EVALUATION OF INTERACTIONS OF SARS-COV-2 STRUCTURAL PROTEINS WITH SPECIFIC ANTIBODIES BY SPR ASSAY

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ABSTRACT

Background: The World Health Organization admitted that the vaccination against Covid 19 limited the deaths, but not the spread of the disease. This requires a method allowing a specific, rapid and accurate diagnosis of the disease. We report a SPR assay that meets the requirements and can be applied no only for SARS Cov-2 diagnosis but as a tool for early diagnosis of otherinfections. (2) Methods: Surface plasmon resonance (SPR) method was used to identify the binding of S/N protein to monoclonal antibodies. N-protein monoclonal antibody (NP mAb), S-protein monoclonal antibody (SP mAb), and receptor bind domain (RBD) antibody were used as recognition molecules. Ligands were deposited by the matrix-assisted laser evaporation (MAPLE) method, which guarantees maximum interaction specificity. (3) Results: We registered S/N protein binding to the corresponding mAbs and S protein to RBD antibody with high sensitivity: the interactions were observed at protein concentration about 130 femtomoles (fM). A very good specificity was observed: the measured S protein binding activity to NP mAb was below the limit of detection (LOD). The same was noticed for N protein binding to SP mAb. (4) Conclusions: The presented SPR assay possesses high sensitivity and selectivity and provides quantitative analysis. This makes it applicable for following the evolution of acute SARS-CoV-2 infection, especially at the early stages of viral replication which can be clinically useful.

Keywords: SARS-CoV-2, spike (S-) protein, nucleocapsid (N-) protein, anti-SARS-CoV-1/2 antibodies, Surface Plasmon Resonance (SPR) assay.

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INTRODUCTION

Despite the significant increase in the numbers of people vaccinated for coronavirus 2019 (COVID-19) disease, additional waves of the pandemic of COVID-19 were registered worldwide. This required a rapid, cost- effective, quantitative, on-site assay that could explore coronavirus 2 (SARS-CoV-2) severe acute respiratory syndrome. This is of particular importance for hospitals and any place where humans spread the virus (1, 2). COVID-19 diagnostic tests that are commercially available can be classified into three groups. The tests in the first group are based on molecular methods involving real-time polymerase chain reaction (RT-PCR), acknowledged as the gold standard for diagnosing COVID-19 (3, 4). However, they require a long turnaround time, well-equipped laboratory facilities, as well as qualified and trained personnel. Furthermore, this group of assays is not suitable for point-of-care testing (5, 6). treatment efficacy monitoring, or identification of a past infection. Serology tests form the second group. They have been established to detect antibodies against the SARS CoV-2 virus in the infected patients (7, 8). Antigen detection methods belong to the third group. They are designed to detect specific SARS-CoV-2 structural proteins (nucleocapsid protein (NP) and spike proteins (SP)). Antigen tests provide a fast and on-side diagnosis but have insufficient sensitivity, as compared to RT-PCR (9, 10).
Hence, there is a high demand for alternative techniques that are able to provide a diagnosis

with higher reliability and accuracy than the ones used to date. Biosensors based on Surface Plasmon Resonance (SPR) have already proved their feasibility as an accurate and sensitive diagnostic method. In (11) they were shown to be efficient for real time detection of an antigen-antibody interaction. In (12) S- and N-proteins were used on SPR transducer as recognition molecules for detecting SARS-CoV-2 antibodies.

The integration of two methods – generation of local SPR by nanoparticles and SPR excited on a planar chip – provides highly sensitive detection (13). This technique was used to achieve ultrasensitive SARS-CoV-2 N-protein detection (14).

SARS detection by an SPR biosensor was reported in (15) where corona viral surface antigen (SCVme) was immobilized on an SPR transducer. The lower limit of detection has been evaluated at 200 ng/mL for anti-SCVme antibodies within 10 min. Comprehensive reviews of SPR-based sensors for SARS-CoV-2 show recent achievements and limitations (16, 17).

Herein, we report SPR sensing of SARS-CoV-2 N/S-proteins at about 130 fM levels using monoclonal antibodies (mAb) and receptor binding domain (RBD) antibody as ligands immobilized directly (without built-in matrix) on the gold surface of an SPR transducer., Using a variety of SARS-CoV-2 specific antibodies as ligands, we evaluated the possibilities of the CPR assay to study the binding affinity of structural proteins.

