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Development of a rapid and sensitive quantum dot nanobead-based double-antigen sandwich lateral flow immunoassay and its clinical performance for the detection of SARS-CoV-2 total antibodies

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Article Info

Keywords:
Quantum dot nanobeads
Lateral flow immunoassay
Fluorescent detection
SARS-CoV-2
Total antibodies

Abstract

Owing to the over-increasing demands in resisting and managing the coronavirus disease 2019 (COVID-19) pandemic, development of rapid, highly sensitive, accurate, and versatile tools for monitoring total antibody concentrations at the population level has been evolved as an urgent challenge on measuring the fatality rate, tracking the changes in incidence and prevalence, comprehending medical sequelae after recovery, as well as characterizing seroprevalence and vaccine coverage. To this end, herein we prepared highly luminescent quantum dot nanobeads (QBs) by embedding numerous quantum dots into polymer matrix, and then applied it as a signal-amplification label in lateral flow immunoassay (LFIA). After covalently linkage with the expressed recombinant SARS-CoV-2 spike protein (R SSP), the synthesized QBs were used to determine the total antibody levels in sera by virtue of a double-antigen sandwich immunoassay. Under the developed condition, the QB-LFIA can allow the rapid detection of SARS-CoV-2 total antibodies within 15 min with about one order of magnitude improvement in analytical sensitivity compared to conventional gold nanoparticle-based LFIA. In addition, the developed QB-LFIA performed well in clinical study in dynamic monitoring of serum antibody levels in the whole course of SARS-CoV-2 infection. In conclusion, we successfully developed a promising fluorescent immunological sensing tool for characterizing the host immune response to SARS-CoV-2 infection and confirming the acquired immunity to COVID-19 by evaluating the SRAS-CoV-2 total antibody level in the crowd.

1. Introduction

Since early December of 2019 and up to November 18, 2020, over 54 million cases of coronavirus disease 2019 (COVID-19) caused by novel coronavirus (SARS-CoV-2) infection, with over 1.9 million deaths have been reported in 223 countries [1]. The timely and accurate diagnosis of SARS-CoV-2 infections is crucial for effectively managing the infected patients and controlling the epidemic of SARS-CoV-2 in a population [2–4]. Currently, the detection of viral nucleic acid using reverse transcription-polymerase chain reaction (RT-PCR) has been widely regarded as the gold standard for confirming SARS-CoV-2 infection [5–7]. However, the accessibility and reliability of this method was largely compromised by the high test cost, the delayed feedback of test results, the need of specialized instrument, high-level biosafety laboratories and skilled technicians, as well as the high false negative rates (even up to 30 %) [8–11].

Increasing studies indicated that COVID-19 infection can also be determined indirectly by monitoring the host immune response to SARS-CoV-2 infection [12–15]. Serological diagnosis by measuring the level of specific antibodies against SARS-CoV-2 in the host is becoming another
important approach supplemental to assist COVID-19 diagnosis because the antibodies have been reported with nearly 100% positive rate within 2 weeks after symptom onset [16,17]. It has been reported that IgM could be found to be positive in the blood of patients even as early as the fourth day after symptom onset [8,18,19]. In addition, serological detection contributes not only to the better knowledge of the antibody response characteristics to SARS-CoV-2 infection, but also the extent of COVID-19 within the community and the identification of individuals who have immunity and are likely to “protect” against infection [16,20,21].

The total antibodies against SARS-CoV-2 are considered as the most sensitive and earliest serological marker compared to IgM or IgG, and has been recommended as the diagnosis standard for COVID-19 by the World Health Organization (WHO) [22]. Therefore, developing a rapid, sensitive and specific method for detecting total antibodies is capable of serving as a valuable and promising tool to improve the diagnosis of COVID-19 [23–25]. To date, a number of serologic testing strategies, including enzyme linked immunosorbent assay (ELISA) [26], lateral flow immunoassay (LFIA) [27], and chemiluminescent immunoassay (CLIA) [28] have been recently reported for the detection of total antibodies, IgM and IgG to SARS-CoV-2. Among the available immunoassays, LFIA has attracted increasing interest due to its simplicity, convenience, rapidity and low cost [29]. In particular, colloidal gold nanoparticle-based LFIA (AuNP-LFIA) for the detection of SARS-CoV-2 infection has experienced rapid development in a short period and some commercial products approved for serological assays have sprung up in various countries and regions [8,30,31]. However, the widespread use of AuNP-LFIA in aiding the COVID-19 diagnosis is still controversial because of its low sensitivity and high false negative rates [32,33].

