytical Chemistry* 72:1930-1933 (2000); and Martynova et al., *Analytical Chemistry* 69(23):4783-4789 (1997)).

[0005] A common feature of all nucleic acid detection methods in microarray chips and microchannels is the use of labels coupled to target specific probes.

5 Typically, these labels are molecules that fluoresce, change, or produce color to indicate target hybridization to a probe (Ramsay, G., *Nature Biotech* 16:40-44 (1998)). Nanoparticles such as magnetic beads (Edelstein et al., *Biosensors & Bioelectronics* 14:805 (2000)), liposomes (Esch et al., *Analytical Chemistry* 73:2952-2958 (2001)) and gold particles (Taton et al., *Science* 289:1756-1760 (2002) and Cao
10 et al., *Science* 297:1536-1540 (2002)) have also been used as labels. In most cases, these particle-labelled assays have proven to be more sensitive as they offer a means for further signal amplification that is not possible with conventional labels. Taton et al., for instance, use silver reduction to enhance visualization of gold particles in their assay (Taton et al., *Science* 289:1756-1760 (2002)). The least expensive and perhaps
15 the simplest signal amplification scheme has been achieved with liposomes. Liposomes are phospholipid vesicles that entrap hundreds of thousands of marker molecules to provide a large signal amplification and enhanced sensitivity, 3 orders of magnitude greater than single fluorophore detection (Lee et al., *Analytica Chimica Acta* 354:23-28 (1997)).

20 [0006] The present invention is directed to molecular biological, immunological, microbial, receptor-based detection in a microfluidic system.

SUMMARY OF THE INVENTION

[0007] The present invention relates to a method for detecting or quantifying
25 an analyte in a test sample. This involves providing at least one test mixture where the test mixture includes a test sample, potentially containing analyte, a capture conjugate, and a marker conjugate. The capture conjugate includes a capture support and a first binding material, where the first binding material is selected to bind with a portion of the analyte. The marker conjugate includes a particle, a marker, and a
30 second binding material, where the second binding material is selected to bind with a portion of the analyte other than the portion of the analyte for which the first binding material is selected. Reaction is permitted to occur in the test mixture between

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analyte present in the test sample and the first and second binding materials, thereby forming a product complex comprising analyte present in the test sample, the capture conjugate, and the marker conjugate. The reacted test mixture is exposed to a capture device having non-specific affinity for the capture support, whereby product complex
5 present in the reacted test mixture is immobilized from the reacted test mixture. The presence or amount of the marker from the immobilized product complex is detected using a detection assembly and correlated with the presence or amount, respectively, of the analyte in the test sample.

[0008] The present invention also relates to a microfluidic test device for
10 detecting or quantifying an analyte in a test sample. This device includes a non-absorbent substrate having at least one inlet and outlet extending therethrough and a non-specific capture device located at, upstream of, or downstream of the analysis portion. The inlet and outlet are connected by at least one microchannel imbedded in the substrate, where the at least one microchannel comprises an inlet portion and an
15 analysis portion.

[0009] Another aspect of the present invention relates to a method for detecting or quantifying an analyte in a test sample. This method includes providing at least one test mixture which includes a test sample potentially containing an analyte, a capture support complex including a capture support and a first member of
20 a first coupling group, a first binding material selected to bind with a portion of the analyte and comprising a second member of the first coupling group, a marker complex which includes a particle, a marker, and a first member of a second coupling group, and a second binding material selected to bind with a portion of the analyte other than the portion of the analyte for which the first binding material is selected
25 and comprising a second member of the second coupling group. Reaction is permitted to occur in the at least one test mixture between the first and second members of the first coupling group, between the first and second members of the second coupling group, and between analyte present in the test sample and the first and second binding materials. As a result, a product complex comprising analyte
30 present in the test sample, the capture support complex, the first binding material, the marker conjugate, and the second binding material is formed. The reacted test mixture is exposed to a capture device having non-specific affinity for the capture

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support so that product complex present in the reacted test mixture is immobilized from the reacted test mixture. The presence or amount of the marker from the immobilized product complex is detected using a detection assembly and correlated with the presence or amount, respectively, of the analyte in the test sample.

- 5 **[0010]** Another aspect of the present invention relates to a method for detecting or quantifying an analyte in a test sample. This involves providing at least one test mixture including a test sample potentially containing an analyte, a capture conjugate comprising a capture support and a first binding material, where the first binding material is selected to bind with a portion of the analyte, and a marker conjugate, comprising a particle, a marker, and an analyte analog. Competition is permitted to occur in the at least one test mixture between analyte present in the test sample and the analyte analog for the first binding material. As a result, a product complex, including the capture conjugate and the marker conjugate, is formed. The reacted test mixture is exposed to a capture device having non-specific affinity for the capture support so that product complex present in the reacted test mixture is immobilized from the reacted test mixture. The immobilized product complex is detected using a detection assembly. The presence or amount of the marker from the immobilized product complex is correlated with the presence or amount, respectively, of the analyte in the test sample.
- 10 **[0011]** Microfluidics combined with a liposome signal amplification scheme, in accordance with the present invention, promises an inexpensive solution to the heightened need for technology that can rapidly and accurately detect pathogenic organisms in environmental, clinical, and food samples in the wake of recent threats of bioterrorism. Liposome technology has been used in analogous membrane detection systems with great success (Baeumner et al., *Analytical Chemistry* 74:1442-1448 (2002); Esch et al., *Analytical Chemistry* 73:3162-3167 (2001); and Rule et al., *Clinical Chemistry* 42:206-1209 (1996), which are hereby included by reference in their entirety). Esch et al. have recently reported gains in sensitivity by converting a liposome-based membrane detection assay for *Cryptosporidium parvum* to a microfluidic format (Taton et al., *Science* 289:1756-1760 (2002), which is hereby incorporated by reference in its entirety).
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BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Figure 1 shows the principle scheme of the biosensor of the present invention based on DNA/RNA hybridization.

5 [0013] Figures 2A-B show the fabrication of the polydimethylsiloxane ("PDMS") microchannels using a silicon wafer as mold and a second silicon wafer as a lid to control flatness and thickness of the PDMS layer. Figure 2A shows these components in an arrangement where the PDMS layer is between the first and second silicon wafers, as would be the case where the PDMS layer is being formed. Figure
10 2B shows an exploded view where the first silicon wafer, the PDMS layer, and second silicon wafer are separated from one another after the PDMS layer is formed.

[0014] Figures 3A-B show the channel layouts for fluorescence and electrochemical detections. Figure 3A shows the channel layout for fluorescent method of signal detection. The enlarged area 106 of the channel near outlet 110 is
15 the detection zone, and the channel with inlet 102 is the main hybridization channel. Figure 3B shows the channel layout for an electrochemical method of signal detection. The wider channel 106 between where inlet 102 and inlet 108 merge and where the outlet 110 represents the detection zone.

[0015] Figure 4 shows the assembly of the microfluidic channel device 20.
20 PDMS layer 22 with the microchannels 24 is laid onto a glass plate 26 in order to provide a cover for the channel structure.

[0016] Figure 5 shows the assembly of microfluidic device 32 in a housing. The PDMS 22-glass plate 26 structure is held together by applying slight pressure using a housing that consists of two plates 28 and 4 – 8 screws 30.

25 [0017] Figures 6A-B show interdigitated ultramicroelectrode arrays ("IDUA") in accordance with the present invention.

[0018] Figure 7 shows a device for fluorescent detection in accordance with the present invention.

[0019] Figure 8 shows the positioning of a magnet in a capture zone of a two-
30 channel microfluidic device in accordance with the present invention. Fluorescence microscopy was used to measure the fluorescence in the detection zone (capture zone) on top of the magnet. While other fluorescence detection devices can be used, a

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fluorescence microscope was chosen here in order to observe the different steps in the analysis optically while the reactions were occurring.

[0020] Figure 9 shows a device for electrochemical detection in accordance with the present invention.

5 [0021] Figure 10 shows the positioning of a PDMS microchannel layer on top of an IDUA transducer on the glass plate in accordance with the present invention.

[0022] Figure 11 shows a simplified block diagram of the analysis system instrumentation.

[0023] Figure 12 shows the original potentiostat circuit. The IDUA potential
10 is set with a 1.2V voltage reference (V_{ref}) and adjusted with a $1M\Omega$ potentiometer. The sensor output is first converted to a voltage, amplified, and output to an LCD or a data-logger connected to a computer. The gain of the current-to-voltage amplifier is adjusted with the switch S2.

[0024] Figure 13 shows the sensor output versus time for detection of 0.1M of
15 potassium ferri- and ferrohexacyanide. Channel 1 graphs the bias potential held constant at 400mV, while channel 2 is the current-to-voltage amplifier output in mV.

[0025] Figure 14A shows the dose response curve for ferri/ferrohexacyanide detection. Figure 14B shows an expanded view of Figure 14A for concentrations of 0, 0.1, and 1 μM .

20 [0026] Figure 15 shows a microcontroller program flow. Operation is interrupt driven where the microcontroller ("MCU") stays in low power mode until an interrupt occurs. It then enters active mode and performs the event requested by the interrupt.

[0027] Figure 16 shows the fluorescence images of the captured
25 superparamagnetic beads with no RNA is in the sample (background) and when there is a complex with target RNA and bound nonlysed (A) / lysed liposomes (B).

[0028] Figure 17 shows the fluorescence intensity vs. amount of liposomes.

[0029] Figure 18 shows the fluorescence intensity vs. amount of magnetic bead.

30 [0030] Figure 19 shows the standard curve for the determination of lower limit of detection. Error bars correspond to 3 x Standard Deviations.

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[0031] Figure 20 shows the IDUA's response in the microchannel upon the injection of 20 nL, 50 nL, 100 nL of 10 μM $\text{Fe}^{2+}/\text{Fe}^{3+}$. Buffer flow rate is 1 $\mu\text{L}/\text{min}$. Buffer background signal - 0.27 ± 0.01 nA, 20nL signal - 0.95 ± 0.03 nA, 50nL signal - 2.06 ± 0.05 nA, 100nL signal - 4.15 ± 0.1 nA.

- 5 [0032] Figure 21 shows the signal response of an IDUA in the microchannel in the presence and in the absence of an analyzed RNA.

DETAILED DESCRIPTION OF THE INVENTION

- 10 [0033] The present invention relates to a method for detecting or quantifying an analyte in a test sample. This involves providing at least one test mixture where the test mixture includes a test sample, potentially containing analyte, a capture conjugate, and a marker conjugate. The capture conjugate includes a capture support and a first binding material, where the first binding material is selected to bind with a
- 15 portion of the analyte. The marker conjugate includes a particle, a marker, and a second binding material, where the second binding material is selected to bind with a portion of the analyte other than the portion of the analyte for which the first binding material is selected. Reaction is permitted to occur in the test mixture between
- 20 analyte present in the test sample and the first and second binding materials, thereby forming a product complex comprising analyte present in the test sample, the capture conjugate, and the marker conjugate. The reacted test mixture is exposed to a capture device having non-specific affinity for the capture support, whereby product complex present in the reacted test mixture is immobilized from the reacted test mixture. The
- 25 presence or amount of the marker from the immobilized product complex is detected using a detection assembly and correlated with the presence or amount, respectively, of the analyte in the test sample. In a preferred embodiment, the marker is released from the immobilized product complex after the exposing and before the detecting steps.

- [0034] The term "analyte" is meant to include the compound or composition
- 30 to be measured or detected. It is capable of binding to the first and second binding materials. Suitable analytes include, but are not limited to, antigens (e.g., protein antigens), haptens, cells, and target nucleic acid molecules. A preferred analyte is a

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target nucleic acid molecule. The present invention is applicable to procedures and products for determining a wide variety of analytes. As representative examples of types of analytes, there may be mentioned: environmental and food contaminants, including pesticides and toxic industrial chemicals; drugs, including therapeutic drugs and drugs of abuse; hormones, vitamins, proteins, including enzymes, receptors, and antibodies of all classes; prions; peptides; steroids; bacteria; fungi; viruses; parasites; components or products of bacteria, fungi, viruses, or parasites; aptamers; allergens of all types; products or components of normal or malignant cells; etc. As particular examples, there may be mentioned T₄; T₃; digoxin; hCG; insulin; theophylline; leutinizing hormones; and organisms causing or associated with various disease states, such as *Streptococcus pyrogenes* (group A), Herpes Simplex I and II, cytomegalovirus, chlamydiae, etc. The invention may also be used to determine relative antibody affinities, and for relative nucleic acid hybridization experiments, restriction enzyme assay with nucleic acids, binding of proteins or other material to nucleic acids, and detection of any nucleic acid sequence in any organism, i.e., prokaryotes and eukaryotes. A more preferred analyte is a target nucleic acid molecule found in an organism selected from the group consisting of bacteria, fungi, yeast, viruses, protozoa, parasites, animals (e.g., humans), and plants. Suitable organisms include, but are not limited to, *Cryptosporidium parvum*, *Escherichia coli*, *Bacillus anthracis*, Dengue virus, and Human immunodeficiency virus (HIV-1).

