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Development of a new technology to detect respiratory infectious diseases

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Abstract
The Covid-19 pandemic brought the need to use social masks to prevent the spread of the SARS-CoV-2 virus. However, no reliable and fast method were yet established to detect viral particles and to improve the protective ability of social masks. Through color changes, colorimetric biosensors can be used as a rapid and easily approach to detect virus. Gold nanoparticles (AuNP) are known to have excellent optical properties and huge research potential. The new SARS-CoV-2 has the ability of entering human body cells, namely through a second pathway of entry – the sialic acid (SA) receptor. In order to respond to the emergency and to contribute to the diminishing of the spread of SAR-CoV-2, we developed a colorimetric biosensor based on the functionalization AuNP by sialic acid (SA) (SA-AuNP), as a new and effective textile coating layer, to provide a direct indication of the protective capacity of social masks. To do that, AuNPs (10 nm) were functionalized with SA (SA-AuNP), in three different concentrations (50-50, 30-70 and 20-80, respectively) to select the optimal concentration for respiratory virus detection. Fourier-transform infrared spectroscopy (FTIR) and Scanning Electron Microscope with a Transmission Detector (STEM) analyses confirmed SA-AuNPs binding. FTIR results showed a well-established bond, through matches of peaks of SA-AuNPs. Bindings between the compounds were more evident in 50-50 concentration of SA-AuNP. In the 30-70 SA-AuNP the STEM images show some superposition of the nanoparticles and not so evident binding, as in the 20-80 concentration. Still, between these last two concentrations, the 30-70 is the one that shows the best results since it is visible some circular points larger than the others. To achieve the goal, the concentrations 30-70 and 50-50 of SA-AuNP were impregnated (Textile Foulard) in two substrates of different compositions, a cellulosic and a synthetic one. However, in this technique there are many parameters, such as drying time and temperature, which were varied to understand which the best procedure was to obtain the biosensor. The development of these smart mask demonstrated as a sensitive, rapid, and simple way to ensure a greater individual protection against viruses that attack the respiratory tract, and further reduce their contagion and preventing their spread. This biosensor will provide information about the state of contamination by SARS-CoV-2 and other viruses that cause breathing problems.

Keywords
face mask — biosensor — SARS-CoV-2 virus — sialic acid

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Introduction

Severe acute respiratory coronavirus 2 (SARS-CoV-2), causing the coronavirus disease 2019 (COVID-19), has spread rapidly worldwide, affecting society on several levels: in public health, populations, social structures, and the economy, among others [1, 2]. This situation required a special attention from the World Health Organization (WHO) and other entities.

An effective strategy to combat and control the Covid-19 pandemic aims to develop methods highly capable of identifying and isolating individuals infected by Sars-Cov-2 [2]. In order to prevent the spread of the virus, the entire population was advised to use personal protective equipment, including face masks. This is an object of great importance and that has safeguarded us since, practically, the beginning of the pandemic. However, no reliable and fast method has yet been established to detect viral particles and to improve the protective capability of social masks.

Biosensors are analytical tools that convert a biological signal into a visible response and have been widely researched and developed as a tool for medical and pharmaceutical fields, among others [3, 4]. Thus, we seek to reduce the risk of contamination from this virus by increasing the effectiveness of mask protection, using a intelligent colorimetric biosensor incorporated into the mask that detects the presence of the virus. This direct indication is given through a colorimetric reaction, by which each user is informed of the need for mask replacement or the presence of virus.

For the development of this biosensor we relied on the use of gold nanoparticles (AuNPs), since research has noted that nanoparticles can be used as colorimetric probes to develop versatile biosensors, taking into consideration the unique optical properties of nanoparticles that can cause color changes detectable directly to the naked eye [5].

AuNPs have been studied extensively over the years and have various biomedical applications. They provide excellent platforms for the development of colorimetric biosensors as their chemical, optical and electronic properties are unique depending on their size, they are very easily functionalized, enjoy easy synthesis and, can exhibit different colors depending on their diameter, shape, and aggregation [6, 7]. From a morphological point of view, CoVs are enveloped viruses containing a single-stranded ribonucleic acid (RNA) viruses [8]. In this sense, SARS-CoV-2 belongs to the genus β-coronavirus characterized by being of crown-like, enveloped, and single-stranded RNA (+ssRNA) viruses [9].

Hoffmann et al. founded that SARS-CoV-2 use the receptor angiotensin-converting enzyme 2 (ACE2) for entry into host cells [10]. An alternative pathway to the entry of the virus into human cells by ACE-2 is the sialic acid which is present on the surface of lung epithelial cells and thus is involved in the primary binding of the virus. Haemagglutinin is a surface protein that is present on several viral species, such as influenza virus. Based on the ability of sialic acid to bind to certain viruses and the similarities that influenza virus has to SARS-CoV-2 virus, the development of a colorimetric sensor to detect SARS-CoV-2 virus using functionalized AuNPs is possible. This detection method is quick and easy to use and it is based on the binding between the envelope protein, haemagglutinin, and sialic acid, thus being able to detect the presence of SARS-CoV-2, and in turn reduce the spread of infection [11]. Given the symptoms and the diagnostic methodology used, we can say that the SARS-CoV-2 virus is similar to the influenza virus, and therefore this colorimetric biosensor gives us information on the state of contamination by SARS-CoV-2, but can also detect other types of respiratory viruses, which is a great advantage since it gives us the information of a safe environment or a contaminated environment.

