Supporting Information for:

Thermochemiluminescent Semiconducting Polymer Dots as sensitive nanoprobe for reagentless immunoassay

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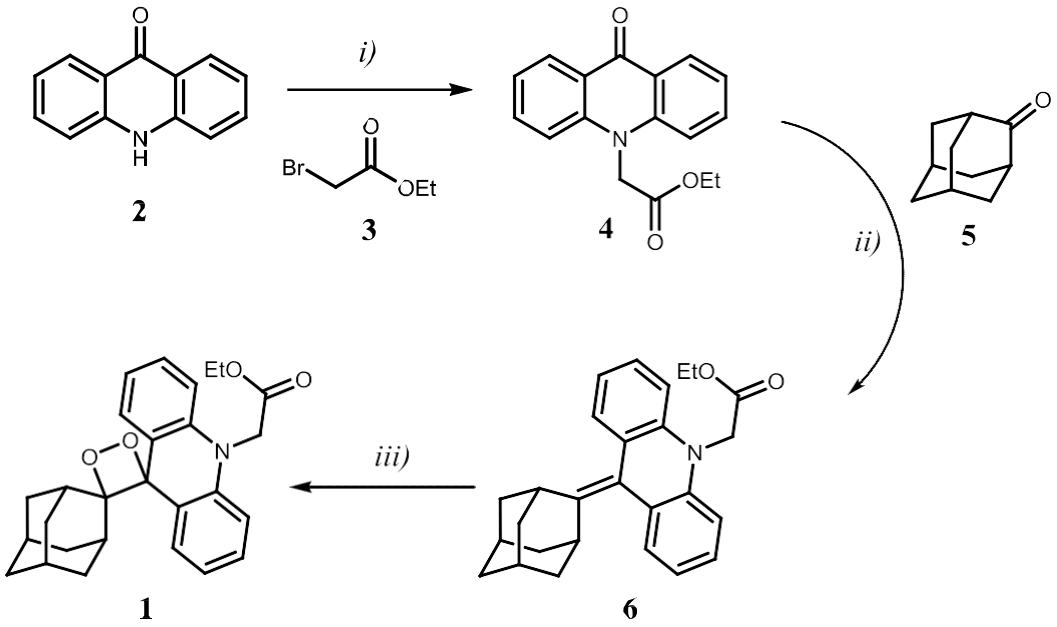
General remarks

Purification of TCL-Pdots-SA was carried out through filtration over a 400 µm cutoff filter and centrifugation, using a 100 K molecular weight cutoff centrifugal membrane (Amicon Ultra-4, Ultracel - 100K). Then, nanoparticles were further purified by size exclusion chromatography, using Sephacryl S-300 HR resin.

Spectroscopic properties, in terms of absorption and emission spectra were recorded using a UV- Vis spectrophotometer (Varian Cary 50) and a Uv-Vis spectrofluorimeter (Carian Cary Eclipse). The ^{1}H and ^{13}C-NMR spectra were recorded on 400 NMR instrument with a 5 mm probe. All chemical shifts have been quoted relative to deuterated solvent signals. Dynamic light scattering (DLS) experiments were conducted using a Malvern Zetasizer NanoZS while TE images were acquired using a Philips CM100 transmission electron microscope (Philips/FEI Corp., Eindhoven, Holland). Fluorescence images of TCL-Pdots-SA conjugated with Biotin-IgG, before and after the eluition step, were obtained using an epi-fluorescence microscope with a 100x, 1.3 oil immersion objective and with a 532 nm diode laser, while the efficiency of FRET mechanism was calculated by Fluorescence Lifetime measurements, using a FluoTime 100 spectrometer (PicoQuant, PicoHarp 300). TCL signal was acquired using a portable battery-operated CCD camera (model MZ-2PRO, MagZero, Pordenone, Italy) equipped with a thermoelectrically cooled monochrome CCD image sensor and an objective (low distortion wide angle lenses 1/3 in. 1.28 mm, f 1.8) obtained from Edmund Optics (Barrington, NJ). TCL images were analyzed using an open

source image processing program (ImageJ). For evaluation of the signal-to-noise (S/N) ratios of the images, signals (S) were calculated by averaging the pixel intensity over the analyzed area, while noise (N) was taken as the standard deviation of the mean pixel intensity in a dark image area.

