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* Supporting Information

ABSTRACT: Stable and efficient conjugates between antibodies and gold nanoparticles (GNP-Ab) are sought to develop highly sensitive and robust biosensors with applications in medicine, toxicology, food safety controls, and targeted drug delivery. Several strategies have been proposed for directing the antibody attachment to GNPs thus preserving antibody activity, including covalently coupling the antibody to a polymer grafted on GNP surface and exploiting the high affinity of bioreceptors as mediators for the binding. Both approaches also allow for shielding GNPs with a protective layer that guarantees the robustness of the conjugate. Notwithstanding, antibodies freely adsorb to GNP with high binding efficiency. The nonspecific adsorption is far more simple, fast, and inexpensive than any mediated coupling. Therefore, it is preferred for most applications, although it is considered to produce GNP-Ab with a limited activity. In this work, we compared three strategies for producing GNP-Ab, such as (i) covalent coupling mediated by a chemical layer, (ii) affinity-based binding mediated by a biomolecular layer composed of Staphylococcal protein A, and (iii) direct attachment via adsorption. The so-prepared GNP-Ab were employed as probes in a colorimetric lateral flow immunoassay (LFIA) for measuring salivary cortisol as a model biosensor that relies on the use of active GNP-Ab conjugates. Unexpectedly, the biosensors fabricated using the three probes were completely comparable in terms of their ability to measure salivary cortisol. Furthermore, we observed that the sensitivity of the LFIA primarily depended on the amount of the antibody bound to GNPs rather than on the method by which it was bound. The probes prepared using both the direct adsorption approach and mediated coupling via the biochemical mediator enabled development of point-of-care devices for the fast, sensitive, and reliable measurement of human salivary cortisol.

KEYWORDS: biosensor, point-of-care testing, colloidal gold, Staphylococcal protein A, InnovaCoat GOLD, antibody conjugation, antibody labeling, colorimetric LFIA, Immunochromatographic Test

INTRODUCTION

Lateral flow assay (LFA) is among very rapidly growing strategies for point-of-care (POC) testing. It owes its popularity to several benefits, including on site usability, rapidity, user-friendly format, long-term stability that, moreover, does not require special storage conditions, and relative cost efficiency. The lateral flow assay technology is also very versatile as it combines a number of variants such as test formats, recognition elements, signal reporters, and detection systems. These features make LFA particularly attractive in different fields, including clinical, veterinary, food safety, forensics, and environmental analysis.

Although nucleic acid-based receptors are increasingly employed as recognition elements,[1,2] most existing LFAs exploit the unique properties of the antigen−antibody interaction to enable highly sensitive and selective analysis (lateral flow immunoassay, LFIA).[4−6] Several labels and

detection systems are used in LFIA, including fluorescent, chemiluminescent, and enzymatic probes.[2−4] Nevertheless, the most popular detection is the visual one based on colored probes, because its simplicity is ideally suited for on-site operation. Colloidal gold nanoparticles (GNPs) are the most widely employed probes in color-based (visual) lateral flow assays, due to several benefits of this kind of label. GNPs are (i) highly colored (i.e., the superficial resonant plasmon of GNPs with diameters between 20 and 40 nm strongly absorbs in the vis region and makes them appear red); (ii) easily and cost-efficiently produced as monodisperse nanoparticles of controllable dimensions; (iii) biocompatible; (iv) able to conjugate directly to biologically active molecules by noncovalent and covalent attachment, with, in addition, easy purification and easy verification of successful conjugation. The choice of the probe strongly affects the sensitivity of the LFIA, not merely as the effect of the intrinsic properties of the

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detection system employed. The efficiency of the binding between the recognition element and the signal reporter, the preservation of the molecular recognition properties, and the directionality of the conjugation are among factors ultimately affecting the characteristics of the assay.

Colloidal gold nanoparticles to be employed in LFIA development are routinely synthesized by tetrachloroauric acid reduction with sodium citrate that also stabilizes gold nanoparticles by preventing their aggregation.[7] Citrate-capped GNPs are relatively stable in low-ionic strength media, thanks to the electrostatic repulsion of the citrate capping; however, they tend to aggregate upon salt addition. The conjugation of biologically active molecules to GNPs is realized via covalent and noncovalent linkages.[8,9] Although it is generally admitted that approaches based on noncovalent binding have several weaknesses, the simplicity and rapidity of the GNP-antibody (GNP-Ab) conjugation via noncovalent binding explain the persistent utilization of this approach in the preparation of LFIA probes. Proteins (and particularly, immunoglobulins) spontaneously adhere to the surface of colloidal gold nanoparticles capped by citrate through several types of noncovalent interactions.[8] Therefore, the direct adsorption of antibodies onto citrate-capped GNPs can be exploited to prepare GNP-Ab probes. Usefully, antibodies adsorbed onto the nanoparticle surface create a layer of proteins that significantly contributes in stabilizing the colloid and protect it from aggregation. Conventionally, therefore, the optimal conditions for the preparation of GNP-Ab probes (i.e., pH and antibody/GNP ratio) are decided upon GNP stabilization.[10] However, the group of Dzantiev demonstrated that the optimal antibodies/GNPs ratio for achieving high sensitivity of the LFIA is not always equal to the stabilizing concentration.[11,12] Similarly, we found that reducing the amount of the antibody (compared to the amount required for stabilizing GNPs) strongly increased assay sensitivity.[13,14] In those works, we applied a dual layer strategy for GNP-antibody conjugation: first, we coated GNPs with a secondary antibody for

stabilization, and then exploited the secondary antibody to bind to the specific antibody, which was used at the concentration that guaranteed optimal sensitivity.