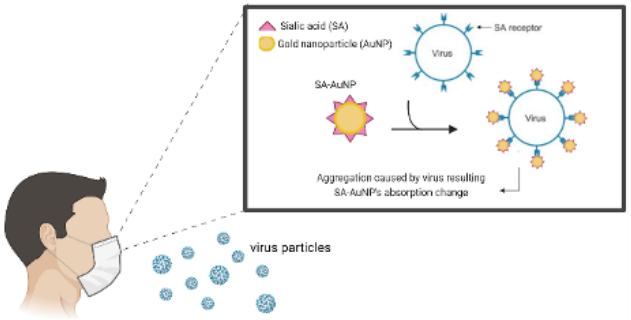


Figure 1. Representative scheme of the SA-AuNPs system incorporated in the masks.

The biosensor developed for the SARS-CoV-2 virus is based on the incorporation of AuNPs functionalized with an enzyme, ACE2 or a biomolecule that recognizes the virus, sialic acid (Figure 1) [12]. Subsequently this biosensor will be impregnated on a textile surface and incorporated into the mask.

1. Methods & Materials

1.1 Gold Nanoparticles with Protein (ACE2)

1.1.1 Reagents

Gold nanoparticles (AuNP, Product No. 752584-00ML) were purchased from Sigma Aldrich. Gold nanoparticles had 10nm of diameter and were stabilized in 0.1 M Phosphate buffer saline solution (PBS); The protein used was ACE2 (5 mg/ml); 10% (w/v) NaCl solution; PBS (0.5x and 1x); Sodium phosphate buffer; Acetic Acid.

1.1.2 pH Optimization and Enzyme Preparation

At this stage, 500μL of the AuNPs solution was added to an eppendorf, and the basal isoelectric pH of the enzyme was adjusted (pH=5.36), using acetic acid and sodium phosphate as needed; Then the enzyme was prepared for use, it was lyophilized and to start its use it was hydrated using PBS (0.5x and 1x), 10μL of the enzyme solution was added to the previous eppendorf, and left to incubate for 10 minutes at room temperature; subsequently 500μL of NaCl was added and left to incubate again for 10 minutes at room temperature.

Samples with sub-optimal pH and amounts of protein will aggregate faster after the addition of NaCl, this phenomenon

may have been observed by a color change of the solution from red to purple/blue. Therefore, if no color change will occur, the samples have ideal conjugation conditions [13].

1.1.3 Incorporation of AuNPs with the enzyme

Next, added another 11μL of enzyme to the previous solution, and stirred for 45 minutes in the centrifuge at a speed of 14.6 x 1000rpm; after centrifugation, and finally resuspended the pellet in PBS. After preparing the samples as indicated, we proceeded to the confirmation techniques for functionalization of AuNPs with ACE2.

1.2 Gold Nanoparticles with sialic acid

1.2.1 Reagents

Gold nanoparticles (AuNP, Product No. 752584-00ML) were purchased from Sigma Aldrich. Gold nanoparticles had 10nm of diameter and were stabilized in 0,1 M PBS solution. *N*-acetylneuraminic acid, 99%, a predominant form of sialic acid (SA, CAS: 131-48-6, 100MG) was used to funcionalize AuNP and was acquired from Thermo Scientific. SA has a powder form and a molecular weight of 309.271 g/mol, so deionized water was used for solution preparation.

1.2.2 Preparation of the SA stock solution

10 mL stock solution was prepared, with a concentration of 1.00×10^{-3} mol/L. Thereby, 3.0927 mg of sialic acid was weighed and dissolved in 10 mL of deionized water.

1.2.3 SA-AuNPs functionalization

In the functionalization phase of AuNPs with SA, different assays were performed according to Table 1, in which we varied the amount of nanoparticles, sialic acid, and deionized water. After the preparation of these solutions, two conditions were studied: stirring the samples for 20 minutes at 7200 rpm, without heating (20^{o}C) or with heating (80^{o}C), to understand if the temperature would interfere with the functionalization. After performing the stirring, the sample was centrifuged for 20 minutes at 6300 rpm, the pellet was collected and stored in deionized water for later use [14].

Table 1. Functionalization of AuNPs with SA – volume proportions.

V_{AuNPs} (0.1 mmol/L) (μL)	V_{SA} (1 mmol/L) (μL)	V_{H_2O} (μL)
200	5	95
	10	90
	20	80
	60	40
	40	60
	80	20
	100	0
175	125	0
50	50	0

After preparing the samples, confirmation for functionalization of the AuNPs with SA was performed by FTIR and TEM techniques.

1.2.4 Fourier transform infrared (FTIR) spectroscopy

The FTIR technique was used to understand whether the AuNPs are functionalized, through the presence or absence of chemical bonds. Surface functional groups of SA-AuNPs were characterized by FTIR on an ATR cell [11]. To perform this technique, it was previously necessary to freeze-dry the samples for a more careful analysis, and to ensure that no noise is present.

The FTIR analysis was performed in the laboratory of FCUP—DQB - Lab&Services, precisely in the equipment PerkinElmer, model Spectrum Two (ATR module). Four scans per spectrum were performed at a wavelength between 4000 - 400 cm^{-1} [15].

1.2.5 Scanning Electron Microscopy with a Transmission Detector (STEM)

The STEM analysis, based on the TEM technique with a transmission detector, is an Ultra high resolution scanning Electron Microscope with field emission, with an integrated system for X-ray microanalysis (EDS - energy dispersive spectrometer) and analysis of electron backscatter diffraction patterns (EBSD - Electron Backscatter Diffraction).

The equipment used was the NanoSEM - FEI Nova 200 (FEG/SEM); EDAX - Pegasus X4M (EDS/EBSD), this has several detectors and possible analysis, the operating resolution in transmission of STEM analysis is 0.8 nm at 30 kV, regarding the image obtained the processor resolution is up to 3584 x 3094 pixels [16]

AuNPs were functionalized with AS (SA-AuNP) at three different concentrations (50-50, 30-70 and 20-80, respectively) to select the ideal concentration for respiratory virus detection, which functionalization was confirmed using mentioned technique.

