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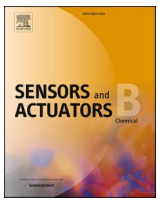

SARS-CoV-2 N protein IgG antibody detection employing nanoporous anodized alumina: A rapid and selective alternative for identifying naturally infected individuals in populations vaccinated with spike protein (S)-based vaccines

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ABSTRACT

Methods to detect naturally infected individuals, especially during or after a pandemic, are valuable in populations with a high rate of vaccination. Having in mind that after the COVID-19 pandemic people has been vaccinated against the virus by Spike protein (S)-based vaccines, we present in this paper a novel nanosensor based on gated nanoporous anodic alumina (NAA) material to detect naturally infected individuals in populations with high rates of vaccination. The nanosensor developed is based on a protein-capped nanomaterial for the identification of IgG antibodies that can detect nucleocapsid protein (N) of SARS-CoV-2. The NAA material has been loaded with an indicator (Rhodamine B (RhB)) and the pores of the material have been blocked with SARS-CoV-2 nucleocapsid protein (N) attached to a specific aptamer. In presence of antibodies against this antigen, the pores are uncapped, triggering the dye release and a fluorescent signal as a result. The biosensor has been tested *in vitro* and simulated serum for IgG detection, proving a detection limit of 1 μg/mL. Moreover, specificity assays with N proteins from other coronaviruses have proved the robustness and efficacy of this nanosensor. Finally, the system has been tested on samples from patients that contained SARS-CoV-2 antibodies, demonstrating its potential for the discrimination of individuals that have been vaccinated or infected by SARS-CoV-2 virus.

1. Introduction

SARS-CoV-2 virus produced in December 2019 the multi-systemic COVID-19 disease. It was initially reported in the city of Wuhan (China), producing a public health emergency [1–3]. Although mostly patients of COVID-19 can be asymptomatic, the most frequent symptoms are cough, fever, and myalgia or fatigue, when appearing, but it has demonstrated can also be deadly for certain population groups [4]. In

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this regard, elderly individuals and those with poor immune function have the highest mortality rate[5]. Furthermore, the prominent presence of asymptomatic carriers increased the viral spread in a large degree among population, resulting in a rocketing rise of global infection numbers [6]. SARS-CoV-2 is included in the same virus family of MERS-CoV and SARS-CoV, pathogenic coronavirus that caused unprecedented nosocomial outbreaks in 2003 and 2012, respectively [7]. It has a genome of 30 kb that encodes one non-structural polypeptide and four proteins, i.e. the envelope (E), spike (S), nucleocapsid (N) and membrane (M) proteins [8]. From them, N protein possess a sequence similarity of 90.6 % in relation to SARS-CoV homolog protein and possess high immunogenicity. Therefore, N protein is a frequent target for possible vaccines and serological assays [9]. According to genomic data and the fact that early cases were detected in the market Huanan in Wuhan, SARS-CoV-2 emergence may be a natural selection involving mammalians (e.g., bats and pangolins) as host reservoir resulting in a possible zoonotic transfer to humans [10,11]. Since this disease produced a massive number of infections and deaths globally in just a couple of months, the 11$^{th\ of}$ March 2020 the WHO proclaimed the COVID-19 pandemic, which prompted most nations to work on a rapid response for this world-wide threat. Firstly, prevention strategies were key and since then, research institutions around the world dedicated significantly effort to develop effective strategies to handle this concerning situation by developing new diagnostic tests, drugs, and vaccines to combat SARS-CoV-2 [12–14].

During the pandemic, an intense study of SARS-CoV-2 was produced, having consequently a rapid progression of clinical trials for new COVID-19 vaccines. Since S protein serves as a key element to enter cells through angiotensin converting enzyme II (ACE2) membrane receptor[3], several RNA-based or protein-based vaccines (e.g., BNT16b2 by Pfizer and BioNTech, mRNA-1273 by Moderna, NVX-CoV2373 by NovaVax) are focused on providing protection by this antigen [15], for which individuals vaccinated generated antibodies. On the other hand, N protein facilitates RNA replication and virus particles assembly and release[9], and it is located inside the virus capsid. Immunological response against the latter antigen can be generated by direct SARS-CoV-2 infection or several whole-virus-based vaccines (e.g., CoronaVac by Sinovac Biotech, Covaxin by Bharat Biotech, KoviVac by Chumakov Center)[15], unlike S protein mRNA vaccines. In this regard, antibody detection is commonly used as a screening method to evaluate immunological protection in general population. Around the world, S Protein RNA-based or protein-based vaccines have been the most commonly used, whereas whole-virus-based vaccines have been used considerably less [16]. Therefore, the identification of N protein antibodies would be a valuable method of identification of naturally infected individuals in populations with a high rate of vaccination where vaccines against S Protein have been used.

