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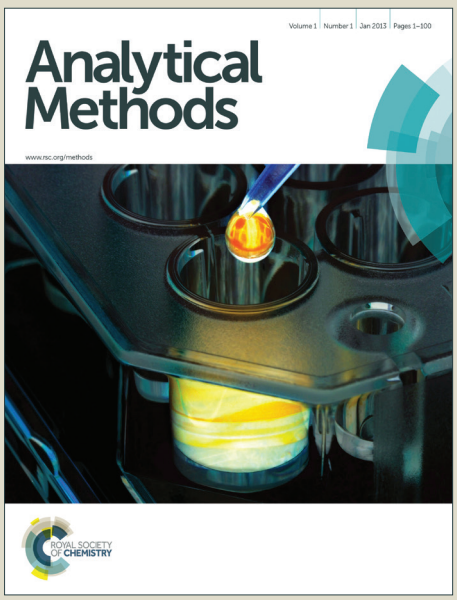





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# A fast and highly sensitive method for the detection of canine distemper virus by naked eye

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An easy, low cost method for the detection of canine distemper virus (CDV) that requires no instrumentation and uses gold nanoparticle-labeled antibodies is presented. The proposed method provides linear results between the concentration and signal in the range of 0 to 1.5 μg/L with a detection limit of 0.7 ng/mL. The method was successfully applied in the detection of the CDV virus in real samples of urine and, the results from these studiescorrelate with values obtained using conventional methods.Our results offerthe possibility of easy, rapid and reliable clinical diagnosis of CDV by monitoring the colorimetric change and measuring linear surface plasmon resonance (LSPR) changes in urine samples. The rapid visual detection of the virus on a test assay may offer a simple and cost-effective alternative to highly complex and instrument-dependent commercial methods.

## AUTOR INFORMATION

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The canine distemper virus (CDV) is a highly contagious disease.[1] This pathogen has high rates of mortality with lower mortality rates observed only in cases of canine rabies,[2] but the eradication of this virus is considered impossible because of its wide range of hosts, which includes terrestrial carnivores. However, CDV can be preventedthrough vaccination. The high mortality rate of CDV necessitates the acceleration of diagnostic procedures to quarantine infected individuals and start the appropriate treatment early. [3] The broad spectrum of clinical signs, which are similar to the signs observed in other respiratory and enteric diseases of dogs, hampers clinical diagnosis of canine distemper and requires laboratory confirmation. Some techniques used to diagnose CDV include serology tests,[4] indirect fluoroimmunoassay,[5] histopathology,[6] reverse transcription-polymerase chain reaction (RT-qPCR),[4] immunofluorescence[7] and immunohistochemistry.[8] However, each of these tests has limitations. The serological techniques are limited only allowing diagnosis of CDV once the infected animal died from distemper and may or may not provide measurable antibody titers.[9,10] The technique of virus isolation in cell culture is specific but time-consuming and can result in false-positive results if the animal is not in the acute phase of the disease.[5] Histopathology is not specific and normally requires a confirmatory test. All of these techniques have the additional limitations of high cost, being time-consuming and requiring extraction of nucleic acids and genome amplification using polymerase chain reaction (PCR) or RT-PCR.

More recently, metallic nanoparticles have been successfully employed as molecular-recognition elements and amplifier biosensors, in addition to serving as components in nanoscale optical devices.[6] Immunoassays using gold nanoparticles have attracted much attention because of their size- and shape-dependent optical properties, which differ significantly from those of the bulk metals.[11] Gold nanoparticles are of great interest for label-free sensing because their extinction spectrum is highly sensitive to the dielectric constant of the surrounding medium.[12] According *Selvakumar et al*., gold nanoparticles are ideal materials for the production of biosensors since they present between all unique electronic and optical properties, making them suitable for application in immunoassays.[13] Biosensors using gold nanoparticles are more easier and stable to work than those using a conventional system with enzymes or fluorescent, so that they are bioconjugation easily with various ligands such as nucleic acids, aptamers and anticorpos.[14-16] Localized surface plasmon resonance (LSPR) biosensors employ noble metal nanoparticles as an alternative to surface plasmon resonance (SPR) sensors

