Peptide-Integrated Superparamagnetic Nanoparticles for the Identification of Epitopes from SARS-CoV-2 Spike and Nucleocapsid Proteins

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ABSTRACT: The COVID-19 pandemic, caused by the fast transmission and spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is currently considered a serious health problem, requiring an effective strategy to contain SARS-CoV-2 dissemination. For this purpose, epitopes of the SARS-CoV-2 spike (S) and nucleocapsid (N) proteins were identified by bioinformatics tools, and peptides that mimic these epitopes were chemically synthesized and then conjugated to superparamagnetic nanoparticles (SPMNPs). Three peptides from S protein and three from N protein were used as antigens in a conventional enzyme-linked immunosorbent assay (ELISA) against serum samples from COVID-19-positive patients, or from healthy donors, collected before the pandemic. Three peptides were effective as antigens in conventional peptide-based ELISA, achieving 100% sensitivity and specificity, with high accuracy. The best-performing peptides, p2pS, p1pN, and p3pN, were associated with superparamagnetic nanoparticles (SPMNPs) and were used to perform nanomagnetic peptide-based ELISA. The p2pS−SPMNP conjugate presented 100% sensitivity and specificity and excellent accuracy (area under the curve (AUC) = 1.0). However, p1pN and p3pN peptides, when conjugated to SPMNPs, did not preserve the capacity to differentiate positive sera from negative sera in all tested samples, yet both presented sensitivity and specificity above 80% and high accuracy, AUC > 0.9. We obtained three peptides as advantageous antigens for serodiagnosis. These peptides, especially p2pS, showed promising results in a nanomagnetic peptide-based ELISA and may be suitable as a precoated antigen for commercial purposes, which would accelerate the diagnosis process.

KEYWORDS: superparamagnetic nanoparticles, peptide, COVID-19, serodiagnosis, antigens, SARS-CoV-2

1. INTRODUCTION

The pandemic caused by the 2019 coronavirus disease (COVID-19), an infectious disease, is now considered a major challenge for global health due to high mortality and morbidity rates.1 In this sense, the better understanding of the disease current situation and the development of strategies to contain the virus spreading is urgently required.2 Despite the significant investment in research, there are no specific clinical treatments registered for COVID-19 so far. Furthermore, even considering the best scenario, with vaccines against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), part of the population may still be infected and develop symptomatology.1−3

Therefore, diagnosis tests are a good front-line strategy to recognize infected people and limit the spread of coronavirus. Serological tests allow the evaluation of the population exposed to the virus, as well as the determination of heterogeneity and the duration of humoral immune response. The early detection of the infection is decisive as it supports the search for more appropriate therapies.5

The diagnostic methods to detect SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV), a virus that presents a high structural similarity with SARS-CoV-2, are based on spike (S) and nucleocapsid (N) structural proteins.7,8 The S protein is a SARS-CoV-2 glycoprotein that protrudes
extensively from the virus envelope. Also for other CoVs, this protein is responsible for facilitating the virus invasion by binding to the membrane surface receptor on host cells. The receptor-binding domain (RBD) of S glycoprotein interacts directly with the peptidase (PD) domain of the host cell receptor, the angiotensin-2 converting enzyme (ACE2). The S protein is located on the viral surface and has been reported as highly immunogenic.9,10 The N protein binds to gRNA, and is involved in virus–host interaction by facilitating the virus multiplication and dissemination.11–15 The N protein has immunogenic activity and is abundantly expressed during the infection of these diseases.7,8,14–16 Both S and N proteins have been previously described as potential coating antigens for COVID-19 serodiagnosis.17,18

Nevertheless, many currently available detection tests of COVID-19 are based on molecular data related to the SARS-CoV proteins, which leads to cross-reactivity, low detection of IgM and IgG in the first days of infection, and low sensitivity. A few enzyme-linked immunosorbent assay (ELISA) kits use the N protein as an antigen, which has 92% amino acid homology with SARS-CoV and therefore presents the same issues mentioned above. The reverse transcription-polymerase chain reaction (RT-qPCR) is considered the gold standard test to diagnose COVID-19. However, this test may present a false-negative diagnosis due to many factors, such as difficulty for sample collection and the correct period of analysis.6,19

