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Original Article

Design and Fabrication of Rapid Diagnostic Test Based on Gold Nanosensors to Detect SARS-CoV-2

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Abstract

Background and Aims: Early detection of COVID-19 can decrease the motality rate. The diagnostic tools available so far have been based on a) detection of viral genes, b) detection of human antibodies and c) detection of viral antigens, among which detection of viral genes by RT-PCR is recognized as the most reliable method. In order to speed up the detection process and reduce the false negative results presented, it is necessary to design an accurate and fast test.

Methods: A specific probe against N gene was designed and then gold nanoparticles were synthesized by citrate reduction method. The properties of the synthesized Nano systems were determined in terms of size, surface charge, morphology and accuracy of nanoparticle formation using dynamic light scattering device, zeta potential, transmission electron microscope and ultraviolet light spectroscopy. The designed probe was added to gold nanoparticles after activation. Negative and positive samples were taken from patients and added to nanoparticles containing probes.

Results: Due to the free the sulfhydryl group and the high bond energy between the gold and this group, after the probe was added to the nanoparticles, change in color was observed in the nanoparticle solution. UV Spectrum and DLS results showed 4 nm red shift and change in size from 11 to 16 nm, respectively. In the presence of the SARS-CoV-2 RNA, AuNPs-probe would bound with RNA particles, resulting in a space barrier against salt particle on the other hand in negative samples, the salt bridge would be formed and agglomeration of AuNPs and a change in color would be observed by naked eye .

Conclusion: The results show a clear way to design rapid detection for other pathogens.

Keywords: Biosensor, Colorimetric detection, Gold Nanoparticles, Rapid test, SARS-CoV-2

Introduction

In December 2019, several people in Wuhan, China, were hospitalized with severe flu-like symptoms. To identify the cause, scientists conducted metagenomic RNA sequencing on samples from these patients. This analysis revealed a new virus belonging to the Coronaviridae family, with its genome showing over 89% similarity to SARS-like bat viruses. Initially named "2019-nCoV" or Novel Coronavirus 2019, the virus was later officially designated "SARS-CoV-2" and the associated disease as "COVID-19" by the International Committee on Taxonomy of Viruses, reflecting its genetic makeup and resemblance to other known coronaviruses (1, 2).

Coronaviruses are among the largest RNA viruses known, characterized by their single-stranded, positive-sense RNA genome. SARS-

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CoV-2, the virus responsible for COVID-19, has a genome of approximately 29.9 kilobases, encoding essential structural and non-structural proteins. Its genetic blueprint includes the ORF1ab polyprotein, which is critical for viral replication and transcription. Additionally, the genome encodes key structural proteins: the envelope (E) protein, membrane (M) protein, nucleocapsid (N) protein, and the spike (S) protein. The spike protein, in particular, facilitates viral entry by binding to the angiotensin-converting enzyme 2 (ACE2) receptor on host cells, initiating infection. The ORF1ab polyprotein is processed into functional units, including RNA-dependent RNA polymerase and proteases, which are essential for viral replication and assembly, highlighting its importance in the viral life cycle (3).

Clinical symptoms manifest post human exposure to SARS-CoV-2 virus, with transmission occurring from infected individuals to healthy individuals via droplets and airborne particles released during sneezing and coughing (4-6). Symptoms of corona disease consist of various clinical signs like fever, dry cough, sneezing, shortness of breath, sore throat, headache, chest pain, hemoptysis, myalgia, fatigue, nausea, vomiting, diarrhea, abdominal pain, as well as loss of smell and taste (4-8). Patients could be showing no symptoms at all or experience symptoms that vary from mild to severe and critical (4, 7, 9). People with risk factors and underlying diseases like old age, smoking, diabetes, high blood pressure, cardiovascular disease, obesity, chronic lung disease, and kidney disease are more likely to experience higher rates of infection and mortality from the SARS-CoV-2 virus (4, 5, 10).

Corona virus has been identified as one of the most lethal viruses for humans because of its high death rate of 36% by MERS-CoV in 2012 and 10% by SARS-CoV in 2002-2003 (11-13). As of now, approximately 233 million individuals globally have been infected by the SARS-CoV-2 virus, resulting in 4.6 million deaths (33). Due to the high transmission rate of the virus and the current need for specialized labs and trained staff, a rapid, inexpensive, and accurate on-site diagnostic method is essential for effective virus detection (2, 3).