In connection with SARS-CoV-2 detection, aptamers have been key elements for the development of accurate and rapid tests with the aim to detect viral structural proteins of this coronavirus by colorimetric and fluorometric methods [17–19]. These single-stranded nucleic acid molecules, produced SELEX techniques have the ability to interact with proteins with high affinity and selectivity, leading to a huge advance on therapeutics and diagnostics fields thanks to a wide array of applications [20]. Development of aptasensors to detect SARS-CoV-2 has mainly focused on N protein and S protein by optical and electrochemical sensors [21], aptamer-linked immobilized sorbent assays (ALISA) [22], and lateral flow devices [23], which can be more effective and easier to deploy than antibody-based methods [24]. Besides, aptamers have been used for therapeutics in COVID-19 treatment over antibodies due to their high affinity for structural proteins and absence of antibody-dependent enhancement (ADE) increasing risk when using these strategies [25].

Recent advances in nanotechnology have improved the design and characterization of numerous nanoparticles and nanomaterials for biosensing applications by attaching different affinity probes (e.g., antibodies, aptamers, ssDNA) [26]. Several types of nanomaterials (e.g., gold nanostructures, polystyrene nanoparticles, graphene, quantum dots) have been used to design effective, rapid biosensors to detect chemicals, biomolecules, and microbial pathogens accurately in just a few minutes/hours, resulting in a wide variety of nanotechnology-based biosensors [27–30]. Among materials used in biosensing approaches, Nanoporous Anodic Alumina (NAA) arises as a propitious tool to detect a broad varieties of endogenous or pathogen biomolecules (e.g., DNA, RNA, proteins) [31–36]. NAA supports fabrication consists of aluminium anodization to produce at the surface a layer of oxide that provides the material with different properties [37]. Furthermore, under specific conditions, a porous structure arises on the material surface, which confers ideal properties to different applications [38]. NAA supports can be reused several times since can be calcinated. Moreover, those supports are also stable in aqueous environments, which renew the material by erasing organic material from their surface [39]. NAA materials have been proved to be viable in biosensing applications employing nucleic acids (DNA, RNA) as molecular gatekeepers to control the signal delivery upon stimuli input[30 33].

Considering worldwide-distributed vaccines are mostly based on S protein, N protein has been the main focus of several antibody detection systems developed in recent studies [40,41]. This approach combined with S protein antibodies detection can provide a more meaningful, complete information about the seroconversion than single antigen-based assays[42–44]. This kind of multiplex analysis of antibodies can be useful to detect whether tested individuals have been naturally infected by SARS-CoV-2. However, the complexity can trade off the reliability for the cost-effectiveness of the test. Based on the above, here we study the application of NAA combined with a covalently attached aptamer[17] and the SARS-CoV-2 N protein as a gated support to develop a rapid antibody detection test to recognize IgG antibodies produced by produced by SARS-CoV-2 N protein. Since the proposed system uses N protein as unique antigen, it makes a suitable, fast, and reliable test to extract information about antibody response against this viral polypeptide. Results show a time of 20 minutes to perform the test, with an LOD of 1 μg of antibodies mL^{-1} with high specificity against another common coronavirus. Moreover, the sensor was validated in clinical samples.

2. Material and methods

2.1. Reagents and chemicals

Rhodamine B (RhB), 3-(Triethoxysilyl) propyl isocyanate, triethylamine (TEA), tris (hydroxymethyl) aminomethane (TRIS), and hydrochloric acid have been purchased from Merk (Spain). The aptamer sequence NH_2-$(CH_2)_6$-5'-GCAATGGTACGGTACTTCCGGATGCGGAAA CTGGCTAATTGGTGAGGCTGGGGCGGT-3' reported previously in the literature[17] was provided by Integrated DNA Technologies (IDT, USA). The Simulated Blood Serum was purchased from Biochemazone (Canada). InRedox (CO, USA; article number AAO/Al-25×75–005–010) provided the NAA supports. Nucleocapsid protein and antibodies were purchased from Sino Biological (China). Human serum samples were obtained from Microbiology Department of University and Polytechnic La Fe Hospital (HUP La Fe; Valencia, Spain) by authorized personnel.

