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The preparation of dual-functional hybrid nanoflower and its application in the ultrasensitive detection of disease-related biomarker

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ABSTRACT

In this work, dual-functional streptavidin (SA)-horseradish peroxidase (HRP) hybrid nanoflowers, integrating the functions of biological recognition and signal amplification, were prepared through facile one-pot green synthesis method. The prepared SA-HRP-$Cu_3(PO_4)_2$ hybrid nanoflowers loaded abundant HRP and simultaneously exhibited enhanced catalytic activity, stability, and durability compared with free enzyme, which fits greatly well with the requirement of signal tag for bioassay. Besides, due to the general SA-biotin linking interaction, the SA-HRP-$Cu_3(PO_4)_2$ hybrid nanoflowers possess universal capture ability to the biotinylated antibody. Hence, combined with enzyme-linked immunosorbent assay (ELISA), the dual-functional hybrid nanoflowers were used to construct a colorimetric sensor for the ultrasensitive detection of alpha-fetoprotein (AFP). The detection limit is 78 pg/mL, which is far superior to commercial ELISA kits. This presented approach holds great promise to develop on-demand hybrid system for a variety of applications ranging from biosensor and biomedicine to biocatalytic process.

1. Introduction

Colorimetric sensors, with the advantages of low-cost, simplicity and ease of measurement, have been extensively used to detect variety of targets including metal ions (Liu et al., 2011), small molecules (Liu et al., 2011; Liu and Lu, 2006), nucleic acids (Mao et al., 2016; Wu et al., 2015, 2016), proteins (Wei et al., 2007) and especially for the detection of disease-related biomarkers (Chen et al., 2015). However, those clinically important protein biomarkers are often at a very low concentration, which is a challenge for their assay. To address this challenge, vast endeavors have been attempted to improve the sensitivity of colorimetric sensors through constructing various signal amplification strategies (Xu et al., 2009; Zhao et al., 2015). Among them, enzyme-based signal amplification has been the most commonly used technique for bioassay, for instance enzyme linked immunosorbent assay (ELISA) (Ye et al., 2014). And the key is to load a large amount of enzymes and simultaneously maintain their activity.

Recently, a new type of organic-inorganic hybrid nanoflowers made of protein and $Cu_3(PO_4)_2$ have received significant investigative interests due to their facile preparation (one-step coprecipitation method) and large surface-to-volume ratio (Ge et al., 2012; Zeng and Xia, 2012). Notably, when an enzyme was used as the protein component, the nanoflowers exhibited significantly enhanced catalytic activity, stability and durability (Cui et al., 2016; Yin et al., 2015; Zhang et al., 2015). This strategy of constructing protein-inorganic nanoflowers has provided a blueprint for other hybrid system. For example, the horseradish peroxidase (HRP)-$Cu_3(PO_4)_2$ hybrid nanoflower has been employed as a colorimetric platform for visual detection of hydrogen peroxide and phenol (Lin et al., 2014), and the glucose oxidase (GOx)-$Cu_3(PO_4)_2$ hybrid nanoflower has been applied to degrade organic pollutants (Huang et al., 2015). According to recent development, multi-protein co-embedded nanoflowers have attracted much attention for the combination of different proteins to achieve comprehensive functions. GOx-HRP-$Cu_3(PO_4)_2$ hybrid nanoflowers, for example, were prepared to achieve one-step two-enzyme cascade catalytic reaction (Sun et al., 2014). Additionally, concanavalin A (Con A)-GOx-$Cu_3(PO_4)_2$ hybrid nanoflowers were successfully used for on-site detection of food pathogen (Ye et al., 2016). Based on these achievements, protein-inorganic nanoflower would be a promising tool in biomedical fields and bioanalytical process (Hua et al., 2016; Lee et al., 2015; Wei et al., 2016; Yu et al., 2015). However, the previously designed nanoflowers are limited in the material universality. Therefore, hybrid nanoflowers with improved versatility should be designed.

