3D-Printed Supercapacitor-Powered Electrochemiluminescent Protein Immunoarrays

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Supporting Information

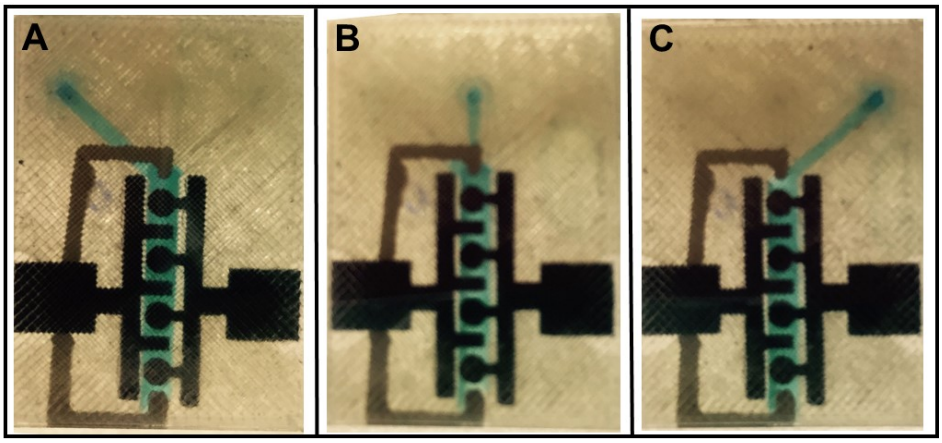


Fig. S1. Bottom view of main array showing reservoir's outlet leading to microfluidic channel. (A) Blue color food dye solution released from reservoir 1 reaching a common microfluidic channel (B) Blue color food dye solution released from reservoir 2 reaching a common microfluidic channel (C) Blue color food dye solution released from reservoir 3 reaching a common microfluidic channel.