Covalent linking of antibodies to GNPs has been shown to overcome major limitations of the noncovalent single layer approach. The covalent coupling of antibodies is attained by means of superficial functional groups that were inserted previously on GNPs. Typically, bare GNPs are reacted with short heterobifunctional linkers provided with a thiol, which is sticky and adheres to the gold surface. The linker forms a protective layer that shields the GNP and yields a chemical functionality, which allows for the subsequent reaction with amino-, carboxyl-, or glycosyl- groups of antibodies.[8,9,15−17] Advantages of the covalent over the noncovalent coupling approach are that (i) generally, the covalent binding requires fewer antibodies; (ii) there is a lower risk of GNP aggregation during conjugation; (iii) the binding is not dependent on pH and ionic strength of the conjugation buffer; (iv) the binding mechanism is known so there is no need to carry out multiple trial conjugations; and (v) different cross-linking strategies can be applied according to particular requirement (Table 1). Furthermore, the covalent approach allows for orientation of

Table 1. Comparisons of Requirements and Attributes of GNP-Ab Probes Prepared According to Different Strategies

	covalent coupling to chemical layer	dual layer	(SpA-mediated) direct attachment by adsorption
risk of aggregation during coupling	low	low	high
dependence of binding efficiency on environment	not dependent	not dependent	salt and pH dependent
need to carry out multiple trial conjugations	no	yes	yes
antibody amount required	low and f ixed	from low to high	high and dependent on GNP stability rather than on required sensitivity
orientation of coupling	oriented	oriented	random

Figure 1. Strategies for coupling antibodies to gold nanoparticles: a) through direct adsorption; b) by covalent attachment to functional groups of polymers that adhere to GNP surface via S-G interaction; and c) by covering GNP with a protein receptor (Staphylococcal protein A, SpA), which then binds to the antibody.

the antibodies while direct adsorption produces GNP conjugates with randomly oriented antibodies (Figure 1a, b). Mustafaoglu et al. reported an interesting “reverse” approach, in which the antibody was modified by site-specific mutagenesis to incorporate a high-affinity tag for the subsequent binding with the gold surface. In this way, the tag predominantly interacted with GNPs and oriented the antibodies on their surface.[18] The limitation of the approach is clearly the need for genetically modifying the antibody, which is rarely feasible and largely cost-demanding.

A third route surpassing the dualism between random direct adsorption and oriented covalent coupling of the antibody to gold nanoparticles is represented by the use of a mediator, such as in the dual layer approach discussed above.[13,14,19,20] In this approach, the mediator is a protein that is randomly adsorbed onto GNPs to form an underlying biochemical layer. The biochemical layer stabilizes GNPs and provides anchoring points for the following attachment of the antibody (Figure 1c). In theory, most of the benefits reported for the covalent coupling of antibodies to GNPs are also valid for the dual layer strategy (Table 1). As a viable alternative to the use of secondary antibodies, Tripathi and Driskell reported the use of

staphylococcal protein A (SpA) to form the stabilizing layer.[21] They compared the activity of antibodies attached to GNPs by direct adsorption and by SpA-mediated binding. According to their measurement, only ca. 25% of the passively adsorbed antibodies were able to bind to the antigen, whereas, remarkably, more than 90% of the antibodies bound through protein A were available for the binding. SpA-linked GNPs have already been employed as probes in LFIA, used as a point of care diagnostics of infectious diseases.[22,23] In this application, the probe assured versatility to the assay, mainly because of the capability of the receptor to recognize immunoglobulins from different species.

To confirm advantages of approaches based on the oriented over random attachment of antibodies to GNPs and to compare covalent with protein A-mediated coupling, we prepared three GNP-Ab probes and compared their performance in a model LFIA. To this aim, we developed a facile and rapid LFIA for measuring cortisol levels in human saliva. Cortisol (F) is a glucocorticoid hormone secreted by the adrenal cortex in response to physical and psychological stress. F is regulated by the hypothalamus−pituitary−adrenal axis, and has effects on virtually all tissues and processes in the human body.[24] Abnormal increases in cortisol levels inhibit inflammation, depress the immune system, and increase fatty and amino acid levels in blood. Hypo- and hyper-cortisolism are measured in the clinical practice to diagnose endocrine disorders.[25] However, the most dominating effect on cortisol variation comes from psychological/emotional stress, which is why cortisol is popularly called the “stress-hormone”.[26] Cortisol levels are measured in plasma, urine, saliva, and hair. Since saliva can be obtained stress-free and independently from medically trained personnel, salivary cortisol has been regarded as a reliable tool for physiological stress detection.[27] Levels of salivary cortisol are routinely measured by

Figure 2. Schematic of the LFD for measuring salivary cortisol: saliva is applied in correspondence of the sample pad, flows through the probe pad (where it suspends the probe, i.e., the conjugate between the anticortisol antibody and GNPs) and through the migration membrane toward the adsorbent pad. The probe first encounters the salivary cortisol (F) and binds to it. The unbound probe is captured at the test line by the immobilized antigen (BSA-F) (a). The excess probe reaches the control line, where both unbound and bound probes are captured by the secondary antibody, and two colored lines form (b). The increase of salivary cortisol in the sample inhibits the binding of the probe to BSA-F, and the color of the test line decreases proportionally until disappearance (c).

immunoenzymatic assays, such as Enzyme-linked Immunosorbent Assay (ELISA).[28] However, efforts are being made to develop analytical devices to quantify stress and related abnormalities in environmental condition. Furthermore, F secretion follows a circadian rhythm, and several other subjective factors can affect its levels (i.e., eating patterns, physical activity, etc.). Hence, the need for point-of-care detection has been highlighted as essential to provide personalized healthcare.[26,29]

The schematic of the lateral flow device (LFD) developed in this work to measure F is depicted in Figure 2 and comprises (from bottom to top): the sample pad, which is the region that comes into contact with the sample; the probe pad, which holds the GNP conjugate; the migration membrane, where the immuno-reactions needed to execute the test take place; and the adsorption pad, which helps the flow of the samples and

reagents. The LFD was initially developed by using the probe with covalently linked antibodies. The performance of other probes (obtained by direct adsorption and by the dual layer strategy) was then evaluated by applying them to the same device, so that only the way of preparation of the probe varied among experiments. Using efficient bioreagents (the anticortisol antibody and the capturing antigen, i.e., cortisol-BSA) and materials (especially, a sample pad suitable for saliva),[30] we were able to investigate the properties of the different GNP-Ab conjugates and, moreover, evaluating how and how much these differences reflected in the performance of the assay. We also developed a colorimetric LFD for measuring salivary cortisol with potentially useful application for the onsite monitoring of stress in different contexts.