2.2. Study population and collection

Selected patients were adults (age ≥18) with anti-SARS-CoV-2 IgG positive determined by LIAISON® XL (Diasorin Iberia SA, Madrid, Spain) and COVID-19 RT-PCR positive.

To obtain samples tested positive by SARS-CoV-2, we investigated patients that were hospitalized in emergency rooms of COVID-19 patients at HUP La Fe in Valencia, Spain. The study excluded patients that were pregnant, breastfeeding or that did not consent to form part of the study. Trained study personnel collected a single blood sample from each consenting participant.

A total of 13 blood samples (3 mL) from patients tested COVID-19 positive and 18 tested COVID-19 negative through IgG and RT-PCR were included to antibody response study. All samples were transported at ambient conditions and stored at 2–4 °C until testing, which took place within 48 h of sample collection. The study was authorized at ethical committees of the HUP La Fe for drug research 2020–463–1 and 2021-012-1.

2.3. General techniques

Fluorescence experiments were performed with a Synergy H1 microplate reader (BioTek, Winooski, VT, USA). Energy Dispersive X-ray spectroscopy (EDX) and Field Emission Scanning Electron Microscopy (FESEM) analyses were carried out with a A ZEISS Ultra 55 microscope. Thermogravimetric analyses were conducted with a balance from TGA/SDTA 851e Mettler Toledo (Mettler Toledo Inc., Schwarzenbach, Switzerland). Conditions of measurements employed air with a flow of 80 mL/min. The program for heating involved a gradual increase of the temperature (10 °C/min from 20 to 1000 °C), having 2 heating steps (100 °C and 1000 °C) during 30 min.

2.4. Preparation of NAA materials S1, S2 and S3

A representative Scheme for the synthesis of materials **S1**, **S2** and **S3** supports is depicted in Scheme S1 (Supporting information). **S1** supports were prepared with 24 NAA disks (2 mm of diameter). Those disks were mixed with a solution of 1.5 mM RhB dye in CH_3CN, and the mixture was leaved with agitation 24 h, with the aim to introduce the RhB on the pores of NAA. Finally, the outer surface of NAA disks was modified employing 3-(triethoxysilyl) propyl isocyanate (10 μM). For that purpose, the mixture was stirred during 6 h.

S2 was prepared mixing **S1** supports with 1.5 mM RhB in CH_3CN, 5 mM aptamer, and 20 mM TEA. Solution was mixed during 3 h at ambient conditions.

Material **S3** was obtained after immersion of **S2** in PBS (pH 7.5) containing 1 μM of nucleocapsid protein, mixing 2 h the solution at ambient conditions. Finally, the unbound protein was removed from **S3** through washes with PBS.

2.5. Recognition assay protocol

The fluorescence emission of RhB released by **S3** material in the solution was measured as an indicator in presence of anti-SARS-CoV-2 IgG antibody. For that purpose, three independent **S3** disks were taken for triple replication. Experiments were conducted with anti-SARS-CoV-2 IgG antibody as input and without antibody as control and **S3** disks were dipped in 1 mL of PBS buffer. Materials were mixed at ambient conditions, taking aliquots at different defined times. The RhB released was quantified monitoring its fluorescence (λ_{exc} =555 nm, λ_{em} =575 nm).

2.6. Selectivity studies

To carry out selectivity studies, anti-N-protein antibodies from SARS-CoV, MERS-CoV, HKU-1, OC43, NL63, and 229E were tested as potential competitors. Assays were performed in 1 mL. **S3** supports were mixed at 25ºC for 20 min. A material without the presence of antibody was used as control. Finally, fluorescence of RhB in solution was quantified (λ_{exc} =555 nm, λ_{em} =575 nm). Experiments were performed in PBS or simulated human blood serum.