In this work, we prepared the streptavidin (SA) and HRP-$Cu_3(PO_4)_2$ hybrid nanoflower through facile one-pot green synthesis strategy, simply adding SA and HRP to a copper ion solution in PBS

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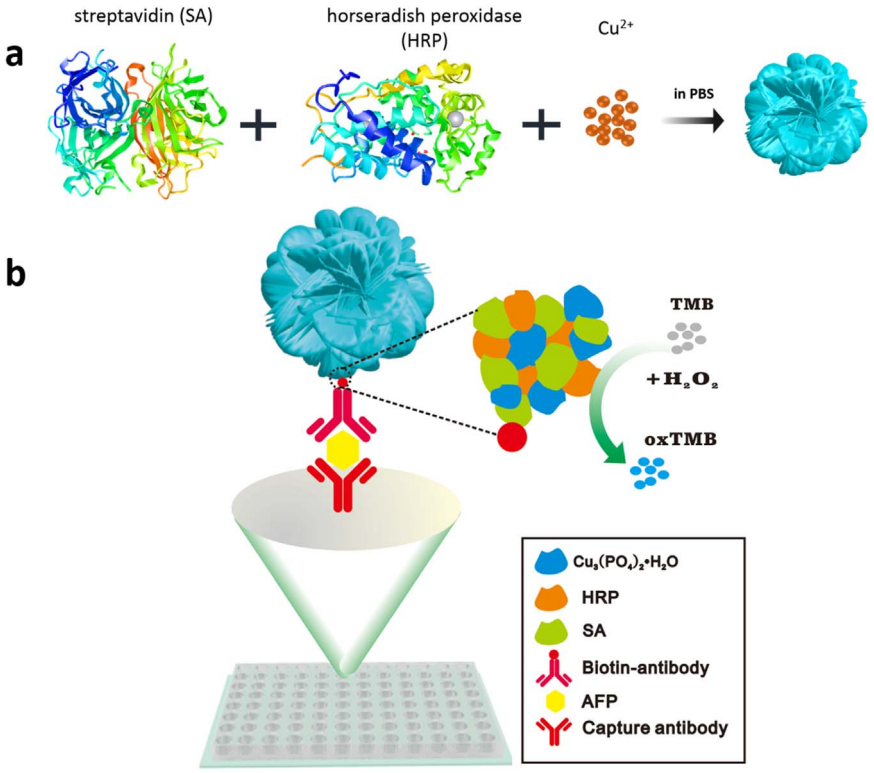

Scheme 1. (a) Synthesis process of SA-HRP hybrid nanoflowers; (b) Schematic illustration of ultrasensitive ELISA for AFP detection based on the dual-functional hybrid nanoflowesrs.

buffer for certain time at room temperature, as illustrated by Scheme 1. The flower-like structures were formed mainly for the coordination between amine group of the protein and copper ions (Zeng and Xia, 2012). It is worth noting that this synthetic approach does not involve any toxic elements, extreme harsh conditions and complex synthesis procedure. Therefore, the biological substances (SA and HRP) employed in the synthesis suffer from less manipulation compared with other conventional methods to maintain the activity of the immobilized protein. Moreover, in the SA-HRP-$Cu_3(PO_4)_2$ hybrid nanoflower, SA was selected as the recognition unit due to its high affinity to biotin, which is a classic system generally used in ELISA. Meanwhile, HRP, which serves as the signal amplification unit, can effectively catalyze the oxidation of 3, 3′, 5, 5′-tetramethylbenzidine (TMB) to a blue-colored product in the presence of H_2O_2. Therefore, the introduction of SA and HRP brings the nanoflower a dual function: the specific capture ability to biotinylated antibody, and enhanced enzymatic activity and stability for producing signal amplification. The prepared SA-HRP nanoflower was further used in ELISA to construct a simple but powerful colorimetric sensor for AFP assay.

2. Experimental

2.1. Reagents and materials

Human alpha-fetoprotein (AFP), anti-human AFP monoclonal antibody (capture Ab), biotin-labeled AFP antibody (biotin-Ab), AFP ELISA kit were purchased from Linc-Bio Science Co. Ltd. (Shanghai, China). Horseradish peroxidase (HRP), copper (II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$), 3, 3′, 5, 5′-tetramethylbenzidine (TMB) liquid substrate system for ELISA, phosphate-buffered saline (0.1 M phosphate buffer, 0.15 M sodium chloride, pH 7.4, at 25 °C), Tween 20, and potassium chloride were purchased from Sigma–Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA) was obtained from Roche (Los Angeles, CA, USA). Streptavidin (SA) was purchased from Amresco (Solon, OH, USA), and 96-well high-binding ELISA strip plates were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Human serum sample was supplied by Zhongnan Hospital of Wuhan University (Wuhan, China). All of the other reagents were of analytical grade. Ultrapure water from a Millipore water purification system (Billerica, MA, USA) was used in all of the assays. All oligonucleotide with different sequences were synthesized and HPLC purified by Sangon Biotechnology Co., Ltd. (Shanghai, China). The sequences of the oligonucleotide used in this work are listed as followed:

B-DNA-F: 5′-biotin-AAAAAA-FAM-3′

DNA-F: 5′-AAAAAA-FAM-3′

2.2. Synthesis and characterization of SA-$Cu_3(PO_4)_2$, HRP-$Cu_3(PO_4)_2$, and SA-HRP-$Cu_3(PO_4)_2$ hybrid nanoflowers

Synthesis of SA-$Cu_3(PO_4)_2$ hybrid nanoflowers (SA nanoflowers): typically, 20 μL of aqueous $CuSO_4$ solution (120 mM) was added to 3 mL of PBS (0.1 mM, pH 7.4) containing different concentrations of SA, followed by incubation at 25 °C for 18 h. The prepared nanoflower precipitate was collected through centrifugation (10,000 rmp for 5 min) and washed with ultrapure water three times.

