Silver and Gold enhancement methods for lateral flow immunoassays

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Abstract: Sensitivity is the main concern at the development of rapid test by lateral flow immunoassays. On the other hand, low limits of detection are often required at medical diagnostics and other field of analysis. To overcome this drawback, several enhancement protocols have been described. In this paper, we have selected different silver enhancement methods and one dual gold conjugation, and we critically compared the amplification produced when applied to a gold-nanoparticle based lateral flow immunoassay for the detection of prostate specific antigen (PSA). The highest amplification was obtained by using an immersion method based on a solution of silver nitrate and hydroquinone/citrate buffer in proportion 1:1. Under these conditions, the system is capable of detecting PSA within 20 min at levels as low as 0.1 ng/mL, with a 3-fold sensitivity improvement .

Keywords: Lateral Flow Immunoassay Dipstick, Enhancement procedures, Silver enhancement, Dual gold conjugate

1. Introduction

Nowadays, there is a need to develop rapid, simple, and cost-effective tests capable of being performed by unskilled operators in areas such as diagnosis of diseases in developing countries or emergency rooms [1]. In this way, lateral flow immunoassay test (LFIA) represent a well-established and very appropriate technology when applied to a wide variety of point-of-care (POC) or in-field use applications. However, the detection sensitivity of the LFIA is lower than the others immunoassays based on fluorescent, radioactive, and enzyme-colorimetric methods (ELISA) [2] and a higher sensitivity is often required in medical diagnostics. Many studies have attempted to improve the sensitivity of the immunoassay, such as by using different labels (colloidal metal nanoparticles [3-6], enzymes [7-9], magnetic beads [10-12], quantum dots [13-14], etc.), coupling with transducters or by amplifying the detection signals. Gold nanoparticles (AuNP) are commonly used because they can be easily conjugated with biomolecules. Furthermore, they allow signal amplification in biosensing where they serve as labels [15]. One strategy to follow in order to enhance the signal is the use of larger nanoparticles. However, while colloidal gold particles smaller than 15 nm were found to be too small for producing an intense color, AuNP larger than 60-70 nm are more unstable, forming aggregates after several days of storage at 4ºC [16]. They also require larger concentrations of antibodies in the conjugation, increasing costs. Tang et al. [17] developed a novel LFIA for the screening of aflatoxin B2 in food samples. The detector reagent consisted of magnetic nanogold microspheres (with nano-Fe_2O_3 particles as core and AuNP as shell) bio-functionalized with monoclonal anti-AFB2 antibodies. The visual detection limit obtained with this approach was about threefold lower compared to a conventional immunodipstick test using AuNP. Parolo et al. [18] discussed the development of an enhanced LFIA based on the use of AuNP in combination with the enzymatic activity of the HRP when it is coupled to the detection antibody. A detection limit of 310 pg/mL was achived using this approach. Recently, H. Chon et al. [19] have carried out a similar approach using AuNP-assisted enzyme for detection of pathogens in food, adding the substrate in the cross-flow direction. With this approach, the limit of detection can be increased by about 1000-fold. AuNP can act as a catalyst and reduce copper and especially silver ions into their respective metals in the presence of a reducing agent. The reaction leads to the deposition of copper and silver on the gold surface as nucleation site and enlargement of the size of AuNP,

resulting in a remarkable signal amplification in the test and control line of the immunostrip [20]. The silver staining can be carried out in an incubation mode by immersing the membrane in the enhancement silver after performance the LFIA [21]. Silver enhancement technology was developed by Yang et al. [22] based on the use of two pads where $AgNO_3$ and the reduction agent were fixed. These two approaches have the inconvenient of involving additional operation steps in the assay, increasing the time and the difficulty to use them outside of the laboratory. To overcome this technical limitation Choi et al. [23] developed a new method to detect Troponin I utilizing two AuNP-antibody conjugates in different pads. The 1st AuNP conjugate was blocked with BSA and it was immobilized at the conjugate pad with an antibody against the antigen chosen. The 2nd AuNP anti-BSA conjugate was designed to bind only with the 1st AuNP conjugate and to enhance the signal. The LFIA method developed in this study is useful since it is a rapid one step analysis with a signal 100-fold more sensitive than the normal LFIA. Similarly, Wiriyachaiporn et al. [24] have recently proposed a double-targeted nanogold using the 2nd gold conjugate with anti-biotin instead of anti-BSA. With this approach they demonstrated a 4-fold lower LOD in the detection of influenza virus. Other approaches based on the use of two different AuNPs make use of DNA as crosslinker agent [25,26].