2.7. Blood serum sample collection and preservation

Two blood samples were stored in tubes that contained a clot activator and a serum gel separator and both were centrifuged (3500 rpm; 10 min). The serum from one of the samples was kept refrigerated to determine in less than 24 h the levels of antibodies against SARS-CoV-2 present. The remaining sample was stored frozen (-80°C) for the rest of the determinations.

2.8. Techniques used for antibody determination

The test was performed with the LIAISON® XL, automated chemiluminescent immunoassay (CLIA) with a separate determination of IgG and IgM. Measurements were carried out on the LIAISON® XL analyzer following instructions provided by the manufacturer's instructions.

3. Results and discussion

3.1. Design and characterization of the system

The proposed sensor is based on NAA supports loaded with RhB at the pores and containing at the external surface 3-(triethoxysilyl) propyl isocyanate to yield nanomaterial **S1**. Subsequently, aptamer NH_2-$(CH_2)_6$-5'-GCAATGGTACGGTACTTCCGGATGCGGAAACTGGC-TAATTGGTGAGGCTGGGGCGGT-3', which proved to have high affinity and specificity for N protein of SARS-CoV-2,[17] was attached covalently to the material through a urea bond, resulting in support **S2**. Finally, pores were blocked by N protein, since it binds to the attached aptamer due to the affinity of the aptamer selected to the N protein [45,46] giving the gated solid **S3** (Scheme 1).

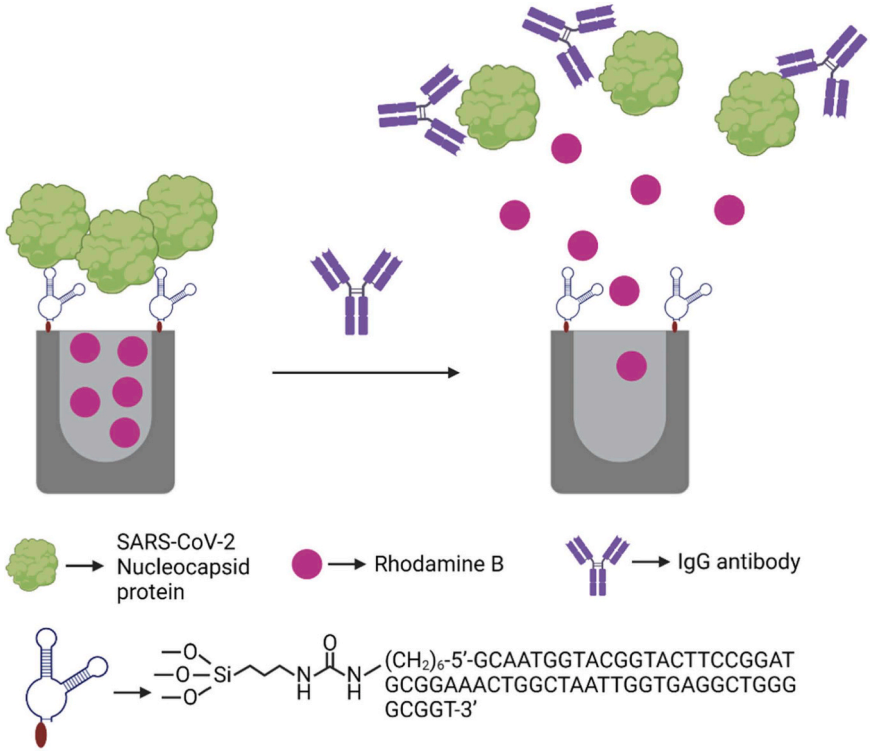
In the absence of anti-N-protein antibody, the SARS-CoV-2 N protein is expected to prevent RhB releasing on the medium by blocking **S3** pores. The mechanism of detection (monitorization of RhB released) relies on the differences of the affinity of the N-protein by the aptamer anchored at the surface and the IgG antibody of SARS-CoV-2, based on competitive displacement immunoassays. For a proper displacement, the target antibody must have higher affinity to the N protein than the aptamers attached to the surface. These differences on the affinity have been already described by Chen et al.,[17] which performed a Western Blot assay comparing the affinities of the aptamer and antibodies used in this work for the N protein. On that study, authors showed that both antibodies and aptamers were able to interact with N protein, yet the affinity of the antibody was slightly higher in comparison by the aptamer.[17] Having this in mind, N protein is expected to detach from the pores of our nanosensor in presence of anti-N-protein antibody, causing the displacement of the blocking system and the delivery of the dye to the medium (Scheme 1).