1.3 Textile Impregnation

This process was developed by the companies involved in this project - Clothius and RDD-Textile, in which they selected 3 samples (all jersey) with different compositions and weights (g/m2).

- Sample 1 - 1 cut: 50% Organic Cotton 50% Recycled Cotton
- Sample 2 - 2 cuts: 100% Organic Cotton
- Sample 3 - 3 cuts: 100% BCI Cotton

Initially, it was necessary to prepare the samples, and therefore, an alkaline boil was made in all samples to remove any type of impurity, resins, grease, finishing that they already had or other substances that could still exist in the textile substrate and that could interfere in the good uniformity and impregnation of the samples. In order to understand the expression rate (ET) (%) or pick-up, for each type of sample a square (10 x 10 cm) was cut and the material was weighed before and after being placed in the intended solution. To impregnate the virus recognition biosensor, the NPsAu functionalized with AS were used. Seven samples of each were cut: one worked as a blank (alkaline boil only), 2 samples were impregnated with the concentration of 30-70 SA-AuNPs and 2 samples were impregnated with the concentration of 50-50 NPsAu-AS.

The expression rate (ET) was calculated using the equation mentioned below, using a small portion of the prepared solution, and placed in the Foulard.

$$ET(\%) = \frac{\text{Mass of wet substrate (g)} - \text{Mass of dry substrate (g)}}{\text{Mass of dry substrate}} \times 100$$

(1)

After impregnation, the samples were placed in the oven for 3 min at 95^{o}C and then FTIR analysis was performed on all samples.

2. Results & Discussion

2.1 ACE2-AuNPs functionalization

2.1.1 Development of the incorporation of AuNPs with ACE2

When using the ACE-2 enzyme, some difficulties appeared regarding the development of the protocol because there was no bibliographic support, however the sample was prepared according to our research, in the first phase of the procedure in the pH optimization and enzyme preparation, initially we had a pink mixture (Figure 2A), after adding the NaCl we obtained a more purplish color (Figure 2B), according to the bibliographic source after adding the NaCl the color should not change if we were in ideal conjugation conditions, despite having followed all the steps, there was a color change.

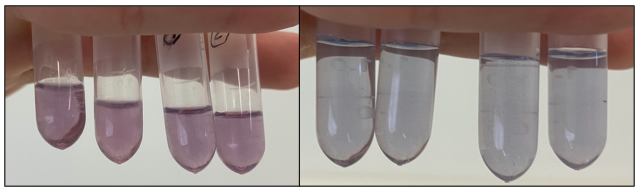


Figure 2. Nanoparticles Solution (left) with ACE2; (right) with ACE2, after the addition of NaCl.

In the phase of incorporation of AuNPs with the enzyme, we performed the centrifugation mentioned above, however we could not obtain any pellet and the samples remained the same color, and also there was no change in the different concentrations prepared (PBS 0.5x and 1x). Despite these results, it was decided to analyze the sample using FTIR.

2.1.2 FTIR analysis of the ACE2-AuNPs sample

Initially, a FTIR analysis was performed on a water sample (Attachments 1), which served as a means of comparison. In figure 4, the blanks containing only the enzyme (blue and yellow line) are different from the other ACE2 solutions with the nanoparticles, but the graph indicates that the transmittance values obtained were very low, detecting mostly noise. One of the reasons for this could have been due to the fact that samples were very diluted as they presented the consistency in solution. Thus, the functionalization of the AuNPs with ACE2 may not have happened. It was also noted that the graph with the water sample had many similarities with the ACE2 graph.

2.2 SA-AuNPs functionalization

2.2.1 Development of the incorporation of AuNPs with SA

In the development of the biosensor, the first step was to prepare a stock solution, and then start mixing the AuNPs solution (500μL) with sialic acid (100μL). Initially the protocol was performed as indicated in 2.2, with the centrifugation and heating steps, where the initial color of the nanoparticles was reddish (Figure 4A). After the addition of SA with 20 minutes of stirring at room temperature the mixture continued with the same color (Figure 4B) and when mixing AuNPs with SA at 80^{o}C stirring for 20 minutes the mixture changed its color to pink (Figure 4C). After 1 day of its preparation, the samples were kept at room temperature and have changed color to a stronger pink color as is shown in Figure 4D.

Following these results, the color change occurred whether the sample was heated or not this could give us indications that functionalization occurred. Regarding centrifugation, the pellet could not be obtained even at duplicated concentrations. As such, to optimize the process, samples preparation was performed in a different way, simply mixing the AuNPs with the SA, without stirring, without centrifugation, and without heating. In this sense, 3 different concentrations of the SA-AuNPs mixture were chosen: 50-50, 30-70 and 20-80. In order to validate the functionalization of AuNPs with SA, Fourier transform infrared spectroscopy (FTIR) and scanning transmission electron microscope (STEM) analysis were performed.

2.2.2 FTIR analysis of the SA-AuNPs sample

In the FTIR analysis, the samples were previously freeze-dried, analyzing the graph (Figure 5) the SA-AuNPs mixture and the SA sample appear very similar to each other. However, the AuNPs peaks were not identified, suggesting the functionalization of AuNPs with SA.

2.2.3 STEM analysis of the SA-AuNPs sample

In Figure 6, the points delimited in red show the bonding established between AuNPs and SA, most evident at the 50-50 SA-AuNPs concentration (Figure 6A). The darker points marked in blue correspond to the overlap of the AuNPs. At the 30-70 SA-AuNPs (Figure 6B) and 20-80 SA-AuNPs (Figure 6C) concentrations, the STEM images show some overlap of the nanoparticles and not so evident binding, this overlap is acceptable for the 30-70 AS-AuNP concentration.