In this study, one new protocol of silver enhancement was developed with the aim of amplifying the signal of colloidal gold on the test strip after the standard assay, avoiding therefore the silver solution handling by the end user. This consisted on immobilizing the silver salt in a separated pad. Results were compared with those obtained by using other protocols reported at the literature. The thick silver layer on the gold nanoparticles endowed particular features, such as colorimetric, electrical and electrochemical properties for quantitative biosensing [27]. Silver has been used as electrochemical label for immunoassays [28,29], and therefore this protocol could be useful to explore the coupling of the strip with an electrochemical transducer in future work. A novel gold enhancement system was also developed based on the use of biotin-neutravidin bridge. While other methods reported in the literature use secondary antibodies such as anti-BSA or anti-biotin to perform the amplification [23, 24], in this work a new method is presented, potentially more advantageous than others reported previously, because it involves a neutravidin protein instead a specific antibody. As a

model antigen for this immunoassay research, prostate specific antigen (PSA), biomarker of prostate cancer, was used.

2. Material and methods

2.1. Chemicals and reagents

Mouse monoclonal anti-PSA antibodies HS-5 (capture antibody) and HS-8 (detection antibody) were produced by Healthsens (Oviedo, Spain). Anti-mouse IgG was purchased from Sigma-Aldrich (Spain). PSA standard solution, calibrated against International Standard Stamey 9010 was obtained from Fujirebio Diagnosis. Gold nanoparticles (AuNP) of 40-nm and 20-nm were purchased from BBInternational (UK) to conjugate the antibody or neutravidin. Bovine serum albumin (BSA), Biotin-conjugated bovine serum albumin, $AgNO_3$, hydroquinone, citric acid monohydrate and sodium citrate dehydrate were purchased by Sigma-Aldrich. Neutravidin was purchased by Thermo Fischer Scientific (Massachusetts,USA). Other reagents used in this study were of analytical grade.

Nitrocellulose membranes (UniSart CN95) were purchased from Sartorius (Spain). Other materials used were: glass fiber sample pads (GFCP001000, Millipore, Germany), backing cards (KN-V1080, Kenoshatapes, Netherlands) and absorbent pads (Whatman, USA).

Based on previous results, the sample buffer consisted of 10mM PBS pH 7.4 with 0.05% Tween-20 and 2% BSA.

2.2. Equipment

An IsoFlow reagent dispensing system (Imagene Technology, USA) was used to dispense the detection line. A guillotine Fellowes Gamma (Spain) was used to cut the strips. To analyze the enhancement procedure, the strip was scanned before and after this step using a HP Officejet Pro 8500A scan. The optical density of the capture image from the signal monitoring window was digitized using ImageJ 1.48v software.

2.3. Labelling antibody with colloidal gold

The desirable concentration of the antibody to stabilize the gold nanoparticles was found following the titration of gold colloid procedure as described in reference [30]. Then, 100μL of 150 μg/mL HS-8 anti-PSA was added to 1.5mL of gold solution. After shaking for 1h, 100μL of the blocking solution Biotin-conjugated BSA (40% v/v ; prepared by mixing 40 μL of 1 mg/mL Biotin-conjugated BSA with 60 μL of 1 mg/mL BSA in phosphate buffer (PBS 10mM, pH 7.4) was added to the mixture to block the residual surfaces of antibody-colloidal gold conjugated. After 20 min of reaction, the solution was centrifuged at 10,000 rpm for 20 min. The supernatant was discarded and the pellet was resuspended in PBS with 10% sucrose and 1% BSA (100 μL). The product (AuNPs-HS-8 conjugate) was then stored at 4ºC until used.

2.4. Characterization of nanoparticles conjugates

Size distribution and ζ-potential were carried out with a Zetasizer Nano ZS (Malvern) equipped with a solid-state He-Ne laser (λ= 633) for monitoring the conjugation process.

2.5. Preparation of immunostrip

The nitrocellulose membrane (NC, 25 mm-wide) was incorporated onto a plastic backing card to give robustness to the membrane. The test zone of the strip was prepared dispensing a desired volume of 1 mg/mL mouse monoclonal HS-5 anti-PSA and anti-IgG to form the test (T) and control (C) lines respectively with the dispenser IsoFlow onto NC membrane at a dispensing rate of 0.100 μL/mm and was dried for 20 min at 37ºC. The sample pad and the absorbent pad were then settled onto the backing card with an overlap between them of around 2 mm. The complete strip was cut into individual 4 mm strips. The strip was assembled as describe in Figure 1.

2.6. Lateral flow immunoassay

PSA standard solutions at concentrations of 0, 0.1, 0.5, 1, 4 and 10 ng/mL were prepared by diluting a PSA stock solution in the sample buffer. The amount of BSA and Tween-20 was optimized for this assay, obtaining the best results when 2% BSA and 0.05% Tween-20 were added to the PBS. The volume of AuNPs-HS8 conjugate was

also optimized, looking for a compromise between sensitivity and reagent costs. For dipstick analysis, samples were transferred into the microtube containing 10 μL of AuNPs-HS-8 conjugate and homogenized (final volume 100 μL). Then the dipstick was added into the microtube and the sample was allowed to run for 10 min (Figure 1). The performance of the immunostrip relied on non-competitive assay formats. PSA in the sample was sandwiched between an anti-PSA antibody immobilized on the strip (Test line, T) and the AuNP-conjugated antibody. The unbound AuNP-conjugates migrated further to be captured by anti-mouse immunoglobulin antibodies (Control, C) for system functional verification. Once the test was run, the line intensities were recorded by scanning the images.