[0035] The term "binding material" is meant to include a bioreceptor molecule such as an immunoglobulin or derivative or fragment thereof having an area on the surface or in a cavity which specifically binds to and is thereby defined as complementary with a particular spatial and polar organization of another molecule -- in this case, the analyte. Suitable binding materials include antibodies, antigens, nucleic acid molecules, aptamers, cell receptors, biotin, streptavidin, and other suitable ligands. When the analyte is a target nucleic acid molecule, the first binding material can be a nucleic acid molecule (e.g., reporter probe, selected to hybridize with a portion of the target nucleic acid molecule) and the second binding material can be a nucleic acid molecule (e.g., capture probe, selected to hybridize with a separate portion of the target nucleic acid molecule), or other moiety, such as an antibody or other agent capable of binding to and interacting with the analyte.

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[0036] Antibody binding materials can be monoclonal, polyclonal, or genetically engineered (e.g., single-chain antibodies, catalytic antibodies) and can be prepared by techniques that are well known in the art, such as immunization of a host and collection of sera, hybrid cell line technology, or by genetic engineering. The binding material may also be any naturally occurring or synthetic compound that specifically binds the analyte of interest.

[0037] The first and second binding materials are selected to bind specifically to separate portions of the analyte. For example, when the analyte is a nucleic acid sequence, it is necessary to choose probes for separate portions of the target nucleic acid sequence. Techniques for designing such probes are well-known. Probes suitable for the practice of the present invention must be complementary to the target analyte sequence, i.e., capable of hybridizing to the target, and should be highly specific for the target analyte. The probes are preferably between 17 and 25 nucleotides long, to provide the requisite specificity, while avoiding unduly long hybridization times and minimizing the potential for formation of secondary structures under the assay conditions. Thus, in this embodiment, the first binding material is reporter probe, which is selected to, and does, hybridize with a portion of target nucleic acid sequence. The second binding material, referred to herein as a capture probe for the nucleic acid detection/measurement embodiment, is selected to, and does, hybridize with a portion of target nucleic acid sequence other than that portion of the target with which reporter probe hybridizes. The capture probe may be immobilized in a capture portion of the microchannel or on a magnetic bead. In addition, the first and second binding materials (reporter and capture probes) should be capable of no or limited interaction with one another. Techniques for identifying probes and reaction conditions suitable for the practice of the invention are described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (1989), which is hereby incorporated by reference in its entirety. A software program known as "Lasergene", available from DNASTAR, or similar products may optionally be used.

[0038] The method of the invention employs marker complexes which include a particle, a marker, and one member of a coupling group. Suitable particles include liposomes (the marker may be encapsulated within the liposome, or incorporated in

the bilayer), latex beads, gold particles, silica particles, dendrimers, quantum dots, magnetic beads (e.g., antibody-tagged magnetic beads and nucleic acid probe-tagged magnetic beads), or any other particle suitable for derivatization. Where multiple marker complexes are used, the marker in each complex may be the same or different.

5 [0039] The use of liposomes as described in the present application provides several advantages over traditional signal production systems employing, for example, enzymes. These advantages include increased signal intensity, shelf stability, and instantaneous release of signal-producing markers, as described in Siebert et al., *Analytica Chimica Acta* 282:297-305 (1993); Yap et al., *Analytical*
10 *Chemistry* 63:2007 (1991); Plant et al., *Analytical Biochemistry* 176:420-426 (1989); Locascio-Brown et al., *Analytical Chemistry* 62:2587-2593 (1990); and Durst et al., Eds., *Flow Injection Analysis Based on Enzymes or Antibodies*, vol. 14, VCH, Weinheim (1990), each of which is hereby incorporated by reference in its entirety.

[0040] Liposomes can be prepared from a wide variety of lipids, including
15 phospholipids, glycolipids, steroids, relatively long chain alkyl esters; e.g., alkyl phosphates, fatty acid esters; e.g. lecithin, fatty amines, and the like. A mixture of fatty materials may be employed, such as a combination of neutral steroid, a charge amphiphile and a phospholipid. Illustrative examples of phospholipids include lecithin, sphingomyelin, and dipalmitoylphosphatidylcholine, etc. Representative
20 steroids include cholesterol, chlorestanol, lanosterol, and the like. Representative charge amphiphilic compounds generally contain from 12 to 30 carbon atoms. Mono- or dialkyl phosphate esters, or alkylamines; e.g. dicetyl phosphate, stearyl amine, hexadecyl amine, dilaurylphosphate, and the like are representative.

[0041] The liposome vesicles are prepared in aqueous solution containing the
25 marker, whereby the vesicles will include the marker in their interiors. The liposome vesicles may be prepared by vigorous agitation in the solution, followed by removal of the unencapsulated marker. Alternatively, reverse phase evaporation plus sonication can be used. Further details with respect to the preparation of liposomes are set forth in U.S. Patent No. 4,342,826 and PCT International Publication No. WO
30 80/01515, both of which are hereby incorporated by reference in their entirety.

[0042] The concentration of electrolytes in the medium will usually be adjusted to achieve isotonicity or equi-osmolality (or up to about 50 to about 100

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mmol/kg hypertonic) with the solution in the interior of liposomes to prevent their crenation or swelling.

[0043] With some increased complexity of the excitation waveform applied by the electroanalyzer, electrochemical measurement in accordance with the invention
5 may also be carried out using stripping voltammetry, employing, for example, liposome encapsulated metal ions for detection and measurement.

[0044] Moderate, and desirably substantially constant, temperatures are normally employed for carrying out the assay. The temperatures for the assay and production of a detectable signal will generally be in the range of about 4-65°C, more
10 usually in the range of about 20-38°C, and frequently, will be about 15-45°C.

[0045] The solvent for the test mixture will normally be an aqueous medium, which may be up to about 60 weight percent of other polar solvents, particularly solvents having from 1 to 6, more usually of from 1 to 4, carbon atoms, including alcohols, formamide, dimethylformamide and dimethylsulfoxide, dioxane, and the
15 like. Usually, the cosolvents will be present in less than about 30-40 weight percent. Under some circumstances, depending on the nature of the sample, some or all of the aqueous medium could be provided by the sample itself.

[0046] The pH for the medium will usually be in the range of 2-11, usually 5-9, and preferably in the range of about 6-8. The pH is chosen to maintain a significant
20 level of binding affinity of the binding members and optimal generation of signal by the signal producing system. Various buffers may be used to achieve the desired pH and maintain the pH during the assay. Illustrative buffers include borate, phosphate, carbonate, tris, barbital, and the like. The particular buffer employed is usually not critical, but in individual assays, one buffer may be preferred over another. For
25 nucleic acid analytes, it is necessary to choose suitable buffers. Such buffers include SSC, sodium chloride, sodium citrate buffer, and SSPE (sodium chloride, sodium phosphate, EDTA).

[0047] This method can be carried out with the bioanalytical microsystem which includes a sample preparation module and a biosensor module. This entire
30 system is preferably produced in a microfluidic platform.

[0048] The principle of the biosensor of the present invention is based on DNA/RNA hybridization system and liposome signal amplification (Figure 1). As

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shown in Figure 1, two sets of probes hybridize specifically with the target RNA. This system is described with reference to a generic probe designed to bind to four Dengue virus serotypes, and four specific probes are designed to bind to the four serotypes only (Wu et al., "Detection of Dengue Viral RNA Using a Nucleic Acid Sequence-based Amplification Assay," *J. Clin. Microbiol.* 39:2794-2798 (2001), which is hereby incorporated by reference in its entirety). The reporter probe is coupled to liposomes with an encapsulated fluorescent dye or an electrochemically active compound and can hybridize to a specific sequence of the target RNA. The second specific probes (capture probes) are immobilized on the surface of superparamagnetic microbeads via biotin-streptavidin interaction. Target RNA is amplified using the isothermal nucleic-acid-sequence-based amplification ("NASBA") reaction. Liposomes with reporter probes and beads with capture probes are incubated with amplified target sequence prior to introducing the mixture into the microchannel where the sandwich complexes subsequently are captured on the magnet for fluorescence or electrochemical detection.

[0049] The microfluidic device of the present invention can be designed to carry out fluorescent or electrochemical methods for signal detection. The approach for construction of the microfluidic device was based on providing precise sample handling in terms of volume and flow-rates, zero-dead volume at inlet and outlet points (no sample losses during the analysis and 100% waste disposal), ability to disassemble the device for replacing the microfluidic channel or transducer parts. Liposome, Dengue virus RNA, reporter and capture probes, and hybridization and washing buffers were used as optimized in experiments previously carried out in the development of membrane strip-based biosensors for Dengue virus detection (Baeumner et al., "A Biosensor for Dengue Virus Detection: Sensitive, Rapid and Serotype Specific," *Analytical Chemistry*, 74(6):1442-1448 (2002) and Zaytseva et al., "Multi-Analyte Single-Membrane Biosensor for Serotype-Specific Detection of Dengue Virus," *Anal. Bioanal. Chem.* 380:46-53 (2004), which are hereby incorporated by reference in their entirety).

[0050] Microfluidic channels can be fabricated as raised structures on 4 inch silicon wafers using standard photolithography processes. A 1 mL of freshly prepared 7:1 by volume mixture of silicone elastomer and silicone elastomer curing

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agent (Sylgard, 184 Silicone elastomer kit) was poured onto the silicone template and covered with another flat silicone wafer. Covering the resulting polydimethylsiloxane (PDMS) layer with a silicone wafer allowed thickness and thickness uniformity of the layer to be controlled. The obtained sandwich structure (Figure 2) was cured in an oven at 65°C for 2 h. The cured PDMS layer was peeled off the wafer and channels were manually cut out. The PDMS layer was 170 micrometer thick. The channels were 50 mm deep and the width was varied from 100 to 500 micrometer.

[0051] Microfluidic channels employed in the microfluidic device of the present invention should fulfill the following requirements: 1) The geometry and dimensions of a channel should be suitable to avoid large pressure drops in the liquid flow upon entering it. Experimentally, it was found that 100 micrometer wide, 50 micrometer deep channel satisfied this requirement; 2) The channel should have a small region with 5 times slower linear fluid flow compared to the rest of it. In this region, magnetic beads that are utilized during the analysis are captured. The signal transducer is placed downstream of the captured beads area; and 3) The PDMS layer with embedded channels structure should have inlet and outlet holes that go vertically through the entire width of the PDMS and that have a diameter not bigger than the width of the adjacent channel. These appropriate inlet dimensions are especially needed in order to avoid volumes of stagnated flow during the analysis.

[0052] Typical channel geometries with dimensions in micrometers are presented in Figure 3. Figures 3A and 3B show 2 embodiments of the present invention where each has inlets 102 and 108 leading to detection section 106 and ultimately to outlet 110. In Figure 3A, inlet 102 has a circuitous region 104 which gives the materials passing through more residence time to undergo reaction.

[0053] The formation of the PDMS 22 with embedded channels 24 is shown in Figures 2A to 2B. As shown in Figure 2A, PDMS 22 with embedded channels 24 is formed by molding between top mold plate T and bottom mold plate B. PDMS 22 is formed with embedded channels 24, which are longitudinally exposed along one surface of PDMS 22. This structure is recovered by removing it from mold plates T and B, as shown in Figure 2B. PDMS 22 with embedded channels 24 is mounted to glass plate 26 to form unit 20, as shown in Figure 4. Such mounting causes the longitudinally exposed channels 24 in PDMS to be covered by glass plate 26.

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[0054] For the microfluidic device with fluorescent detection, glass plate 26 is clear. In the case of electrochemical detection, glass plate 26 is provided with patterned interdigitated ultramicroelectrode arrays (IDUAs) which are in fluid communication with channels 24 so that material passing through channel 24 can be in contact with and analyzed by the IDUAs.

[0055] Leak tight sealing was achieved by applying pressure from above PDMS layer 22 and beneath glass plate 26. For this purposes two plates 28 and 4-8 screws 30 were used as shown in Figure 5. In the case of optical detection, at least the plate 28 adjacent to glass plate 26 is transparent to permit visualization within channel. Alternatively, the optical detection device can be installed between plate 28 and glass plate 26.

[0056] It should be noted that the upper plate has tubing 32 and 34 glued into the locations that line up with inlet 102 and outlet 110 of the PDMS device. The pressure applied onto the PDMS-glass plate device also provides a seal for the PDMS-Plexiglas interface. Initially, metal tubing was used in the Plexiglas inlet and outlet holes. However, due to high background signal during the electrochemical detection, these were replaced with plastic tubing.

