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Automated 3D-Printed Microfluidic Array for Rapid Nanomaterial-Enhanced Detection of Multiple Proteins

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Abstract

We report here the fabrication and validation of a novel 3D-printed, automated immunoarray to detect multiple proteins with ultralow detection limits. This low cost, miniature immunoarray employs electrochemiluminescent (ECL) detection measured with a CCD camera and employs touch-screen control of a micropump to facilitate automated use. The miniaturized array features prefilled reservoirs to deliver sample and reagents to a paper-thin pyrolytic graphite microwell detection chip to complete sandwich immunoassays. The detection chip achieves high sensitivity by using single-wall carbon nanotube–antibody conjugates in the microwells and employing massively labeled antibody-decorated RuBPY–silica nanoparticles to generate ECL. The total cost of an array is \$0.65, and an eight-protein assay can be done in duplicate for \$0.14 per protein with limits of detection (LOD) as low as 78–110 fg mL^{−1} in diluted serum. The electronic control system costs \$210 in components. Utility of the automated immunoarray was demonstrated by detecting an eight-protein prostate cancer biomarker panel in human serum samples in 25 min. The system is well suited to future clinical and point-of-care diagnostic testing and could be used in resource-limited environments.

Abstract

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Notes

The authors declare no competing financial interest.

Reliable early detection is currently the best hope for successful cancer patient outcomes.^{1–3} Measuring protein biomarkers overexpressed into blood due to cancer has great early diagnostic potential^{1,4–8} that is still largely untapped in the clinic.⁹ Enzyme-linked immunosorbent assays (ELISA) have long served as the method of choice for single protein diagnostic tests with limits of detection (LOD) typically in the 3–40 pg/mL range.^{10,11} More recently, bead-based optical and electrochemiluminescent (ECL) methods have been marketed for multiplexed protein assays but do not improve on ELISA LODs.^{12–15} A single-protein counting technology known as Simoa features automation and LODs of 4–200 fg mL^{–1} for multiplexed assays.¹⁶ However, reliable bioanalytical devices that offer automated, low cost, highly sensitive, multiplexed assays for clinical protein detection are still lacking. Immunoarrays have been reported that measure small numbers of proteins with accuracy, reliability, and automation but usually lack one key attribute, usually low cost or high sensitivity.^{17–22}

Thus, low cost, automated diagnostic platforms that can rapidly detect multiple cancer biomarkers with high sensitivity and specificity will be valuable tools for future personalized patient care of cancer and other diseases, as well as for research applications requiring measurement of low abundance proteins.⁹ In the present paper, we describe a new, low cost 3D-printed array for multiple protein detection using automated reagent delivery with a simple user interface for rapid assays.

Desktop 3D-printers can be used to rapidly design, optimize, and fabricate low cost, high performance microfluidic devices.^{22–27} 3D-printing enables rapid prototyping of single-unit devices while avoiding expensive masters or masks necessary for the alternative methods of microfluidic device fabrication such as photo lithography and soft lithography.^{28–30} Device assembly tasks required when using precision cutting, molding, and machining for fabrication are minimized by 3D-printing to produce nearly complete microfluidic devices.

Plans for 3D-printed objects are developed using computer-aided design (CAD) software, and the CAD file is processed to generate print instructions that are uploaded to the printer.^{23,24} Optimization is achieved at a fraction of cost and time of lithography, and the final optimized prototype becomes the usable device. While lithography can presently achieve better resolution than 3D-printing, ongoing advances in 3D print resolution and speed are underway.³¹ However, stereolithographic (SLA) 3D-printers can achieve channel widths of 150 μ m and structural features of 95 μ m³² and are well suited for many bioanalytical microfluidic devices. Recent analytical applications include smartphone-controlled biosensors,^{33–37} electrochemical sensors,^{38,39} optics for SPR,⁴⁰ and other biomedical sensors.⁴¹

