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Enhanced lateral flow immunoassay using gold nanoparticles loaded with enzymes

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ABSTRACT

The use of gold nanoparticles (AuNPs) as labeling carriers in combination with the enzymatic activity of the Horseradish Peroxidase (HRP) in order to achieve an improved optical lateral flow immunoassay (LFIA) performance is here presented.

Briefly in a LFIA with an immune-sandwich format AuNPs are functionalized with a detection antibody already modified with HRP, obtaining an "enhanced" label. Two different detection strategies have been tested: the first one following just the red color of the AuNPs and the second one using a substrate for the HRP (3 different substrates are evaluated), which produces a darker color that enhances the intensity of the previous red color of the unmodified AuNPs. In such very simple way it is gained sensitivity (up to 1 order of magnitude) without losing the simplicity of the LFIA format, opening the way to other LFIA applications including their on-demand performance tuning according to the analytical scenario.

KEYWORDS: Lateral flow immunoassay; Human immunoglobulin; gold nanoparticle; horseradish peroxidase; 3,3′,5,5′-Tetramethylbenzidine (TMB); 3-Amino-9-ethylcarbazole (AEC); 3,3′-Diaminobenzidine tetrahydrochloride (DAB) with Metal Enhancer.

1. INTRODUCTION

The detection of protein is of tremendous interest in diagnostics, since many biomarkers for many diseases are actually proteins and their early detection could save many lives, especially in the third world countries (Mabey et al., 2004). In fact, whereas in the industrialized world many complex and expensive techniques are available, in third world countries these techniques cannot be used because of their high cost and the lack of trained personnel (Yager et al., 2006; Ellerbee et al., 2009). The same situation can be found in small ambulatory, remote regions and battlefields. In this context the development of easy to use, point of care (PoC) and cheap biosensors is essential. A possible answer to this demand is the Lateral Flow Immunoassay (LFIA), which is a platform that can be defined as ASSURED: affordable, sensitive, specific, user-friendly, rapid and robust, equipment free and deliverable to end-users (Peeling et al., 2006; "http://www.who.int/std_diagnostics/about_SDI/priorities.htm," n.d.). LFIA is based on the recognition of one or more analytes of interest, mainly proteins, by using antibodies. The antibodies are fixed onto a nitrocellulose membrane and they interact with the analyte either in sandwich or competitive formats using a proper label. The main advantage compared with other immunosensors is that the entire assay can be done in one step and in few minutes (Posthuma-Trumpie et al., 2009). On the other hand the limit of detection (LoD) is generally not as good as other assays like ELISA; this limitation is the main reason to the lack of extensive use in clinical laboratories, even being one of the most used PoC biosensors, since the first pregnancy test sold in 1970s.

On the other hand, nanomaterials (NMs) and especially nanoparticles have been widely used in many biosensors ranging from protein (de la Escosura-Muñiz et al., 2010) to nucleic acid (Alegret et al., 2005) detection and with different techniques (both optical and electrochemical) due to their unique properties which make them excellent labels

and carriers. Gold nanoparticles (AuNPs) are the most used NM in LFIA (Lou et al., 2011; Yang et al., 2011) but not the only one; actually also magnetic nanoparticles, quantum dots (QDs) (Zou et al., 2010; Zhu et al., 2011), liposomes (Edwards and Baeumner, 2006; Shukla et al., 2011), carbon nanotubes (Wang et al., 2009, 2012; Abera and Choi, 2010), carbon nanoparticles (Ornatska et al., 2011) have also been used for such purposes. Although the peculiar properties of materials help to increase the sensitivity of the LFIA, this must be further increased to expand the application range in diagnostics. One of these advantageous properties consists in their ability to act as carriers of a high number of enzymes so as to increase their availability to catalyze the detection reaction. This approach was already performed in different biosensors like ELISA (Ambrosi et al., 2010) and lateral flow for nucleic acid (He et al., 2011)(Mao et al., 2009).