Synthesis of HRP-$Cu_3(PO4)_2$ hybrid nanoflowers (HRP nanoflowers): typically, 20 μL of aqueous $CuSO_4$ solution (120 mM) was added to 3 mL of PBS (0.1 mM, pH7.4) containing different concentrations of SA, followed by incubation at 25 °C for 18 h. The prepared nanoflower precipitate was collected through centrifugation (10,000 rmp for 5 min) and washed with ultrapure water three times.

Synthesis of SA-HRP-$Cu_3(PO_4)_2$ hybrid nanoflowers (SA-HRP nanoflowers): typically, 20 μL of aqueous $CuSO_4$ solution (120 mM) was added to 3 mL of PBS (0.1 mM, pH 7.4) containing different concentrations of SA and HRP at a ratio of 1:10, followed by incubation at 25 °C for 18 h. The prepared nanoflower precipitate was collected through centrifugation (10,000 rmp for 5 min) and washed with ultrapure water three times.

2.2.1. . Characterization

The size and morphology of prepared nanoflowers were characterized by Scanning Electron Microscope (SEM, HITACHI S-4800),

Transmission Electron Microscope (TEM, JEM-2100F), X-ray Diffraction Spectrometry (XRD, D8 ADVANCE) with Cu Kα radiation (l ¼ 1.5418 A), and fluorescence microscopy (Kratos Ltd. XSAM-800).

2.3. SA performance test of the SA-HRP nanoflower

50 μL of 10^{-7} M B-DNA-F was incubated with SA nanoflower, HRP nanoflower, and SA-HRP nanoflower for 2 h, respectively. And 50 μL of 10^{-7} M DNA-F was incubated with SA-HRP nanoflower for 2 h. After centrifugation (10,000 rmp) for 5 min, precipitate was collected and washed with ultrapure water three times for fluorescence microscopy observation, while the supernatant was stored for Fluorescence spectrum analysis.

2.4. HRP performance test of the SA-HRP nanoflower

For the catalytic activity test, different kinds of nanoflowers were added to TMB liquid substrate. For the storage stabilities test, free HRP and SA-HRP nanoflowers were stored at the same condition for certain time, then added to TMB liquid substrates. For the thermal stability test, free HRP and hybrid nanoflower were first heated at different temperatures, then added to TMB liquid substrates. For the relative activity against cycle times test, a certain amount of nanoflowers were added to TMB liquid substrates, and then record the absorbance value. After that, the reused nanoflowers were separated from the mixture through centrifugation (10,000 rmp for 5 min) and washed with deionized water three times for next circle.

For the kinetics assays of hybrid nanoflower, the TMB-H_2O_2-HRP system was performed with 5 mM H_2O_2 and different concentration of TMB (50–500 μM). All the reactions were monitored in time scan mode at 650 nm by the UV–vis spectrophotometer. A series of initial reaction rates was calculated and applied to the double reciprocal of Michaelis-Menten equation, $1/v=(K_m/V_{max})\cdot(1/[S])+1/V_{max}$, where v is the initial velocity, [S] is the concentration of substrate, K_m is the Michaelies-Menten constant and V_{max} is the maximal reaction velocity.

2.5. Sandwich SA-HRP nanoflower-based ELISA for AFP detection

The capture antibody of human AFP (50 μL, 0.02 mg/mL) was first attached to the bottom of 96-well high-binding ELISA strip plates. 1% BSA was used as blocking solution for 2 h at 37 °C to prevent nonspecific adsorption. After washing with PBS-Tween buffer (10 mM, 150 mM NaCl, 0.1% Tween 20, pH 7.40), different concentrations of AFP (50 μL) were added to the 96-well plates and incubated for 2 h. This step was followed by rinsing with 300 μL of PBS-Tween buffer three times (all of the binding steps described below were followed by a rinsing step). Next, the Biotin-Ab (50 μL, 0.02 mg/mL) was bound to AFP through an immunoreaction for 2 h, followed by 30 min incubation with the prepared SA-HRP nanoflowers (50 μL, 0.02 mg/mL). Finally, the ready-to-use TMB (50 μL) was added and oxidized by H_2O_2 for 15 min at 37 °C. After adding 2 M H_2SO_4 to end the change of color in the microplate, the supernatant was collected and diluted to 350 μL. And then the absorbance of the diluted supernatant at 450 nm was measured by UV-2250 spectrophotometer (Shimadzu, Japan) using a 1 cm path length quartz cuvette.

