

SUPPLEMENTARY INFORMATION

1. Experimental procedures

Procedure S1. Preparation of azide-functionalized MWCNTs

Firstly, MWCNTs were carboxylated. 25 mg of MWCNTs were suspended in 85 mL of deionized water and ultrasonically stirred for one hour. Then, 16 mL of 65% w/w nitric acid were added and the mixture was kept under reflux for one hour. Once cooled at room temperature, the solid was centrifuged at 4000 rcf for 10 minutes. Finally, the resulting cMWCNTs were washed with deionized water until washing liquids reached pH 7, and dried under nitrogen stream. Thereafter, carboxyl groups were activated; 20 mg cMWCNTs were suspended in 2.8 mL of 25 mM MES buffer, pH 6.5, containing 21.4 mg EDC and 23.8 mg NHSS, and magnetically stirred for two hours in the dark. For the preparation of azide-functionalized-MWCNTs, 4.5 μ L of 11-azide-3,6,9-trioxaun-decan-1-amine were added, and the mixture was magnetically stirred for 48 h in the dark. Then, the product was centrifuged at 4000 rcf for 10 minutes, and sequentially washed with 10 mM PBS of pH 7.0 and deionized water. Finally, the product was dried at 37 °C.

Procedure S2. Preparation of alkyne-functionalized IgG using succinimidyl-3-propiolate

In this step, the method described by Qi¹ was applied with slight modifications (Figure S1). Succinimidyl-3-propiolate was synthesized² by dropping 10 mL of a solution containing 1.14 g N,N'-dicyclohexylcarbodiimide (DCC) onto another solution containing 0.39 g of propiolic acid and 0.64 g of N-hydroxysuccinimide (NHS) in 30

mL of ethyl acetate. The resulting solution was magnetically stirred at 4 °C for 8 h. Next, the solution was filtered through a Büchner funnel and then, the solvent was removed using a rotary evaporator. The obtained residue was dissolved in 15 mL ethyl acetate and the resulting solution was transferred to a separatory funnel and sequentially washed by shaking 2 min each with 15 mL of a NaCl saturated aqueous solution (40 % w/w), 15 mL of a NH₄Cl saturated aqueous solution (60% w/w), and 15 mL of de-ionized water. After this, water traces from the organic solution were removed by addition of a small quantity of solid sodium sulfate and, once filtered, the solvent was eliminated from the solution using a rotary evaporator. The resulting product was recrystallized by dissolving repeatedly in the minimum volume of ethyl acetate, at 20°C. Finally, the solvent in the last solution was evaporated at 37°C in a rotary evaporator, and the obtained solid was stored in a vial at 8°C until used. The succinimidyl-3-propiolate was identified by NMR.

From the as obtained succinimidyl-3-propiolate, a 1 mg/mL solution was prepared in 250 mM carbonate buffer of pH 9.0. An aliquot of 250 µL from this solution was then mixed with 125 µL of a 2 mg/mL anti-rabbit-IgG solution and diluted with the same carbonate buffer up to 1 mL. The resulting solution was maintained for 24 h at 4 °C under continuous stirring. Thereafter, the solution was filtered through an Amicon® Ultra 10 K centrifuge filter by centrifugation at 1400 rcf and 8 °C for 10 min. Five washing steps were then applied using each time 500 µL of 10 mM PBS of pH 7.0 at 8°C, and centrifuge 10 min at 14,000 rcf, followed by turning the filter and centrifuge again 10 min at 1000 rcf. Finally, the obtained product was dissolved in 125 µL of 10 mM PBS of pH 7.0.