[0057] In order to accommodate a magnet required to capture magnetic beads during the analysis in the capture zone, a groove can be made in the upper Plexiglas plate. The distance between the magnet and the upper wall of the PDMS channel can be precisely controlled by the depth of the groove and the thickness of the PDMS layer. These parameters and the strength of the magnetic field have a great influence on the ability to quantitatively capture beads during the analysis under varying flow rates. The closer the magnet is positioned with respect to the upper wall of the microchannel, the higher flow rates can be used during the analysis. In the microfluidic device of the present invention, the magnet (35DNE1304-NI, Magnet Applications, Inc.) is placed at a distance of 270 μm from the upper wall of the channel. This allows all the beads (1 μm diameter) to be captured at a linear flow rate of 0.2 m/min or 5 $\mu\text{L}/\text{min}$.

[0058] The capture device can be any device which achieves non-specific binding (i.e. does not involve use of any of the above-described binding materials). A magnetic field generating device or a filter with a binding material are particularly

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preferred capture devices. Any suitable solid support can be utilized as the capture support to which the capture device has an affinity. It is particularly preferred to use magnetic beads as the capture support, while the capture device comprises a magnetic field generating device. In this embodiment, as shown in Figure 1, a paramagnetic particle with a capture probe specific to a target material is contacted with a sample potentially containing the target material under conditions which will permit the target to bind to the capture probe. The resulting complex is then removed from the sample mixture with the magnet. When the filter alternative to a magnetic field generating device is selected, the arrangement of Figure 5 must be modified so that the filter is in communication with channel 24. Preferably, they can be in a position aligned with the position of magnet 36 (which would not be present in such an embodiment) relative to inlet 102 and outlet 108.

[0059] When a filter is used as the capture device, any porous material having a pore size of from about 0.1 μm to about 100 μm , preferably from about 1 μm to about 30 μm , which allows an aqueous medium to flow therethrough can be used. The pore size has an important impact on the performance of the device. The pore size has to be larger than the mean diameter of the marker. Also, the pores should not be too large so that a good volume to surface ratio can be obtained and to hold back the magnetic, polymer, or silica beads coupled to capture probes. Additionally, the filter could function as a conventional filter and retain large particles. Thus, liposomes bound to silica or other particles (the silica or other particles being too large to fit through the filter) would be retained but all other liposomes would pass through the filter. As a result, the amount of target present could be measured by the amount of liposomes bound via target to silica or other particles retained on the filter.

[0060] Suitable filter membranes for the device and methods of the present invention include nitrocellulose membranes, nitrocellulose mixed esters, mylar membranes, polysulfonfyl based membranes, plain filter paper, glass fiber membranes, and membranes of any plastic material with defined pore size, such as polycarbonate filters, porous gold, and porous magnetic material. It can also be fabricated using microfabrication tools directly inside the microchannel using photoresist materials, such as SU-8 or also PDMS. The filter membranes can be of a variety of shapes, including rectangular, circular, oval, trigonal, or the like.

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[0061] When the optical detection embodiment of the present invention is utilized, an optical marker is immobilized in the liposome. Suitable optical markers include a fluorescent dye, visible dyes, bio- or chemi-luminescent materials, quantum dots, and enzymatic markers. A qualitative or semi-quantitative measurement of the presence or amount of an analyte of interest may be made with the unaided eye when visible dyes are used as the marker. The intensity of the color may be visually compared with a series of reference standards, such as in a color chart, for a semi-quantitative measurement. Alternatively, when greater precision is desired, or when the marker used necessitates instrumental analysis, the intensity of the marker may be measured directly on the membrane using a quantitative instrument such as a reflectometer, fluorimeter, spectrophotometer, electroanalyzer, etc.

[0062] When using liposomes as the particle, the amount of marker material present can be measured without lysis of the liposomes. However, lysis can be used to enhance such visualization. This may be accomplished by applying a liposome lysing agent. Suitable liposome lysing materials include surfactants such as octylglucopyranoside, sodium dioxycholate, sodium dodecylsulfate, saponin, polyoxyethylenesorbitan monolaurate sold by Sigma under the trademark Tween-20, and a non-ionic surfactant sold by Sigma under the trademark Triton X-100, which is t-octylphenoxypolyethoxyethanol. Octylglucopyranoside is a preferred lysing agent for many assays, because it lyses liposomes rapidly and does not appear to interfere with signal measurement. Alternatively, complement lysis of liposomes may be employed, or the liposomes can be ruptured with electrical, optical, thermal, or other physical means.

[0063] A suitable arrangement for the embodiment of the present invention using optical detection is shown in Figures 7 to 8. In operation, as particularly shown in Figure 8, a test mixture containing a test sample potentially containing the target analyte, a capture conjugate which includes paramagnetic beads, and a marker conjugate is injected through inlet 102. The passage leading from inlet 102 can have a circuitous configuration 104 to provide more residence time for these reactants to contact one another, permitting formation of a product complex which includes the target analyte, the capture conjugate, and the marker. Once the test mixture reaches magnet 112, the product complex is immobilized. Wash liquid is injected into inlet

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102 (or 108) which ultimately leads to magnet 112 so that immobilized product complex can be treated to remove unbound marker (e.g., liposomes) which is discharged through outlet 110. If magnet 112 is located in optical detection region 106, such optical detection can take place with the washed product immobilized on magnet 112. Alternatively, whether detection region 106 is located at magnet 112 or downstream of it, the immobilized product complex can be treated such as by agents injected through inlet 108 to release the marker. For example, if the marker is a liposome containing a fluorescent dye, an agent which will disrupt the liposome is injected through inlet 108. As a result, the presence of target analyte in the test sample can be detected by an optical reader. Such detection can also be achieved if detection region 106 is upstream of magnet 112 by causing reverse flow conditions in the channel after marker release.

[0064] Interdigitated ultramicroelectrode arrays ("IDUA") can be fabricated on glass wafers using standard photolithographic and lift-off techniques. A typical IDUA was produced by evaporation deposition of 70nm Ti followed by 500 nm Au on patterned Pyrex glass wafers (7740, Corning, NY). IDUAs with different dimensions were studied as signal transducers in oxidation-reduction reaction of the potassium ferro/ferrihexacyanide, $\text{Fe}^{2+}/\text{Fe}^{3+}(\text{CN})_6$, pair. It has been shown that both, the background noise and the specific signal depend on the microelectrode's finger/gap ratio as well as on the total amount of fingers (Min et al., "Characterization and Optimization of Interdigitated Ultramicroelectrode Arrays as Electrochemical Biosensor Transducers," *Electroanalysis*, 16(9):724-729 (2004), which is hereby incorporated by reference in its entirety). The IDUA designed with 3.8 μm wide fingers and 2.5 μm wide gaps with a total of 1000 electrode fingers demonstrated the best characteristics in terms of sensitivity and signal to noise ratio. Typical microphotographs of IDUAs are present in Figure 6.

[0065] The general principles described above have been used for the device assembling.

[0066] IDUAs fabricated on glass plates are used as signal transducers for the electrochemical signal detection scheme. During assembly, the PDMS channel is positioned on the glass in such a way that the IDUA detection zone is located

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downstream of the capture zone. In addition, the PDMS channel should be on top of the active microelectrode fingers (Figure 10).

[0067] When the electrochemical detection embodiment of the present invention is utilized, an electroactive species, such as potassiumhexaferrocyanide and potassium hexaferriicyanide, is encapsulated in the marker, e.g., liposomes. The microchannel is placed above reusable electrodes, such as an interdigitated electrode array, as described above. After lysis of the liposomes, the quantity of the electroactive species is determined.

[0068] Suitable electrochemical markers, as well as methods for selecting them and using them are disclosed, for example, in U.S. Patent No. 5,789,154 to Durst et al., U.S. Patent No. 5,756,362 to Durst et al., U.S. Patent No. 5,753,519 to Durst et al., U.S. Patent No. 5,958,791 to Roberts et al., U.S. Patent No. 6,086,748 to Durst et al., U.S. Patent No. 6,248,956 to Durst et al., U.S. Patent No. 6,159,745 to Roberts et al., U.S. Patent No. 6,358,752 to Roberts et al., and co-pending U.S. Patent Application Serial No. 10/264,159, filed October 2, 2002, which are hereby incorporated by reference in their entirety. Briefly, the test device may be designed for amperometric detection or quantification of an electroactive marker. In this embodiment, the test device includes a working electrode portion(s), a reference electrode portion(s), and a counter electrode portion(s) in the microfluidic device. The working electrode portion(s), reference electrode portion(s), and counter electrode portion(s) are each adapted for electrical connection to one another via connections to a potentiostat. The test device can instead include a working electrode portion and a counter electrode portion. Alternatively, the microfluidic device may be designed for potentiometric detection or quantification of an electroactive marker. In this embodiment, the device includes an indicator electrode portion(s) and a reference electrode portion(s). The indicator electrode portions and reference electrode portions are adapted for electrical connection to potentiometers. In another embodiment, the test device may include an interdigitated electrode array positioned to induce redox cycling of an electroactive marker released from liposomes upon lysis of the liposomes.

[0069] Suitable electroactive markers are those which are electrochemically active but will not degrade the particles (e.g., liposomes) or otherwise leach out of the

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particles. They include metal ions, organic compounds such as quinones, phenols, and NADH, and organometallic compounds such as derivatized ferrocenes. In one embodiment, the electrochemical marker is a reversible redox couple. A reversible redox couple consists of chemical species for which the heterogeneous electron transfer rate is rapid and the redox reaction exhibits minimal overpotential. Suitable examples of a reversible redox couple include, but are not limited to, ferrocene derivatives, ferrocinium derivatives, mixtures of ferrocene derivatives and ferrocinium derivatives, cupric chloride, cuprous chloride, mixtures of cupric chloride and cuprous chloride, ruthenium-tris-bipyridine, potassium ferrohexacyanide, potassium ferrihexacyanide, and mixtures of potassium ferrohexacyanide and potassium ferrihexacyanide. Preferably, the electrochemical marker is encapsulated within a liposome, in the bilayer, or attached to a liposome membrane surface.

[0070] A suitable arrangement for the embodiment of the present invention using electrical detection is shown in Figures 9 to 10. In operation, as particularly shown in Figure 10, a test mixture including a test sample potentially containing the target analyte, a capture conjugate 102. Again, although not shown in Figure 10, the passage leading from inlet 102 can have a circuitous configuration to provide more residence time for these reactants to contact one another, permitting formation of a product complex which includes the target analyte, the capture conjugate, and the marker. Once the test mixture reaches magnet 112, the product complex is immobilized. Wash liquid is injected into inlet 102 which ultimately leads to magnet 112 so that immobilized product complex can be treated to remove unbound marker (e.g., liposomes). As shown in Figure 10, when electrical detection region 106 containing IDUA 114 is located downstream of magnet 112, the immobilized product complex can be treated by agents injected through inlet 108 to release the marker. For example, if the marker is a liposome containing a fluorescent dye, an agent which will disrupt the liposome is injected through inlet 108. As a result, the presence of target analyte in the test sample can be detected by the IDUA 114. IDUA 114 is formed from interdigitated fingers 116 and 118 extending from connectors 120 and 122, respectively.

[0071] As hereinabove indicated, the assay may be qualitative (presence or absence of certain level of target) or quantitative or semi-quantitative. The

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preparation of suitable standards and/or standard curves (the term "standard curve" is used in a generic sense to include a color chart) is deemed to be within the scope of those skilled in the art from the teachings herein.

[0072] In one embodiment, the test device includes multiple capture portions, each of which is modified to bind a distinctive second binding material specific for one of several analytes. Thus, each analyte may be determined by assignment of each conjugate/analyte to its own measurement portion for concentration and measurement. Alternatively, the conjugate of each of the analytes to be determined in this embodiment of the present invention, may include a marker which is distinctly detectable from the other markers. With different encapsulated dyes (e.g., fluorescent dyes) or quantum dots, the results of the assay can be "color coded". In particular, a multi-wavelength detector can be used in a capture portion.

[0073] As a matter of convenience, the present device can be provided in a kit in packaged combination with predetermined amounts of reagents for use in assaying for an analyte or a plurality of analytes. Included within the kit are stabilizers, buffers, and the like. The relative amounts of the various reagents may be varied widely, to provide for concentration in solution of the reagents which substantially optimizes the sensitivity of the assay. The reagents can be provided as dry powders, usually lyophilized, including excipients, which on dissolution will provide for a reagent solution having the appropriate concentrations for performing the assay. The kit or package may include other components such as standards of the analyte or analytes (analyte samples having known concentrations of the analyte).

[0074] The present invention also relates to a microfluidic test device for detecting or quantifying an analyte in a test sample. This device includes a non-absorbent substrate having at least one inlet and outlet extending therethrough and a non-specific capture device located at, upstream of, or downstream of the analysis portion. The inlet and outlet are connected by at least one microchannel imbedded in the substrate, where the at least one microchannel comprises an inlet portion and an analysis portion.

[0075] The non-absorbent substrate is formed from a material like quartz, glass, polymethylacrylate, polydimethyl siloxane, or polymeric materials.

[0076] The microfluidic test device can additionally include an incubation portion upstream of the analysis portion.

