

Paper-Based Microfluidics Immunoassay for Detection of Canine Distemper Virus

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ABSTRACT

Paper-based devices present low-cost and are versatile, making them very attractive for clinical analysis. To manufacture those devices wax patterns are printed on paper surface and upon heating the wax permeates through the entire thickness of the paper, creating hydrophobic barriers that delimit test areas. Antibodies produced in rabbits against canine distemper virus (CDV) were physically adsorbed on the surface of gold nanoparticles (AuNPs) and incubated with CDV viral antigens, forming the immunocomplex. Anti-CDV antibodies were immobilized into the microchannels by physical adsorption, forming the test region. The test solution containing conjugated AuNPs was applied at the bottom of the microchannel and it was eluted with a phosphate buffer solution 0.01 M pH 7.4. When the solution containing the AuNPs reached the test zone the recognition of antigens contained on the immunocomplex occurred with the consequent development of a red line, which represents a positive outcome for the test. This method demonstrated the success of physical immobilization of antibodies on AuNPs and the physical immobilization of antibodies on cellulose's surface. This colorimetric assay brings simplicity and versatility to clinical analyses, presenting potential for CDV diagnosis.

Key words: paper-based devices; wax printing; point-of-care testing; gold nanoparticles; canine distemper virus.

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INTRODUCTION

Paper-based microdevices provide low-cost, fast and portable analysis, being suitable for application in clinical analysis in needy regions^{1,2}. Results from paper-based tests can be obtained on-site, on qualitative and semi-quantitative basis, or digitalized and send to an analysis center, for quantitative analysis by digital means³. The use of wax printing is an inexpensive and practical method for manufacturing microfluidic paper devices and requires only a wax printer and a hot plate or a lab oven. The printer deposits the wax onto the surface of the paper, and the hot plate melts the wax, which permeates through the entire thickness of the paper. This process creates hydrophobic barriers and produce channels suitable for individualized chemical reactions⁴.

Gold nanoparticles (AuNPs) have been extensively used in bioassays due to their optical properties and compatibility with biomolecules⁵. Colloidal solutions of AuNPs can exhibit different colorations, depending on their size, shape, and solvation, which is attributed to the surface plasmon resonance phenomena⁵. Several methods have been developed to optimize the production of AuNPs with controlled size and shape⁶ and surface ligands, to distinct applications⁷. AuNPs exhibit excellent affinity with various biomolecules, mostly through the interaction between those biomolecules with thiol and amino groups and the surface of AuNPs⁷, being one of the preferential attachment point^{8,9}. Different organic molecules and biomolecules can be used to increase specificity of functionalized AuNPs bioassays with the target molecules, showing the versatility of the method¹⁰.

The Turkevich method is the most common chemical route to produce AuNPs and uses tetrachloroauric acid (HAuCl₄) and sodium citrate to reduce Au (III) cations to metallic Au in aqueous solution. Other synthetic methods use different reducing agents, distinct reducing condition (photochemical reduction) or different reactional conditions, such as pH and temperature¹¹. The size of the nanoparticles can be tuned controlling the nucleation and growth of the nucleus formed at the beginning of the gold salt reduction by changing reaction temperature, reactional time and precursor concentration¹¹.

The use of AuNPs in immunoassays is based mainly on the combination of nanoparticles with biomolecules of interest, such as DNA, antibodies, amino acids, small peptides, among others^{7,12,13}, particularly antibodies, which enable the detection of specific antigens widely used in clinical diagnosis^{11,14}. One strategy for the bioconjugation of gold nanoparticles is physical adsorption through electrostatic interactions, which allow interactions between surfaces with opposing charges. Under physiological conditions antibodies have an overall positive charge¹⁵, interacting electrostatically with the nanoparticles when in contact with them⁷. This method is common in laboratories as it is simple and efficient. Besides the fact that this is the easiest approach to immobilize antibodies onto the AuNPs surface, it results in a random distribution of antibodies, what can diminishes the binding affinity between the antibody and antigen, resulting in loss of sensitivity in bioassays⁷. This inconvenience is minimized in qualitative and semi-quantitative readouts, such as in this low-cost bioassay, when in comparison with quantitative measurements.