NAA materials were evaluated through FESEM and EDX techniques. Representation of FESEM images showed the that the pores of NAA were disordered (Supplementary Material, Figure S1). NAA disks had 2 mm of diameter and pore densities around $9{\cdot}10^{11}$ cm^{-2}, showing a funnel-shaped structure at the entrance of the pores that decreased gradually, starting with 25 ± 5 nm at the outer surface of NAA and shrinking to 5 nm at the bases of NAA, having a length of 10 μm. The silver disks gain an intense pink color once RhB was loaded into the pores.

EDX spectroscopy shows the content of organic matter of **S2** and **S3** (Table 1). **S1** had a high carbon content, ascribed to the RhB present into the NAA material. Nitrogen indicates the presence of RhB in the support. The carbon content of solid **S2** is higher than **S1**. Moreover, the presence of phosphorus (P) indicates the incorporation of the aptamer to the NAA. Finally, material **S3** had a similar organic matter content, conserving the P/Al value for the aptamer, and slightly increasing the N/Al due to the presence of the capping N protein.

3.2. Delivery kinetics

Recognition of antibodies IgG SARS-CoV-2 antibodies employing support **S3** produces an opening of the blocked pores, delivering RhB to the medium and producing the optical signal. The behaviour of material **S3** to N protein IgG antibody was tested in PBS buffer (pH 7.5). Dye delivered in the solution was determined through fluorescence



Scheme 1. Design and working principle of the nanosensor prepared.

Table 1
Atomic element ratios on the different supports.

	C/Al	N/Al	P/Al
S0	0.29 ± 0.04	-	-
S1	0.47 ± 0.04	0.06 ± 0.02	-
S2	0.64 ± 0.06	0.06 ± 0.01	0.02 ± 0.01
S3	0.58 ± 0.14	0.08 ± 0.01	0.02 ± 0.01

measurements of aliquots that were collected at certain times.

Fig. 1A shows little RhB emission is recorded in absence of the anti-N-protein antibody, which suggests the pores are notably closed by N protein and the aptamer anchored to the surface. On the contrary, when antibody is present, a fluorescence emission enhancement is recorded, which is attributed to dye releasing to the solution (Fig. 1A). After 20 minutes, approximately all the RhB able to be released was liberated to the medium. This observed response agrees with the higher affinity of the antibody to the capping N protein in comparison with the aptamer, which causes a displacement of the antigen from the pores.[17] As both antibodies and aptamers interact with N protein, it can be pictured that N protein binds to aptamer and antibody in an equilibrium manner that let the pore alternate between opened and closed, letting the dye release gradually. Delivery behaviour of this system is quite similar to other optical NAA nanosensors [47], which proves NAA supports are a versatile framework to develop a wide variety of strategies in biomedical diagnostics.

3.3. Sensitivity and specificity

The sensitivity of the system was evaluated by monitoring the RhB fluorescence in presence of different concentrations of SARS-CoV-2 N protein IgG antibody. NAA supports were immersed in PBS for each antibody concentration, measuring fluorescence at 575 nm after 20 minutes of constant stirring. Experiments were measured in