3. Results and discussion

3.1. Characterization of the SA-HRP hybrid nanoflowers

Morphology of the SA-HRP hybrid nanoflower was illustrated by scanning electron microscope (SEM) and transmission electron microscope (TEM) images. As shown in Fig. 1a, most of the SA-HRP hybrid nanoflowers are uniform with an average size of 5 μm. The high-resolution SEM image of the SA-HRP nanoflower shows that they have hierarchical structures with high surface-to-volume ratios, which really seem like they are assembled from hundreds of nanopetals (Fig. 1b). Whereas, in the absence of protein, large crystals rather than nanoflowers were formed (Fig. S1). A TEM image of a single nanopetal is presented in Fig. 1c, and a high-resolution TEM image of the crystal structure of the nanopetal is shown in Fig. 1d. The X-ray powder diffraction (XRD) analysis was performed to demonstrate the inorganic component of the SA-HRP nanoflower (Fig. S2), and the X-ray

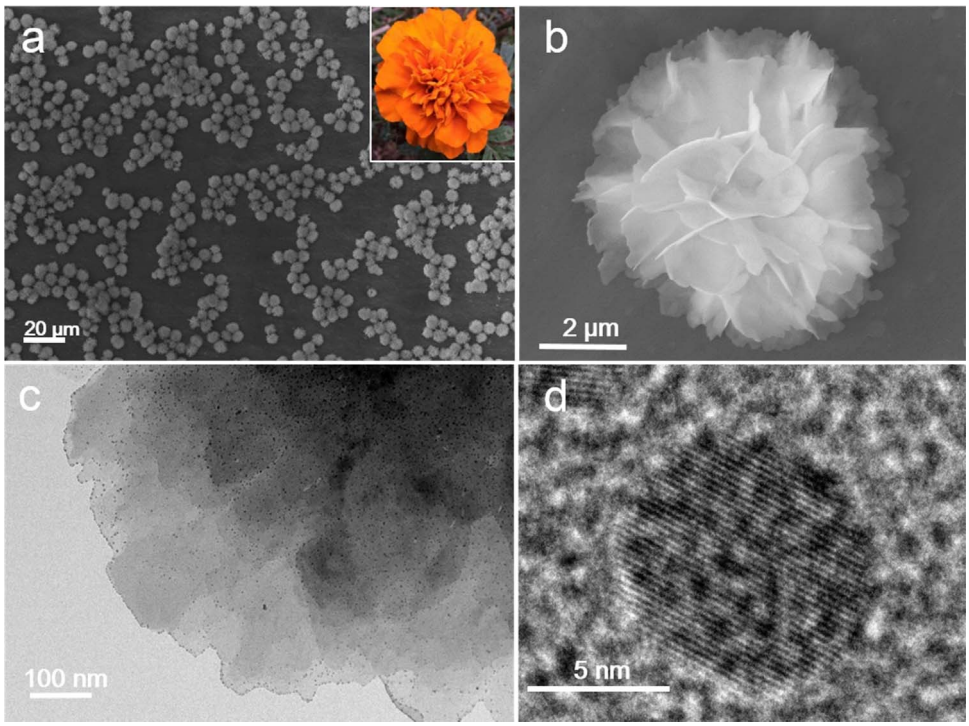

Fig. 1. (a) SEM images of SA-HRP-$Cu_3(PO_4)_2$ hybrid nanoflowers. (b) High-resolution image of SA-HRP-$Cu_3(PO_4)_2$ hybrid nanoflowers. (c) TEM image of SA-HRP-$Cu_3(PO_4)_2$ hybrid nanoflowers. (d) High-resolution TEM image of SA-HRP-$Cu_3(PO_4)_2$ hybrid nanoflowers.