[FIGURE 1]

2.7. Silver enhancement procedure

2.7.a. Immersion

The immersion protocol for silver enhancement was carried out according to the literature with some variations [31]. A solution of silver nitrate (0.3 % w/v in water) and other of hydroquinone (3 % w/v in 0.5 M citrate buffer pH 4.0) were prepared and stored at room temperature in the dark. Just before use, the enhancing solution was freshly prepared by 1:1 mixing of the two solutions. 10 min after the addition of the sample, the NC strip was dipped into the microtube containing 100 μL of this solution for 10 min. The strip was then scanned to analyze the signal intensity.

2.7.b. Sandwich immunochromatographic assay

The protocol was executed as described elsewhere [22]. A glass fiber pad (4x10 mm) was washed with distilled water and immersed in a silver nitrate solution (0.3 % w/v in water). Another pad with the same dimensions was saturated with the reducing agent (3% w/v hydroquinone in 0.5 M citrate buffer pH 4.0). Both pads were dried at room temperature in the dark and kept in those conditions until use. After 10 min of the sample adding, the test zone on the NC membrane was covered by the silver pad, and then the reducer pad was placed above it. Once the pads were located forming a sandwich in the strip, 100 μL of distilled water was added to the pads. After 10 min, the strip was scanned to measure the optical density of the stain.

2.7.c. Conjugated pad modified with silver

In order to perform this assay, the silver salt for silver enhancement was incorporated into the device itself. A glass fiber sample pad (4x10 mm) was washed with different solutions to study the release of the silver from the pad and was immersed in a silver nitrate solution (0.3 % w/v in water). It was dried at room temperature in the dark and placed in the strip in the place of the sample pad. The strip should be kept in the dark until use. The test was carried out as described in part 2.5. After running the test, 50 µl of 3 % w/v hydroquinone in 0.5 M citrate buffer pH 4.0 were added in the microtube. Line intensities were recorded by scanning images after 10 min.

2.8. Gold enhancement procedure

In this assay, two AuNP conjugates were used. The first conjugate contained a monoclonal antibody against the analyte in the same way as in previous assays. The second conjugate is prepared by conjugation of 20-nm AuNP with neutravidin (the conjugation protocol is the same that in the case of AuNP with HS-5 described in part 2.3, after the optimization of the neutravidin concentration required to stabilize the gold). This procedure was designed to bind only the first primary AuNP conjugate to improve the signal. The test was carried out as described in part 2.5. Once the test was run, 8 µL AuNP-neutravidin conjugate, previously optimized, was added to the microtube containing PBS buffer with 0.05% Tween (final volume 50 µL). Line intensities were recorded by scanning images after 10 min.

3. Results and Discussion

3.1. Characterization of nanoparticles conjugates

Dynamic Light Scattering (DLS) measurements were carried out to confirm the conjugation reaction between the gold nanoparticles and the antibody or neutravidin in each case. This technique allows monitoring the size variation of the nanoparticles after the conjugation reaction. Comparing with TEM, the DLS values are expected to be larger due to the double layer which goes into the calculations of the hydrodynamic diameter of the particles in solution. The results showed that the hydrodynamic sizes of

the conjugates were 85.60 nm (PDI 0.180) and 43.88 (PDI 0.230) for AuNP-HS8 and AuNP-Neutravidin respectively. The data from DLS shows that the conjugates are monodisperse (Figure 2). The absence of additional peaks, together with the PDI value obtained, indicate the not presence of aggregates in the solution. The radious observed following conjugation is consistent with the steps carried out, including blocking with BSA.

To determinate their stability, ζ-potential measures were carried out. To remain stable, they must have enough repulsion to keep the particles apart. The results showed a ζ-potential of -20 mV and -23 mV for AuNP-HS8 and AuNP-Neutravidin respectively, indicating that the conjugates were stable.

[FIGURE 2]

3.2. Silver immobilization optimization

The first step to carry out the silver enhancement was to study the silver immobilization process itself. In order to optimize the silver release from the sample pad, it was treated with different solutions before the immobilization process (water, PBS 10mM with 0.05% Tween and PBS 10mM with 1%BSA). Only in the case that the sample pad was treated with BSA, the grey signal produced when the hydroquinone/citrate solution was added in the second step appeared. This could be explained because in the others methods either the immobilization process was less effective or the silver was strongly bond to the sample pad and couldn't be released from it. Additional experiments were carried out by modifying the sample pad with PBS 10mM with 1%BSA before the silver immobilization.

3.3. Gold enhancement procedure

Different volumes of AuNP-Neutravidin and different sample buffers were tested in order to obtain the higher amplification. The best results were obtained when 8 μL of AuNP-Neutravidin were added to PBS buffer with 0.05% Tween. Higher volumes resulted in a high background, hindering the signal.