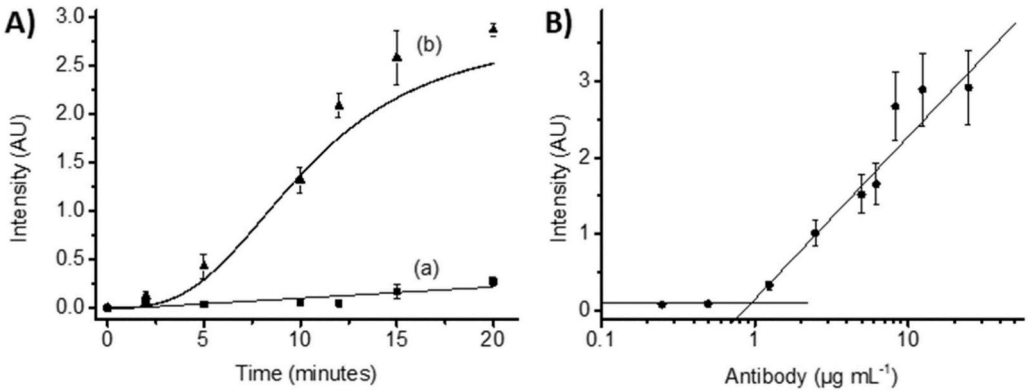


Fig. 1. A) Rhodamine B released from **S3** in PBS (pH 7.5) as a function of the time. (a; black squares) shows delivery of dye in absence of IgG antibody, whereas (b, black triangles) represents dye release when the target antibody (6.20 μg/mL) was present. B) Rhodamine B released by **S3** as a function of the concentration of IgG antibody against N protein.

triplicate. Study comprised antibody concentrations in the range 250–25 μg mL^{-1}. As shown in Fig. 1B, the dye delivered is directly related to antibody concentration, following a dose-dependent release. The detection limit (LOD) was 1 μg mL^{-1}, estimated taking into account the intersection of the represented lines in the Fig. 1B. Despite our LOD is not as low as in other systems for SARS-CoV-2 antibody detection [48–50], NAA supports is a versatile, rapid, and customizable material for antibody detection based on aptamer-protein interaction. In addition, the studies on the specificity of this system (*vide infra*) prove an excellent capacity for discerning viral antibodies from different coronavirus that might coexist with SARS-CoV-2. Moreover, calibration curve shows an IC50 value of ca. 5 ug/mL. Having in mind the typical IgG molecular weight of 150 kDa, this would correspond to an IC50 value of ca. 33 nM, which is in agreement with affinity constants of other anti DNA aptamers reported, with K_D values between 10^{-7}–10^{-12} M [51,52].

The specificity of **S3** was tested when anti-N-protein IgG antibodies of other coronaviruses were present. In this case, we used SARS-CoV, MERS-CoV, 229E, NL63, OC43, and HKU1 anti-N-protein IgG antibodies at concentration 6.20 μg/mL in PBS. As shown in Fig. 2A, NAA solid releases negligible cargo when non-SARS-CoV-2 antibody is present, except SARS-CoV IgG, which cross-reacts with N protein of SARS-CoV-2 as it is reported in other studies by ELISA assay [53]. In addition, we tested the robustness of this selectivity in simulated blood serum as a preview for real samples (Fig. 2B). Similar behaviour of our material in these two media shows a degree of consistency in the system against the presence of certain interferents. Mild COVID-19 shares a great number of general symptoms with common cold human coronaviruses (e.g., cough, fever, muscular pain, and headache) [54]. In addition, these coronaviruses are widely distributed around the world with a high prevalence [55]. Thus, it may hinder an accurate identification of SARS-CoV-2 according to symptomatology as only criteria. Therefore, an accurate, fast, and reliable test like the system presented in this report might be of importance to discriminate between SARS-CoV-2 and other frequent cold human coronaviruses.

3.4. SARS-CoV-2 detection in patient samples

Clinical application of **S3** is an important step to test our sensor for the detection of SARS-CoV-2 antibody. Therefore, sensing capability of the material was studied employing blood serum samples from the HUP La Fe previously analyzed by ELISA reference method to confirm the presence or absence of anti-N-protein antibodies. Moreover, samples were also analyzed by RT-PCR to confirm if the patients were infected by SARS-CoV-2 at the moment of the sample extraction. Moreover, it should be important to remark that the study was performed with samples of patients collected between March and May 2021, period in which vaccination was starting and was taking place mostly only with S-protein-based vaccines (on that moment no whole-protein vaccines were available). A total of 31 patients participated in this study (Table 2), having 13 patients confirmed to have SARS-CoV-2 N antibodies by ELISA. The remaining 18 were employed as negative controls. It is noteworthy that all positive tested 13 patients for SARS-CoV-2 antibodies by ELISA also presented COVID-19-related symptoms and were tested positive by RT-PCR, corroborating that the 13 patients were infected by SARS-CoV-2, whereas negative controls showed no respiratory disorder and were negative tested by RT-PCR. All vaccinated patients were inoculated with S-protein-based vaccines before this study. After data collection, a threshold value of 0.25 AU of fluorescence (Average value of negatives +1σ) was established to discriminate between positive and negative patients. Samples were measured in triplicate with the nanomaterial **S3,** by introducing individual plates in the undiluted blood serum and fluorescence of RhB in the medium was measured after 20 minutes. Fig. 3 shows that in positive samples there is a higher dye delivery than in negative controls, leading to a notable distinction between both groups. Same results were obtained by ELISA and RT-PCR reference methods for viral infection and antibody detection at the hospital and with **S3** in all the 31 tested samples (Table 3). These data suggest that material **S3** can be a suitable support for recognition of SARS-CoV-2 N antibodies, and the vaccination status does not interfere with the test output. Interestingly, the history of a patient