Recent studies have suggested that the use of fluorescent materials with highly luminescent intensity as alternative LFIA label to AuNPs is beneficial to improving the analytical sensitivity [34,35]. As a novel fluorescent nanomaterial, quantum dot nanobeads (QBs) have been well demonstrated with great potential in enhancing target detection through LFIA based on their high luminescence and resistance to matrix interference [36,37]. Hence, in this work, we design and develop a QB-based LFIA (QB-LFIA) for the detection of total antibodies to SARS-CoV-2 in human serum. The QB-LFIA employs a double-antigen sandwich immunoassay format (Scheme 1), in which the expressed recombinant SARS-CoV-2 spike protein is applied to capture target antibodies at the test (T) line of the strip and conjugate with the QBs to prepare the detecting probe. After preparation, the obtained QB-LFIA was demonstrated with approximately one order of magnitude improvement in sensitivity compared to AuNP-LFIA. We further evaluated the role of QB-LFIA in monitoring the dynamic changes of antibody responses to SARS-CoV-2. In brief, this work proves that our designed QB-LFIA can serve as a promising tool for evaluating the context of humoral immune response of COVID-19-infected patients, and confirming the acquired immunity to COVID-19 by monitoring the SARS-CoV-2 total antibody level.

2. Experiment section

2.1. Materials and apparatus

Recombinant SARS-CoV-2 spike protein (RSSP) was purchased from China Sino Biological Inc. (Beijing, China) Mouse anti-digoxin (DIG) monoclonal antibody (mAb) and goat anti-mouse IgG were obtained.
from Abcam. Octadecylamine coated CdSe/ZnS QDs with the emission wavelength at 615 nm was bought from XingShuo NanoTech, Ltd. (Suzhou, China). The sample pad, conjugate pad, absorbent pad, nitrocellulose (NC) membrane, and PVC backing card were obtained from Schleicher and Schuell GmbH (Dassel, Germany). N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC-HCl), poly(methyl methacrylate) (PMMA), poly(maleic anhydride-alt-1-octadecene) (PMAO), sodium dodecyl sulfate (SDS), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Chemical (St. Louis, MO). Mu-propyl)-(3-(Dimethylamino) NHCl), poly(methyl methacrylate) (PMMA), poly(maleic anhydride-alt-1-octadecene) (PMAO), sodium dodecyl sulfate (SDS), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Chemical (St. Louis, MO).

0.01 M phosphate buffer (PB, pH 7.4) was prepared by adding NaOH (Sigma Aldrich Chemical, St. Louis, MO). The as-prepared QBs emulsion was evaporated at 70 °C for 2 h. The conjugate pad was prepared by spraying QB@RSSP (75 μg/mL) with 5 μL/cm spray volume and QB@DIG-mAb conjugate (25 μg/mL) with 3 μL/cm through the BioJet Quant3000k dispenser, followed by drying with a vacuum dryer at 37 °C for 2 h. Meanwhile, 1 mg/mL RSSP solution and 2 mg/mL mouse anti-digxogenin mAb solution were sprayed onto the NC membrane as the T line and C line with a density of 0.4 μL/cm, respectively. The as-prepared NC membrane was then stored at 37 °C for drying overnight. All of the elements were then assembled on the PVC backing card with an overlap of 2 mm each other to ensure the solution moving through the strip during test. Finally, the QB-LFIA strip preparation process for AuNP-LFIA strip was the same as that of QB-LFIA test strip except for using AuNP@RSSP and AuNP@DIG-mAb to replace QB@RSSP and QB@DIG-mAb to spray the conjugate pad. The detection results were obtained by collecting the optical densities of the strip on the test line and control line via a commercial HG-8 strip reader (Shanghai Huguo Science Instrument Co., Ltd., Shanghai, China).

