**Electrochemiluminescence (ECL) Amplification Strategies for Sensitive Detection of Cancer Protein Biomarkers using [Ru-(bpy)$_3$]$_2^+$ Doped Silica Nanoparticles**

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**Supplementary Information (ESI)**

**Synthesis of [Ru-(bpy)$_3$]$_2^+$-doped silica nanoparticles (RuBPY-silica):**

Uniform ([Ru-(bpy)$_3$]$_2^+$)-doped silica (RuBPY-silica) nanoparticltes were synthesized by using a water-in-oil (W/O) microemulsion method with protocol reported by Tan and co-workers. The stock solution was prepared by dissolving [Ru-(bpy)$_3$]$_2^+$ solid powder in pure water to make the 0.04 M ([Ru-(bpy)$_3$]$_2^+$) solution. 1.80 g Triton-X 100, 1.80 mL n-hexanol and 7.50 mL cyclohexane were mixed well and 340 µL of 0.04 M [Ru-(bpy)$_3$]$_2^+$ solution was added into the mixture. After 30 minutes stirring, 60 µL fresh ammonium hydroxide (28-30% wt) and 100 µL TEOS (tetraethylorthosilicate) were added to this mixture. The final solution was stirred vigorously for 24 hours in the dark and later extracted with acetone to obtain precipitation. The precipitates were washed with ethanol three times and water one time, and dried in low-temperature oven for one hour and then under room temperature for one day. The final products RuBPY-silica were weighed (15-25 mg yields) and collected in a small vial for characterization and further modification.

**Fabrication of SWCNT Immunosensors.**

SWCNT forests were assembled and characterized by AFM and Raman spectroscopy as reported previously. Briefly, SWCNTs were first carboxyl-functionalized and shortened by sonicitation in 3:1 HNO$_3$/H$_2$SO$_4$ for 4 h at 70 °C. These dispersions were filtered, washed with water, dried, and dispersed in DMF. Abraded, ordinary basal plane pyrolytic graphite (PG) disks ($A = 0.16$ cm$^2$) were prepared for forest assembly by forming a thin layer of Nafion and Fe(OH)$_3$ on their surfaces. After immersion of these substrates into 1-15 week aged DMF dispersions of shortened SWCNTs, vertical assemblies of SWCNTs were formed (forests), which were then dried in a vacuum for 18 h. Immunoassays were performed same protocol reported previously, but using the RuBPY-silica-antibody label instead of enzyme labels. The carboxylic groups of nanotube ends were activated by placing 30 µL of freshly prepared 400 mM 1-(3-(dimethylamino)-propyl)-3-ethylcarbodiimide hydrochloride (EDC) and 100 mM N-hydroxysulfosuccinimide (NHSS) in water onto SWCNT electrodes, and washed off after 10 mins. This was followed by 3 h incubation with 20 µL of 2 nmol mL$^{-1}$ (0.04 mg mL$^{-1}$) capture antibody (Ab$_1$) in 0.01 M PBS buffer (pH 7) containing 0.05% Tween-20 at 37°C. The electrode was then washed with 0.05% Tween-20/ PBS buffer (pH 7) and PBS buffer (pH 7) for 3 minutes each. Washing steps used here and below were adapted from the procedure previously recommended to block nonspecific binding (NSB) by treating surfaces with attached antibody with protein and detergent at every step of the assembly and assay. The capture anti-PSA/SWCNT electrode sensor constructed as described above was incubated for 1.15 h after treating with 20 µL of 2% BSA. This step is followed by washing with 0.05% Tween-20/PBS buffer (pH 7) and PBS buffer (pH 7) for 3 minutes each. The next step was to incubate the above assembly with treatment with antigen (PSA) in undiluted calf serum. Each electrode was treated with different concentrations of antigen (0.04 ng mL$^{-1}$ to 5ng mL$^{-1}$). This step is followed by washing with 0.05% Tween-20/PBS buffer (pH 7) and PBS buffer (pH 7) for 3 minutes each. The next step was to incubate the inverted sensor with a 10 µL drop of (the concentration ranged from 0.1 pmol mL$^{-1}$ to 4 pmol mL$^{-1}$) RuBPY-silica-anti-PSA (Ab$_2$) in buffer containing 2% BSA and 0.05% Tween-20/PBS buffer (pH 7) for 1.15 h, followed by washing in 0.05% Tween-20/PBS buffer (pH 7) and PBS buffer (pH 7) for 3 minutes each. The immunosensor was then placed in an electrochemical cell containing 10 mL of 100 mM Tripropylamine + 0.05% Triton-X 100 + 0.05% Tween-20 in 0.2 M Phosphate buffer (pH 7.5) for ECL and electrochemical measurements were performed. Controls were SWCNT-anti-PSA immunosensor omitting addition of PSA.