According to the results presented, both by FTIR and TEM analysis, the concentrations 30-70 and 50-50 SA-AuNPs were chosen to be impregnated (Foulard Textile) on two substrates of different compositions, one cellulosic and one synthetic. However, in this technique there are many parameters, such as drying time and temperature, which were varied in order to understand which the best procedure was to obtain the biosensor.

2.3 Textile Impregnation

In this project, the impregnation of the samples into fabrics was performed at Clothius and RDD Textiles, as indicated above, and the results described below were obtained. Functionalized nanoparticle solutions with 30-70 and 50-50 concentrations of SA-AuNPs were impregnated (Textile Foulard) on substrates of different compositions. For this purpose, 3 samples with different compositions and weights were selected: 50% organic cotton 50% recycled cotton, 100% organic cotton and BCI cotton.

Regarding the ET calculation (shown in Table 2), the results were not very favorable, since the company used a TE of 70%, and in this case values above this percentage were obtained. Therefore, we proceeded with the 70% because the company's Foulard is formatted for this percentage.

Given the results obtained, it was observed that the peaks become more intensified after impregnation thus demonstrat-

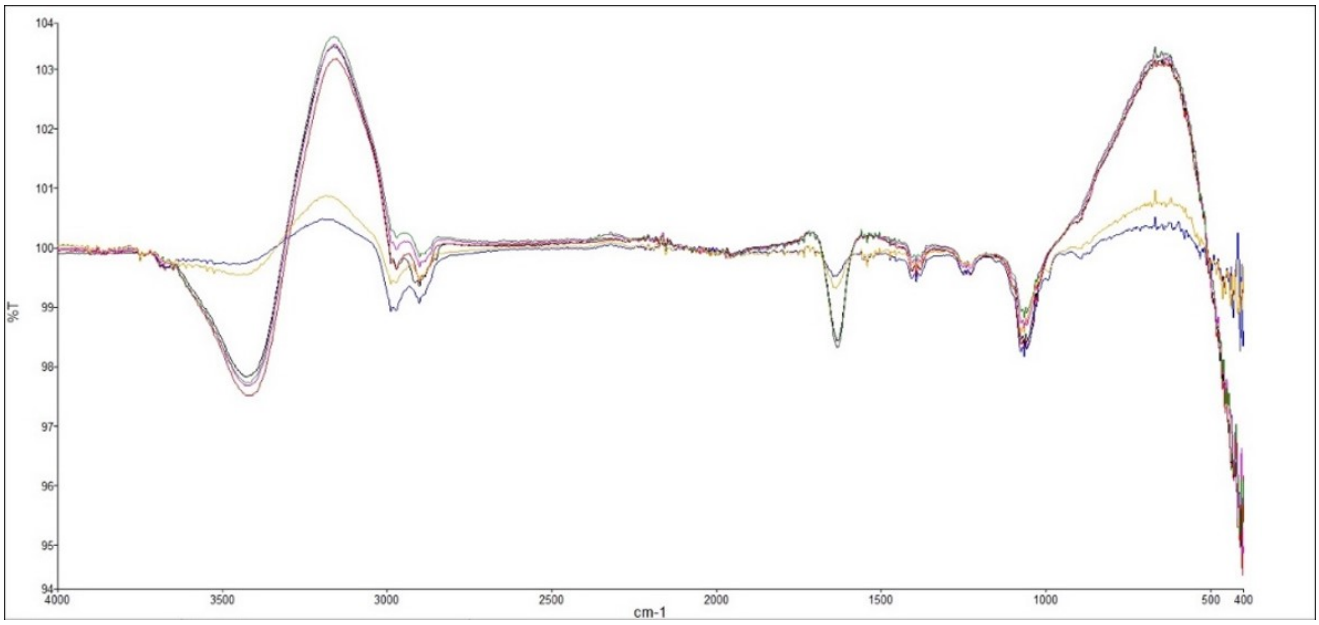


Figure 3. FTIR analysis of the various intended samples, samples with the blanks: ACE2 0.5x Blank (blue line) and ACE2 1x Blank (yellow line) and samples with the ACE2 plus nanoparticles: ACE2 0.5x (black and red line) and ACE2 1x (pink and green line).

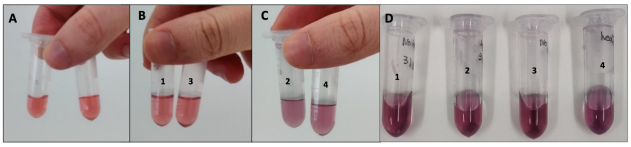


Figure 4. Functionalization of AuNPs with AS:(A) Color of NPs without SA treatment; (B) Color of NPs + SA without heating, stirred for 20 min at room temperature - Sample 1 and 3; (C) Color of NPs + SA with heating, stirred for 20 min at 80^{o}C- Sample 2 and 4; (D) Samples 1,2,3 and 4 after 1 day.

Table 2. Calculation of the TE for the different samples.

Sample	Sample weight (g)		TE (%)
	Dry	Wet	
1	0.304	0.521	71
2	0.407	0.728	78
3	0.415	0.739	78

ing that there really was binding of the SA-AuNPs mixture with the different types of cotton.

3. Conclusions

Biosensors can be an answer to combat infectious diseases, in this context when placed on face masks. This project was developed in the context of the COVID-19 pandemic, with financing to develop a biosensor with an indicator for the presence of the virus, which may have an impact on the spread of certain infectious diseases. For the development of this biosensor, we tested two approaches, as mentioned above: protein (ACE2) and sialic acid (SA). According to our results, we performed tests to confirm the functionalization of AuNPs to the two alternatives, by FTIR and STEM analyzes.