Our research team has 3D-printed microfluidic devices that include an electrochemical hydrogen peroxide sensor, electrochemiluminescent DNA sensor,⁴² a genotoxicity chemistry reactor with DNA damage end point,⁴³ and prototype immunoarrays for proteins.^{22,44,45} Earlier, we used polymer molding to develop ultrasensitive microfluidic immunoarrays that detect multiple proteins by combining massively labeled antibody-coated magnetic beads with nanostructured electrochemical sensors.^{46–48} We used precision cutting and machining to develop microfluidic ECL arrays combining antibody-coated silica nanoparticles

containing 0.5 million $\text{Ru}(\text{bpy})_3^{2+}$ (RuBPY) ions with single-walled carbon nanotube (SWCNT) forest sensors.⁴⁹ Both methodologies detect up to four proteins with ultralow detection limits (LOD) of 5–100 fg mL⁻¹ in less than 1 h.

In the current paper, we describe a low cost, miniature 3D-printed immunoarray to detect multiple proteins using ECL with CCD camera measurement. We integrated an automated micropump controller with the unibody 3D-printed device to deliver samples and reagents. This immunoarray measured eight prostate cancer biomarker proteins in human serum in 25 min with LODs as low as 85–110 fg mL⁻¹. We verified accuracy for the eight proteins in serum and successfully demonstrated analysis of human serum samples.

EXPERIMENTAL SECTION

Chemicals and Materials.

Poly(diallyldimethylammonium chloride) (PDDA), poly(acrylic acid) (PAA), bovine serum albumin (BSA), 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimidehydrochloride (EDC), and *N*-hydroxysulfosuccinimide (NHSS) were from Sigma. Human prostate cancer patient serum samples were from George Washington University (GWU) Hospital under IRB ethical approval. Calf serum as a surrogate for human serum was used to dissolve standard proteins for calibrations.

$\text{Ru}(\text{bpy})_3^{2+}$ -silica nanoparticles (RuBPY–SiNP) were prepared by a previously described method,⁵⁰ had a diameter of 110 ± 13 nm from TEM (Figure S1A, SI) and are stable for one month or more at 4 °C. RuBPY–SiNPs of about 100 nm were found to provide a good balance between mobility in a microfluidic system while retaining good detection sensitivities. RuBPY–SiNP was coated successively with PDDA and PAA followed by attachment of detection antibodies (Ab_2) using EDC–NHSS to link amine groups of antibodies to COOH groups of PAA on the RuBPY–SiNP surface.⁵⁰ Optimized concentrations of Ab_2 used for attachment to RuBPY–SiNP were 8 $\mu\text{g mL}^{-1}$ for PSA, PSMA, IGFBP-3, VEGF-D, and PF-4, 5 $\mu\text{g mL}^{-1}$ for IGF-1 and CD-14, and 3 $\mu\text{g mL}^{-1}$ for GORM-1. Once Ab_2 was covalently attached, bicinchoninic acid (BCA) total protein assays⁵¹ were used to determine the number of antibodies per particle, which gave Ab_2 :RuBPY–SiNP ratios of ~32:1 in all cases. An optical absorbance assay measured 400 000 RuBPYs per nanoparticle. $\text{Ru}(\text{bpy})_3^{2+}$ concentration in the silica nanoparticle dispersions was estimated using a calibration curve for dissolved $\text{Ru}(\text{bpy})_3^{2+}$ absorbance at 457 nm. The number of $\text{Ru}(\text{bpy})_3^{2+}$ ions per particle was obtained by dividing the $\text{Ru}(\text{bpy})_3^{2+}$ concentration found by the total number of $\text{Ru}(\text{bpy})_3^{2+}$ silica nanoparticles.^{21,50} The resulting Ab_2 –RuBPY–SiNP particles were incubated in 2 mg mL⁻¹ BSA to minimize nonspecific binding, washed, and stored at 4 °C. In the assay, ECL was generated from RuBPY–SiNP by applying 1.0 V vs Ag/AgCl from an on-board three-electrode potentiostat using 500 mM tripropylamine (TPA) coreactant with 0.05% Tween-20 (T20) and 0.05% Triton-X in 0.2 M phosphate buffer (See Scheme S1, ECL pathway).

