- Supporting information -

Direct vs. mediated coupling of antibodies to gold nanoparticles: the case of salivary cortisol detection by lateral flow immunoassay

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EXPERIMENTAL SECTION

Materials

The monoclonal anti-cortisol antibody produced in mouse and the antigen (cortisol linked to bovine serum albumin, BSA-F) were kindly supplied by EuroClone Spa (Milano, Italy). The secondary anti-mouse immunoglobulin antibody, BSA, cortisol standard, and Staphylococcal protein A (SpA) were purchased from Sigma-Aldrich St. Louis, MO, USA).

Other chemicals were purchased from VWR International (Milan, Italy).

Millipore High Flow (HF) 180, absorbent cellulose pad and glass fiber probe pad were obtained from Merck Millipore (Billerica, MA, USA). The Whatman GF/DVA sample pad was obtained from Whatman International Ltd (Maidstone, England). The LFIA cassettes were furnished by Kinbio (China).

Gold nanoparticles: synthesis and characterization

Citrate-capped GNPs were synthesized according to the method of Turkevic and Frens [32]. Briefly, 1 mL of 1% w/v sodium citrate was added to 0.01% of boiling tetrachloroauric acid under vigorous stirring. The color of the solution changed gradually from light yellow to red thus confirming the successful formation of gold nanoparticles.

GNPs were characterized by UV-vis spectroscopy on a Varian Cary 1E spectrophotometer (Agilent Technologies, USA) and by transmission electron microscopy using a Jeol 3010-UHR (Jeol Ltd, Japan) high resolution transmission electron microscope (HR-TEM) equipped with a LaB6 filament operating at 300 kV and with an Oxford Inca Energy TEM 300 X-ray EDS analyzer. For TEM imaging, a drop of the GNPs aqueous suspension was put on a copper grid covered with a lacey carbon film for the analysis.

The GNP resulted almost spherical in shape, with a sharp SPR band centered at 525 nm. The mean diameter was calculated for TEM images, by averaging 180 nanoparticles and resulted to be 25 ± 4 nm.

Flocculation stress test

Conventionally, the optimal pH and amount of antibody for conjugation by adsorption should be established beforehand by the flocculation stress test [10].

The test consists in adding concentrated sodium chloride to GNP-antibody conjugates obtained by varying pH and GNP/Ab ratios. Sodium chloride is known to promote aggregation of not completely shielded GNPs, thus aggregation of a GNP-antibody conjugate upon addition of NaCl is considered as a proof of uncomplete shielding of the GNP surface by the antibody. And the best combination of pH and Ab/GNP ratio is defined as the one providing a stable GNP-Ab conjugate.

According to the stress test, the optimal conditions for conjugating the anti-cortisol antibody to GNPs were pH 8.0 and 10 µg of Ab per 1ml of OD1 GNP, respectively.

Fabrication of the LFIA device

The capturing antigen (BSA-F) and the secondary anti-mouse antibody (0.5 mg/ml) were applied to the nitrocellulose (NC) membrane to form the test and control lines, respectively. Reagents were dotted at 1 µL cm^{-1} by means of a XYZ3050 platform (Biodot, Irvine, CA, USA), equipped with BioJet Quanti™ 3000 Line Dispenser for non-contact dispensing, keeping a distance of 4 mm between the lines. The signal reporters (GNP conjugates) were absorbed onto the glass fiber conjugate pad previously saturated with GNP storage buffer. The pad was dipped into GNP-SpA solution (optical density 1) and dried for 3 hours at room temperature. NC membranes were dried at 37°C for 60 minutes under vacuum, layered with sample, conjugate and absorbent pads (Fig. 2), cut into strips (4.2 mm width) by means of a CM4000 guillotine (Biodot, Irvine, CA, USA) and

inserted into plastic cassettes to fabricate the ready-to-use LFIA device. Cassettes were stored in the dark in plastic bags containing silica at room temperature until use.

Stability

With the aim of evaluating the shelf-life of the GNP-Ab conjugates, accelerated ageing experiments were carried out [42.]. LFIA cassettes, including the three GNP-Ab conjugates were kept at 37°C for 7 days and tested on day 0, 1, 3 and 7. On each day, the running buffer (blank) and cortisol standard prepared in the running buffer at 1 and 10 ng ml^{-1} were analyzed in duplicate. LFIA devices were stored in the dark and with desiccant added.

