Electronic Supplementary Material (ESI) for Chemical Communications

Plasmonic-driven Thermal Sensing: Ultralow Detection of Cancer Markers

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Supporting Information

1. Materials and General Experimental Methods

Hydrogen tetrachloroaurate (III) hydrate (99.9%) was supplied from Strem Chemicals. Sodium thiosulfate, N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride crystalline (EDC), N-hydroxysulfosuccinimide sodium salt (Sulfo-NHS>98%), Tetramethylbenzidine liquid substrate system for ELISA (TMB), Albumin from bovine serum (BSA 98%), Streptavidin from *Streptomyces avidinii* were purchased from Sigma Aldrich. Polyethylene glycols, α-mercapto-ω-carboxy polyethylene glycol ($HS\text{-}C_2H_4\text{-}CONH\text{-}PEG\text{-}O\text{-}C_3H_6\text{-}COOH$ MW = 5000 g/mol) and α-methoxy-ω-amino polyethylen glycol ($MeO\text{-}PEG\text{-}NH_2$ MW = 750 g/mol) were obtained from Rapp-Polymere. 2-(N-morpholino)ethanesulfonic acid (MES 98%) was purchased from Alfa Aesar. Carcinoembryonic antigen (CEA), and monoclonal mouse antibodies anti-CEA (capture antibody *MAb3C1* clone, detection antibody *MAb3C6* clone, detection antibody biotin-labelled *MAb3C6-biotin* clone, and detection antibody HRP-labelled *MAb3C6-HRP* clone) were purchased from HyTest. Column chromatography was performed on sephadex PD-10 desalting columns from GE Healthcare Life Sciences. Coomassie (Bradford) protein assay kit, for protein determination, was supplied from Thermo Scientific. Nitrocellulose membrane 0.05 μm was obtained from Millipore. UV-Vis absorption spectra were performed on a Varian Cary 50 spectrophotometer.

2. Synthesis of PEGylated NPRs

NPRs nanoparticles were synthesized as previously described elsewhere. Briefly, 100mL of $HAuCl_4$ 2 mM (aq) are mixed with 120 mL of freshly prepared 0.5 mM $Na_2S_2O_3$ for 9 minutes at RT under mild stirring conditions, prior to a second addition of a volume between 20-50 mL of $Na_2S_2O_3$ 0.5 mM, for tuning the LSPR in the range of 750-1075 nm. Prior to functionalization of NPRs with different proteins, NPRs were derivatized with HS-PEG-COOH (5000 g/mol). 10 mL (ca. 1.6 mg) of NPRs were supplemented with 1 mg of thiolated PEG. The pH of the solution was adjusted to 12 with a concentrated solution of NaOH. NPRs wee left overnight to react (ligand-exchange) under mild stirring conditions. Finally, the excess of reagents was removed washing PEGylated NPRs by centrifugation for 15 minutes at 10000 rpm and resuspending the pellets in Milli-Q water. Figure S1 shows a representative SEM micrograph and the corresponding UV-Vis-NIR spectrum.

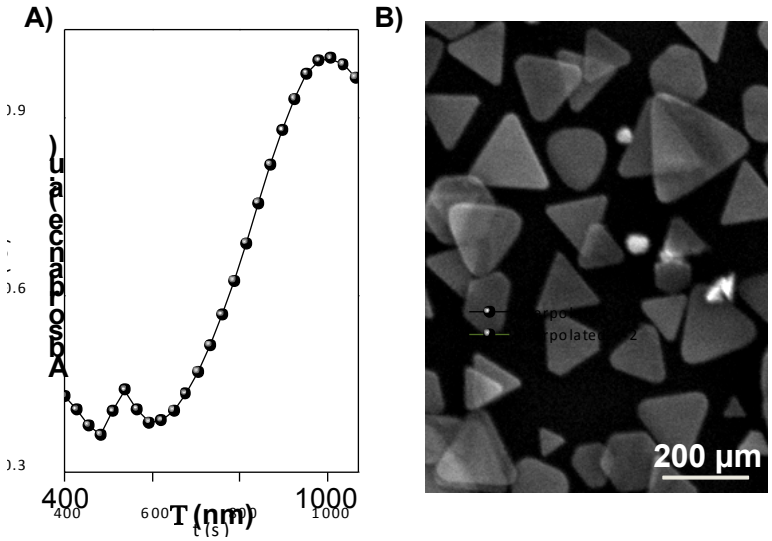

Figure S1. Characterization of PEGylated AuNPrs; (A) UV-Vis-NIR spectrum of PEGylated Au NPRs; (A) electron microscopy image of nanoprisms shows the triangular shape of the probe.