Because of this, the development of individual more efficient, faster, and accurate diagnostic methods is essential. Several studies have demonstrated that the use of nanotechnology, such as superparamagnetic nanoparticles (SPMNPs), can increase the diagnostic performance, offering new possibilities for the development of faster and more sensitive tests, with more accurate results in a short period of time, at a low cost.20–22 SPMNPs are hysteresis-free, ideal for biomedical application, presenting high saturation magnetization value, and can be quickly moved in the desired direction; also, SPMNPs can be easily decorated with biomolecules.20,29 A low-cost immunoassay based on magnetic beads was developed to detect antibodies against SARS-CoV-2, enabling a faster detection (in minutes) than conventional ELISA assays.30 Furthermore, a magnetic chemiluminescence enzyme immunoassay (MCLIA) using a synthetic peptide from SARS-CoV-2 S protein, presented promise in the detection of specific antibodies, IgG and IgM, without reaction crossover, evaluated in a cohort of 276 infected confirmed patients, with a rate of 81.52% positive.31 Another serodiagnosis is to measure titers of IgM and IgG anti-SARS-CoV-2, using a chemiluminescent immunoassay (CLIA) with magnetic beads coated with SARS-CoV-2 nucleocapsid protein and spike protein, with sensitivity between 93 to 100% and specificity of 75 to 90.9% (95% confidence intervals (CI)) to IgM and with sensitivity between 88.2 to 100% and specificity of 100% (95% CI) to IgG.32 A flow immunoassay using magnetic beads for the detection of IgG antibodies against SARS-CoV-2 was also validated.33 All diagnoses recently applied prove that this technology has been successful in detecting SARS-CoV-2 antibody. On the other hand, the serodiagnosis still needs improvement to guaranty appropriate sensitivity and specificity. Furthermore, the identification of epitopes by bioinformatics has been a useful tool for the development of antigens to detect specific antibodies against SARS-CoV-2.30,31,34–37 However, the epitope predictions made so far from SARS-CoV-2 proteins are essentially linear, based on the primary structure of these proteins.30,31,34,35–37 This can lead to cross-reactions due to the high similarity degree between protein primary sequences of other coronaviruses, which would impair detection. Moreover, most antigenic determinants recognized by B cells and antibodies are conformational epitopes because the regions of interaction between antigen and antibody involve amino acids that are not continuous in the primary sequence of protein but assemble in the protein when it folded, mimicking the epitope region as in the structural conformation of the protein.38 They are useful as antigen coatings in ELISA. Our work describes for the first-time conformational epitopes from SARS-CoV-2 N and S proteins; after predicting epitope regions, we proposed peptides that mimic this region and validated these peptide in vitro assays as ELISA serodiagnosis to COVID-19 and then propose a conjugation of these peptides with superparamagnetic nanoparticles (Scheme 1). Therefore, in this work, we developed a peptide-based ELISA as well as a nanomagnetic peptide-based ELISA against SARS-CoV-2. Our findings show the critical importance of computational identification from the S and N protein conformational epitope as an immune diagnosis coating against SARS-CoV-2.
2. EXPERIMENTAL SECTION

2.1. Bioinformatics Analysis. 2.1.1. Sequence Alignment. The primary sequences of proteins S (YP_009724390.1) and N (YP_009724397.2) from SARS-CoV-2 (NC_045512.2) were evaluated by the BLAST-p program and aligned with other primary sequences of structural S and N proteins from different SARS-CoV-2 strains, and also other CoVs, such as SARS-CoV, by the Clustal Omega server.

2.1.2. Specific B Cell Epitope Prediction. 2.1.2.1. Linear Epitope Prediction. S and N protein epitopes were predicted using a combination of two algorithms: (i) Emini surface accessibility scale from B Cell Epitope Prediction from Immune Epitope Database (IEDB), with the following parameters: window size = 16 and threshold = 1.0; and (ii) ABCpred Server, using the following parameters: threshold = 0.80, window length = 14 for N protein and 16 for S protein. The results of IEDB and ABCpred analysis were combined, and the outcomes were used to design the conformational epitopes in three-dimensional (3D) structures.

2.1.2.2. Conformational Epitope Prediction. Structural data of epitopes regions on SARS-CoV-2 S protein were designed on 3D structure Protein Data Bank (PDB): 6X8R. The 3D structure of SARS-CoV-2 N protein was modeled in SWISS-MODEL server. The conformational epitopes contain a combination of amino acids from different protein regions selected according to specific B cell linear epitope prediction using SwissPDBviewer.