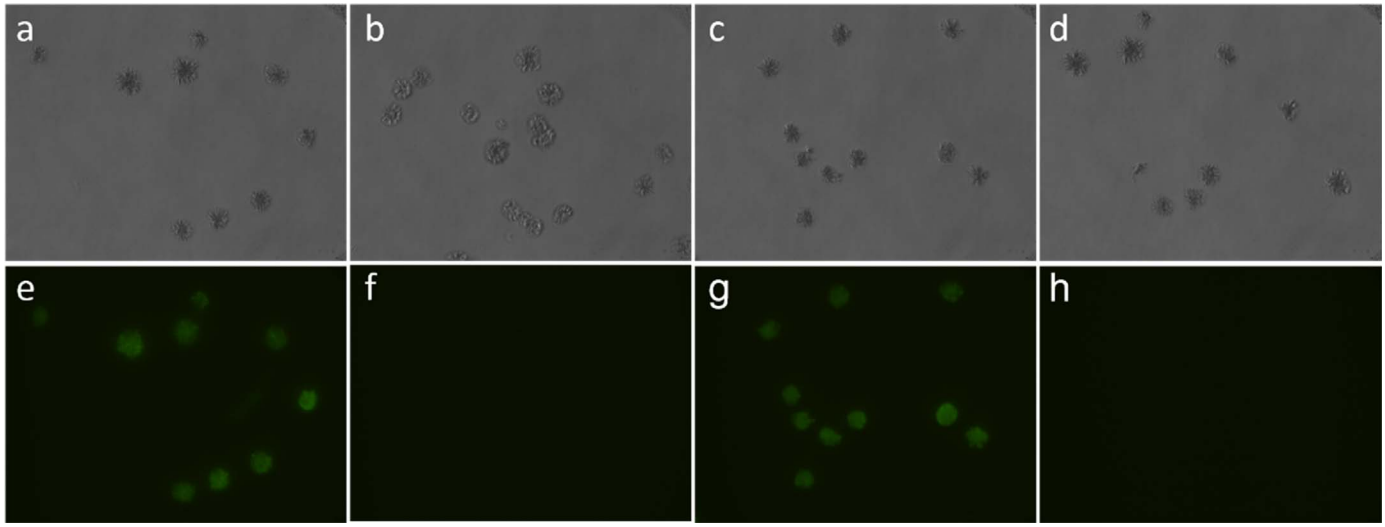

Fig. 2. Fluorescence microscopy images of different hybrid nanoflowers. (a-d) Bright-flied images of SA nanoflowers with B-DNA-F, HRP nanoflowers with B-DNA-F, SA-HRP nanoflowers with B-DNA-F, SA-HRP nanoflowers with DNA-F, respectively. (e-h) Dark-field images SA nanoflowers with B-DNA-F, HRP nanoflowers with B-DNA-F, SA-HRP nanoflowers with B-DNA-F, SA-HRP nanoflowers with DNA-F, respectively. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

diffraction pattern of the nanoflower power matched well with that of $Cu_3(PO_4)_2{\cdot}3H_2O$ according to the JCPDS card (PDF#22-0548). The energy dispersive X-ray spectroscopy (EDX) experiment revealed the distribution of five typical elements including Cu, P, C, O, and N (Fig. S3) in the SA-HRP-$Cu_3(PO_4)_2$ nanocomposite. To evaluate the weight percentage of different components in the hybrid system, thermogravimetric analysis (TGA) was shown in the Fig. S4, revealing that the weight percentage of organic component (i.e., SA and HRP) of the untreated nanoflowers was 11.45%. The immobilization efficiency, defining as the ratio of the amount of immobilized protein to the total amount of protein introduced, was determined to be 59.76%, by measuring the free protein in the supernatant using the Bradford protein assay (see Supporting Information).

3.2. Performance test of SA in the SA-HRP hybrid nanoflower

To test the performance of SA in the SA-HRP hybrid nanoflower, B-DNA-F, DNA modified with biotin at one end and FAM at the other end was incubated with different type of nanoflowers, followed by centrifugation. As shown in the Fig. 2, green fluorescent flowers can be observed with both SA nanoflower (Fig. 2a) and SA-HRP hybrid nanoflower (Fig. 2c) in the dark field, while no green fluorescence can be observed with the HRP nanoflower (Fig. 2b). Besides, DNA-F, DNA only fluorescently labeled with FAM was also incubated with SA-HRP nanoflower, and no green fluorescence can be seen after centrifugation (Fig. 2d), which demonstrated no nonspecific absorption during the course. As we can see, the SA still remained great capture ability during the immobilization. This result were also confirmed by fluorescent spectrum (Fig. S5).