Table 2
Patient information (in numbers and percentages) classified according to gender, age group, vaccination, and symptomatic or not.

Variable	User	
	n	%
Gender		
Female	10	32.2
Male	21	67.8
Age		
≤30	0	0
31–40	1	3.2
41–50	4	12.9
51–60	2	6.4
61–70	6	19.4
71–80	8	25.8
81–90	9	29
91–100	1	3.2
Vaccinated		
Yes	9	29
No	22	71
Symptomatic		
Yes	13	41.9
No	18	58.1
Total	**31**	**100**

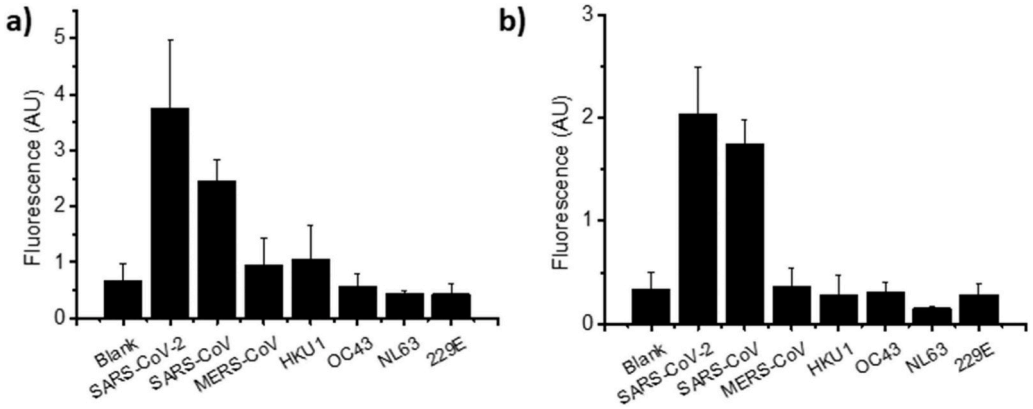


Fig. 2. RhB delivered from **S3** produced by 6.20 μg/mL of specific IgG antibodies for N protein of SARS-CoV-2, SARS-CoV, MERS-CoV, HKU1, OC43, NL63 and 229E. Experiments were accomplished at 20 min in buffered media (PBS, pH 7.5). (A) and in simulated human blood serum (B).

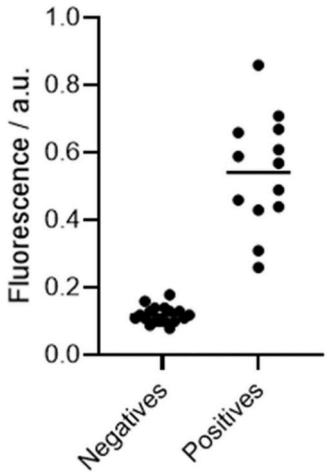


Fig. 3. Dye released in human blood serum samples. Scatter plot reveals much lower fluorescent signals in negative samples (n = 18) than the fluorescence observed with samples tested positive (n = 13). Measurements were taken after 20 minutes.

that showed negative results with our method and also with ELISA and RT-PCR, was infected 7.5 months before the sample collection, indicating that after this long period of time the IgG N-protein antibodies were not present. This evidence is in agreement with data reported nowadays, which also confirm that IgG SARS-CoV-2 antibodies are remaining in our body only after 3–4 months succeeding the infection or subsequent to vaccination [56]. Therefore, our method is valid for the discrimination of patients as long as the antibodies are present, like the ELISA technique but having in mind the advantages and simplicity for this application in rapid diagnostics. Moreover, the data also suggest that **S3** is a suitable candidate to identify naturally infected people in regions with high rate of vaccination with S-protein based vaccines.