2.2. Peptide Synthesis. Peptide sequences were manually synthesized by Solid Phase Peptide Synthesis using Fmoc chemistry, as described in ref 45, with some adaptations. In this technique, the N-terminus of each amino acid was protected by 9-fluorenylmethoxycarbonyl (Fmoc). Moreover, an additional protecting group on the side chains was applied to avoid side reactions. A 6 mL syringe containing 0.001 M hydrophobic polystyrene resin (200 μm) was used throughout the synthesis steps. The C-terminal amino acid residue is anchored in an insoluble solid support via its carboxylic acid group to a rigid amide resin. The Fmoc group is removed by 25% 4-methylpiperidine in dimethylformamide (DMF) in quick washes, intercalating methanol and DMF were made, followed by 30 min. Then, four deprotection cycles of 5 min incubation followed by an additional overnight freezing step, and the supernatant was subsequently discarded. Following that, the peptide was washed with cold ethyl ether and overnight freezing (−20 °C) was recovered by centrifugation. The first step of centrifugation (10 000 g, for 10 min at 4 °C) was performed after the overnight freezing step, and the supernatant was subsequently discarded. Following that, the peptide was washed with cold ethyl ether, reentrifuged (10 000 g, for 10 min at 4 °C), and lyophilized after the supernatant removal. The synthetic peptides were analyzed by a mass spectrometer coupled to a high-performance liquid chromatography (HPLC) system. We performed peptide characterization to mass confirmation. The peaks from chromatographic separation were submitted to matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analyses. The characterization with MS analysis was performed using a mass spectrometer MALDI-TOF-TOF AutoFlex III (Bruker Daltonics) instrument, calibrated with Peptide Calibration Standard II (Bruker Daltonics), and α-cyano-4-hydroxycinnamic acid was used as the matrix. The FlexControl software controls the MS in positive/relector mode.

2.3. Ethical Approvals. 2.3.1. COVID-19 Patients. Sera samples (n = 25) from patients admitted to São José Hospital, Criciúma/SC, Brazil, who tested RTq-PCR-positive for SARS-CoV-2 and volunteered to participate in this study, were recruited from November 2020 to March 2021. Written consent was obtained from all patients. This study was carried out in accordance with the Declaration of Helsinki, in accordance with the ethical principles for research involving humans, and was approved by the Human Research Ethics Committee of São José Hospital, protocol number CAP 313846.0.1001.5364.

2.3.2. Negative Control Samples. The serum specimens (n = 18) of healthy individuals were selected from Universidade do Extremo Sul Catarinense bank, Criciúma/SC, Brazil. Those serum samples were collected before the 2020 outbreak of COVID-19 pandemic and were used as the negative control.

2.4. Peptide-Based ELISA. ELISA plates (Costar 3590, Corning) were coated with 100 μL/well of peptides (0.05 μg/μL) in coating buffer (sodium carbonate buffer, pH 9.6), overnight at −4 °C. Next, the plates were washed twice with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) and blocked with 200 μL/well of 5% bovine serum albumin protein (BSA) in PBS-T for 1 h at 37 °C. After the incubation period, the wells were washed three times and the COVID-19-positive or negative serum samples, diluted 1:100 in a solution of 0.5% BSA in PBS-T, were added to each well (100 μL/well) and incubated at 37 °C for 1 h. The wells were then washed four times with PBS-T and incubated with 100 μL/well of anti-human polyclonal IgA, IgM, and IgG conjugated with peroxidase (Sigma-Aldrich), diluted 1:5000 in 0.5% BSA in PBS-T at 37 °C for 1 h. After washing four times with PBS-T, colorimetric detection was made by p-phenylenediamine solution (0.2 μg/μL in citrate buffer, pH 5.2) with 0.05% hydrogen peroxide (100 μL/well). The reaction was incubated for 20 min in the dark at room temperature and stopped with 2 M sulfuric acid. The optical density at 492 nm (OD492) was measured by a SARS-CoV-2 total antigen (DEVAX, Brazil) was used as a control to coat the ELISA plate with 100 μL/well (0.05 μg/μL) in the coating buffer.

2.5. Synthesis of Superparamagnetic Nanoparticles (SPMNPs). SPMNPs were synthesized by the co-precipitation method, as previously described by Feuser and collaborators. Iron(III) chloride hexahydrate (FeCl3·6H2O) and ferrous sulfate (FeSO4·7H2O) were dissolved in a Baker (molar ratio of 1:2) containing distilled water under mechanical stirring at 900 rpm. Ammonium hydroxide (99%) solution (11 mL) was then rapidly added to the solution, which was maintained at agitation for 1 h. The produced SPMNPs were separated using a neodymium magnet and washed three times with deionized water to remove any impurity.