2.4. Preparation of QB-LFIA test strip

The QB-LFIA test strip was fabricated according to a previous work [40]. Scheme 1 shows the construction of QB-LFIA test strip for the detection of total antibodies against SARS-CoV-2, which consists of five sections: sample pad, conjugate pad, NC membrane, absorbent pad and the PVC backing card. The sample pad was treated in 0.01 M PBS containing 1 % BSA and 0.2 % Tween-20 and dried at 60 °C for 2 h. The conjugate pad was prepared by spraying QB@RSSP (75 μg/mL) with 5 μL/cm spray volume and QB@DIG-mAb conjugate (25 μg/mL) with 3 μL/cm through the BioJet Quant3000k dispenser, followed by drying with a vacuum dryer at 37 °C for 2 h. Meanwhile, 1 mg/mL RSSP solution and 2 mg/mL mouse anti-digxogenin mAb solution were sprayed onto the NC membrane as the T line and C line with a density of 0.4 μL/cm, respectively. The as-prepared NC membrane was then stored at 37 °C for drying overnight. All of the elements were then assembled on the PVC backing card with an overlap of 2 mm each other to ensure the solution moving through the strip during test. Finally, the QB-LFIA strip preparation process for AuNP-LFIA strip was the same as that of QB-LFIA test strip except for using AuNP@RSSP and AuNP@DIG-mAb to replace QB@RSSP and QB@DIG-mAb to spray the conjugate pad. The detection results were obtained by collecting the optical densities of the strip on the test line and control line via a commercial HG-8 strip reader (Shanghai Huguo Science Instrument Co., Ltd., Shanghai, China).

2.5. Procedure of the detection of total antibodies against SARS-CoV-2 using QB-LFIA

The strip sealed in a plastic bag was opened before use. The whole blood from the patient was centrifuged at 3000 rpm for 10 min to remove red cells. The obtained serum solution was then placed at 4 °C for further use. When the refrigerated serum sample was tested, it should be brought to room temperature before testing. During testing, 40 μL of serum samples containing an unknown concentration of total antibodies against SARS-CoV-2 were pipetted into the sample well of the strip, followed by adding 80 μL of running buffer (1 × PBS, 0.01 M, pH 7.4; with 6 % BSA) to drive capillary action along the strip. After 15 min, the fluorescence intensities of T and C lines were recorded by a portable fluorescent strip reader.

2.6. Data analysis

The total antibodies against SARS-CoV-2 were tested using our test strip at the First Affiliated Hospital of Nanchang University, with 69 clinical positive and 53 clinical negative patient serum samples. All detection data was recorded and analyzed. The sensitivity and specificity of our test strip were calculated with the following equations [41]:

Sensitivity (%) = 100 × [true positive/(true positive + false negative)];
Specificity (%) = 100 × [true negative/(true negative + false positive)]

3. Results and discussion

3.1. Synthesis and characterization of QBs

Highly luminescent QBs were synthesized by an evaporation-assisted emulsion self-assembly method [42], in which a trichloromethane-based
organic phase containing octadecylamine coated CdSe/ZnS QDs, PMMA, and PMAO and aqueous phase containing SDS were used as to prepare the emulsion system. After the evaporation of trichloromethane, the 615 nm emitting QBs were obtained via centrifugation. The resultant QBs were then characterized by using TEM imaging, DLS analysis, UV–vis absorption spectra, and fluorescent spectra. TEM image in Fig. 1A shows that the synthesized QBs exhibit a uniform spherical shape of size 180 nm with a typical QD-polymer structure, wherein numerous isolated QDs are compactly embedded in the polymer matrix. DLS analysis (Fig. 1B) reveals that the average hydrodynamic diameter of the resultant QBs is about 185 nm with a polydispersity index of 0.054, suggesting an excellent monodispersity of the prepared QBs. UV–vis absorption spectra (Fig. 1C) display that the absorption peak of the QBs is the same as that of the original QDs, indicating a negligible effect of the encapsulation on the absorption spectra of QDs. Fluorescence spectra in Fig. 1D present the QBs have a maximal fluorescent emission at 618 nm, which is similar to that of the individual QD without an obvious peak shift. However, under the same particle concentration, the fluorescent intensity of QBs is about 2800-time higher than the corresponding QD, implying the huge potential of the proposed QBs with significantly enhanced emissions as a signal amplification label for improving the detection sensitivity of conventional LFIA.