3D-Printed Microfluidic Device.

A Form 2 (Formlabs) stereolithographic (SLA) desktop 3D-printer was used to achieve high resolution ($\sim 25 \mu\text{m}$) with low surface roughness.³² CAD files of the arrays were prepared using 123D software (Autodesk), converted to the appropriate format, and then uploaded to the printer to fabricate the desired object. Clear photo curable resin (GPCL02 Formlabs clear resin) was used to produce compact plastic arrays with internal chambers to hold assay reagents, sample, and detection chip. Freshly printed arrays were cleaned by flushing, bathing, and sonicating in isopropanol for 10–15 min to remove uncured resin, followed by air drying. Before use, arrays were coated with acrylic spray (Krylon) to improve optical clarity from slightly opaque to 90% transmittance to visible light.⁴² The reference electrode, Ag/AgCl, and counter electrode platinum wires were then inserted into grooves printed so that they will lie opposite along the microwell row in the detection array in the fully assembled array

The complete printed array has dimensions (*L*) length = 40 mm, (*W*) width = 35mm, and (*H*) height = 3.5 mm (Figure 1A). Internal reagent and sample chambers have dimensions of $25 \times 2 \times 1 \text{ mm}$ (*L* x *W* x *H*) and volumes of $48 \pm 2 \mu\text{L}$. The detection channel has an open bottom that is converted into a closed detection chamber by bonding it with adhesive onto a paper-thin pyrolytic graphite microwell detection chip cut from pyrolytic graphite sheet (PGS, Panasonic).⁵² The detection chamber is $30 \times 2 \times 0.6 \text{ mm}$ (*L* x *W* x *H*) to accommodate the sample and reagents volumes to be delivered from upstream. Chamber sizes were optimized to efficiently contain reagent and sample with low dead volumes. The volume of the detection chamber is slightly less than $50 \mu\text{L}$ and accommodates a 16-microwell detection chip, with the microwells separated in space and preassigned for duplicate measurements of each analyte protein (Figure 1B). Sample and reagents were preloaded into the array using a micropipette prior to the immunoassay and stored at 4°C until use. No reagent degradation was found for 1 or 2 day storage.

To minimize nonspecific binding (NSB) of sample constituents to the immunoarray, we incubated arrays with 2 mg mL^{-1} bovine serum albumin (BSA) for 1 h prior to use. Reagents and samples were added into their chambers via small loading ports present on the top of the array. By placing a pipet tip into these ports, assay reagents were loaded with good reproducibility. Vent holes were strategically placed at the end of each chamber so that solutions fill uniformly and efficiently, while air is expelled. Vent holes and loading ports were closed with single-sided transparent tape to form an airtight continuous channel to sequentially deliver reagents downstream to the detection chamber.

The detection chamber is completed by attaching the nanostructured pyrolytic graphite sheet (PGS) microwell chips via double-sided adhesive under the 3D-printed detection channel. These detection chips were prepared by cutting $70 \mu\text{m}$ thick PGS (Panasonic Industrial Devices and Solutions, P113689 ND) to desired sizes to fit array dimensions. The pyrolytic graphite chip was patterned by printing an inkjet toner microwell template onto glossy paper⁵³ and then heat transferring onto the pyrolytic graphite to make 16 microwells that are about 10–15 nm deep and can hold volumes of $\sim 1 \mu\text{L}$. Densely packed single-walled carbon nanotube (SWCNT) forests were assembled in the microwells using previously described methods^{54,55} to increase surface area and enhance amounts of capture antibodies (Ab_1)

attached. Figure S1B,C shows SEM images of the surface of the PGS sheets with microwells prepared from hydrophobic printer toner. Figure S1C shows the surface of PGS inside a microwell region. Tapping mode atomic force microscopy (AFM) showed increased roughness from the bare PGS surface (35 nm) to the SWCNT forest surface from vertically aligned SWCNTs (47 nm), (Figure S1D,E). The terminal carboxyl groups of SWCNTs were activated using EDC–NHSS to attach capture antibodies (Ab_1) by amidization, with a resulting decrease in the AFM roughness factor to 38 nm. (Figure S1F). Chips with Ab_1 attached are stable for at least 1 week at 4 °C.