In this article we discuss the development of a LFIAs based on the use of AuNPs not only as labels but also as carriers of enzymatic labels. AuNPs produce red bands at the detection and control lines of the LFIA when acting as direct labels; but if they are coupled with an antibody modified with HRP they can also act as carriers. 3,3′,5,5′-Tetramethylbenzidine (TMB); 3-Amino-9-ethylcarbazole (AEC); 3,3′-Diaminobenzidine tetrahydrochloride (DAB) with Metal Enhancer as substrates of the HRP are evaluated since they produce insoluble chromogens which cannot be moved by the flow, concentrating the color at the lines. The developed LFIAs offer two different detection alternatives: one produced just by the red color of the AuNPs and one more sensitive produced by the substrate of the HRP achieving an ‘on-demand’ tuning of the biosensing performance. Its application for protein detection, after related optimizations, could open the way to several uses with interest in diagnostics, safety and security between other fields.

2. EXPERIMENTAL SECTION

2.1. Materials

All the materials used for the production of the LFIA strips were purchased from Millipore (Billerica MA 08128; USA): sample and absorbent pads (CFSP001700), conjugation pad (GFCP00080000), detection pad (SHF2400425) and the backing card (HF000MC100). A guillotine Dahle 533 (Germany) was used to cut the strips. The sample pad buffer consisted in 10mM PBS pH 7.4 with 5% BSA and 0.05% Tween20; the conjugation pad buffer was 2mM borate buffer pH 7.4 with 10% of sucrose; the antibody buffer was 10 mM phosphate pH 7.4. An IsoFlow reagent dispensing system (Imagene Technology, USA) was used to dispense the detection and control lines. A strip reader (COZART – SpinReact, U.K.) was used for quantitative measurements.

Hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4.3H_2O$, 99.9%) and trisodium citrate ($Na_3C_6H_5O_7.2H_2O$) were purchased from Sigma-Aldrich (Spain) to synthesize AuNPs. A Transmission Electron Microscope (TEM) Jeol JEM-2011 (Jeol Ltd, Japan) and a scanning electron microscope Merlin®FE-SEM (Carl Zeiss, Germany) were used to characterize the AuNPs.

For the AuNP conjugation: the borate buffer solution (BB) was prepared with 0.1M boric acid and adjusted to pH 9.2 with 5M NaOH. Blocking buffer solution consisted in mQ water with 1 mg/mL bovine serum albumin (BSA). The stirrer used was a TS-100 Thermo shaker (Spain). A thermostatic centrifuge Sigma 2-16 PK (Fisher Bioblock Scientific, France) was used to purify the conjugates of AuNPs/antibodies.

Human IgG whole molecule (I2511), anti-human IgG whole molecule (produced in goat; I1886), anti-human IgG γ chain specific HRP modified (produced in goat; A6029)

and all the chemical reagents (analytical grade) used for the preparation of buffer solutions were purchased from Sigma-Aldrich (Spain). Anti-goat IgG (produced in chicken; ab86245) was purchased from Abcam (U.K.).

The HRP substrates: TMB (T0565); AEC (AEC101); DAB with Metal Enhancer (D0426) were purchased from Sigma-Aldrich (Spain).

mQ water, produced using Milli-Q system (>18.2MΩcm^{-1}) purchased from Millipore (Sweden), was used for the preparation of all the solutions.

2.2. Methods

Preparation and modification of gold nanoparticles

AuNPs of 20 nm of diameter were prepared according with the citrate reduction of $HAuCl_4$, method pioneered by Turkevich (Turkevich, 1951), as stated in the *supplementary material*.

The prepared AuNPs were further modified with the antibody anti-human IgG γ chain specific HRP modified. The functionalization process is described in the *supplementary material*. The AuNPs/antibody conjugates were further centrifuged at 14000 rpm for 20 min at 4º C in order to concentrate them 5 times in 2 mM borate buffer pH 7.4 with 10% of sucrose.