3.4. Comparison of enhancement procedures

We compared four signal amplification methods involving gold, silver or both: Immersion in silver hydroquinone/citrate solution, Sandwich immunochromatographic assay, Conjugated pad modified with silver and Gold enhancement procedure (Figure 3). In order to compare these different enhancement strategies, and based in previous experiments, we selected a low PSA concentration, which produced a weak red line in the test line, almost undetectable when it was scanned. The concentration chosen was 0.5 ng/mL. All the assays were performed by triplicate and were scanned in grey scale with a scan resolution of 2400ppp. The results are shown in Figure 4. The test line intensity was analyzed using ImageJ software.

[FIGURE 3 and 4]

Referring to Figure 4, we see that, basing on naked-eye, the higher amplification was obtained using the immersion method. These results were confirmed using the ImageJ software, which showed 3-fold amplification related to the signal obtained when only gold is used. The optical density in this case is higher than in the other amplification methods. The results obtained when the sandwich method was used were good too, but the process is more tedious because it needs more user manipulation to put the two modified pads over the test and control lines. Moreover, it produces a higher background that in the case of the immersion. When silver is immobilized in the conjugate, the signal obtained in the test line is not homogeneous when the first binding assay with AuNP-HS8 is carried out. This is probably due to the non-homogeneous presence of BSA and silver nitrate in the sample pad. This effect also results in a lower sensitivity even before the enhancement step. The assay performed using two gold nanoparticles produced good results. However, they do not reach the amplification level of the silver immersion method. This approach has got the advantage of that it can be performed in a single step, mixing the two conjugates in the microtube before dipping the strip in . The amplification, however, is a bit worst, due probably to some steric impediments.

In view of these results, it can be concluded that the immersion in silver and hydroquinone/citrate solution produced low background, and yielded a relatively strong

signal, in a simple step consisting in mixing two solutions previously prepared. Therefore this was selected as the best amplification method for further assays. A photograph showing the red and grey-dark test and control lines produced by both gold (primary) and the best silver enhancement method is shown in Figure 5.

[FIGURE 5]

3.5. Calibration curve with silver enhancement

Several standards were prepared by spiking the buffer solution with various concentrations of PSA, and they were assayed by the test strip. Detection limit for the first step (AuNP-HS8 as label) was 0.5 ng/mL by eye-naked. To improve detection limit, the tested strip was dipped in the silver enhancer solution. Using this approach, 0.1 ng/ml of PSA was easily detected by naked-eye. Very low non-specific binding is shown in the absence of PSA in the silver enhancement step. The line intensities were recorded before and after the silver enhancement using the HP scan and the intensities were measured using ImageJ software. Results are comparatively shown in Figure 6.

[FIGURE 6]

4. Conclusion

An enhanced lateral flow immunoassay was successfully developed using different silver staining and one dual gold method as signal amplification strategy to detect PSA. The best results were obtained when the strip was dipped in a second step in a solution containing silver nitrate and hydroquinone/citrate buffer in proportion 1:1. A 3-fold improvement in sensitivity with a detection limit of 0.1 ng/mL of PSA could be achieved by using this approach. The proposed method is simple, convenient, and low cost. The performance of the assay was simple and can be completed in 20 minutes.

This study demonstrated a proof-of-concept of different LFIA amplification procedures, which could be potentially used in other applications.

5. Acknowledgments

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6. Conflict of interest

All authors declare no conflict of interest

7. References

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Figure captions

Figure 1.

A) Schematic representation of the Lateral Flow Immunoassay Dipstick. Specific antibodies against prostate specific antigen (PSA) (Test, T) and anti-mouse immunoglobulin antibodies (Control, C) are immobilized on the membrane. **B)** Detection of PSA before the amplification procedure. PSA, if present in the sample, form a complex with AuNP-conjugated antibodies and are captured onto the membrane by the immobilized antibodies. C) Dipstick procedure for sample analysis

Figure 2. .

Hydrodynamic size distribution profiles of different gold nanoparticles solutions before (denoted as AuNP 40 nm and AuNP 20 nm) and after the conjugation with antibody or neutravidin (denoted as AuNP-HS8 and AuNP-Neu, respectively)

Figure 3.

A) Silver enhancement procedure. Reducing reagent and silver ions are applied on the membrane and silver clusters are formed around the AuNP. B) Gold enhancement procedure. A second AuNP-conjugated is added on the membrane and it binds with the AuNP-conjugated antibody immobilized in the test line.

Figure 4.

Results obtained by enhancement process. In each cell of the table, the strip before the amplification process is on the left, and the strip after the amplification is on the right. The strips are accompanied by corresponding signal intensity peaks generated by ImageJ software A) Immersion. B) Sandwich immunochromatographic assay. C) Conjugated pad modified with silver. D) Gold enhancement procedure. Bottom row show the amplification obtained in each case. Both images and the image analysis are representative of three individual experiments.