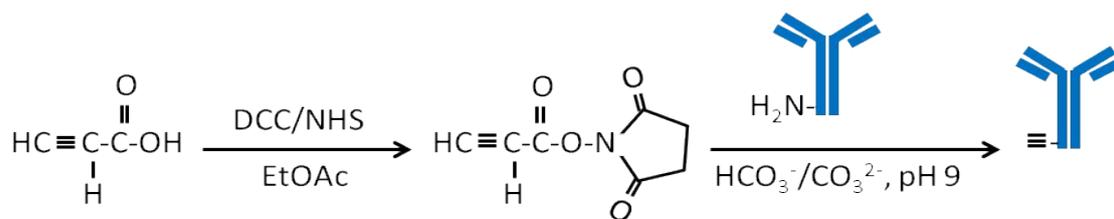


Figure S1. Preparation of alkyne-functionalized IgG using succinimidyl-3-propiolate

Procedure S3. Preparation of alkyne-functionalized IgG using sodium peryodate

The method described by Le³ was applied (Figure S2). Briefly, 50 μ L of a 20 mM sodium peryodate solution prepared in 0.1 M sodium acetate buffer (pH 5.5) were mixed with 800 μ L of a 2 mg/mL anti-rabbit-IgG solution and the resulting solution was maintained in an ice bath and in the dark for 30 min. Next, the mixture was filtered through an Amicon® Ultra 10 K centrifuge filter by centrifugation at 14,000 rcf and 4 °C for 15 min. Three washing steps were then applied using each time 500 μ L of Coupling Buffer at 4 °C, and centrifuge 15 min at 14000 rcf, followed by turning the filter and centrifuge 2 min at 1000 rcf. Finally, the resulting product was incubated for 2 h at 25 °C with 500 μ L of Coupling Buffer containing 8.8 mg of ethynyl hydrazide. Once filtered through another Amicon® Ultra 10 K centrifuge filter by centrifugation at 14,000 rcf and 4°C for 15 min, five washing steps with 500 μ L of Coupling Buffer were applied. Next, a last washing with 0.1 M PBS of pH 7.4 at 4°C was performed, and centrifuge 15 min at 14,000 rcf, followed by turning the filter and centrifuge 2 min at 1000 rcf. Finally, the obtained product was dissolved in 800 μ L of 10 mM PBS of pH 7.0.

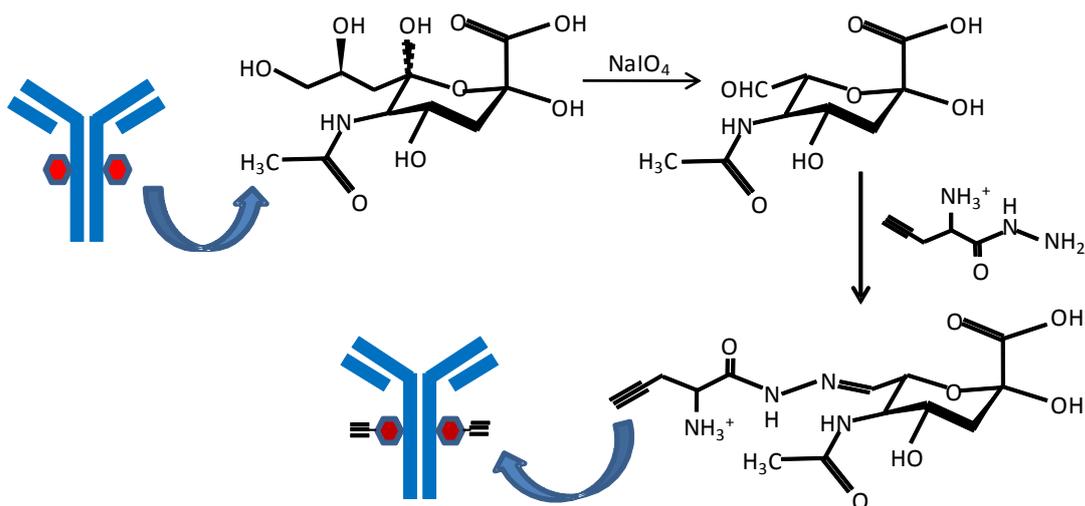


Figure S2. Preparation of alkyl-functionalized IgG using sodium peryodate

2. Characterization of the “click” synthesis by IR spectrophotometry

The IR absorption spectra obtained for the characterization of azide-functionalized-MWCNTs and the alkyne-anti-IgG binding are depicted in Figure S3.