**Preparing a ECL based bioconjugate:**

RuBPY-silica nanoparticles (97± 8 nm diameter, avg. pore size 4 nm) were used to making ECL labels. RuBPY-doped silica nanoparticles (2 mg mL$^{-1}$) were added to 2 mg mL$^{-1}$ aqueous polydiallyldimethylammonium chloride (PDDA) to make a total volume of 1.0 mL for 20 min, then centrifuged for 4 min. at 8000 rpm to remove nanoparticles from the PDDA solution followed by 3 washings with distilled water, and 2 min. centrifugation at 8000 rpm. The thin layer of PDDA (~0.5 mg) was used to avoid blocking pores of the nanoparticles. The microbeads were then resuspended in 1.0 mL of 1.0 mg mL$^{-1}$ poly(acrylic acid) (PAA) solution for 20 min, followed by the same centrifugation and washing steps. The PAA layer was added to place carboxylic acid groups on the outer side of the nanoparticles for linking to Ab$_2$ via EDC-NHSS amidization. This dispersion was then mixed with 1 mL of freshly prepared 400 mM EDC and 100 mM NHSS in PBS buffer at room temperature for 10 min. The resulting mixture was centrifuged at 15,000 rpm for 5 min, and the supernatant discarded. The buffer wash was repeated to remove excessive EDC and NHSS. Secondary anti-PSA antibody (Ab$_2$) at 8 µg/mL and PAA/PDDA/RuBPY-silica nanoparticles at 2 mg/mL were added to the mixture and shaken at 200 rpm for 8 hr at room temperature. The reaction mixture was then centrifuged at 15000 rpm at 4 °C for 10 min, and supernatant was removed. 1 mL PBS buffer (pH 7.0) was added to the
solid conjugate remaining in the vial, mixed well, and centrifuged again at 15000 rpm at 4 °C for 10 min, and the supernatant was discarded. This step was repeated 3–4 times, which removes free Ab2 leaving the RuBPY-silica-Ab2 nanoparticles at the bottom. This step was repeated 4 more times. 1 mL of 0.05% Tween-20/PBS buffer (pH 7.0) was then added to the bioconjugate precipitate collected to form a homogeneous dispersion, and stored in refrigerator at 4 °C.

Characterization of RuBPY-silica-Ab2 bioconjugate

TEM 20 mg RuBPY-silica was dispersed in pure water with a final concentration of 20 mg ml⁻¹. One drop of this dispersion was placed on a silica wafer and dried under air for a day to prepare for TEM characterization. TEM was performed before the RuBPY-silica nanoparticles were coated with polyelectrolytes (PDDA and PAA). The size distribution was estimated by analyzing 102 nanoparticles. The observation showed that the average size of the particles was 97 ± 8 nm (Fig. S1).

Characterization of RuBPY-silica-Ab2 nanoparticles by Atomic force microscopy (AFM):
RuBPY-silica nanoparticles modified with polyelectrolyte were characterized with AFM. The observation showed that the average size of the particles was 97 ± 8 nm (n = 42) (Fig. S2), suggesting that the original nanoparticles are not increased much in size by addition of the polyelectrolyte layers. This is consistent with measurements of layers of PDDA and PAA on solid surfaces, which suggest a polyelectrolyte film of ~1 nm.

Ab2/RuBPY-silica particle ratio

The molar concentrations of secondary anti-PSA antibody (Ab2) and [([Ru-(bpy)₃]²⁺)] in the ECL based bioconjugate was deduced by a fluorescence experiment. The calibration curve for known concentrations of Ab2 and [([Ru-(bpy)₃]²⁺)] were plotted against fluorescence (Fig S3). The native fluorescence spectra for concentrations of Ab2 and [([Ru-(bpy)₃]²⁺)] were 1.0906 and 4.9 respectively (Fig S4). The fluorescence spectrum for control at 280 nm wavelength was 0.0378, which includes 2% BSA, PBS-Tween-20 (pH 7.0) without addition of secondary anti-PSA antibody (Fig S4a). However no fluorescence spectrum was obtained from control at 457 nm wavelength (not shown). The fluorescence values for Ab2 and [([Ru-(bpy)₃]²⁺)] after subtracting the values from control were 0.071 and 4.9 respectively which corresponds to 28 nM (0.0045 mg ml⁻¹, M.W. of Ab2: 158 kDa) and 1.6 mg ml⁻¹ (2.14 nM, M.W. of Tris(2,2′-bipyridine)dichlororuthenium(II) hexahydrate: 748.62) respectively (Fig S3, S4).
Fig. S3 Influence of concentration of secondary anti-PSA antibody and [Ru-(bpy)_3]^{2+} on fluorescence intensity a) at 280nm wavelength with [secondary anti-PSA antibody] range: 6.25 – 50 nM. b) at 457 nm wavelength with RuBPY-silica nanoparticles range: 0.1 – 2mg mL^{-1}.

