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# SUPPORTING INFORMATION

# Electrochemiluminescent immunoassay enhancement driven by carbon nanotubes

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## 1. Experimental procedure.

**Materials.** Tri-n-propylamine (TPrA, MW = 143.27 g/mol, ≥98% V/V), sodium phosphate monobasic dihydrate ($NaH_2PO_4 \cdot 2H_2O$, MW = 119.98 g/mol, ≥99%), sodium phosphate dibasic ($Na_2HPO_4$, MW = 141.96 g/mol, ≥99%), phosphoric acid ($H_3PO_4$, MW = 98,00 g/mol, ≥85%), dimethyl sulfoxide (DMSO, anhydrous, MW = 78.13 g/mol, ≥99.9%), MES sodium salt (MW = 217,22 g/mol, ≥99%), phosphate-buffered saline (PBS), N-hydroxysuccinimide (NHS, MW = 115.09, ≥98%), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, MW = 191.70, ≥98%), and biotin (MW = 244.31, ≥99%) were purchased from Sigma-Aldrich. 2.8 μm and 4 μm beads coated with streptavidin were purchase by ThermoFisher scientific (Dynabeads beads), and antibody labeled with biotin and $Ru(bpy)_3^{2+}$. Double-walled carbon nanotubes (DWCNT) were produced using the CVD method and purchased from Nanocyl S.A. (Sambreville, Belgium).

**Synthesis of double walled carbon nanotubes (f-CNTs) functionalized with $Ru(bpy)_3^{2+}$ complex (CNT-Ru).** Reagents and solvents purchased from Sigma Aldrich and Riedel-Fluka were used as received. DWCNTs were firstly oxidized using a mixture of nitric and sulfuric acids as previously reported.[1] Then 100 mg of oxidized DWCNTs (***ox*-DWCNT**) were sonicated for 1 hour in 100 mL of DMF to obtain a homogeneous dispersion. 150 mg of paraformaldehyde and 150 mg of the corresponding amino acid **(A)** were added 4 times (every 24 hours) to the suspension, which was stirred upon heating at 115 °C for 5 days. After being cooled to room temperature, the suspension was sonicated for 30 minutes and filtered on a Teflon membrane (Millipore, JHWP, 0.1 μm). The black solid was washed by redispersion/filtration in DMF, MeOH and $Et_2O$. The obtained product (97 mg) was dried under vacuum overnight. 30 mg of **DWCNT 1** were sonicated for 30 minutes in 20 ml of anhydrous DMF with a molar excess of DIEA. Then, 100 equivalents of EDC were dispersed in 20 mL of anhydrous DMF and added to the mixture, that was stirred under Argon 1 hour at rt. After the addition of 100 equivalents of NHS, the solution was stirred under Argon overnight at rt. The solution was sonicated for 30 minutes and filtered on a Teflon membrane (Millipore, JHWP, 0.1 μm). The black solid was washed by redispersion/filtration in DMF, i-PrOH and $Et_2O$. The obtained product (27 mg) was dried under vacuum overnight. 8 mg of **DWCNT 1'** were sonicated for 50 minutes in 8 mL of anhydrous DMF with a molar excess of DIEA. 2 mg of $Ru(bpy)_3^{2+}$ amine derivative were dissolved in 2 mL of anhydrous DMF and added to the reaction mixture, which was sonicated for 20 minutes and stirred under Argon for 24 hours at 45 °C. The mixture was sonicated and filtered on a Teflon membrane (Millipore, JHWP, 0.1 μm). The black solid was washed by redispersion/filtration in DMF, MeOH and $Et_2O$. The obtained product (6 mg) was dried under

vacuum overnight. To remove Boc protecting group, **DWCNT 2** were treated with HCl:1,4-dioxane (1/2) overnight, affording free terminal amines which increase the solubility of the compound and are available for subsequent functionalization steps.

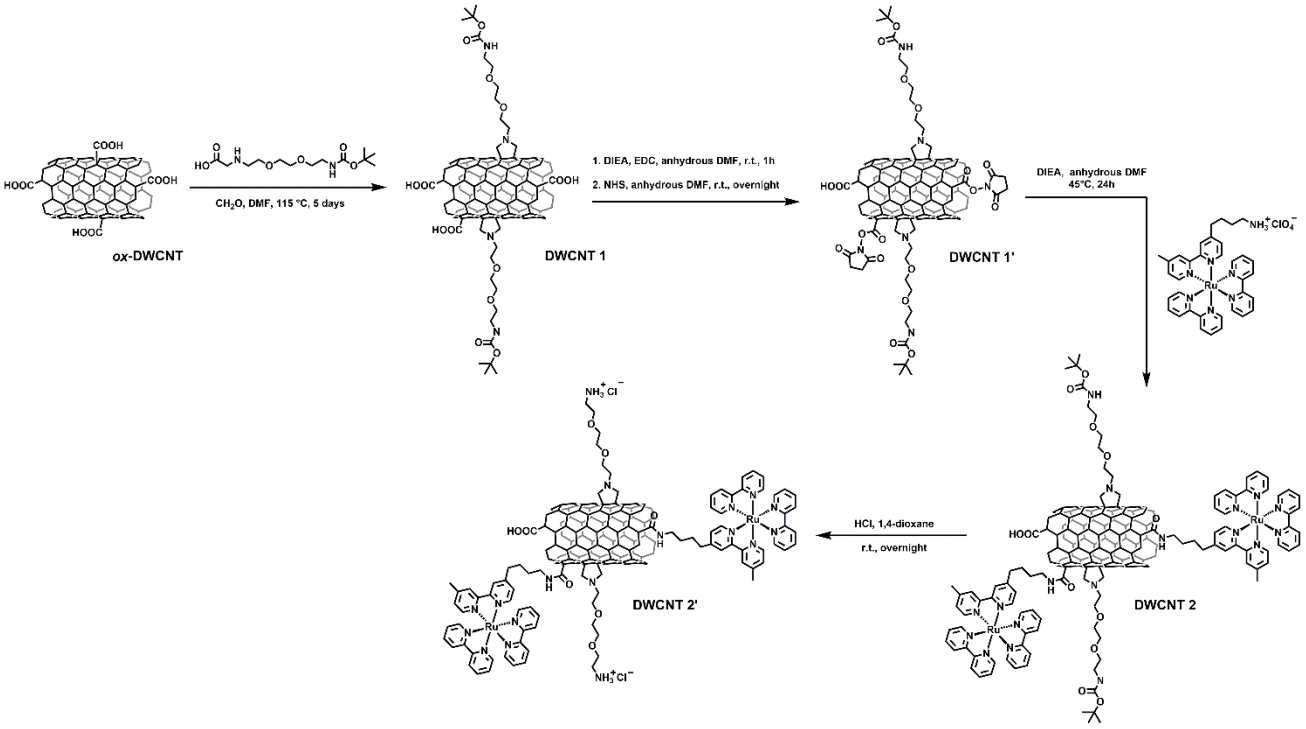


**Figure S1.** Schematic of synthetic strategy for DWCNT 2'.

The amount of Ru present in the nanomaterial was than quantified with ICP-MS spectrometry.