2.6. Peptide–SPMN Conjugation. SPMNPs were conjugated with different peptides. All peptides used for conjugation with SPMNPs (p2pS, p2pN, and p3pN) were anchored with cysteine at the ends. Therefore, SPMNPs were also conjugated with cysteine for further characterization of possible interaction between cysteine and SPMNPs. For conjugation, free cysteine (0.05 μg/μL) and peptides (0.1 μg/μL) solution were prepared. Subsequently, the solutions were added drop by drop in a coated beaker SPMNPs (320 μg/mL) and the mixture was sonicated for 2 h using an ultrasonic bath. Subsequently, cysteine-SPMNPs were exposed to neodymium magnetic and washed with PBS to remove free cysteine.

2.7. Characterization. SPMNPs were characterized by transmission electron microscopy (TEM, JEM-1011 TEM, 100 kV) and dynamic light scattering (DLS) (Nanosizer, Malvern Instruments), and the surface charge was determined by ζ potential measurements (Zetasizer, Malvern Instruments). For the DLS and ζ potential analyses, the samples were diluted in distilled water and analyzed in triplicate at 25 °C (pH ~ 7). A vibration sample magnetometer (VSM) was used to measure the hysteresis loops of the obtained SPMNPs. The peptides (0.1 μg/μL) were individually conjugated.
with SPMNs (320 μg/mL), drop by drop, in an ultrasonic bath for 2 h.

2.8. Nanomagnetic Peptide-Based ELISA. Conventional 96-well plates (KASVI) were coated with 100 μL/well of peptide–SPMNPs conjugated through magnetic precipitation by neodymium magnet (8 × 1.5 mm^2) applied to each well for 5 min (it was applied in all washing steps). The following steps were performed as described in ref 20 with some adaptations. Serum samples diluted 1:100 in 0.5% BSA in PBS-T were added to the wells (100 μL/well) and incubated for 1 h at 37 °C. Next, the wells were washed three times with PBS-T and incubated with 100 μL/well of anti-human polyvalent IgA, IgM, and IgG conjugated with peroxidase (Sigma-Aldrich), diluted 1:5000 in 0.5% BSA in PBS-T, for 1 h at 37 °C. After washing three times with PBS-T, the colorimetric detection was performed with 100 μL/well of o-phenylenediamine solution (0.2 mg/mL in citrate buffer, pH 5.2) with 0.05% hydrogen peroxide and incubated for 20 min in the dark at room temperature. The reaction was stopped with 2 M sulfuric acid, and the OD492 was determined by a SpectraMax M3 (Molecular Devices, San Jose, CA) plate spectrophotometer.

2.9. Statistical Analyses. Data were analyzed using GraphPad Prism software, version 8.0.1. A p-value less than 0.05 was considered statistically significant. To differentiate negative samples from positive samples against peptides tested as antigens to detect SARS-CoV-2, a cutoff value was determined considering the mean of negative control samples (healthy patients) plus 3 standard deviation (SD). Receiver operator curves (ROC) were determined, and the area under the curve (AUC) was calculated individually to determine sensitivity (Se), specificity (Sp), and accuracy of each peptide, confidence intervals (CI) using a 95% confidence level (95% CI), and to analyze the

Table 1. Conformational Peptides Sequences According to the S Protein (YP_009724390.1), Amino Acid Position, and Physicochemical Properties of Synthetic Peptides

| peptide | peptide sequence in S protein position | isotropic mass | isoelectric point (pH) | water solubility |
|---------|---------------------------------------|----------------|-----------------------|-----------------|
| p1pS   | V134Y144Y145H146K147N148K150W152G181K182N185G184N189F186K187N211L212V213R214D215L216P217W64H66V62H69 | 3250.72 | 10.00 | poor |
| p2pS   | Y473A475G476S477T478P479N481G482V483E484F490L492Q493L455Y421D420K424 | 1902.09 | 4.37 | poor |
| p3pS   | E773D775K776Q779E780Q784K786Q787I788Y789K790P792P807P809S810K811P812S813K814 | 2255.56 | 9.31 | good |

Figure 1. Conformational epitopes on SARS-CoV-2 S protein 3D structure (PDB: 6XR8A). p1pS is denoted as yellow, p2pS as green, and p3pS as red.

Figure 2. Conformational epitopes from SARS-CoV-2 N protein on 3D structure models of NTD and CTD. p1pN is denoted as pink, and p2pN as blue in the N-NTD 3D model. p3pN is highlighted in purple in the N-CTD 3D model.
3. RESULTS

3.1. Conformational Epitope of SARS-CoV-2 S and N Proteins. Following the analysis of primary sequences of S (YP_009724390.1) and N (YP_009724397.2) proteins from SARS-CoV-2 (NC_045512.2) by the BLAST-p program, the epitopes were predicted by two different algorithms, from IEDB and ABCpred servers. The results were combined and used to design the conformational epitopes in the 3D structures.