The eight proteins in the prostate cancer biomarker panel are listed in Table S1. Capture antibodies for each protein were attached to SWCNT wells at 100 $\mu\text{g mL}^{-1}$ concentration. Antibody-coated arrays were incubated with 2% BSA for 1 h at room temperature to minimize NSB.

The 3D-printed array features nine chambers, one inlet port, and one outlet port leading to the interconnected detection chamber. Chambers holding different solutions are in series with an air-filled chamber to avoid intermixing of reagents. The first reagent chamber next to the pump inlet port is filled with tripropylamine (TrPA) ECL coreactant, and the second and third chambers are filled with wash buffer: 10 mM PBS + 0.05% Tween 20, pH 7.4. These last two chambers are not separated by an air gap, since the two solutions are identical and intermixing is not an issue. The fourth chamber holds air followed by the fifth chamber containing a dispersion of the ECL detection nanoparticles, Ab_2 –RuBPY–SiNP. This sixth chamber is another air gap, followed by a wash buffer chamber. The eighth chamber is air-filled followed by the final chamber containing sample or standard solution directly before the detection chamber (Figure 1A). The inlet port is connected to the programmed micropump, which facilitates sequential delivery of reagents on an optimized time schedule.

Automation and User Interface.

A touch-screen operated controller system employs simple user commands to start the assay. The micropump user interface uses a voltage-controlled oscillator (VCO) latch and a digital-to-analog convertor (DAC) latch. The VCO controls on and off cycles of the micropump and times assay events. The DAC controls amplitude and frequency of the micropump, which controls flow rate. Three DACs are connected to three submicrocontrollers to control three micropumps to enable three simultaneous assays. Another DAC was used to equip a three-electrode potentiostat to apply 1.0 V vs Ag/AgCl to drive the ECL detection reaction (see SI).

Components are integrated with an Arduino microcontroller and the touch LED screen to input user commands. A small 4.5 V rechargeable lithium ion battery powers the control system and drives ECL. Once the controller is turned on using an on/ off toggle switch, the user can start the immunoassay with a preset program or press a program button to enter new settings to control flow rates and timing of immunoassay steps. The start button initiates the immunoassay; the “IDLE” button turns into “RUN”, which is an indication of pump initiation. A digital timer indicating step number is displayed so that the user can monitor assay progress (Figure 2). When the immunoassay is complete, the screen shows “Done” and “Measure OFF” is displayed. At this point, no voltage is applied, but once the user

touches the “Measure OFF” button, the screen turns to a green “Measure ON” sign that applies voltage for the ECL reaction with CCD camera data collection. While the ECL potential is being applied, “Measure ON” is displayed for 180 s to indicate that detection is in progress (Figure 2). In developing assays for the immunoarrays, we optimized conditions to attain the best analytical performance characteristics including high sensitivity, wide dynamic range, ultralow detection limits, and good reproducibility.

Assay Procedure.