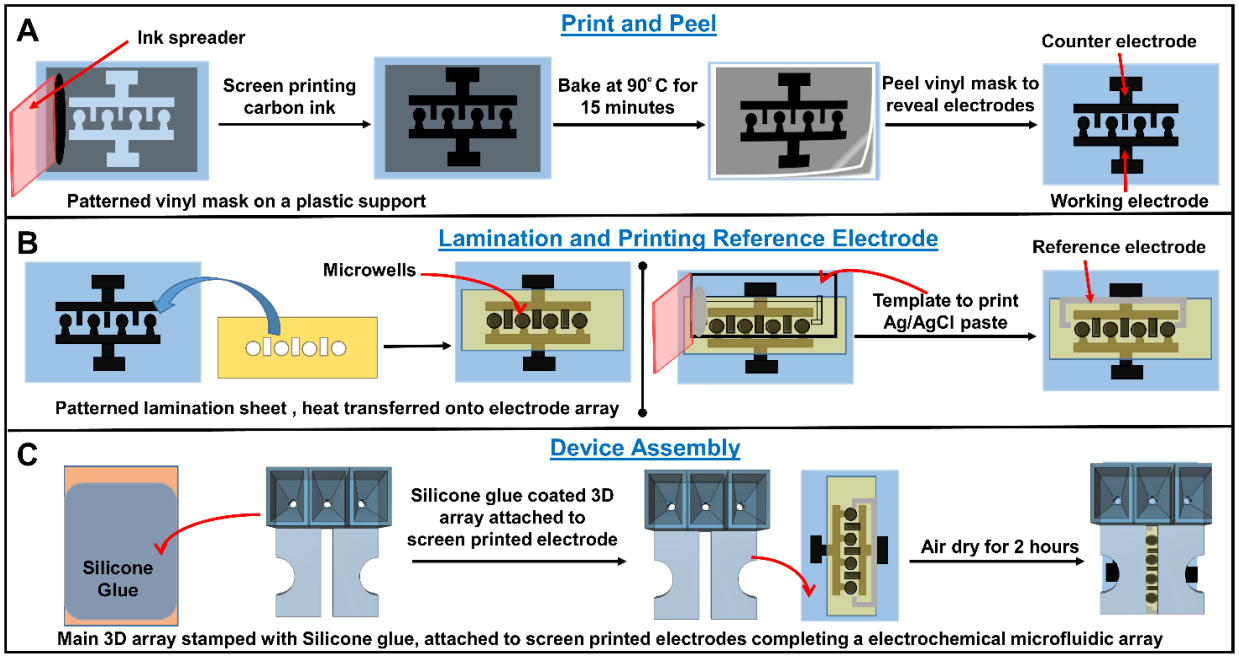


Fig. S2. Schematic representation of steps for screen printing carbon electrodes and assembling the immunosensor chip. (A) Technique to screen print carbon graphitic ink over the vinyl electrode template using an ink spreader followed by heating to form solid screen printed electrodes. (B) Laminating the exposed electrodes to leave 4 microwells followed by screen printing Ag/AgCl reference electrode. (C) Printed electrodes glued to complete immunoarray device.

Synthesis of $[Ru(bpy)_3]^{2+}$ -doped silica (RuBPY-Silica) nanoparticles

$[Ru(bpy)_3]^{2+}$ -doped silica (RuBPY-Silica) nanoparticles were synthesized by water in oil (W/O) microemulsion.[1] 0.04 M $[Ru(bpy)_3]^{2+}$ stock solution was prepared by dissolving $[Ru(bpy)_3]^{2+}$ in pure water. 340 µL of stock was mixed with 1.8 g of Triton X 100, 1.8 mL of n-hexanol and 7.5 mL of cyclohexane and stirred for 30 minutes. 60 µL of fresh ammonium hydroxide (28-30 % weight) and 100 µL of tetraethylorthosilicate (TEOS) were added to the above mixture and stirred for 24 hours in dark. Acetone was added to the reaction mixture to precipitate out RuBPY doped SiNPs followed by centrifugation and washing with ethanol and water for three times. This precipitate was vacuum dried at room temperature overnight. The final product was weighed and stored in dark at 4ºC. These particles were further characterized using transmission electron microscopy (TEM).

Characterization of RuBPY silica nanoparticles by TEM

2.0 mg of RuBPY particles were dispersed in 1 mL of pure water and further diluted five times to prepare the sample for TEM. A drop of this dispersion was added on to carbon coated copper TEM grid and dried under vacuum. The average diameter of these particles was 117 ± 10 nm. These RuBPY particles were modified to prepare ECL label. (Figure S3A & B)