Preparation of the LFIA strips

First the sample pad was prepared dipping it into a sample pad buffer solution and then drying it at 60º C for 2 hour. The conjugation pad was prepared soaking it with the AuNPs/antibody conjugate, prepared as described above, and then drying it at room temperature under vacuum for 2 hours. Finally the antibodies anti-human IgG whole molecule and anti-goat IgG, diluted in the antibody buffer at a concentration of 1 mg/mL, were spotted onto the detection pad to form the detection and control lines

respectively, using the IsoFlow reagent dispensing system. The detection pad was dried at 37º C for 1 hour.

The different pads were subsequently laminated onto the baking card with an overlap between them of around 2 mm, in order to allow the sample to flow (see figure 1). Finally they were cut 8 mm wide and stored in dry conditions at 4º C up to a week (longer storage times were not assayed, although it is known that these kind of strips, like i.e. those used for pregnancy tests can be stored for many weeks)

(Preferred position for Figure 1)

LFIA procedure

Sample solutions were prepared diluting different amounts of Human IgG (HIgG) in PBS, obtaining different analyte concentration: 0.5 ng/mL, 5 ng/mL, 50 ng/mL, 500 ng/mL. PBS without analyte was considered as blank. The assay procedure consisted in first dispensing of 200 μl of sample solution onto the sample pad and waiting during 15 minutes untill the flow is stopped. Then 200 μl PBS were dispensed in order to wash away the excess of AuNPs/antibody. The strips were read with the strip reader to obtain the calibration curve corresponding to the AuNPs used as 'direct' labels. After the first reading step, the LFIA strips were dipped for 5 min into the different HRP substrates and washed with mQ water to stop the reaction and prevent a saturation of the signals. The strips were finally read again with the reader.

3. RESULTS AND DISCUSSION

3.1. AuNP/antibody conjugates characterization

The AuNPs were characterized first by UV-Vis analysis to calculate their concentration (it resulted $2.1x10^{-4}$ M) and to have an estimation of their size, which resulted to be around 20 nm in diameter since the wavelength of the peak was 520 nm (de la Escosura-Muñiz et al., 2011) (figure 1S-A). Then they were also visualized by transmission electron microscopy (TEM) to have a more accurate size distribution, giving as result a diameter of 21 ± 3 nm (figure 1S-B).

The detection lines obtained for different concentrations of HIgG were visualized also by scanning electron microscopy (SEM). In the figure 1S-C it is possible to see the AuNPs (the white spots) in the test line of a LFIA after detecting 500 ng/mL of HIgG, whereas figure 1S-D shows another zone of the detection pad without AuNPs.

3.2. Evaluation of the LFIA performance using AuNPs as both direct labels and carriers of enzymatic labels

The prepared LFIA strips gave rise to two different signals. The first one consists in the red color of the AuNPs, whereas the second one corresponds to the color of the chromogen produced by the enzymatic activity of the HRP. The red color of the AuNPs enables to detect up to around 50 ng/mL of HIgG for naked eyes. The strip reader gives as output a % value corresponding to the intensity of the lines: stronger the color lower is the % value (figure 2). A linear relationship between this value and the logarithm of the HIgG concentration was found (see Supplementary Material). The limit of detection using the reader was calculated as the concentration of HIgG corresponding to three times the standard deviation of the isof 2 ng/mL Although the obtained sensitivity can be considered adequate for some applications it is not enough for others, whose required detection limits are lower. For this reason the TMB, AEC and DAB with metal enhancer were tested as HRP substrates in the developed LFIA.

The first HRP substrate tested was the TMB. As stated in the *experimental section* the TMB used in these experiments produced an insoluble blue-violet chromogen, which is deposited at the level of the control and test lines (as shown in figure 2); in this way the color is not dispersed along the strip. After stopping the HRP activity the strips were read with the strip reader. The results obtained are shown in the graph of figure 2, where the improvement in the sensitivity seems to be clear. By using non-modified AuNPs as labels it was difficult to distinguish between a blank and 5 ng/mL of HIgG with the naked eye; on the other hand using the TMB the difference between the blank and the samples is evident, even at 5 ng/mL. The limit of detection obtained using the reader (calculated as explained before; see the quantification equation at the Supplementary Material) was of 200 pg/mL..