Canine distemper virus (CDV) is an infectious disease caused by a virus, of the *Morbillivirus* genus, *Paramyxoviridae* family, that infects domestic dogs and carnivores¹⁶. The main symptoms are vomiting, diarrhea and respiratory affections, besides affecting the nervous system, leading the seizures, paraplegia, coma, and death¹⁶. Due to the fact that this disease presents a high mortality rate, its rapid diagnosis becomes extremely relevant. Among the most common diagnostic methods, the polymerase chain reaction (PCR)¹⁷ and the immunoassays¹⁸ are

prominent, but immunochromatography assays arise as a new alternative for rapid diagnosis of CDV¹⁹. The functionalities of nanoparticles and the low cost of paper-based microdevices were combined to prospect a rapid and inexpensive test to CDV.

MATERIALS AND METHODS

Paper microdevice production

The platform was designed in the CorelDRAW software with a layout similar to that used in conventional lateral flow tests. The sheets of Whatman N° 1 chromatographic paper were then cut to size A4 and inserted into the Xerox Phaser 8560 printer. Wax patterns were printed on the surface of the paper, creating the channels. Then, the printed microdevices were taken to a thermal press and heated for 2 min at a temperature of 150 °C to melt the wax on the surface and allow it to permeate to into the paper. The steps of manufacturing the paper microdevices are shown in Figure 1.

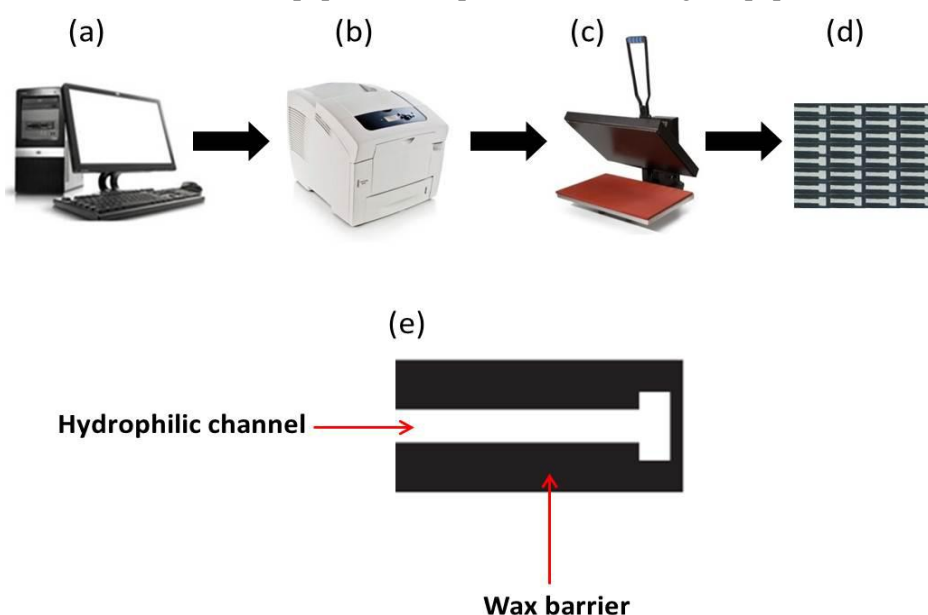


Figure 1. Steps involved in the production of the paper microdevices. (a) Designing layout. (b) Wax printing on the surface of the paper. (c) Heating wax printed paper with a heat press. (d) Several microdevices printed on a single sheet. (e) Example of a finished lateral flow microdevice.

Using the CorelDRAW software, microchannels of diameters ranging from 1 to 6 mm were designed in order to evaluate the most effective microchannel that lead to the smaller migration time.

Gold nanoparticles (AuNPs) synthesis

In order to synthesize particles with larger diameters and spherical shape two different methods were used: the Turkevich and the sodium borohydride methods.