EXPERIMENTAL SECTION

Covalent Conjugation of Anticortisol Antibody to Innova-Coat GOLD (GNP-cvt-Ab). GNPs to be covalently conjugated to the anticortisol antibody were purchased from Innova Bioscience, Ltd. (Cambridge, U.S.A.). GNPs were declared to have diameters of 20 nm and were supplied as covered by a trademark shell that allows for the facile coupling to antibodies and also for stabilizing GNPs.[31] The coupling chemistry was based on a one-step covalent binding of carboxyl from GNPs with aminogroups of the antibody. GNPs. Anticortisol antibody conjugation to commercial GNPs (GNP-cvt-Ab) was carried out by following supplier's instruction.[32]
The optimal amount of the Ab to be conjugated with 50 μL of GNP with optical density (OD) 20 (which corresponded to 1 mL of GNPs OD 1), was found to be 1 μg. This amount was in agreement with the one suggested by the supplier.

Preparation of GNPs and GNP-Ab Conjugates via Direct Adsorption. Naked GNPs with an SPR band at 525 nm and mean diameter of ca. 25 nm (Figure S1 of the Supporting Information, SI) were prepared by tetrachloroauric acid reduction with sodium citrate.[33] Details of the synthesis and the characterization of GNPs are reported in the SI.
For the conjugation with the anticortisol antibody (Ab) by passive adsorption, the optimal pH and Ab/GNP ratio were established by the flocculation stress test.[10] According to the results of the flocculation test (see SI), 10 mL of naked GNPs was adjusted to pH 8.0 by sodium carbonate (50 mM, pH 9.6), and then added to 100 μg of Ab. The mixture was reacted for 30 min at 37 °C. BSA (1% in borate buffer 20 mM pH 8.0) was added and reacted for 10 min at 37 °C to saturate the uncovered GNP surface. The so-prepared GNPads-Ab_{10} conjugates were recovered by centrifugation (14 000 rpm, 15 min) and washed twice with borate buffer pH 8.0 supplemented with 0.1% BSA (washing buffer). Finally, GNP-ads-Ab_{10} were resuspended in GNP storage buffer (borate buffer pH 8.0 with 1% BSA, 0.25% Tween 20, 2% sucrose, and 0.02% sodium azide added) and stored at 4 °C until use.
In order to have the same amount of anticortisol antibody linked to the GNPs as for the GNP-cvt-Ab, a second conjugate was prepared by following the same protocol as that described above, but using 10 μg of anticortisol antibody per 10 mL of GNPs (GNP-ads-Ab_1) instead of 100 μg.

GNP-Ab Conjugation Mediated by SpA. Conjugation of anticortisol antibody to GNPs mediated by SpA was achieved in two steps. First, SpA was passively adsorbed onto GNPs following the same protocol described above, except for the pH of the all the buffers (which was 7.4 instead of 8.0) and of the naked GNPs (which was adjusted to 6.0 instead of 8.0). GNP-SpA conjugates were recovered by centrifugation, washed once with the washing buffer and reconstituted in the washing buffer after a second centrifugation step (0.5 mL). Then, 1 mL of the GNP-SpA conjugate (OD 1) was incubated with 1 μg of anticortisol antibody for 30 min at 37 °C to

form the GNP-SpA-Ab conjugate. The unreacted antibody was removed by centrifugation and one washing of the pellet, which was finally recovered in the storage buffer.

The influence of the stabilizing layer on the LFIA performance was studied by varying the SpA amount adsorbed onto GNPs. Accordingly, five GNP-SpA-Ab conjugates were prepared that differed for the SpA adsorbed (from 0.6 to 1.4 μg per mL of GNPs by 0.2 μg steps).

Probe Characterization. The hydrodynamic diameter distribution of the suspensions prepared was evaluated using a Zetasizer instrument (Zetasizer Nano-ZS, Malvern Instruments, U.K.) based on the dynamic light scattering (DLS) technique. The measurements were performed after checking that the automatic attenuator was between 6 and 9. The results were expressed as hydrodynamic diameter distribution in intensity (average of mean values of 5 measurements),

Electrophoretic Light Scattering (ELS) technique (Zetasizer Nano-ZS, Malvern Instruments, U.K.) was used to determine the zetapotential of the sample suspensions. The bare GNP was measured undiluted, while functionalized GNPs were diluted ca. 10 times depending on the initial concentration (absorption range 0.8−1) prior to light scattering measurements.

Spectral scans were obtained from a double-beam ultraviolet−visible spectrometer (Cary 1E, Agilent Technologies, U.S.A.); wavelengths were collected from 200 to 800 nm.

LFIA Execution and Signal Quantification. Details on the LFD fabrication are described in the SI. Briefly, the antigen (cortisol linked to BSA, BSA-F) and a secondary antimouse immunoglobulin antibody formed the test and control lines, respectively. The strips comprised a glass fiber sample pad (Whatman GF/DVA), a glass fiber probe pad (GF from Millipore), a nitrocellulose membrane (Millipore HF180), and a cellulose adsorbent pad. Strips were inserted in a plastic cassette provided with a sample well and a reading window.