### 3.2. Parameter optimization

As shown in Scheme 1, the QB-LFIA for the detection of total antibodies was developed through a double-antigen sandwich immunoassay format, wherein the recombinant SARS-CoV-2 spike protein expressed by mammalian cells was applied as the captured element sprayed onto the T line of the strip and the detected element conjugated onto the surface of QBs to prepare the QB@RSSP probe. To obtain the best target detection, several key parameters affecting the detection sensitivity of QB-LFIA, including the solution pH for the conjugation of QBs and RSSP, the saturated labeling content of RSSP on the surface of QBs, and the RSSP concentration sprayed at the T line, were systematically investigated and optimized. We first studied the effect of solution pH varied from 5.5 to 8 on the conjugation of QBs and RSSP. The result in Fig. 2A shows that the highest fluorescent intensity of 3025 ± 120 was obtained at the solution pH of 6.0, indicating that 6.0 is the optimized pH for the conjugation of RSSP with QBs. Then, we further studied the effect of the labelled amount of RSSP on the sensitivity of QB-LFIA by adding different amounts of RSSP ranging from 30 μg to 1 mg QBs, respectively. Fig. 2B shows that the fluorescence intensity at the T line obviously increases from 752 ± 31 to 2958 ± 96 with increasing the labelling amount of RSSP from 30 μg to 100 μg. However, with the further increase of RSSP amount, the fluorescence intensity gradually decreased, which is likely due to the stereo-hindrance effect from the oversaturated conjugated RSSP on the QB surface that maybe cause the blocking of binding sites against SARS-CoV-2 antibodies. Therefore, 100 μg of RSSP per mg of QBs was selected as the optimal conjugation concentration. Finally, we investigated the influence of the RSSP concentration immobilized at the T line on the detection sensitivity of QB-LFIA by spraying different concentrations of RSSP from 0.5 to 2.5 mg/mL on the NC membrane. The result in Fig. 2C displays that when the RSSP concentration is at 1.5 mg/mL, the maximum fluorescent intensity of 3524 ± 98 is achieved at the T line. Thus, 1.5 mg/mL is chosen as the optimum concentration.

### 3.3. Design and construction of QB-LFIA

To determine the baseline of the QB-LFIA method for real sample detection, we measured the cutoff value for SARS-CoV-2 positive detection, in which the cutoff value was defined as the mean of fluorescent intensity at the T line (Fl2) plus three-fold standard deviation of twenty RT-PCR confirmed clinic negative samples. Under the optimal condition, the cutoff value for QB-LFIA was calculated as 49.7 (Table S1). Therefore, the real serum sample is detected to be positive with Fl2 > 49.7; otherwise, it is considered to be negative. On the basis of the diagnostic criteria, we further investigated the advantage of our QB-LFIA over traditional AuNP-LFIA in detecting SARS-CoV-2. For direct comparison, we benchmarked the performance of QBs in LFIA against ~40 nm AuNPs with the same set of RSSP and materials. The AuNP-LFIA strip for real serum sample detection shares the same protocol as the QB-LFIA strip. Four different clinic positive serum samples...
from four RT-PCR confirmed COVID-19 patients were serially diluted using the running buffer to a series of sample solutions containing different concentrations of total antibodies, and then all obtained diluted samples were simultaneously analyzed by the proposed QB-LFIA and the AuNP-LFIA. The results in Table 1 and Fig. S1 present that the QB-LFIA method could detect the sample with a positive response with a maximum dilution of 10 folds for Sample 1, 80 folds for Sample 2 and 3, and 160 folds for Sample 4, whereas the AuNP-LFIA can only respond the original Sample 1 without dilution, a 10-fold dilution for Sample 2 and 3, and a 20-fold dilution for Sample 4. These results indicate that the developed QB-LFIA is more sensitive than the AuNP-LFIA with about one order of magnitude improvement, suggesting that our QB-LFIA method is capable of serving as a promising biosensing tool for detection the total antibodies even at lower concentrations.

### 3.4. Cross-reaction evaluation

The cross-reaction is an important indicator in avoiding the presence of false positives [43]. Thus, the cross-reaction of the QB-LFIA strip with other antibodies was further studied by testing the clinic positive serum samples containing anti-influenza A virus antibodies, anti-influenza B virus antibodies, anti-chlamydia pneumoniae antibodies, anti-mycoplasma pneumoniae antibodies, anti-respiratory syncytial virus antibodies, anti-hepatitis B surface antibodies and anti-hepatitis C surface antibodies. Each antibody was tested three times. The results in Table S2 reveal that there was no cross-reaction between our QB-LFIA strip and these antibodies.