3. Derivatization of PEGylated-NPRs with proteins: antibody anti-CEA (NPRs@anti-CEA$_{det}$) and streptavidin (NPRs@streptavidin)

PEGylated NPRs were functionalized with antibody monoclonal mouse anti-CEA (detection antibody *MAb3C6*). Briefly, 0.5 mg of NPRs was incubated with 1 mL of an activation solution, i.e. 1.5 mM EDC and 3.5 mM Sulfo-NHS, in MES buffer 10 mM pH 6 for 30 minutes at 37ºC. The activated NPRs solution was run through a PD-10 column using 10mM MES pH 6 as the exchange buffer, to remove the excess of EDC/sulfo-NHS. Then, 2.5 µg of Ab anti-CEA$_{cap}$ (5 µg Ab per mg NPRs) was added to the activated NPRs and the mixture was stirred at 37ºC for 1 hour. Subsequently, the NPRs activated carboxylic groups were blocked with 50mM MeO-PEG-NH_2 MW 750, at 37ºC for 2 hours in a final volume of 1.5 mL. Finally, antibody-functional NPRs were then washed out of antibody excess by centrifugal precipitation at 6000 rpm for 5 minutes; then the pellets were resuspended in 1mL MES 10mM pH 6 and the NPRs@anti-CEA$_{det}$ were stored at 4ºC. The same protocol was followed to prepare NPRs nanoparticles derivatized with 1,25 µg of streptavidin (NPRs@streptavidin). The amount of antibody (or streptavidin) immobilized onto the NPRs surface was quantified by a fast colorimetric protein determination kit (Bradford assay).

4. Antibody and streptavidin biological activity of derivatized NPRs: NPRs@anti-CEA$_{det}$ and NPRs@streptavidin.

The biological activity of antibody and streptavidin immobilized onto NPs has to be assayed since protein conformation changes following immobilization can prevent their normal biological activity. A dot-blot assay was carried out to check the antigen-antibody and streptavidin-avidin recognition by means of functionalized NRPs (NPRs@anti-CEA$_{det}$ and NPRs@streptavidin). A drop of 2 µL of CEA or biotin labelled anti-CEA *(MAb3C6-biotin)* at different concentrations was spotted onto a nitrocellulose membrane. After the membrane was completely dried, 0.25 mL of blocking buffer (PBS buffer with 0.5% Tween and 5 mg/mL BSA) was added for 1 hour at 37ºC. Then, the blocked membranes were incubated (37ºC for 30 minutes) with 0.1 mL of 15 µg/mL NPRs@anti-CEA$_{cap}$ or NPRs@streptavidin. Finally, membranes were washed three times with PBS buffer with 0.5% Tween for 5 minutes and were left to air dry at room temperature. The membranes were place onto the thermosensitive support and were irradiated 1 minute with a fiber-coupled NIR laser (CW 1064 nm, irradiated area of *ca*. 1 mm^2, average intensity 115 W/cm^2). Imprinted thermal responses can be visualized clearly in Fig. S2, proving that the biological activity of proteins remains intact. Fig. S2 proves that derivatized NPRs (NPRs@anti-CEA (A) and NPRs@streptavidin (B)) can recognize specifically molecules of CEA or biotin-labelled anti-CEA captured in the nitrocellulose membranes, enabling detecting low concentration of the analyte (1 ng/mL). The structure of proteins (anti-CEA or streptavidin) immobilized onto the NPRs was not dramatically distorted as they still maintain their biological activity. The membrane without the analyte did not develop any signal because unspecific interactions between nanoparticles surface and the membranes were efficiently blocked. Moreover, the PEG coverage of NPRs prevented completely unspecific interactions between NPRs and other proteins immobilized in the membranes and thus, negative controls did not developed any signal on the thermosensitive surface after laser irradiation.