The arrays were filled with immunoreagents and sample and were then connected to the automated interface module. Pumps and pumping cycles are initiated by pressing “start”. Step 1 flows sample to detection chamber; the stopped-flow incubation occurs and then washing. Step 2 delivers ECL RuBPY–SiNP dispersion, incubates, washes, and then delivers coreactant TrPA. For detection, 1.0 V vs Ag/AgCl was applied from the onboard potentiostat with the array under a CCD camera in a dark box for ECL generation and measurement. Captured ECL light is analyzed with appropriate software to relate light intensities to concentration of analyte.^{43,44} Micropumps were optimized to deliver reagents reproducibly at designated timed intervals at $130 \pm 5 \mu\text{L min}^{-1}$ flow rates. When more than one assay is desired, synchronization of three micropumps is ensured by adjusting frequency and amplitude via the user interface. Optimized incubation times for sample and label in the multiplexed detection chamber were 10 min. For the duplex protein detection, the incubation time for the sample is 14 min and for the RuBPY label is 12 min, enabling a slightly better LOD. ECL was generated at 1.0 V vs Ag/AgCl for 180 s camera accumulation times using 500 mM tripropylamine in 200 mM PBS + 0.05% Tween 20 + 0.05% Triton-X at pH 7.5. Relative ECL intensities were obtained by analyzing the light intensities of sample concentrations and dividing by the signal for protein-free controls.

RESULTS

Development of an assay for an eight-protein prostate cancer biomarker panel in serum was chosen to evaluate the new 3D-printed immunoarray. About 160 000 prostate cancers are predicted for men in the U.S. in 2017 with 27 000 deaths.⁵⁶ Current diagnosis of prostate cancer using the prostate specific antigen (PSA) biomarker test with a decision threshold of 4 ng mL^{-1} yields a clinical specificity of 63% and sensitivity of 35% for disease detection^{57,58} and can result in overdiagnoses leading to unnecessary surgery. Also, the serum PSA test is unable to identify life-threatening aggressive forms of the disease.⁵⁹ Our long-term goal is to establish a panel of prostate cancer serum biomarkers to facilitate distinguishing aggressive from indolent forms of the disease.^{60–62}

Members of the eight-protein biomarker test panel (Table S1) were chosen from prior literature. A subset of this panel was shown to facilitate surgical decisions for prostate cancer patients. Vascular endothelial growth factor-D (VEGF-D) is implicated in prostate cancer angiogenesis.^{63,64} Insulin-like growth factor IGF-1 and IGF-binding protein IGFBP-3 are implicated in cell survival.⁶⁵ Serum monocyte differentiation antigen CD14 (CD14) is an inflammation marker.⁶⁶ Golgi membrane protein 1 (GOLM-1) is a prostate cancer gene fusion protein.^{67,68} We also included general prostate cancer biomarkers PSA, platelet

factor-4 (PF-4), and prostate specific membrane antigen (PSMA), for which we had previously developed multiplexed immunoassays in serum.⁶⁹

Initially, two-protein assays were used to optimize reproducibility and test compatibility of antibody pairs selected. A single two-antibody RuBPY–SiNP particle was used for detection of each protein pair. Duplex assays coupled PSA and PSMA, VEGF-D and PF-4, IGF-1 and CD-14, and IGFBP3 and GOLM-1 (Table S1) as simultaneously detected pairs. Relative ECL responses from the 3D-printed immunoarray with three trials of controls and 1 pg mL⁻¹ PSA and PSMA in undiluted calf serum gave an average relative standard deviation (RSD) of $\pm 5\%$ for well-to-well ($n = 8$) and $\pm 8\%$ for array-to-array ($n = 3$). We constructed calibration curves for all eight proteins in duplex assays and found reproducible ECL signals with RSDs ranging from ± 7 – 13% . ECL signal intensities were divided by ECL intensity from protein-free controls and expressed as relative ECL intensities, which were plotted against concentration for calibration (Figure S2, see Figure 3 for typical raw data). Dynamic ranges were from 0.1 to 1000 pg mL⁻¹ for PSA, PSMA, VEGF-D, IGF-1, CD-14, and IGFBP-3 and 0.1 to 10 000 pg mL⁻¹ for GOLM-1 and PF-4. Limits of detection of 78–100 fg mL⁻¹ were obtained for all eight analyte proteins in 35 min assays.

Multiplexed Detection.