In relation to FTIR analysis, the samples with ACE2-AuNPs gave us the indication that this approach was not the best since in the graph presented it was not evident the binding of AuNPs to ACE2, only noise was evident, it was concluded that this is a more sensitive protein and the fact that it has 7 days of viability after hydration may interfere with the process. For the SA-AuNPs samples, the concentrations chosen for optimal impregnation were 30-70 and 50-50 SA-AuNPs, since functionalization of the gold nanoparticles with sialic acid was evidently observed. After the FTIR analysis, STEM was also performed which confirmed the functionalization of AuNPs with SA.

It is important to mention that for the ACE2 sample lyophilization was not performed, as this process is time consuming and cost inefficient for certain proteins, and so it was only performed on the samples with the SA, and the promising results being evident, STEM analysis was not found necessary for ACE2, as the process was continued using the SA, a cheaper and easier to work with approach.

For textile impregnation, according to the data provided to us by the companies, the FTIR analysis concluded that the peaks become more intensified after impregnation, thus demonstrating that binding of the SA-AuNPs mixture with the different cotton samples occurred. The impregnation was validated using preliminary results, however user validation has not yet been performed due to lack of time.

Since sialic acid is a biomolecule present in humans and AuNPs are harmless to humans, according to toxicity studies [18], it is believed that the biosensor will also be safe for use in humans, however sensitivity testing would be required to confirm this theory. This biosensor has been successfully developed, this will provide information about the state of contamination by SARS-CoV-2 and other viruses that cause breathing problems. The facemask used, with textile composition, will have the same use indications and it is intended to develop a disposable mechanism to adapt the biosensor to the mask, in order to maintain the extended use of this type of

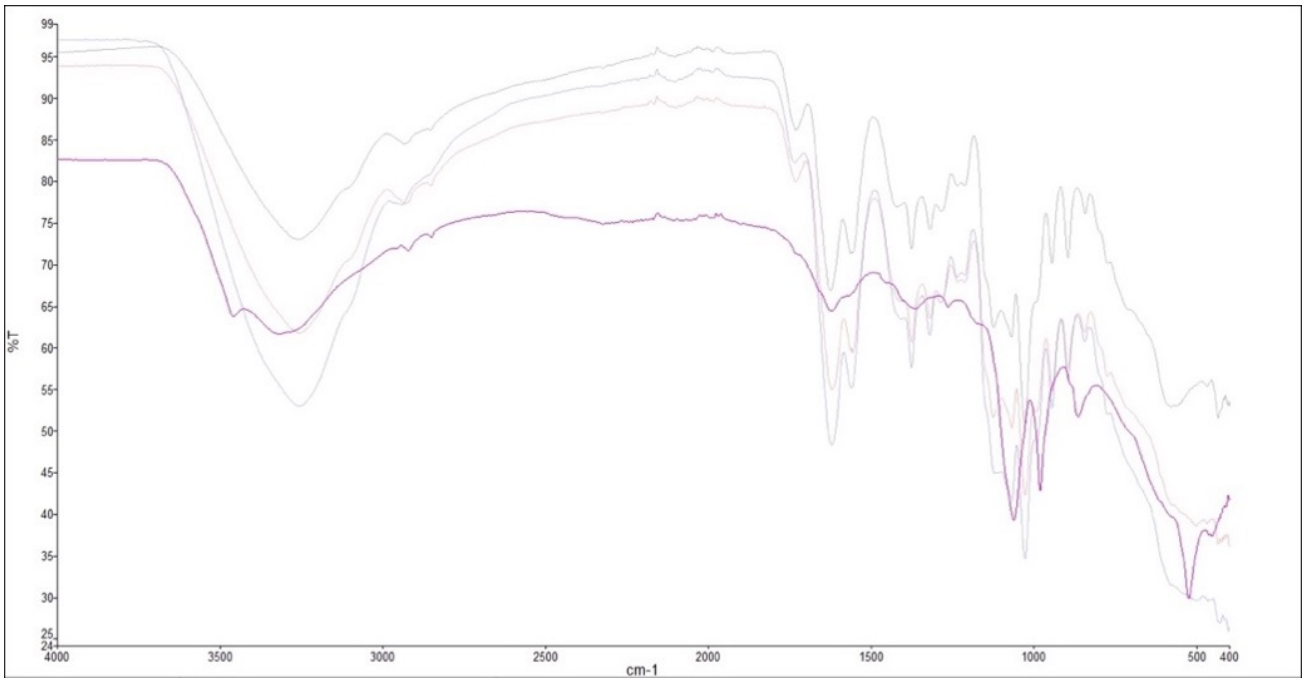


Figure 5. FTIR analysis of specific samples: lyophilized nanoparticles (pink line), sialic acid (black line), NP+AS mixture with different concentrations, 50-50 (red line) and 30-70 (blue line).

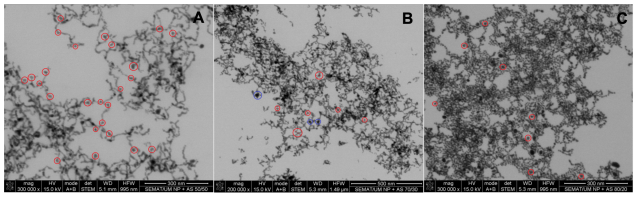


Figure 6. STEM analysis: (A) AuNPs+SA 50-50 mixture, (B) AuNPs+SA 30-70 mixture and (C) AuNPs+SA 20-80 mixture.

protection equipment.

The development of this biosensor demonstrated as a sensitive, rapid, and simple way to ensure a greater individual protection against viruses that attack the respiratory tract, and further reduce their contagion and preventing their spread.

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Competing Interests

The authors have no competing interests to declare.