Polymerase chain reaction (PCR), reverse transcription (RT-PCR) and antigen-based tests are employed in diagnosing pandemic infections. Differentiating between direct (RNA) and indirect (spike protein, IgG and IgM) detection methods is essential (14). The enzyme-linked immunosorbent assay (ELISA) remains a widely used biochemical method for detecting specific antibodies or antigens in blood samples. However, advancements in diagnostic technologies have driven the development of nanomaterial-based methods aimed at achieving higher accuracy, simplicity, cost-effectiveness, and rapid detection. Among these innovations are reverse transcription-mediated isothermal amplification (RT-LAMP) integrated with nanoparticle-based biosensors (RT-LAMP -NBS), which combine amplification and detection in a single step. Field-effect transistor (FET)-based biosensors, including graphene field-effect transistors (Gr-FET), leverage the high sensitivity of graphene and its ability to detect specific antibody-antigen interactions, such as the binding of SARS-CoV-2's spike protein S1 to host molecules.

Additionally, dual-purpose plasmonic biosensors have emerged, utilizing a combination of plasmonic photothermal (PPT) effects and localized surface plasmon resonance (LSPR) to enhance the sensitivity and specificity of viral detection. These techniques represent significant strides in rapid, reliable diagnostics for emerging pathogens like SARS-CoV-2 (15).

Lateral flow immunoassays (LFIAs) are chromatographic assays that are qualitative and similar to traditional pregnancy tests. They follow a non-competitive two-site format like the ELISA test and can be conducted on-site by either a specialist or the patients themselves, although they have limited sensitivity (16).

Recent progress in nanotechnology and the utilization of nanomaterials in biosensors manufacturing has resulted in enhanced efficiency, bio sensitivity, and performance of these devices, thanks to their outstanding conductivity, photoelectrochemical properties, and potential for platform miniaturization (17-21). Gold nanoparticles are one of the nanomaterials commonly utilized in the detection and

treatment of cancer, bacteria, and viruses due to their distinctive qualities like surface chemistry, adjustable size and shape, high biocompatibility, simple synthesis, and easy functionalization (22). In this study, we designed and developed a new Au-nanoprobe to identify the SARS-CoV-2 N gene in RNA extracted from samples taken from patients.

Methods and Materials

Probe Design (Antisense Oligonucleotide Design)

Two single-stranded oligonucleotide sequences were synthesized, complementing region 5˝ of the N genes, respectively, and then were thiolated at the 5˝ end (Table 1). Alignments was done for finding the conserved nucleotides on all of SARS-2 sequences in EBI –EMBL site (https://www.ebi.ac.uk/Tools/msa/clustalo/).
Probes were designed after determination of the reaction conditions such as GC%, Melting temperature (Tm), the length of the probe and the product range based on online tools. (https://www.eurofinsgenomics.eu/en/ecom/tools/pcr primer-design/).

Preparation of Gold Nanoparticles (AuNPs)

Synthesis method that we used for AuNPs production was citrate reduction method (23). Briefly, 200 mg of HAuCl4.3H2O (99.9%, Sigma-Aldrich) was solved in 5 ml ultrapure water (procured from Milli-Q Direct 8 system) then added to 100 ml Round-boomed flasks containing 50 mL of ultrapure water under vigorous stirring until the aqueous solution starts boiling. After boiling, 1% sodium citrate (99%, Sigma-Aldrich) solution was slowly added to the solution, which made solution's color changed from yellow to purple and red at the end. After that temperature switched off and we let solution stir for another 10 min and cool down naturally. After that we put the solution in an Ice container and pour in different container to store in the dark at 4 C until further uses.

AuNPs Characterization

The hydrodynamic diameters and Zeta Potential of AuNPs before and after probe addition were measured by NANO-flex and ZETA-check respectively (particle Metrix). The morphology of AuNPs before and after probe insertion was studied using transmission electron microscopy (TEM) with a Leo 906 Zeiss Germany at a 100 kV operating voltage. The absorbance spectra of AuNPs were obtained using a Cecil Ultraviolet-visible spectrophotometer (Cambridge, United Kingdom) with an optical resolution of 0.01nm full width at half maximum (FWHM). Using a 45×12.5×12.5 mm path quartz cuvette, the spectrum response was measured in 2 nm steps from 200 to 800 nm.

