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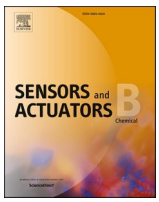

Bio-hybrid nanoarchitectonics of nanoflower-based ELISA method for the detection of *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus is one of the crucial bacterial pathogens that cause food poisoning and human infectious diseases. Therefore, the establishment of a rapid and sensitive detection method for *S. aureus* is of great significance for the diagnosis, prevention, and control of the diseases caused by this pathogen. To this end, we have established a bio-hybrid nanoarchitectonics of nanoflower-based ELISA method for the detection of *S. aureus* with high specificity and sensitivity. The core of the method is to prepare a specific cell wall binding domain (CBD)-horseradish peroxidase (HRP)-$Cu_3(PO_4)_2$ three-in-one organic-inorganic hybrid nanoflower complex through nanoarchitectonics. Detection consists of two main steps: we firstly coated an anti-*S. aureus* polyclonal antibody into the wells of a 96-well plate to specifically capture *S. aureus*, and then added customized CBD-HRP-$Cu_3(PO_4)_2$ organic-inorganic hybrid nanoflower to the system. Due to the high specificity of the CBD from lysin PlyV12 of bacteriophage ϕ1 with *S. aureus*, a stable antibody-bacterium-hybrid nanoflower complex was formed. Then, the signal amplification of HRP enzyme activity in the hybrid nanoflower was carried out by using the TMB (3,3′,5,5′-tetramethylbenzidine)-hydrogen peroxide reporting system. The method has been proved to be capable of specifically detecting *S. aureus* ranging from 10^1 to 10^6 conoly-forming unit (CFU)/mL linearly with a detection limit down to 6 CFU/mL, which is arguably one of the best for all detection methods reported to date.

1. Introduction

Staphylococcus aureus is widely present in the natural environment and can be considered a representative of Gram-positive bacterium. They are spherical in shape, arranged in clusters like grapes, and mostly form smooth and golden-yellow colonies on agar medium [1]. *S. aureus* can produce enterotoxin, hemolysin, leukocidin, coagulase, and other virulence factors under certain conditions [2], which can easily lead to food poisoning and hospital infections. In fact, *S. aureus* is a major food-borne pathogen, causing approximately 25% of food poisoning events; when food is contaminated with a certain amount of *S. aureus*, it can cause serious nausea, vomiting, abdominal pain, diarrhea, fever, and other symptoms. In severe cases, it can be fatal. *S. aureus* is also the most common pathogen in human purulent infections; it can also cause serious enteritis, pneumonia, endocarditis, and even toxic shock, sepsis, etc. [3]. Since enterotoxins are the main pathogenic factor of *S. aureus* and have high thermal stability, it is thus rather difficult to abolish its toxicity merely through heat treatment. Therefore, it poses a huge threat to food safety and public health. To improve and strengthen its detection and monitoring, it is still necessary to develop a faster, more specific, and higher sensitive methods for detecting *S. aureus*.

There have been many methods for detecting *S. aureus* to date, which can be roughly divided into the following three categories: viable bacteria counting method, nucleic acid detection method, and immunological detection method. The viable bacteria counting method based on microbial culture and colony counting is the "gold standard" of *S. aureus* detection. However, it takes a long time and cannot meet the needs of rapid on-site detection [4,5]; nucleic acid-based detection methods such as real-time quantitative polymerase chain reaction, loop-mediated isothermal amplification technology, nucleic acid probe technology and so on, have high sensitivity, but are susceptible to interference from impurities such as proteins, polysaccharides and other bacteria, and require specific equipment and experienced personnel [6,7]. Therefore, its application is subject to certain restrictions; immunoassay detection

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technologies, such as enzyme-linked immunosorbent assay (ELISA), immunofluorescence, colloidal gold immunoassay, immuno assay based on superparamagnetic nanoparticles [8,9], etc., are simple and fast, and are currently the relatively mature and general methods. Among them, the enzyme-based ELISA is the most widely used. Furthermore, it is important to select a bio-recognition element that can specifically bind with the detection target in the ELISA method [10,11]. There are currently many kinds of bio-recognition elements of *S. aureus* available, such as antibody [3,12,13], aptamer [14–16], and cell wall binding domain (CBD) of bacteriophage lysin [17–19], etc. The combination of bio-recognition elements with peroxidase such as horseradish peroxidase (HRP) can not only specifically recognize and bind to *S. aureus*, but also amplify the detection signal and improve the detection limit.

In 2012, a new type of organic-inorganic hybrid nanoflower comprising protein and Cu(II) ion was reported and attracted widespread attention [20]. Bio-hybrid nanoarchitectonics of such nanoflower quickly show unique advantages [21,22]. Studies have shown that when enzymes are used as the protein component of hybrid nanoflowers, the special structure of nanoflowers can increase the contact area between the substrate and immobilized enzyme, thereby improving the catalytic activity of the enzyme [23,24]. This novel strategy for constructing organic-inorganic hybrid nanoflowers inspired the developments of other hybrid systems. For example, by assembling HRP and $Cu_3(PO_4)_2.3H_2O$ into hybrid nanoflowers, the enzymatic activity of the encapsulated HRP is increased by 506% compared with the free enzyme, which can realize the highly efficient detection of hydrogen peroxide and phenol [25]. By incorporating α-amylase and $CaHPO_4$ into nanoflowers, α-amylase was shown to exhibit higher enzymatic activity [26]. When the *Escherichia coli* O157:H7 antibody-HRP nanoflowers was applied to the ELISA, the detection limit was found to reach 60 conoly-forming unit (CFU)/L, which is far better than the commercial ELISA [27]. Since the first report in 2012, organic-inorganic hybrid nanoflowers have been widely used in many fields such as biosensing [22,28–31], biocatalysis [25,32,33] and others [34,35].