Fig. S4 Fluorescence emission spectra obtained at a) 280nm wavelength showing, A) Control, which includes 2% BSA, PBS-Tween-20 (pH 7.0) without addition of secondary anti-PSA antibody, B) [Secondary anti-PSA antibody] in ECL based bioconjugate. b) 457 nm wavelength showing [Ru-(bpy)_3]^{2+} in ECL based bioconjugate. Control, which includes 2% BSA, PBS-Tween-20 (pH 7.0) without addition of [Ru-(bpy)_3]^{2+} did not give fluorescence emission spectrum.

Fig. S5 UV Visible spectroscopy showing absorbance (A) obtained for 10 mg mL^{-1} of RuBPY-silica nanoparticles at λ= 465 nm.

UV Visible spectroscopy was performed to find the exact amount of [Ru-(bpy)_3]^{2+} in the particles (Fig S5). The absorbance (A) obtained was 0.39 (Fig S5). The concentration (c) of [Ru-(bpy)_3]^{2+} in 10 mg mL^{-1} of RuBPY-silica nanoparticles was calculated from eq 1 (Beer-Lamberts law) to get 2.67 × 10^{-5} moles L^{-1} (ε = 14600L mol^{-1} L^{-1}).

\[ A = ε b c \]  

Considering 100 times dilution and M.W. of Tris(2,2'-bipyridine)dichlororuthenium(II) hexahydrate (748.62) we were able to calculate the concentration of [Ru-(bpy)_3]^{2+} in 10 mg mL^{-1} of RuBPY-silica nanoparticles to be 2.0 mg mL^{-1}. 

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Therefore concentration of \([\text{Ru}-(\text{bpy})_3]^{2+}\) in 1.6 mg ml\(^{-1}\) of RuBPY-silica nanoparticles in the ECL based bioconjugate should be 0.32 mg ml\(^{-1}\).

Dispersion viscosity was used to determine the number of RuBPY-silica nanoparticles per volume. The theoretical relationship between the viscosity of a dilute solution of spherical nanoparticles and the volume fraction of suspended nanoparticles \((\Phi)\) was fit into the following equation: \(^2\) The viscosity of the RuBPY-silica nanoparticle dispersion \((h)\) was obtained. The viscosity \((h_0)\) of the solvent (water) without nanoparticles was 1.00 (so \(h/h_0\) is the relative viscosity), and \(\Phi\) is the volume fraction of nanoparticles, which was calculated as 0.00031 (eq 2).

\[
\frac{h}{h_0} = 1 + 2.5 \Phi \quad (2)
\]

The number of RuBPY-silica nanoparticles (diameter \((d)\): 97 nm) per volume was obtained, which is \(6.5 \times 10^{11}\) mg\(^{-1}\) (eq 2). Therefore number of RuBPY-silica particles per volume for 1.6mg of \([\text{Ru}-(\text{bpy})_3]^{2+}\) (present in ECL based bioconjugate) are 1.04 \(\times 10^{12}\) (eq 3, Fig S3b and S4b).

\[
N = \Phi \left[\frac{4}{3}\pi \left(\frac{d}{2}\right)^3\right] \quad (3)
\]

The number of molecules of Ab\(_2\) in ECL based bioconjugates is \(1.7 \times 10^{13}\) (multiplying number of moles of Ab\(_2\) (28 pmoles) by Avogadro number (Fig S3a, S4a). Therefore, the ratio between Ab\(_2\) to silica nanoparticles was confirmed about 16:1.

\([\text{Ru}-(\text{bpy})_3]^{2+}\) / RuBPY-silica particle ratio

The number of \([\text{Ru}-(\text{bpy})_3]^{2+}\) ions present in 1.04 \(\times 10^{12}\) RuBPY-silica particles is \(2.57 \times 10^{17}\) (multiplying number of moles of \([\text{Ru}-(\text{bpy})_3]^{2+}\) (427 nmoles) by Avogadro number). Therefore we can predict that each RuBPY-silica particle contains \(2.47 \times 10^2\) \([\text{Ru}-(\text{bpy})_3]^{2+}\) molecules.