LFIA for cortisol was carried out at room temperature, by applying 60 μL of cortisol standard diluted in the running buffer (phosphate buffer 20 mM, pH 7,4, 1% BSA, 0.1% tween 20) or 60 μL of saliva samples to the sample well, waiting for 10 min, and recording results. The signals generated at the test and control lines, due to GNP-Ab binding to immobilized antigens and secondary antibodies, were measured by acquiring the images of the LFD by a portable scanner (OpticSlim 550 scanner, Plustek Technology GmbH, Norderstedt, Germany) and quantifying the intensity of the color on each line with QuantiScan 3.0 software (Biosoft, Cambridge, U.K.).

The performance of the different probes was compared according to the following points: (1) intensity of coloring of the test (and control) lines, and (2) sensitivity of the LFIA for measuring cortisol. Accordingly, cortisol standard solutions (0, 1, and 10 ng mL^{-1}) were prepared in the running buffer and applied to the various LFDs including the different probes. The signals produced at the test (T) and control (C) lines were singularly quantified for point 1. Furthermore, the T/C ratio, normalized for the signal of the blank, was plotted toward cortisol concentration as an estimation of the assay sensitivity (point 2).

Analytical Figures of Merits of the LFIAs Based on GNP Conjugates Prepared by Direct Adsorption and the Dual-Layer Strategy.

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Saliva samples were collected form eight healthy adult subjects as detailed in the SI. Considering the circadian variation of cortisol,[34] samples were collected at 1 pm, so that natural levels of cortisol were expected to be between 1 and 4 μg L^{-1}. Reference values of cortisol in saliva samples were measured by a commercial enzymatic immunoassay kit produced by Salimetrics (CA, U.S.A.).[35] Five out of the eight recruited subjects provided enough sample volume to allow dividing it into portions. One portion was analyzed directly, and the others were fortified with known amounts of cortisol to generate samples with high levels of the hormone. In summary, a

total of 13 saliva samples were made available, which varied for the donor or for the cortisol level. Each sample was analyzed by means of both the LFDs including GNP-ads-Ab_1 or GNP-SpA-Ab as the signal reporter.

The limit of detection (LOD), lower limit of quantification (LLOQ), and upper limit of quantification (ULOQ) were calculated as the 90% (IC10), 85% (IC85), and 15% (IC15) inhibitory concentrations, respectively.[36]

Saliva samples used for preliminary comparative studies within the different GNP conjugates were collected from a single subject in different days and pooled for allowing the execution of several assays while minimizing matrix variability.

RESULTS AND DISCUSSION

LFIA for Salivary Cortisol Based on Covalently Coupling the Anticortisol Antibody to GNPs. The InnovaCoat GOLD produced by Innova Bioscience Ltd. was used to prepare the probe exploiting covalent coupling of Ab and GNPs. The probe was incorporated in a colorimetric LFD for measuring salivary cortisol. The principle of the LFD functioning is depicted in Figure 2. Briefly, the sample was added in correspondence with the sample pad. From here, it was transferred by capillarity to the probe pad, where it resuspended the probe. The sample and the probe flowed across the detection membrane and reacted with the recognition elements anchored in zones defined as test and control lines, respectively. In particular, the test line was formed by cortisol linked to BSA (BSA-F) and, therefore, captured the free GNP-Ab, while the control line was formed by a secondary antibody that captured any GNP-Ab (free and bound to salivary cortisol). The amount of free GNP-Ab bound to BSA-F, and, therefore, the color of the test line was inversely correlated to the amount of cortisol in the sample. Qualitatively, the more intense the color of the test line, the lower was the level of cortisol in the sample (Figure 2a, b) and vice versa (Figure 2c, b). By measuring the color at the test line (T) and normalizing it by the color at the control line (C), it was possible also to correlate quantitatively the signal to the concentration of cortisol.[30]

The probe with the anticortisol antibody covalently coupled to GNP (GNP-cvt-Ab) was obtained by coupling carboxylic groups of functionalized GNPs to amines of the anticortisol antibody via a one-step coupling protocol optimized by the manufacturer.[31] The probe was prepared by following the manufacturer's instructions without modifications. As such, we checked the optimal amount of anticortisol antibody to be conjugated. Different Ab amounts (0.2, 1, and 4 μg) were added to 0.05 mL of GNPs with optical density equal to 20 to produce three GNP-cvt-Ab probes. The optimal amount of Ab, defined as the one providing better sensitivity of the LFIA for salivary cortisol, corresponded to the dose suggested by the manufacturer (1 μg, Figure S2).

Once the covalent probe was obtained, we fabricated an LFD for measuring salivary cortisol based on it. Optimal concentrations of reagents and other relevant parameters (such as time of reaction, amount of sample to be applied, and so on) were decided according to the following criteria: signals at the test (T) and control (C) lines above 50 color units (which corresponds to a clearly visible red color when observed by the naked eye), T/C ratio for the blank above 1, and the half maximal inhibitory concentration (IC50) was between 2 and 5

ng mL^{-1}. This last criterion was based on the intended application of the LFIA for measuring cortisol in human saliva, where reference levels vary between 1 and 15 ng mL^{-1}, as a function of the circadian rhythm of the hormone.[34] Best conditions were selected by a checkerboard titration approach, using standard solutions of cortisol diluted in the running buffer, except for the signal check, which was carried out by running pooled saliva. The as-defined optimal LFIA comprised GNP-Ab diluted to OD 3 and preadsorbed onto the probe pad in order to have 4 μL of GNP-Ab per strip. A typical calibration curve for the "covalent" LFIA, obtained as detailed in the Experimental Section, is shown in Figure S3a. The LOD, working range (LLOQ-ULOQ), and IC50 were calculated as follows: 0.2 ng mL^{-1}, 0.3−7.4 ng mL^{-1}, and 1.2 ± 0.14 ng mL^{-1}, respectively. According to the analytical figures of merits, the developed LFIA was adapted for measuring levels of cortisol in human saliva, as the LOD and range of detection matched requirements for clinical applications.[37]

Dual Layer Probe: Coupling Mediated by SpA.