Turkevich method

In a 250 mL round-bottom flask, 25.4 μmol of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ were added to 95 mL of water, and this solution was heated to boiling point under stirring. Under these conditions, 5 mL of a sodium citrate solution (1% m/m) was heated to 90 °C and quickly injected into the reaction medium. After 10 min, this solution was cooled down to room temperature and the stirring was kept for another 15 min. The colloidal solution was stored as-synthesized without further purification²⁰.

Martin method

An aqueous solution consisting of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (50 mmol L^{-1}) and HCl (50 mmol L^{-1}) was prepared. Another aqueous solution contained NaOH (50 mmol L^{-1}) and NaBH_4 (50 mmol L^{-1}). For the synthesis of the AuNPs, $100 \mu\text{L}$ of the $\text{HAuCl}_4/\text{HCl}$ solution were added to a test tube containing 9.6 mL of water at $25 \text{ }^\circ\text{C}$, under agitation using a vortex-type mixer. $300 \mu\text{L}$ of the $\text{NaBH}_4/\text{NaOH}$ solution was quickly injected into the $\text{HAuCl}_4/\text{HCl}$ solution. The solution changed from light yellow to orange and then to red, indicating the formation of AuNPs, then the stirring was stopped after 1 min and the solution was stored in dark for further surface functionalization²¹.

AuNPs Characterization

Colloidal AuNPs solutions were characterized by UV-Vis spectroscopy using a JASCO V-630 series instrument. The presence of the surface plasmon resonance band (SPR) near 520 nm indicates nanoparticles formation. The concentration of particles was calculated by using the extinction coefficient from the literature⁸.

The morphology of the nanoparticles was analyzed at the Structural Characterization using a PHILIPS CM120 transmission electron microscope (TEM) operating at 120 kV and a JEOL JEM-2100 transmission electron microscope operating at 200 kV . The samples were prepared to analysis as follows: a small portion of the sample was dispersed in isopropanol, and $10 \mu\text{L}$ of this dispersion was then dropped on a TEM copper grid containing a Formvar® film coated with a carbon sputtering. The solvent was allowed to evaporate slowly at room temperature, and the sample was kept in a vacuum for at least 24 h . The diameter of approximately 150 nanoparticles was performed using ImageJ software to estimate the average particle diameter.

Production of rabbit polyclonal antibodies against CDV and its partial purification

The production of rabbit polyclonal antibodies against CDV (anti-CDV) was approved by Ethics Committee on Animal Use of Universidade Federal de São Carlos under No. 086/2012. Anti-CDV polyclonal was generated by immunizing rabbits with intradermal injections of 1.0 mL of viral suspension (respectively, 1.0 mg mL^{-1}) mixed with 1.0 mL Freund's complete adjuvant, in order of determine the concentration of viral suspension. Subsequent injections of viral suspension mixed with incomplete adjuvant were given weekly for a period of 4 weeks and then monthly for a period of 3 months. The rabbits were bled 7 days after the last dose. The immunoglobulin fractions of both sera were separated by precipitation with ammonium sulfate (1.56 mol L^{-1} ; $\text{pH } 6.5$). Desalinization of the protein solution was performed using a dialysis sack (Sigma-Aldrich) with Molecular Weight Cut-Off (MWCO) of 12 kDa against PBS buffer solution (0.01 mol L^{-1} , $\text{pH } 7.4$)²². Immunoglobulin fractions were stored at $-70 \text{ }^\circ\text{C}$ until use. This desalinization procedure by selective and passive diffusion through a semi-permeable membrane is common for protein purification, buffer exchange in protein solutions and also for sample preparation for ion exchange chromatography. Desalinization was used here to avoid gold nanoparticles aggregation during conjugation with anti-CDV.