Article Information

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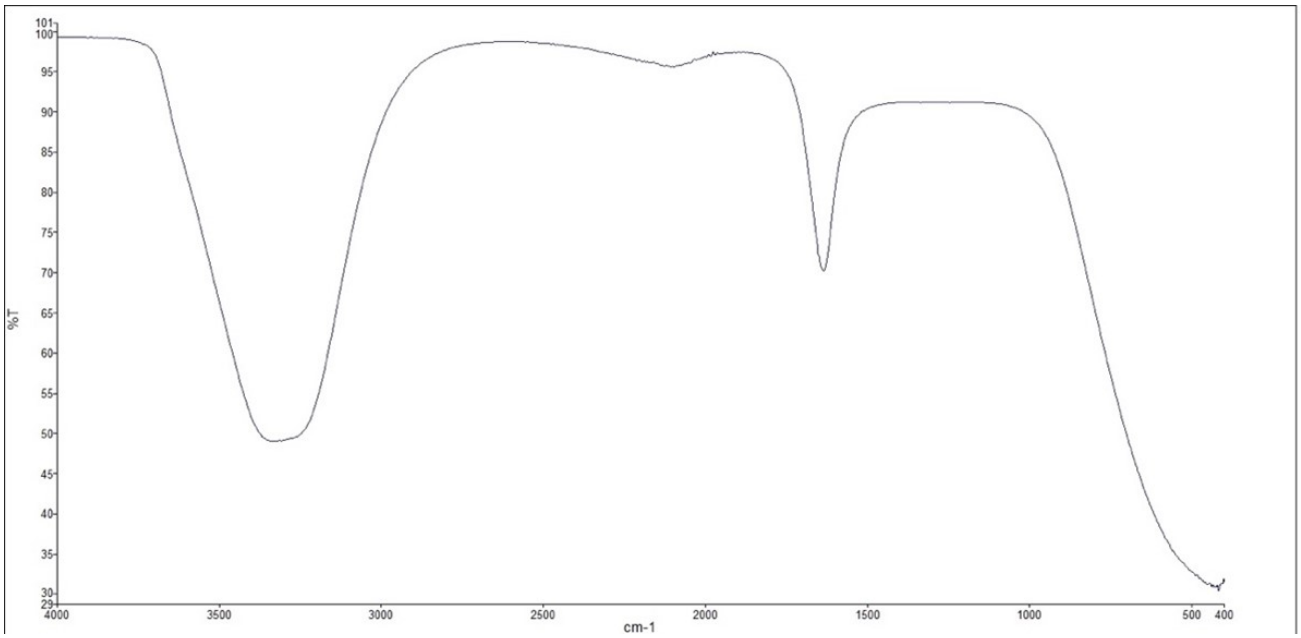


Figure 7. FTIR analysis of the water sample.

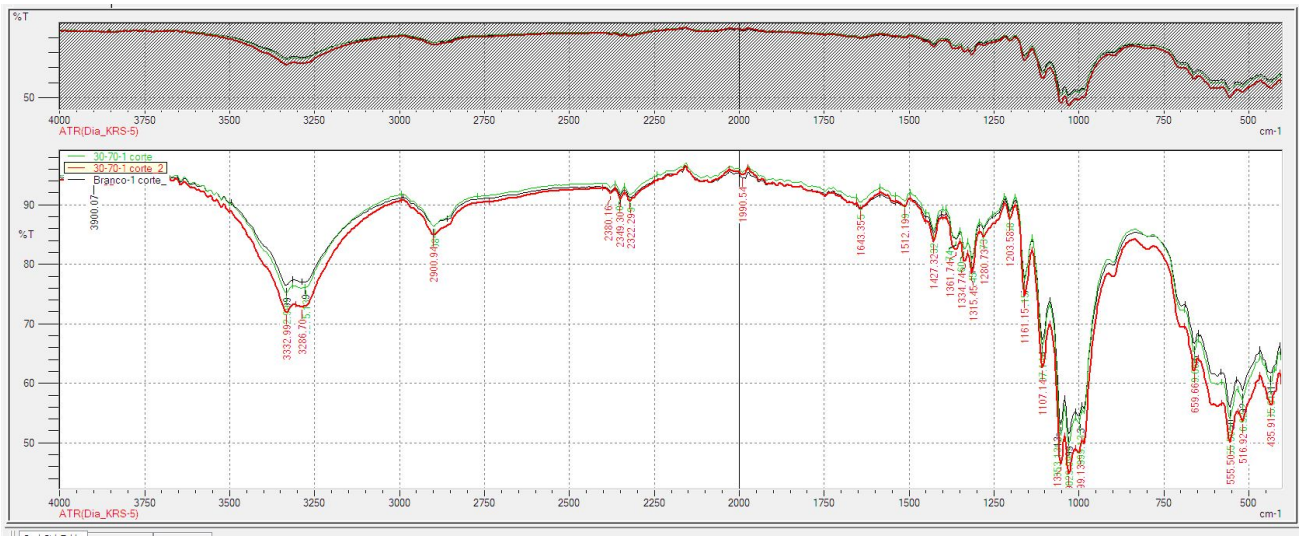


Figure 8. Sample 1: 30-70 SA-AuNPs (green, red) + White (50% organic cotton 50% recycled cotton) (black).