Figure 5. Comparison of the test lines obtained in a Lateral Flow Immunoassay Dipstick for PSA (0.5 ng/mL). A) Antibody labelled with AuNP (40nm). B) Silver enhancement signal using the immersion method

Figure 6 Detection of PSA with varying concentrations by LFIA Dipstick and the silver enhancement by immersion. The data points were obtained in triplicate and error bars represent the standard deviations of triplicate runs. Representative examples of results obtained in the strips before and after the silver enhancement process are shown on the right.

Figure1

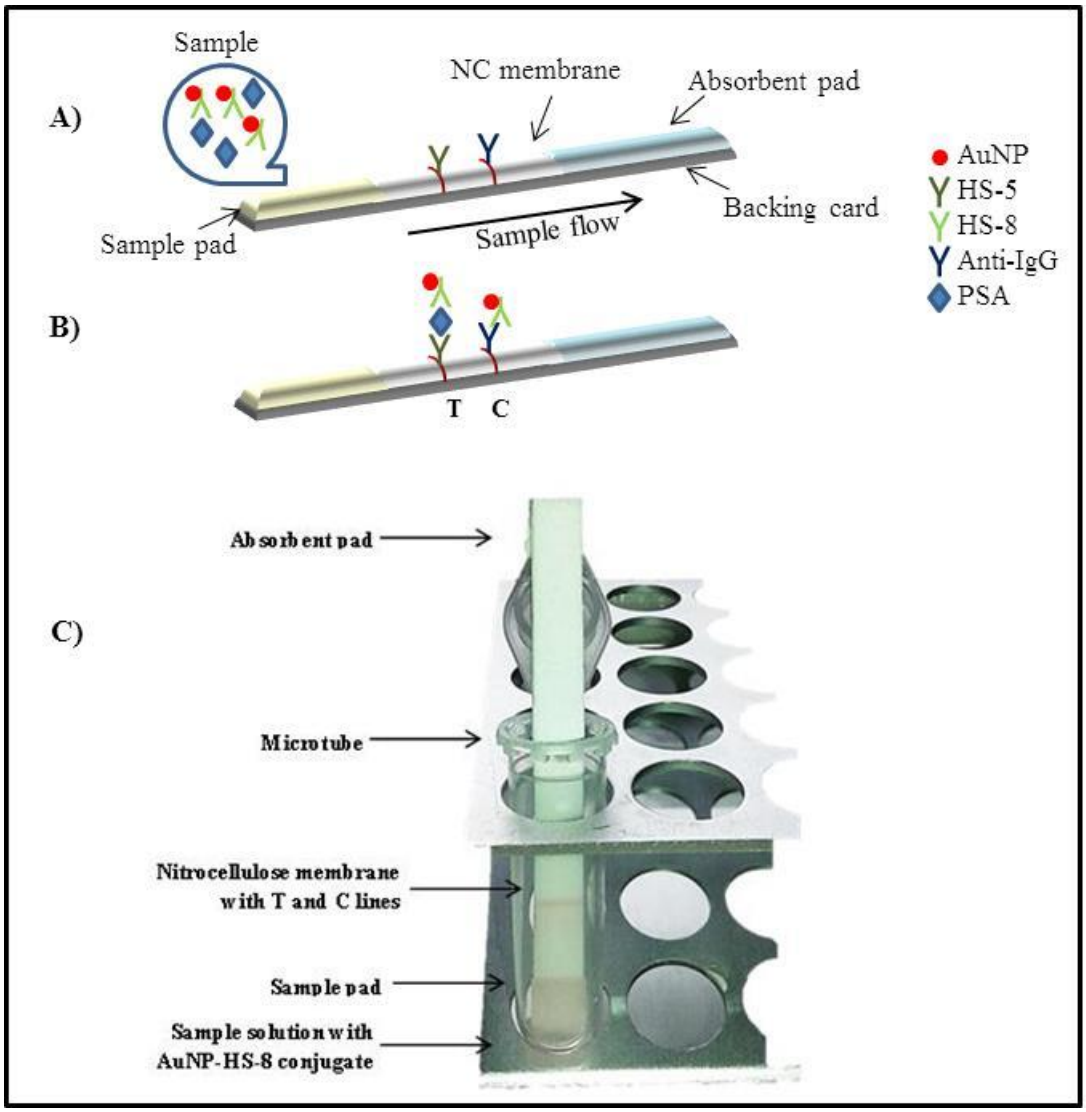

Fig. 1 A) Schematic representation of the Lateral Flow Immunoassay Dipstick. Specific antibodies against prostate specific antigen (PSA) (Test, T) and anti-mouse immunoglobulin antibodies (Control, C) are immobilized on the membrane. B) Detection of PSA before the amplification procedure. PSA, if present in the sample, forms a complex with AuNP-conjugated antibodies and are captured onto the membrane by the immobilized antibodies. C) Dipstick procedure for sample analysis