In this study, we prepared a fused protein tdTomato-$(G_4S)_3$-CBD that contains a red fluorescent protein tdTomato, a long flexible Gly4Ser linker $(G_4S)_3$, and a CBD of the bacteriophage lysin PlyV12 derived from bacteriophage ϕ1 [36,37], which were incorporated together with HRP and $CuSO_4$ to form a three-in-one hybrid nanoflower. Due to the high specific binding ability of CBD with *S. aureus*, and the enhanced stability and activity of HRP, the bio-hybrid nanoarchitectonics of nanoflower allowed us to develop a novel ELISA method for efficient and sensitive detection of *S. aureus* (Fig. 1A). In the experiment, we first coated the wells of a 96-well plate with anti-*S. aureus* polyclonal antibody, which can specifically capture *S. aureus* with a high affinity. After washing away other substances in the sample, we then applied bovine serum albumin (BSA) to block nonspecific binding site on the well, followed by adding prepared CBD-HRP-$Cu_3(PO_4)_2$ three-in-one hybrid nanoflower to the system. Through its CBD component, this hybrid nanoflower can specifically bind to the captured *S. aureus* to form an antibody-bacterium-nanoflower complex. Finally, we used the TMB (3,3′,5,5′-tetramethylbenzidine)-hydrogen peroxide reporting system to amplify the signal to achieve the rapid and sensitive detection of *S. aureus* (Fig. 1B).

2. Material and methods

2.1. Materials and reagents

The rabbit polyclonal anti-*S. aureus* antibody (ab20920) was purchased from Abcam (Cambridge, USA). HRP and TMB chromogen solution were purchased from Beyotime Biotechnology (Shanghai, China). $CuSO_4.5H_2O$ was obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). The protein Quantification Kit BCA was purchased from Yeasen Biotech Co., Ltd (Shanghai, China). All other reagents are of analytical grade. Phosphate buffered saline (PBS) contains 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 (pH 7.4). All aqueous solutions were prepared using ultrapure water as needed. *E. coli*

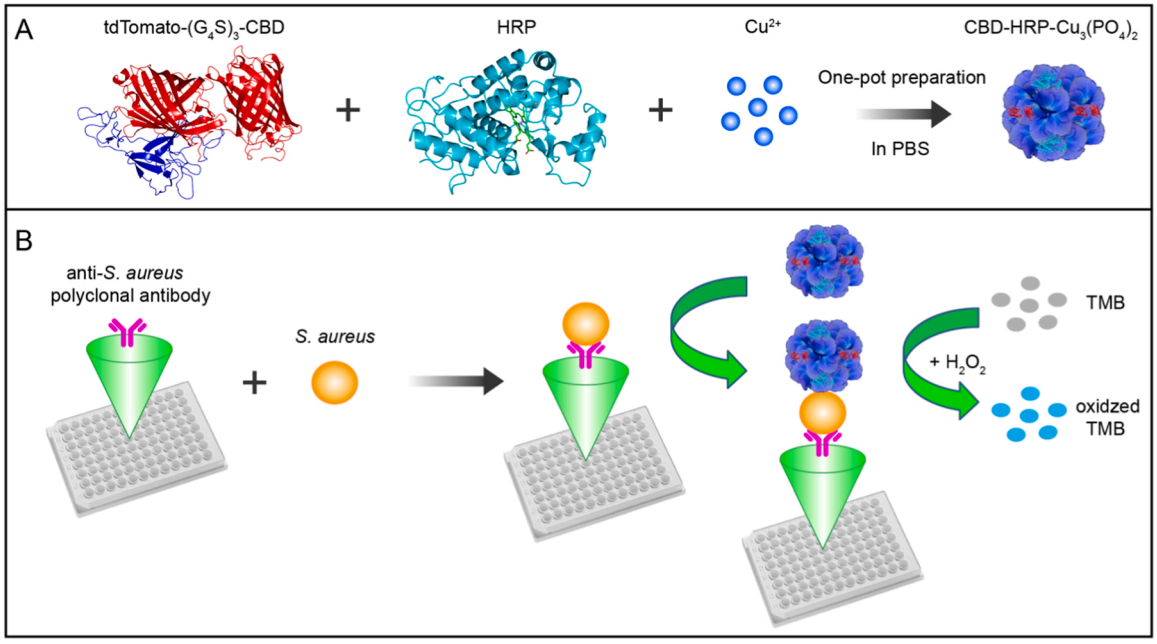

Fig. 1. Schematic diagram of *S. aureus* detection. (A) Preparation of CBD-HRP-$Cu_3(PO_4)_2$ three-in-one nanoflower. Through a one-pot preparation method, the recombinant protein tdTomato-$(G_4S)_3$-CBD, HRP, and $CuSO_4$ were co-incubated to form the hybrid nanoflower. (B) Detection of *S. aureus* by the three-in-one hybrid nanoflower-based ELISA. First, the anti-*S. aureus* polyclonal antibody was coated in the wells of a 96-well plate to specifically capture *S. aureus* in the samples, in which the hybrid nanoflower was then added to form the antibody-antigen (bacterium)-nanoflower complexes. Finally, the TMB (3,3′,5,5′-tetramethylbenzidine)-H_2O_2 reporting system was applied to amplify the signal and rapidly and sensitively detect *S. aureus*. That is, in the presence of H_2O_2, the HRP in the hybrid nanoflower can oxidize TMB to form oxidized TMB, and turning the transparent solution to blue. The intensity of the color is positively correlated with the concentration of *S. aureus* in the sample.