| Sample | Ru concentration (μg/mg) |
|---|---|
| **CNT-Ru** | 1.57 |

**Table S1.** Amount of Ru present in the DWCNT 2'.

**DWCNT 2' biotinylation.** Biotin was dissolved in a MES buffer 0.1 M solution (pH = 6.5) and added for 20 minutes to a MES buffer solution containing EDC and NHS. 0.4 mg of DWCNT 2' was suspended in 40 μL of DMSO, sonicated for 20 minutes, and then added to the solution containing biotin, EDC, and NHS. The mixture was brought to 1 mL with MES buffer and maintained under rotation at 25 ºC overnight. 200 μL of MES buffer solution containing biotin, EDC, and NHS was added to the mixture, which was then maintained under rotation for 72 hours (Figure S2). Afterwards, the solution was dialyzed for 24 hours at 25 ºC in deionized water with 3–4 changes of water. Finally, the obtained solution of biotinylated DWCNT 2' (CNT-Ru) was diluted in 100 mL of PBS 0.01 M.

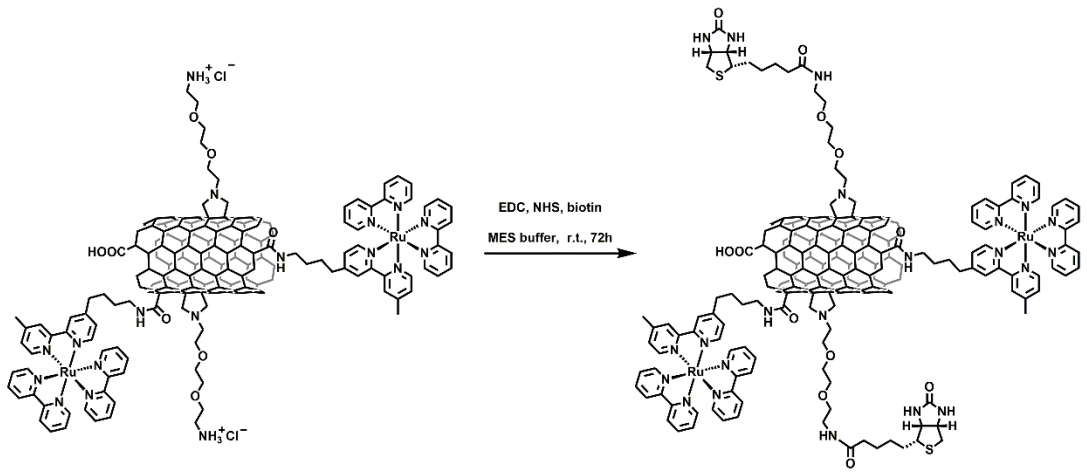


**Figure S2.** Schematic of biotinylation of DWCNT 2'.

**Beads functionalization**[2]. To enable the ECL imaging measurements, streptavidin-coated beads with a diameter of 2.8 μm were functionalized with biotinylated CNT labeled with the $[Ru(bpy)_3]^{2+}$ complex (CNT-Ru). The magnetic beads solution (diameter 2.8 μm; Dynabeads beads, ThermoFisher scientific, total surface area of $7 \times 10^9$ $\mu m^2$, 6 mL) was poured in a 20 mL vial, and the beads were collected using a magnet for 2 minutes. Afterwards, the supernatant was discharged and 18 mL of CNT-Ru solution (1.6 μM of Ru) in phosphate buffer saline (0.01 M) was added, followed by 2 hours incubation at 37 °C under rotation to form the biotin-streptavidin bond. The solution was separated with a magnet and the supernatant discharged. The whole procedure was repeated five times. Beads@Ru were obtained using the same procedure but using Free Conjugate (antibody labeled with biotin and $Ru(bpy)_3^{2+}$) instead of the CNT-Ru solution.

**Antibody labeled with biotin and $Ru(bpy)_3^{2+}$.** We derivatized the antibody (IgG from Vector Laboratories) incubating a solution of 1 mg/mL of antibody in PBS with 85 mol equiv. of EDC, NHS, $Ru(bpy)_2$-bpy-CO-OSu (Ruthenium-butanic acid-ester from Cyanagen) and biotin. After the incubation of 90 minutes the solution is purified removing ruthenium complex and biotin in excess through centrifugation with Millipore Amicon Ultra 0.5 mL centrifugal filter devices, with 50 000 cutoff membrane.[4]

**ECL imaging measurements.** The ECL/optical imaging of beads@CNT-Ru and beads@Ru, deposited on the working electrode and collected by a magnet, was performed using a solution of 0.2 M PB, 180 mM TPrA, and polidocanol surfactant (pH 6.9) in a PTFE homemade electrochemical cell comprising a Pt working (0.16 $cm^2$), Pt counter, and Ag/AgCl (3 M KCl) reference electrodes. For microscopic imaging, an epifluorescence microscope from Nikon

(Chiyoda, Tokyo, Japan) equipped with an ultrasensitive EMCCD camera (EM-CCD 9100−13 from Hamamatsu, Hamamatsu Japan) was used with a resolution of 512 × 512 pixels and a size of 16 × 16 μm$^2$. The microscope was enclosed in a homemade dark box to avoid interferences from external light. It was also equipped with a motorized microscope stage (Corvus, Märzhauser, Wetzlar, Germany) for sample positioning and with long-distance objectives from Nikon (100×/0.80 /DL4.5 mm and 40×/0.60 /DL3.6mm). Additionally, the integrated system included a potentiostat from AUTOLAB (PGSTAT 30) triggered with the camera. Images were recorded during the application of a constant potential of 1.4 V (vs. Ag/AgCl 3M KCl) for 4 s with an integration time of 8 s. Integrated ECL intensity was obtained through software ImageJ, integrating the signals from ECL images with squares of 50x50px for 2.8 μm and 4 μm beads. TOF parameter will allow the normalization of ECL intensity integrated for the number of luminophores on beads surface.

**Characterization of f-CNTs.**

**Transmission electron microscopy (TEM).** TEM images were taken on a Philips EM 208 electronic microscope operating at 100 kV. Sample were prepared suspending the compound in DMF or water and depositing some drops of the solution on copper grids covered by a carbon film of 3.00 mm. Solvent was dried overnight under vacuum.

**Thermogravimetric analysis (TGA).** TGA analysis were run on the instrument TGA Q500 from *TA Instruments* using $N_2$ gas flux (25 mL·min$^{-1}$ flow rate) starting with an isotherm at 100 °C for 20 min and then heating at a rate of 10 °C·min$^{-1}$ up to 1000 °C. Each sample were analysed at least 2 times and the average value of weight loss was reported. Data were analysed by evaluating the ratio between number of carbon atoms and the functional groups obtained by the relation: $[(100 - A)/12]/(A/B)$; where $A$ is the weight loss and $B$ is the molecular weight of the organic moiety.