4. Conclusions

The preparation of rapid, precise, and accurate tests for antibody recognition of SARS-CoV-2 is essential to control the disease in future outbreaks. On that regard, nanomaterials have been combined with aptamers and binding proteins, obtaining a fluorescence nanosensor that can recognise antibodies against the N protein of SARS-CoV-2. NAA support is filled with RhB and capped with a DNA-protein complex made by N protein of SARS-CoV-2 and an aptamer that possess high affinity against this viral protein. When antibodies against N protein are present, this protein is displaced and the pores are unblocked, producing the delivery of the RhB dye. This biosensor shows a LOD of 1 μg mL^{-1} of immunoglobulins in PBS. Importantly, the system shows high selectivity for antibodies that selectively recognise SARS-CoV-2 N protein in comparison with other immunoglobulins against common cold coronaviruses N protein. The reported system was successfully applied in clinical samples to detect antibodies in blood serum of individuals infected by SARS-CoV-2 virus. In addition, it showed total coincidence with the standard serological method ELISA and there was no interference with vaccination status of the patient. This approach may also inspire the development of new protein-binding detection systems for pathogen testing.

CRediT authorship contribution statement

Eva Calabuig: Writing – review & editing, Investigation. **María Dolores Gómez:** Writing – review & editing, Investigation. **María Ángeles Tormo-Mas:** Writing – review & editing, Supervision. **Javier Pemán:** Writing – review & editing, Supervision. **Yoel Esteve-Sánchez:** Writing – original draft, Methodology, Investigation, Formal analysis.

Table 3
Summary of blood samples results analysed measured employing **S3** and ELISA as reference method. Vaccination status of patients is also showed.

# Sample	Reference method (ELISA)	S3	Vaccinated	PCR TEST
1	-	-	YES	-
2	-	-	YES	-
3	-	-	NO	-
4	-	-	YES	-
5	-	-	YES	-
6	-	-	YES	-
7	-	-	NO	-
8	-	-	NO	-
9	-	-	YES	-*
10	-	-	NO	-
11	-	-	NO	-
12	-	-	NO	-
13	-	-	NO	-
14	-	-	NO	-
15	-	-	NO	-
16	-	-	YES	-
17	-	-	NO	-
18	-	-	YES	-
19	+	+	NO	+
20	+	+	NO	+
21	+	+	NO	+
22	+	+	NO	+
23	+	+	NO	+
24	+	+	YES	+
25	+	+	NO	+
26	+	+	NO	+
27	+	+	NO	+
28	+	+	NO	+
29	+	+	NO	+
30	+	+	NO	+
31	+	+	NO	+

* Patient infected by SARS-CoV-2 7.5 months before sample collection. Negative results indicate that after that period of time, no IgG Antibodies against N-protein SARS-CoV-2 virus are present on the body.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.snb.2024.136378.

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Ramón Martínez Máñez is a full Professor of Inorganic Chemistry in the Department of Chemistry at the Polytechnic University of Valencia (UPV), is the Head of the Interuniversity Research Institute for Molecular Recognition and Technological Development (IDM) in Valencia and the Scientific Director of the Biomedical Research Center Network in Bioengineering, Biomaterials and Nanomedicine (CIBER BBN). He has more than 595 publications (h-index 61). He has co-authored one scientific reference book published by Wiley in 2010 and is the co-author of 25 book chapters. He holds supervised 33 Ph.D. theses and more than 38 patents. Recently he received the Research Excellence Award 2016 from the Real Sociedad Española de Química (RSEQ) and in 2018 he received the in 2018 he received the King Jaume I Award for New Technologies. He has been an active researcher in the fields of chromo-fluorogenic probes to detect ions and molecules of biological and environmental importance, some of which are based on hybrid nanomaterials. Moreover, he has also been working on the design of innovative gated nanodevices for controlled drug release using external stimuli.

Elena Aznar is currently lecturer at the UPV and she develops her research activity in the Mixed Unit of Nanomedicine and Sensors of the Hospital La Fe Health Research Institute. During the last years, Dr. Elena Aznar has focused her interest on the development of new

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