MATERIALS AND METHODS
Reagents and materials
All the chemicals and reagents used were of analytical grade. We used the following SARS-CoV-2 specific structural proteins for evaluation of the bimolecular interaction:

1. SARS-CoV-2 Spike S1 subunit protein fused to a C-terminal poly-histidine (6x Histidine) tag with a tri-amino acid linker (Molecular weight (Mw) ~ 123 kDa) were purchased from InvivoGen Company USA. Stock solutions for the experiments were prepared at initial concentration 100 μg/ml in endotoxin and nuclease-free water (DEPC-treated water, ThermoFisher Scientific, USA). Aliquots were prepared and stored at −20°C until use. Working concentrations were propagated in DEPC-treated water in the concentration range 13 fM – 13 pM.

2. SARS-CoV-2 nucleocapsid protein fused to a human IgG1 Fc tag with a TEV (Tobacco Etch virus) sequence linker (Mw ~ 79 kDa) were purchased from InvivoGen Company, USA. Stock solutions were prepared at initial concentration 100 μg/ml in DEPC-treated water. Aliquots were stored at −20°C until use. Working concentrations of the stock solution were propagated in DEPC-treated water in the concentration range 0.0025 – 2.5 μg/ml.

3. Anti-SARS-CoV-1/2 NP antibody, clone 1C7C7 ZooMAb® mouse monoclonal (mAb) (Sigma-Aldrich, USA) (Mw ~ 46 kDa) was prepared at a working concentration 2.5 μg/ml in DEPC-treated water and then stored at −20°C until use.

4. Anti-SARS-CoV-2 SP antibody (SP mAb) cleavage site (Lot No 9091), raised against a peptide corresponding to 12 amino acids near the center of SARS CoV-2 Spike glycoprotein; purchased from Sigma-Aldrich, USA.

5. SARS-CoV-2 (COVID-19) Spike glycoprotein RBD Antibody (Lot No 9087) raised against a peptide corresponding to 19 amino acids near the carboxyterminus of SARS-CoV-2 Spike glycoprotein RBD SPR chips were incubated for 20 minutes in N and S protein solutions of different concentrations at room temperature, then washed with deionized water (< 2μS/cm), after which the liquid phase was removed by centrifugation.

SPR method
The fundamental principles of SPR method rely on the propagation of plasmon wave along the interface of a thin, metal layer (commonly gold) and a dielectric. SPR biosensing takes advantage of the local refractive index changes of the transducer surface when monitoring molecular interactions between the target analyte and the immobilized biological receptor.

In contrast to the prism-coupling method, widely used in SPR biosensors, we use grating-based SPR. Fig. 1A illustrates what kind of a transducer is used in our study – this is a gilded diffraction grating. The gratings were supplied by DEMAX Ltd, Sofia, Bulgaria; for the purposes of the experiment we covered them with about 110 nm gold film coating obtained by vacuum evaporation. SPR conditions were fulfilled for P- polarized light beam that illuminated the
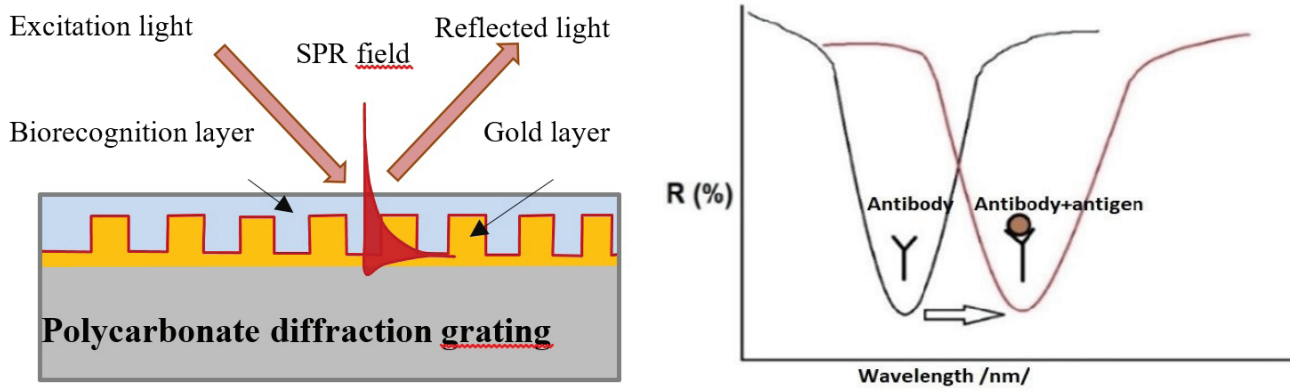
Figure 1. SPR principle: A/ SPR biochip: gilded diffraction grating with immobilized antibody. B/ resonance wavelength shift occurs when proteins bind to antibody.