**Fluorescence spectroscopy.** Emission spectra were collected on a Cary ECLIPSE spectrofluorimeter in 1 cm thick quartz cuvettes.

**Characterization of beads 2.8 μm functionalized with CNT-Ru.**

**Scanning electron microscopy (SEM)** Scanning electron microscopy images were obtained using a Thermo Fisher Scientific Helios 450s electron microscope. The images are acquired using an acceleration voltage of 1 kV and a beam current of 6-13 pA, in high vacuum and in high-resolution mode.

**Raman spectroscopy** Raman spectra were recorded with a Renishaw Invia Raman spectrometer equipped with a green laser (λ=532 nm) and plotted by means of the Wire 4.3 software.

**Laser scanning confocal microscopy** Confocal microscopy images were acquired with a Zeiss LSM 510 Confocal Microscope equipped with a pulsed diode laser of 405 nm excitation wavelength. To allow proper comparison between different samples, the same microscope parameters (master gain, digital offset, pinhole dimension, laser power) were employed in all the experiments. For the images acquisition a 63x (1.40 N/A) oil objective with 575 nm LP filter was installed, and the pinhole was adjusted at 136 μm. Data analysis was performed using ImageJ 1.53c software (Wayne Rasband, National Institutes of Health). To compare the fluorescence intensity of different samples, the mean grey value of beads populations (n=126) was measured using a fixed area circle. The background signal was calculated measuring the mean grey value of empty areas of the images (same fixed area circle).

**Functionalization of 4 μm beads with $Ru(bpy)_3^{2+}$ (4beads@Ru) and CNT-Ru complex (4beads@CNT-Ru):**[2]

To enable the ECL imaging measurements, streptavidin-coated beads with a diameter of 4 μm were functionalized with biotinylated CNT labelled with $[Ru(bpy)_3]^{2+}$ complex (CNT-Ru). The magnetic beads solution (diameter 4 μm; Dynabeads beads, ThermoFisher scientific) 665.1 μL, (total surface area of 7 $x10^9$ $\mu m^2$) was poured in a 20 mL vial, and beads were collected using a magnet for 2 minutes. Afterwards the supernatant was discharged and 18 mL of CNT-Ru solution (1.6 μM of Ru) in phosphate buffer saline (0.01 M) was added, followed with 2 hours incubation at 37 °C under rotation to form the biotin-streptavidin bond. The solution was separated with a magnet and the supernatant discharged. The whole procedure was repeated five times. 4Beads@Ru were obtained using the same procedure but using free conjugate (antibody labelled with biotin and $Ru(bpy)_3^{2+}$) instead of CNT-Ru solution.

**Turnover frequency (TOF)**

TOF is the number of photons emitted in 1 s by a single luminophore.[3] It is defined as:

$$TOF = \frac{\int_0^t ECL_{Ru@bead} - \int_0^t ECL_{bead}}{Fluorescence\ intensity\ x\ integration\ time}$$

ECL emission ($ECL_{bead@Ru}$) was quantified by integration of ECL images, as obtained with the CCD camera for a time (*t*) of 8 s.

Integrated ECL noise ($ECL_{bead}$) was subtracted from the integrated ECL emission for each bead dimension. $ECL_{bead}$ was measured without beads presence in images.

Integration time is the time when the potential 1.4 V is applied, and it is of 4 s.

Software ImageJ was used to integrate the signals from ECL images with squares of 50×50px for 4 μm and 2.8 μm beads.

Fluorescence intensity is the mean grey value of 126 beads images obtained using laser confocal microscopy technique.

## 2. Fluorescence spectroscopy of f-CNTs.

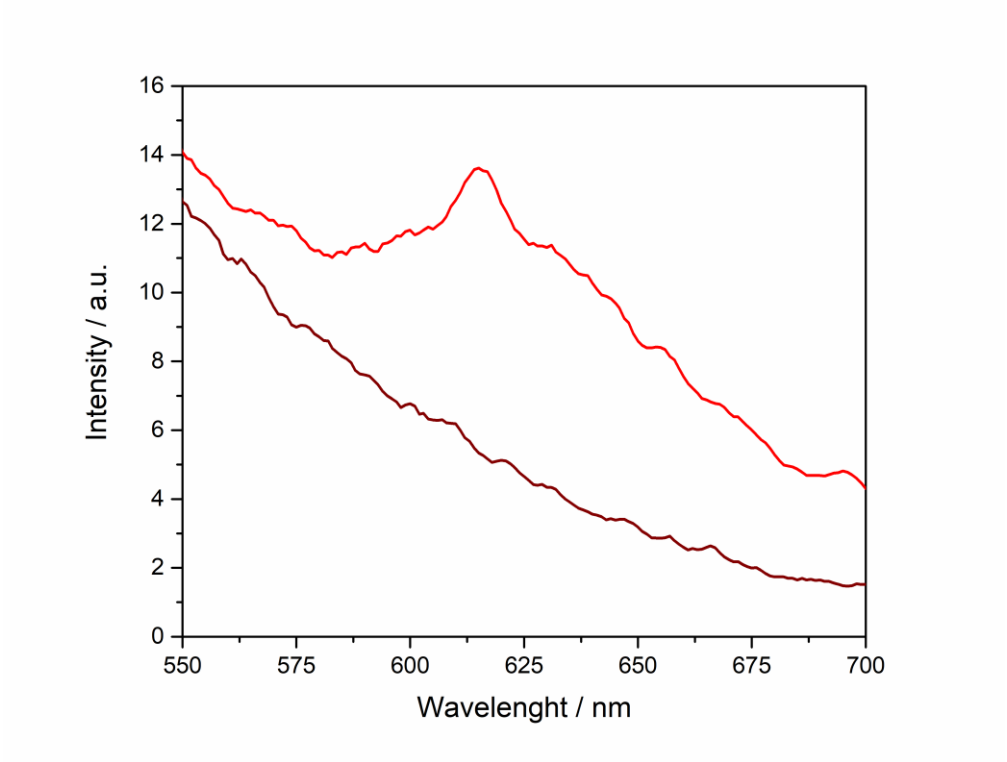


**Figure S3**. Emission spectra of a DMF solution of ox DWCNTs (dark red line) and CNTs labelled with $[Ru(bpy)_3]^{2+}$ complex (named DWCNT 2' light red line).