The conformational epitopes of SARS-CoV-2 S protein were designed on the 3D structure (PDB: 6XR8A), as shown in Figure 1. These epitopes contain a combination of amino acids from different protein regions. Table 1 shows the amino acid sequences obtained for three peptides that mimic the conformational epitopes from S protein and their physicochemical properties.

| peptide | peptide sequence in S protein position | isotropic mass | isoelectric point (pH) | water solubility |
|---------|---------------------------------------|---------------|------------------------|-----------------|
| p1pS    | G170K169L167T165G164Q163P168L161Q160V158I146A155A149N154N153N150T148H145D144P142T141N140L139G137E136 | 2602.84       | 5.32                   | poor            |
| p2pS    | P59S62L61D60K59M58K57G56D55G54R52I51R50R49T48                    | 1730.02       | 10.91                  | good            |
| p3pS    | K248K249S250A251A252E253A254S255K256K257P258R259Q260K261R262T263A264 | 2297.69       | 11.76                  | good            |

Figure 3. Serological reactivity using synthetic peptides as antigen for serodiagnosis of COVID-19. Antigens (peptides) from S protein (A) and N protein (B). Polyvalent conjugated was diluted 1:5000 in 0.5% BSA in PBS-T. Orange dots represent the optical density values for each positive sample, and blue dots represent the optical density values for each negative sample. These values were used to determine receiver operator curves (ROC). The cutoff values (dotted lines) for positive (COVID-19 sera (n = 25)) and negative (healthy donors (n = 18)) samples were determined by calculating ROC.

3.2. SARS-CoV-2 Proteins Epitopes as Tools for COVID-19 ELISA Diagnosis. To determine the specific antibody detection against epitopes from SARS-CoV-2 structural proteins, serum specimens (n = 25) of COVID-19 patients were analyzed and compared to serum specimens of healthy donors, considered as negative control (n = 18). All peptides were analyzed individually for IgM, IgA, and IgG detection by peptide-based ELISA (Figure 3). Of the three S protein peptides, only p2pS was able to distinctly recognize serum samples from COVID-19-positive patients (Figure 3A). Regarding N protein, two of the three peptides, p1pN and p3pN, were capable of correctly recognizing the positive sera from COVID-19 patients (Figure 3B). The OD492 values were considered positive when greater than the OD492 mean from negative control plus 3 SD.

The predicted conformational epitope is from N different protein regions (NTD and CTD). Table 2 shows the sequences obtained for three peptides that mimic the conformational epitopes from N protein and their physicochemical properties.
diagnostic. The AUC value determines the performance of the model at distinguishing between positive and negative samples for COVID-19. A model has an appropriate measure of separability when the AUC is near to 1. The ROC for all

**Table 3. Receiver Operating Characteristic (ROC) Parameters of Polyvalent (IgA, IgM and IgG)-Specific Detection Using p1pS, p2pS, p3pS, p1pN, p2pN, or p3pN as Antigen for COVID-19 Serodiagnosis**

| antigen | AUC | P value | cutoff | Se (%) | 95% CI | Sp (%) | 95% CI |
|---------|-----|---------|--------|--------|--------|--------|--------|
| p1pS | 0.8280 | 0.0002 | <1.492 | 92.31 | 75.86–98.63 | 61.11 | 38.62–79.69 |
| p2pS | 1.0 | <0.0001 | <0.526 | 100.0 | 86.68–100.0 | 100.0 | 82.41–100.0 |
| p3pS | 0.8926 | <0.0001 | <0.756 | 80.00 | 60.87–91.14 | 73.78 | 51.21–88.19 |
| p1pN | 1.0 | <0.0001 | <0.354 | 100.0 | 85.69–100.0 | 100.0 | 82.41–100.0 |
| p2pN | 0.9533 | <0.0001 | <0.853 | 84.00 | 65.35–93.60 | 88.89 | 67.20–98.03 |
| p3pN | 1.0 | <0.0001 | <1.342 | 100.0 | 86.68–100.0 | 100.0 | 82.41–100.0 |

*Sera samples of patients with COVID-19 (n = 25), and from healthy donors (n = 18), collected before the 2020 pandemic, were used in peptide-based ELISA to determine the sensitivity (Se), specificity (Sp), confidence Interval (95% CI), and area under the curve (AUC) by ROC analysis.

**Figure 4.** Determination of the receiver operator curves (ROC). ROC of S protein (A) and N protein (B) peptides.

**Figure 5.** (A) TEM images of cysteine-SPMNPs. (B, C) Magnetic properties of SPMNPs and cysteine-SPMNPs. (D) Magnetic separation (cysteine-SPMNPs) without and (E) with neodymium magnet.