grating at an incidence angle of about 35 degrees. Typically, the resonance was excited in the range 690-710 nm for a bare grating having 80 nm high grooves at a distance of 1.55 µm from one another. More details about our SPR system can be found in (18). We elaborated three type SPR biochip that represent the grating with immobilized NP mAb, SP mAb or RBD antibody of certain thickness, as shown in Fig. 1A. The wavelength, at which the plasmon wave is excited, shifts significantly when structural proteins interact with the ligand, as illustrated in Fig. 1B. This wavelength shift corresponds to the number of interacting molecules, therefore SPR assay provides a quantitative assessment of interaction.

RESULTS AND DISCUSSION
Antibody immobilization
The main disadvantage of SPR biosensors is their low specificity. Even one hundred percent specificity of the ligand does not guarantee high specificity of the biosensor. This is due to the built-in matrix required for ligand immobilization. Protein immobilization is a delicate procedure – proteins tend to unfold and denature upon contact with metals and most other artificial substrates. However, these statements are valid for conventional methods of immobilization. We use another approach for ligand immobilization. The matrix-assisted pulsed laser evaporation (MAPLE) method has been successfully applied for deposition of proteins. MAPLE immobilized proteins without using built-in matrix and preserving their bioactivity. Then, specificity of reactions depends only on specificity of the ligand. In a previous publication (19) we showed that this technique provides deposition of intact molecules, as well as high accuracy and sensitivity of detection (20).

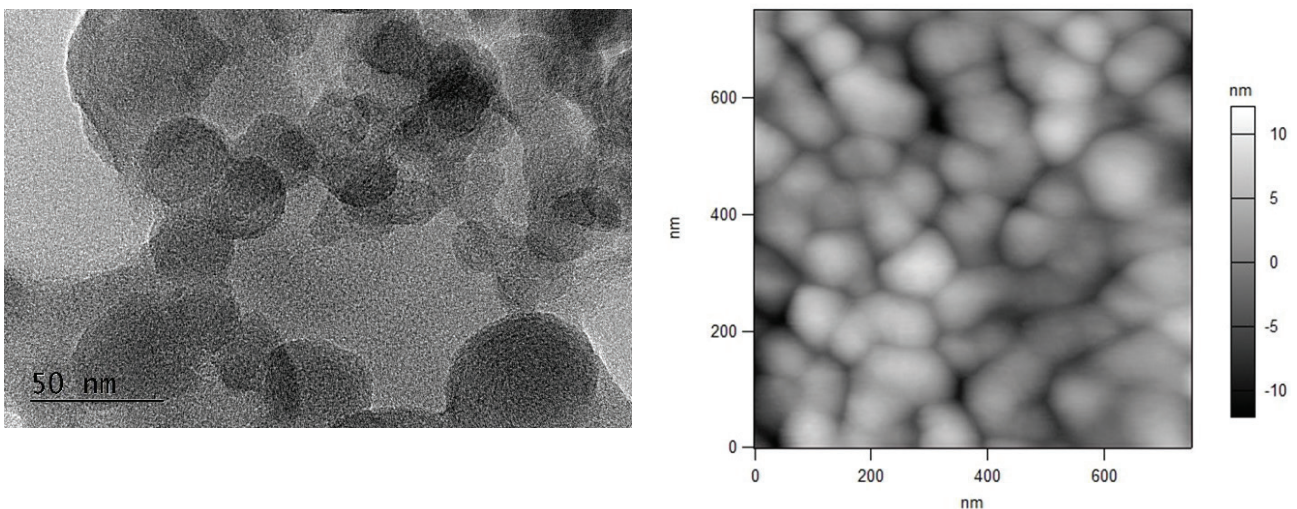For antibodies deposition we used frozen targets consisting of 19.2 pM/ml antibodies dissolved in