because the highly localized electromagnetic fields that occur at nanoparticle surfaces can enable improved detection of nanoscale biological analytes. LSPR can sensitively monitor binding events in real time and has been used to detect a variety of processes, including biomolecular interactions (antigen–antibody, DNA–DNA, protein-enzyme), and for typical detection times. Also, the LSPR peak position is strongly dependent on the size and shape of the nanoparticles, as well as on the dielectric constant of the environment. It was previously demonstrated that changing the interparticle distance radically changes the gold nanoparticle LSPR peak position. Normally gold nanoparticles show a maximum absorption at ca. 520 nm. After the bioconjugation, they start forming aggregates, which shifts the absorption band to ca. 600–800 nm. This change can be observed by the naked eye or measured quantitatively with an ultraviolet-visible (UV–Vis) spectrophotometer. This behavior has been utilized for sensing of various biological substances and models such as immunoglobin,[15] Alzheimer's disease,[16] *E. coli* and salmonella,[17] nuclease activity,[18] DNA,[19] influenza virus[20] and bacteriophages.[21]

In this study, we describe a simple and colorimetric new method to detect CDV, using real-time binding of an antigen to an antibody-gold-nanoparticle conjugate.In contrast to the previously mentioned conventional methods for the detection of CDV, the methodology provides a convenient means of realizing rapid and sensitive CDV-specific antibody detection. In addition, this methodologyis fast, non-toxic, and biocompatible and can partially mimic conditions in whole serum samples for clinical use, as compared to conventional ELISA-based protocols. The great advantage of this new method is that the samples used for diagnosis are urines collected in a non invasive manner not being painful for the animal. Another advantage of this technique as compared to other existing is that based on a colorimetric analysis using gold nanoparticles which have unique characteristics compared with other materials, such as unique ability to good biocompatibility and easy functionalization with proteins that make them ideal as labeling tags or reading platforms in biosensors.[22]

Antibody immobilization on the gold nanoparticles (AuNPs) was investigated using LSPR spectroscopy. Antibodies were labeled with 25-nm AuNPs through electrostatic and covalent interactions between the antibody side chains and nanoparticle surfaces. AuNPs were synthesized according to standard wet chemical methods using sodium citrate as a reducing agent.[23]A colloidal gold solution appears intensely red in color. The transition electron microscopy (TEM) image in Figure S1A shows mono dispersed gold

nanoparticles with an average particle diameter of 23 nm (see Figure S1B). A characteristic LSPR band of the AuNPs is shown in the UV-Vis spectrum at 525 nm, which confirms the presence of nanoparticles (see Figure S1C). To verify that the antibodies areattached to the gold nanoparticles, extinction measurements of the gold nanoparticles were taken before and after the conjugation step (Figure 1A). The gold nanoparticles exhibited an extinction peak at 525 nm. The next step of the methodled to a peak that shifted 6 nm, indicating the self-assembled monolayer modification. The representative LSPR peak after the treatment with 1:1 EDC/NHS shifted to 533 nm (green line). Following antibody conjugation, the extinction peak shifted to 616 nm, indicating the attachment of the antibody. This shift is attributed to an increase in the local refractive index due to the antibody and is the result of an increase in the local refractive index of the medium surrounding the gold nanoparticles, as shown in the blue line. When the anti-CDV conjugated gold nanoparticles were measured, the LSPR band intensity decreased and continued to remain lower than that of the LSPR bands before the addition of anti-CDV. This behavior is caused by the interaction of the antibody with the colloidal gold particles. The agglutination of the gold probe with the anti-CDV antibody IgG reduces the surface of nanoparticles; consequently, the surface plasmon resonance is decreased. In comparing the nanoparticle spectra before and after antibody immobilization, there was a displacement of the peak at 97 nm because the ratio of the LSPR peak increases when the samples are illuminated with unpolarized light. This change in the optical spectrum suggests that some of the nanoparticles may be attached in an end-on configuration. Following CDV conjugation there was an extinction peak shift of 30 nm. As shown in Figure 1B, a clear colorimetric change is observed when CDV was added to the antibody-conjugated gold nanoparticles. This colorimetric change is mainly due to the aggregation of the antibody-conjugated gold nanoparticles, as shown in Figure 1A, followed by an extinction peak shift of 30 nm. We have not observed any color change upon the addition of serum albumin (BSA) protein or the negative control (see supporting Figure S2B). The colorimetric data demonstrate that our assay is highly sensitive to CDV and can distinguish it from other proteins. All the experiment, since from the preparation of solutions to be used to the adsorption of the urine sample, lasted about 2:30 hours.