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peptide-based ELISA are shown in Figure 4, and Table 3 shows cutoff and ROC parameters. Peptides p2pS, p1pN, and p3pN exhibited specificity and sensitivity profiles of 100% (Figure 4 and Table 3) and were able to correctly detect and differentiate positive and negative samples. The p1pS has a sensitivity level of >90% but not enough capacity to correctly detect positive samples without potential cross-reaction, due to the specificity level around 60% (Table 3). On the other hand, both p3pS and p2pN have AUC ≥ 0.89, level of sensitivity ≥80%, and specificity ≥70% (Figure 4 and Table 3); however, these peptides were not able to distinguish all positive samples from negative samples.

Synthetic peptide-based ELISA was performed against the human serological panel (COVID-19 patients and health individuals) for each antigen (peptide). The optical density values for all samples were used to individually determine the ROC, based on the sensitivity and specificity of each antigen in serodiagnosis.

After that, we selected the best-performing peptides (p2pS, p1pN, and p3pN) to proceed with the nanomagnetic ELISA, which consists of the use of nanoparticles as a support for ELISA, conjugating the peptides individually with the SPMNPs. To mimic the possible interactions of peptides and SPMNPs, all characterization was performed with cysteine-SPMNPs. As demonstrated in Figure 5, cysteine-SPMNPs presented a size range of 5−20 nm (Figure 5A). Cysteine-SPMNPs had a positive ζ potential of 18 mV while free SPMNPs presented a negative ζ potential of −28 mV. The positive value shown is related to the protonation of amino and carboxylic groups on the surface of MNPs.47 An increase in particles size distribution was also observed to free SPMNPs (34 ± 5 nm) and cysteine-SPMNPs (54 ± 5 nm). Modifications in ζ potential and nanoparticle size indicate successful cysteine conjugation on the surface of MNPs.48 SPMNPs and cysteine-SPMNPs presented high saturation magnetization (80 and 74 emu/g) and superparamagnetic properties (Figure 5B,C).28,49

Figure 6. Synthetic peptide−SPMNP conjugates as antigen for faster ELISA serodiagnosis of COVID-19 and its ROC. (A) Serological reactivity using synthetic peptide−SPMNP conjugates as antigen for faster ELISA serodiagnosis of COVID-19. The polyvalent conjugated was diluted 1:5000 in 0.5% BSA in PBS-T. Orange dots represent the optical density values for each positive sample, and blue dots represent the optical density values for each negative sample. Those values were used to determine ROC. The cutoff values (dotted lines) for positive (COVID-19 sera (n = 25)) and negative (healthy donor (n = 18)) samples were determined by calculating ROC. (B) Determination of ROC of S and N protein peptide−SPMNP conjugates. Nanomagnetic peptide-based ELISA was performed against the COVID-19 patients and healthy individuals for each antigen with the peptides p2pS, p1pN, and p3pN. The optical density values for all samples were used to individually determine the ROC, based on the sensitivity and specificity of each antigen in serodiagnosis.

Table 4. Parameters of ROC Analysis of Nanomagnetic Peptide-Based ELISA Polyvalent (IgA, IgM, and IgG)-Specific Detection Using p2pS, p1pN, or p3pN as Antigen for COVID-19 Serodiagnosis

| antigen | AUC       | P value | cutoff  | Se (%) | 95% CI  | Sp (%) | 95% CI   |
|---------|-----------|---------|---------|--------|---------|--------|----------|
| p2pS    | 1.000     | <0.0001 | <0.930  | 100.0  | 86.68−100.0 | 100.0  | 82.41−100.0 |
| p1pN    | 0.9256    | <0.0001 | <0.940  | 84.00  | 65.35−93.60 | 88.89  | 67.20−98.03 |
| p3pN    | 0.9844    | <0.0001 | <0.445  | 96.00  | 80.46−99.79 | 88.89  | 67.20−98.03 |

Sera samples of patients with COVID-19 (n = 25), and from healthy donors (n = 18), collected before the 2020 pandemic, were used in nanomagnetic peptide-based ELISA to determine the sensitivity (Se), specificity (Sp), confidence interval (95% CI), and area under the curve (AUC) by ROC analysis.
that the synthesized cysteine-SPMNP s can be easily separated when a neodymium magnet is applied. These data are closer to the value of saturation magnetization of bulk magnetite (90–100 emu/g), suggesting that the SPMNP s are formed mostly of magnetite.26,51 As expected, a slight reduction was observed when the cysteine was conjugated in SPMNP s; however, the cysteine-SPMNP s showed a sufficient magnetization saturation to be moved quickly by a neodymium magnet.26,48

The peptide p2pS–SPMNP conjugate exhibited specificity and sensitivity profiles of 100% (Figure 6 and Table 4) and was capable of correctly distinguishing positive from negative samples. In addition, the difference between the positive and negative samples increased compared to conventional ELISA. p1pN and p3pN presented sensitivity and specificity levels >80% and accuracy >0.9 (Table 4). However, it was insufficient to correctly distinguish all positive samples from negative samples (Figure 6).