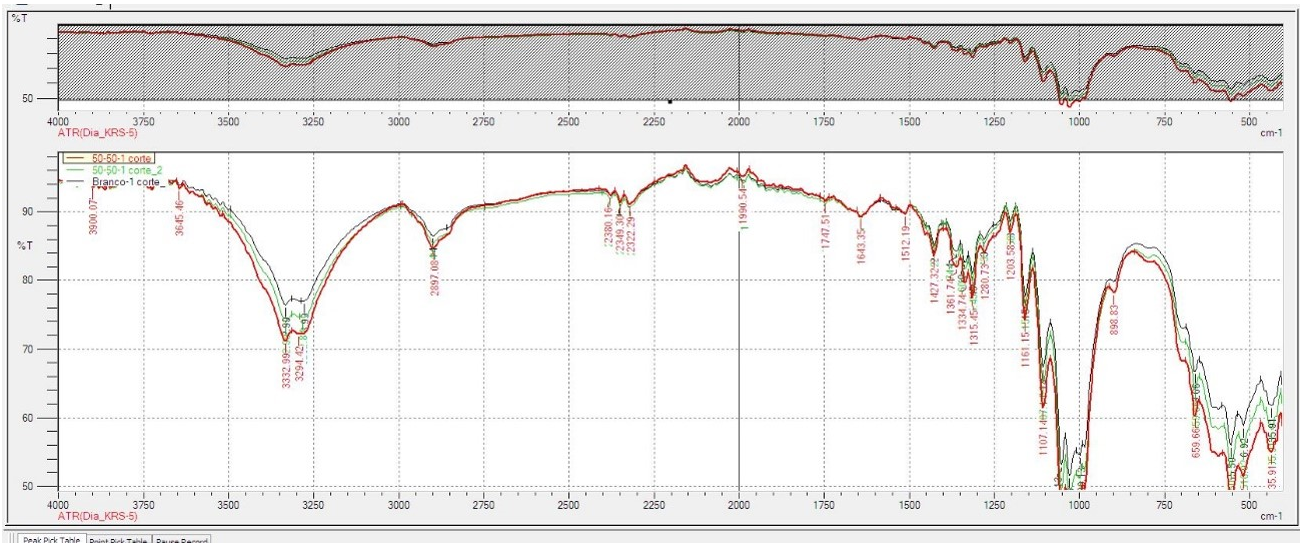


Figure 9. Sample 1: 50-50 SA-AuNPs (red, green) + White (50% organic cotton 50% recycled cotton) (black).

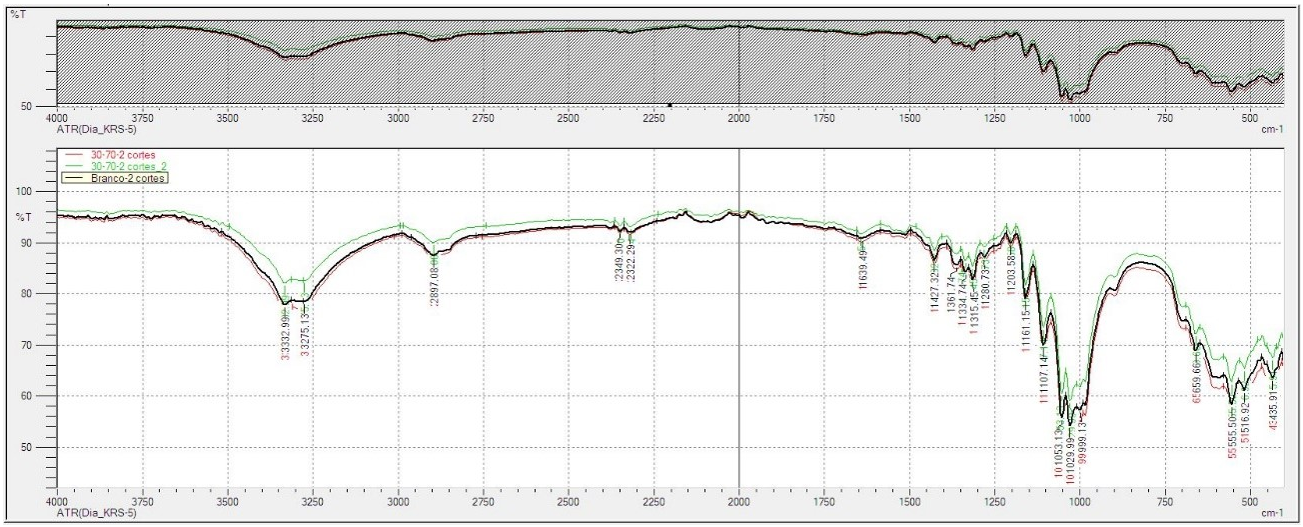


Figure 10. Sample 2: 30-70 SA-AuNPs (red, green) + White (100% organic cotton) (black).

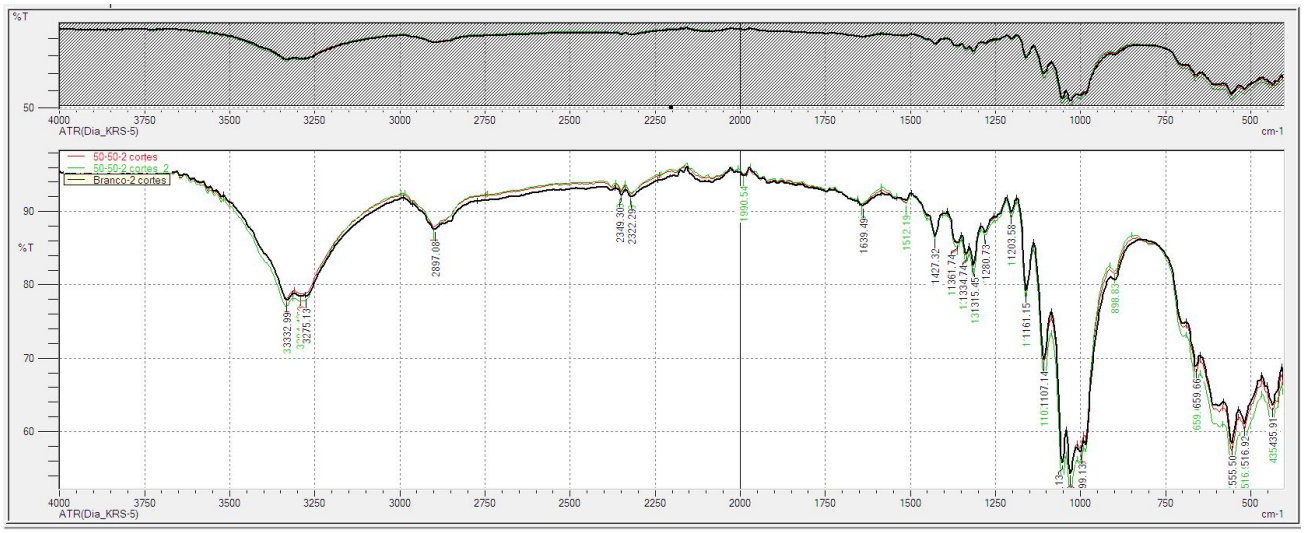


Figure 11. Sample 2: 50-50 SA-AuNPs (red, green) + White (100% organic cotton) (black).