[0077] When the capture device and the analysis portion are at the same location, the complex containing analyte, capture conjugate, and marker conjugate
5 can be detected at the capture device. When the analysis portion is downstream of the capture device, marker is released from the complex immobilized to the capture device and detected as it moves with fluid in the direction from inlet 102 to outlet 110. In a third embodiment, the analysis portion is located upstream of the capture device so that when marker is released from the immobilized complex, it is carried to
10 the analysis portion by then reversing flow of fluid in the direction from outlet 110 to inlet 102.

[0078] The electrochemical detection assembly comprises a microcontroller-based analysis system. An example of such a system is described in the following paragraphs.

15 [0079] The current instrumentation is at once a potentiostat for electrochemical detection, a data acquisition/storage system, and a controller for the active components (such as the pump actuator and electromagnet) of the microfluidic biosensor. Requirements for portability, low power consumption, and a small form factor are achieved with an electronic design that uses as few components as possible.

20 [0080] The heart of the system is the low power, highly integrated MSP430FG439 microcontroller ("MCU") from Texas Instruments. Texas Instruments produces a large range of devices that differ only in terms of the number of I/O pins, integrated peripherals, memory, and price. The underlying architecture of all the MCUs are the same. Thus, code written for one MCU will work on all MCUs
25 with a few changes to the initialization setup. The flexibility offered by MCU choice allows the manufacture of inexpensive basic analysis systems as well as deluxe systems using the same code-base. Furthermore, the system can be easily upgraded with an advanced MCU.

[0081] The MSP430 has 4 main sections – CPU, memory, clock, and
30 peripherals. See Figure 11. The CPU performs all the calculations and data manipulation.

[0082] The MSP430FG439 has 60KB of program memory and 2K SRAM. Program memory is flash and self-programmable. This feature allows about 100 data files to be stored for 1-minute measurements taken in 1 sec intervals. The storage capacity can be increased with extra non-volatile memory modules.

- 5 [0083] The clock system is very flexible and allows the device to operate in a very low power mode at 32 KHz for unattended periodic measurements for instance, and up to a fast 8 MHz for data acquisition, analysis, transmission, and display in real time.

- [0084] Most of the system's functionality is provided by the MCU's
10 peripherals. The MSP430FG439 has a built in liquid crystal display ("LCD") controller, 1 universal synchronous asynchronous receiver transceiver ("USART"), an 8 channel 12-bit analogue-to-digital-conversion ("ADC") port, 2 channel 12 bit digital-to-analogue conversion ("DAC") port, 3 operational amplifiers, a built in supply voltage supervisor, 6 general input/output (I/O) ports and 4 timers. In this
15 application, the basic timer is used to maintain a real time clock and time stamps for logged data. It also supplies the LCD frame frequency rate. Timer A is used to generate alarm and distinctive status beeps on a buzzer. Timer B is used to generate PWM outputs used to control peripherals external to the MCU. Any of the timers can be set to keep track of the interval and duration of measurement. The watchdog timer
20 can also reset the device when errors occur during operation.

- [0085] The firmware for the MCU is written mainly in C and compiled with the open-source MSPGCC compiler for the MSP430 line of microcontrollers. The microcontroller is in-circuit programmable via a JTAG interface. The current design calls for the JTAG headers to be left in the circuit so that the firmware can be
25 upgraded and easily debugged. However, the interface can also be removed to prevent tampering. Writing the code in C offers another distinctive advantage to this system, with the addition of a hardware configuration file for the parts and peripherals, any capable microcontroller can be substituted for the MSP430 line of microcontrollers.

- 30 [0086] The other major components of the system are the analogue chain couplings to the ADC and DAC channels of the MCU. Each ADC channel is coupled to a programmable gain current-to-voltage amplifier. The amplifier converts the

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current induced in an IDUA sensor to a voltage and amplifies it. The signal is then captured and logged by the analogue-to-digital converter of the MCU. The potential signal is converted back to current in software before display.

5 [0087] The built-in DAC peripheral supplies a bias potential of up to 2.5V for the IDUA. The potential is adjusted by a user via the user interface described in more detail below.

10 [0088] The ADC and DAC analogue chains form a potentiostat for the electrochemical detection scheme of the biosensor. As mentioned earlier, this circuit was derived from a standalone analogue version that was thoroughly tested. Figures 12, 13, 14A, and 14B show the original potentiostat circuitry and the results of electrochemical detection of the redox pair potassium ferri/ferrohexacyanide on a gold IDUA at a potential of 400mV. The IDUA had 400 fingers. The fingers were on average 1000Å high and 2µm wide with 0.9 µm gaps size between them. The resistance of the current-to-voltage amplifier was set to 200 KΩ (sensor current =
15 voltage/200000). Unless specified, measurements were taken at 1 second intervals for a duration of 1 minute.

[0089] The operation of the microcontroller-based device is interrupt driven. For the most part, the MCU stays in low power mode. In this mode, the real time clock is on but most of the peripherals are turned off. The device enters active mode
20 only in response to interrupts generated by pressing one of the push buttons; communication received on the USART; a power-on reset; low battery alarm; or any of the timers. The operations are summarized in the Figure 15.

[0090] The device is battery powered. When connected, a power-on reset starts up the MCU. It goes through an initialization sequence and preps its peripherals
25 and timers. The MCU then enters and stays in the main loop of low power mode with bursts of activity generated by other interrupts.

[0091] Every interrupt received is processed in order of priority. Each interrupt wakes the MCU up and puts it in active mode to perform whatever activity is required. Once all the instructions have been processed in active mode, the MCU
30 goes back into low power mode to wait for the next event.

[0092] Four push buttons generate interrupts that turn the LCD display on or off, initiate measurements, initiate a change in the parameters, and puts the device in

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monitor mode where the device wakes up periodically to take a measurement at predetermined intervals. The functions are not fixed and can be re-programmed as needed.

[0093] The device also wakes up when it receives an input on its USART port.

5 This input may be a request to retrieve logged data for instance. A low battery interrupt disables most of the MCU activity and generates alarms that may include — beeping and/or flashing the low battery sign on the LCD. The watchdog timer generates an interrupt if there is a problem with the execution of an instruction. This interrupt will cause the device to re-initialize itself with default parameters and
10 notify the user accordingly.

[0094] The user interface currently includes an LCD, a serial connection to a computer, 4 buttons as well as connections to a keypad. The interface also includes a cross-platform graphical user interface (“GUI”) with access to the underlying platform's internet capabilities that a client may use to change measurement or control
15 parameters, upload/download data, and visualize sensor output. The modular design of the system allows other communication schemes such as ethernet, infrared, and wireless to be easily integrated as needed.

[0095] The GUI provides an easy to use menu-driven interface for adjusting sensor potential, full scale measurement range, measurement interval, communication
20 settings and setting the correct time. Currently, sensor potential may range from 0 to 1500mV. Full scale range (+/-) may be 10nA to 1mA. Measurement interval is a minimum of 0.5 seconds at the moment.

[0096] There is no restriction on measurement duration if data storage is not required of the MCU. The capacity of the MCU at the moment is restricted to 6000
25 data points. Duration thus depends on capacity. Thus, for 1sec intervals, measurement duration should not exceed 100mins. Capacity can be increased to 30000 data points in the MCU's flash memory. Also, as mentioned earlier, capacity can be increased with external dataflash.

[0097] The GUI also allows the user to watch sensor signals change in real
30 time on a graph or graph data downloaded from the MCU. The data can also be saved as comma delimited files for viewing and analysis in third party applications.

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[0098] As described above, the method and device of the present invention can be used in a variety of assays, such as competitive binding assays and sandwich assays, as described in U.S. Patent No. 5,789,154 to Durst et al., U.S. Patent No. 5,756,362 to Durst et al., U.S. Patent No. 5,753,519 to Durst et al., U.S. Patent No. 5,958,791 to Roberts et al., U.S. Patent No. 6,086,748 to Durst et al., U.S. Patent No. 6,248,956 to Durst et al., U.S. Patent No. 6,159,745 to Roberts et al., U.S. Patent No. 6,358,752 to Roberts et al., co-pending U.S. Patent Application Serial No. 09/698,564, filed October 27, 2000, and co-pending U.S. Patent Application Serial No. 10/264,159, filed October 2, 2002, which are hereby incorporated by reference in their entirety.

[0100] Another aspect of the present invention relates to a method for detecting or quantifying an analyte in a test sample. This method includes providing at least one test mixture which includes a test sample potentially containing an analyte, a capture support complex including a capture support and a first member of a first coupling group, a first binding material selected to bind with a portion of the analyte and comprising a second member of the first coupling group, a marker complex which includes a particle, a marker, and a first member of a second coupling group, and a second binding material selected to bind with a portion of the analyte other than the portion of the analyte for which the first binding material is selected and comprising a second member of the second coupling group. Reaction is permitted to occur in the at least one test mixture between the first and second members of the first coupling group, between the first and second members of the second coupling group, and between analyte present in the test sample and the first and second binding materials. As a result, a product complex comprising analyte present in the test sample, the capture support complex, the first binding material, the marker conjugate, and the second binding material is formed. The reacted test mixture is exposed to a capture device having non-specific affinity for the capture support so that product complex present in the reacted test mixture is immobilized from the reacted test mixture. The presence or amount of the marker from the immobilized product complex is detected using a detection assembly and correlated with the presence or amount, respectively, of the analyte in the test sample. In a

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preferred embodiment, the marker is released from the immobilized product before the exposing and after the detection steps.

[0101] The components and steps used to carry out this aspect of the present invention are substantially the same as those described above.

5 [0102] Another aspect of the present invention relates to a method for detecting or quantifying an analyte in a test sample. This involves providing at least one test mixture including a test sample potentially containing an analyte, a capture conjugate comprising a capture support and a first binding material, where the first binding material is selected to bind with a portion of the analyte, and a marker
10 conjugate, comprising a particle, a marker, and an analyte analog. Competition is permitted to occur in the at least one test mixture between analyte present in the test sample and the analyte analog for the first binding material. As a result, a product complex, including the capture conjugate and the marker conjugate, is formed. The reacted test mixture is exposed to a capture device having affinity for the capture
15 support so that product complex present in the reacted test mixture is immobilized from the reacted test mixture. The presence or amount of the marker released from the immobilized product complex is detected using a detection assembly and correlated with the presence or amount, respectively, of the analyte in the test sample. In a preferred embodiment, the marker is released from the immobilized product
20 before the exposing and after the detection steps.

[0103] In this embodiment of the present invention, an analyte analog is used, because this embodiment involves a competitive binding assay format. Thus, the term "analyte analog" is meant to include an analog which binds to the capture conjugate. When an analog is employed, however, it is necessary that the particular
25 characteristics of the analyte necessary for recognition by the first binding material in the competition reaction be present in the analyte analog conjugated with the marker complex.

[0104] In all other respects, the components and steps used to carry out this aspect of the present invention are substantially the same as those described above.

EXAMPLES

Example 1 -- Investigation of Liposome Lysis using the Fluorescence Detection Approach.

5 [0105] Through inlet 102 of the microfluidic device shown in Figure 8 and described above, a sample mixture containing complexes of bead - target RNA - liposome is introduced. A mixture containing liposomes encapsulating sulforhodamine B, magnetic beads, target RNA, and a hybridization buffer (60% formamide, 6xSSC, 0.8% Ficoll type 400, 0.01% Triton X-100, 0.15M sucrose) was
10 injected through inlet 102. Captured beads were washed from unbound liposomes by injecting a washing buffer (10% formamide, 3xSSC, 0.2% Ficoll type 400, 0.01% Triton X-100, 0.2M sucrose) into inlet 102. At this point, signals can be detected using the CCD camera connected to the fluorescence microscope. Exposure times were optimized (1 sec), and signals were analyzed using Image Pro Express software.
15 Alternatively, in order to increase the signal to noise ratio by lysing liposomes and obtaining a significantly higher fluorescence signal due to the released sulforhodamine B dye, a solution of 25 mM β -octyl glucopyranoside (OG) was injected into inlet 108 to perform liposome lysis. The fluorescence intensity of the dye released from liposomes was measured by means of a CCD camera connected to
20 the microscope. The device can be operated at preprogrammed volume flow rates from 0.01 to 80 μ L/min. Fluorescence of nonlysed (Figure 16A) and lysed (Figure 16B) liposomes is detected.

Example 2 -- Optimization of RNA Detection in the Microfluidic Channels.

25 [0106] A series of experiments was performed in order to optimize the detection of RNA in the microfluidic channels. These experiments were done without any liposome lysis and were monitored using the fluorescence microscope. The amount of liposomes (1.61 OD value for 1/100 dilution in PBS+ sucrose buffer, pH
30 7.0, osmolality 630 nmol/kg) with immobilized reporter probe (Figure 17), beads with immobilized capture probe (Figure 18) and washing buffer (up to 14 μ L) were optimized with respect to signal to noise ratio. Therefore, the limit of detection was obtained for the analysis of Dengue virus RNA. The amount of reporter probe was

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0.013mol% from the total amount of lipids. The biotinylated capture probe was immobilized on the surface of the beads (Dynabeads MyOne Streptavin) following the manufacturer protocol. 1 mg of the beads binds approximately 3,000 pmoles of free biotin.