## 3. TEM characterization of f-CNTs.

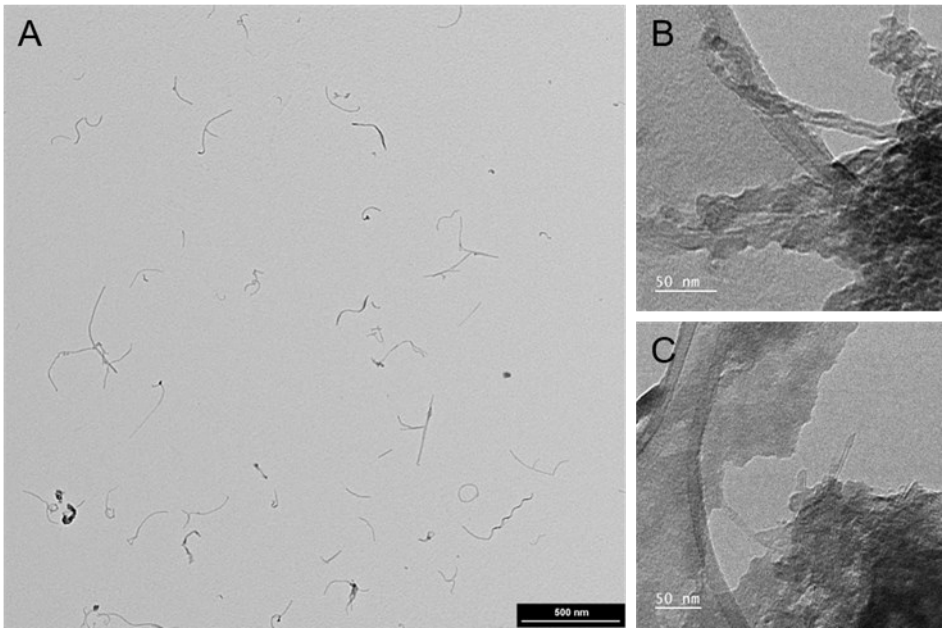


**Figure S4.** TEM images of DWCNT 1 (A) and DWCNT 2' (B, C) dispersed in DMF. Scale bar 500 nm (A) and 50 nm (B, C).

## 4. TGA analysis of f-CNTs.

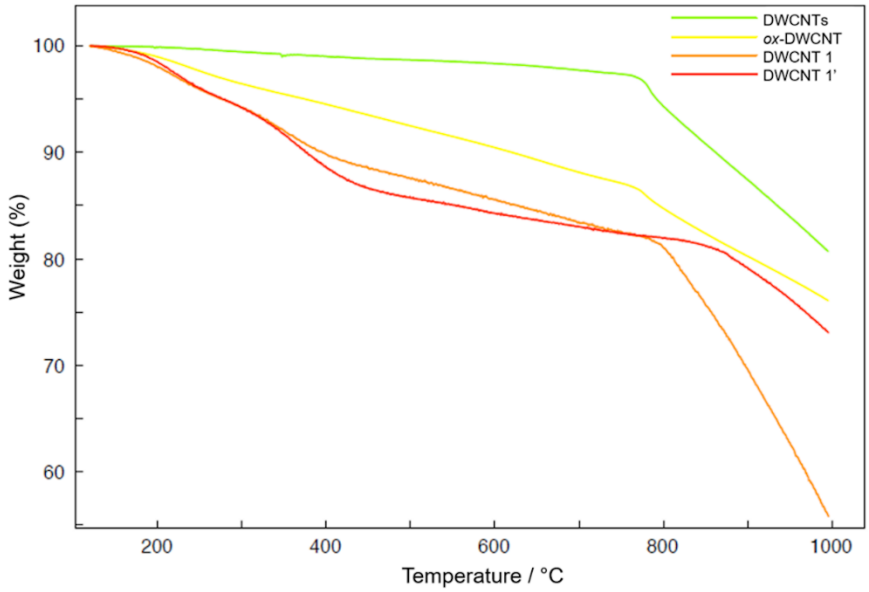


**Figure S5.** Comparison between TGAs of pristine DWCNTs, *ox*-DWCNT, DWCNT 1 and DWCNT 1' show the introduction of different organic moieties that decompose between 200 and 400 degrees. The weight loss corresponds to 1.7 mmol/g for *ox*-DWCNT, 186 μmol/g for DWCNT 1 and 480 μmol/g for DWCNT 1'. TGAs are performed under $N_2$.