Once we optimized and characterized performance with duplex assays, we progressed to detecting all eight proteins simultaneously in duplicate. The eight different capture antibodies were immobilized in two individual SWCNT sensor microwells each in the order of IGF-1, PSA, PF-4, CD-14, VEGF-D, GOLM-1, PSMA, and IGFBP-3 (Figure 3). A detection label dispersion was prepared by mixing the duplex Ab₂–RuBPY–SiNP assay labels, label 1 for PSA and PSMA, label 2 for VEGF-D and PF-4, label 3 for CD-14 and IGF-1, and label 4 for GOLM-1 and IGFBP-3. These four nanoparticle types were mixed in equal proportions and delivered to the detection chamber after analyte protein binding to complete the sandwich immunoassay. As the levels of these proteins in human serum samples allowed (Table S1), we sacrificed detection limits slightly by shortening incubation periods to achieve a shorter assay time of 25 min.

In the eight-protein assay, the pump delivers sample or a mixture of standard proteins from the sample chamber to the detection microwells, followed by stopped-flow incubation for 10 min, then washing with PBS at pH 7.4. The pump then delivers RuBPY–SiNP dispersion to the detection microwells, stop-flow incubates for 10 min, then washes with 10 mM PBS and PBS Tween 20 (pH 7.4). TPrA solution is then delivered and flow stopped, and ECL responses are measured by the CCD camera in a dark box while applying 1.0 V vs Ag/AgCl to the detection chip. A representative recolorized ECL image of calibration results (Figure 3) shows increased ECL light with increase in analyte protein concentrations. Figure 4 shows the resulting calibration graphs for all eight proteins, six of which were linear, and the remaining two were slightly curved but still fully acceptable for reliable sample concentration determinations. Acceptable dynamic ranges from 0.5 pg mL⁻¹ to 10 ng mL⁻¹ were obtained for all proteins with LODs from 110 to 500 fg mL⁻¹ for this multiplexed protocol; the difference in ECL signals for each biomarker was represented as their sensitivities (Table S5). Among the eight proteins under study, calibration curves for CD-14

and VEGF-D showed a nonlinear curve fitting; these nonlinear curve fittings are common and acceptable in ligand binding assays.⁷⁰ Accuracy of the measurements was evaluated using spiked serum and by measuring recovery. Known concentrations of all target proteins from 750 fg mL⁻¹ to 2.5 ng mL⁻¹ were spiked into diluted human serum, and percent recovery was determined from the measured concentration. Percent recovery values for individual proteins (Table S2) were in the analytically acceptable range⁷¹ of $100 \pm 14\%$, demonstrating reasonably good accuracy, and the absence of significant antibody cross reactivity.

Single-protein ELISA assays of all eight proteins were not possible due to the lack of sufficient sample volumes. However, we compared PSA concentrations in serum samples using the multiplex immunoarray with ELISA data supplied by George Washington University Hospital and found good correlation between the two methods (Figure S3).

Human Serum Samples.

We used the multiplexed immunoarray to measure the prostate cancer protein panel in 12 human serum samples. Gleason scores (GS) are used to grade prostate cancer tumors,⁷² and 6 or less means that the tumor tissue is well differentiated and likely to grow slowly, suggesting a less aggressive form of cancer. A Gleason score of 7 is intermediate, and 8 or more is categorized as the most aggressive. The patient samples included four cancer-free individuals, four samples with nonaggressive (indolent) forms of prostate cancer with GS 6, and four aggressive forms of prostate cancer with GS 8. We label cancer-free (CF) samples as CF1 to CF4, indolent cancer samples as I1 to I4, and aggressive samples as A1 to A4. Guided by normal serum levels of the biomarkers (Table S1), we diluted serum samples up to 750-fold in PBS prior to analysis to bring ECL responses into the linear or sensitive curvilinear ranges of the calibration curves. Concentrations of all eight biomarkers in the samples are shown in Table S2. Clustered multiple variable (CMV) graphs (Figure 5) show visual comparisons of multiple biomarker concentrations obtained from the multiplexed assays, which clearly segregate into the subgroups of cancer-free vs cancer patient.⁷³ The central boxes, in blue for cancer-free and black for cancer patients, indicate values that lie in lower to upper quartile (25th to 75th percentile). The horizontal line in the each box represents median values, while the vertical lines and the box ends represent the range from minimum to maximum concentration.