K12 MG1655, *Bacillus subtilis* 168 and *Pseudomonas aeruginosa* 2555 were stored in our laboratory. *S. aureus* 25923 was provided by Professor Xinmiao Fu (Fujian Normal University,). pET28a-tdTomato-$(G_4S)_3$-CBD was kindly provided by Professor Hang Yang [3] (State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences).

2.2. Protein expression and purification

The pET28a-tdTomato-$(G_4S)_3$-CBD was transformed into *E. coli* BL21 (DE3). This plasmid contains the DNA sequences for encoding red fluorescent protein tdTomato, the fused flexible linker G_4S, and the sequences for encoding the CBD domain of the lysin PlyV12 from bacteriophage ϕ1. When the OD_{600} of the bacterial culture reached around 0.5, Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to the culture at the final concentration of 0.5 mM to induce the protein expression overnight at 18 °C. Harvested cells were suspended in the binding buffer (20 mM Tris-HCl pH 8.0, 20 mM imidazole, and 0.5 M NaCl). After ultra-sonication, the proteins were purified from the supernatant using Ni-NTA affinity column. The purified proteins were dialyzed against 0.01 M PBS buffer (pH 7.4) to remove residual imidazole and then analyzed by SDS-PAGE. The protein concentrations were quantified by the BCA protein quantification kit.

2.3. Preparation of the CBD-HRP-$Cu_3(PO_4)_2$ hybird nanoflower

The CBD-HRP-$Cu_3(PO_4)_2$ hybrid nanoflower was prepared according to the work of Ge et al. with some modifications [20]: 20 μL of aqueous $CuSO_4$ solution (120 mM) was added to 3 mL of PBS (10 mM, pH 7.4) containing different concentrations of the CBD and HRP, followed by incubation at 25 °C for three days. The prepared hybrid nanoflower precipitate was collected by centrifugation (4, 000 × *g* for 3 min) and washed with ultrapure water three times. The solution was then divided into small aliquots and stored at − 20 °C.

2.4. Characterization of the CBD-HRP-$Cu_3(PO_4)_2$ hybrid nanoflower

For scanning electron microscope (SEM) observation, the prepared hybrid nanoflower suspension was filtered and dried on the membrane (pore size, 0.1 mm) and sputter coated with gold in a EM ACE200 low vacuum coater (Leica, Wetzlar, Hesse, Germany), and then characterized under a SU-8010 SEM (HITACHI, Tokyo, Japan). The components of the hybrid nanoflower were analyzed under a D8 Advance X-ray diffraction spectrometry (Bruker, Billerica, MA, USA). The 2θ scanning ranges range from 10° to 80° with a scanning speed of 0.1° S^{-1}. Confocal microscopic images were acquired with TCS SP8 confocal laser scanning microscope (Leica, Wetzlar, Hesse, Germany).

2.5. Enzymatic activity assay of the CBD-HRP-$Cu_3(PO_4)_2$ hybrid nanoflower

For the catalytic activity test, the hybrid nanoflower was added to TMB liquid substrate and react for 15 min before 2 M H_2SO_4 was added to stop the reaction, and then the absorbance was measured at 450 nm.

For thermal stability test, free HRP and the hybrid nanoflower wrapping HRP were first heated at different temperatures, and then analyzed after the addition of TMB liquid substrates. For storage stability test, free HRP and the hybrid nanoflower were stored at the same condition for a certain period of time, and then analyzed after the addition of TMB liquid substrates.

2.6. Detection of S. aureus by the CBD-HRP-$Cu_3(PO_4)_2$ hybrid nanoflower-based ELISA

We first used coating buffer to dilute anti-*S. aureus* polyclonal antibody to 8 μg/mL, took 100 μL to coat each well of the high binding affinity 96-well plate (Jet Biofil, Guangzhou, Guangdong, China), and then placed it at 4 °C overnight. After the coating is completed, the plate was washed 3 times with PBS with 0.05% Tween 20 (PBST) for 3 min each time, and then 200 μL 2% BSA was added to each well for blocking at 37 °C for 1 h. We then used 200 μL PBST to wash the plate 3 times, followed by adding 100 μL of *S. aureus* at different concentrations to each well, and further incubated the plate at 37 °C for 30 min; after washing with 200 μL PBST for 4 times and adding 100 μL CBD-HRP-$Cu_3(PO_4)_2$ nanoflower, the solution were incubated at 37 °C. After another washing with 200 μL PBST for 4 times, TMB substrate was added, and the color was developed at 37 °C for 30 min. Then, 50 μL of 2 M H_2SO_4 was added to stop the color development. Finally, a Spark multimode microplate reader (Tecan, Männedorf, Zurich, Switzerland) was used to detect the absorbance at 450 nm.

2.7. Recovery experiment

Firstly, 1×10^8 *S. aureus* was prepared. Then pure milk (Bright, Shanghai, China) was used for gradient dilution, so that the final concentrations of *S. aureus* were set at 1×10^5, 1×10^4, 1×10^3, and 1×10^2 CFU/mL. Each 100 mL of milk contained 3.2 g protein, 3.8 g fat, 4.7 g carbohydrate, 60 mg sodium, 100 mg calcium and other microelements. Afterwards, 100 μL of each sample containing *S. aureus* at the above concentrations was taken, and the absorbance at 450 nm was detected with the optimized ELISA detection system. Subsequently, the recovery rate was calculated based on the measured concentration and the added *S. aureus* concentrations.