Staphylococcal protein A is a highly stable surface receptor with a molecular weight of 42 kDa. It is known that SpA is able to bind immunoglobulins G (IgG) from several mammalian species. SpA has high affinity for the Fc portion of IgG; however it is able to bind also to the Fab portion of IgG. The binding ratio of protein A-IgG complexes has been estimated from 1:1 to 1:5, depending on the method used to measure it.[38]

SpA is extensively employed as a highly specific affinity medium for isolating IgG from plasma and serum.[39] However, it has been used also as a broad-selective capturing agent in ELISA[40−42] and LFIA.[30] The advantage of the employment of SpA instead of species-specific secondary antibodies relies in the ability of SpA to detect immunoglobulins from different animals within a single assay, which attributes versatility to the assay.

Apart from the high affinity and versatility, SpA is also particularly efficient in shielding GNPs and preventing their aggregation.[21] We observed that, in general, a smaller quantity of the protein was required for GNP stabilization compared to other proteins usually employed at this purpose, such as antibodies and BSA (data not shown). The only precaution needed for obtaining stable and reproducible GNP-SpA conjugates was maintaining the pH below 7.5 during conjugation.[30]

In this work, the GNP-SpA conjugate was used with the purpose of binding GNPs with the anticortisol antibody, exploiting the high affinity of SpA toward mouse immunoglobulins. Therefore, after the attachment of SpA to the naked GNPs through passive adsorption and the saturation of GNP free surface with BSA ("stabilizing layer"), the GNP-SpA were incubated with the anticortisol antibody to form the "specific layer" (Figure 1).

In order to compare the strategy employing a double layer of proteins (dual layer approach) to the covalent coupling approach, we coupled GNPs to the anticortisol antibody using the same GNP/Ab ratio (1 mL GNP OD1/1 μg Ab) as for the GNP-cvt-Ab, and fabricated an LFD similar to the one

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described above, except for the GNP-Ab probe that was replaced by the GNP-SpA-Ab (the amount of the probe was

kept constant).
Preliminary, we studied the effect of modifying the stabilizing layer, while keeping the GNP amount and GNP/Ab ratio constant. By increasing the SpA amount from 0.6 μg to 1.2 μg, we observed a significant increase of the signal of the test line. However, when SpA was further increased, the color intensity of the test line sharply decreased (Figure S4a). The signal at the control line was almost unaffected by the SpA amount in the stabilizing layer, thus the T/C ratio followed the trend of the T line signal. The ability of free cortisol to inhibit the binding of the probe to the test line was also affected by the stabilizing layer, and the sensitivity largely depended on the SpA amount attached to the GNP. The percent inhibition - calculated as the ratio of signals (T/C) obtained from cortisol standards at 1 (S_1) and 10 ng mL^{-1} (S_{10}) compared to the signal of the blank (S_0)←showed a clear dependence on SpA amount, with a sharp decrease corresponding to increasing SpA from 0.8 to 1 μg, followed by an increase when SpA further incremented (Figure S4b).
Interestingly, the stabilizing amount of SpA, defined as the minimum amount required for completely shielding GNPs and preventing their aggregation induced by salt[10] was 1.4 μg. Nevertheless, the optimal performance both in terms of the signals (which represents the maximum availability of the anticortisol antibody for the binding to the immobilized antigen) and sensitivity (which again is related to the orientation of the anticortisol antibody and, more generally, to its availability for binding to cortisol) were reached by working with less SpA than the quantity required for GNP stabilization. This finding is in agreement with the observations of Safenkova et al., who attributed the positive effect of decreasing the bioaffinity reagents to the organization of the protein layer at the interface with gold.[12] It is worthwhile noting that the choice of an adequate stabilizing layer had a dramatic impact on the LFIA sensitivity. Therefore, contrarily to what happens for GNP-cvt-Ab, multiple trial experiments are required when developing LFIA based on probes prepared by the dual layer strategy.
As a compromise between sensitivity and intensity of signals, we used 1.2 μg SpA for further experiments.
The standard curve for cortisol quantification obtained with the GNP-SpA-Ab probe is shown in Figure S3b. Analytical figures of merits were comparable to those achieved by employing the probe with the antibody covalently attached

Figure 3. Signals (S) generated at the test (T) and control (C) lines by probes prepared: (a) by adsorbing 1 (GNP-ads-Ab$_1$) and 10 μg (GNP-ads-Ab$_{10}$) of anticortisol antibody to 1 mL of GNP, and (c) by mediated coupling via chemical (GNP-cvt-Ab) or biochemical (GNP-SpA-Ab) layers.
The sensitivity of the LFIAs employing the different probes was estimated by applying standard solutions of cortisol and measuring the corresponding inhibition rate (b, d).

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(i.e., LOD 0.13 ng mL^{-1}, working range 0.2−6.3 ng mL^{-1}, IC50 0.8 ± 0.02 ng mL^{-1}). This finding indicates that the benefits achieved through covalent coupling of antibodies can be also attained by the dual layer strategy. In particular, the use of a biochemical mediator enabled the efficient stabilization of GNPs and the correct orientation of the specific antibody, ultimately yielding to a highly active probe.