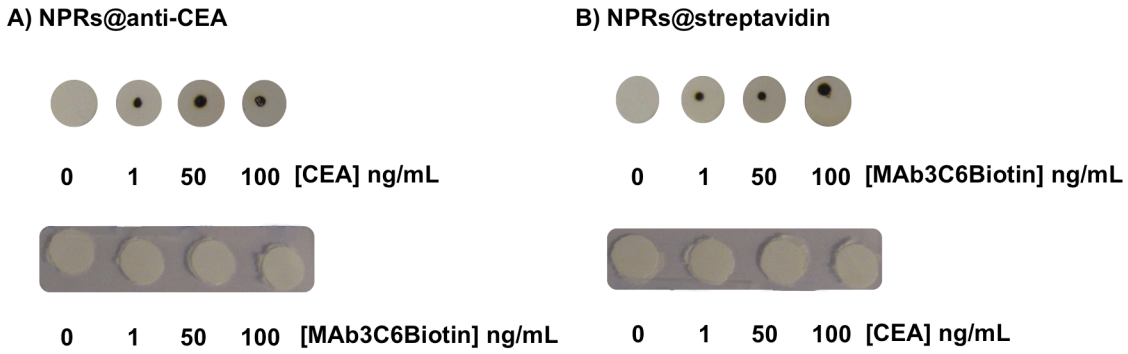

Figure S2. Experimental results using a direct immune-dot-blot assay to ensure the biological activity of the proteins attached to the NPRs: (A) membranes with different concentrations of CEA were incubated with NPRs@anti-CEA probing the antibody-antigen recognition after the antibody immobilization onto nanoparticle surface. Alternatively, in the lower panel, membranes with biotin-labelled anti-CEA *MAb3C6-biotin* were incubated with NPRs@anti-CEA as a negative control to check the specificity of the assay. (B) Membranes with different concentrations of biotin-labelled anti-CEA *MAb3C6-biotin* were incubated with NPRs@streptavidin probing the biotin-streptavidin recognition after the streptavidin immobilization onto the nanoparticle surface. Alternatively, membranes with CEA were incubated with NPRs@streptavidin as a negative control to check the specificity of the assay.

5. NPRs immunoassay

For strategy 1 (Scheme 1A): A coating solution of 50 µg/mL antibody anti-CEA (capture antibody *MAb3C1*) was prepared in bicarbonate buffer 50 mM pH 9.5 and 2 µL was spotted directly onto nitrocellulose membranes. After 15 minutes the membrane was blocked with 0.25 mL of BSA 5 mg/mL dissolved in PBS buffer with 0.5% Tween, at 37ºC for 1 hour. Then, the blocked membrane was incubated with 0.1 mL of sample (CEA) for 30 minutes at 37ºC. After the antibody-antigen recognition, the membrane was washed twice with PBS buffer with 0.5 % Tween for 5 minutes, followed by incubation at 37ºC for 30 minutes with 0.1 mL of functionalized nanoparticles NPRs@anti-CEA_{det} 20 µg/mL (dissolved in blocking buffer PBS with 0,5% Tween and 5 mg/mL BSA). Finally the membrane was washed four times with PBS buffer with 0.5 % Tween for 5 minutes and they were left to air dry at room temperature. The membranes were place onto the thermosensitive support and were irradiated 10 seconds with a fiber-coupled NIR laser (CW 1064 nm, irradiated area of *ca*. 1mm^2, average intensity 115 W/cm^2). The result was read visually for the appearance of a spot in the thermosensitive surface after the laser irradiation.

For strategy 2 (Scheme B): A coating solution of 50 µg/mL antibody anti-CEA (capture antibody *MAb3C1*) was prepared in bicarbonate buffer 50mM pH 9.5 and 2 µL was spotted directly onto the nitrocellulose membrane. After 15 minutes the membrane was blocked with 0.25mL of BSA 5mg/mL dissolved in PBS buffer with 0.5%Tween, at 37ºC for 1 hour. Then, the blocked membrane was incubated with 0.1mL of sample (CEA) for 30 minutes at 37ºC. After the antibody-antigen recognition, the membrane was washed twice with PBS buffer with 0,5% Tween for 5 minutes, followed by incubated at 37ºC for 30 minutes with 0.1 mL of detection antibody, biotin-labelled antibody *MAb3C6-bitoin* 2 µg/mL in PBS buffer with 0,5% Tween. Then, the membranes were rinsed twice with washing buffer (PBS + 0.5% Tween) and incubated at 37ºC for 30 minutes with 0.1 mL of functionalized nanoparticles NPRs@streptavidin 20 µg/mL (dissolved in blocking buffer PBS with 0.5% Tween and 5mg/mL BSA). Finally the membrane was washed four times with PBS buffer with 0,5% Tween for 5 minutes and were left to air dry at room temperature. The membranes were place onto the thermosensitive support and were irradiated 10 seconds with a fiber-coupled NIR laser (CW 1064 nm, irradiated area of *ca*. 1mm^2, average intensity 115W/cm^2). The result was read visually for the appearance of a spot in the thermosensitive surface after the laser irradiation.