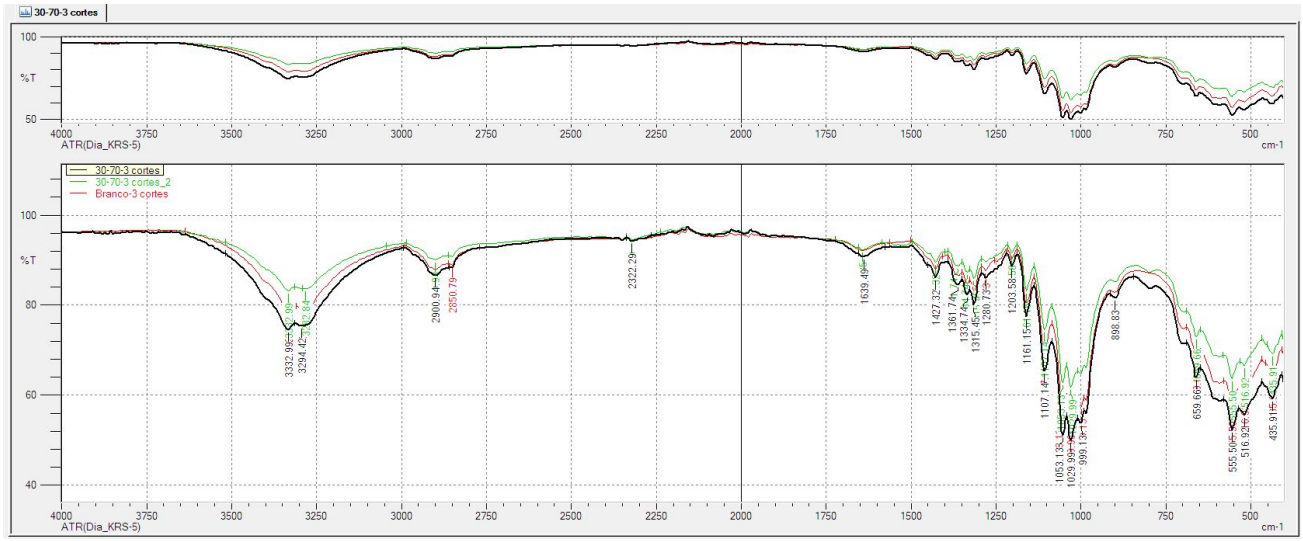


Figure 12. Sample 3: 70-30 SA-AuNPs (black, green) + White (100% cotton BCI) (red).

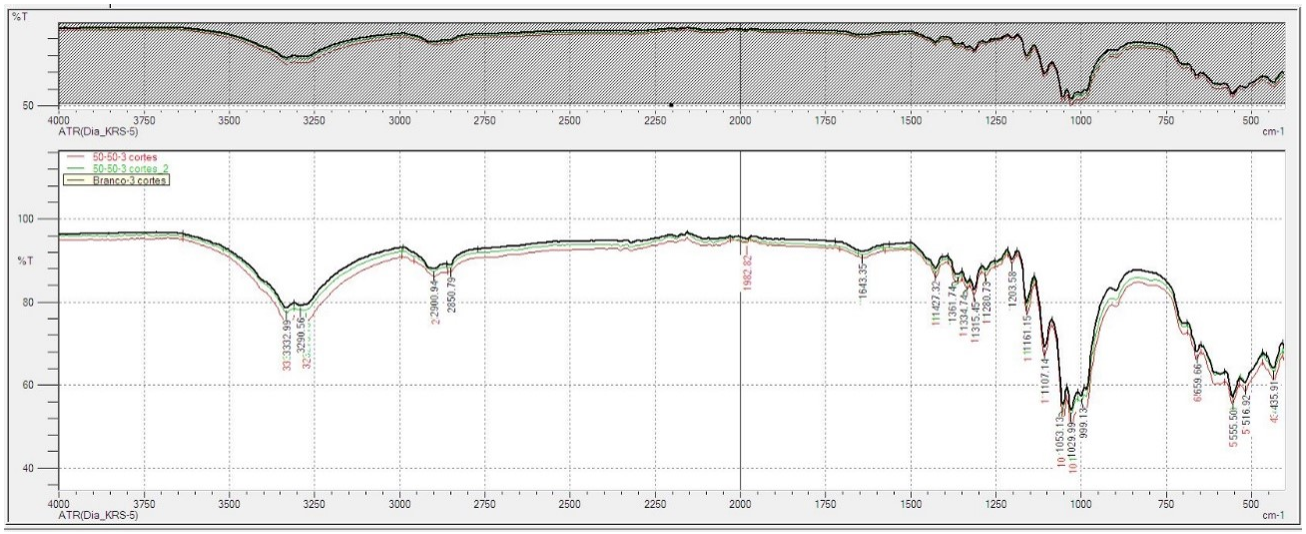


Figure 13. Sample 3: 50-50 SA-AuNPs (red, green) + White (cotton BCI) (black).