3.3. Performance test of HRP in the SA-HRP hybrid nanoflower

For the enzyme-based signal amplification strategies, it is essential to load a large amount of enzymes and simultaneously maintain the enzyme activity (Ye et al., 2016). Previous studies demonstrated that the process of forming the nanoflower can accumulate abundant proteins (Wei et al., 2016). Then the performance of HRP in the SA-HRP hybrid nanoflower was carefully investigated and the result was depicted in Fig. 3, including the catalytic activity, stability, and durability. Different kinds of nanoflowers were added into the PBS buffer (pH 7.4) containing the TMB liquid substrate. As depicted in Fig. 3a, the HRP nanoflower and the SA-HRP nanoflower can catalyze the TMB liquid substrate from clear into blue at room temperature, whereas no obvious change happened in the cuvette containing HRP-free nanoflower. According to reported literatures (Yang et al., 2015; Lin et al., 2014; Huang et al., 2015), the catalytic activity of peroxidase was evaluated by using TMB as the substrate in the presence of constant concentration of H_2O_2. As shown in Fig. S6, upon adding free HRP, absorbance at 650 nm increased slowly, whereas, in hybrid nanoflower system, the absorbance platform could be achieved within 80 s, indicating the high catalytic efficiency of hybrid nanoflower. To further investigate the enzyme kinetics, by calculating from Lineweaver-Burk plots (Fig. S6), the Michaelis-Menten constant (K_m) of free HRP and hybrid nanoflower were 0.1038 and 0.0536 mM, respectively, which reflects that the hybrid nanoflower have stronger affinity for substrate in comparison with free HRP. The stability of the hybrid nanoflower was further compared with that of free HRP. The hybrid nanoflower maintained more than 80% of its catalytic activity after one month of storage in PBS at room temperature, while the free HRP only retained about 30% of its initial activity (Fig. 3b). In addition, the stability against high temperature and the durability in cycle test were further investigated. As shown in Fig. 3c, SA-HRP nanoflower showed stable performance at temperature as high as 70 °C, whereas free HRP lost a large part of its catalytic activity at the temperatures higher than 50 °C, suggesting that the immobilization of the enzymes in the nanoflower had enhanced stability. For the protein is mainly located in the core of the nanoflower (Ge et al., 2012; Zeng and Xia, 2012), in some way, the copper phosphate petals serves as protective layers to maintain the activity of HRP in the hybrid nanoflower in higher temperature (60 °C, 70 °C, 80 °C). In addition, Fig. 3d is the plot of relative activities against the number of catalytic reaction time. Clearly, the nanoflower lost only 25% of its catalytic activity over the course of six rounds of reaction, which demonstrates the durability of hybrid nanoflowers.

3.4. Performance of the SA-HRP hybrid nanoflower-based ELISA for AFP assay

Integrating recognition unit and signal amplification unit, the dual-functional SA-HRP hybrid nanoflower was further used to construct the colorimetric sensor for ultrasensitive detection of disease-related biomarker. As indicated in Scheme 1b, AFP was used as a model protein in this work. The 96-well microplate was attached with the capture Ab through physical absorption and blocked with BSA. In the