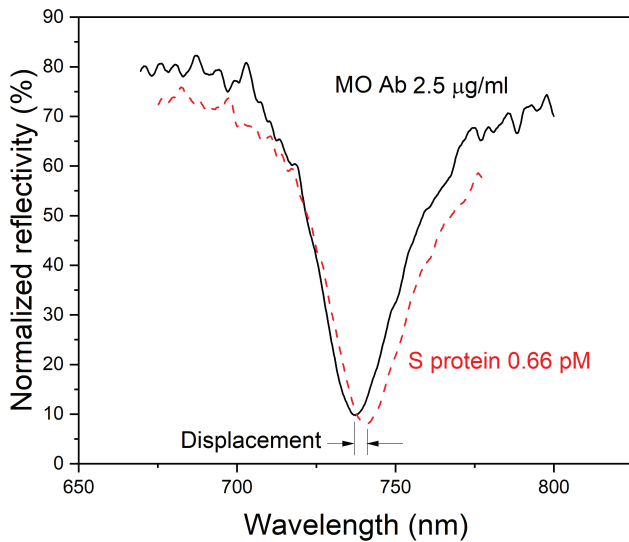**Figure 2.** Mab Layer A/ TEM images of mAb layer. B/ AFM image of the same layer.

Figure 3. SPR detection of antigen–antibody interactions.

Figure 4. SPR assay with immobilized specific NP mAb: detection of S/N-proteins with different concentrations.

DEPC water. This concentration was established after many experiments as MAPLE technology requirements against detection sensitivity tradeoff. Details regarding the MAPLE technique and the parameters of the immobilization procedure can be found in (19).

Characterization of the mAb layer

The layer of anti-SARS-CoV-2 antibodies was deposited with a thickness of about 110 nm – a well controllable parameter of the MAPLE technique. At this thickness SPR ensures a maximum sensitivity of detection, since the field of plasmon wave entirely penetrates the deposited layer. Specificity is guaranteed, since the deposited layer consists only of antibody molecules. The only but significant problem is whether the deposited molecules are bioactive. To check this, we studied the deposited film by well adopted techniques for nanolayer characterization as Transmission Electron Microscope (TEM) (21) and Atom Force Microscope (AFM) (22). Fig. 2A shows TEM images of the MAPLE-deposited sensing layer covering the metal surface. The layer is uniform, dense and antibodies molecules are identified, which is confirmed by the AFM – Fig. 2B shows that the deposited molecules are well shaped. This is a convincing evidence that intact direct immobilization was performed and bioactivity of deposited layer has to be expected.
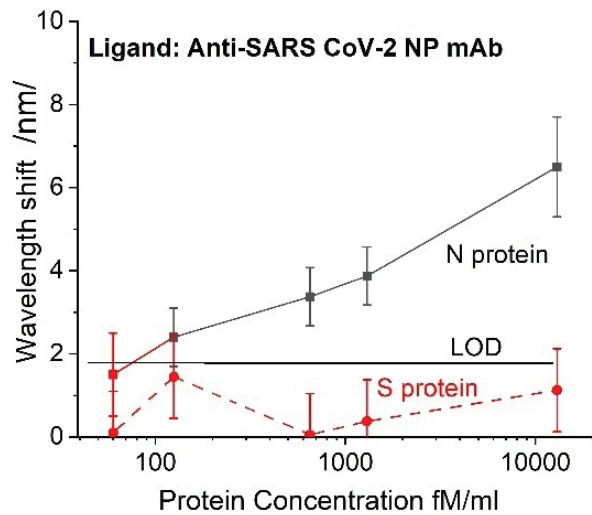
SPR-based assay

SPR is a very effective label-free technique for registering the real-time interaction of two binding molecules. It can provide useful information on the interaction's specificity and binding affinity. Today, it is used in many other life science areas.