Collection of human saliva samples

Eight adult subjects were recruited for the study, 3 males and 5 females, aged between 23 to 48 years. Healthy subjects were included, without applying any other exclusion or partitioning criteria. Participants to the study were kindly invited not eat, brush their teeth and do strong exercise in the 2 hours that preceded the saliva collection.

Saliva samples were collected at 1 pm by using the SalivaBio Children's Swab from Salimetrics (Salimetric LLC, CA, USA) and following supplier's instructions. In details, each subject was requested to rinse the mouth with water, wait for 10 minutes and then put the swab under the tongue for 3 minutes. The swab was placed in the upper part of the collector, immediately refrigerated at -20°C for at least 24 hours. After thawing, saliva was recovered by centrifugation of the swab (15 min at 2000 x g) and immediately analyzed.

Reference values of cortisol in saliva samples were measured by a commercial enzymatic immunoassay kit produced by Salimetrics (CA, USA) [34].

Saliva samples used for preliminary comparison within the different GNP conjugates were collected from a single healthy adult subject. Samples were collected in different days (at 1 pm) as

described above and pooled for allowing the execution of several assays while minimizing matrix variability.

Coupling mediated by secondary antibody: preparation of the GNP-2ndAb-Ab conjugate

The performance of the new conjugate were checked and compared to those of the GNP conjugates previously described.

FIGURES

Figure S1 UV-vis spectrum (a) and TEM image (b) of gold nanoparticles obtained through the citrate reduction method.

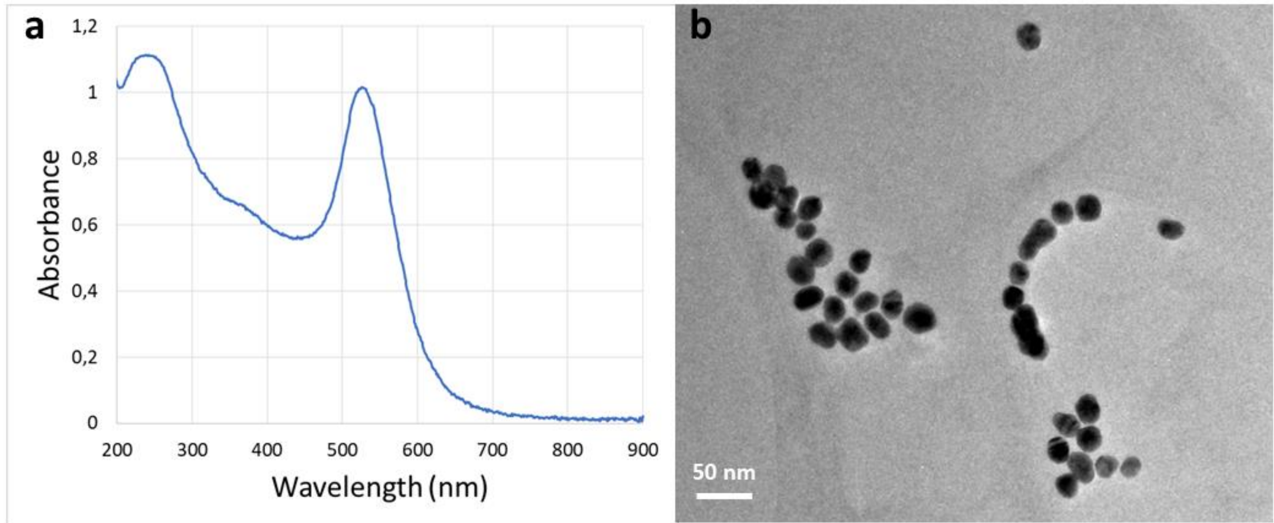

Figure S2. Optimization of quantity of anti-cortisol antibody to be coupled to GNP via covalent linking. Increasing the antibody amount led to increase the absolute signals (a), whereas the sensitivity of the LFIA (b) decreased. The quantity of 1 μg was chosen as the best compromise.