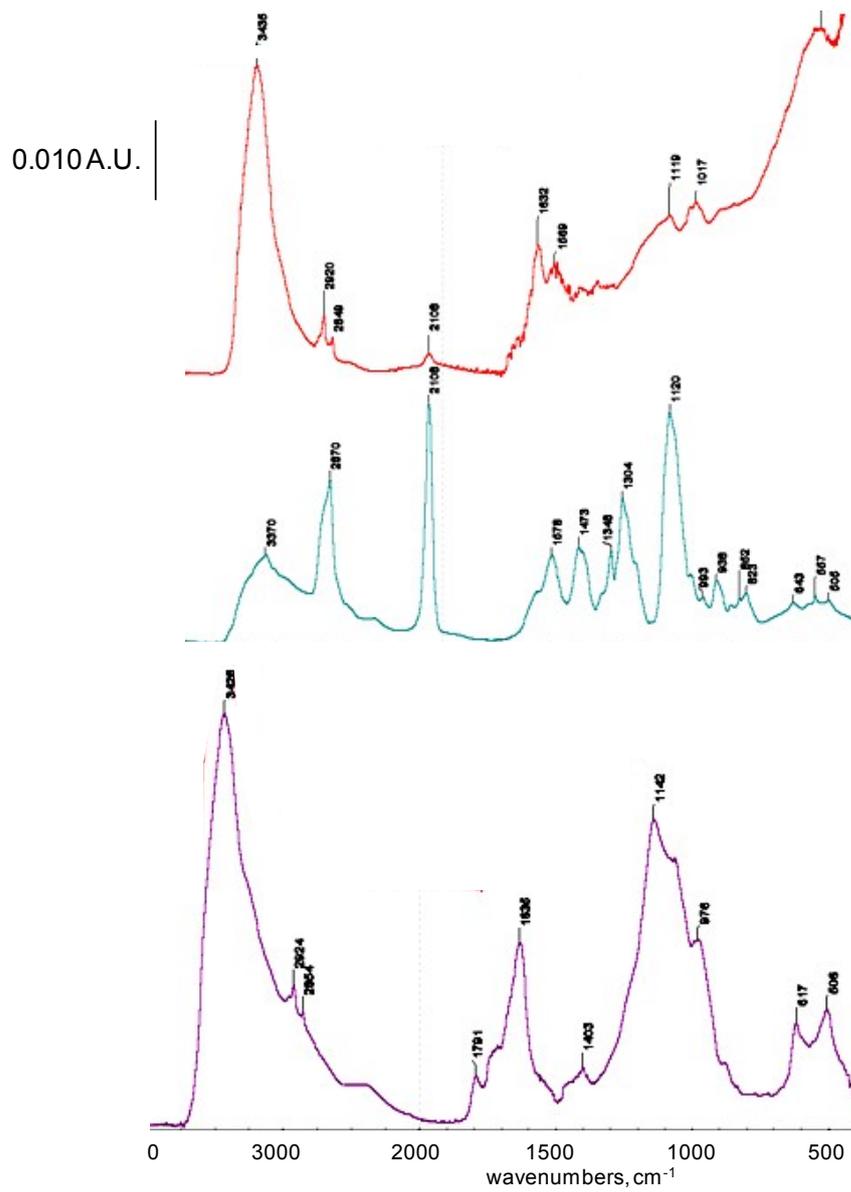


Figure S3. IR spectra of azide-MWCNTs (red), commercial azide (blue), and IgG-alkyne-azide-MWCNTs (purple).

3. Optimization of the variables involved in the preparation and performance of the immunosensor

a) Effect of the IgG-alkyne-azide-MWCNTs loading on the SPCE

The influence of the IgG-alkyne-azide-MWCNTs conjugate loading on the SPCE surface was evaluated by checking the amperometric responses obtained with different immunosensors prepared with conjugate contents of 2.5, 5.0, or 10.0 $\mu\text{g/mL}$ for 0 (unspecific) and 100 pg/mL TGF- β 1. The conjugate contents corresponded to the IgG concentration used for alkyne-functionalization and further conjugation with 0.25 mg/mL azide-MWCNTs. The immunosensors were prepared (Section 2.3.1) by sequential addition of the following solutions (5 μL each): IgG-alkyne-azide-MWCNTs, casein, 10 $\mu\text{g/mL}$ anti-TGF, 1 $\mu\text{g/mL}$ Biotin-anti-TGF, and 1/500 diluted poly-HRP-Strept. The obtained results are displayed in Figure S4a, showing that the largest specific-to-unspecific current ratio was obtained for 5 $\mu\text{g/mL}$ conjugate. Therefore, this value was selected for the preparation of the immunosensor.