## 5. Additional Raman spectra of beads@Ru, beads@CNT-Ru and $Ru(bpy)_3^{2+}$.

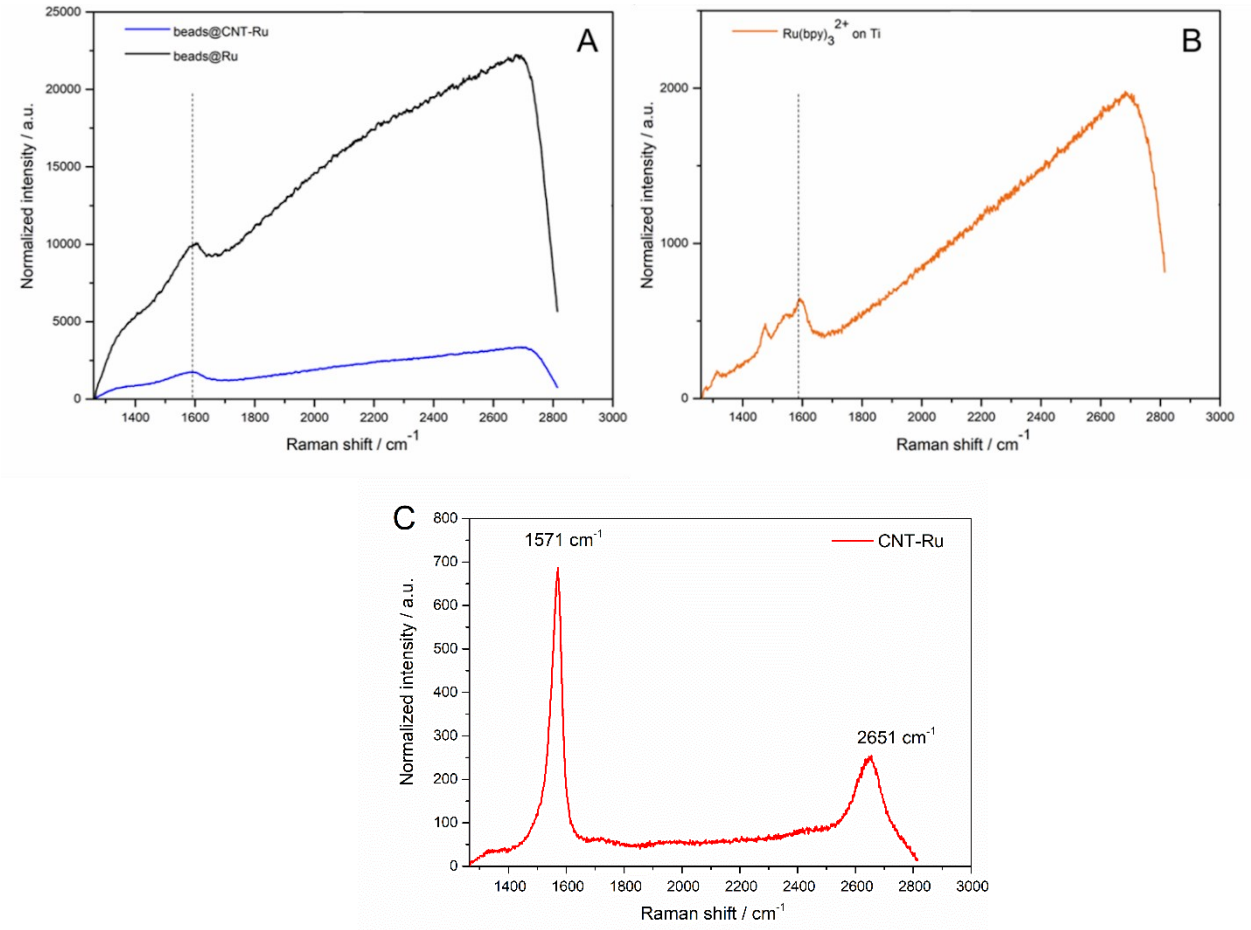


**Figure S6.** Raman spectra of (A) beads functionalized with a biotinylated antibody labelled with $Ru(bpy)_3^{2+}$ complex 2.8 µm beads@Ru (black line) and CNT-Ru 2.8 µm beads@CNT-Ru (blue line); (B) Raman spectra of $Ru(bpy)_3^{2+}$ dropcasted on titanium surface. C) Raman spectroscopy analysis of CNT-Ru. Raman spectra were recorded using a 532 nm green laser (100% laser power) and 1 second of exposition time.

Due to the high fluorescence and broad signal of Ru complex at 1600 $cm^{-1}$, the Raman signals of carbon nanotubes are hindered. Therefore, the characterization of CNT-Ru by Raman spectroscopy is not possible.

## 6. SEM analysis of beads@Ru and beads@CNT-Ru.

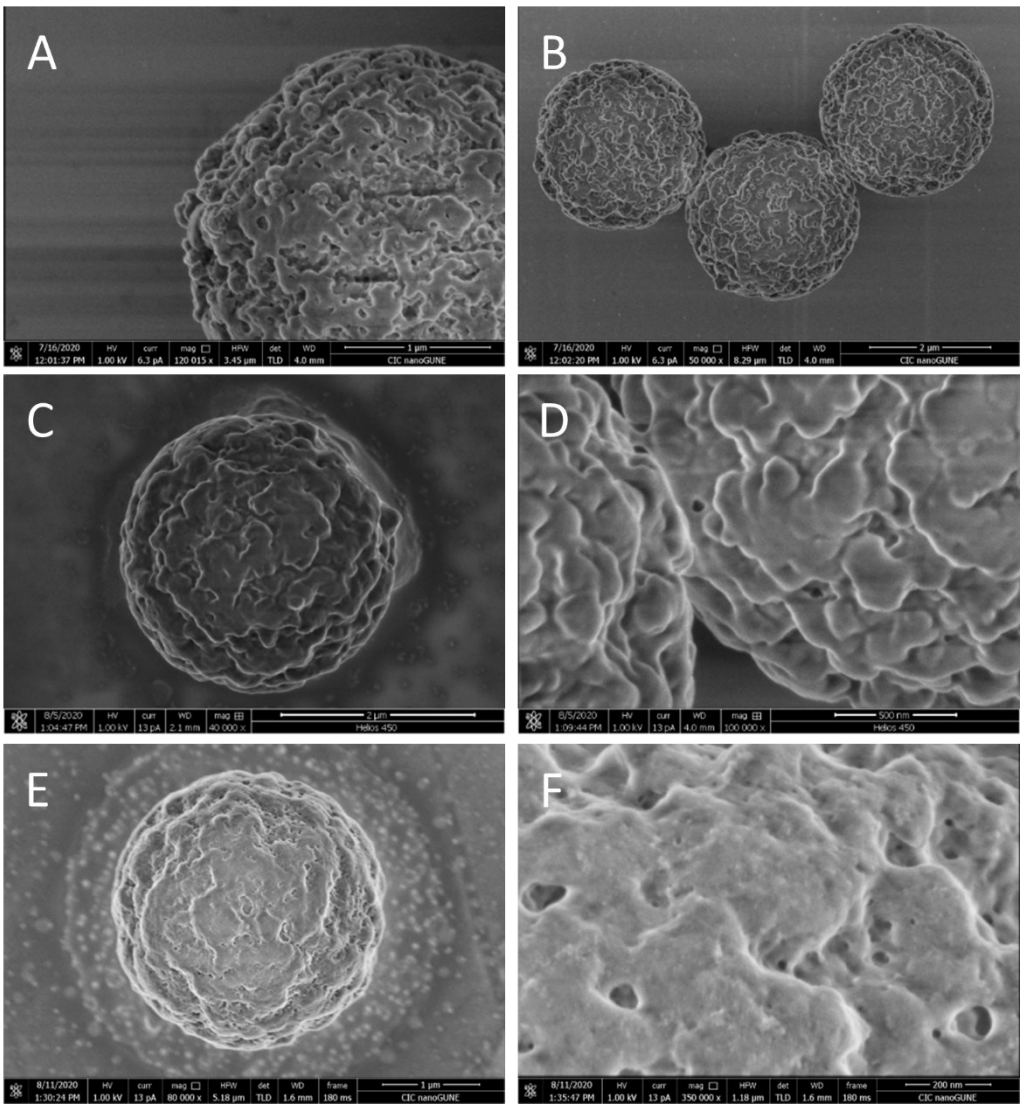


**Figure S7.** SEM images of bare 2.8 µm beads (A,B), 2.8 µm beads@Ru (C,D) and 2.8 µm beads@CNT-Ru (E,F).

The SEM images of beads@CNT-Ru show a smoother surface than bare 2.8 µm bead surface, which evidence a surface modification.