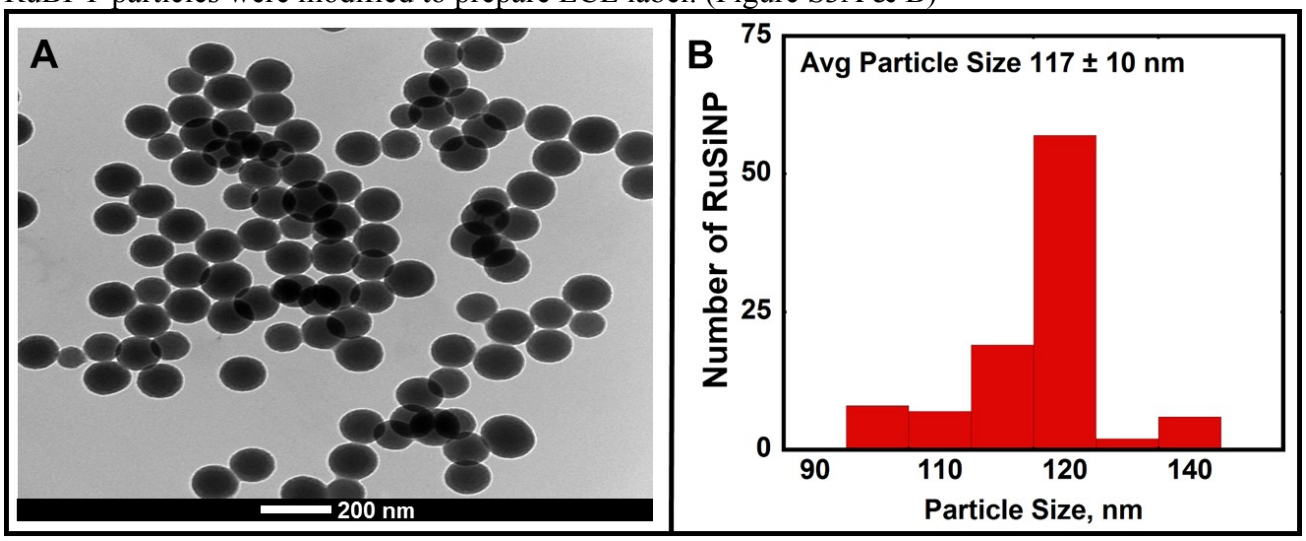


Fig. S3. (A) TEM image of RuBPY-silica nanoparticles on a 200 nm scale bar. (B) Size distribution of RuBPY silica nanoparticles with an average diameter of 117 ± 10 nm.

RuBPY-Silica nanoparticle preparation

2 mg mL^{-1} RuBPY silica particles were first sonicated for 1 min to remove any loosely bound $[Ru\text{-}(bpy)_3]^{2+}$ from the particles and separated using centrifugation at 10,000 rpm for 10 min. Layer of polydiallyldimethylammonium chloride (PDDA) was formed by adding 0.33 mg mL^{-1} of aqueous PDDA solution. The mixture was allowed to stand for 15 min to form a thin layer. This was further centrifuged at 9,000 rpm to remove excess and subsequently washed three times with water. Later these particles were suspended in 0.33 mg mL^{-1} of polyacrylic acid (PAA) for 15 min to form a carboxylic acid groups on the surface of the nanoparticle. Excess PAA was removed by centrifugation and washing three times with water. Carboxylic acid groups present on the surface were used to bind the desired protein using (400 mM-100 mM) EDC-NHSS amidization. After leaving the nanoparticles with EDC-NHSS mixture for 10 min the excess was removed and washed with water as explained earlier at 9,000 rpm. To the modified particles, Ab_2 was added as optimized. The prepared particles with Ab_2 on modified RuBPY silica nanoparticles are incubated overnight on a rotor. The resultant mixture with excess proteins was removed by centrifugation at 8,000rpm in a refrigerated centrifuge and washed 3X with PBS buffer at 7.4 pH. The resultant bioconjugate is finally dispersed in 1 mL of 2 % BSA in 0.05 % Tween-20/PBS buffer (pH 7.4) to block any

nonspecific binding during the immunoassay also helping in forming a uniform dispersion. Multiplexed label was prepared by mixing equal volumes of PSA Ab_2 (8 μg mL^{-1}) prepared particles, PSMA Ab_2 (7.5 μg mL^{-1}) and PF-4 Ab_2 (7.5 μg mL^{-1}) prepared particles.

Estimating the number of RuBPY in Si particles

Relation between viscosity of a dilute solution of spherical nanoparticles and the volume fraction of suspended nanoparticles (Φ) is as shown in equation 1, where h is the viscosity of the nanoparticle suspension and h_0 is the viscosity of the solution without nanoparticles. Viscosity of the nanoparticle suspension was estimated to be 1.0078 and the viscosity of pure solvent is 1.00. Substituting the values in equation 1, we get the value of volume fraction (Φ), to be 0.00031.

$$h/h_0 = 1 + 2.5\Phi \qquad (1)$$

The number of nanoparticles (N) per volume was obtained by using equation 2, where r is the radius of the particles in cm. The diameter of the nanoparticles was estimated using transmission electron microscopy to be 117 ± 10 nm. Substituting these values in equation 2 we calculated number of particles to be 3.4 X 10^{11} per mL of solution. $N = \Phi /(4/3\pi r^3)$ (2)