Synthesis of 1,2-dioxetane 1



Scheme S1: synthetic approach to 1,2-dioxetane **1**.

Compound **1** was obtained following a three-steps synthetic strategy (Scheme S1):

Step i: According to a previously reported procedure,[1] a solution of 9-(10H)-Acridanone **2** (300 mg, 1.5 mmol) in anhydrous DMF (7 mL) was added to a suspension of NaH (78 mg, 1.95 mmol) in anhydrous DMF (3mL). The mixture was stirred for 30 min at room temperature first, then, it was cooled to 0 °C and ethyl 2-bromoacetate **3** (267 mg, 2.4 mmol) and tetrabutylammonium iodide mg, 0.015 mmol) were added. The solution was stirred for further 24 h at room temperature and then poured into cold water. The precipitate was collected by filtration, dried under vacuum, and purified by flash chromatography on silica gel using 8:2 (v/v) cyclohexane/ethyl acetate as the eluent to obtain compound **4** (186 mg, Y = 44%). The obtained spectroscopic data were in agreement with the literature data.[1]

Step ii: Compound **6** was synthesized according to the previously reported procedure.[2] Under a nitrogen atmosphere, $TiCl_4$ (1M in dichloromethane, 4.33 mmol) was added to a suspension of

zinc powder (626 mg, 9.58 mmol) in anhydrous THF (11 mL) at 0 °C, and the suspension was stirred for 10 min under reflux. A solution of ketone **4** (200 mg, 0.71 mmol) and 2-adamantanone **5** (106 mg, 0.71 mmol) in anhydrous THF (3 mL) was added dropwise over a period of 30 min. The reaction mixture was refluxed for 45 minutes. Then, it was cooled to room temperature, quenched with water and extracted with AcOEt (3 x 10 mL). The combined organic layers were dried over sodium sulfate and evaporated under vacuum. The crude product was purified by flash chromatography on silica gel, using 8:2 (v/v) cyclohexane/ethyl acetate as the eluent to obtain compound **6** (252 mg, Y = 89%). The obtained spectroscopic data were in agreement with the literature data.[1]

Step iii: The 1,2-dioxetane **1** was synthesized according to the previously reported procedure.[2] Alkene **6** (30 mg, 0.075 mmol) and Methylene Blue (1.5 mg, 0.005 mmol) were dissolved in CH_2Cl_2 (3 mL). The solution was cooled to -20 °C and subjected to an oxygen atmosphere (1 atm, balloon). The solution was stirred at the same temperature under irradiation using a 500 W halogen lamp equipped with an UV cut-off filter (0.5% transmission at 550 nm). The irradiation was continued for 2 h, after which, the reaction mixture was purified by a rapid filtration on a 5 mm layer of silica gel, using cooled (-40 °C) CH_2Cl_2 as eluent, and the filtered solution was evaporated under vacuum at 0 °C, to give 1,2-dioxetane **1** (32 mg, Y = 98%). The obtained spectroscopic data were in agreement with the literature data.[1]

Synthesis of Termochemiluminescent Semiconductive Polymer dots (TCL-Pdots-SA)

The carboxylic units of TCL-Pdots were linked to Streptavidin according to the following procedure: 80 μL of polyethylene glycol (5% w/v PEG, MW 3350) and 80 μL of concentrated HEPES buffer (1 M) were added to a solution of TCL-Pdots (80 ppm in MilliQ water). Then, 120 μL of Streptavidin (2 mg/mL) was added to the solution along with 80 μ of freshly- prepared EDC solution (10 mg/mL in MilliQ water). After 3 hours of stirring at room temperature, BSA (10% (w/v), 80 μL) was added to the Pdots solution and the mixture was stirred for 30 minutes. Lastly, Triton X-100 (2.5% (w/v), 80
μL) was added to the mixture of TCL-Pdots-SA to stabilize the nanoparticles solution. The streptavidinated NPs solution was concentrated to 1 mL by centrifugation through a 100 K molecular cutoff membrane first, then purified by size exclusion chromatography using Sephacryl HR-300 gel media.