The observations of the LSPR band changes were further confirmed through the characterization of each step of the experiment using transmission electron microscopy (TEM). To perform the analysis, each solution was deposited in specific metals gratings

of equipment and was viewed on the day after its deposition, in order to completely dry, thereby excluding possible interfering. Figure 2A(A) shows a representative TEM image of the gold nanoparticles with average diameter of 25 nm. After modification with MUA and EDC/NHS, the nanoparticles were measured to have a diameter of 48 nm (Figure 2A(B)), which confirmed an increase in shell thickness. Figure 2A(C) show the adsorption of the antibody through covalent binding between the thiol groups and the amines present inthe antibody. After the interaction between the antibody and CDV, the nanoparticleshave a diameter of 200 nmbecause CDV is very large with a diameter of approximately 100 nm (Figure 2A(D)). Comparing the results obtained using UV-Vis spectrometry and its reduction in the intensity of peak absorbance, we conclude that the results are consistent with the images observed using TEM. Our TEM image shows clearly that the antibody-conjugated gold nanoparticle and CDV interaction is highly specific. As a result, a colorimetric change is observed from red to yellow in color (as shown in Figure 1B). To confirm the results obtained in the characterization through TEM, we realize all the experimental step using surface plasmon resonance (SPR). Analyzing the SPR graphic (Figure 2B), we can observe all binding process which occurs throughout the experiment. The point A is the injection of EDC/NHS on the gold surface of the sensor that is modified with MUA, after this step washing of the surface was performed using a phosphate buffer solution pH 7.4 to remove weakly bound material (point B). In the point C was added to the antibody solution that remained for 1h, followed by washing (point D). To subtract the point D to point C, we can see that there was a shift in the RU for 301.24 milledegree with correspond to 2.5 ng/mm$^2$ of antibody adsorbed in gold nanoparticles, because an increase in the plasmon resonance angle of 120 millidegrees corresponds to an average material layer growth of 1 ng mm$^{-2}$.[24] After this washing step, we can observe that there was a small decay in the graph compared to the baseline (point C to D) confirming that there was a link between the antibody and nanoparticles. Point E represents the injection of BSA solution used to block the remaining spaces between antibodies, followed by washing (point F). The point G is the injection of negative urine (negative control), after the washing process it is possible to observe that there was a total decay of the line equaling the baseline, confirming the negative control (points G to H). The regeneration of the sensor surface was performed using glycine solution, which removed all the antigen prevailingonly bindinguntil antibodies, followed by washing steps (points I and M to J and N). The injection of positive sample with the presence of the virus occurred at points K and O,

which after the washing in points L and P, occurred a small decay signal showing that occurred specific binding between the antigen/antibody. All washing steps were performed using phosphate buffer solution pH 7.4.

To determine the optimal concentration of antibodies for the experiment, tests varying the dilutions of the antibody were performed (see Figure S3A). The ability of these gold nanoparticle-conjugated antibodies to bind specifically to an antigen and enhance the LSPR sensor response was measured by monitoring the LSPR shift upon antibody binding at several different concentrations of CDV. Hence, the sensitivity of the methodology was examined (Figure 3A); a plot of the changes in absorbance as a function of the anti-IgG concentration shown in Figure 3B indicates that the CDV detection limit was 0.7 ng/mL. Within the insert in the figure, the linear range of the CDV concentration as well as the linear fit of the plotted graph is shown (Figure 3B). The relative standard deviation (RSD) is 6.5%, showing a good reproducibility of the immunosensor preparation. The LSPR band intensity of the gold nanoparticles decreased and continued to remain lower than that of the LSPR bands before the addition of anti-CDV (Figure 5A). This observation is due to the fact that the CDV is much larger in size (~100nm) than the gold-nanoparticle bioprobes modified by the anti-CDV antibody (88 nm), and several antibody-conjugated gold nanoparticles are attached to the CDV surface. A similar result was also reported by *Wang and Irudayaraj* through varying the concentration of *E. coli*, in which a higher concentration of the sample had a smaller absorbance intensity and higher scattering due to the large number of compounds present in the sample. Similar behavior of IgG was found with CDV; the absorbance signal decreases as the CDV concentration increases. This indicates excellent sensitivity for a plasmonic nanosensor.[25,26] We normalized each pair of spectra to the absorbance at the maximum extinction before CDV attachment, which allowed for an easy comparison of the relative extinction increases after CDV attachment for each pair with the aim of exploring the selectivity for Au-LSPR. Similar virus needs to be seriously considered because they could be provided a false positive response. We investigated the effects of Ophidian Paramyxovirus (OPMV) (1), Parvovirus (2) Paramyxovirus (3), and Newcastle vírus (4) on the immunosensor response (see Figure 1). There was no change of color for all virus tested. These results demonstrate that the methodology was selective to CDV in the presence of other similar virus.