### 3.5. Evaluation of the tolerance to real sample interference

The complex properties of real samples covering various interference factors maybe result in the negative influence on the detection accuracy

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**Table 1**

A direct comparison of QB-LFIA and AuNP-LFIA in testing a series of diluted samples from four different clinic positive serum samples collected from four RT-PCR confirmed COVID-19 patients. (For interpretation of the references to colour in this table legend, the reader is referred to the web version of this article.)

| Dilution-fold | Sample 1 | Sample 2 | Sample 3 | Sample 4 |
|---------------|----------|----------|----------|----------|
|               | AuNP-LFIA| QB-LFIA  | AuNP-LFIA| QB-LFIA  |
| 0             | +        | +        | +        | +        |
| 10            | –        | –        | –        | –        |
| 20            | –        | –        | –        | –        |
| 40            | –        | –        | –        | –        |
| 80            | –        | –        | –        | –        |
| 160           | –        | –        | –        | –        |

*Note: (+) positive test, (−) negative test. The “+” highlighted in red indicate the lowest detection limit of the two methods.*
and reliability of the QB-LFIA strip. Therefore, we systematically analyzed the tolerance of the QB-LFIA strip to real sample interference by adding a series of clinically common interfering substances into the clinic SARS-CoV-2-positive and SARS-CoV-2-negative serum samples, respectively. Each interference was tested three times. The results in Table S3 show that the QB-LFIA assays for all positive and negative samples were not affected by all these interfering substances, indicating an excellent anti-interference capacity of the established QB-LFIA strip.

3.6. Method validation

Prior to the clinical use, the reliability of the QB-LFIA strip for SARS-CoV-2 detection was further confirmed using a commercial colloidal gold test strip kit (Guangzhou Wondfo Biotech Co., Ltd, China). Twenty-four clinic positive serum samples were determined simultaneously by these two approaches. The results in Table S4 display that all samples were tested as positive by our developed QB-LFIA, which is highly consistent with the test results obtained by this commercial test kit, thus verifying that the SARS-CoV-2 detection by our developed QB-LFIA method is reliable.

3.7. Clinical performance study

Given its improved detection, low cross-reactivity, and strong anti-interference capacity, the QB-LFIA was further extended for a mandatory clinical usage efficacy evaluation by performing a set of clinical studies. A total of 122 serum samples collected from 69 clinically confirmed SARS-CoV-2-infected patients and 53 non-SARS-CoV-2-infected patients were tested for total antibodies against SARS-CoV-2. The clinical tests were performed at the First Affiliated Hospital of Nanchang University, and approved by the medical ethics committee of the First Affiliated Hospital of Nanchang University (Nanchang, China). The written informed consents were obtained from each enrolled subject before testing. The sensitivity and specificity of the QB-LFIA test strip were measured and summarized in Table 2. Of the 69 serum samples from COVID-19 patients, 67 samples were detected to be positive with 2 false negatives, providing a sensitivity of 97.1 %, whereas all serum samples from 53 non-COVID-19 patients were tested to be negative without false positives, giving a specificity of 100 %. Meanwhile, all 69 SARS-CoV-2-positive serum samples were tested using our developed AuNP-LFIA strip. The results in Table S5 reveal that among 69 positive samples, 59 samples were tested as positive, 7 samples were tested as suspected with weakly positive, and 3 samples were identified as false negative. The above results indicate the superiority of our QB-LFIA over traditional AuNP-LFIA in responding the presence of SARS-CoV-2 total antibodies in serum. In addition, Table 3 shows that the developed QB-LFIA is comparable to or even better than most of the previously reported LFIA methods.