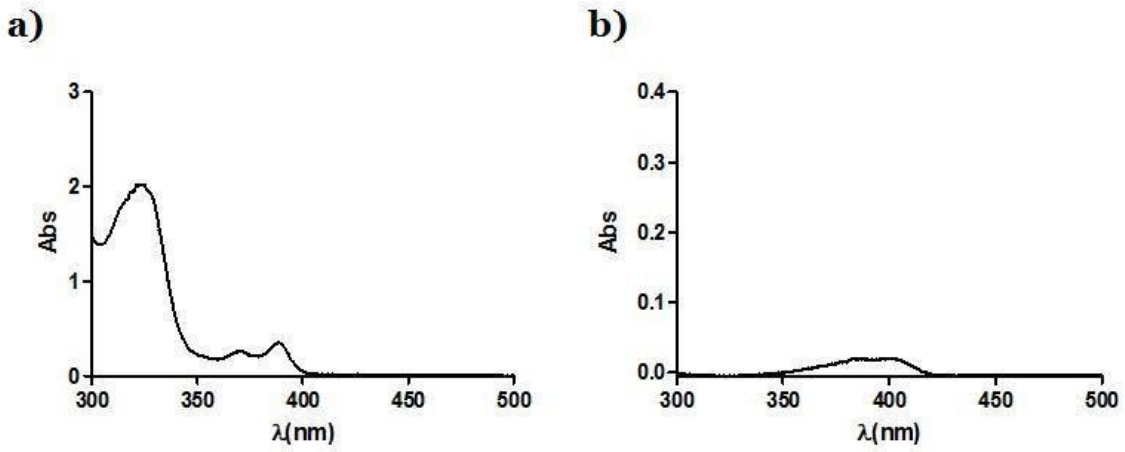


Figure S1: a) Uv-vis absorption of 1,2-dioxetane **1** (323 nm) and ketone **2** (387 nm) in THF solution b) Absorption spectrum of the filtrate solution, showing only the presence of a very small amount of ketone **2** (probably generated during the synthesis of TCL-Pdots-SA in water.

The fully encapsulation of 1,2-dioxetane **1** inside Pdots was confirmed by both the TCL emission from Pdots after bioconjugation and the UV-vis absorption spectrum (Figure S1b) of the wasted solution collected after centrifugation, which has shown the absence of dioxetane **1** in the filtrate.

Synthesis of PS-NH_2-2 derivative and FRET experiments

PS-NH2-**2** was obtained following a two-steps synthetic strategy. First, the ester functionality of ketone **2** underwent a basic hydrolysis to restore the carboxylic acid unit. Then, an EDC-catalyzed condensation reaction between PS-NH_2 and ketone **2**-COOH yielded the functionalized polymer PS-NH_2-**2**. According to a previously reported procedure,[3] ketone **2** (0.4 mmol) was dissolved in ethanol (8mL), and the solution was cooled to 0 °C. Then, a solution of NaOH (0.5 M) was added dropwise and the mixture was stirred at room temperature. After 2 hours, the solution was poured into cold water (30 mL) and the pH was adjusted to 4-5 value using HCl (8 M). The precipitate was collected and used in the next step without further purifications.