AuNP conjugation with anti-CDV antibodies

Anti-CDV antibodies were physically adsorbed on the surface of gold nanoparticles (AuNPs). 1.5 mL of gold nanoparticles solution were adjusted to $\text{pH } 9.0$ with 10 mmol L^{-1} borate buffer $\text{pH } 9.2$. Then, under constant stirring, $100 \mu\text{L}$ of the antibody solution ($100 \mu\text{g mL}^{-1}$) were added and this solution was incubated under agitation for 20 min at 650 rpm . Then, $100 \mu\text{L}$ of an aqueous bovine serum albumin (BSA)

solution ($1 \mu\text{g mL}^{-1}$) was added and the stirring was continued for another 20 min at 650 rpm. Finally, the solution was centrifuged at 14,000 rpm during 20 min. The supernatant was removed and the conjugated AuNPs precipitate was resuspended in 300 μL of a 2 mmol L^{-1} borate buffer (pH 7.4) ²³.

Immunochromatographic trials

The immobilization of anti-CDV antibodies in cellulose occurred by manual deposition onto the microchannel forming a line. This process used 5 μL of anti-CDV solution ($100 \mu\text{g mL}^{-1}$) diluted at the ratio 1:4 in carbonate-bicarbonate buffer (0.2 mmol L^{-1} , pH 9.6), and the microplate containing the antibodies was maintained for 1 h at 37°C and overnight at 4°C .

As long as the purpose of this test was to identify the canine distemper virus antigens, CDV antigens ($635 \mu\text{g mL}^{-1}$) were mixed at the ratio 1:2 with AuNPs conjugated with anti-CDV antibodies after sensitization. The immunocomplex was captured by the immobilized anti-CDV antibodies on the test line. Five microliters of the immunocomplex were applied at the bottom of the device, which were eluted with 200 μL of PBS buffer solution (0.01 mol L^{-1} , pH 7.4) to permeate through the whole channel, being detected at the test line.

RESULTS AND DISCUSSION

Nanoparticle synthesis

The synthesis of gold nanoparticles is easily followed by eye. Initially, the $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ solution presented a yellow color, but upon the formation of gold nanoparticles, the solution changed to a red color. Depending on the synthesis method AuNPs with different sizes are obtained. The Turkevich method ²⁰ generated spherical particles with an average diameter of 17.3 nm (Figures 2B, 2C, and 2D), while the Martin method ($\text{NaBH}_4\text{-NaOH}$) ²¹ generated smaller spherical particles, with an average diameter of 5.4 nm (Figures 2A, 2C, and 2D). Figure 2D shows the UV-Vis spectrum of Turkevich AuNPs and Martin AuNPs before and after anti-CDV conjugation, showing that the conjugation process held colloidal stability for both methods. Since the conjugation of AuNPs with anti-CDV antibodies approach used in this work was based on unspecific electrostatic interactions ⁷, some parameters modulate the adsorption efficiency, such as: nanoparticles size, surface charge, pH and antibody concentration ²⁴. As IgG molecules are relatively large ($\sim 150 \text{ kDa}$), increasing the size of AuNPs also increases their total surface area, which could improve the adsorption ²⁵. Indeed, AuNPs synthesized via Turkevich method displayed bigger diameter than Martin nanoparticles and, in principle, has the ability to conjugate higher amount of IgG per particle. Therefore, anti-CDV/AuNPs from Turkevich method were chosen to be used in the immunochromatographic assays.