These results on a limited data set suggest that VEGF-D, CD-14, PF-4, and PSMA differentiate well between prostate cancer and cancer-free patient samples. Receiver operating characteristic (ROC) plots can provide estimates of accuracy for clinical diagnostic predictions.⁷⁴ Preliminary ROC analyses were done for the 12 sample data set (see SI), and results suggest the possibility that this panel may be useful in distinguishing between prostate cancer and cancer-free patients and perhaps even between aggressive and indolent cancer patients. These results are encouraging but still very preliminary, and hundreds of samples will need to be analyzed before definitive conclusions can be drawn.

DISCUSSION

The results above successfully demonstrate a miniature 3D-printed ECL immunoarray operated automatically by a touch-screen interface for simultaneous multiplexed detection of proteins in aqueous samples. Results for an eight-protein prostate cancer biomarker panel demonstrate that the immunoarray can achieve sensitive multiplexed protein detection with LODs of $\sim 110 \text{ fg mL}^{-1}$ and dynamic ranges of 0.5 pg mL^{-1} to 10 ng mL^{-1} (Figures 3 and 4). Spike and recovery tests confirmed accurate measurement of target proteins in diluted human serum (Table S2). Using sample dilutions to bring protein concentrations into the dynamic ranges, as little as $1 \mu\text{L}$ of serum was used for an eight-protein, 25 min assay. Lower detection limits of $78\text{--}100 \text{ fg mL}^{-1}$ and wider dynamic ranges up to 0.1 pg mL^{-1} to 10 ng mL^{-1} can be obtained by using longer incubations resulting in 35 min assay times, demonstrated in duplex assays for the eight biomarkers. Here, better performance in the lower protein concentration range is most likely due to allowing more time for antibodies on sensors and detection particles to incubate and bind with the analyte proteins.⁷⁵

The immunoarray requires only a few touch-screen commands to start and complete the immunoassay automatically, at minimum “RUN” and “Measure ON” commands (Figure 2). Assay parameters for pump on–off cycles that regulate incubation times can be changed with touch-screen commands if required. The automated immunoarray is robust and can in principle be adapted to sandwich assays for any analyte macromolecule, for which two selective binding agents with two distinct epitopes are available, e.g., antibodies or aptamers. Good reproducibility was achieved with RSD ranging from ± 1 to $\pm 13\%$ for the eight proteins, with most RSDs less than $\pm 8\%$ for well-to-well multiplexed detection and $\pm 8\%$ for array-to-array ($n = 3$).

The cost of fabricating the 3D-printed arrays was about \$0.65 in materials, and the total assay cost for a single multiplexed assay including all the immunoreagents to detect eight proteins in duplicate was $\sim \$1.10$, or $\$0.14$ per protein. Low volume reagent chambers ($50 \mu\text{L}$) minimize the overall cost per assay. Microwells on these detection chips are important to facilitate SWCNT assembly and antibody attachment without cross contamination. The microwells are rapidly fabricated by our computer print–heat transfer method.⁵³ (Figure 1B).

3D-printing enabled fast development and optimization of unique design features like strategically arranged air gap chambers, vent holes for reproducible liquid loading, and interconnected inlet and outlet ports leading to a detection channel with low dead volume that streamlines multitask immunoassay operations. 3D-printing also facilitates reproducible mass production of disposable microfluidic devices. For example, we printed 22 fully functional microfluidic devices at a time on an SLA desktop printer and post-treated them all within 6.5 h ($\sim 30 \text{ min/array}$). Thus, when working with human samples, diagnostic platforms can be disposable and destroyed after the assay to avoid pathogen contamination issues. A new array is readily plugged into the automated control system to analyze the next sample. The arrays can also be reused if desired following an extensive cleaning (see SI).

