Colorimetric Test for Fast Detection of SARS-CoV-2 in Nasal and Throat Swabs

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Supporting Information Placeholder

ABSTRACT: Mass testing is fundamental to face the pandemic caused by the coronavirus SARS-CoV-2 discovered at the end of 2019. To this aim, it is necessary to establish reliable, fast and cheap tools to detect viral particles in biological material so to identify the people capable to spread the infection. We demonstrate that a colorimetric biosensor based on gold nanoparticle (AuNP) interaction induced by SARS-CoV-2 lends itself as an outstanding tool for detecting viral particles in nasal and throat swabs. The extinction spectrum of a colloidal solution of multiple viral-target gold nanoparticles – AuNPs functionalized with antibodies targeting three surface proteins of SARS-CoV-2 (spike, envelope and membrane) - is redshifted in few minutes when mixed to a solution containing the viral particle. The optical density of the mixed solution measured at 560 nm was compared to the threshold cycle (Ct) of a Real Time-PCR (gold standard for detecting the presence of viruses) finding that the colorimetric method is able to detect very low viral load with a detection limit approaching that of RT-PCR. Since the method is sensitive to the infecting viral particle rather than to its RNA, the achievements reported here open new perspective not only in the context of the current and possible future pandemics, but also in microbiology as the biosensor proves itself to be a powerful though simple tool for measuring the viral particle concentration.

Since its identification in China in late 2019, the SARS-CoV-2 epidemic has spread rapidly worldwide affecting millions of people, thus pushing the World Health Organization (WHO) to declare a COVID-19 outbreak a global health emergency. Mass testing is fundamental to identify and isolate clusters in order to limit and eventually eradicate SARS-CoV-2.¹ The gold standard for diagnosing COVID-19 infection is a reverse transcription real-time polymerase chain reaction (RT-PCR)² that is able to detect the virus genetic material (RNA) in samples collected via nasopharyngeal swab. Currently, only qualitative RT-PCR assays are available that yield positive/negative results without providing information about the viral load. Due to its complexity, RT-PCR tests are performed in certified laboratories, are time consuming, require experienced personnel, and can hardly lend themselves for mass screening.³–⁶ Huge efforts are put in overcoming such a bottleneck thereby making nucleic acid amplification suitable for Point Of Care tests, but the variety of methods⁷ and the lack of any commercial solution demonstrates that the gap between research and real applications is still to be filled.⁸ The main reason for that has to be found in the detection principle (RNA-extraction, reverse transcription and amplification) whose complexity, though greatly reduced by several approaches (e.g. Loop-mediated Isothermal Amplification (LAMP) and Recombinase Polymerase Amplification (RPA) or CRISPR-based detection) is far from being used for quick POC tests.

Lateral flow assays (LFA)s are among the actual biosensing platforms for home tests and potentially for mass screening,³–⁶ but the relatively poor sensitivity inherent to this technology⁹ makes urgent the quest for a different approach.¹⁰ Biosensors based on metal nanoparticles are often proposed because of their unique optical properties, which makes them potentially suitable to develop easy-to-use and rapid colorimetric diagnostic tests for point-of-care applications or even for home use.¹⁵ Due to its surface chemistry and given its biocompatibility gold is generally preferred to other metals.¹⁶ The physical process underlying this class of biosensors is the Localized Surface Plasmon Resonance (LSPR) that consists of coherent and non-propagating oscillations of free electrons in metal nanoparticles arising when they interact with an electromagnetic wave whose frequency resonates with the plasmonic one.¹⁷,¹⁸ Colorimetric detection based on gold nanoparticles (AuNPs) takes advantage of the color change occurring in a colloidal suspension from red to blue as a result of LSPR coupling among the nanoparticles.¹⁹ AuNP aggregation can be regulated using biological mechanisms such as antigen-antibody (Ab) interac-
Figure 1. (a) Sketch of the SARS-CoV-2 and functionalized AuNPs. SARS-CoV-2 proteins (spike, membrane and envelope) and their corresponding antibody (S, E, and M) are highlighted in dark red, light violet and gray, respectively. The inset shows the pink colloidal solution containing the anti-SARS-CoV-2 functionalized AuNPs (f-AuNPs). (b) The f-AuNPs surround the virion forming a nanoparticle layer on its surface. Their interaction leads to a shift of the resonance peak in the extinction spectrum and, hence, to a color change visible in the inset. (c) Extinction spectra reporting the OD of f-AuNP colloidal solution mixed with samples from patients with different viral load. At very low virion concentration (curve C<sub>5</sub>) the extinction spectrum is not distinguishable from the spectrum of f-AuNPs (black continuous line). At intermediate virion concentration (curve C<sub>7</sub>) the extinction spectrum is slightly redshifted and its difference with the “control” (f-AuNPs) produces the curve C<sub>7</sub>-C<sub>5</sub> that evidences the contribution entailed by the virion. At high virion concentration (curve C<sub>15</sub>), the extinction spectrum peaks at 560 nm as for C<sub>15</sub>-C<sub>7</sub>. The agreement between the curve C<sub>7</sub> and the simulated spectrum (gold continuous line, scaled to the experimental one) from a dielectric sphere (100 nm diameter) surrounded by smaller AuNPs (20 nm diameter) confirms the interpretation of the extinction spectra as due to nanoparticle aggregation.