To evaluate the practicality of the methodology using gold nanoparticle-labeled antibodies, our results were compared with pre-existing nested RT-qPCR methodology using 14 positive urine samples. The average positive hits are in the range of 98.0%, which further indicates that the developed immunosensor possesses an excellent specificity and its performance meets the requirements for a sensitive determination of CDV in urine samples. This methodology has the potential to provide rapid and valuable information of presence of CDV as an alternative to time-consuming and expensive instrumentation analysis.

## Conclusions

In this work, we demonstrate the simple synthesis of gold nanoparticles modified for the detectionof CDV. Binding of CDV resulted in a redshift in the LSPR absorption peak, and a good correlation was observed between the wavelength shift and concentration, which demonstrates the feasibility of the method for the quantitative measurement of CDV. The method exhibited a linear range for CDV between 0 and 1.5 μg/mL with a lower detection limit of 0.7 ng/mL. The method has been shown to be simple, reliable, sensitive, selective and cost-effective for the measurement CDV in urine samples. The most important characteristic of the method is direct detection by the “naked-eye”, which makes it more convenient than other methods that rely on advanced instrumentation. With the results obtained in this work, our future prospects are to employ the use of this methodology for the diagnosis of other pathogens in different areas as a food, environment, and medical research.

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## Figure Caption

**Fig. 1** UV-Vis spectrum showing the absorbance and wavelength of the sample of gold nanoparticles (black line), MUA (red line), EDC / NHS (green line), antibody (blue line) and CDV (pink line)**.** The colorimetric tests show gold nanoparticles (A), after addition of antibody (B), a positive sample (C) and a negative sample (D), respectively. Inteferent test: Ophidian Paramyxovirus (OPMV) (1), Parvovirus (2) Paramyxovirus (3), and Newcastle vírus (4).

**Fig. 2 A)** TEM images of gold nanoparticles (A) and gold nanoparticles modified with self assembled-monolayer (B), antibody (C) and CDV (D). **B)** SPR graph showing the steps of the experiment and all process of adsorption and desorption on the surface of the sensor. The point A is injection of EDC/NHS, point C is adsorption of antibody, point E is solution of BSA, point G is injection the negative urine sample (negative control), points I was the glycine injections, K was the positive urines samples with the presence of CDV virus and the points B, D, F, H, J, and L are the washing steps using phosphate buffer pH 7.4.

**Fig. 3** Variations of the CDV concentrations. A) A sample of CDV diluted in 1 mL of 100.0 μg $L^{-1}$ in 10 mM PBS to 0.0 mL PBS (pink line), 20.0 mL PBS (black line), 50.0 mL PBS (red line), 100.0 mL PBS (green line) and 150.0 mL PBS (blue line). B) Calibration curve for CDV.