Optimization of [Ab\(_1\)]

Immunoassays were done by using the SWCNT forest structures on PG disks and optimizing the capture antibody concentration [Ab\(_1\)] used for attachment by using 100 mM TPrA as a coreactant and measuring ECL. The results showed that the ECL signal between the control \([\text{PSA} = 0 \text{ ng mL}^{-1}\] and the sample \([\text{PSA} = 5 \text{ ng mL}^{-1}\] increased as [Ab\(_1\)] decreased (Fig. S6). The [Ab\(_1\)] was lowered till a significant difference was observed between the samples (red) and the control (blue). Four [Ab\(_1\)] were tried for performing an immunoassay, which includes 0.33 mg ml\(^{-1}\), 0.258 mg ml\(^{-1}\), 0.165 mg ml\(^{-1}\) and 0.04 mg ml\(^{-1}\). The difference in the ECL signal, between the control and the sample, was highest for [Ab\(_1\)] = 0.04 mg ml\(^{-1}\) (Fig. S6a). Therefore this [Ab\(_1\)] was used to build the test immunosensors. To enhance the ECL signal between the control and sample further, Tween-20 (0.05%) and Triton-X (0.05%) was added to 100 mM TPrA used as coreactant. Approximately a 10 fold increase in ECL was observed as compared with the immunoassay where TPrA alone was used as coreactant (Fig. S6b).

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**Fig. S6** ECL signals for SWCNT modified PG incubated with PSA \([\text{PSA}]: 5 \text{ ng mL}^{-1}\) in 10 \(\mu\)L undiluted newborn calf serum for 1.15 h, then anti-PSA RuBPY-silica nanoparticles in 2% BSA and 0.05% Tween-20 for 1.15 h. Controls (blue) used were SWCNT-anti-PSA immunosensor omitting addition of PSA with 10 ng ml\(^{-1}\). (a) At different Ab\(_1\) concentration (0.33 mg ml\(^{-1}\), 0.258 mg ml\(^{-1}\), 0.165 mg ml\(^{-1}\), 0.041 mg ml\(^{-1}\)) in the presence of 100 mM TPrA in 0.2 mM phosphate buffer pH 7.5. (Symbols in red, controls are blue)(b) Influence of detergents on ECL yield: on left, 100 mM TPrA in 0.2 mM phosphate buffer (pH: 7.5) and on right 0.05% Tween 20 + 0.05% Triton-X 100 + 100 mM TPrA in 0.2 mM phosphate buffer (pH: 7.5).
Table 1. Average photon count (ECL signal) with standard deviation (STD) and relative standard deviation (RSD) obtained from ECL simultaneously with amperometry at 0.95 V vs SCE for immunoassay on SWNT immunosensor in the presence of 0.05% Tween 20 + 0.05% Triton-X 100 + 100 mM TPrA in 0.2 M phosphate buffer (pH: 7.5).

<table>
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<th>[PSA] ng mL⁻¹</th>
<th>Relative ECL STD</th>
<th>RSD</th>
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Fig. S7 ECL signal obtained at 0.95 V vs SCE with [PSA] range: 0.4-5 ng mL⁻¹ in the presence of 0.05% Tween 20 + 0.05% Triton-X 100 + 100 mM TPrA in 0.2 M phosphate buffer (pH: 7.5). of SWCNT forest immunosensor with attached capture antibody incubated with 5 ng mL⁻¹ PSA in 10 μL undiluted calf serum for 1.15 h after treating with 20 μL of 2% BSA for 1.15 h, followed by addition of 10 μL Ab₂-RuBPY-silica in 2% BSA and 0.05% Tween-20 for 1.15 h. Every step was followed by washing with 0.05% Tween-20 and PBS buffer for 3 minutes each. a) Influence of time on cumulative ECL counts at 1.05 V for 10 s for [PSA] range: 0.4-40 ng mL⁻¹. b) Plots of ECL intensity verses the nano-molar concentration of PSA showing Influence of PSA concentration on ECL signal at 10 s for [PSA] range: 0.4-40 ng mL⁻¹. Control is immunoassay omitting addition of PSA. Error bars show standard deviations (n = 4). Detection limit = 0.4 ng mL⁻¹, which is control plus three times the standard deviation.

References