3. Results

3.1. Characterization of the CBD-HRP-$Cu_3(PO_4)_2$ hybrid nanoflower

To verify whether the CBD-HRP-$Cu_3(PO_4)_2$ hybrid nanoflower was assembled successfully, we first used a SEM to characterize the newly prepared CBD-HRP-$Cu_3(PO_4)_2$ hybrid nanoflower. As can be seen from the SEM image shown in Fig. 2A and B, the CBD-HRP-$Cu_3(PO_4)_2$ hybrid nanoflower forms an excellent three-dimensional nanostructure with a size of about 16 μm and a hierarchical structure of petals (Fig. 2 A). It appears to consist of hundreds of nano-petals (Fig. 2B), with a shape similar to that of the mature fruit of cotton (Fig. 2A). In PBS, the proteins CBD and HRP induce the nucleation of copper phosphate crystals to form a "petal" scaffold, which acts as a "glue" to bind the "petals" together to form a bifunctional CBD-HRP-$Cu_3(PO4)_2$ hybrid nanoflower that can correlate specific binding with amplified signal.

Furthermore, we also characterized the chemical element of the prepared hybrid nanoflower; X-ray powder diffraction data analysis shows that the characteristic peaks of hybrid nanoflower are consistent with those from $Cu_3(PO_4)_2.3\,H_2O$ (Fig. 2C). Energy-dispersive X-ray spectroscopy analysis shows that the hybrid nanoflower contains Cu, O, P, S and other characteristic elements, which is consistent with the characteristic of prepared hybrid nanoflower element composition (Fig. 2D). In addition, the strong peak near 2 keV is that of impurity from Si, which comes from the silicon plate on which the sample is coated.

To detect whether CBD-HRP-$Cu_3(PO_4)_2$ hybrid nanoflower can specifically bind to *S. aureus*, we incubated the purified recombinant protein tdTomato-$(G_4S)_3$-CBD with *E. coli, P. aeruginosa, B. subtilis* and *S. aureus*, respectively, and then removed the unbound proteins by centrifugation, and observed the images with a fluorescence microscope. The results are shown in Fig. S1: There is no red fluorescence on the samples of *E. coli* (Fig. S1A), *P. aeruginosa* (Fig. S1B), and *B. subtilis* (Fig. S1C), while that of *S. aureus* has obvious red fluorescence (Fig. S1D), indicating that the fluorescent protein tdTomato labeled CBD can specifically bind to *S. aureus*, but cannot bind to other bacteria such as *E. coli, P. aeruginosa*, and *B. subtilis.*

Furthermore, we incubated the prepared CBD-HRP-$Cu_3(PO_4)_2$ hybrid nanoflower with *E. coli, P. aeruginosa, B. subtilis* and *S. aureus* at 37 °C for 30 min, followed by filtering the incubated sample with a

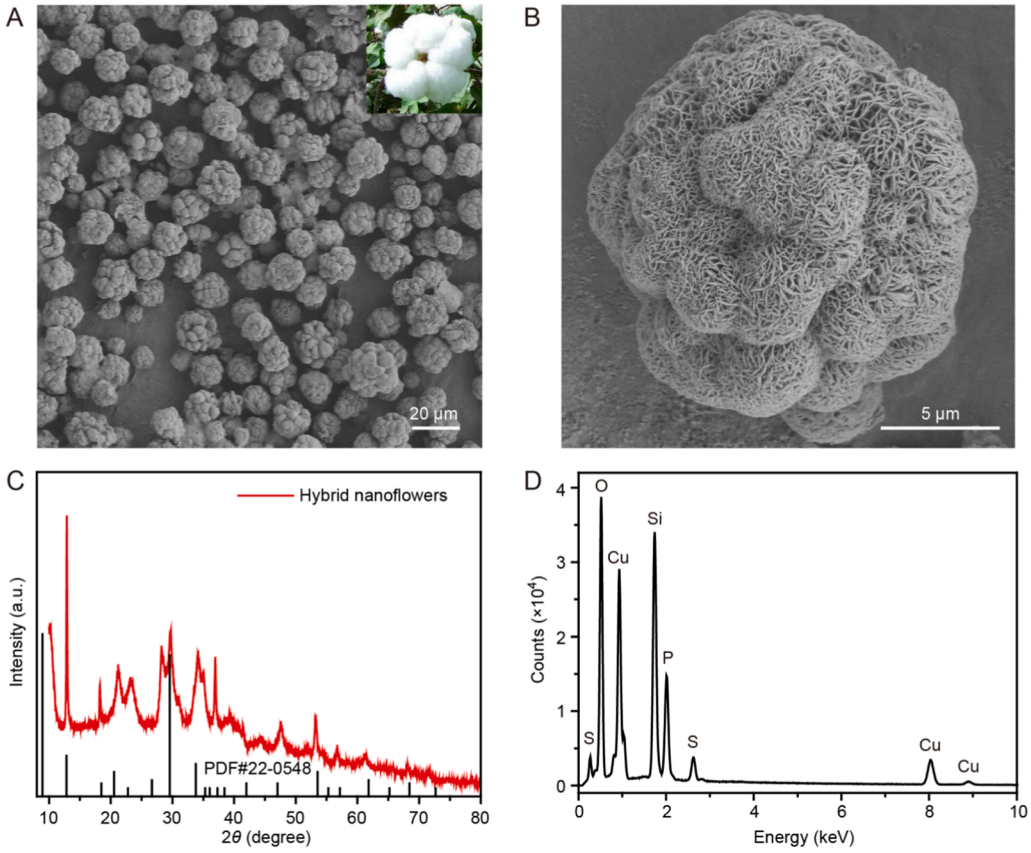

Fig. 2. Characterization of CBD-HRP-$Cu_3(PO_4)_2$ hybrid nanoflower. (A) SEM images of the CBD-HRP-$Cu_3(PO_4)_2$ hybrid nanoflower. In the upper right corner is the image of mature cotton fruit. (B) An enlarged view of the CBD-HRP-$Cu_3(PO_4)_2$ hybrid nanoflower. (C) The X-ray powder diffraction patterns of the hybrid nanoflower are consistent with those listed in the International Centre for Diffraction Data (ICDD) (PDF#22–0548). (D) Energy-dispersive X-ray spectroscopy spectrum of chemical element distribution of the hybrid nanoflower.