Theoretically, a SPR sensing structure having three different ligand-analyte modes has been proposed in (23): (i) the monoclonal antibodies (mAb) as ligand and the SARS-CoV-2 virus spike RBD as analyte, (ii) the virus spike RBD as ligand and the virus anti-spike immunoglobulins (IgM, IgG) as analyte and (iii) the specific RNA probe as ligand and the virus single-stranded RNA as analyte.

In our study we realized experimentally three ligand-analyte strategies: (i) NP mAb as ligand and NP as analyte, (ii) SP mAb as ligand and SP as analyte, (iii) RBD antibody as ligand and SP as analyte.

Various modifications of the SPR platforms targeting amplification of the signal have bee applied for SARS-Cov-2 detection. A SPR assay with a graphene layer was proposed in (24) for the detection of SARS-Cov-2 N proteins. The reported LOD has been evaluated to 1.02 pM.

In (25) was reported LOD of 0.22 pM in protein detection by photothermal enhanced plasmonic biosensor. The LOD achieved in (14) was 85 fM in N protein detection by nanoparticle-enhanced SPR. A record sensitivity was reported in (26) – 12 fg/ml in the detection of S protein by SPR excited in a
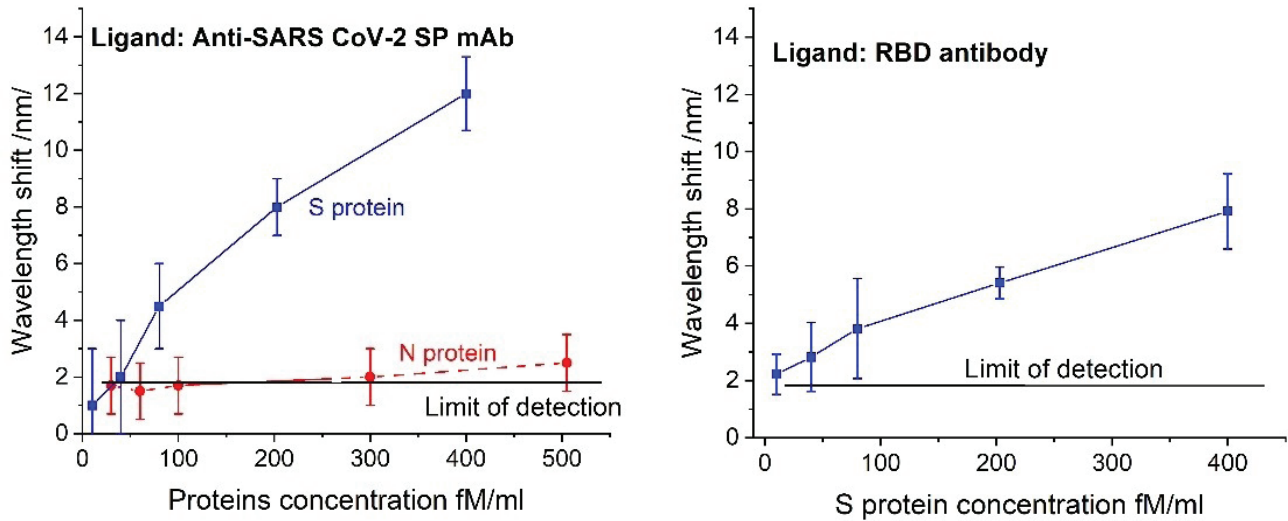
Figure 5. SPR wavelength shift as a function of proteins concentrations for chips functionalized with: A/ SARS CoV-2 SP mAb. B/ RBD antibody.

multilayer structure including graphene.

The SPR-based assay presented here was designed to study the binding affinity between structural SARS-CoV-2 S-and N-proteins and a specific anti-SARS-CoV-2 mAbs and RBD antibody.

Three MAPLE deposition procedures were performed to functionalize SPR gratings. A total of 75 SPR biochips were examined in order to establish the measurement accuracy, out of which 30 were NP mAb-functionalized, 30 were SP mAb-functionalized, and 15 were functionalized with RBD antibody.