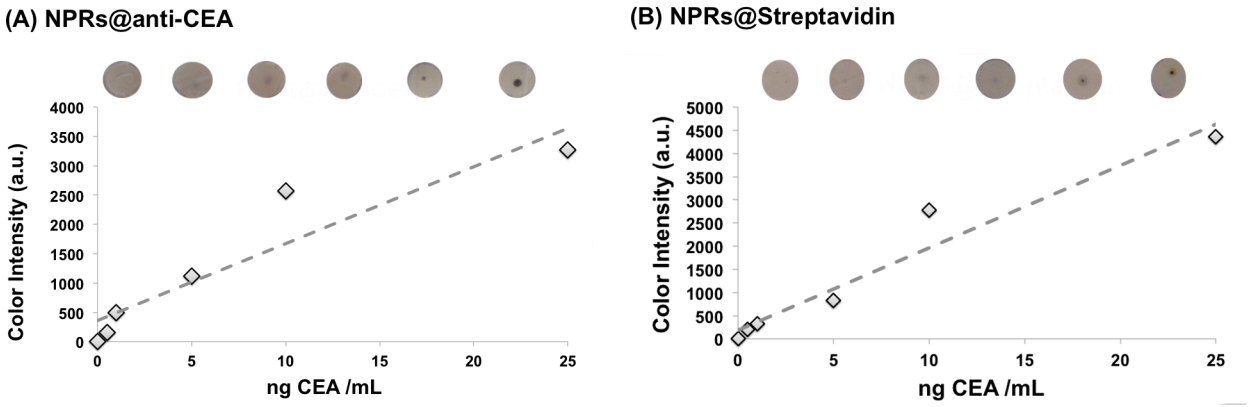

Figure S3. (A) Strategy 1, NPRs immunoassay for CEA detection: capture anti-CEA (*MAb3C1*) + CEA + NPRs@anti-CEA. (B) Strategy 2, NPRs immunoassay for CEA detection: capture anti-CEA (*MAb3C1*) + CEA + detection biotin-labelled anti-CEA (*MAb3C6-bitoin*) + NPRs@streptavidin. The colour intensity of the immune-dot blot was measured using Image J® software.

6. AuNPs immunoassay: negative control for Au NPs other than NPRs.

Spherical gold nanoparticles AuNPs (average diameter 14 nm, surface plasmon band at 519 nm (Fig. S4(A)) were synthesized following the Turkevich-Frens procedure and derivatized with polyethylene glycol (HS-PEG-COOH). NPs were functionalized with detection anti-CEA (*MAb3C6*) following the same protocol as for NPRs@anti-CEA. These AuNPs@anti-CEA were tested to detect CEA performing experiments equivalent to those performed with NPRs@anti-CEA (described in NPRs immunoassay, strategy 1). Fig. 4(B) shows that no signals were developed for different CEA concentrations, using AuNPs@anti-CEA as signal transducer, after the laser irradiation. This fact is due to AuNPs absorbs light at different wavelengths and thus, thermal signals can be only developed using NPs that absorb light at the same wavelength of laser emission.