Functionalization of AuNPs with probe

Conjugating surface of gold nanoparticles with antibodies,functional groups and probes can be done by physical and chemical interactions. Chemisorption via thiol derivatives is one the well-known chemical intercations for AuNPs surface modification (24). There is a high affinity between Au atoms and thiol group, therefore we capped our probe with thiol group to make a covalent Au-S bond. Before Functionalization of AuNPs, ssDNA probe needed to be activate as company protocol. Briefly, 10 µL of 1 N DTT (purchased from Sigma-Aldrich) was added to aqueous probe solution and incubated at room temperature then wash and vortex three times with ethyl acetate (purchased from Sigma-Aldrich) to remove unwanted thiol fragments and excess DTT. Due to unstability of sulfhydryl group, Probe addition to AuNPs and Functionalized it needed to be done immediately. Then a salt aging method was used to determine to check enough probe particles attached to the surface of gold nanoparticles.

Sample Collection

The collection of clinical specimens for SARS-CoV-2 was done in accordance with CDC guidelines for handling and testing clinical specimens for SARS-CoV-2 (). Patients' samples were considered for the study if they provided written informed consent and clinical evidence of COVID-19 was confirmed by a PCR test. The Ethics Committee of Iran Uni-

versity of Medical Sciences approved the study (ethics code: IR. IUMS.REC.1399.329).
SARS-CoV-2 was detected in symptomatic patients using a real-time reverse transcriptase assay using nasopharyngeal and oropharyngeal swab and sputum samples.

RNA Extraction
The QIAamp DSP Virus Kit (Qiagen GmbH, Hilden, Germany) was used to extract RNA according to the manufacturer's instructions. In a nutshell, the Qiagen protease was added to the lysis tube and mixed with 500 µL of sample. Following that, the carrier RNA-containing lysis buffer was added to the tube and pulse-vortexed. For 15 minutes, the tube was incubated at 56 °C. Ethanol was added to the tube and incubated at room temperature for 5 minutes. The lysate was injected into columns that were connected to a vacuum system. Following the washing steps, the tube was centrifuged at full speed for 1 minute to dry the membrane completely. RNA was extracted from the membrane and stored at -80 °C in 50 µL of elution buffer.
The Novel Coronavirus (2019-nCoV) Nucleic Acid Diagnostic Kit (Sansure Biotech, Changsha, China) was used to detect SARS-CoV-2 using a qualitative multiplex real-time PCR assay, as directed by the manufacturer. All samples were treated with a reaction mastermix that included the PCR mix (primers, probes, dNTPs, MgCl2, RNasin, and buffer) as well as the enzyme mix (RT and Taq). Each tube was filled with 20 µL of the sample. The test also comprised a positive control, a negative control, and an NTC (no-template control). The assay was run on a Rotor-Gene Q qPCR machine (Qiagen, Hilden, Germany) with the following cycle parameters: 1 cycle reverse transcription at 50 °C for 30 minutes, 1 cycle cDNA pre-denaturation at 95 °C for 1 minute, 45 cycles of denaturation at 95 °C for 15 seconds, and an annealing-extension at 60 °C for 30 seconds (fluorescence collection). If typical S-shaped amplification curves were seen at Ct 40, the results were considered positive.

Au-nanoprobe Hybridization and Color Detection
40 µL probe was added to 160 µL AuNPs to make our Au-nanoprobe. Then three negative and three positive samples with different Cts were selected from patient with extracted RNAs. Our final step is to add salt to the mixture of our nanoprobe and each sample for colorimetric detection.We expect that there are no color change for positive sample but negative and blank sample should show a change in color from red to dark blue.

Results

Preparation and Characterization of AuNPs
Characterization of AuNPs
AuNPs were prepared by citrate reduction method. Hydrodynamic Diameter and Morphology of AuNPs were characterized by Dynamic Light Scattering (DLS) and TEM respectively. TEM results confirmed that our AuNPs had spherical shape. To measure DLS, we prepared the solution of AuNPs in distilled water. DLS revealed that the average size of AuNPs was 11 nm, as shown in Figure 2. Also Zeta potential was measured -35 mV which is due to presence of citrate group on gold nanoparticles surface. UV Spectrophotometer Result showed a peak at 524 nm. Size of nanoparticle was calculated by Heiss and approved DLS results.