4. DISCUSSION

The outbreak of COVID-19 is a global health threat and a fast and accurate detection method for the disease is urgently required. A sensitive and specific immune diagnosis is helpful to track SARS-CoV-2 exposure, becoming an epidemiological vigilance tool. Therefore, despite a negative molecular test result, performing serological tests is recommended in patients with suspected COVID-19. This combination balances the limitations of each method, as RT-PCR compensates the low sensitivity of the serological assay in the COVID-19 acute phase, while the serological assay compensates the low sensitivity of RT-PCR in the final phase of the disease.25,52,53

Serological tests could contribute to the detection of IgA and IgM in the infection acute phase, in addition to identifying asymptomatic and recovered patients by detecting persistent IgG.54 S and N proteins have been previously described as potential coating antigens for COVID-19 serodiagnosis.17,18 In addition, many diagnostic methods for SARS-CoV and MERS-CoV were also based on these proteins.7,8

In this study, we designed conformational epitopes from S (YP_009724390.1) and N (YP_009724397.2) proteins by bioinformatic tools. The predicted sequences were used as antigens in a peptide-based ELISA against SARS-CoV-2 for COVID-19 immune diagnosis. Conformational peptides are advantageous since they allow the combination of epitope regions and maintenance of protein region conformation, which may lead to a better interaction with specific antibodies and decrease cross-reaction.

The p2pS peptide presented the best performance among all tested sequences. p2pS was capable of differentiating the true positives from true negatives, obtaining 100% sensitivity, 100% specificity, and accuracy (AUC) 1.0. Although the p1pS and p3pS peptides were not able to correctly differentiate all positive samples from negative samples, p1pS showed a sensitivity of 92.31%, a specificity of 61.11%, and an accuracy (AUC) of 0.8280, and p3pS presented an 80% sensitivity, a 73.78% specificity, and an accuracy (AUC) of 0.8926 (Table 3 and Figures 3A and 4A). The better performance of p2pS peptide may be associated with its localization in the receptor-binding domain (RDB) (R319−F434) region of S protein (Table 1). The S RBD mediates the interaction with the host cell receptor, the angiotensin-2 converting enzyme (ACE2). This domain has been shown to be a common target for human antibodies, and it is poorly conserved between SARS-CoVs and other human coronaviruses, which makes it a promising antigen in serodiagnosis and to justify the high sensitivity and specificity observed in the tests. In samples evaluated by Piccoli et al., it was observed that the RBD was the target of 90% neutralizing antibodies, with the highest IgG detection and lower detection found in the S2 subunit.25 Furthermore, the S1 subunit, which contains the RBD, showed a more specific detection of antibodies against SARS-CoV-2 than the entire S protein.7

Most humans have antibodies against endemic human coronaviruses, mainly for four common cold-causing coronaviruses, HCoV-OC43, HCoV, NL63, and 229E, which circulate in the population. Therefore, excellent specificity is crucial to avoid false-positive results.7,17,53

Regarding the N protein, two (p1pN and p3pN) out of the three designed peptides showed encouraging results, as they were able to distinguish COVID-19-positive from -negative samples. Both p1pN and p3pN peptides showed 100% sensitivity and specificity, with 1.0 accuracy. Although p2pN peptide was not able to correctly distinguish all positive from negative samples, analysis of results showed sensitivity and specificity values greater than 80%. The p3pN peptide sequence is conserved in SARS-CoV N protein (YP_009825061.1), which can lead to cross-reactions and compromise specificity; however, further studies are needed to confirm that. Nonetheless, the use of peptides is advantageous as different antigens can detect different antibodies, which potentially overcome the limitations of false-positive or false-negative results and consequently increase the performance of serodiagnosis tests.16 Serologic assay using SARS-CoV-2 peptides was able to detect antibodies in most samples from COVID-19 convalescent patients, as shown by Ryzhov et al.36 Antibodies against S protein RBD, as well as against N protein, appear first in the course of infection, and for that reason, the three best-performing peptides, p2pS, p1pN, and p3pN, could be appropriate markers in the early serodiagnosis of COVID-19.17,58