5

Example 3 -- Electrochemical Analysis of Liposome Capture in the Microfluidic Device.

[0107] To test the IDUA response in microfluidic system, as shown in
10 Figure 10, different volumes (20nL – 100nL) of 10 μ M potassium hexaferrocyanide/potassium hexaferricyanide solution were injected at flow rate of 1 μ l/min into inlet 108, while buffer solution was introduced at flow rate of 1 μ l/min through inlet 102. The typical result of the IDUA response in the single continuous run is provided in Fig. 20.

15 [0108] These results demonstrated that indeed the system based on the IDUA is capable of a fast response to the electrochemical composition changes inside the channel. The delay time between injection and the maximum signal reached was about 5-7 sec. In all the experiments the IDUA itself demonstrates a good reproducibility and the ability to function for prolonged periods of time without
20 mechanical cleaning.

[0109] The typical results of RNA analysis by means of electrochemical detection is present in Figure 21. In this experiment, 2 μ l of Dengue serotype 4 amplicon (1:100 dilution) was incubated with 1 μ g of supermagnetic beads with attached capture probe and 1 μ l of liposomes (150 mM potassium
25 ferro/ferrihexacyanide encapsulant solution).

[0110] Hybridization mixture was injected into inlet 102 at 3 μ l/min. After all the beads were captured on the magnet and washed with 15 μ l buffer, 25mM solution of OG was injected into inlet 108 at 0.8 μ l/min to lyse liposomes. Electrochemical responses of the IDUA in the presence and in the absence of RNA in the hybridization
30 mixture are present in Figure 21. The signal response of the IDUA to the presence of RNA can be estimated at its peak value or as an integral value of the whole curve (Table 1).

Table 1

| | Area | Peak height, nA | Retention time, sec |
|------------|------|-----------------|---------------------|
| RNA | 1069 | 28 | 128 |
| Background | 200 | 4.2 | 128 |

[0111] A microfluidic biosensor for the highly specific and sensitive detection
5 of pathogens via their nucleic acid sequence has been developed. The biosensor
module employs the two alternative methods of detection, fluorescent or
electrochemical. A microfabrication approach allows one to use microliter amounts
of reagents to perform a single analysis. The microfluidic system was tested and
optimized with a model Dengue virus target sequence. It has been shown that as low
10 as 0.5 fmol of the synthetic target can be detected using a microfluidic platform,
fluorescence detection method, and nonlysed liposomes.

[0112] Although preferred embodiments have been depicted and described in
detail herein, it will be apparent to those skilled in the relevant art that various
modifications, additions, substitutions, and the like can be made without departing
15 from the spirit of the invention and these are therefore considered to be within the
scope of the invention as defined in the claims which follow.

- 30 -

WHAT IS CLAIMED:

1. A method for detecting or quantifying an analyte in a test sample comprising:
 - 5 providing at least one test mixture comprising:
a test sample, wherein the test sample potentially contains an analyte;
a capture conjugate, wherein the capture conjugate comprises a capture support and a first binding material, wherein the first binding material is
10 selected to bind with a portion of the analyte; and
a marker conjugate, wherein the marker conjugate comprises a particle, a marker, and a second binding material, wherein the second binding material is selected to bind with a portion of the analyte other than the portion of the analyte for which the first binding material is selected;
15 permitting reaction to occur in the test mixture between analyte present in the test sample and the first and second binding materials, thereby forming a product complex comprising analyte present in the test sample, the capture conjugate, and the marker conjugate;
exposing the reacted test mixture to a capture device having
20 non-specific affinity for the capture support, whereby product complex present in the reacted test mixture is immobilized from the reacted test mixture;
detecting the presence or amount of the marker from the immobilized product complex using a detection assembly; and
correlating the presence or amount of the marker from the
25 immobilized product complex with the presence or amount, respectively, of the analyte in the test sample.
2. The method according to claim 1, further comprising:
30 releasing the marker from the immobilized product complex
after said exposing and prior to said detecting.

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3. The method according to claim 1, wherein each of the first and second binding materials is an antibody, an antigen, a nucleic acid sequence, an aptamer, or a cell receptor.

5 4. The method according to claim 1, wherein the analyte is a target nucleic acid molecule, the first binding material is a capture probe selected to hybridize with a portion of the target nucleic acid molecule, and the second binding material is a reporter probe selected to hybridize with a portion of the target nucleic acid molecule other than the portion of the target nucleic acid molecule for which the
10 capture probe is selected.

5. The method according to claim 4, wherein the target nucleic acid molecule is found in an organism selected from the group consisting of bacteria, fungi, yeast, viruses, protozoa, parasites, animals, and plants.

15 6. The method according to claim 1, wherein the particle is selected from the group consisting of liposomes, latex beads, gold particles, silica particles, dendrimers, quantum dots, and magnetic beads.

20 7. The method according to claim 6, wherein the particle is a liposome and the marker is encapsulated in said liposome.

8. The method according to claim 1, wherein the marker comprises an electroactive marker.

25 9. The method according to claim 8, wherein the electroactive marker is a reversible redox couple.

10 30 10. The method according to claim 9, wherein the detection assembly is an electrochemical detection assembly.

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11. The method according to claim 10, wherein the electrochemical detection assembly comprises an electrode array comprising a first conductor having a plurality of fingers and a second conductor having a plurality of fingers, wherein the fingers of the first conductor are interdigitated with the fingers of the second conductor, the first and second conductors are electrically connected to one another via a voltage source and readout device, and the array is positioned to induce redox cycling of the electroactive marker.

12. The method according to claim 1, wherein the marker comprises an optical marker.

13. The method according to claim 12, wherein the optical marker is a fluorescent dye.

14. The method according to claim 12, wherein the detection assembly is an optical detection assembly.

15. The method according to claim 2, wherein the capture support is a solid support.

16. The method according to claim 15, wherein the solid support is a magnetic bead and the capture device comprises a magnetic field generating device.

17. The method according to claim 1, wherein the capture device is a magnetic field generating device or a filter.

18. The method according to claim 1, wherein said method is carried out in a microfluidic device comprising:
a substrate having an inlet, an outlet, and a channel extending between the inlet and the outlet, the capture device and the detection assembly being in or proximate to the channel.

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19. The method according to claim 18, wherein the channel is longitudinally-exposed on a surface of the substrate, said microfluidic device further comprising:

5 a cover plate attached to the surface of the substrate and covering the channel.

20. A microfluidic test device for detecting or quantifying an analyte in a test sample comprising:

10 a non-absorbent substrate having at least one inlet and outlet extending therethrough, said inlet and outlet connected by at least one microchannel imbedded in the substrate, wherein the at least one microchannel comprises an inlet portion and an analysis portion and

15 a non-specific capture device located at, upstream of, or downstream of the analysis portion.

15

21. The microfluidic test device according to claim 20, wherein the non-absorbent substrate is formed from a material selected from the group consisting of silicon, quartz, glass, polymethylacrylate, polydimethyl siloxane, and polymeric materials.

20

22. The microfluidic test device according to claim 20, wherein the microchannel further comprises an incubation portion upstream of the analysis portion.

25

23. The microfluidic test device according to claim 20, wherein said capture device is upstream of the analysis portion.

24. The microfluidic test device according to claim 20, wherein the capture device is at the analysis portion.

30

25. The microfluidic test device according to claim 20, wherein the capture device is a magnetic field generating device or a filter.

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26. The microfluidic test device according to claim 20, wherein the analysis portion comprises an electrochemical detection assembly.

5 27. The microfluidic test device according to claim 26, wherein the electrochemical detection assembly comprises an electrode array comprising a first conductor having a plurality of fingers and a second conductor having a plurality of fingers, wherein the fingers of the first conductor are interdigitated with the fingers of the second conductor, the first and second conductors are electrically connected to
10 one another via a voltage source and readout device, and the array is positioned to induce redox cycling of the electroactive marker.

 28. The microfluidic test device according to claim 26, wherein the electrochemical detection assembly comprises a microcontroller-based analysis
15 system.

 29. The microfluidic test device according to claim 20, wherein the analysis portion comprises an optical detection assembly.

20 30. The microfluidic test device according to claim 20, wherein the microchannel is longitudinally-exposed on a surface of the substrate, said microfluidic test device further comprising:

 a cover plate attached to the surface of the substrate and covering the channel.

25

 31. A method for detecting or quantifying an analyte in a test sample comprising:

 providing at least one test mixture comprising:

 a test sample, wherein the test sample potentially contains an

30 analyte;

 a capture support complex, wherein the capture support complex comprises a capture support and a first member of a first coupling group;

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a first binding material, wherein the first binding material is selected to bind with a portion of the analyte, and wherein the first binding material comprises a second member of the first coupling group;

5 a marker complex, wherein the marker complex comprises a particle, a marker, and a first member of a second coupling group; and

a second binding material, wherein the second binding material is selected to bind with a portion of the analyte other than the portion of the analyte for which the first binding material is selected, and wherein the second binding material comprises a second member of the second coupling group;

10 permitting reaction to occur in the at least one test mixture between the first and second members of the first coupling group, between the first and second members of the second coupling group, and between analyte present in the test sample and the first and second binding materials, thereby forming a product complex comprising analyte present in the test sample, the capture support complex, 15 the first binding material, the marker conjugate, and the second binding material;

exposing the reacted test mixture to a capture device having non-specific affinity for the capture support, whereby product complex present in the reacted test mixture is immobilized from the reacted test mixture;

20 detecting the presence or amount of the marker from the immobilized product complex using a detection assembly; and

correlating the presence or amount of the marker from the immobilized product complex with the presence or amount, respectively, of the analyte in the test sample.

25 32. The method according to claim 31 further comprising:
releasing the marker from the immobilized product complex after said exposing and prior to said detecting.

30 33. A method for detecting or quantifying an analyte in a test sample comprising:

providing at least one test mixture comprising:

- 36 -

a test sample, wherein the test sample potentially contains an analyte;

a capture conjugate, wherein the capture conjugate comprises a capture support and a first binding material, wherein the first binding material is
5 selected to bind with a portion of the analyte;

a marker conjugate, wherein the marker conjugate comprises a particle, a marker, and an analyte analog;

permitting competition to occur in the at least one test mixture between analyte present in the test sample and the analyte analog for the first binding
10 material, thereby forming a product complex comprising the capture conjugate and the marker conjugate;

exposing the reacted test mixture to a capture device having non-specific affinity for the capture support, whereby product complex present in the reacted test mixture is immobilized from the reacted test mixture;

15 detecting the presence or amount of the marker from the immobilized product complex using a detection assembly; and

correlating the presence or amount of the marker from the immobilized product complex with the presence or amount, respectively, of the analyte in the test sample.

20

34. The method according to claim 33 further comprising:

releasing the marker from the immobilized product complex after said exposing and prior to said detecting.