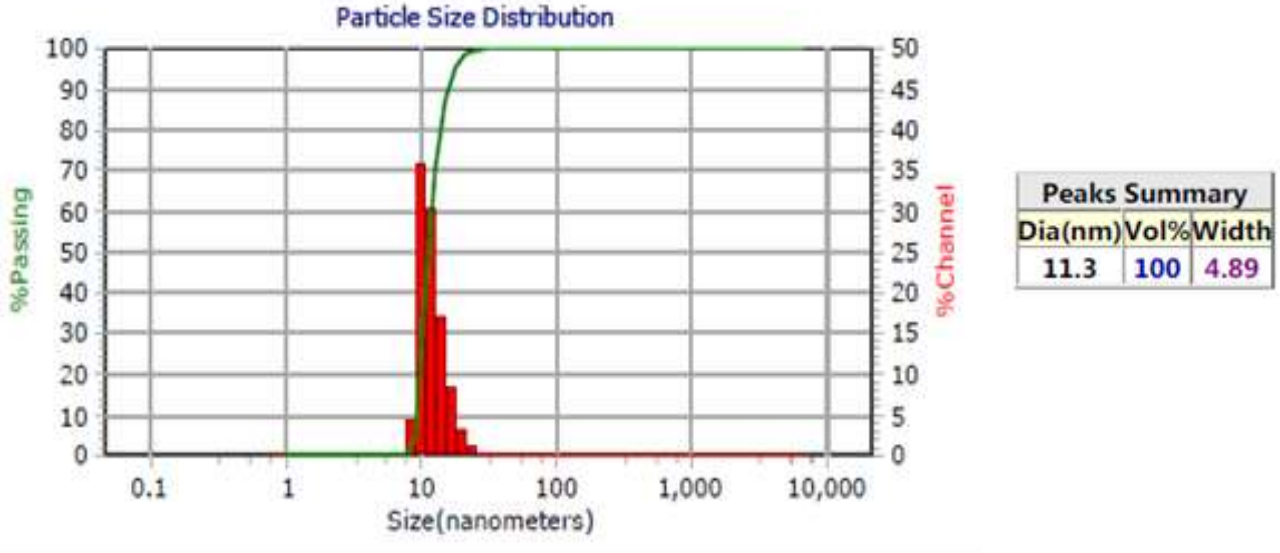
Characterization of Au-Nano Probe
Due to presence of the probe, hydrodynamic Diameter, size and UV Spectrophotometer peak got changed.Fig.3 shows TEM analysis of the Au-nanoprobe and hydrodynamic diameter was measured by DLS and showed 16 nm and a change was observed in Zeta potential to -55 mV. UV Spectrophotometer showed a 4 nm shift in plasmon peak result in 528 nm.

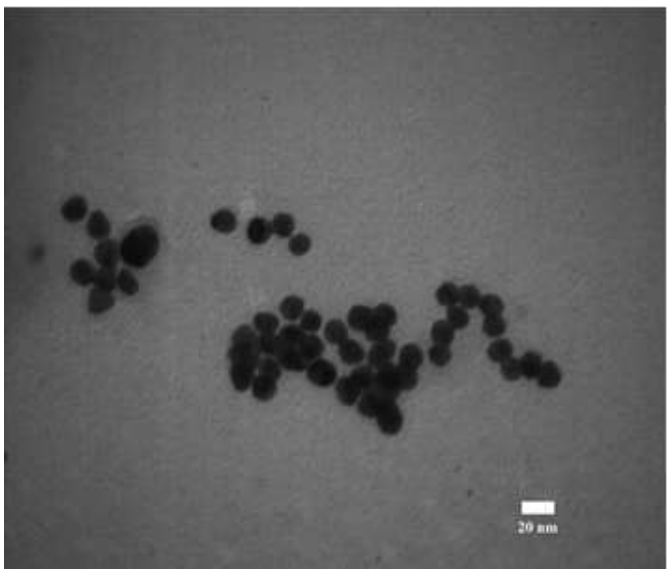
Au-Nanoprobe Hybridization and Color Detection
According to material and method section in positive samples probe can attach to RNA therefore when we add salt, due to space barrier on AuNP-probe surface there are no space for attachment of salt ions therefore our nanoparticles stay intact but in negative and blank samples there are no RNAs to attach to our probe so salt ions can bind to surface of

Table 1. Oligonucleotide sequence and properties

Oligo Name	Oligo Sequences	Base count	MW (Da.)	T_m
N	Thiol-C6-TAG AAG CCT CAG CAG CAG ATT	21	6767	59

Fig 1. Schematic for probe attachment on gold nano-particles surface by chemical interaction between thiol group and Au atoms.