In our results, we concomitantly detected polyvalent antibody (IgA, IgM, and IgG) in serum samples from hospitalized patients with different clinical outcomes of COVID-19, and on different days of collection from the onset of symptoms. The three peptides (p2pS, p1pN, and p3pN) were able to identify and distinguish between positive and negative sera in distinct phases of the disease, showing the potential application of these antigens for the detection of different immunoglobulins, which may indicate different stages of the infection. In a previous study using the ELISA method as serodiagnosis for COVID-19, the detection of anti-SARS-CoV-2 IgA, IgG, and IgM on late infections showed high analytical performances, with values of sensitivity and specificity higher than 90%. Furthermore, simultaneous detection of anti-SARS-CoV-2 IgM, IgG, and IgA could significantly reduce costs and time of analysis.17 The evaluation of serodiagnosis showed that IgA responses were detectable for both S and N antigens in hospitalized patients; however, IgM was detectable almost exclusively when S protein remained as an antigen.55 The detection of IgM and IgG antibodies is low in the first seven days of infection, showing a rapid increase in IgM detection, then IgG, with maximum detection on day 20, followed by a plateau of IgG from day 20 to 35 after the onset of symptoms and a gradual decline in IgM positivity.4,59 Moreover, serologic tests in which IgM and IgG detection are combined showed increased general sensitivity.
The development of an early, faster, and accurate diagnosis is critical to guide the appropriate decision-making process regarding resources and health support, or adequate quarantine for COVID-19 patients. Therefore, the peptides with the best performance in conventional peptide-based ELISA were tested by nanomagnetic peptide-based ELISA. Nanomagnetic peptide-based ELISA methodology provides a faster performance, without overnight coating and blocking steps, decreasing the total assay processing time. SPMNPs used as magnetic material presented nanometric size with superparamagnetic behavior due to low value of coercivity and remanent magnetization at room temperature (hysteresis-free).

The p2pS peptide conjugated to SPMNPs exhibited expressive performance, with an increase in antibodies detection and in the capability to distinguish between positive and negative sera (Figure 5), maintaining the promising results of 100% sensitivity and specificity and excellent accuracy, AUC 1.0 (Table 4), in a shorter time process (approximately 2.5 h) than conventional ELISA (approximately 21 h). However, p1pN and p3pN peptides, when associated with SPMNPs, did not maintain the ability to properly differentiate positive samples from negative samples (Figure 5), even so both presented sensitivity and specificity above 80% and a high accuracy, AUC > 0.9 (Table 4). In this sense, these N protein peptides need further adjustments to improve their sensitivity and specificity in nanomagnetic ELISA.

Nanotechnology application in diagnostics is advantageous, once it increases the performance of diagnostics, in addition to being an alternative to accelerate the conventional diagnostics processing time. The application of iron magnetic nanoparticles in ELISA for diagnosis of viral infections, such as H1N1 virus, showed satisfactory results in the study by Oh et al. A recombinant SARS-CoV-2 nucleocapsid protein was immobilized on magnetic beads for the detection of anti-SARS-CoV-2 antibodies. Fast performing indirect chromogenic ELISA, with approximately 11 min of runtime and an ROC (of 165 samples, indicated that this magnetic bead ELISA outperformed the conventional method. This system presented 97% sensitivity and 100% specificity. Our results could guide the development of faster and accurate serodiagnosis tests for COVID-19 and support an appropriate decision about resources and health support, or adequate quarantine and isolation period.

5. CONCLUSIONS

Bioinformatic for epitopes identification has proven to be a useful tool to guide the development of coating antigens for immunoassays for SARS-CoV-2 indirect detection. Conformational epitopes are capable of mimicking the structural protein, being useful as antigen coating in ELISA. The ELISA based on the peptides designed from S and N proteins showed high performance, resulting in three peptides as promising antigens for COVID-19 serodiagnosis. These peptides, highlighting the p2pS, showed encouraging results when tested in nanomagnetic peptide-based ELISA, which is an advantageous technique, a less time-consuming process, and provides better detection of antibodies than the conventional peptide-based ELISA. Therefore, this may be suitable as a precoated antigen for commercial purposes, which would accelerate the diagnosis process. However, more studies are needed as the small number of patient samples analyzed and the insufficient information on their clinical status limited our work.

As a perspective, we suggest the adaptation of these peptide SPMNPs integrated for rapid immunoassay diagnostics to allow the application of these antigens in point-of-care tests. The identification of asymptomatic infections and the epidemiology of the infection can play an important role in the curbing of SARS-CoV-2 spread.

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