Figure2

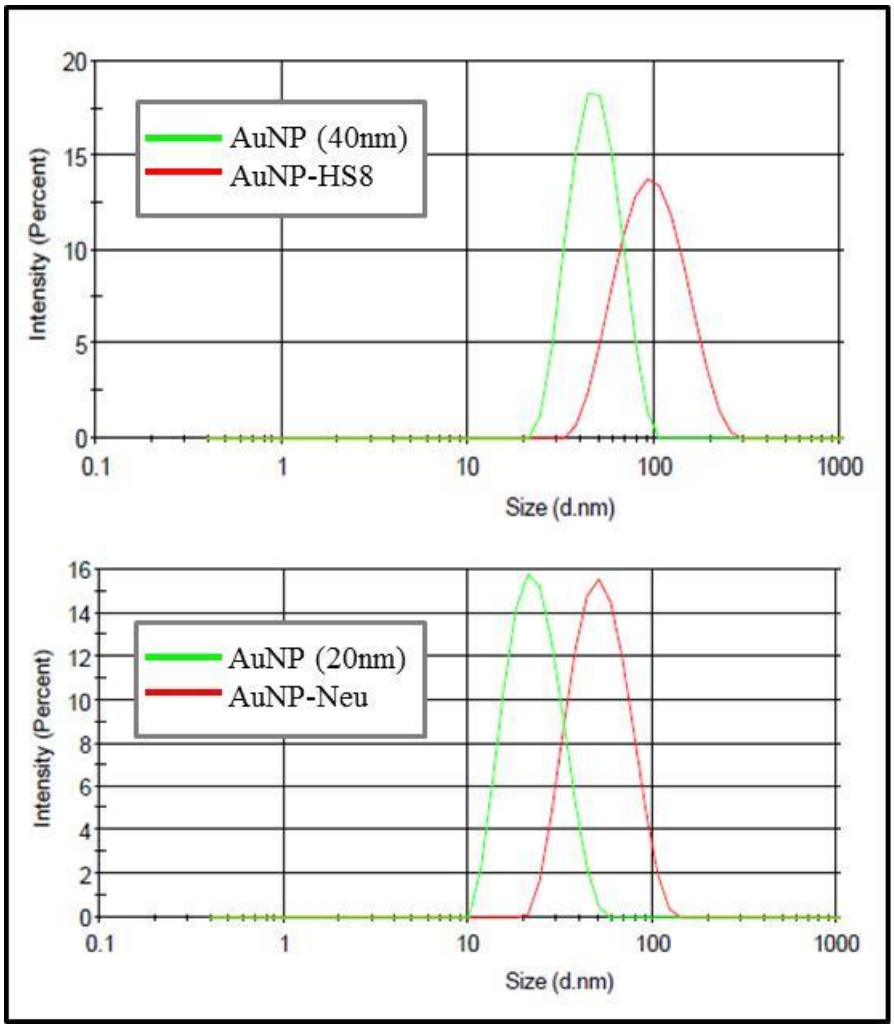

Fig 2. Hydrodynamic size distribution profiles of different gold nanoparticles solutions before (denoted as AuNP 40 nm and AuNP 20 nm) and after the conjugation with antibody or neutravidin (denoted as AuNP-HS8 and AuNP-Neu, respectively)

Figure3

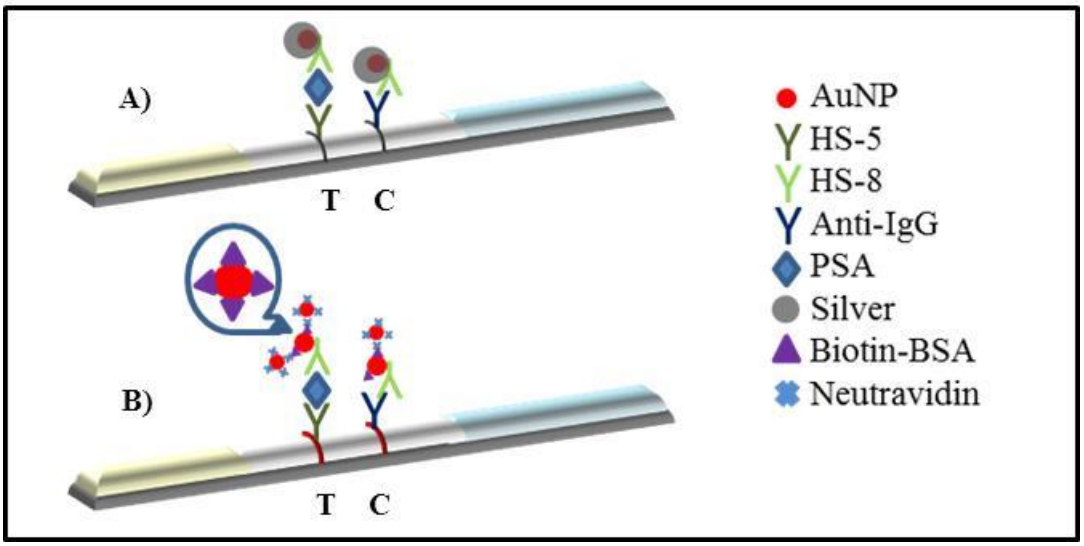

Fig. 3A) Silver enhancement procedure. Reducing reagent and silver ions are applied on the membrane and silver clusters are formed around the AuNP. B) Gold enhancement procedure. A second AuNP-conjugated is added on the membrane and it binds with the AuNP-conjugated antibody immobilized in the test line.