b) Effect of the type of blocking agent

Three different substances commonly used as blocking agents were tested to minimize unspecific adsorptions onto IgG-alkyne-azide-MWCNTs/SPCE after anti-TGF immobilization. Blockers solutions prepared at the typical concentrations used in the fabrication of immunosensors: 25 % milk powder, 1 % casein and 5% BSA, in 0.1 M PBS of pH 7.4, were evaluated with the results shown in Figure S4b. Best specific-to-unspecific current ratio was obtained using casein as blocking agent solutions. 60 min incubation time was shown to be sufficient for an effective blocking.

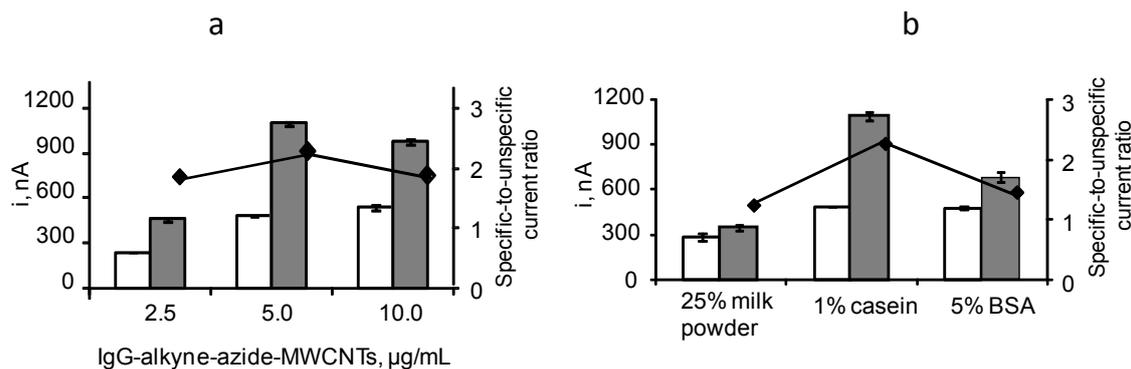


Figure S4. a) Effect of the IgG-alkyne-azide-MWCNTs loading on the SPCE: 5 μ L IgG-alkyne-azide-MWCNTs; 5 μ L 1% casein, 30 min. b) Effect of the type of blocking agent: 5 μ L blocking agent; 5 μ L 5 μ g/mL IgG-alkyne-azide-MWCNTs. 0 (white) or 100 (dark grey) pg/mL TGF- β 1; 5 μ L 10 μ g/mL anti-TGF, 60 min; 5 μ L 1 μ g/mL Biotin-anti-TGF, 60 min; 5 μ L 1/500 diluted poly-HRP-Strept, 20 min.

c) Effect of the type of enzymatic labeling of the detection antibody

Peroxidase-streptavidin polymer (poly-HRP-Strept) has been used as an efficient labeling tool to achieve electrochemical signal amplification in particular when compared with responses obtained with conventional HRP-Strept conjugates⁴. This comparison is shown in Figure S5a for Biotin-anti-TGF-TGF- β 1-anti-TGF-IgG-alkyne-azide-MWCNTs/SPCE immunosensors using HRP-Strept or poly-HRP-Strept as enzyme labels. As expected, remarkably larger currents were measured for TGF- β 1 when poly-HRP-Strept was employed as label because of the multiple HRP molecules in the polymer available to be involved in the biocatalysis of the enzyme substrate.

d) *Effect of the poly-HRP-Strept concentration.*

Figure S5b shows the dependence of the measured current with the poly-HRP-Strept dilution factor over the 1/250 to 1/2000 range. The specific response for 100 pg/mL TGF- β 1 decreased slightly with the dilution factor while the best specific-to-unspecific current ratio was obtained for a 1/500 poly-HRP-Strept dilution and, therefore, this value was selected for further work. Furthermore, an incubation time of 20 min was enough to get an effective binding with the biotinylated secondary antibody.