The Photochemical Immobilization Technique (PIT) is a surface functionalization procedure that only requires UV activation of the Abs and leads to a high density functionalized surface within minutes. PIT has proven itself to be effective in tethering Abs upright not only on flat surfaces, but also on AuNPs, which were used either to labelballast small antigen or to realize a colorimetric biosensor for detecting IgGs and estradiol. In the latter cases, the presence of the antigen was detected as a change in the absorbance that can be easily measured by a spectrophotometer or even by naked eye. An approach relying on nanoparticle aggregation induced by the presence of the antigen was also used to detect the influenza A virus, but no clinical application was reported to demonstrate the effectiveness of the whole procedure in clinical cases.

Here, we report on the realization of a colorimetric biosensor that can be used for COVID-19 mass testing with sensitivity and specificity higher that 95% as demonstrated by a comparative analysis carried out on a total of 94 samples (45 positive and 49 negative), tested by standard RT-PCR in the Virology Unit of A.O.U. Federico II/Department of Translational Medicine of the University of Naples “Federico II”. The detection scheme is shown in Figure 1a and consists of a colloidal solution of PIT-functionalized AuNPs (f-AuNPs) against three surface proteins of SARS-CoV-2: spike, envelope and membrane (S, E, and M, respectively, in Figure 1a). AuNP fabrication (20 nm diameter), characterization and functionalization are described in the Supporting Information (see sections S1-S5), that contains a scalable procedure to realize the colloidal solution for COVID-19 test. In this approach, the sample was a solution of Universal Transport Medium (UTM, Copan Brescia, Italy), in which the specimen was dipped after its collection from the patient and without any additional treatment (see Supporting Information S6). Our test was carried out by mixing 50 μL of the f-AuNP colloidal solution with 100 μL of sample and 100 μL of ultrapure water. The presence of the viral particles (virions) induced the formation of a nanoparticle layer on its surface (Figure 1b) that led to a redshift of the optical density (OD) in the extinction spectrum of the solution (Figure 1c). When the viral load was relatively high, i.e. C<sub>7</sub> < 15 (Supporting Information S7), the color change from red to purple was visible even by naked eye (Figure 1a-b).

The extinction spectrum of f-AuNPs reported in Figure 1c (black continuous line) is not distinguishable from the spectrum of a mixed solution with a sample having C<sub>7</sub> = 32 (dashed light blue curve, C<sub>32</sub>). On the contrary, red shift is observed for a sample with C<sub>7</sub> = 15 (blue continuous line, C<sub>15</sub>), which becomes much more noticeable when C<sub>7</sub> = 7 (red continuous line, C<sub>7</sub>). The contribution of the virion (surrounded by nanoparticles) to the extinction spectrum can be deduced by subtracting the spectrum of f-AuNPs from C<sub>15</sub>. In fact, the curve C<sub>15</sub>-C<sub>7</sub> shows a peak at a wavelength comparable to that exhibited by C<sub>7</sub> (approximately 560 nm).

To further confirm that the spectrum C<sub>7</sub> arose from f-AuNPs surrounding SARS-CoV-2, we simulated the virion as a 100 nm diameter dielectric sphere of 1.45 refractive index. A change-
In order to test the validity of the colorimetric biosensor, we analyzed real samples previously examined by RT-PCR. The samples were from 45 positive to SARS-CoV-2 patients for which $C_t\leq 35$ and 49 negative patients ($C_t > 35$). For all of them, we measured the optical density at 560 nm ($OD_{560}$) by a commercial microplate reader (Figure 2a). It is quite evident the correlation between the $C_t$ value (reported on the top scale) of the positives (red circles) and $OD_{560}$, whereas all the negatives (identified by a progressive number in the bottom scale) are randomly distributed providing a low performance test associated to the colorimetric biosensor. In fact, with such a threshold we get 96% and 98% for sensitivity and specificity, respectively. This result is even more remarkable if we consider that $C_t > 30$ correspond to a very low viral load for which the infection aptitude is questioned. To this aim, it is important noticing that in the early phase of the infection, high viral loads are detected by RT-PCR in upper respiratory specimens with low $C_t$ reads (20, with our assay). Positive results with high $C_t$ readouts pose a diagnostic challenge, since they do not necessarily indicate active infection by a replicating virus. It has been observed using viral culture that patients with high $C_t$ RT-PCR results with protracted positivity are not infectious, suggesting that the assay likely detects non active viral particles such as genetic material present in remnants of inactive virus thereby making our approach of high diagnostic value.