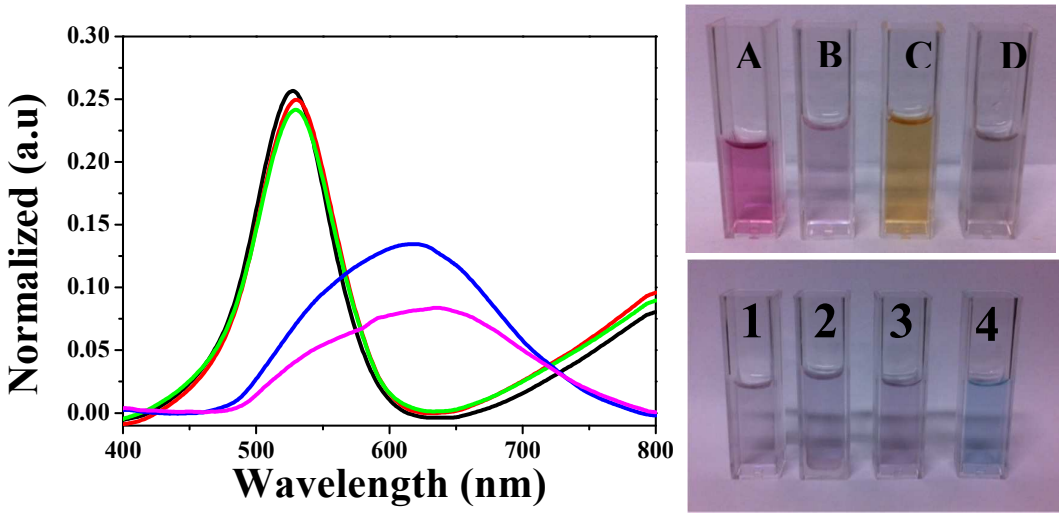
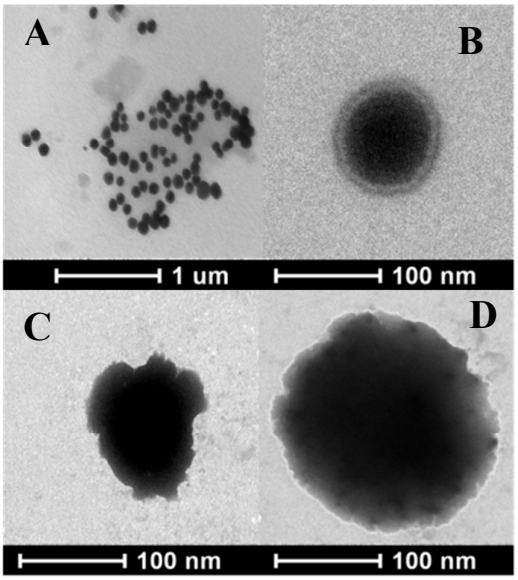



**Fig 1.**



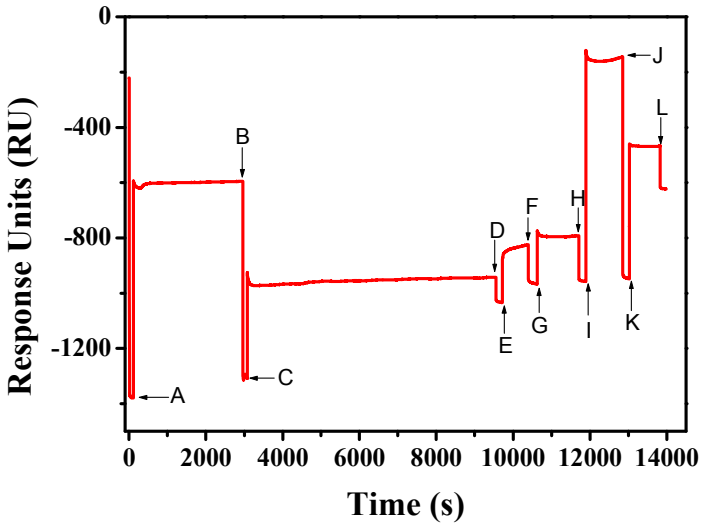




**Fig. 2.**

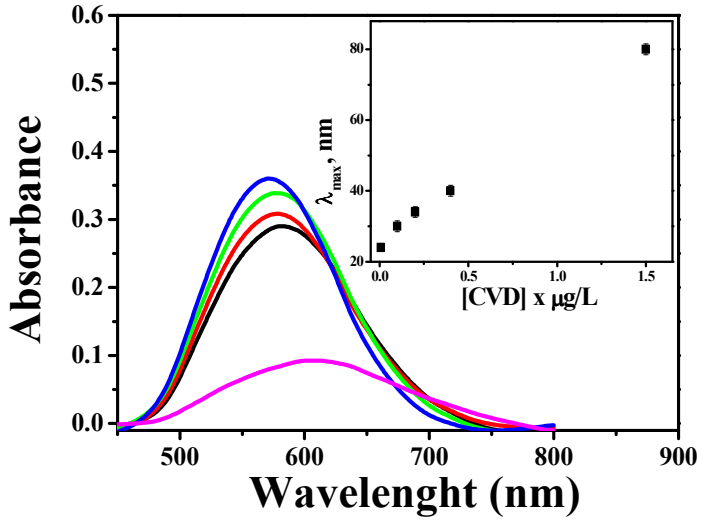



**Fig. 3**.