The condensation reaction was carried out dissolving ketone **2** (34 mg, 0.135 mmol), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 1 eq.) and N-Hydroxy- succinimide (NHS, 1 eq.) in DMF (3 mL). After 30 minutes of stirring, PS-NH_2 (0.13 eq.) was added and the mixture was further stirred at room temperature for 16 h. Then, the solution was poured into cold water (10 mL) and the precipitated was filtered and purified by flash chromatography on silica gel, using using 8:2 (v/v) cyclohexane/ethyl acetate as the eluent. The fraction containing the desired product (95 mg, Y = 80%)

was characterized by both ^{1}H NMR (Figure S2) and UV-vis absorption spectra (Figure S3). The first has shown typical signal of polystyrene hydrogens,[4] while the absorption spectrum has confirmed the presence of the ketone **2** (covalently bonded to PS chains).

In order to calculate the efficiency of FRET mechanism, we synthesized two different PS-based nanoparticles, following a previously reported procedure.[5] Specifically, 0.1 mL of a solution of PS-NH_2-**2** (6 mg/mL) in THF was diluted to 7 mL and quickly injected in 10 ml of Milli-Q water under sonication. In a second experiment, 0.1 mL of PS-NH_2-**2** (6 mg/mL) and 0.5 mL of CN-PPV (1mg/mL) were mixed together, diluted to 7 mL with THF and quickly injected into 10 mL of Milli- Q water, under sonication. Both nanoparticle solutions were filtered by a 400 µm cellulose filter, centrifugated using a 100 K molecular weight cutoff centrifugal membrane and purified by size exclusion chromatography using Sephacryl HR-300 gel media.

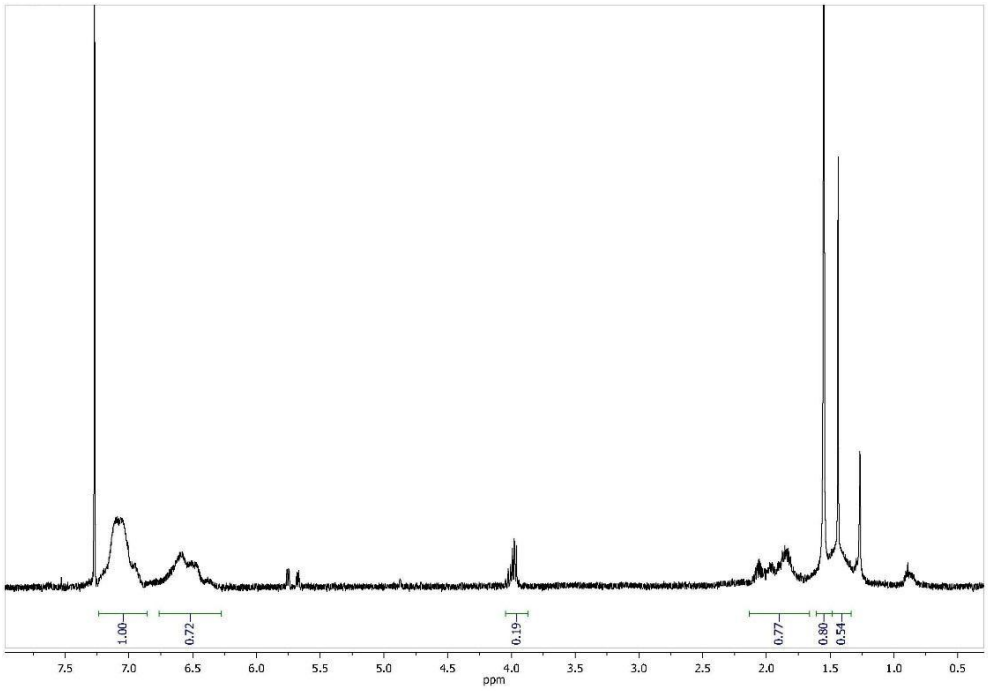


Figure S2: ^{1}H NMR spectrum of the fraction containing the desired product PS-NH_2-**2**.