## 7. ECL images from beads@CNT-Ru

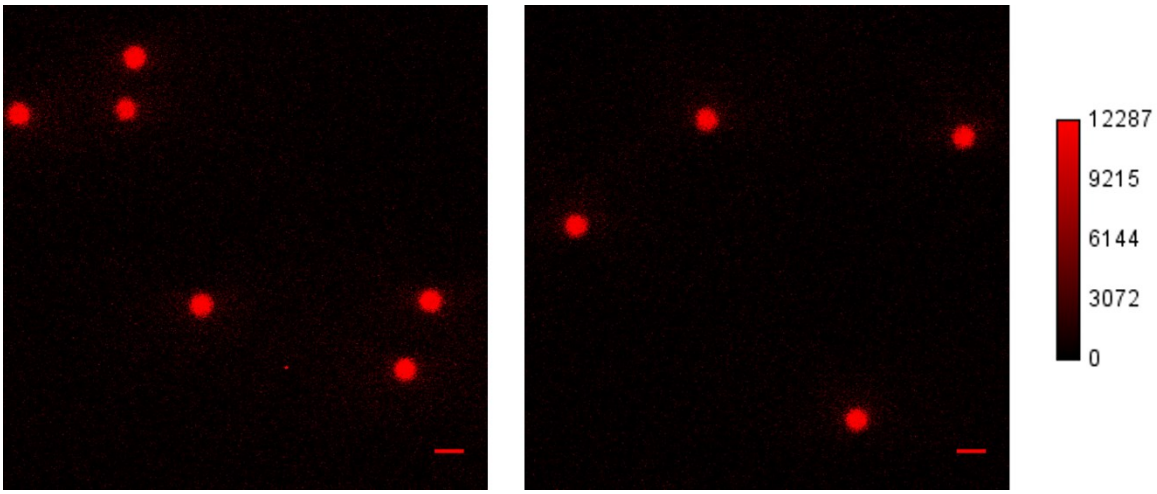


**Figure S8.** ECL imaging of 2.8 μm single-bead labeled with CNTs labeled with $[Ru(bpy)_3]^{2+}$ (beads@CNT-Ru). The images were obtained by applying a constant potential of 1.4 V (vs. Ag/AgCl) for 4 s in 180 mM TPrA and 0.2 M phosphate buffer (PB). Pt wire was used as counter electrode. EMCCD camera was coupled with a potentiostat. Integration time, 8 s; magnification, X100; scale bar, 5 μm.

## 8. Laser scanning confocal microscopy.

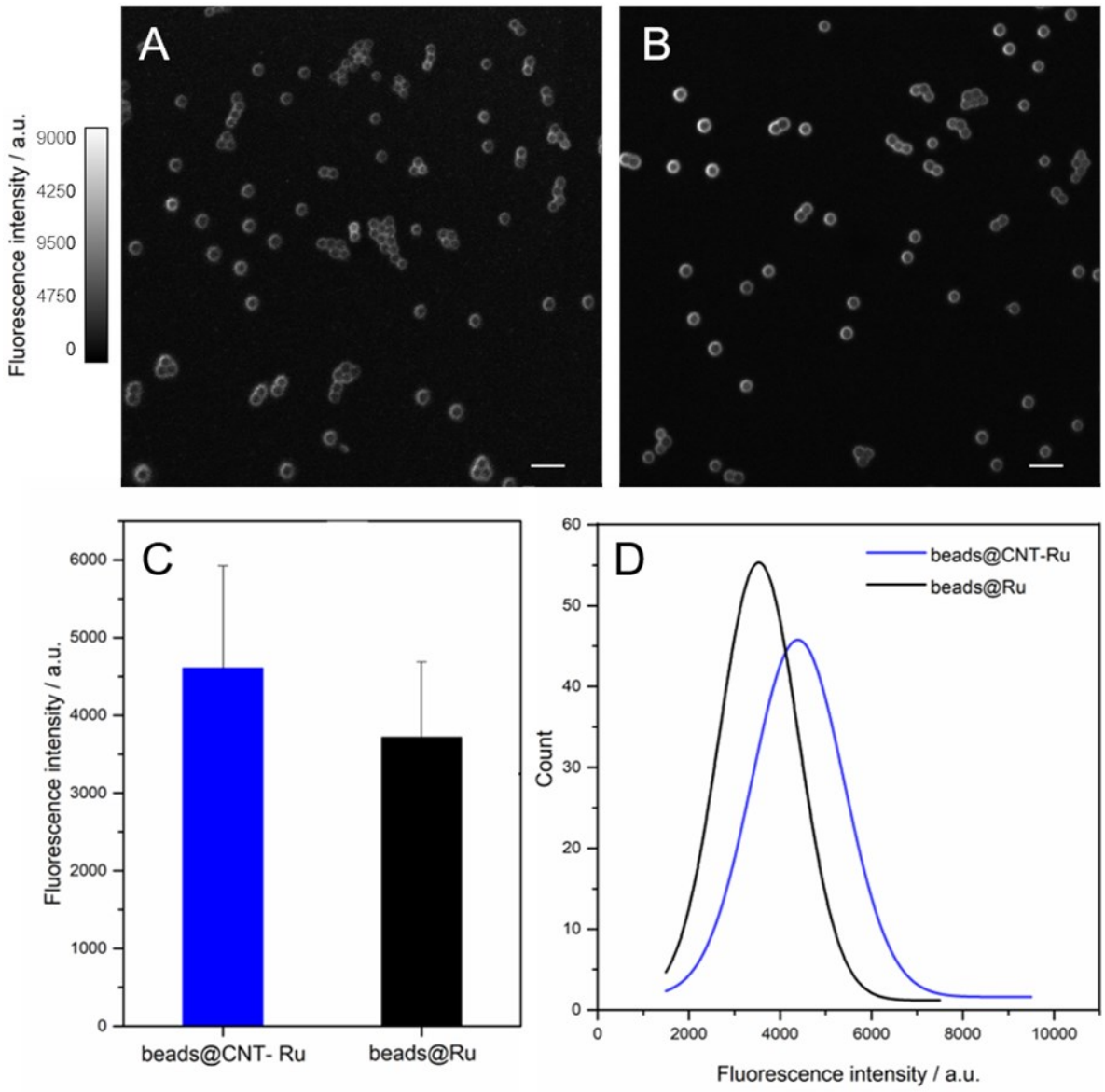


**Figure S9.** Laser scanning confocal microscopy images of (A) 2.8 µm beads@Ru and (B) 2.8 µm beads@CNT-Ru. Laser 405 nm, filter 575 nm LP, pinhole 136 µm, section 1 µm. Scale bar 10 µm. (C) Histograms of mean fluorescence intensity expressed as mean grey value of 2.8 µm beads@CNT-Ru (blue) and 2.8 µm beads@Ru (black). Error bars show the standard deviation (n=126). (D) Fluorescence intensity gaussian distribution of beads@CNT-Ru and beads@Ru.

## 9. ECL measurements with DBAE co-reactant.

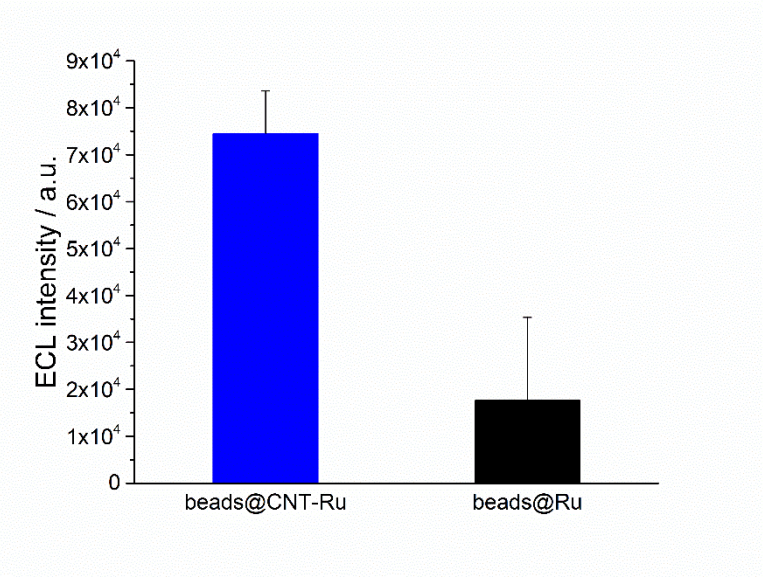


**Figure S10.** Comparison between ECL intensity values of 2.8 µm beads@Ru (black) and 2.8 µm beads@CNT-Ru (blue). Error bars show the standard deviation (n=5). They were obtained by applying a constant potential of 1.4 V (vs. Ag/AgCl) for 4 s in 180 mM DBAE and 0.2 M phosphate buffer (PB). Pt wire as counter electrode. EMCCD camera coupled with a potentiostat. Integration time, 8 s.