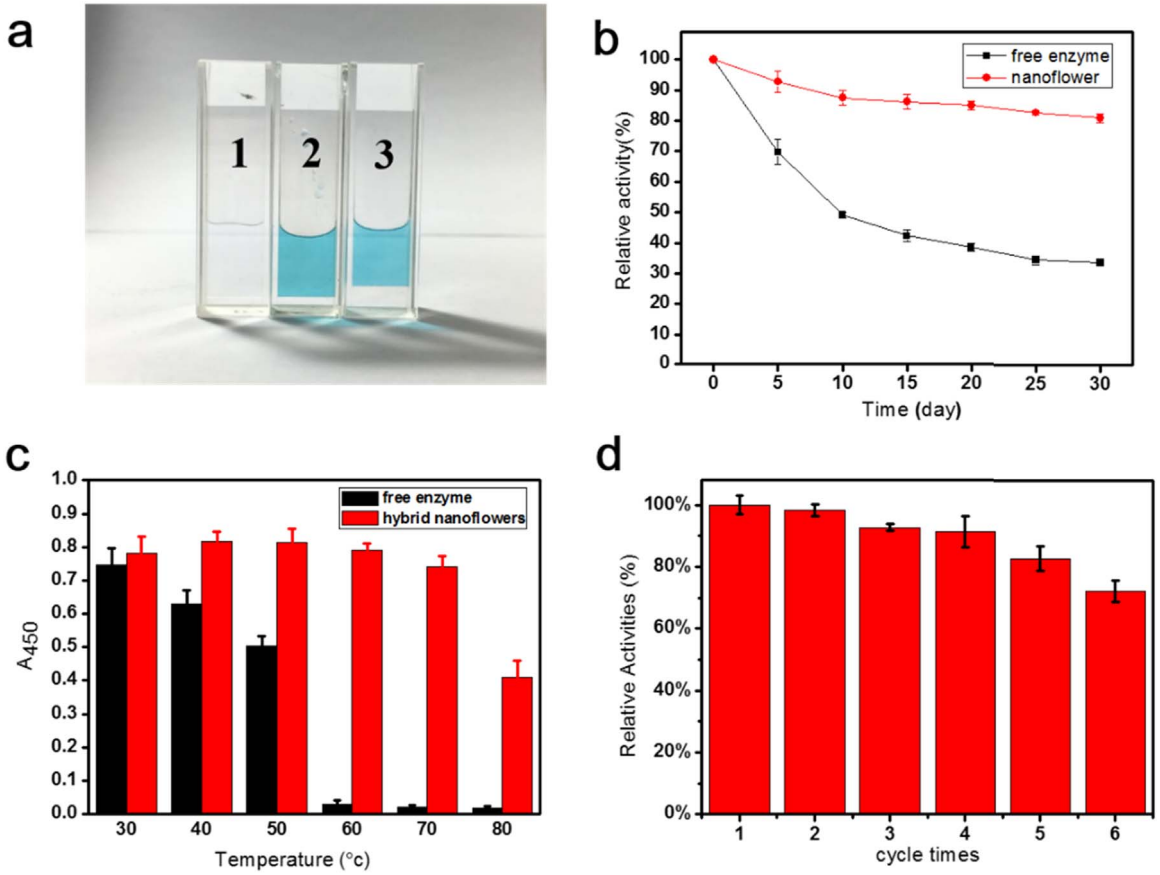

Fig. 3. (a) Peroxidase catalytic activity images of different nanoflowers (1) SA nanoflowers; (2) HRP nanoflowers; (3) SA-HRP nanoflowers. (b) Storage stabilities of free HRP and SA-HRP hybrid nanoflowers in PBS (pH 7.4) at room temperature. (c) The thermal stability of free HRP (black bar) and SA-HRP hybrid nanoflowers (red bar) at different temperatures. (d) Relative activity of nanoflowers against cycle times. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

presence of the target protein, the captured target was recognized by the biotin-Ab. Sequentially, the SA of the SA-HRP nanoflower played its recognition role and conjugated to the biotin-Ab through the biotin-SA reaction. And at the same time, the HRP of the SA-HRP hybrids, serving as the signal amplification unit, catalyzed the TMB into the oxidation product of TMB in the presence of H_2O_2, thus the color of the solution changing from clear to blue. The changed color was readily measured by a UV–vis spectrophotometer or even can be observed with naked eye. In the absence of the target, the biotin-Ab and the SA-HRP nanoflowers were washed away, resulting in a low background signal. Therefore, it is able to determine the concentration of the target protein simply by monitoring the intensities of the UV–vis absorbance or observing the change of the color with naked eye.

In the assay, the performance of the colorimetric sensor was affected by the employed nanoflower. For one hand, the recognition efficiency between the biotinylated antibody and the SA-HRP nanoflower was affected by the amount of immobilized SA in the hybrid. For the other hand, the colorimetric signal was directly determined by loading amount of HRP in the hybrid. As shown in the Fig. S7, the maximum ΔA was obtained by employing the SA-HRP hybrid nanoflower with the total amount of 0.3 mg protein at the ratio of 1:10, which was further used in the nanoflower based ELISA.

To detect AFP by this proposed method, different concentrations of AFP in PBS buffer were added. As the increase of the concentration of AFP, the color of the reaction solution gradually changed from the clear into bright blue (Inset in Fig. 4a). These results were further confirmed by UV–vis spectroscopy (Fig. 4a). An obvious increase in the absorption peak at 450 nm was clearly observed with the increase of AFP concentration. Furthermore, quantitative analysis was conducted by comparing the absorbance at 450 nm (A450) in the presence of different concentrations of AFP. A linear relationship was plotted between A450 and the AFP concentration in the range of 0.10–50 ng/mL (R=0.990) (Fig. 4b). The limit of detection (LOD) was calculated to be 78 pg/mL (the LOD was calculated according to the method described by Demchenmko, as illustrated in the ESI), which is comparable to those of reported assays (Table S1). Besides, compared with conventional commercial ELISA kits (Fig. S8), despite slightly inferior reproducibility, the developed immunosensor is more sensitive than commercial ELISA kit.