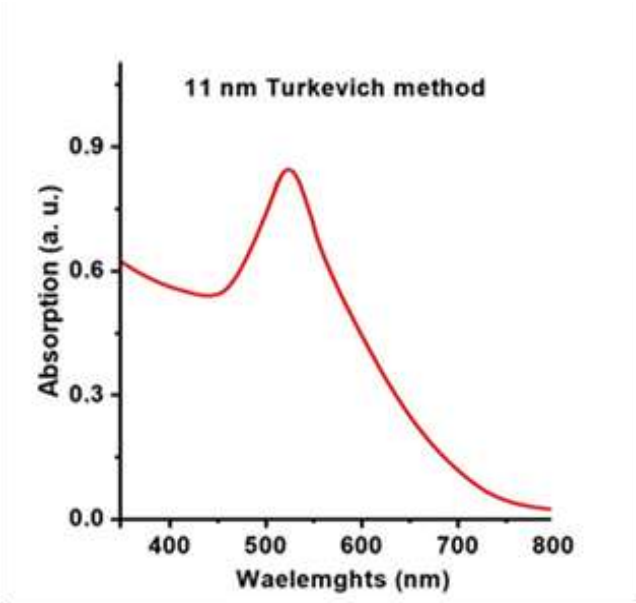


Fig 2. Characterisation of AuNPs: a.DLS result, b. TEM image of the morphology and shape of the gold nanoparticles magnifications. Scale bar: 20 nm, c. UV Spectrophotometer of AuNPs