Direct Coupling of the Antibody to GNPs by Passive

Adsorption. Immunoglobulins strongly interact with citratecapped GNPs by several types of interactions, and these are conventionally exploited for preparing GNP-Ab probes by letting the antibody freely adsorbs onto the naked gold surface. To maximize interactions, usually the reaction is conducted at a pH above the pI of the antibody and the GNP/Ab ratio is decided based on the amount of Ab required for stabilizing GNPs. These parameters are defined as a result of the flocculation stress test.[10] Basically, the conditions assuring the formation of a layer of antibodies that shield GNPs and prevent their aggregation (induced by salt addition in the stress test) are identified as the most suitable for GNP-Ab conjugation. In accord with this conventional approach, we defined the pH and amount of anticortisol antibody for the conjugation to GNPs, which resulted to be 8.0 and 10 μg Ab per mL of GNPs (OD1), respectively (see SI). However, besides the probe prepared in the so-defined optimal conditions (GNP-ads-Ab_{10}), we prepared another GNP-Ab conjugate (GNP-ads-Ab_1) by adsorbing one tenth of the stabilizing amount of antibody (namely, 1 μg Ab per ml of GNPs). The second probe had exactly the amount of antibody used to prepare probes via covalent and SpA-mediated coupling. Unexpectedly, the GNP-ads-Ab_1 probe prepared by using one tenth of the stabilizing amount was perfectly stable both as a concentrated solution and in the dried form. Once applied to the conjugate pad for fabricating the LFD, the dried probe remained stable for at least one month at room temperature. This was probably achieved thanks to the overcoating of the uncovered GNP surface by means of excess of BSA.

The amount of anticortisol antibody adsorbed onto GNP surface had a negligible impact on the signal at the test line (Figure 3a). Instead, the control line was completely absent when using GNP-ads-Ab_{10}. Apparently, the interaction of GNP-ads-Ab_{10} with the test line was so efficient that completely prevent the probe surpassing it. When free cortisol was added to the system (Figure 3b) the difference between the two probes became more evident. In particular, the GNPads-Ab_1 allowed for achieving a significantly better sensitivity compared to GNP-ads-Ab_{10}.

Comparison among Probes Obtained by Covalent Coupling, SpA-Based Dual Layer Strategy and Direct Absorption. To characterize the probes, zeta potential, dynamic light scattering (DLS) and the localized surface plasmon resonance (LSPR) shift were studied. For GNPs of defined size and shape, the LSPR depends on the dielectric constant in the immediate layer to the gold core.[43,44] Changes at the interface due to adsorption or chemical binding are reflected in the shift of the LSPR band.[45−47] Table 2 shows LSPR peaks of the probes prepared in this work and the shift calculated in correspondence of the different layers attached to the GNP surface. Physical adsorption of antibodies on bare GNPs produced the largest red-shift (7−8 nm), while the adsorption of SpA shifted the LSPR peak of 4 nm. Interestingly, the addition of the anticortisol antibody to GNP-SpA did not resulted in further changes of the LSPR band. The unconjugated Innovacoat GOLD GNPs showed an LSPR band centered at 529 nm, which was consistent with grafting from the GNPs a polymer.[43] Also in this case, no LSPR shift was measured upon covalent attachment of the anticortisol antibody to the polymer. Although the contribution of physical adsorption of antibodies to coupling could not

be excluded for GNP-cvt-Ab and GNP-SpA-Ab, the adsorption of antibodies directly to GNP surface strongly affected the LSPR peak position. However, when the covalent linking or the mediated approaches were used, no changes in the LSPR peak were observed. This finding was considered as an indication that, if present, the physical adsorption mechanism minimally contributed to the attachment of antibodies to GNPs for the covalent and SpA-mediated probes.

Stability of bare GNPs was higher at alkaline pH compared to acidic conditions, as expected. The commercial GNPs that were covered with a polymeric layer ending with carboxylic groups showed a largely negative zeta potential. Stability of GNPs functionalized with proteins (either SpA alone, antibodies alone and the combination of the two) were comparable and varied in the range −28.3 ± 0.9 to −30.5 ± 0.4 mV (Table 2).

DLS measurements revealed a marked difference between the GNP-Ab probe prepared by direct adsorption compared to the other two. In particular, the Innovacoat GOLD had a mean hydrodynamic layer of 85 nm (while the gold core was 40 nm in size[32]), which increased of just 11 nm upon reaction with the anticortisol antibody. Considering that antibodies are assimilable to cylinders, 6−7 nm in diameter, and 8 nm long,[48] the thickness is compatible with a monolayer of antibodies. The same observation applies to the GNP-SpA-Ab probe, where the stabilizing layer comprising SpA enlarged particle size more than the following addition of the antibody layer.

Table 2

coupling strategy	material	maximum of LSPR band[a] (nm)	Δλ[b] (nm)	Zeta potential (mV)	hydrodynamic diameter (nm)	layer thickness[c] (nm)
covalent	Innovacoat GNP	529		−41.7 ± 0.4	85	
	GNP-cvt-Ab	528	+0	−29.4 ± 0.5	96	5.5
SpA-mediated	naked GNP(pH 6)	525		−36.1 ± 1.4	38	
	GNP-SpA	529	+4	−28.0 ± 1.1	64	13
	GNP-SpA-Ab	529	+0	−30.5 ± 0.4	75	5.5
direct adsorption	naked GNP (pH 8)	525		−40.5 ± 1.7	39	
	GNP-ads-Ab$_1$	533	+8	−28.3 ± 0.9	84	22.5

[a]Bandwidth = 0.5 nm. [b]Calculated as the LSPR peak difference due to the addition of a new layer. [c]Calculated as (diameter of the resulting GNP − diameter of the pristine GNP)/2[48]

Finally, the physical adsorbed antibodies showed a marked increase of the particle size. The apparent thickness suggested the formation of a multilayer of antibodies, under the hypothesis of the spherical shape of the probe. However, the multilayer speculation was not compatible with the very small amount of anticortisol antibodies adsorbed compared to the saturation amount. Most likely, the random attachment of antibodies to the GNP surface produced protrusions and valley, depending on the orientation of the molecule,[48] so modifying the scatter of light. However, the experiment confirmed the irregular and nonoriented arrangement of the antibody layer for the GNP-ads-Ab$_1$ probe.

One of the main drawbacks attributed to the direct attachment of antibodies to GNPs by adsorption is exactly the random orientation that is reflected in the unavailability of part of the antibodies. Hence, we studied the ability of the passively adsorbed probes to bind to the antigen at the test line and to the free cortisol and compared its performance to those

of the probes obtained by covalent and SpA-mediated coupling strategies.