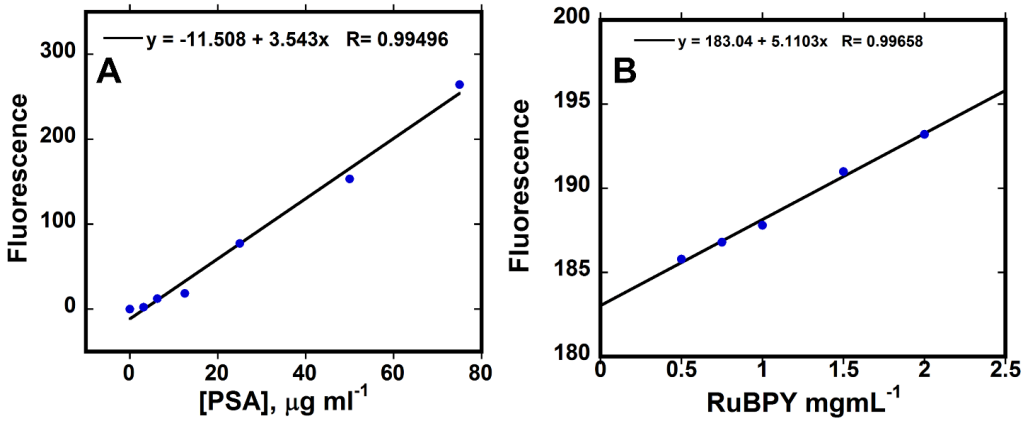


Fig. S4. Influence of concentration of secondary antibody and [$[Ru\text{-}(bpy)_3]^{2+}$] on fluorescence intensity (a) at 280 nm wavelength with [secondary antibody] range: 3.125 - 75 μg mL^{-1}. (b) at 457 nm wavelength with [$[Ru\text{-}(bpy)_3]^{2+}$] range: 0.5 - 2 mg mL^{-1}

Ratio of Ab_2/RuBPY Silica particles in ECL bio-conjugate label

Fluorescence emission spectroscopy was used to calculate the number of antibodies attached to the RuBPY Silica particles. Antibodies contain tryptophan, which excites specifically at 280 nm. Calibration curve was developed for known concentration of Ab_2 as shown in Figure S4A. The unknown concentration of Ab_2 on the bio-conjugate label (PSA-Ab_2, PSMA-Ab_2, PF4-Ab_2 in 2 % BSA, PBS Tween-20; at pH7.2) and a control (2 % BSA, PBS Tween-20; at pH 7.2) was estimated from the linear calibration curves. The concentration of Ab_2 from calibration curve was calculated to be 3.5 μgmL^{-1}. The number of Ab_2 in the ECL bio-conjugate label was calculated to be 1.31 X 10^{13}. Therefore the ratio of Ab_2/RuBPY Silica nanoparticles was estimated to be 38:1.

[$[Ru\text{-}(bpy)_3]^{2+}$] per RuBPY-Si nanoparticle. A calibration curve for different RuBPY was prepared using fluorescence spectroscopy by exciting at 457 nm. The unknown concentration of RuBPY in the bio-conjugate label was determined by extrapolating from the linear calibration curve (Figure S4B). The number of moles were obtained using the molecular weight of Tris(2,2'-bipyridine)dichlororuthenium(II)hexahydrate, 748.62. The number of $[Ru\text{-}(bpy)_3]^{2+}$ ions were estimated using Avogadro's number. Relative fluorescence intensity 192 was obtained for ECL- bio-conjugate label when excited at 457 nm. The amount of $[Ru\text{-}(bpy)_3]^{2+}$ was estimated to be 1.9 mg mL^{-1} and the number of $[Ru\text{-}(bpy)_3]^{2+}$ ions were calculated to be 1.55 x10^{18}. The number of [Ru-

$(bpy)_3]^{2+}$] ions per particle were estimated by dividing with the total number of RuBPY silica nanoparticles in 1 mL of bio-conjugate label dispersion, which is 4.6 X 10^5.