10 μm filter membrane to remove unbound bacteria, and then observed the results with a fluorescence microscope, as shown in Fig. S2: After the hybrid nanoflower was incubated with *E. coli* (Fig. S2A), *P. aeruginosa* (Fig. S2B), or *B. subtilis* (Fig. S2C), it seems that no bacteria can be found on the surface of the hybrid nanoflower. Whereas, some of *S. aureus* were found to be bound on the surface of the hybrid nanoflower (Fig. S2D). This shows that the hybrid nanoflower can specifically bind to *S. aureus*.

Similarly, we also used SEM to directly observe the combination of the prepared hybrid nanoflower with bacteria of *E. coli, P. aeruginosa, B. subtilis* and *S. aureus*, respectively. The SEM characterization showed

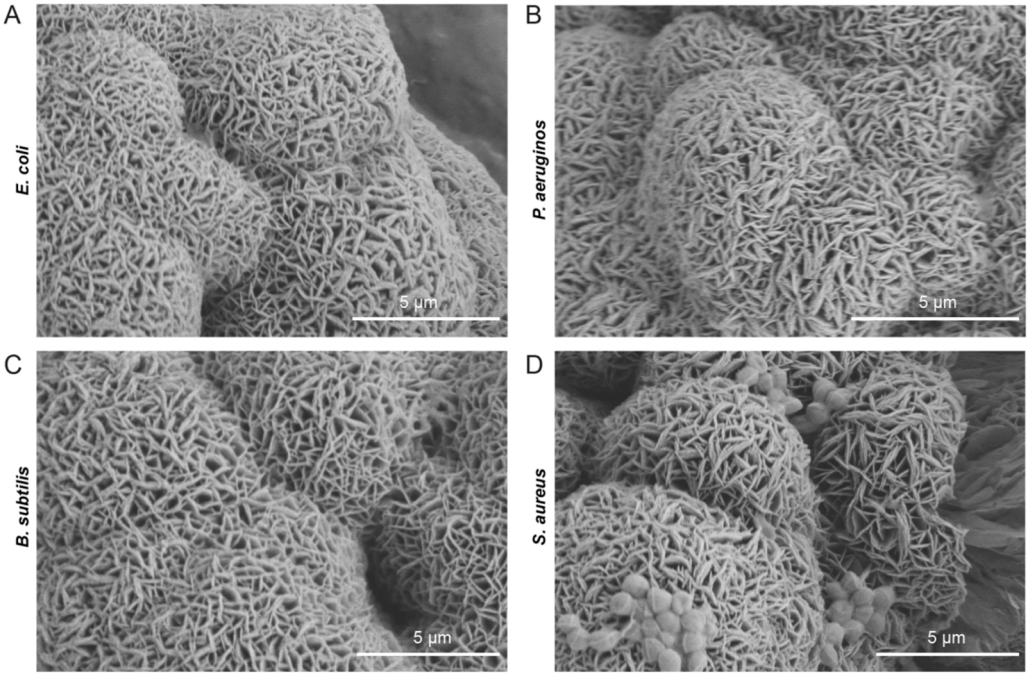

Fig. 3. SEM characterization of the adsorption of bacteria of *E. coli* (A), *P. aeruginosa* (B), *B. subtilis* (C) and *S. aureus* (D) to the CBD-HRP-$Cu_3(PO_4)_2$ hybrid nanoflower.

that when the unbound bacteria were washed off, one could not observe the adsorption of bacteria *E. coli* (Fig. 3A), *P. aeruginosa* (Fig. 3B), or *B. subtilis* (Fig. 3C) on the surface of the hybrid nanoflower. But when hybrid nanoflower and *S. aureus* were treated similarly, one could observe adsorbed *S. aureus* on the surface of the hybrid nanoflower (Fig. 3D), indicating that the hybrid nanoflower and *S. aureus* have specific binding to each other.

These results collectively indicate that we have successfully assembled a CBD-HRP-$Cu_3(PO4)_2$ hybrid nanoflower, which can specifically bind to *S. aureus*.

3.2. The stabilities of HRP in the CBD-HRP-$Cu_3(PO4)_2$ hybrid nanoflower

Generally, free enzymes have some problems such as poor heat resistance and reduced enzyme activity after long-term storage. However, after the enzyme is immobilized, its thermal and storage stabilities are generally improved to some extent. To study whether the HRP stabilities are changed after being wrapped in hybrid nanoflower, we conducted temperature-dependent and time-dependent experiments to compare the HRP activities in the hybrid nanoflower and free enzyme states. The results show that hybrid nanoflower can retain about 2/3 of the HRP activity after being placed at 80 °C for 30 min, while that of the free HRP has dropped to only about 20% of the catalytic activity (Fig. 4A). Simultaneously, the hybrid nanoflower can still retain about 80% of the enzyme activity, while free HRP only retains about 25% of the enzyme activity after 15 days of storage at room temperature (Fig. 4B). These results indicate that wrapping HRP in the hybrid nanoflower can significantly enhance the thermal and storage stabilities of the HRP enzyme.

3.3. Optimization of the detection system

Since many factors will affect the detection performance of the hybrid nanoflower-based ELISA, to improve its specificity and sensitivity for the detection of *S. aureus*, we have optimized several important influencing factors, including the total amount of encapsulated proteins, the ratio of CBD to HRP during the hybrid nanoflower preparation, as well as the antibody concentration coated in the 96-well plates, the hybrid nanoflower concentration, and the incubation time during the detection process.