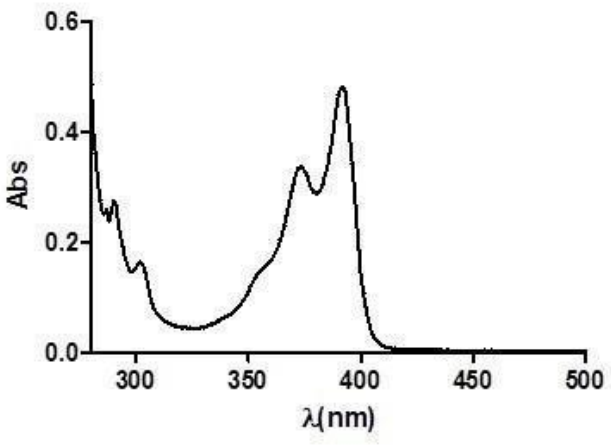


Figure S3: UV-vis absorption spectrum of the fraction containing the desired product PS-NH_2-**2**.

Estimation of the number of dioxetane 1 entrapped within each Pdot

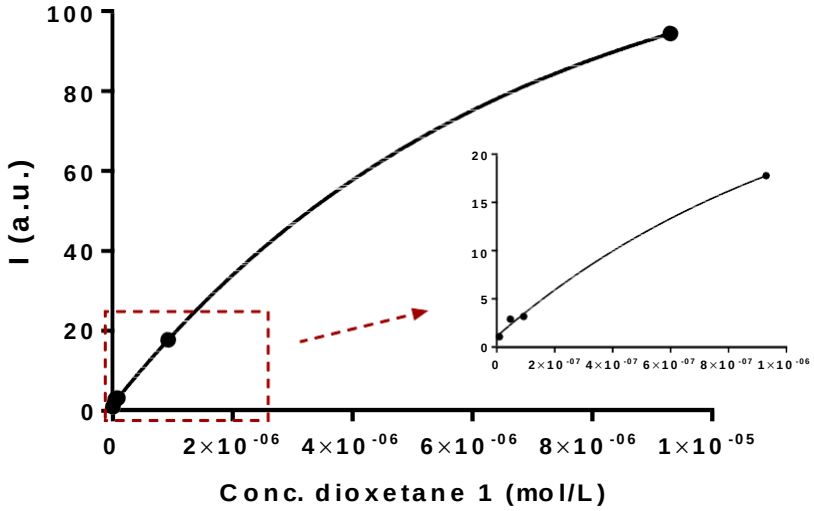


Figure S4: Calibration curve obtained from solutions of dioxetane **1** in THF, at different concentrations.

A calibration curve for the determination of compound **1** inside Pdots was obtained by acquiring the TCL emission from solutions of dioxetane **1** in THF at different concentrations (see Figure S4). Then, the total TCL signal generated by **1** (*i.e.* ketone **2** decomposed inside the NPs) was estimated heating a solution of TCL-Pdots at 110 °C and using a bandpass optical filter (centered at 425 ± 20 nm) during light acquisition through a CCD camera. The TCL signal intensity was corrected by both the area of the emission band and the FRET efficiency (53% and 80% respectively). Lastly, the concentration of

dioxetane **1** entrapped inside the nanoparticles was divided by the Pdots concentration (calculated from absorption measurements).

Estimation of FRET efficiency by TCL emission measurements

The light emitted by ketone **2** or CN-PPV (after FRET process) was collected separately, using two different bandpass optical filters, namely 425 ± 20 nm and 600 ± 40 nm. The first one was centered upon the emission of **2** while the 600 nm filter allowed us to acquire the emission from CN-PPV (see Figure S5).