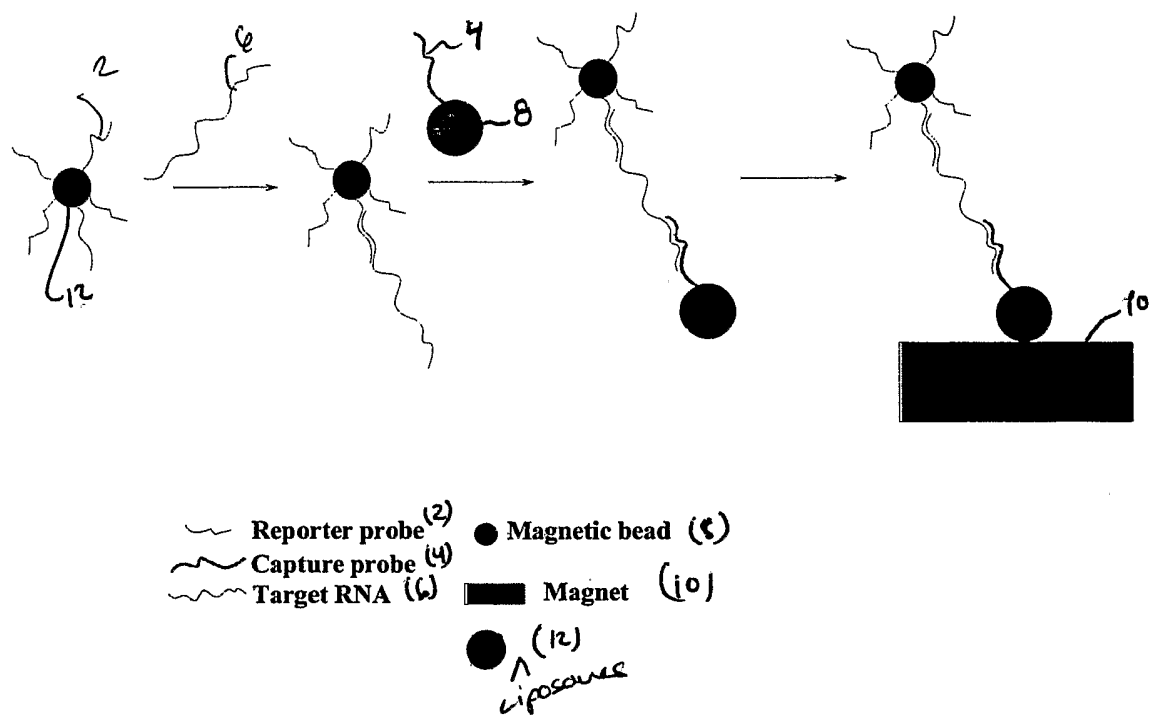
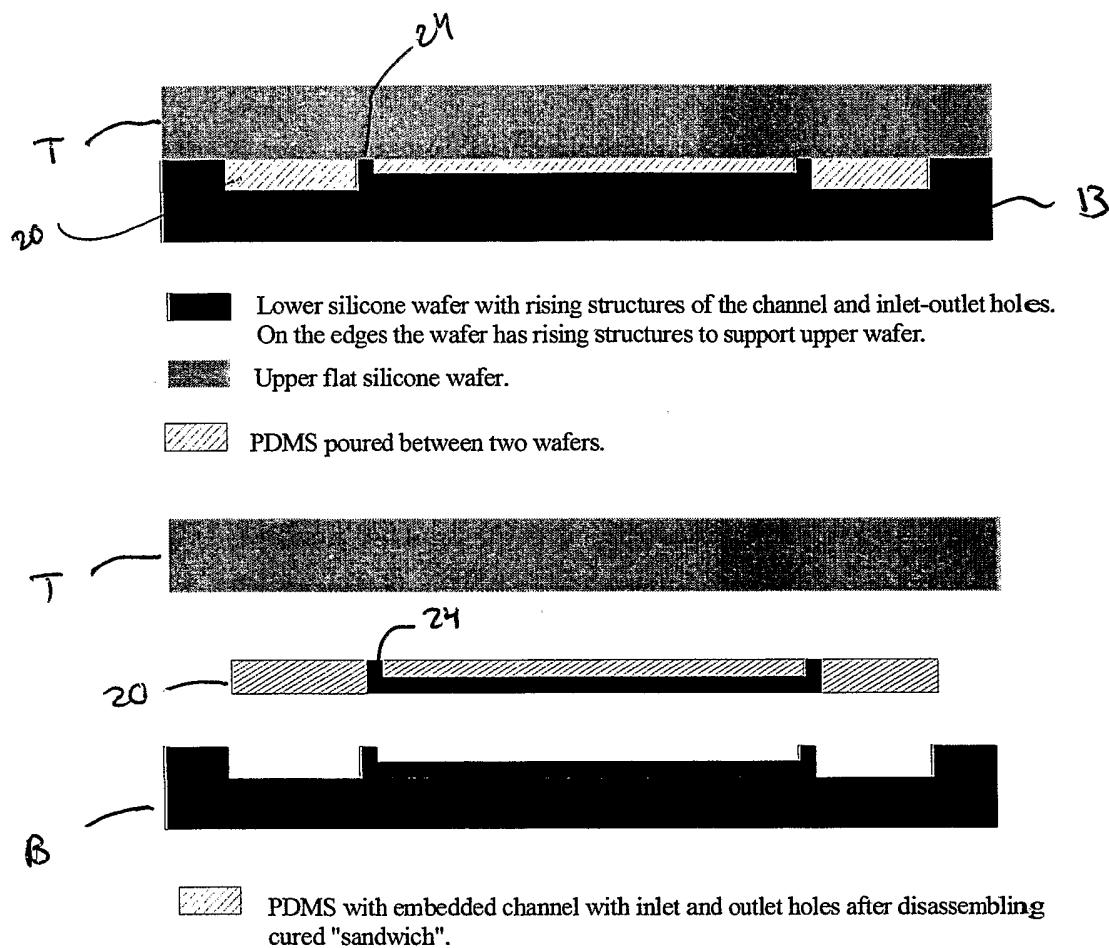


FIGURE 1



FIGURES 2A-B

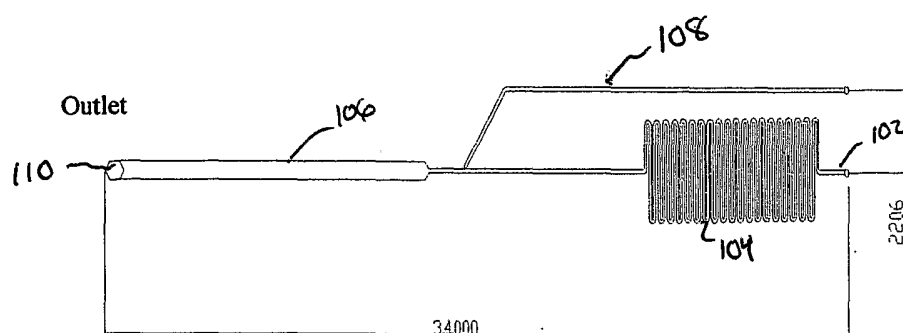


FIGURE 3A

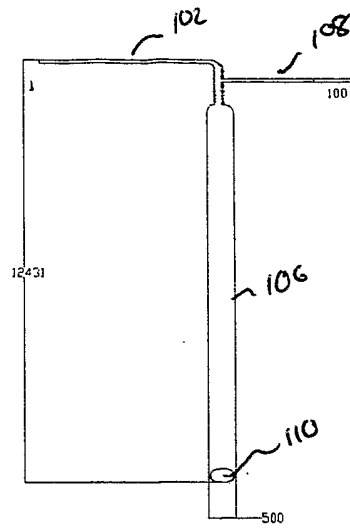
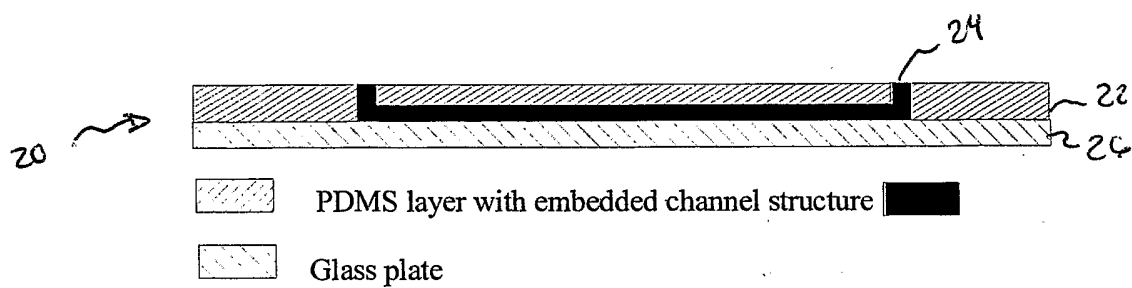


FIGURE 3B

**FIGURE 4**

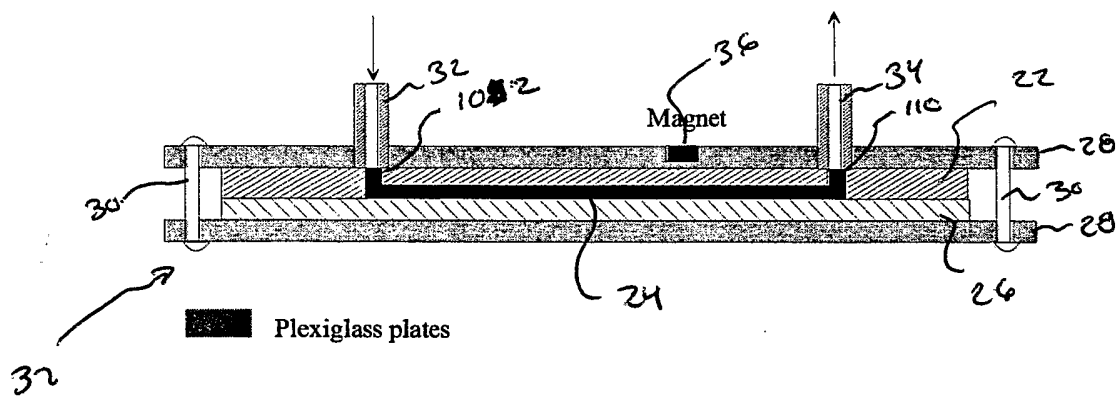
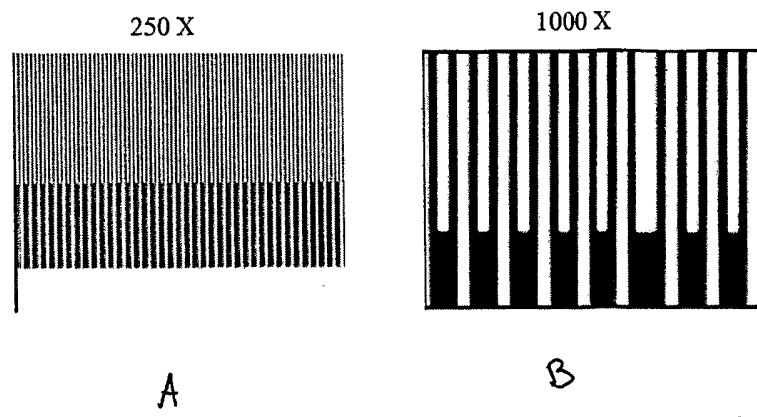


FIGURE 5



FIGURES 6A-B

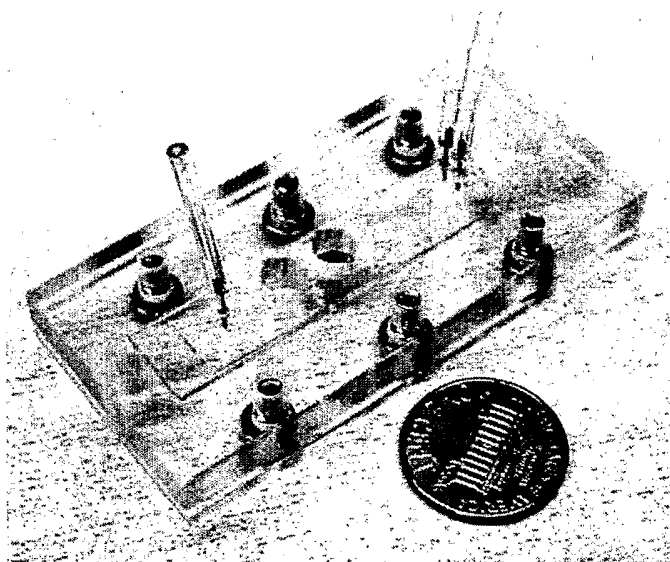
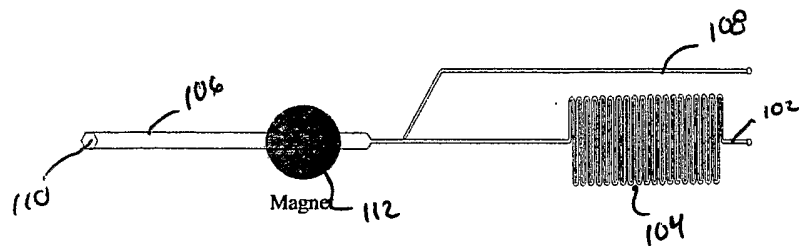