(Preferred position for Figure 2)

The second substrate used was the AEC, which produces an insoluble red chromogen. This substrate was already used by Liu's group in a LF for the detection of nucleic acids (He et al., 2011)(Mao et al., 2009). As in the case of TMB substrate also the AEC enables increasing the sensitivity of the assay, allowing to detect up to 5 ng/mL with the naked eye as shown in figure 2. The limit of detection using the strip reader was in this case 0.31 ng/mL (see equation at the Supplementary Material)..

Finally the DAB with metal enhancer was tested. This HRP-substrate developed a grey/black insoluble compound. Surprisingly, it did not produce any appreciable increment in the sensitivity of the LFIA, being the minimum concentration detected with the naked eye of around 50 ng/mL, as also shown in figure 2. The limit of detection using the reader (see equation at the Supplementary Material) was only a little bit lower (1.6 ng/mL) than the obtained for the unmodified AuNPs (2ng/mL, as explained before).

It is also noticed a higher colored background in the case of the enzymatic reactions, probably due to a not completely washing of the strips.

The reproducibility responses of 50 ng/mL HIgG, n = 3, give a relative standard deviation (RSD) of 1.3% for AuNPs, 5.6% for TMB, 1.5% for AEC and 16% for DAB..

It seems to be clear that the use of the enzymatic reactions catalyzed by the HRP loaded on AuNPs using TMB and AEC as substrates of the enzyme allows an enhancing of the sensitivity of the LFIA of around one order of magnitude compared to the results obtained just from the direct measurement of the AuNPs as non modified optical labels. In particular the TMB was the substrate which gave the best limit of quantification compared with the others (figure 2). TMB is also cheaper compared with DAB Furthermore it has an important advantage compared to the other substrates studied in this work: it is ready to use. In fact both AEC and DAB need to be prepared freshly, using deionized water and mixing of at least two reagents. These characteristics make these two substrates not so suitable for LFIA applications, since they are more time consuming and could increase the possibility of human errors leading to an increase of the irreproducibility of the results. It has also to be noted that the AEC produces a red chromogen which color can be added to the red color of the AuNPs and the background resulted less intense than the one of the TMB.

4. CONCLUSIONS

A LFIA strip, with AuNPs loaded with HRP enzymatic labels was obtained. The use of such a label allows increasing an order of magnitude the limit of quantification in a LFIA for the detection of Human IgG used as model protein. The strips prepared gave two different detection ranges: one less sensitive considering just the red color of the

AuNPs and one more sensitive considering the color produced by the HRP substrates. Three different HRP substrates were tested and the TMB was resulted the most suitable for LFIA applications compared with AEC and DAB with metal enhancer. This result could open the way to the use of LFIA in more diagnostics applications, especially in an ambulatory/laboratory context, due to the lower limit of quantification obtained. Furthermore, the use of various substrates offers the possibility to the 'on-demand' tuning of the sensitivity of the device adapting it to the analytical scenario.

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FIGURE CAPTIONS

Figure 1. (A) Scheme of the LFIA for the detection of HIgG. (B) Detail of the different parts of a LFIA strip and cartoons representing the AuNP modified with the antibody anti-human IgG γ chain specific HRP modified, and the different colors expected for the different substrates (TMB, AEC and DAB) used.

Figure 2. (Left) Photos of the LFIA strips for different concentrations of HIgG and the different substrates. (Right) Graph of the results obtained with the strip reader. Experimental conditions as explained in the text.