**10. Deconvolution of curves CV-ECL combination.**

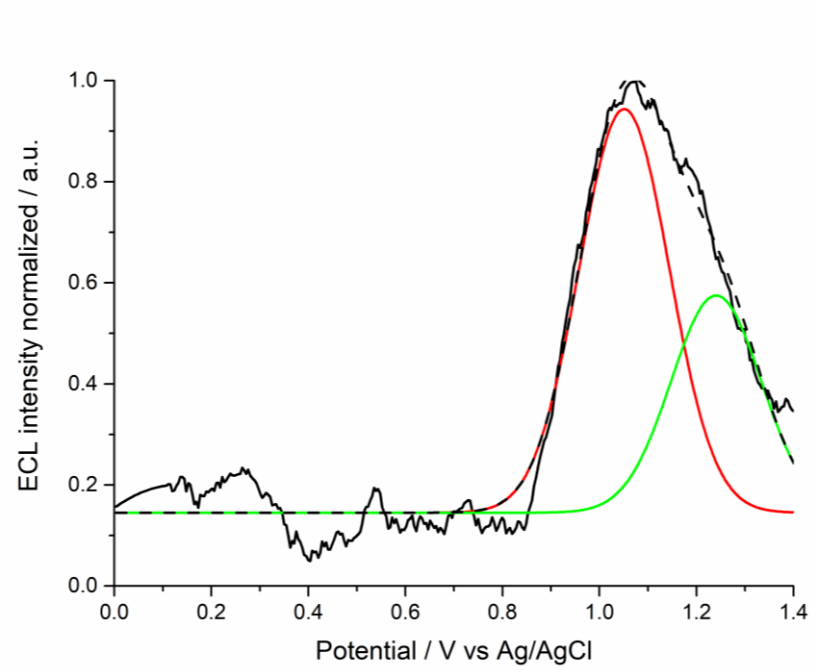


**Figure S11.** Deconvolution of peak at 1 V (red line) and 1.2 V (green line), at which the TPrA and $[Ru(bpy)_3]^{2+}$ were oxidized, respectively. The sum of this two peaks deconvolution was represented by dotted black line. Cyclic-voltammetries were performed on 2.8 μm beads@Ru scanning the potential between 0 V and 1.4 V and acquiring the ECL emission signal each 200 ms. EMCCD camera coupled with a potentiostat. Integration time, 200 ms.

**11. Square wave voltammetries of beads@CNT-Ru and beads@Ru**

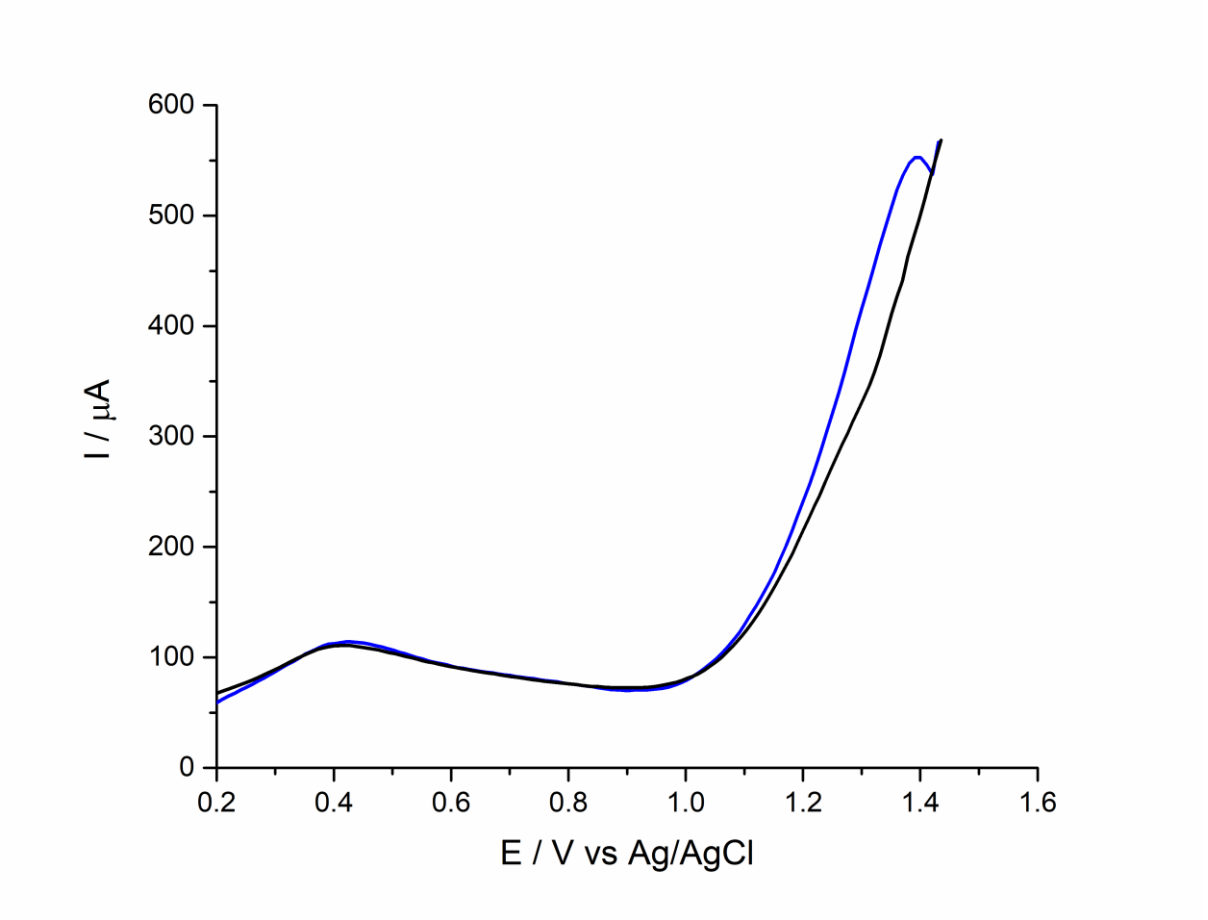


**Figure S12.** Square wave voltammetries performed on 30µL of beads@CNT-Ru (blue line) or beads@Ru (black line), scanning the potential from 0.2 V to 1.45 V (vs. Ag/AgCl) in 0.2 M phosphate buffer (PB), scan rate 0.1 V $s^{-1}$. Pt plate was used as working electrode. Pt wire was used as counter electrode.