Ab_2 Optimization

Three different batches of RuBPY silica nanoparticles were three different concentrations of Ab_2. Here we used PSA Ab_2 with 4 µg mL^{-1}, 8 µg mL^{-1}, 12 µg mL^{-1} and we performed immunoassay with 100 µg mL^{-1} Ab_1 on the surface. Three concentrations of PSA, 0 pg mL^{-1}, 10 pg mL^{-1} and 10 ng mL^{-1} in undiluted calf serum was used on different arrays at 4 µg mL^{-1} of Ab_2. Similar experiments were repeated with rest of the concentrations of the two Ab_2 concentrations. Suitable concentration for immunoassay was determines where we observe highest difference between control and highest concentration Figure S5A. Similar experiments were done for PSMA Figure S5B and Figure PF-4 S5C also.

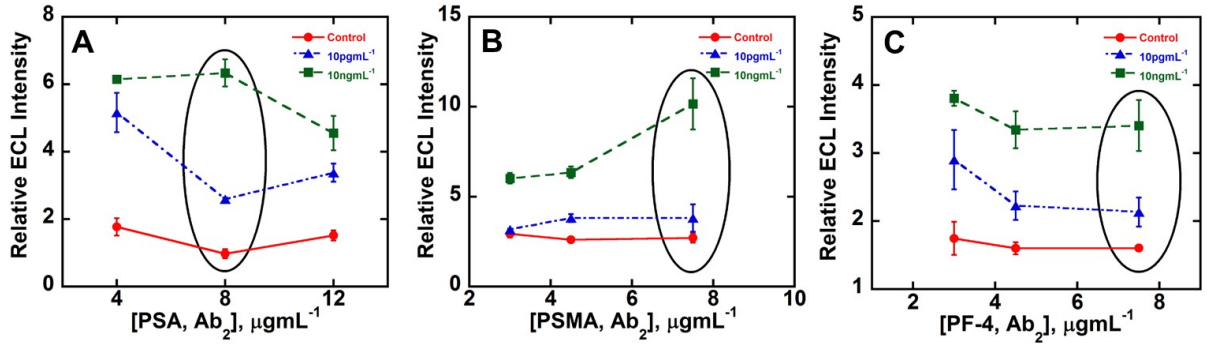
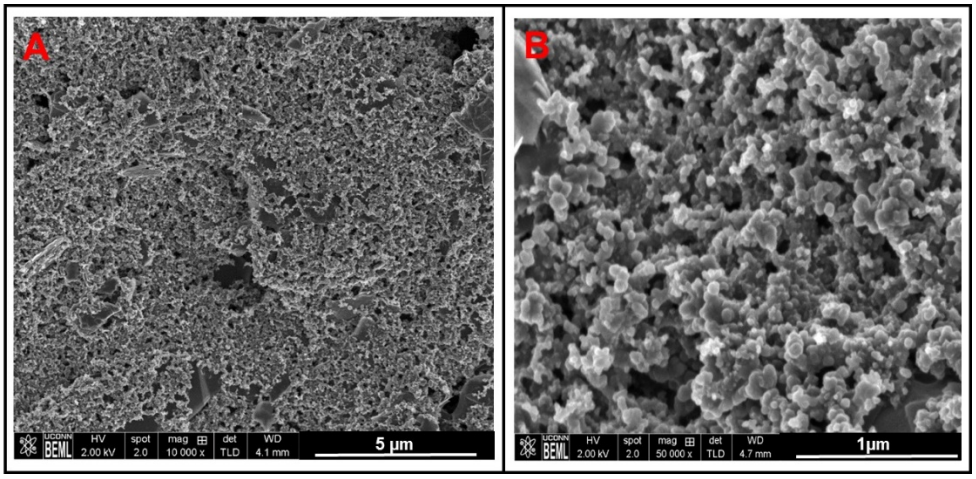


Fig. S5. Optimization of detection antibody (Ab_2) concentrations used to derivatize RuBPY silica nanoparticles. Concentrations in the circle were found to give largest difference between control and protein signal: (A) PSA, optimized Ab_2 8 µg mL^{-1} (B) PSMA, optimized Ab_2 is 7.5 µg mL^{-1} (C) PF-4, Optimized Ab_2 7.5 µg mL^{-1}.