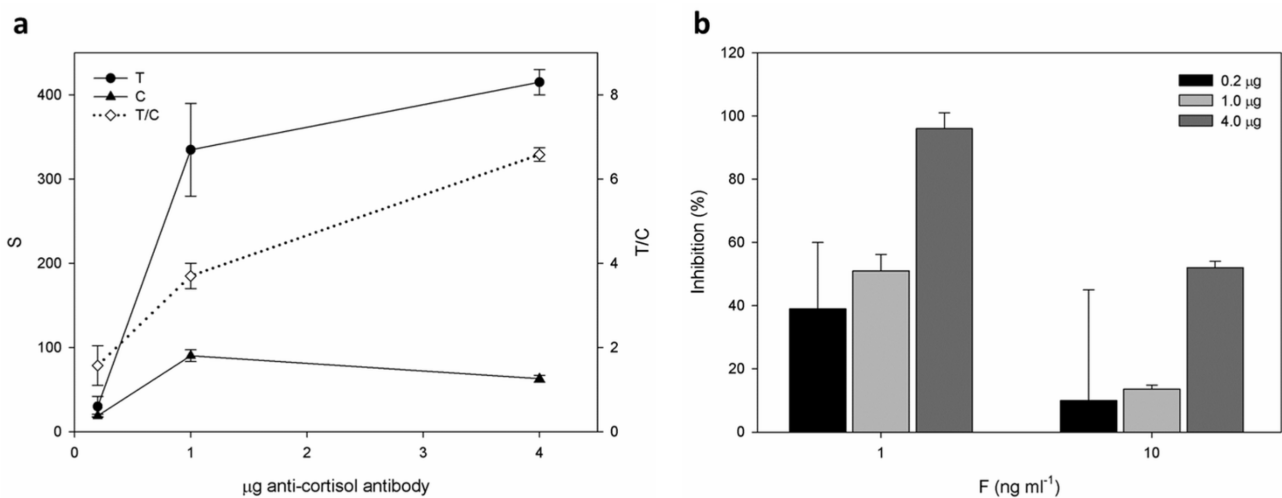

Figure S3. Typical calibration curves obtained from the LFD with: (a) the probe with covalently attached antibody (GNP-*cvt*-Ab); and (b) the dual-layer probe (GNP-*SpA*-Ab). The ratio T/C was plotted towards cortisol concentration. Cortisol standards were prepared in the running buffer.