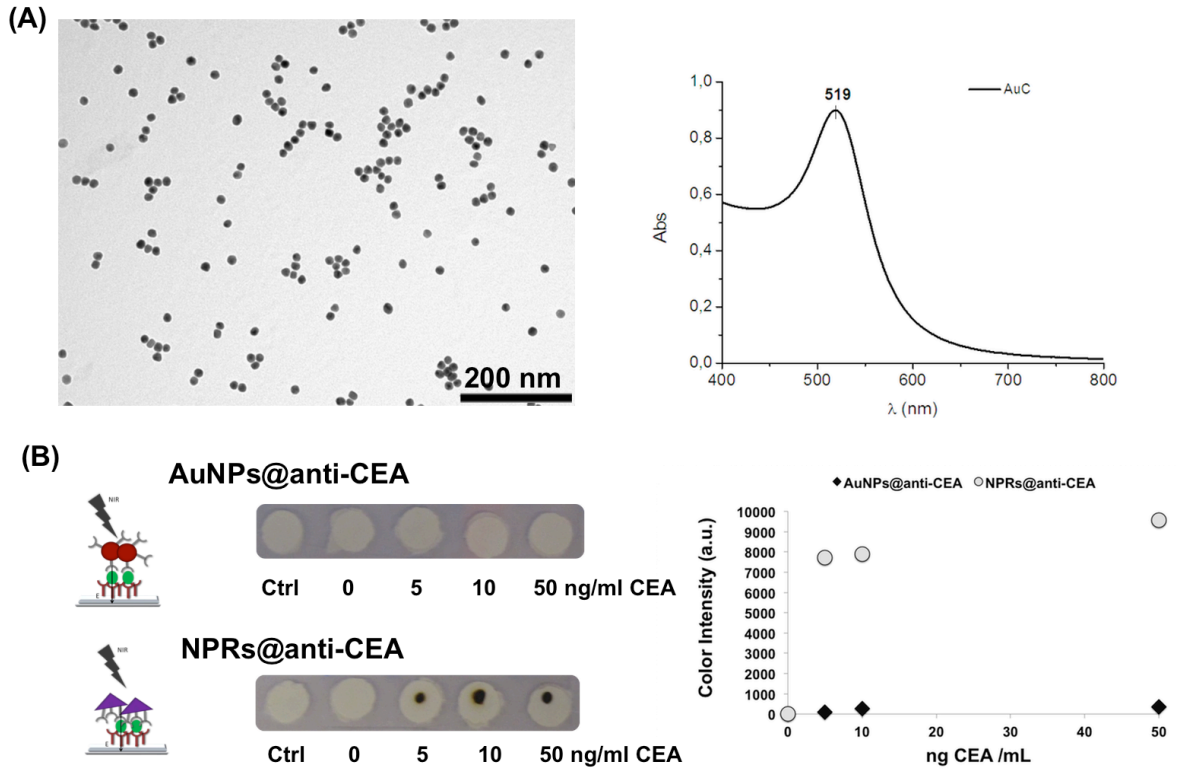

Figure S4. (A) SEM image and UV-Vis spectra of spherical gold nanoparticles (AuNPs average diameter 14 nm). (B) Comparative immune dot-blot assay for CEA detection using spherical gold nanoparticles AuNPs@anti-CEA (maximum absorbance at 519nm) and gold nanoprisms NPRs@anti-CEA (maximum absorbance at 1064nm). The colour intensity of the immune-dot blot was measured using Image J® software.

7. Dot-blot immunoassay

A coating solution of 50 μg/mL antibody anti-CEA (capture antibody *MAb3C1*) was prepared in bicarbonate buffer 50 mM pH 9.5 and 2 μL was spotted directly onto the nitrocellulose membrane. After 15 minutes the membrane was blocked with 0.25 mL of BSA 5 mg/mL dissolved in PBS buffer with 0.5% Tween, at 37ºC for 1 hour. Then, the blocked membrane was incubated with 0.1 mL of sample (CEA) for 30 minutes at 37ºC. After the antibody-antigen recognition, the membrane was washed twice with PBS buffer with 0.5% Tween for 5 minutes, followed by incubation at 37ºC for 30 minutes with 0.1 mL of detection antibody HRP-labelled anti-CEA (*MAb3C6-HRP*) 2 μg/mL (dissolved in blocking buffer PBS with 0.5% Tween and 5 mg/mL BSA). Finally the membrane was washed four times with PBS buffer with 0.5% Tween for 5 minutes and 0.05 mL of HRP substrate TMB was added. The membranes were shacked for 15 minutes until the colour appeared. This experiment aimed at proving the feasibility of colour image analysis to quantify signals on the membranes. Using this approach, we were able to detect CEA up to 50 ng/mL.