We first investigated the influencing factors in the hybrid nanoflower preparation process, including the amounts of encapsulated proteins, the immobilized CBD, and the loading HRP. By comparing the effects of total protein and the ratio of CBD to HRP on the capture and detection performances of the hybrid nanoflower to *S. aureus*, we found that when the total amount of protein is 0.25 mg and the ratio of CBD to HRP is 1/10, the hybrid nanoflower has the best capture and detection efficiency toward *S. aureus* (Fig. 5A).

Next, we investigate some influencing factors in the detection process. We compared the effects of various antibody concentrations coated on the 96-well plate, the influence of hybrid nanoflower concentration, and incubation time of the hybrid nanoflower with *S. aureus* on the detection in the presence or absence of *S. aureus*.

The antibody coated on the 96-well plate was found, as expected, to play an important role in the specific capturing of *S. aureus*. Gradient experiments show that as the antibody concentration increases from 0 to 10 μg/mL, the absorbance value of the experimental group continues to increase until the antibody concentration reaches 8 μg/mL, and leveled off (Fig. 5B). This indicates that 8 μg/mL antibody is enough to saturate the binding of *S. aureus*. Therefore, we choose 8 μg/mL antibody concentration to coat the wells of 96-well plates in the subsequent experiments.

The concentration of the hybrid nanoflower added is also an important parameter. In the detection system, the hybrid nanoflower specifically bind to *S. aureus* through the CBD, and the amount of captured *S. aureus* can be correlated very well with the HRP enzymatic activity. In this test, we have used different concentrations of the hybrid nanoflower at a fixed concentration of *S. aureus* for experiments. The results showed that the absorbance value of the experimental group increased with the increase of the hybrid nanoflower concentration, while the absorbance value in the absence of hybrid nanoflower also started to increase when the hybrid nanoflower concentration is above 20 μg/mL, namely, false positive results seem to start producing (Fig. 5C). Therefore, in subsequent experiments, we restricted the hybrid nanoflower concentration to 20 μg/mL to prevent false positives.

The incubation time of the hybrid nanoflower with *S. aureus* is also closely correlated to the detection sensitivity and specificity. In the experiment, we used a certain amount of hybrid nanoflower concentration to compare the effects of different incubation times on the absorbance. The results showed that the hybrid nanoflower started to produce false positive results after incubation for 40 min. This is possibly due to the fact that the longer the incubation time, the more non-specific binding may result. Therefore, the optimal incubation time for the detection complex is set at 30 min on the safe side (Fig. 5D).

3.4. Detection of S. aureus by the hybrid nanoflower-based ELISA

Under the above optimized conditions, we evaluated the linear range, detection limit, specificity, and recovery rate of the *S. aureus* detecting method.

We first checked the linear range and the detection limit of the method. We set up a concentration gradient of *S. aureus* and determined their absorbance values by the hybrid nanoflower-based ELISA. The

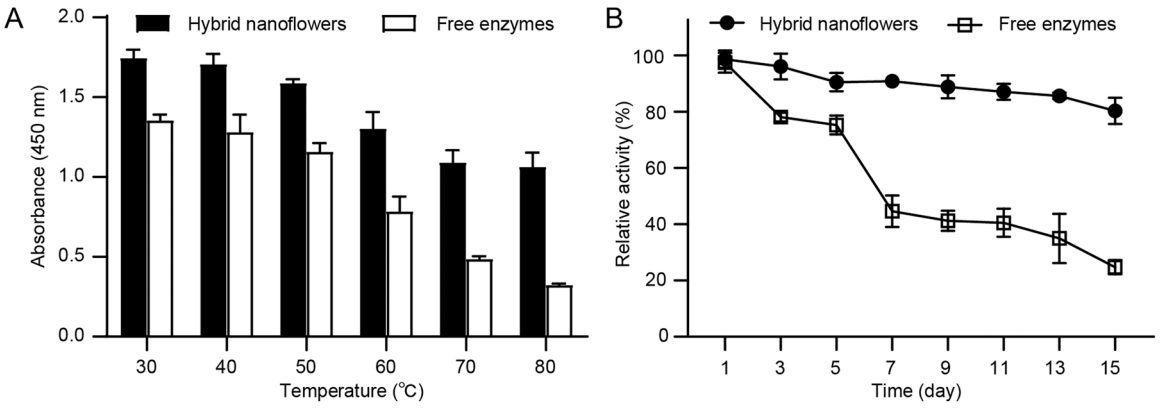

Fig. 4. Stability experiments of the CBD-HRP-$Cu_3(PO_4)_2$ hybrid nanoflower (A) Thermal stability experiment of the hybrid nanoflower. (B) Storage stability experiment of the hybrid nanoflower.