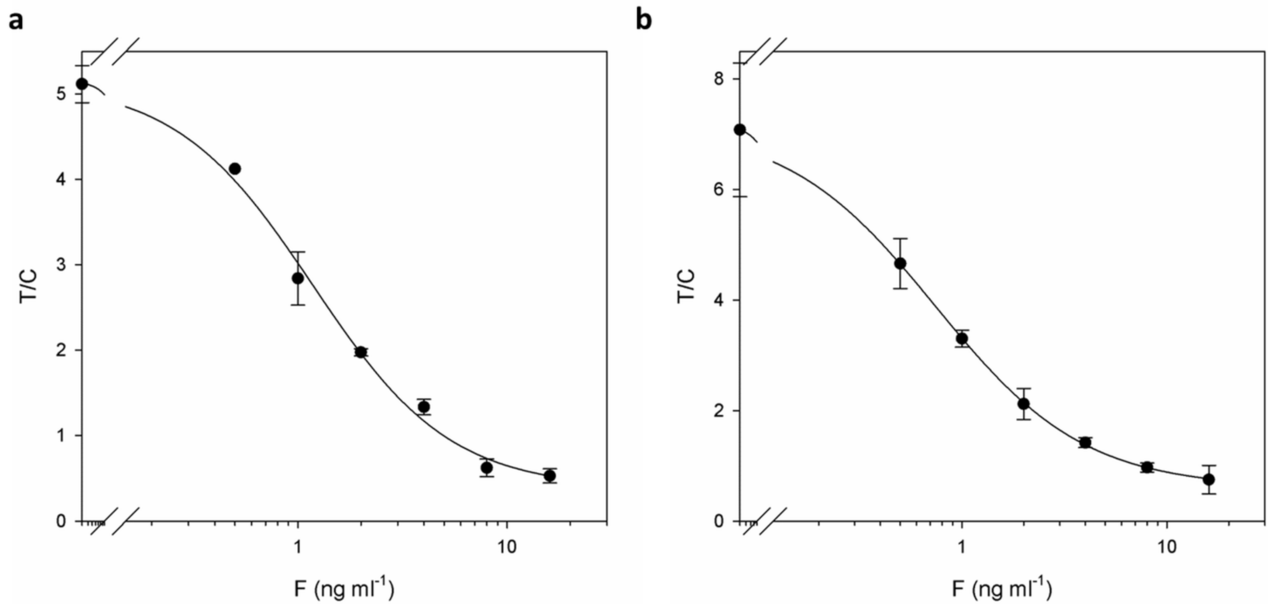

Figure S4. Effect of the SpA amount on absolute signals (a) and sensitivity of the LFIA (b) employing the probe with SpA-mediated attachment of the antibody to GNP (GNP-*SpA*-Ab). The amount of the antibody was kept constant at 1 μg.

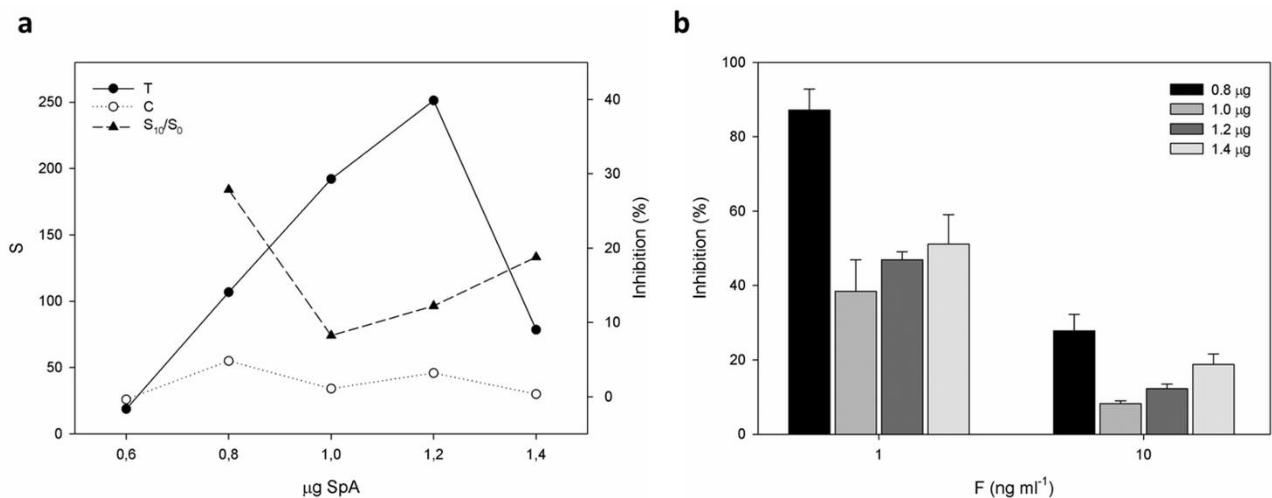

Figure S5. Stability at 37°C of the LFDs including probes prepared by direct adsorption (GNP-*ads*-Ab_1), and by mediated coupling via chemical (GNP-*cvt*-Ab) or biochemical (GNP-*SpA*-Ab) layers: normalized signal (T/C) of the running buffer (S_{blank}) (a); and inhibition rate measured by adding 1 ng ml^{-1} (solid symbols/lines) and 10 ng ml^{-1} (open symbols/ dashed lines) of cortisol to the running buffer (b).