The Receiver Operating Characteristic (ROC) curve from the data shown in Figure 2a is reported in Figure 2b together with the indication of three threshold values, all of them leading to sensitivity and specificity significantly higher than 90%. In particular, the highest threshold value (0.263) leads to 100% specificity while keeping the sensitivity at the remarkable value 94%. Overall, the high performance of the test associated to the colorimetric biosensor is demonstrated by the area under the ROC curve whose value is 0.98. The qualitative difference in the color between positives and negatives can be observed in Figure 2c that shows a picture of the multiwells containing the samples whose analysis is summarized in Figure 2a-b.

To measure the dose-response curve of the biosensor, we assessed the optical density $OD_{560}$ of samples obtained by serial dilutions (1:10) of an initial volume with very high viral load ($C_t = 7$). The results are shown in Figure 3, in which $OD_{560}$ is reported as a function of the relative concentration of SARS-CoV-2.
As a final remark, we point out that the colorimetric solution described here relies on its sensitivity to the virion rather than to its content (RNA). The importance of this is twofold: 1) after the calibration of the optical response, the biosensor lends itself as a powerful tool to quantify the viral load, a non-trivial issue in diagnostic assays in virology; 2) being sensitive only to the virions, the biosensor detects the presence of active viral particles; thus, our method is apt to discriminate between high infective samples from those containing inactive virus.

In conclusion, we realized a colorimetric biosensor based on a colloidal solution of AuNPs (20 nm, OD=1) each of them functionalized with Abs against one of the three surface proteins of SARS-CoV-2 (spike, envelop and membrane). The ratio among the three kinds of functionalized AuNPs was 1:1:1. Although both the ratio and the size of AuNPs are still susceptible to optimization so to allow one to push even further the limit of detection, the current performances of the biosensor would already permit its use as a tool for mass screening since the detection is based on the interaction among the virions and the pAb-functionalized AuNPs (single step detection) without any pretreatment (e.g. RNA extraction and amplification). The comparison of the readout of our biosensor at 560 nm with the threshold cycle (Ct) of a RT-PCR proved that viral loads corresponding to Ct=30 are detected by the colorimetric biosensor. This threshold is of particular importance because it corresponds to a low viral load for which the infecting capacity is likely negligible. Such a good performance has to be ascribed to a high filling ratio of the virion surface that results from the presence of multiple Abs (three proteins are targeted) and an effective AuNP surface functionalization procedure (PIT). In fact, through PIT not only one Fab is always exposed so to make AuNP highly “reactive”, but also the Abs are attached to the surface (side-on position) without any linker (e.g. protein A), the latter being detrimental for the plasmonic interactions among AuNPs on which the colorimetric biosensor is based.

Another remarkable feature of the biosensor described here relies on its sensitivity to the virion rather than to its content (RNA). The importance of this is twofold: 1) after the calibration of the optical response, the biosensor lends itself as a powerful tool to quantify the viral load, a non-trivial issue in diagnostic assays in virology; 2) being sensitive only to the virions, the biosensor detects the presence of active viral particles; thus, our method is apt to assess the actual degree of infectiveness of a specimen. Conversely, the qualitative RT-PCR assays do not allow to clearly discriminate between high infective samples from those containing inactive virus.

As a final remark, we point out that the colorimetric solution described here can be easily modified to target other viruses. Thus, we expect that single-step colorimetric detection of viruses can become a general technique to be used for laboratory applications as well as point-of-care testing.

ASSOCIATED CONTENT

Supporting Information

Materials (S1). Instruments (S2). Gold nanoparticle synthesis (S3). Optical and morphological AuNP characterization (S4). Functionalization (S5). Storage of the samples and validation of
the measurements with PCR (S6). Threshold cycle in PCR measurement and viral load (S7). FDTD optical simulations (S8).

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Notes

The authors declare no competing financial interests.

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