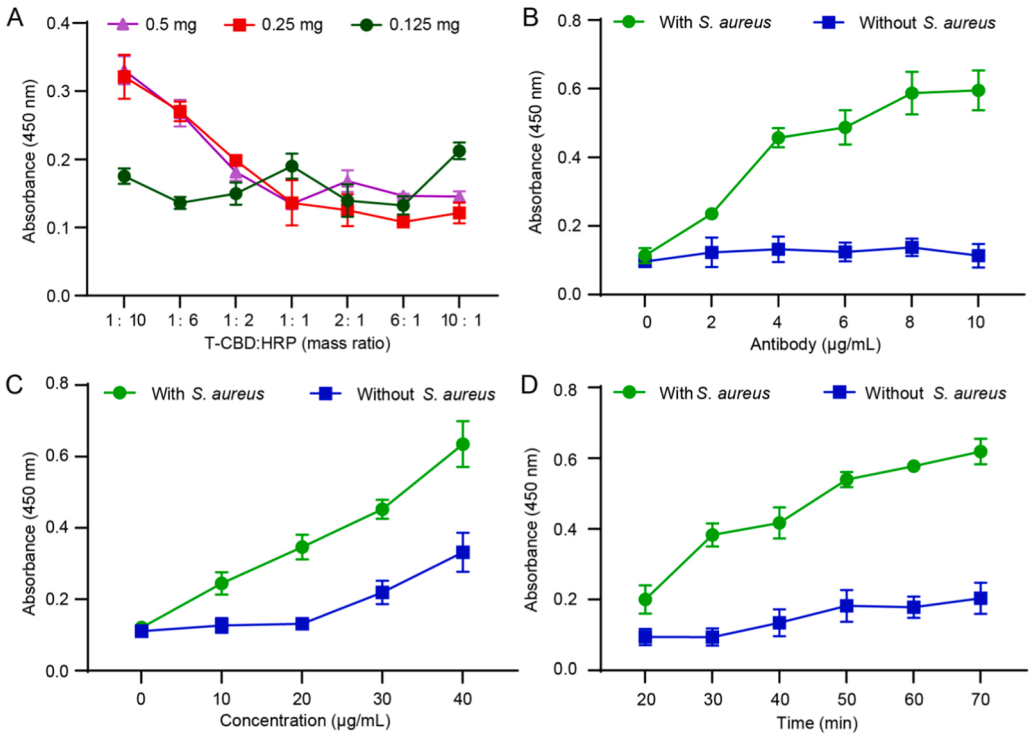

Fig. 5. Optimization of the hybrid nanoflower-based ELISA detection conditions. (A) The total amount of encapsulated protein and the ratio of CBD to HRP during the preparation process of the hybrid nanoflower. (B) The antibody concentration coated on the 96-well plates. (C) The added hybrid nanoflower concentration. (D) Optimization of incubation time.

results showed that the absorbance value increases with the increase of the concentration of *S. aureus*, and the linear response ranges from 10^1 to 10^6 CFU/mL, with a linear equation of $Y = 0.1206X + 0.1007$, $R^2 = 0.9850$ (Fig. S3A). The detection system was highly sensitive to *S. aureus*, with a minimum detection limit of 6 CFU/mL.

Detection specificity is an important indicator for evaluating a detection system. In order to evaluate the specificity of the detection system to *S. aureus*, we selected 10^5 CFU/mL of *E. coli, B. subtilis* and *P. aeruginosa* as the objects to be detected. The results show that only *S. aureus* and the mixed system containing *S. aureus* gave high absorbance values at 450 nm, while those of other bacteria remained roughly unchanged as the control group (Fig. S3B). This result shows that the hybrid nanoflower-based ELISA method shows a high specificity for the detection of *S. aureus*.

Recovery experiments were used to determine whether the detection is affected by other interference factors and to evaluate the reliability and accuracy of the detection method. In this experiment, we added different concentrations of *S. aureus* to pure milk, and then took 100 μL of each for analysis. The results showed that the recovery rates ranged between 94.4% and 97.5% (Table 1), which confirms the high accuracy of the detection method and its great potential in the practical application of detecting the samples contaminated with *S. aureus*.

Table 1
Recovery of *S. aureus* in milk with this proposed method.

S. aureus added (CFU/mL)	*S. aureus* found (CFU/mL)	Recovery (%)	RSD (%)
1×10^2	0.954×10^2	95.4	10.3
1×10^3	0.959×10^3	95.9	2.9
1×10^4	0.975×10^4	97.5	5.1
1×10^5	0.944×10^5	94.4	10.3

4. Discussion

S. aureus is one of the important pathogens that cause food poisoning and various human infectious diseases. Therefore, the establishment and development of a fast, specific, and sensitive detection method for *S. aureus* is of great significance to diagnosis, prevention, and control of the diseases caused by this pathogen. In this work, we established a new hybrid nanoflower-based ELISA method for the detection of *S. aureus*: First, *S. aureus* was specifically captured from samples by *S. aureus*-specific antibodies coated on a 96-well plate, and then the tailored-made CBD-HRP-$Cu_3(PO_4)_2$ hybrid nanoflower with high specificity binding to *S. aureus* is added to the system for the second selective binding. In this way, false positives are greatly reduced, resulting in higher detection specificity. Meanwhile, through the HRP activity of the TMB-H_2O_2 reporting system, the amount of *S. aureus* captured is correlated with the amplified signal to achieve high sensitivity detection of *S. aureus* with a detection limit down to 6 CFU/mL, which is better than most reported detection systems (Table S1). There are probably two reasons for this: 1) We used two specific bio-recognition approaches to improve the selectivity of the assay: the first was to capture *S. aureus* with an antibody coated in a 96-well plate, and the second was to capture *S. aureus* with CBD for specific binding. 2) We chose the bio-hybrid nanoarchitectonics of nanoflower method to increase the detection sensitivity. During the formation of the hybrid nanoflower, the proteins CBD and HRP induce the nucleation of copper phosphate crystals to form "petal" scaffolds, which act as "glue" to bind the "petals" together to form a bifunctional CBD-HRP-$Cu_3(PO_4)_2$ three-in-one hybrid nanoflower. Due to the three-dimensional porous structure and higher surface area-to-volume ratio of the hybrid nanoflower, it is also more favorable for enzymes to contact substrates to improve the efficiency of the catalytic reaction [23, 38].