## 12. ECL emission from CNT without $Ru(bpy)_3^{2+}$

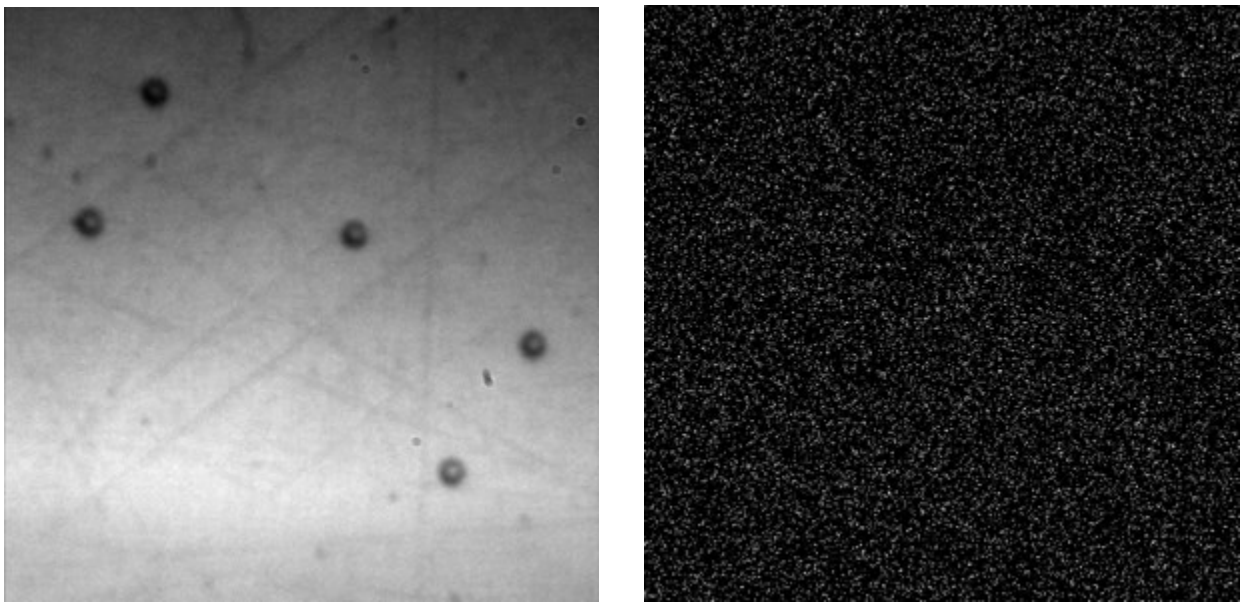

**Figure S13.** (A) Optical and (B) ECL images of beads functionalized with CNT not labelled with $[Ru(bpy)_3]^{2+}$. They were obtained by applying a constant potential of 1.4 V (vs. Ag/AgCl) for 4 s in 180 mM TPrA and 0.2 M phosphate buffer (PB). Pt wire as counter electrode. EMCCD camera coupled with a potentiostat. Integration time, 8 s.

We performed the functionalization of 2.8 µm beads with CNT not labelled with $[Ru(bpy)_3]^{2+}$, using the same procedure of the others functionalization. We didn't observe any ECL emission, confirming the fundamental role of the luminophore in ECL signal generation and the absence of ECL signal from CNTs.

**13. ECL measurements of 4 µm beads@Ru and beads@CNT-Ru.**

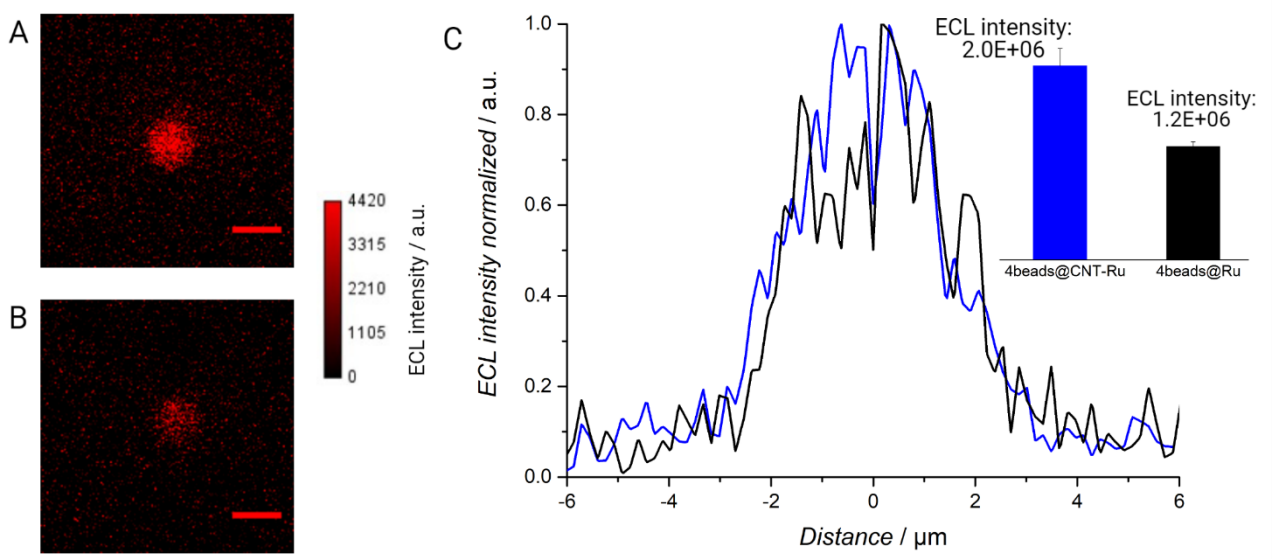


**Figure S14.** (A) Electrochemiluminescence (ECL) imaging of 4 µm beads@CNT-Ru and (B) 4 µm beads@Ru. The ECL images were obtained by applying a constant potential of 1.4 V (vs. Ag/AgCl) for 4 s in 180 mM TPrA and 0.2 M phosphate buffer (PB). Pt wire as counter electrode. EMCCD camera coupled with a potentiostat. Integration time, 8 s; magnification, 100x; Scale bar, 5 µm. (C) Comparison of the beads profile lines normalized (black line, 4beads@Ru; blue line, 4beads@CNT-Ru). Inset of the comparison between ECL intensity integrated values (50x50 pixel square) of 4beads@Ru (black) and 4beads@CNT-Ru (blue). Error bars show the standard deviation (n=12).

## 14. Supplementary Movie.

- **Supplementary Movie**. ECL imaging of 2.8 μm beads@CNT-Ru during a CV scanning in TPrA 180 mM in 0.2M Phosphate Buffer (PB, pH 6.9). Potential 0 - 1.4V vs Ag/AgCl (3M KCl), scan rate 20 mV $s^{-1}$, exposure time 200 ms, and magnification 100x.

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