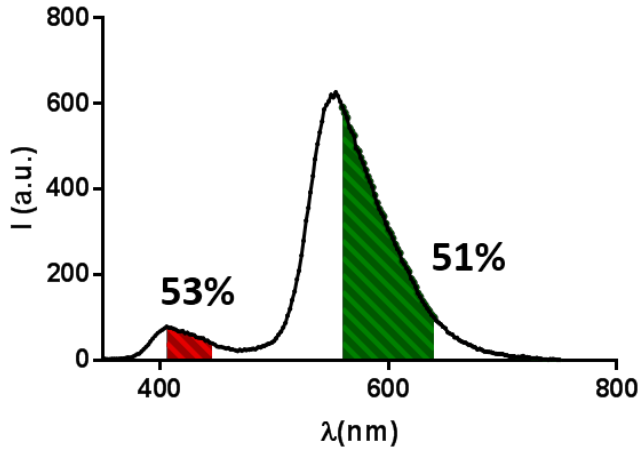


Figure S5: TCL emission spectrum acquired during thermal decomposition of 1,2-dioxetane **1** in presence of CN-PPV. The red and green colored areas represent the wavelengths range collected using the two band-pass optical filters.

Thus, 3 µL of TCL-Pdots-SA solution was spotted onto the mini-heating element (ceramic resistance connected to a power supply) and heated at 110 °C, while acquiring the TCL signal through a portable CCD camera. Both the heating pad and the CCD were placed inside a dark box, to avoid the background signal from the external ambient light. We acquired 30 min images to ensure a complete decomposition of **1** during the analysis, thus measuring the total photons produced. Then, the TCL emission intensities were corrected by the respective areas, namely 53% for ketone **2** and 51 % for CN-PPV, and the TCL signal at 600 nm was multiplied by a correction factor of 1.04, taking into account the different spectral response of the CCD's sensor ICX285 (Figure S6).[6]

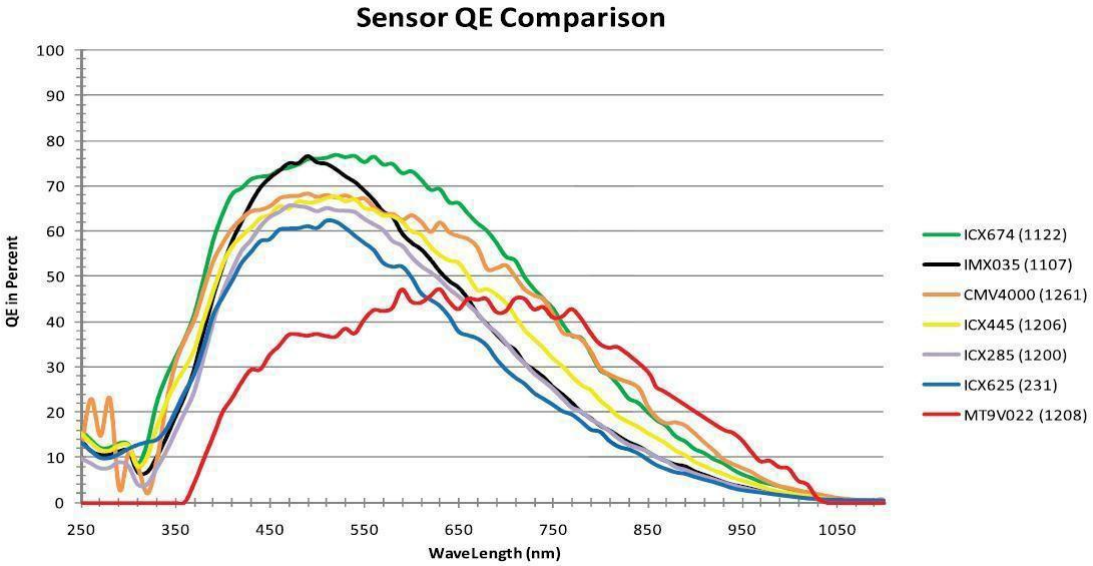


Figure S6: Spectral response of the CCD's sensor used as detector for FRET estimation.