The biochips functionalized with NP mAb were incubated as follows: 15 – with S protein of different concentrations, 15 – with N protein of different concentrations. The biochips functionalized with SP mAb were incubated as follows: 15 – with S protein of different concentrations, 15 – with N protein of different concentrations. I.e. when performing the above-mentioned ligand-analyte strategies (i) and (ii), 3 measurements were provided for each concentration of the N/S proteins in order to assess the accuracy of the measurements. The biochips functionalized with RBD antibody were incubated with S proteins: 3 measurements were provided for each concentration in order to assess the accuracy of the measurements.

After the gilded diffraction gratings were functionalized, the plasmon resonances were measured at six diffrent points on the biochip surface to evaluate the quality of the ligand layer. The spectral position of the resonances at each point was taken as a reference against which the shift due to the antibody – protein interaction was registered.

23. After incubation, the plasmon resonances were measured at the same 6 points on the biochip surface and the resonance wavelength shifts were estimated as differences from the reference resonances. Then the corresponding resonance shift average values and the absolute measurement errors were determined. Therefore, each of the experimental points in the graphs, as well as the errors, are the result of 18 measurements.

Fig. 3 shows an experimentally observed SPR resonance shift for a biochip treated with 0.66. pM/ml SARS-CoV-2 N protein, compared with the resonance of a biochip immobilized with a NP specific anti-SARS-CoV-2 mAb, accepted as reference resonance. The wavelength shift of the plasmon resonance results from the viral N-protein binding to mAb

We evaluated the dependence of the wavelength shift of the incubated chips on the viral structural S- and N-protein concentrations. For this purpose, various concentrations of structural SARS-CoV-2 S- and N-proteins in the concentration range 60 femtomoles/ml (fM/ml) – 13 picomoles/ml (pM/ml) were prepared.

Fig. 4 presents the wavelength shift plotted as a function of the S- and N-protein concentrations for SPR biochip having a ligand specified NP mAb. We observed pronounced mAb – N protein interaction

for concentration above 126 fM. For N-protein concentration of 126 fM the measured spectral displacement was 2.5 nm, which is above the limit of detection (LOD) accounting for measurement error (in this case–0.5 nm). LOD was evaluated by considering the accuracy of the spectrometer as well as the accuracy of the goniometer for setting-up the angle of light incidence. The probability of reliably measuring concentrations lower than 126 fM is small because the SPR displacement is compatible to the LOD and the measurement error increases.

The mAb – S proteins interactions generated an SPR response in the range of measurement accuracy, as illustrated in Fig.4. First of all, this was due to the specificity of the used anti-SARS-CoV-1/2 NP clone 1C7C7 ZooMAb® mouse monoclonal antibody. However, this result also shows the applicability of our method of detection.

Having laser deposited the specific SP mAb and RBD antibodies upon the grating surfaces we provided a similar measurement procedure for structural proteins detection. The results of SPR measurements are summarized in Figure 5.

The S protein detection by specified SP mAb (Fig. 5A) is better expressed than the detection of N protein by mAb (Fig.4), however the LOD is the same – 126 fM. N- proteins binding mAb generated a signal slightly above the detection limit, but within the measurement error zone, as shown in Fig. 5A which is partly due to the direct immobilization of the mAb, but also to its specificity.

S-protein binding the RBD antibody (Fig. 5B) is not so effective as S-protein/mAb binding (Fig. 5A). This fact indicates that RBD antibody affinity is lower that the affinity of SP mAb and hardly can be used for S-protein detection.

CONCLUSIONS

The SPR-assays presented here are able to evaluate a wide range of biomolecular interactions. Its high specificity, partly due to the specificity of immobilized antibodies and to the immobilization method, makes it applicable in a diversity of conditions, especially when studying SARS CoV-2.

The proposed SPR assay could be optimized for any new antibody (monoclonal or polyclonal). Most

importantly, this type of assay design could assist the detection of a variety of viruses.

It is worth mentioning the high sensitivity of about 130 fM achieved in detecting structural proteins. As reported in our recent research (27) the comparison of SPR assay with clinically used ones shows that the SPR method ensures sensitivity and accuracy similar to those of the rapid antigen tests. Therefore, SPR assay is able to detect acute SARS-CoV-2 infection, especially at the early stages of viral replication and can be clinically useful.

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