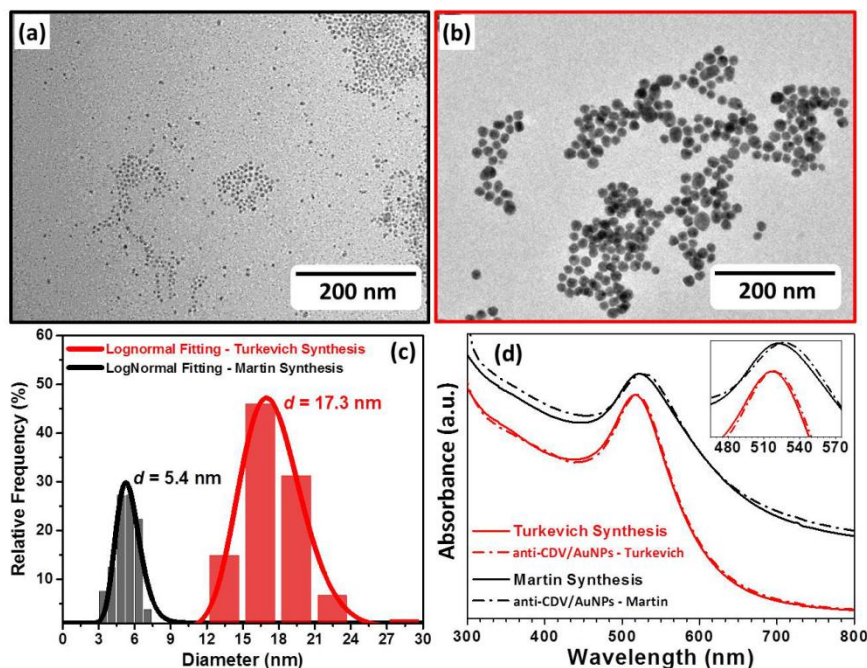


Figure 2. (a) TEM micrograph of AuNPs synthesized via Martin method; (b) TEM micrograph of AuNPs synthesized via Turkevich method; (c) Relative frequency counting and lognormal distribution fitting of both AuNPs samples showing their average diameter (d); (f) UV-Vis spectrum of both AuNPs suspension, Martin synthesis ($8 \times 10^{-8} \text{ mol L}^{-1}$) and Turkevich synthesis ($1.5 \times 10^{-9} \text{ mol L}^{-1}$) before and after anti-CDV conjugation.

Microchannel optimization

In order to evaluate the design of devices that allows the fastest migration rate of liquids into microchannel, six devices with the thicknesses of the hydrophilic channel ranging from 1 to 6 mm (nominal) were placed in contact with 200 μL of red ink for pen (Waterman, Paris), to assess the migration time of the front of the solvent in each microchannel, as shown in Figure 3A to 3D. After melting, the wax spreads into the original area of channel, resulting in channels with smaller width than the nominal values originally printed⁴.

Detection of canine distemper on paper

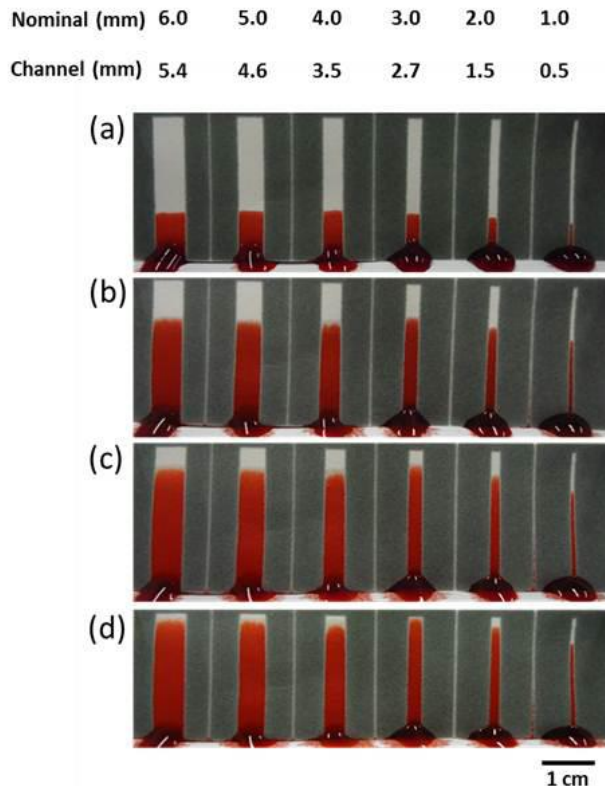


Figure 3. Study of variation of width of the microchannels and fluid migration times. (a) 30 s after the application of the test solution. (b) 1 min 30 s. (c) 2 min 30 s. (d) 3 min 10 s.