FIGURE 1

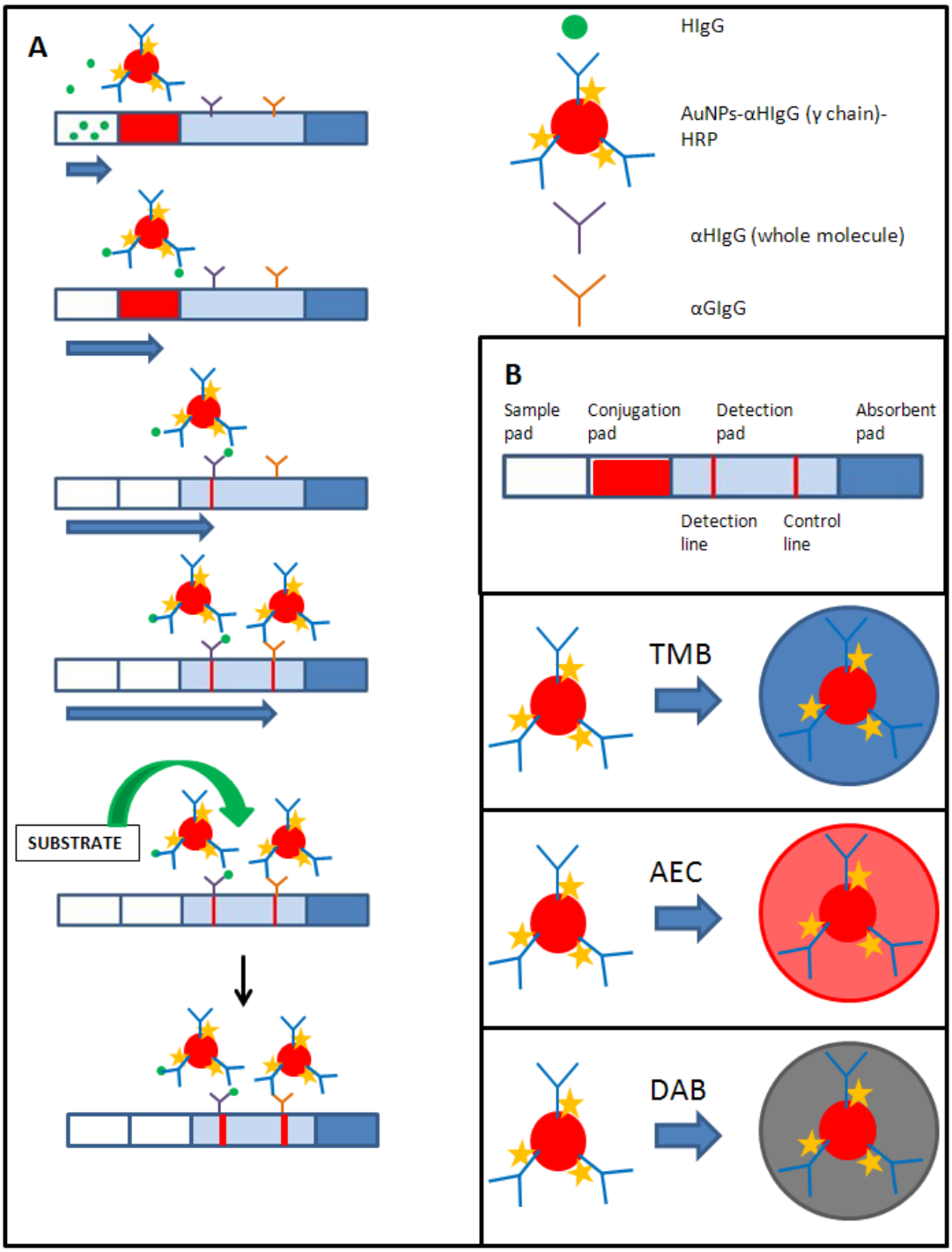
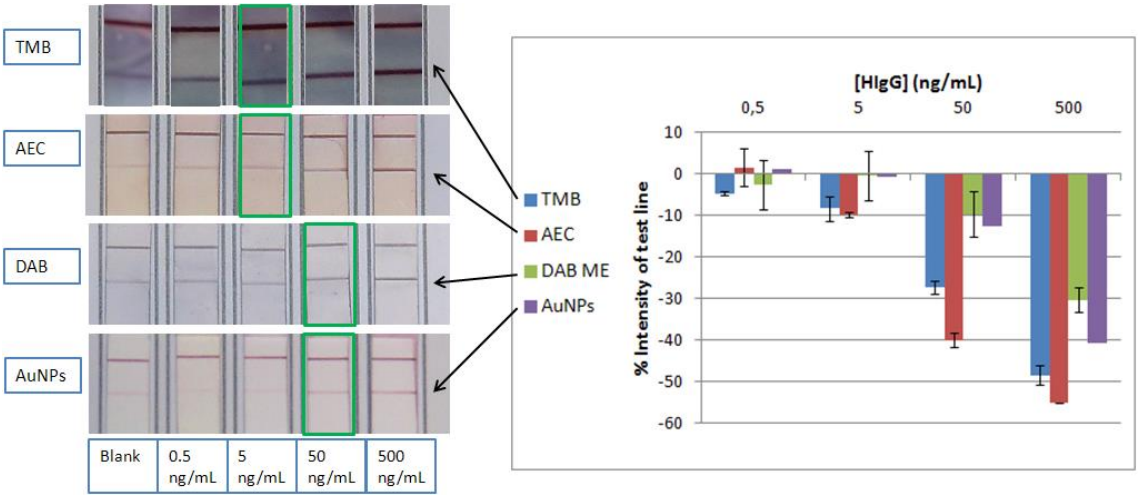