With regard to the binding to the antigen, we observed that probes having the anticortisol antibody well oriented (GNPSpA-Ab and GNP-cvt-Ab) showed the superior capacity of giving specific binding compared to the probe with randomly oriented antibodies. Considering the sum of color intensities at the two lines, apparently the GNP-cvt-Ab probe was more available for binding (both to the antigen and to the secondary antibody) than the other two (Figure 3c). This can be related to a better dispersity of GNPs (and, therefore, to a higher resistance to aggregation) or to a higher extinction coefficient of the commercial GNPs compared to the in-house prepared colloid.

Despite random orientation and apparent lower binding ability, inhibition experiments revealed that the sensitivity of the LFIA exploiting GNP-ads-Ab_1 was comparable to the one observed for the probe with the covalently linked antibody (Figure 3d). The factor predominantly determining the sensitivity appeared to be the quantity of the specific antibody attached to the GNPs rather than the nature of the attachment. Indeed, previous works highlighting superiority of the covalent coupling over direct adsorption approach compared probes having largely different amounts of specific antibody bound, because conventional protocols for the direct adsorption of antibodies to GNPs, which are based on the "GNP stabilization approach", require high quantity of the antibody.[11−14]

The dual layer strategy, which used SpA as the stabilizing layer, enabled improvement of the sensitivity compared to both the direct and the covalent coupling approaches. Despite the optimization of the formulation of the stabilizing layer is critical for the performance of the assay, and requires several experiments, the strategy demonstrated the superior quality of the probe, which showed high binding ability toward the test line and, above all, toward the free cortisol (Figure 3c,d).

The unexpectedly high sensitivity reached with the GNPads-Ab probe was attributed to the use of limited amounts of the antibody, largely lower than the one required for GNP stabilization. As a consequence of the doubtful stability of this kind of conjugate, we checked the stability of the three conjugates by an accelerated aging experiment,[30,48−50] in which the LFDs incorporating the different GNP-Ab conjugates were kept at 37° for 1 week and tested at days 0, 1, 3, and 7. Parameters used to evaluate the stability of the probes were the normalized color intensity (T/C) and the sensitivity measured as the percentage inhibition at two levels of cortisol (1 and 10 ng mL^{-1}). No significant variation of the LFD performance was observed (Figure S5), thus confirming the robustness of the probes, that were substantially comparable.

The comparison of probes was repeated using fortified human saliva as the sample, instead of standard solutions of cortisol. Matrix interference is known to affect LFIA in several ways, which include: the inefficient release of the probe from the probe pad; inducing aggregation of GNPs; slowing down the flow and thus changing the rate of interactions of the probe with reagents forming the lines; and impacting antibody−antigen interaction. Apart from the latter parameter, it is clear that matrix adverse effects vary in their seriousness depending on the nature of the probe. To investigate probe robustness toward matrix interference, saliva was collected from a single

volunteer over several days (at 1 pm), pooled, and fortified with cortisol at two levels (2 and 10 ng mL^{-1}). When using saliva, all three probes produced lower signals at the test line compared to those obtained by using the running buffer. This confirmed that some components of the saliva affected the interaction among anticortisol antibodies and the antigen. The decrease of the binding to the test line was especially relevant for the GNP-SpA-Ab probe, which appeared to be strongly affected by the presence of the salivary matrix. Nevertheless, the trend of inhibition rates was qualitatively similar to the one observed for cortisol standards, where the use of the dual layer strategy, with SpA as the mediator, produced higher sensitivity compared to using the probe with adsorbed antibodies and also to the one with covalently linked antibodies (Figure S6).

LFIA for Detecting Salivary Cortisol: The Way of Coupling Antibodies to GNP Affects Assay Performance?

According to preliminary results on human saliva, the LFDs incorporating GNP-SpA-Ab and GNP-ads-Ab_1 probes showed comparable ability in measuring salivary cortisol at levels corresponding to the reference interval for healthy adults.[34] Cortisol release is known to vary significantly among individuals and also in the same subjects, as the effect of chronobiological rhythm, physical and mental stress, health status, and so on.[24−29] Furthermore, the composition of saliva is strongly dependent on biological inter and intraindividual variability. Therefore, we tried to overcome matrix variability by collecting saliva samples coming from different subjects. We recruited eight adult subjects (3 male and 5 female aged between 23 and 48) and asked to them to collect saliva at 1 pm. The samples which volumes were particularly abundant were analyzed both as collected and fortified to generate highlevel samples. Reference values for natural cortisol content were measured by a commercial ELISA kit, validated for the quantification of human salivary cortisol.[35]

Both probes (GNP-SpA-Ab and GNP-ads-Ab_1) were completely suspended and flowed correctly in the LFD for all checked samples and provided response that correlated with cortisol levels as measured by the reference assay. The correlation between LFIA output (i.e.: the ratio of test and control line signals, T/C) and cortisol levels was satisfactory for both LFDs (Figure 4), as confirmed by the coefficients of determination of the four-parameter logistic equation (Table 3). At first sight, the two probes did not significantly affect the sensitivity of the assay; indeed, the half maximal inhibitory concentrations were quite comparable, considering errors. This reinforced the rejection of the hypothetical weakness of the direct coupling approach via passive adsorption. Likely, the lower sensitivity usually achieved with this kind of probe can be ascribed to the common practice of using large amounts of the antibody for coupling and, above all, on the practice of deciding its quantity according to GNP stabilization rather than on maximizing sensitivity of the LFIA.