After 3:10 min, the channel filling was complete in the platform with 3 mm nominal diameter (2.7 mm channel), and thus this thickness was chosen to perform the lateral flow assay. When the printed microdevices are heated there is a narrowing of the channels due to the lateral spreading of the wax from the hydrophobic barriers, as described by Washburn Equation (Equation 1) ²⁶, characterized by the permeation of melted wax in the porous paper matrix due to capillary flow ⁴. As long as the Washburn Equation is fluid and porous matrix dependent – the melted wax and the paper, respectively – there is a linear relationship between the width of the barrier and the printed line (Equation 2) and the width of the channel and the space between the hydrophobic barriers (Equation 3) ⁴, what allows one to predict the final dimensions of the device during designing steps.

$$L = \left(\frac{\gamma Dt}{4\eta} \right)^{1/2} \quad (1)$$

$$W_B = W_P + 2L \quad (2)$$

$$W_C = W_G - 2L \quad (3)$$

L: Distance percolated by the liquid

η : Liquid viscosity

γ : Surface tension

D: Pore diameter

t: Permeation time

W_B : Width of the barrier
 W_P : Width of the printed line
 W_C : Width of the hydrophilic channel
 W_G : Space between the two printed lines

Immunochemical assays

The simplest and fastest method of immobilization of biomolecules on cellulose support is through direct adsorption, in which the biomolecule of interest is non-covalently attached to the cellulose by intermolecular interactions such as hydrogen bonding, van der Waals, hydrophobic or electrostatic interactions²⁷. However, depending on the complexity of the matrix in which the analytes are, the previously immobilized biomolecules can be removed from their original adsorption site what can affect later the detection step²⁸. The immunocomplex solution (antibodies conjugated with AuNPs and complexed to viral antigens) applied to the paper was eluted with a larger volume of PBS buffer in order to avoid pH variations during the elution and thus preventing migration of the absorbed antibodies.

The immunocomplex solution presented a red coloration on paper that was almost completely absent when the diluent buffer was applied. Only when the fluid front containing the whole immunocomplex reached the previously immobilized antibodies on cellulose surface (the test and control lines, respectively), the anti-CDV immobilized on paper recognized the antigen contained on the immunocomplex and interacted with it, thus forming the red line characteristic of a positive result. Non-specific interactions between the immobilized antibodies on cellulose surface and gold nanoparticles could result in a false positive outcome; however, such a problem is avoided by the recovering of the surface of the conjugated AuNPs with BSA, which blocks the surface of the nanoparticles from undesirable interactions²⁹. If the antigen is absent in the original solution, then the immunocomplex will not interact with the previous immobilized antibodies on the surface of the cellulose and the AuNPs will not concentrate in that test/control region, and there will be no appreciable change in the color of paper to red, thus indicating a negative outcome. The Figure 4 schematizes all stages of a lateral flow assays.