Screen printed carbon electrode characterization using Scanning electron microscopy (SEM)

SEM of the screen printed electrodes modified with polydiallyldimethylammonium chloride (PDDA) and poly acrylic acid (PAA) revealed a highly rough surface that can suitable for



immunoassay Figure S6.

Fig. S6. Scanning electron microscopy (A-B) PDDA/PAA modified SCE at (A) 5 µM scale revealed rough surface (B) 1 µM scale image for SCE.

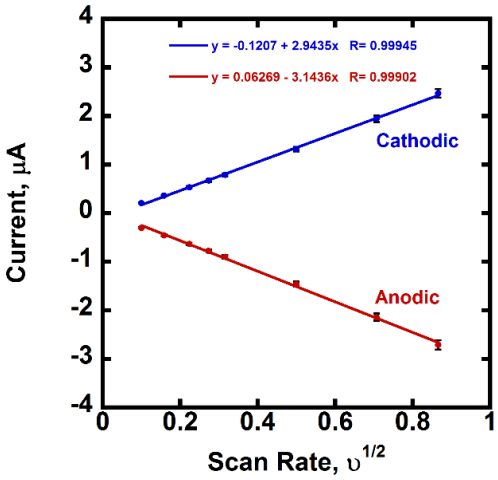


Fig. S7. Cyclic voltammetry characterization of screen printed carbon electrode showing trumpet plot of peak current vs. square root of scan rate.

Ab_1 modified Carbon Sensor

Sensors in the array were coated by layer-by-layer (LbL) deposition (Lvov et al., 2000) of PDDA (2 mg mL^{-1} in water) and PAA (2 mg mL^{-1} in water) for 20 min, giving estimated layer thickness of 1-2 nm (Lvov et al., 1998). Scanning electron microscopy (SEM) showed very rough surfaces for the screen-printed electrodes. (Fig. S6, SI). Carboxylic groups on the outer PAA layer were activated by freshly prepared 400 mM EDC + 100 mM NHSS to immobilize capture antibody (Ab_1) by amidization (Malhotra et al., 2010) using an optimized Ab_1 concentration of 100 µg mL^{-1}.