Figure4

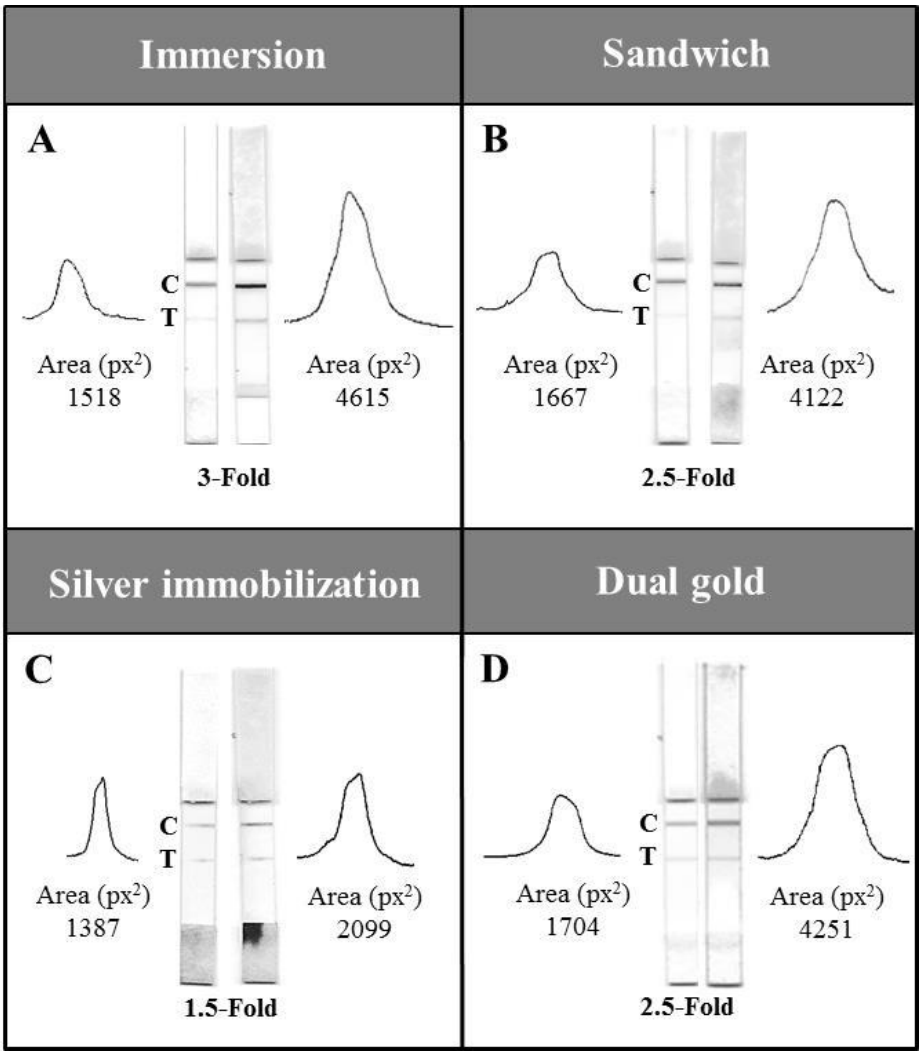

Fig. 4. Results obtained by enhancement process. In each cell of the table, the strip before the amplification process is on the left, and the strip after the amplification is on the right. The strips are accompanied by corresponding signal intensity peaks generated by ImageJ software A) Immersion. B) Sandwich immunochromatographic assay. C) Conjugated pad modified with silver. D) Gold enhancement procedure. Bottom row show the amplification obtained in each case. Both images and the image analysis are representative of three individual experiments.

Figure5

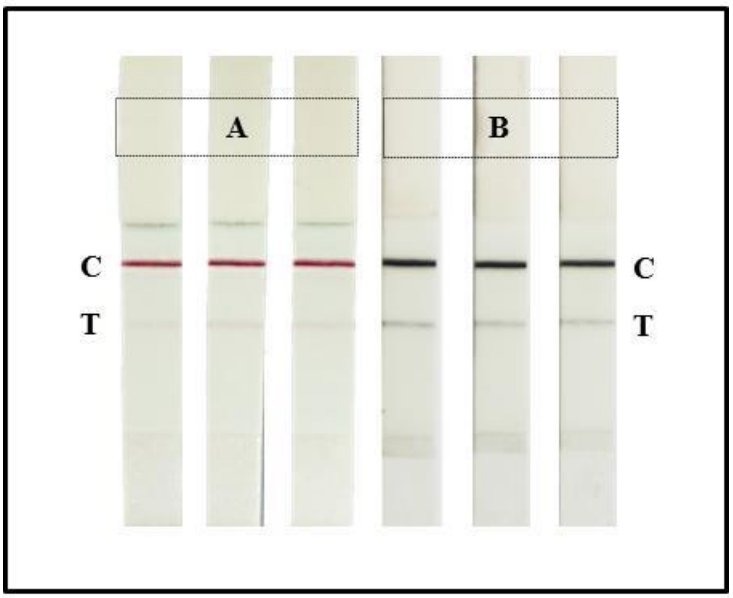

Fig. 5. Comparison of the test lines obtained in a Lateral Flow Immunoassay Dipstick for PSA (0.5 ng/mL). A) Antibody labelled with AuNP (40nm). B) Silver enhancement signal using the immersion method.