Previous work indicated that the SARS-CoV-2-specific antibodies produced in the blood of recovered COVID-19 patients play key roles in protecting the host from secondary infection by the virus and neutralizing or treating virus infection [44,45]. Thus, it is important to detect the levels of antibodies in recovered COVID-19 patients because it is widely regarded as an indicator to evaluate the efficacy of specific antibodies for the prevention and treatment of SARS-CoV-2. Given this, we tested the levels of total antibodies in blood samples collected from 25 recovered COVID-19 patients. Of the 25 samples, 15 convalescent COVID-19 patients were tested positive for antibodies whereas another 10 patients were tested negative by the QB-LFIA strip (Table S6), suggesting the disappearance of antibodies against SARS-CoV-2 in some recovered COVID-19 patients. This result is consistent with previous reports [46]. The lack of specific antibodies to SARS-CoV-2 may be one of the possible reasons why some recovered patients are subjected to the risk for reinfection.

The dynamic changes of serum antibody levels in 12 SARS-CoV-2-infected patients at different disease stages from admission to hospital after diagnosis to discharge from hospital after recovery were monitored. Serial serum samples from these patients were collected and measured for antibody response using our proposed QB-LFIA. The changes in antibody levels were presented via recording the variation of fluorescence response signal at the T line of the strip. The results in Fig. 3 display that with disease progression, the levels of serum total antibodies exhibit an upward trend in 3 patients, show a downward trend in 5 patients, present a first increased and then decreased trend, and have no significant change in 1 patient, implying that the humoral immune response among different patients to infection with SARS-CoV-2 is variable and the difference of antibody level at the later stage was significant among different infected patients. This finding shows that the dynamic monitoring of serum antibody levels is helpful to provide important clinical information on serological testing and protective immunity during SARS-CoV-2 infection.

4. Conclusions

In this work, we develop a novel QB-LFIA for rapid and sensitive detection of SARS-CoV-2 total antibodies in serum and demonstrate its clinical application potential in improving the test effectiveness of total antibody COVID-19 compared to the widely-used AuNP-LFIA. Besides, the developed QB-LFIA strip was further extended for assessing the dynamic variation of antibody responses to SARS-CoV-2 following the course of the disease and confirming the acquired immunity to COVID-19. Collectively, this work demonstrates the feasibility of using the QB-LFIA strip as a valuable public health tool for high-sensitivity monitoring the total antibody level at the population level and understanding the context of antibody response of COVID-19 infection. Nonetheless, this work still has some limitations. Firstly, although the introduction of QBs for developing fluorescent LFIA can improve the detection sensitivity compared with traditional AuNPs, the requirement of the special fluorescent reader partially compromises the portability of LFIA. However, notably, the used amount of RSSP for QB conjugation is only half as much as that for AuNP conjugation, which results in the reduction by 30~40 % of total assay cost. Secondly, the clinic confirmation of our method was conducted via a single-center study. Therefore, the universalization and generalization of our results should be further assessed by performing large-scale clinical trials and studies in multiple medical centers. Thirdly, although the QB-LFIA has good specificity to healthy volunteers and most infected patients by non-coronaviruses-virus, the cross-reaction of this approach to other coronaviruses requires further evaluation. Fourthly, the collected real serum sample numbers are relatively limited and some of them are stored at −20 °C before testing, which may lead to the statistical difference bias in the test results. Thus, we propose to evaluate the detection performance of our QB-LFIA for on-site testing of fresh blood samples. With these improvements, we can believe that the designed QB-LFIA could serve as a promising tool to improve the current in vitro detection of SARS-CoV-2 infection, contributing to preventing the pandemic of COVID-19.

CRediT authorship contribution statement

Yaofeng Zhou: the main data curation, writing original draft, and data curation. Yuan Chen: methodology and data curation. Wenjuan...
Yuanjie Liu: methodology. Hao Fang: methodology. Xiangmin Li: synthesis and characterization of materials. Li Hou: methodology and investigation. Yuanje Liu: investigation. Weihua Lai: experimental design and instruction. Xiaolin Huang: conceptualization, methodology, and reviewing & editing paper. Yonghua Xiong: experimental design, instruction and revision.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

We are grateful for financial support from the National Natural Science Foundation of China (32001788, 31760485, 31901780), the Scientific Research Foundation of Education Department of Jiangxi Province (GJJ200221), the Interdisciplinary Innovation Fund of Natural Science Foundation of Education Department of Jiangxi Province (GJJ16-27060003-ZD01), and Jiangxi Provincial Natural Science Foundation (20202ACB215004).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.snb.2021.130139.

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