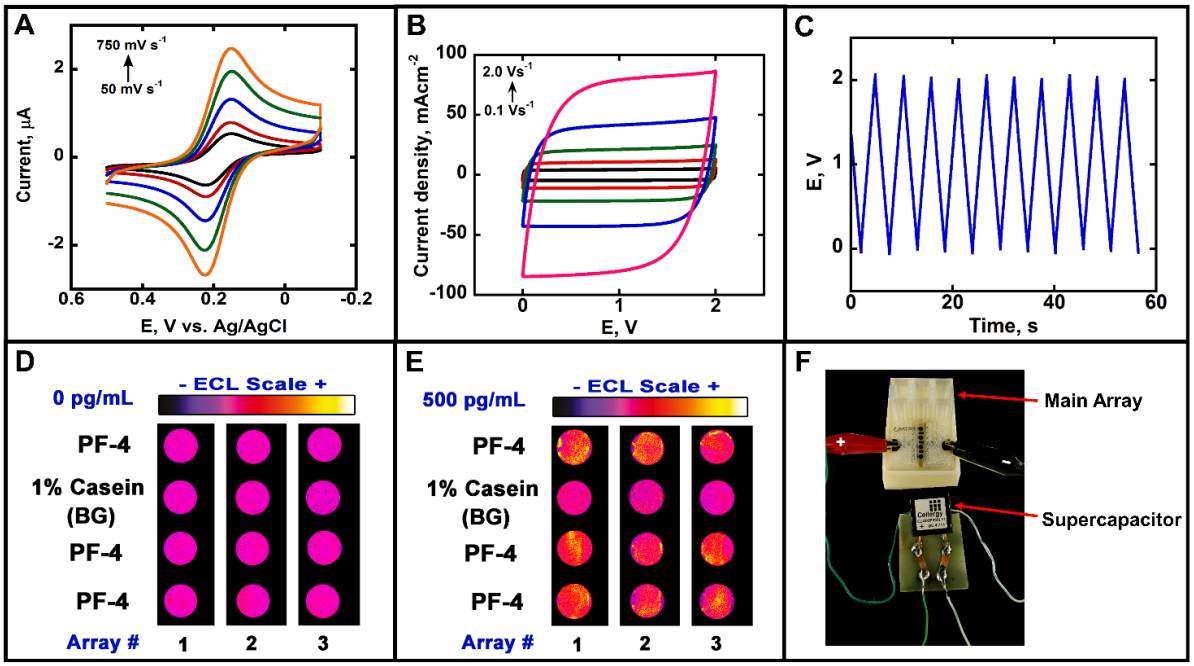


Fig. S8. Electroanalytical characterization of sensors and supercapacitor: (A) Cyclic voltammograms of screen printed carbon electrodes with 0.06 mM FcMeOH in 1 M NaCl, showing oxidation-reduction reversible peak pair separated by ~60 mV at low scan rates. (B) CV's for supercapacitors up to 2 V s^{-1} showing nearly ideal electrical double layer capacitance behavior. (C) Galvanostatic charge-discharge cycles at current density 30 mA cm^{-2}. (D) Recolorized ECL images demonstrating

reproducibility between 9 spots across 3 arrays at 0 pg mL^{-1} PF-4; (E) Recolorized ECL image demonstrating reproducibility between 9 spots across 3 arrays at 500 pg mL^{-1} PF-4. (F) Mounted supercapacitor connected to ECL array inside a dark box.

Single Biomarker detection

Calibration curves were done for the three individual biomarkers in undiluted calf serum (Figure S9). Carbon electrodes were coated with one of the three capture antibodies. Calibration was developed by flowing different concentrations of protein using 3D-printed arrays. Finally ECL images were captured over 60 s in dark box at 1.5 V versus Ag/AgCl in the presence of 0.05 % Tween-20+0.05 % Triton-X 100 and 350 mM TPrA in 0.2 M phosphate buffer, pH 7.4.

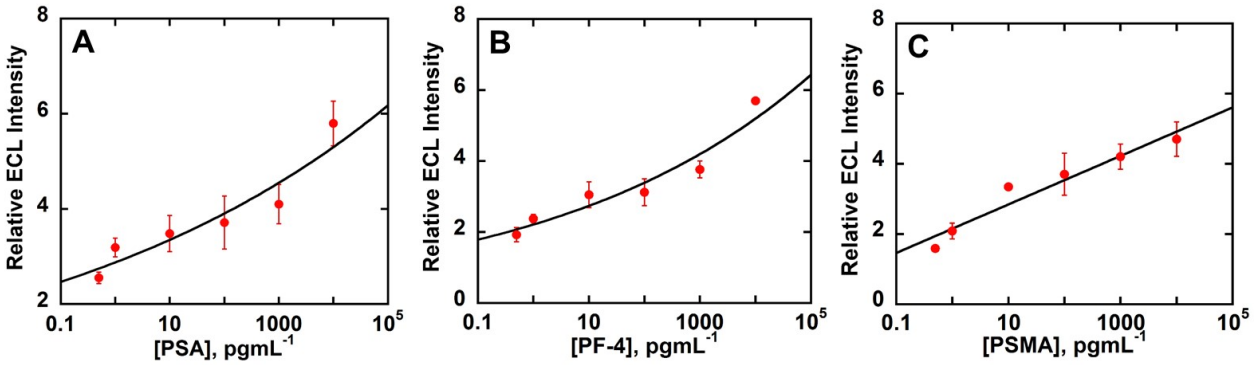


Fig. S9. Calibration curves for single protein detection (A-C) demonstrating good reproducibility: A) PSA from 0.5 pg mL^{-1} to 10,000 pg mL^{-1} B) PSMA from 0.5 pg mL^{-1} to 10,000 pg mL^{-1} C) PF-4 from 0.5 pg mL^{-1} to 10,000 pg mL^{-1}.Error bars show standard deviation, n=3.