A
HIgG
AuNPs-αHIgG (γ chain)-HRP
αHIgG (whole molecule)
αGIgG
B
Sample pad
Conjugation pad
Detection pad
Absorbent pad
Detection line
Control line
SUBSTRATE
TMB
AEC
DAB

FIGURE 2


TMB
AEC
DAB
AuNPs
Blank
0.5 ng/mL
5 ng/mL
50 ng/mL
500 ng/mL
TMB
AEC
DAB ME
AuNPs
[HIgG] (ng/mL)
0,5
5
50
500
% Intensity of test line
10
0
-10
-20
-30
-40
-50
-60

Supplementary material

Enhanced lateral flow immunoassay using gold nanoparticles loaded with enzymes

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Preparation of gold nanoparticles

Gold nanoparticles of 20 nm of diameter were prepared according with the citrate reduction of $HAuCl_4$, method pioneered by Turkevich.(Turkevich, 1951) Briefly: 50 mL aqueous solution of 0.01% $HAuCl_4$ was heated to boiling and vigorously stirred in a 250 mL round-bottom flask; 5 mL of 40 mM sodium citrate were added quickly to this solution. Boiling was continued for additional 10 min. The solution was cooled to room temperature with a continuous stirring for another 15 min. The colloids were stored in dark bottles at 4° C. All glassware used in this preparation was thoroughly cleaned in aqua regia overnight and rinsed with double distilled H_2O and reflux was used for all the procedure.

AuNPs modification with antibodies

The antibody anti-human IgG γ chain specific HRP modified was conjugated to the AuNPs: the pH of 1.5 mL of AuNPs was corrected to 7.4 with borate buffer. Then 100 µL of the antibody solution (100 µg/mL) were added drop by drop and the resulting solution was incubated for 20 min at 650 rpm. Then 100 µL of 1 mg/mL BSA in H_2O mQ were added drop by drop and the stirring was continued for other 20 min at 650 rpm. Finally the solution was centrifuged at 14000 rpm for 20 min. The supernatant was removed and the pellet of AuNPs was re-suspended in 300 µL of 2 mM borate buffer pH 7.4 with 10% of sucrose.