Although the use of immunoassay to detect bacteria is simple and fast, in traditional ELISA, the antibody-based pathogen detection seems to be susceptible to antigen variation [39]. In addition, due to the existence of shared conserved epitopes in close relatives, antibodies

usually have limited specificity for capturing target bacteria. For example, 99% of *S. aureus* isolates have staphylococcal protein A (SPA) on the cell surface, which is one of the target proteins for *S. aureus* detection. IgG has strong binding ability to SPA and can be used to capture *S. aureus*. However, when the *S. aureus* captured by the IgG further binds to the secondary detection antibody, it may compete with IgG for binding to SPA, resulting in the release of captured bacteria and reduction of the sensitivity of traditional ELISA. Therefore, choosing an alternative to replace antibodies can increase the sensitivity of ELISA [3]. In addition to the antibody, the CBDs from bacteriophage lysins, bacterial autolysins, and bacterial exolysins can also specifically bind to target bacteria [40] through bacterial peptidoglycan ligands, with K_D values ranging from nM to pM, which is equivalent to or better than antibody-antigen interaction. Therefore, CBDs can be used as a new type of specific component in capturing bacteria specifically. To date, there have been many studies reported for detecting bacteria using the characteristic CBDs [41]. For examples, biolayer interferometry technology combined with phage lysin LysGH15 was used to detect *S. aureus* [42], lateral flow assay combined with engineered CBD of a phage endolysin was used to detect *B. cereus* [43], and the combination of immunomagnetic separation with enzyme linked CBD of lysin plyV12 was used to detect *S. aureus* [3].

In this experiment, we used the CBD from the bacteriophage lysin PlyV12, because this protein can specifically bind to *S. aureus* and has a binding affinity similar to that of antibody. Here, for the first time, we wrapped the capture element CBD in the hybrid nanoflower to specifically bind to a bacterium for quantitative detection. This has two advantages: It can avoid the use of second antibody and reduce the detection cost because we can just use *E. coli* to express and purify CBD. In addition to CBD of bacteriophage lysin, the CBDs of bacterial autolysins and bacterial exolysins can also be used as specific binding component to capture bacteria. Of course, in the signal amplification part, one can also try to use other enzymes except HRP, or use different signal output methods instead of colorimetric detection. In short, there is still much to explore for the combined use of new specific components.

The core of the method is to prepare a specific organic-inorganic hybrid nanoflower complex through nanoarchitectonics. The CBD-HRP-$Cu_3(PO_4)_2$ three-in-one hybrid nanoflower has the following three advantages: 1) The preparation is easier. Compared with covalent modification, this method can similarly firmly combine CBD and HRP in the hybrid nanoflower by just mixing them. 2) The bio-hybrid nanoarchitectonics of nanoflower endows the method higher detection sensitivity. The hybrid nanoflower has stronger adsorption capacity and efficient loading capacity and therefore contains a large amount of CBD, which ensures the hybrid nanoflower with a relatively high binding ability to *S. aureus*. Thus, after first capturing *S. aureus* by the use of antibody, the signal can be amplified in the presence of a large amount of HRP in the hybrid nanoflower. Simultaneously, the catalytic activity of HRP in the hybrid nanoflower is stronger than that of free enzyme. So, the detection method has higher sensitivity. 3) The hybrid nanoflower has higher thermal and storage stabilities than that general immobilized or free HRP. Therefore, these advantages maximize the detection sensitivity and provide a new method for the rapid detection of *S. aureus*.

Nanoflowers have high loading capacity and can integrate multiple functional components to form multifunctional nanoparticles. Therefore, nanoflowers can specifically bind to the target and exhibit the function of signal transduction and amplification. In short, the hybrid nanoflower-based ELISA strategy described in this paper was simple and convenient, which may replace the enzyme-labeled antibody method commonly used in traditional ELISA, and has great application potential in *S. aureus* detection in food, medical and environmental samples. Furthermore, the method was portable, and the same strategy could be developed to detect other pathogens.

Although organic-inorganic hybrid nanoflowers have satisfactory advantages in a series of applications such as biocatalysis [25,32,33] and biosensors [22,28–31], there are still some challenges and problems for future research. For example, in the preparation process of organic-inorganic hybrid nanoflowers, different kinds of metal ions may affect the activity of proteins/enzymes. Therefore, an in-depth understanding of the interaction between the inorganic phase and organic molecules will provide guidance for the preparation of organic-inorganic hybrid nanoflowers with higher biological activity. In addition, the morphology and size of organic-inorganic hybrid nanoflowers may also affect its performance in many applications. Therefore, precise control of the morphology and size of hybrid nanoflowers may be of great significance in improving its performance.

5. Conclusion

In this study, we established a hybrid nanoflower-based ELISA method for the detection of *S. aureus* with high specificity and sensitivity. The core of the method is to prepare a three-in-one hybrid nanoflower possessing the dual functions of bio-recognition and signal amplification. To improve detection selectivity, we used two specific bio-recognition elements: antibody and CBD. To the best of our knowledge, this is the first time that CBD, a capture element, is used and wrapped in a nanoflower to specifically bind to a bacterium. The combination of hybrid nanoflower and classic ELISA method seems to provide a simple, fast, and sensitive method for the detection of *S. aureus*, with a linear response ranging from 10^1 to 10^6 CFU/mL and a detection limit as low as 6 CFU/mL. Therefore, this method is superior to most detection methods reported so far.

CRediT authorship contribution statement

Wen Yin: Methodology, Data curation, Writing – original draft preparation. **Li Zhu:** Methodology, Validation, Investigation. **Hui Xu:** Methodology, Formal analysis, Data curation. **Qing Tang:** Investigation, Formal analysis. **Yingxin Ma:** Formal analysis, Writing – review & editing. **Shan-Ho Chou:** Writing - review & editing, Supervision. **Jin He:** Conceptualization, Supervision, Writing – review & editing.

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.

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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.2022.132005.

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