The automation module features simple electronics with touch-screen user input that is portable and user-friendly and can be configured for several simultaneous assays. Micropumps were programmed with open source Arduino mega and digital commands to achieve complete automation without complex software. Power is supplied by a low cost rechargeable lithium ion battery allowing up to nine assays with a single recharge. All components are commercially available with a total cost of \$210 for a single-micropump system and \$375 for a three-micropump system. An on-board three-electrode potentiostat allows application of precise voltage for ECL generation powered by the same rechargeable battery.

Thin, semiflexible pyrolytic graphite sheets (PGS) facilitated inclusion of a low cost detection chip. PGS is a unique alternative to bulk pyrolytic graphite, as they are paper thin, highly conductive, cheap, and withstand organic solvents. After microwells were printed on small cut pieces of PGS, they are fully compatible with DMF-based assembly of SWCNT forests that provide nanostructured surfaces for enhanced capture antibody coverage, which enhances sensitivity.^{47,54} Detection antibody-decorated RuBPY–SiNP as detection probes provide 0.4 million Ru(bpy)₃²⁺ labels per bound protein analyte to greatly enhance the ECL response.^{20,49}

Accurate detection of the eight low abundance biomarker proteins in the presence of thousands of other proteins in human serum at concentrations up to mg mL⁻¹⁷⁶ support the high specificity and selectivity of the 3D-printed immunoarray. For clinical samples, sample volume is often a big concern but can be as low as 1 μ L in our assays.

Results for the eight proteins detected in spiked serum and prostate cancer samples show proof of concept for the practical clinical utility of the immunoarray. While the number of samples analyzed is much too small to draw definitive conclusions, these early results (Figures 5, S4,S5, and Table S3) also suggest that there may be a significant future role in prostate cancer diagnostics for this biomarker panel.

In summary, results above demonstrate the fabrication and utility of a novel 3D-printed ECL immunoarray to detect multiple proteins simultaneously with ultralow detection limits. The new low cost immunoarray with touch-screen control enables simple automated operation with low sample volumes and fast assays. This system should be well suited to future clinical and point-of-care diagnostic testing as well as in resource-limited environments. At present, efforts are underway to analyze a much larger number of patient samples to validate the biomarker panel used here for advanced prostate cancer diagnostics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Representations of 3D-printed immunoarray: (A) 3D-printed microfluidic array with chambers to hold sample, wash buffers, detection nanoparticles, and coreactant for ECL generation. Array is shown on left without detection chip and on right bonded to pyrolytic graphite sheet (PGS) microwell detection chip with reagent and sample chambers filled with dye solutions for visualization; (B) representative disposable PGS chip with heat transferred microwells printed using hydrophobic toner ink. Inset illustrates sandwich immunoassay on a single-wall carbon nanotube forest (SWCNT) in one microwell.

Figure 2.

Immunoassay controller with touch-screen user interface for immunoarray. A microfluidic array connected to a micropump is shown with dye-filled reagent chambers and graphite detection chip. Inset figures show multiple immunoassay steps along with messages to inform the user.

Figure 3.

Recolorized CCD images for five arrays showing increase in ECL light with increase in concentration for all eight proteins on a single array with an acquisition time of 180 s at 1.0 V Ag/AgCl in the presence of 500 mM TrPA.

Figure 4.

Calibration data for multiplexed detection in undiluted calf serum from ECL responses at 1.0 V vs Ag/AgCl. (A–H) are multiplex assay calibration curves for IGF-1, PSA, PF-4, CD-14, VEGF-D, GOLM-1, PSMA, and IGFBP-3. Standard deviations for $n = 4$.

Figure 5. Statistical summary of patient sample results using clustered multiple variable graphs for each protein. (A,B) Box-and-whisker plots show data point distribution classified as cancer-free and prostate cancer samples for each biomarker. The plots present lower, upper quartile, and median values along with minimum and maximum ranges.