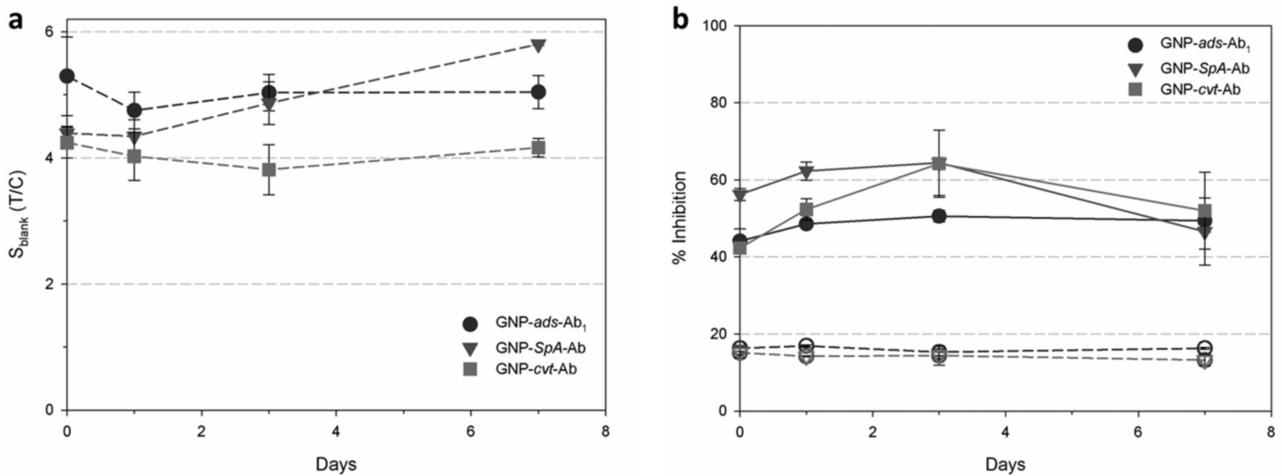

Figure S6. Signals (S) generated at the test (T) and control (C) lines for a pooled saliva sample collected at 1 pm from an adult volunteer (a); and inhibition rate measured by fortifying the saliva with increasing amounts of cortisol (b). Comparison of results obtained in LFDs including probes prepared by direct adsorption (GNP-*ads*-Ab_1), by mediated coupling via chemical (GNP-*cvt*-Ab) or biochemical (GNP-*SpA*-Ab) layers.

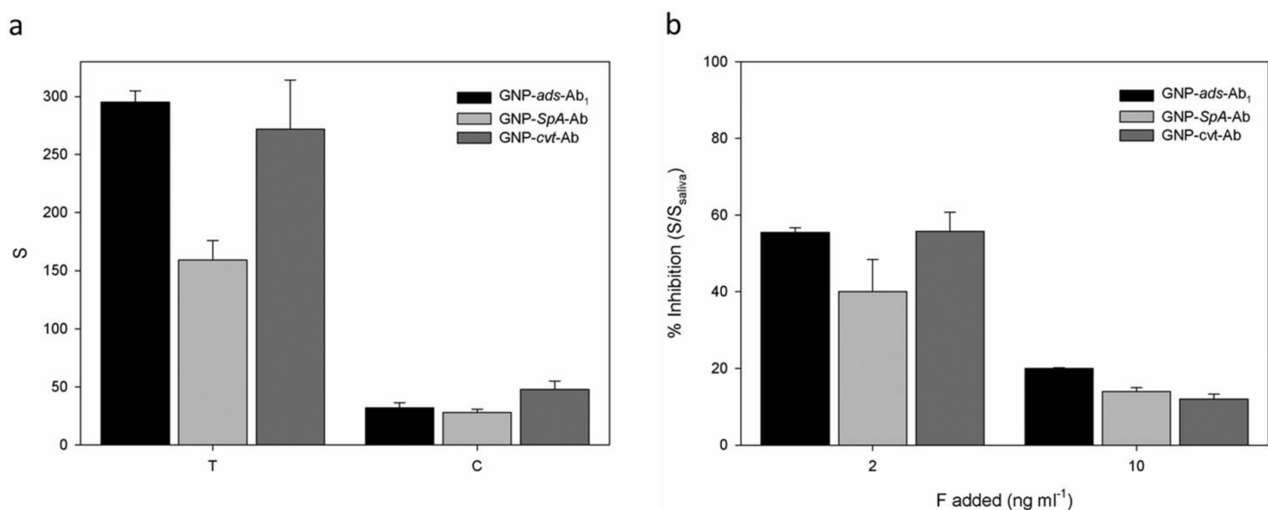

Figure S7. Signals (S) generated at the test (T) and control (C) lines for the running buffer (a) and a pooled saliva sample collected at 1 pm from an adult volunteer (c); and inhibition rate measured by fortifying the running buffer (b) and the pool of saliva with increasing amounts of cortisol (d). Comparison of results obtained in LFDs including probes prepared by mediated coupling employing SpA (GNP-*SpA*-Ab) and a secondary antibody (GNP-*2ndAb*-Ab) biochemical mediators.