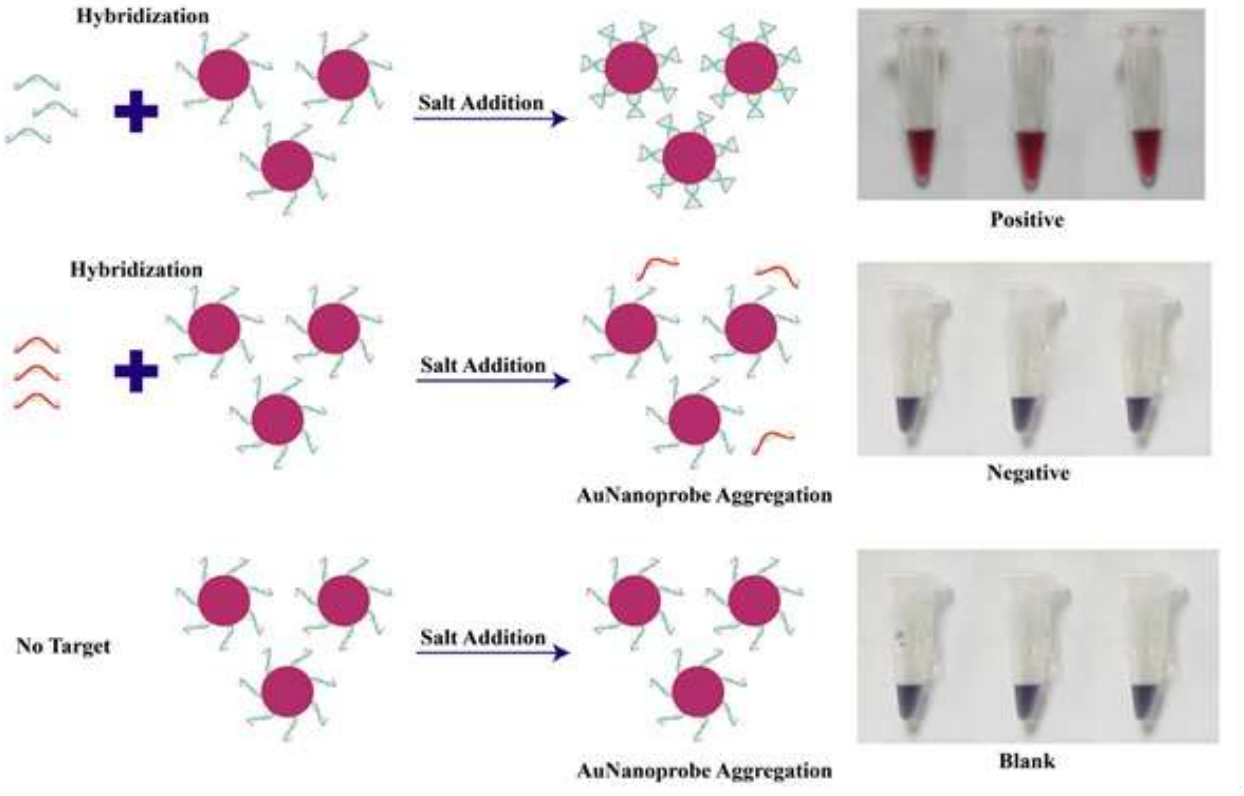


Fig 4. Interaction between Au-probe and positive, negative and blank sample and Colorimetric detection results

our nanoparticles and aggregate them so we observe a color change from red to blue. we add 10 µL of each sample to 200 µL of Au-nanoprobe mixture, then we can observe a color change in negative samples by naked eye due to the salt addition, but positive sample color would not change.

Discussion

In this work, the ability of the AuNPs-probe to detect the SARS-CoV-2 Nucleocapsid Protein (N) using SPR were investigated. First developed rapid tests for SARS-CoV-2 detection were Lateral Flow Immunassay (LFIA) tests using IgG or IgM or both of them. Many studies were done on LFIA based diagnosis but there were some drawbacks (28-33).

The mentioned diagnostic time for these kits is reported between 10-30 minutes. Due to the decrease in IgM levels in the body after the patient recovers and the need to pass an average of two weeks after the patient to measure the mentioned immunoglobulins, the use of IgG and IgM is not a good option for early diagnosis of the disease. Some studies were done on developing sensors for spike protein detection (34-37).

Spike protein has the most mutation rate between SARS-CoV-2 protein therefore is not a good option for probe design. E protein has quit higher sensitivity than N protein therefore make improving biosensor N protein detection a good area for research. Synthesis method that we used for AuNPs production was citrate reduction method. TEM results confirmed that our AuNPs had spherical shape and DLS measurement showed a 11 nm hydrodynamic particle size. UV Spectrophotometer Result showed a peak at 524 nm as we expected.

In the present study, biosen-sing is based on non-cross-linking aggregation therefore unlike cross-linking aggregation there is no need for many probes to detect virus (38). ssDNA probe was design based conserved nucleotides on all of SARS-2 sequences, complementing region 5˝ of the N genes. Probe was thiolated at the end of 5˝. Thiol group help our ssDNA to bound on surface of AuNPs. Probe attachment to AuNPs make a change in hydrodynamic diameter to 16 nm and UV Spectrum to 528

nm. To achieve good result, 160 µL of the AuNP was added to 40 µL probe.
We have 3 microtube of positive samples with different Cts and 3 microtubes of negative samples; then 10 µL of each these samples were added to 200 µL of Au-nano-probe. Next the salt was added to those mixtures, in the presence of the SARS-CoV-2 RNA, AuNPs-probe would bound with RNA particles, resulting in a space barrier against salt particle on the other hand in negative samples, the salt bridge would be formed and agglomeration of AuNPs and a change in color would be observed by naked eye.

Conclusions

To sum up, we have developed a simple, easy to use and cost-effective, AuNP-based biosensor method that allows the colorimetric detection of the SARS-CoV-2 with naked-eye and no need to any expensive instruments. In the present study, an optical biosensor based on surface plasmon resonance was designed and fabricated to detect nucleocapsid (N) protein based on non-cross-linking aggregation. Visible change of color was observed after salt addition in negative samples result and we have no alteration in color for positive samples by naked eye. These results will lead to developing sensors detecting other pathogens.

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Disclosure

None

Conflict of Interest

No conflict of interest is declared.

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Ethics Approval and Consent to Participate

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