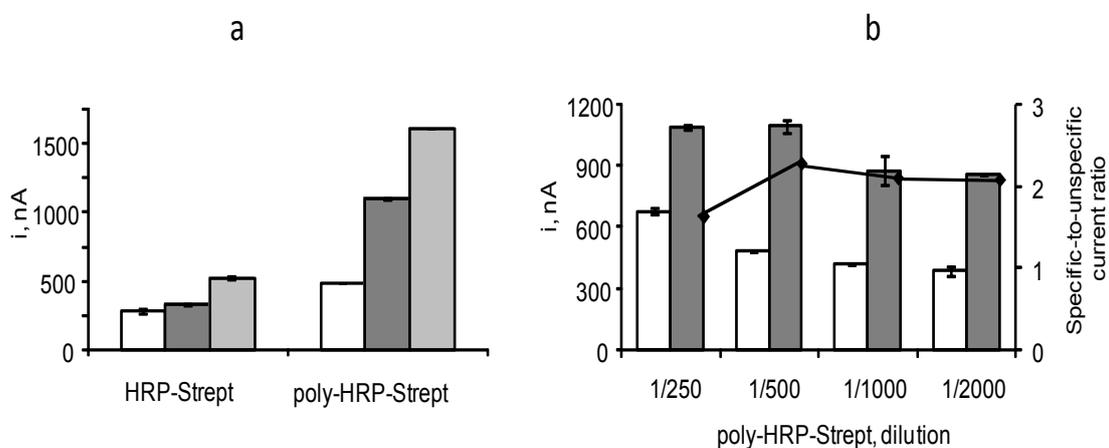


Figure S5. a) Effect of the type of enzymatic labeling of the detection antibody: 0 (white), 100 (dark grey) or 200 (light grey) pg/mL TGF- β 1; 5 μ L 1/2000 diluted HRP-Strept or 1/500 poly-HRP-Strept, 20 min incubation time. b) Effect of the poly-HRP-Strept concentration: 0 (white) or 100 (dark grey) pg/mL TGF- β 1; 5 μ L poly-HRP-Strept, 20 min. 5 μ L 5 μ g/mL IgG-alkyne-azide-MWCNTs; 5 μ L 1% casein, 30 min; 5 μ L 10 μ g/mL anti-TGF, 60 min; 5 μ L 1 μ g/mL Biotin-anti-TGF, 60 min.

e) *Effect of the anti-TGF loading on IgG-alkyne-azide-MWCNTs/SPCE*

The anti-TGF loading was optimized by measuring the specific and unspecific responses with capture antibody concentrations ranging between 5 and 20 μ g/mL.

Figure S6a shows as the largest specific-to-unspecific current ratio was obtained for 10 $\mu\text{g/mL}$. The decrease and further stabilization observed for specific responses at higher antibody concentrations are attributed to probable reaching of the saturation level. Accordingly, 10 $\mu\text{g/mL}$ anti-TGF was selected to construct the immunosensor. Regarding the incubation time for this step, 60 min were selected as an optimal value.

f) Effect of the Biotin-anti-TGF loading on TGF β 1-anti-TGF- IgG-alkyne-azide-MWCNTs/SPCE

The electrochemical responses measured with different immunosensors prepared with Biotin-anti-TGF concentrations ranging between 0.5 and 10 $\mu\text{g/mL}$ were also evaluated. Figure S6b shows as the measured current increased with increasing the Biotin-anti-TGF concentration between 0.5 and 1 $\mu\text{g/mL}$. Larger concentrations produced smaller responses suggesting saturation of the antibodies binding sites. Accordingly, 1 $\mu\text{g/mL}$ was chosen as the conjugate concentration for further work. Regarding the incubation time for this step, a period of 60 min was shown be enough to allow binding of all biotinylated antibodies to TGF- β 1 antigen.