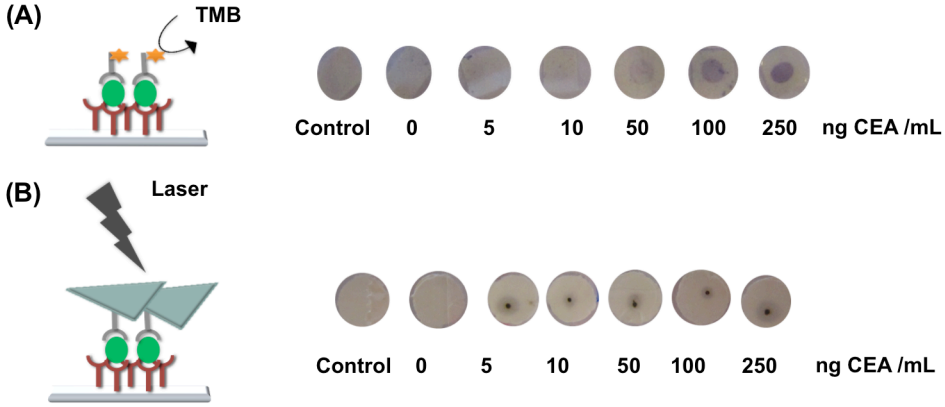

Figure S5. (A) Dot-blot immunoassay for CEA detection: capture anti-CEA (*MAb3C1*) + CEA + detection HRP-labelled anti-CEA (*MAb3C6-HRP*). (B) NPRs immunoassay for CEA detection: capture anti-CEA (MAb3C1)+ CEA + NPRs@anti-CEA.

8. ELISA assay

An ELISA assay, double antibody sandwich immunoassay, was used to validate the detection limit of capture anti-CEA (*MAb3C1*) and detection HRP-labelled anti-CEA (*MAb3C6-HRP*). A polystyrene microtiter plate was coated with 0.1 mL of capture antibody 5 μg/mL MAb3C1 dissolved in bicarbonate buffer 50 mM pH 9.6 and incubated overnight at 4ºC. The plate was emptied and blocked with 0.25 mL of blocking buffer (PBS with Tween 0.5% and 5 mg/mL of BSA) for 1 hour at 37ºC. After the plate was rinsed twice with 0.25 mL of washing buffer (PBS with Tween 0.5%), 0.1 mL of CEA samples at concentrations covering the range from 0 to 500 ng/mL were added in duplicates and incubated for 1 hour at 37ºC. Then, the plate was washed three times with 0.25 mL of PBS buffer with 0.5% Tween for 5 minutes, followed by incubated at 37ºC for 30 minutes with 0.1 mL of detection antibody, HRP-labelled antibody *MAb3C6-HRP* 1 μg/mL in PBS buffer with 0.5% Tween. Finally, the plate was rinsed three times with washing buffer (PBS + 0.5% Tween) and the HRP activity was measured. 0.1 mL of HRP substrate, tetramethylbenzidine liquid substrate system (TMB) was added per well, and optical densities were measured at 630 nm every 30s for 10 min. The calibration curve for CEA determination is displayed in Fig. S6. The linear range covered from 0 to 500 ng/mL with a regression equation of the form y = 0.1452x + 7.9914 and a correlation coefficient of 0.98991. The detection limit was calculated to be 28.7 ng CEA/mL at three times the background noise divided by analytical sensibility.

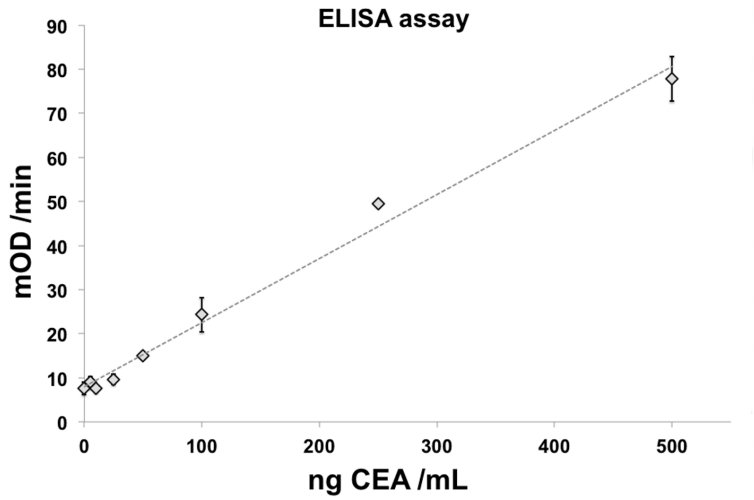

Fig. S6. ELISA sandwich assay for CEA detection (anti-CEA capture *MAb3C1* and HRP-labelled anti-CEA detection *MAb3C6-HRP*) LOD = 28.7 ng CEA / mL.

For the validation of this NPRs-based sensing technique, its limit of detection for CEA marker was compared with the experimental result using an ELISA immunoassay, shown Fig. S6 (LOD = 28,7 ng/mL). According to the experimental results obtained, the value of LOD for NPRs immunoassay (0.01 ng/mL) was about 3000 times more sensitive than the LOD of the enzyme-linked immunoasorbent assay.