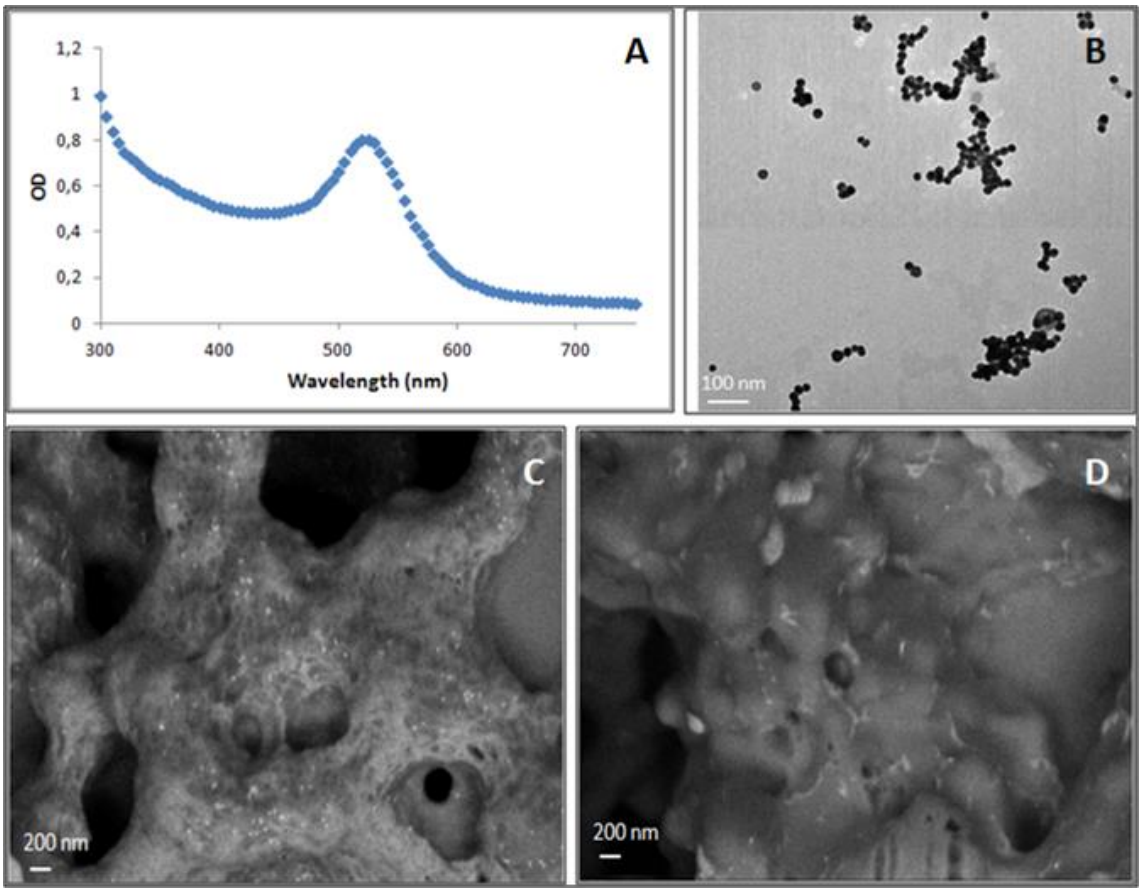

Figure S1. (A) UV-Vis spectra of AuNPs. (B) TEM image of AuNPs. (C) SEM image of the test line of the LFIA detecting 500 ng/mL HIgG; the white spots represent the AuNPs. (D) SEM image of the detection pad without AuNPs.

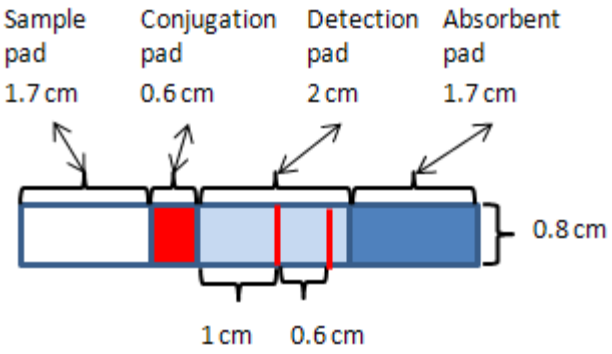

Figure S2. Scheme of the strip sizes.