Figure6

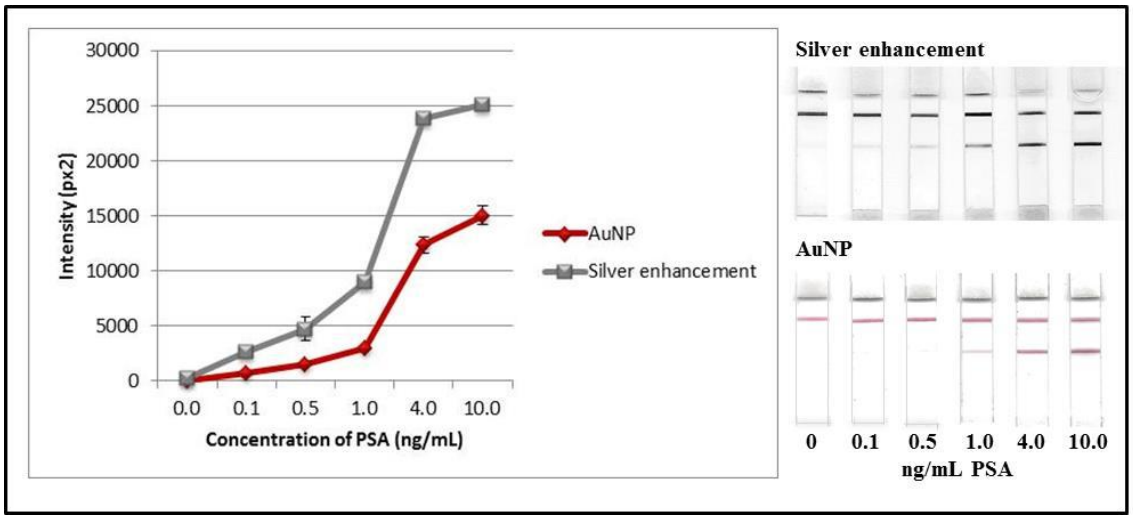

Fig. 6. Detection of PSA with varying concentrations by LFIA Dipstick and the silver enhancement by immersion. The data points were obtained in triplicate and error bars represent the standard deviations of triplicate runs. Representative example of results obtained in the strips before and after the silver enhancement process is shown on the right.

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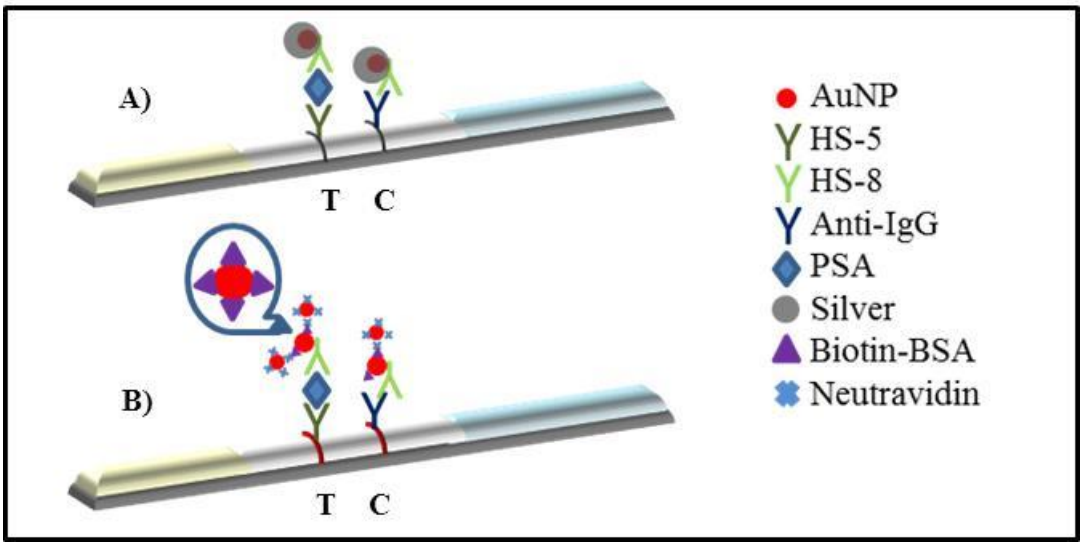
*Graphical Abstract (for review)



Graphical abstract