Finally, the TCL intensities were divided by the respective fluorescence quantum yield (11% for Ketone **2** and 60% for CN-PPV) to calculate the total excited states produced by each specie, and the FRET process efficiency was estimated to be 80 %, which is in good agreement with the result obtained from lifetime experiments.

Determination of activation parameters (Ea and lnA)

In Table S1 are reported the kinetic constants of TCL emission decay, calculated at different temperatures.

Table S1: Kinetic constants for the thermal decomposition of 1,2-dioxetane **1**, occurring at different temperatures.

Temperature (°C)	lnK ± SD[a]
100	-7,11 ± 0,23
110	-6,18 ± 0,29
120	-5,81 ± 0,29
130	-4,88 ± 0,20

[a]Mean ± SD of three independent measurements.

TCL-based non-competitive sandwich-type immunoassay for detection of IgG

An increasing volume of Biotin-IgG (in the range between 0 and 34 μL) was added to a 100 μL Anti- IgG MBs solution in a 1.5 mL eppendorf, and the final volume was diluted to 500 μL using a PBS 1X buffer solution (pH = 7.4) with BSA 5% (w/v). Each sample was incubated at 37 °C for 2 hours. Then, MBs were held against the vial's wall using a magnet, washed three times (washing buffer = PBS 1X with Tween-20 0.1% (v/v)) to remove all the free Biotin-IgG, and 200 μL of TCL- Pdots-SA solution was added along with 300 μL of PBS 1X buffer solution (pH = 7.4) and BSA 5%. Biotin-IgG MBs were let incubating with TCL-Pdots-SA at 37 °C for additional 2 hours, after which MBs were washed three times and dispersed in the elution buffer (50 μL) for 15 minutes, at room temperature. The BSA was used to avoid any non-specific interaction between species, while the elution buffer (solution of Glycine, pH = 10) allowed us to effectively recover the TCL-Pdots-SA/Biotin-IgG MBs, as shown in Figure S7. In fact, the image taken before the elution step shows the presence of micrometric aggregates (MBs), while after using Glycine buffer, we obtained a well dispersed solution of TCL-Pdots- SA/Biotin-IgG.

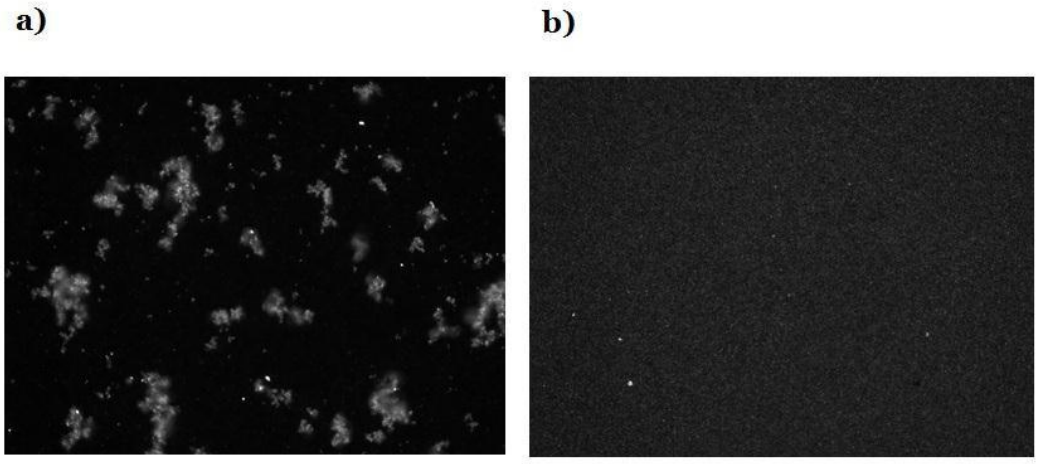


Figure S7: Images of TCL-Pdots-SA-Biotin-IgG MBs a) before and b) after the elution step.

The calibration curve was obtained heating 10 μL of the eluted solution up to 110 $^{\circ}$C and acquiring the TCL emission by a CCD camera.

References

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