FIGURE 7

**FIGURE 8**

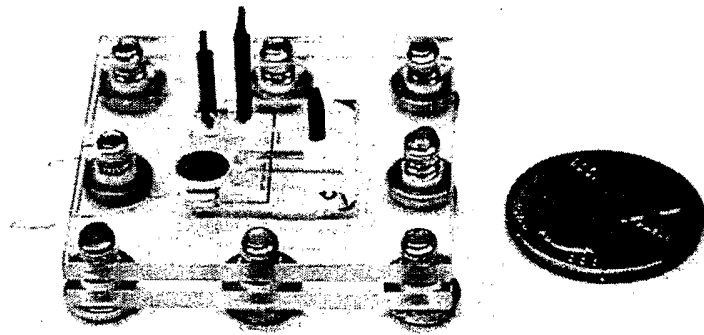


FIGURE 9

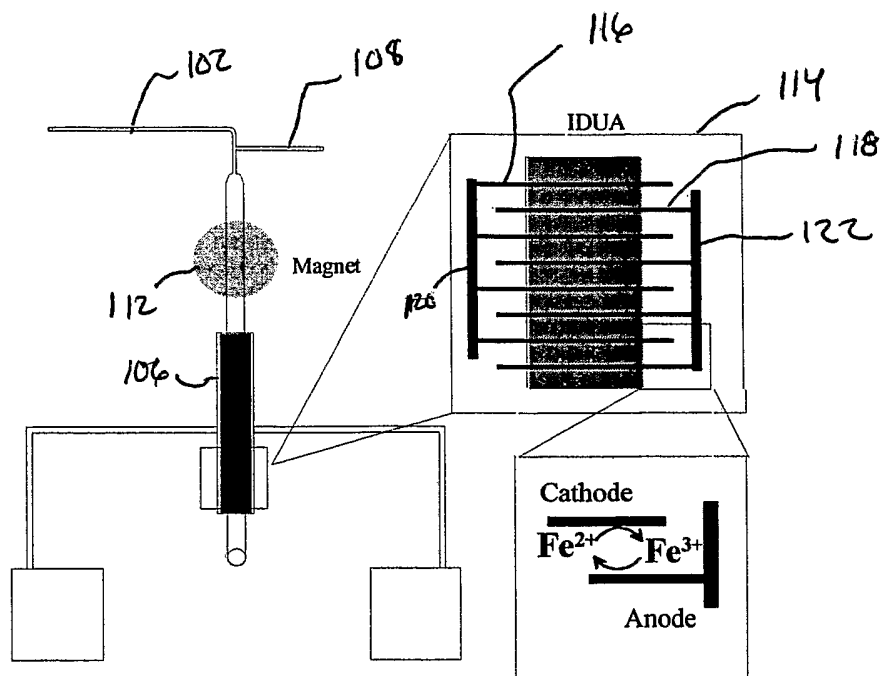
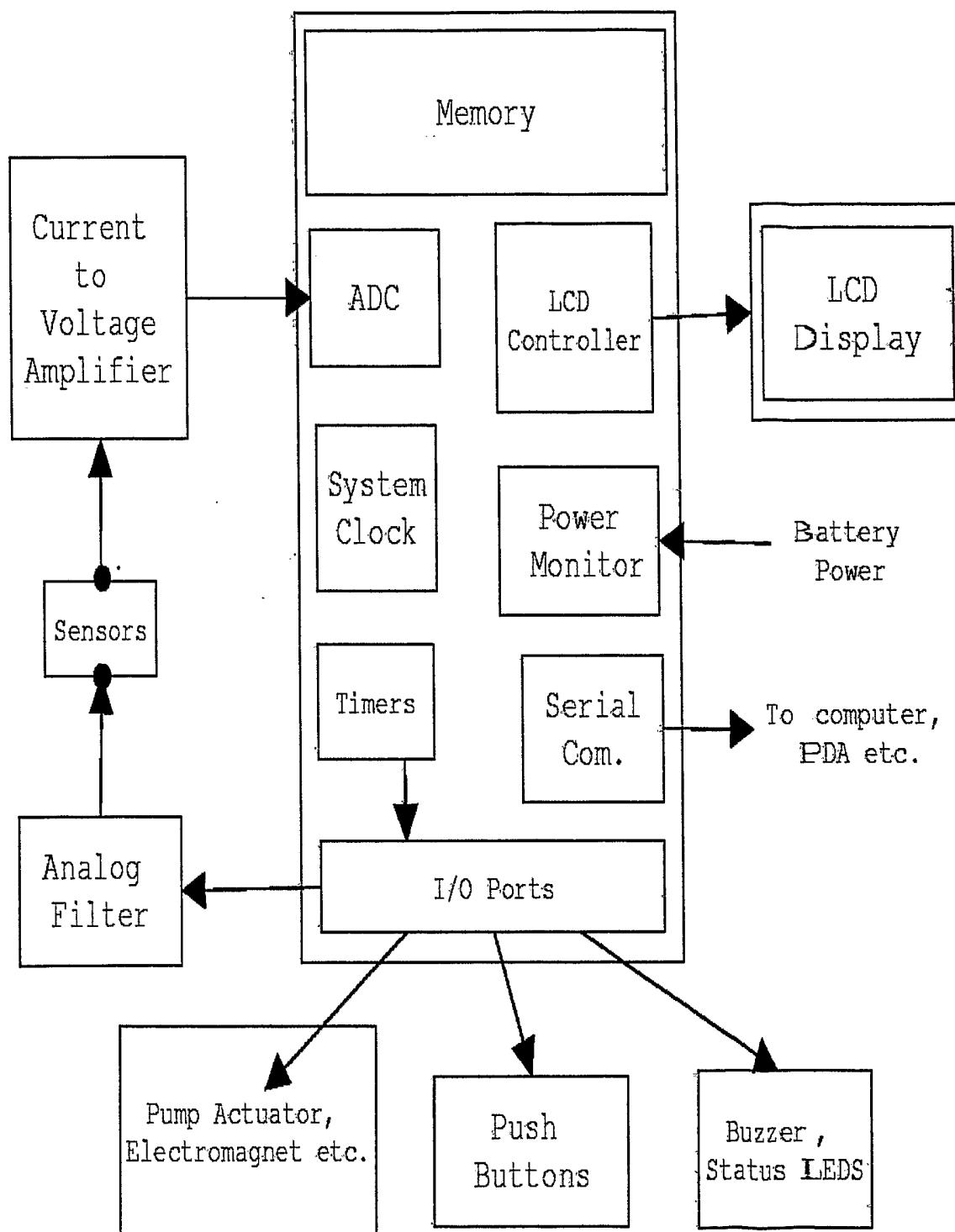