However, looking more attentively, the probes caused some variations to the shape of the correlation curves. The probe with SpA-mediated attachment of the antibody provided a curve with a lower slope and, coherently, a larger working range. Furthermore, the limit of detection was also lowered, though not so remarkably. In general, signals at the test lines were more intense for the LFD including the GNP-ads-Ab_1

probe, whereas the control lines were similar in the two systems. Thus, the slightly lower sensitivity shown by the LFD using the GNP-ads-Ab_1 can be related to a higher amount of available antibody sites. However, the higher background shown by the GNP-ads-Ab_1 and measured by the minimum of the correlation curve, can also account for the worse performance. The broad-selectivity of the biochemical mediator used in this work can be regarded as a limitation, especially for the application of the dual layer strategy to different types of samples (namely, plasma and serum) where the interference of immunoglobulins belonging to the sample will be significant. As an alternative to SpA, species-specific secondary antibodies were also considered as candidate biochemical mediators for GNP-Ab preparation. However, attempts made for preparing GNP-Ab using an antimouse secondary antibody (secondAb) as the mediator for the successive attachment of the anticortisol antibody led to conjugates with limited performance compared to the GNPSpA-Ab probe both analyzing cortisol standards and saliva fortified with cortisol (Figure S7). It should be noted that we did not optimize the amount of the secondary antibody and we merely used the "stabilizing amount" to prepare the GNPsecondAb-Ab probe. As discussed above, neither for the adsorbed antibody nor for the SpA mediator, the best performance in terms of assay sensitivity were reached using the stabilizing amount of the protein. This reinforced our conclusions that the dual layer strategy requires an extensive optimization to obtain an efficient probe.

CONCLUSIONS

Stable and efficient conjugates between antibodies and gold nanoparticles are sought to develop highly sensitive and robust biosensors with disparate applications in medicine, toxicology, and food safety controls. For most applications, the preservation of antibody activity (namely, the preservation of the affinity and the accessibility of binding sites) is of utmost relevance. Several strategies have been proposed for directing antibody attachment to gold nanoparticles, including grafting polymers to/from the GNP surface and covalently coupling the antibody to the polymer, and the use of mediators, such as secondary antibodies and other high-affinity receptors. Notwithstanding, antibodies freely adsorb to the GNP surface with high binding rates and the direct adsorption approach is far more simple, fast, and inexpensive than those discussed above. Therefore, it is preferred for most applications, although this approach is considered to limit the performance or the resulting assay. In this work, we prepared three GNP-Ab conjugates using different conjugation strategies and compared their behavior in a lateral low immunoassay for measuring salivary cortisol, as a model colorimetric biosensor employing GNP-Ab conjugates. Unexpectedly, the analytical figures of merits of LFIA carried out with the three probes were comparable both in buffer and in saliva, provided that the amount of the anticortisol antibody attached to the GNP was kept constant. The sensitivity of the assay was mostly dependent on the amount of the antibody and, for the dual layer probe, on the characteristic of the stabilizing SpA layer. The covalent coupling approach has superior features in terms of robustness toward "environmental factors" (such as pH and presence of additives in the antibody solution) and redundancy

of trial experiments to find optimal experimental conditions for the coupling. Conversely, the direct adsorption strategy benefits of cost-efficiency and simplicity. The dual layer

Figure 4. Correlation curves between normalized signals (S/S_{max}) provided by two LFDs for 13 human salivary samples and the cortisol concentration as measured by a reference ELISA. The two LFDs varied for the probe that was either prepared by the SpA-mediated attachment and by direct adsorption of anticortisol antibodies to GNPs. S_{max} was estimated from interpolation of experimental values by the 4-parameter equation.

Table 3. Analytical Figures of Merits of LFIAs for Measuring Salivary Cortisol Employing Two GNP-Ab Probes

	dual layer SpA-mediated GNP-SpA-Ab	direct attachment by adsorption GNP-dc-Ab1
IC50 (ng mL^{-1})[a]	4.5 ± 0.3	5.0 ± 0.2
slope[a]	−3.7 ± 0.8	−8.2 ± 2.6
R^2[a]	0.984	0.979
LOD (ng mL^{-1})[b]	2.5	3.8
LLOQ − ULOQ (ng mL^{-1})[b]	2.8−7.3	4.0−6.1

[a]From the 4-parameter logistic equation. [b]Calculated as the cortisol levels which give signals corresponding to 90% (LOD), 85% (LLOQ), and 15% (ULOQ) of the maximum signal, respectively.[36]

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strategy can be regarded as a viable alternative to covalent coupling with similar pros and cons, including relatively low cost if generic receptors (as protein A) are used instead of secondary antibodies. However, protein A has the inherent disadvantage of being prone to interference from mammalian immunoglobulins, which can be largely present in other biological fluids, such as plasma, serum, and also milk. This limitation can be overcome by employing species-specific secondary antibodies as biochemical mediators, and completely resolved by resorting to directly adsorbed probes.

ASSOCIATED CONTENT

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.9b11559. Experimental details on: chemicals, gold nanoparticles preparation, and characterization, flocculation test, LFD fabrication, stability study, saliva collection; and figures representing: UV−vis spectrum and TEM image of gold nanoparticles (Figure S1); the optimization of anticortisol amount covalently coupled to commercial GNPs (Figure S2); typical calibration curves obtained from calibrators diluted in buffer for the LFDs including GNP-cvt-Ab and GNP-SpA-Ab (Figure S3), the effect of the SpA amount on absolute signals and sensitivity of the LFIA employing the GNP-SpA-Ab (Figure S4); signals and inhibition rates measured during the accelerated aging of LFDs incorporating different GNP-Ab conjugates (Figure S5); comparison of signals and inhibition rates obtained from LFDs differing for the probe used as measured for a fortified human saliva sample (Figure S6); and evaluation of the signals and sensitivity of the LFD encompassing the GNP-secondAb-Ab (Figure S7) (PDF)

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Notes
All studies were conducted in accordance with principles for human experimentation as defined in the Declaration of Helsinki and the Oviedo convention. Informed consent was obtained from all individual participants involved in the study after they were told of the potential risks and benefits as well as the investigational nature of the study.
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