Detection of canine distemper on paper

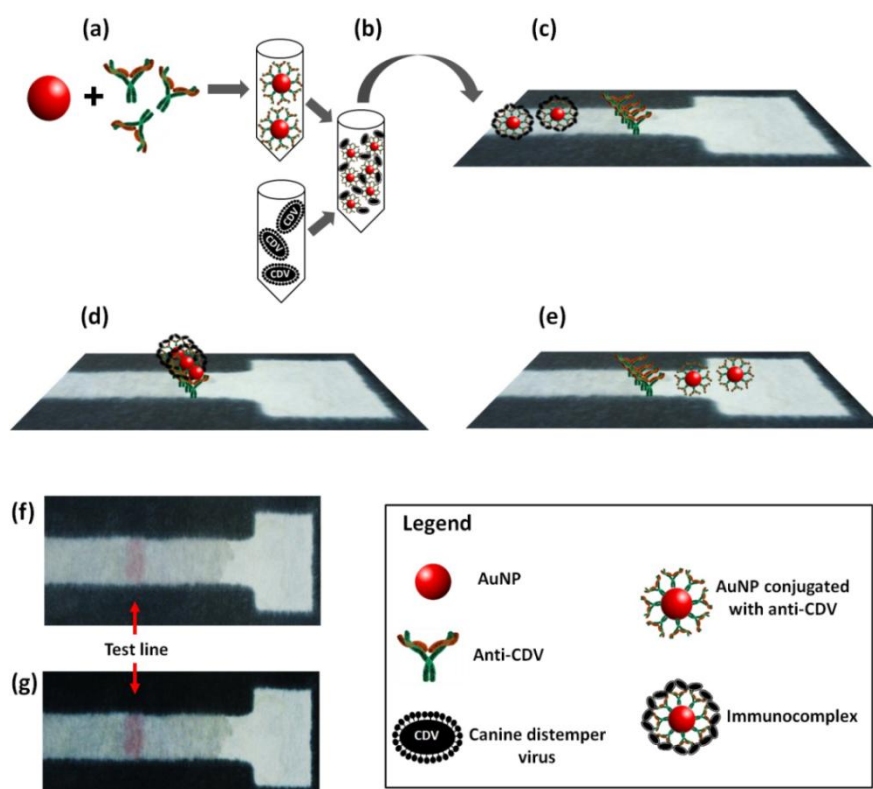


Figure 4. Lateral flow immunoassay on a single layer of paper. (a) Conjugation of gold nanoparticles with anti-CDV. (b) Immunocomplex formation. (c) Anti-CDV antibodies immobilized on cellulose and application of immunocomplex solution at the bottom of microchannel. (d) Recognition of viral antigens contained in the immunocomplex by the immobilized anti-CDV. (e) Anti-CDV antibodies immobilized on cellulose surface and application of gold nanoparticles with antibodies solution (antigens are absent in this case) at the bottom of microchannel, not occurring recognition step between the anti-CDV immobilized with the antibodies in the nanoparticle, and thus there is no change in color in the test line (negative outcome). (f) Photograph of lateral flow positive assay. (g) Photograph of the lateral flow positive assay with an enhanced contrast (digital), for better visualization of the test line.

In the scheme presented in Figure 4 is possible to notice that after the immunocomplex application (Figure 4c) the solution reaches the immobilized antibodies zone on cellulose, and then the bio recognition occurs (Figure 4d)¹⁹, with the consequent development of a red line. The maintenance of the reddish color of the solution suggests the stability of the immunocomplex, corroborating with the spectra presented in Figure 2D. This line indicates that there was a recognition between canine distemper virus and antibodies from the AuNPs, what provides signal intensification and then the red color appears in the test line (Figure 4F; 4G). These results showed that the expected interaction between the immobilized anti-CDV and the immunocomplex from the solution was efficient. Thus, we could monitor the success of the bioconjugation process with gold nanoparticles and the immobilization using bicarbonate/carbonate buffer. conjugation

The next step of this study involves method validation, in order to evaluate the ruggedness of devices, made by the comparison with standard methods (i.e., ELISA)¹⁸. Furthermore, clinical trials should provide relevant information on the performance of test, such as sensitivity, specificity, and accuracy^{30,31}. This new diagnostic tool focuses on the development of simple, cheap, and robust immunoassays, that would be used in a preliminary screening for the disease.

CONCLUSION

Immuno-chromatographic assays using a single layer of chromatographic paper and wax printing arises as a prototype with potential clinical use due to their simplicity and low manufacturing costs. AuNPs synthesis by the Turkevich method resulted in gold nanoparticles with an average diameter of 17.4 nm and a spherical shape ideal for carrying out bioconjugations and for immunological applications. By varying the width of the microchannels we found out that channels with 2.7 mm presented the fastest wetting rates and were chosen as the preferential design for the microdevices. The simplest method of antibody bioconjugation with gold nanoparticles by physical adsorption made it possible to detect canine distemper virus at the test line, indicating the potential use of such devices at home or in veterinary clinics.

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