Patient sample data

Table S1 Results from correlation plots of ECL array assays vs. ELISA for patient samples

Protein	Slope	Intercept	Correlation Coefficient
PSA	1.46 ± 0.16	-1.3627 ± 0.681	0.9767
PSMA	0.77 ± 0.05	0.0081 ± 0.035	0.9900
PF-4	0.99 ± 0.08	0.0015 ± 0.041	0.9883

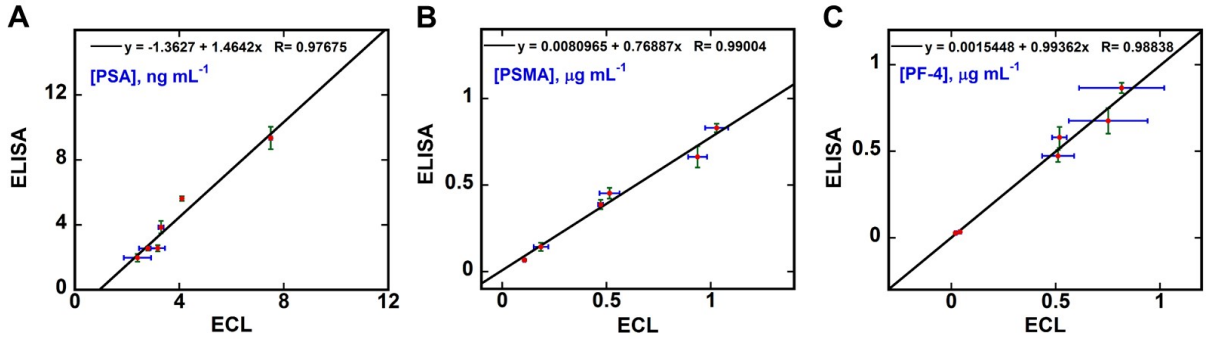


Fig. S10. Linear correlation plots of ECL vs. ELISA validation studies for (A) PSA, (B) PSMA and (C) PF-4.

ELISA kits for PSA (RAB0331) and PF-4 (RAB0402) were from Sigma Aldrich. The PSMA kit (EL008782HU-96) was from Lifeome Biolabs/Cusabio.

Table S2. Comparison between single protein ELISA vs. 3D printed ECL array in this work

Method	ELISA	3D Printed ECL Array
Number of Proteins & Instrumentation	Single Protein Requires Plate reader and Plate washer	Three Proteins Requires CCD camera in a Dark box.
Cost	409$ for PSA,PF-4 each & 567$ for PSMA Per Plate	~€1.20 Per Array + 10$ Supercapacitor + 12$ Solar panel
Assay Time	4 hours 45 minutes	35 minutes
Dynamic range	10 pg mL^{-1} to 2500pg mL^{-1} PSA 20pg mL^{-1} to 15,000 pg mL^{-1} PF-4 39pg mL^{-1} to 2500pg mL^{-1} PSMA	500fg mL^{-1} to 10ng mL^{-1} for PSA, PF-4 & PSMA.
Detection limits	~8 pg mL^{-1} for PSA, ~19 pg mL^{-1} for PF-4 & ~12 pg mL^{-1} for PSMA	300 fg mL^{-1} for PSA, 420 fg mL^{-1} for PF-4 & 535 fg mL^{-1} for PSMA.

Auto CAD files with templates for screen printing of electrodes and lamination along with 123 design files for 3D printing ECL immunoarrays are available at :
http://web2.uconn.edu/rusling/3D_printing.html

Reference

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