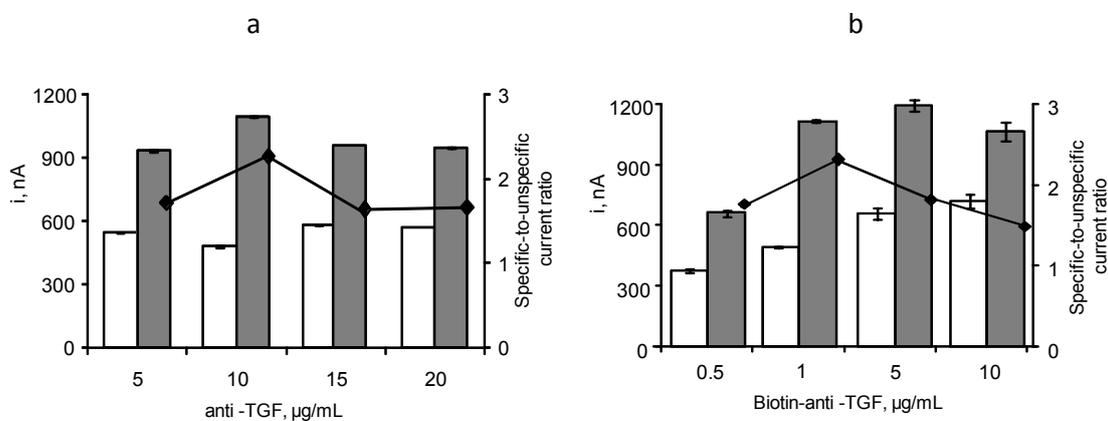


Figure S6. a) Effect of the anti-TGF loading on IgG-alkyne-azide-MWCNTs/SPCE: 5 μL anti-TGF, 60 min; 0 (white) or 100 (dark grey) pg/mL TGF- β 1; 5 μL 1 $\mu\text{g/mL}$ Biotin-anti-TGF, 60 min. b) Effect of the Biotin-anti-TGF loading on TGF β 1-anti-TGF- IgG-alkyne-azide-MWCNTs/SPCE: 5 μL 10 $\mu\text{g/mL}$ anti-TGF, 60 min; 0 (white) or 100 (dark grey) pg/mL TGF- β 1; 5 μL Biotin-anti-TGF, 60 min. 5 μL 5 $\mu\text{g/mL}$ IgG-alkyne-azide-MWCNTs; 5 μL 1% casein, 30 min; 5 μL 1/500 diluted poly-HRP-Strept, 20 min.

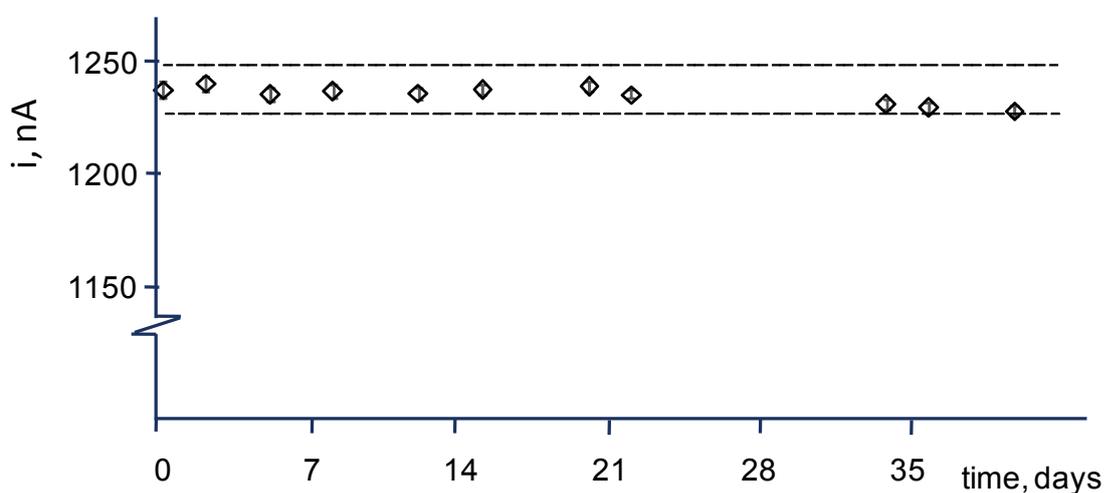


Figure S7. Control chart constructed to evaluate the storage stability of anti-TGF- IgG-alkyne-azide-MWCNTs/SPCE conjugates. Each point corresponds to the mean value of three successive measurements for 125 pg/mL TGF- β 1.

REFERENCES

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