FIGURE 10

**FIGURE 11**

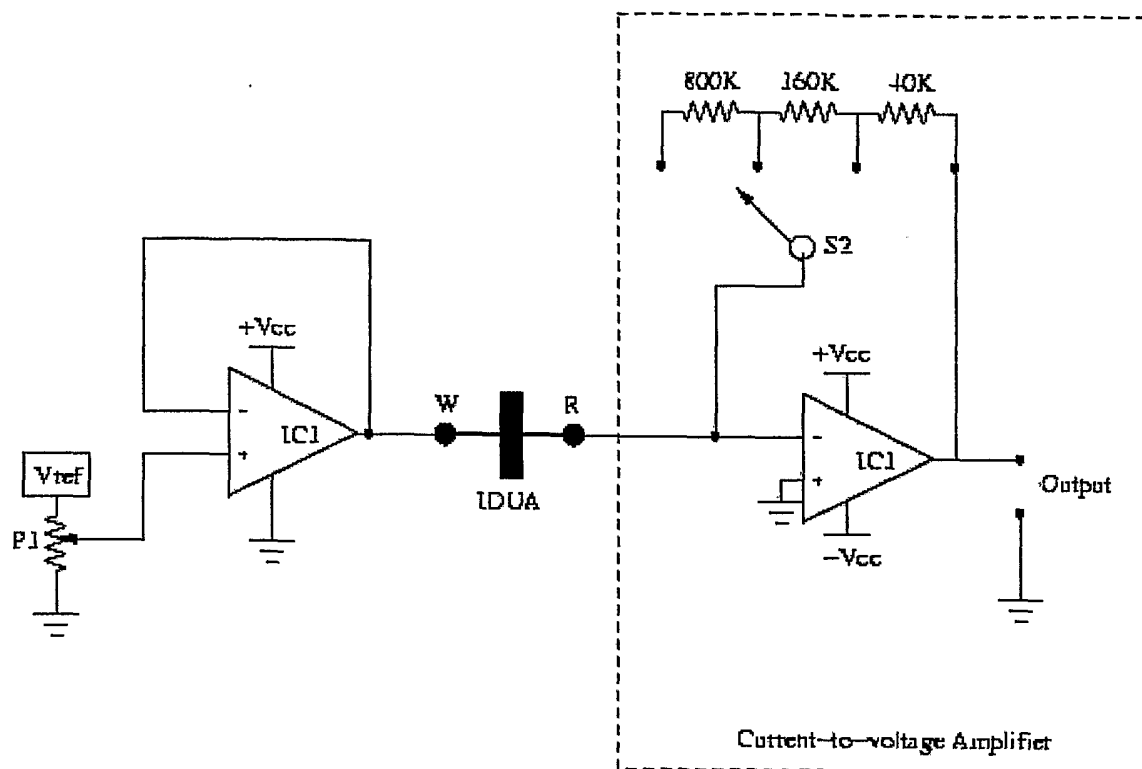


FIGURE 12

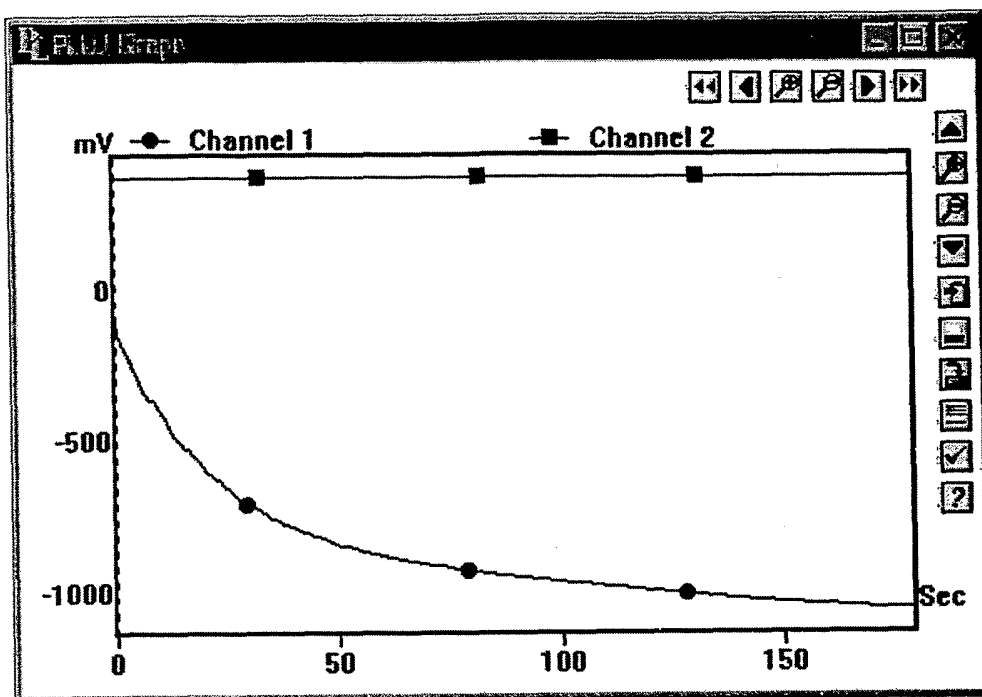


FIGURE 13

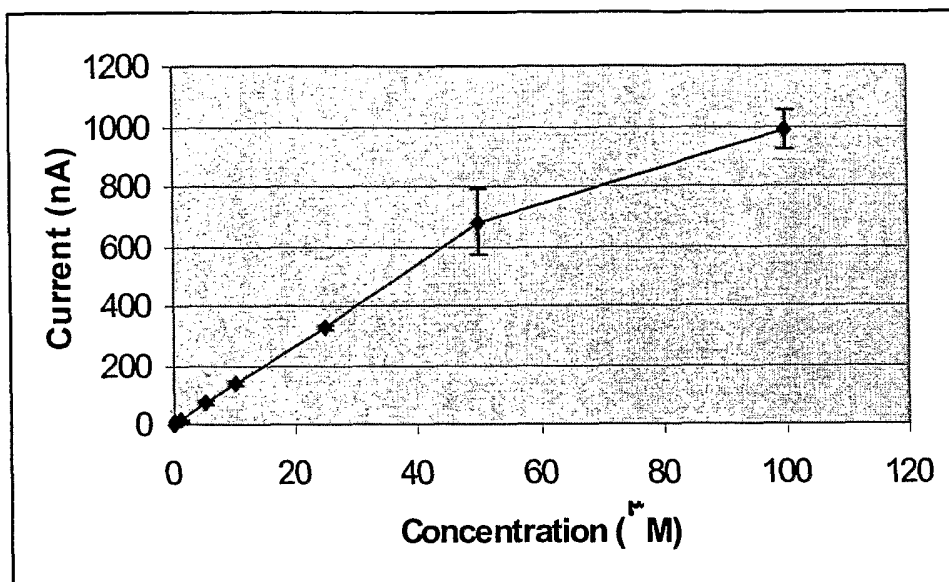
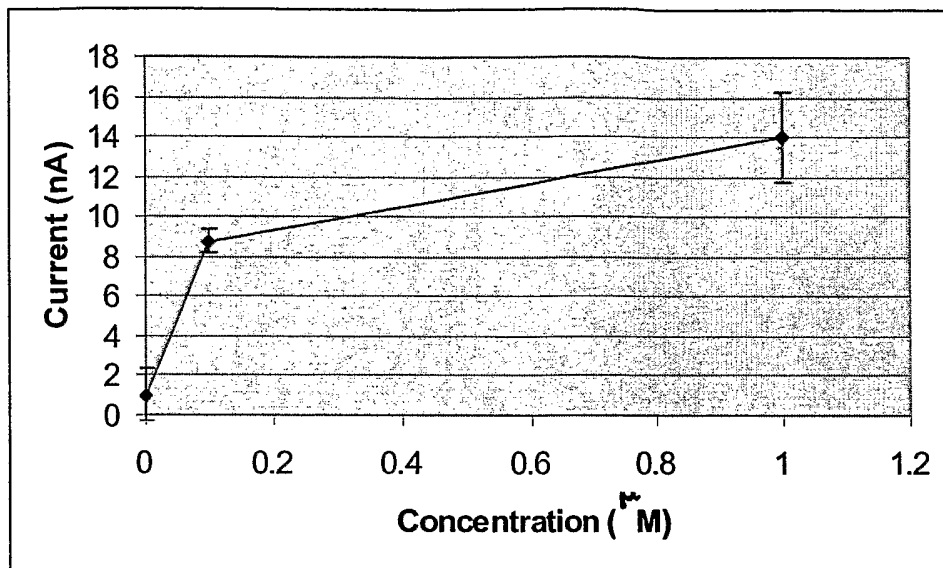
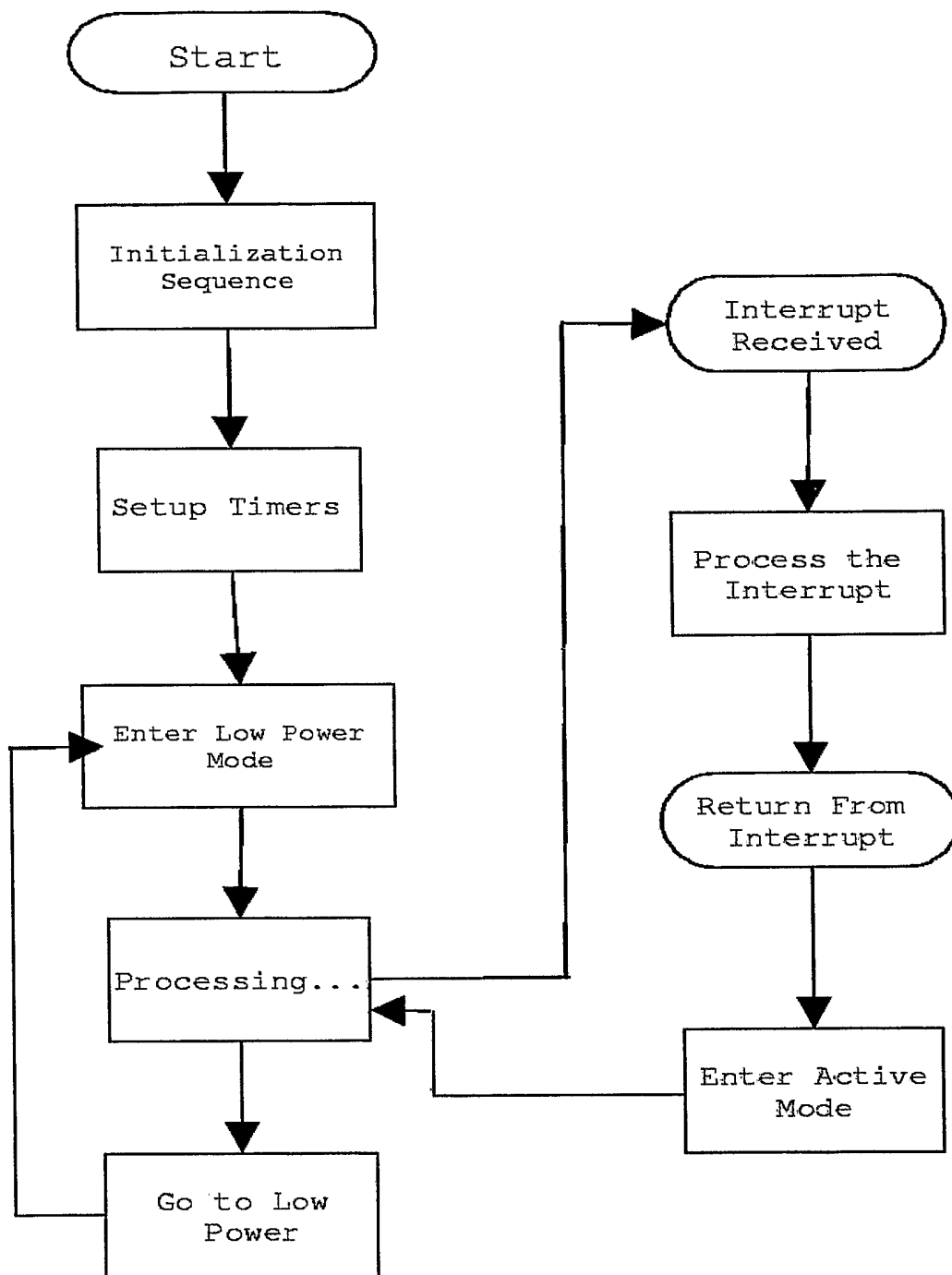
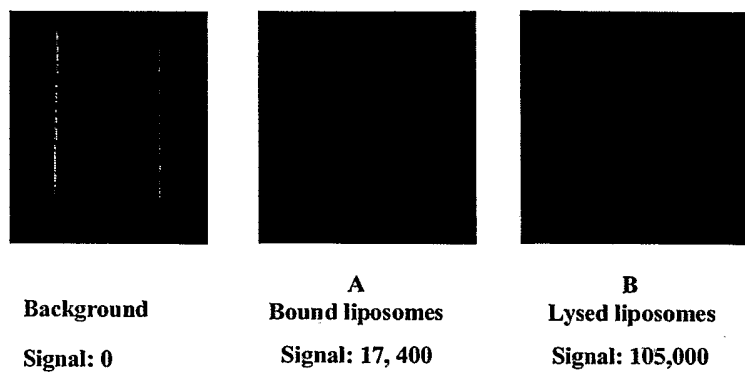
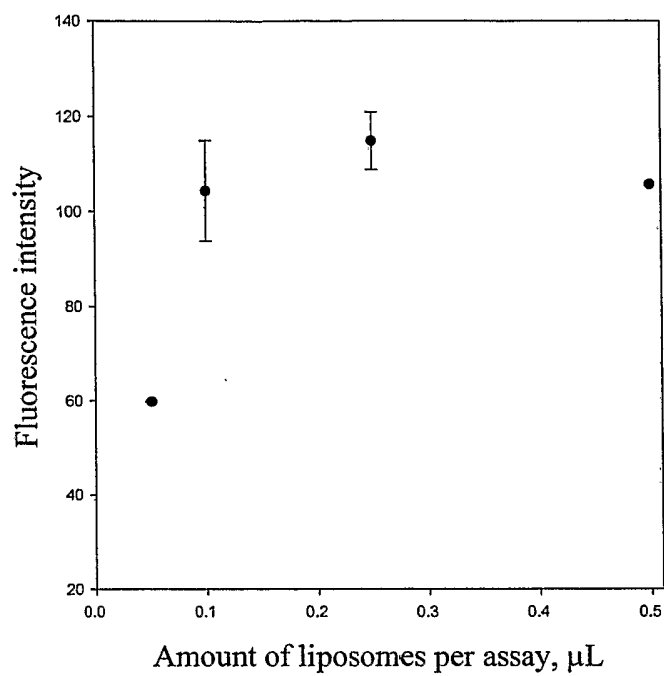


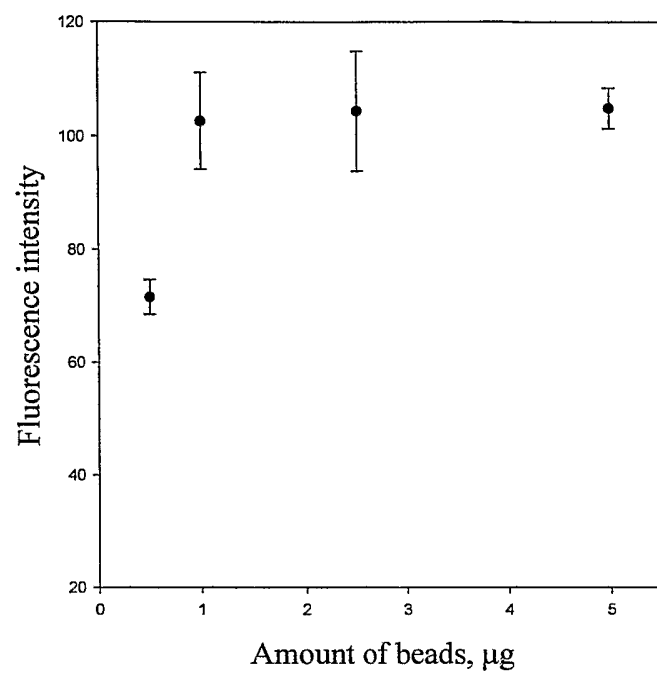
FIGURE 14A

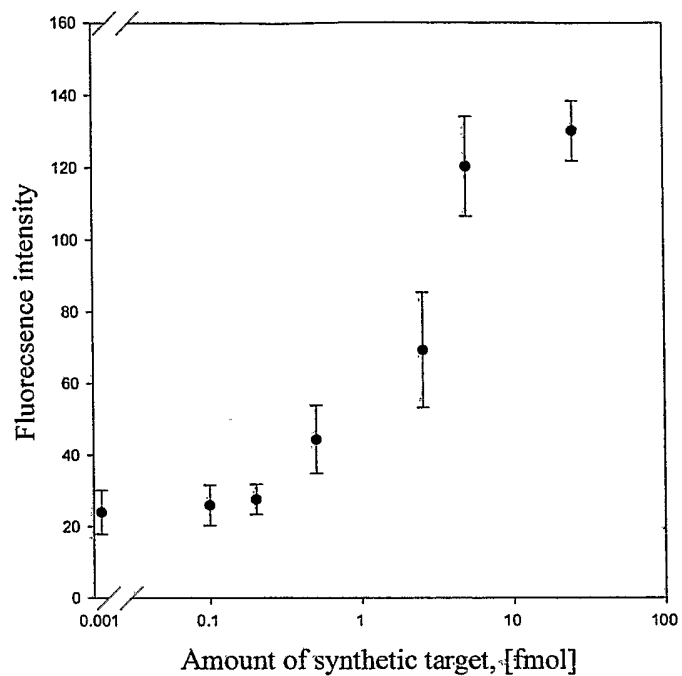
**FIGURE 14B**

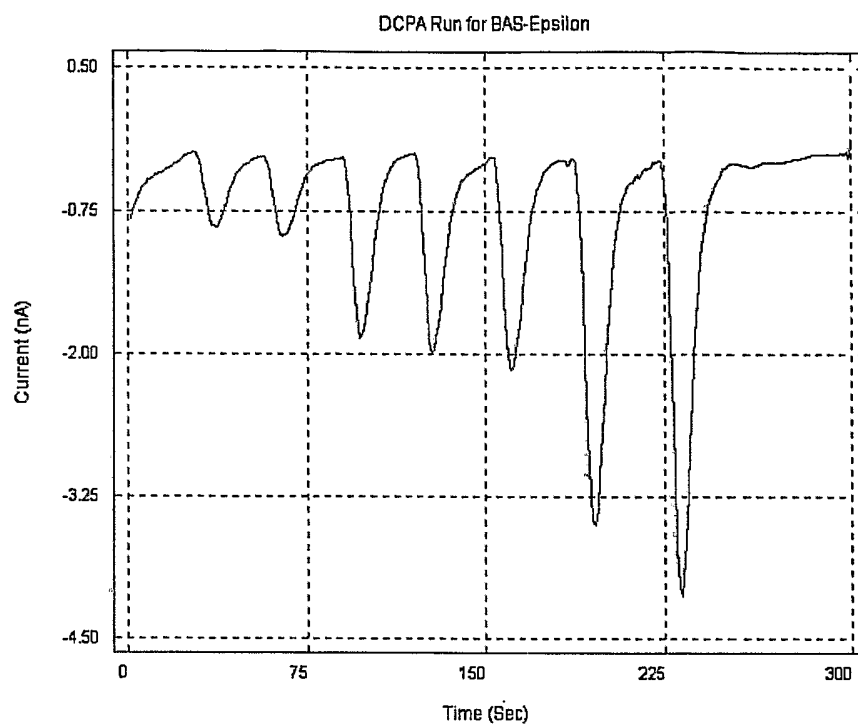
**FIGURE 15**

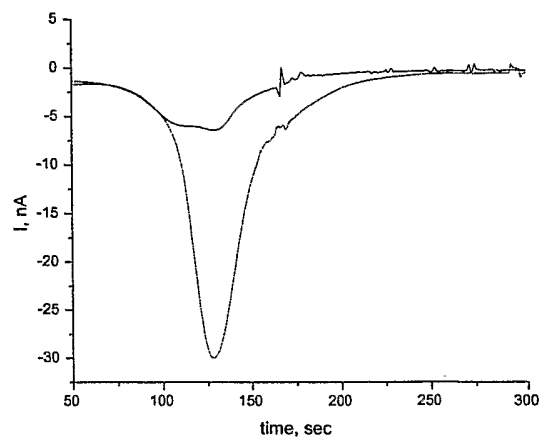
**FIGURE 16**

**FIGURE 17**

**FIGURE 18**

**FIGURE 19**

**FIGURE 20**

**FIGURE 21**