3.5. Real sample analysis

To evaluate the assay toward the target in complex biological matrix, SA-HRP hybrid nanoflower based ELISA was performed to detect AFP in human serum samples. The human serum was used in the detection without any pretreatment. The reaction exhibited an obvious color change from clear to bright blue after the addition of AFP in human serum. The UV–vis absorbance intensities still exhibited a linear to the concentration of AFP throughout the range of 0.10–50 ng/mL (Fig. 4c). To further demonstrate this method is free from the complex sample matrix effect of human serum, 1% healthy human serum was adopted as model matrix and recovery tests were conducted. The results are listed in Table 1. The recovery is between 93.0% and 109.3% and the relative standard deviation values varied from 1.1% to 6.7%. Therefore, this colorimetric method is applicable for the determination of AFP in real sample.

4. Conclusion

In summary, a green synthesis method has been developed for the

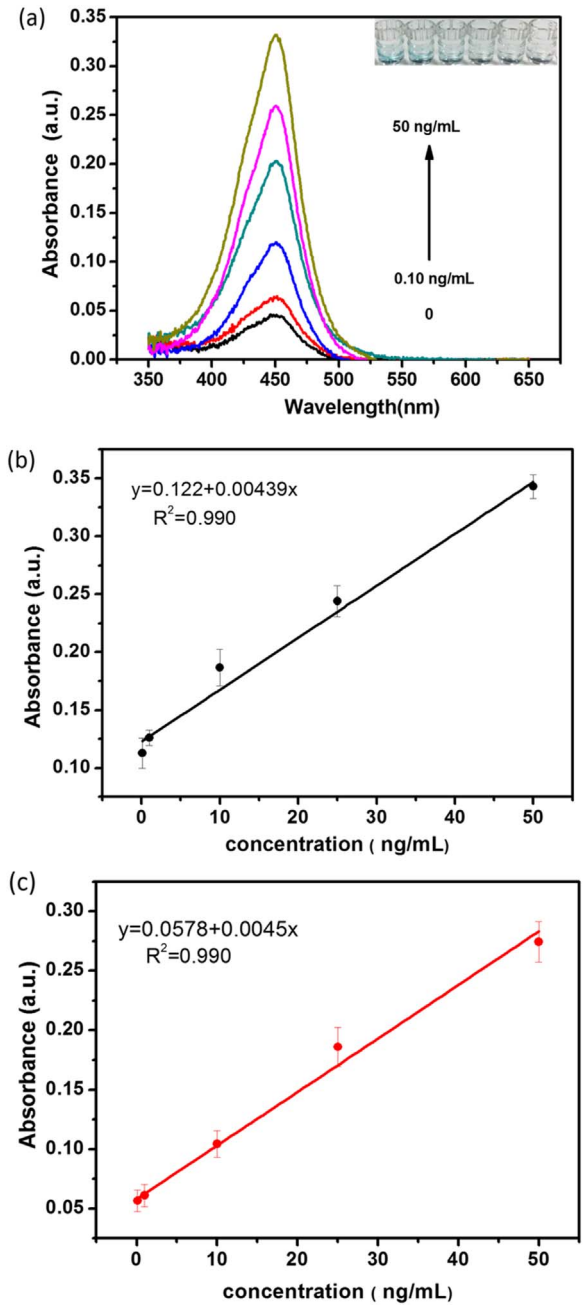

Fig. 4. (a) The UV–vis absorbance spectra at different concentrations of AFP: 0, 0.1, 1, 10, 25, 50 ng/mL. Inset: colorimetric responses of the sensing system in the presence of different concentration of AFP (without the terminal reaction of H_2SO_4). (b) The calibration curve for the determination of the AFP concentration in PBS buffer (pH 7.4). (c) The calibration curve for the determination of the AFP in human serum.

Table 1
Recovery of AFP in healthy human serum with this proposed method.

Sample number	Added (ng/mL)	Found (ng/mL)	Recovery (%)	RSD (%) (n=3)
1	1.00	0.93 ± 0.02	93.0	2.1
2	10.00	10.93 ± 0.73	109.3	6.7
3	25.00	26.71 ± 0.75	106.8	2.8
4	45.00	43.82 ± 0.47	97.3	1.1

preparation of dual-functional SA-HRP-$Cu_3(PO_4)_2$ hybrid nanoflowers integrating the functions of recognition unit and signal amplification unit. Combined the prepared dual-functional SA-HRP hybrid nanoflower with the classic ELISA method, a simple, general and highly sensitive colorimetric sensor has been constructed for AFP determination. The results showed that the SA-HRP-$Cu_3(PO_4)_2$ hybrid nanoflower-based ELISA had a sensitive performance. Furthermore, the preparation strategy of the dual-function SA-HRP-$Cu_3(PO_4)_2$ hybrid nanoflower described in this work can be extended to many other hybrid system. Besides HRP, other kind of enzymes could be employed as the signal amplification unit of the hybrid system. By varying the enzyme, different signal output would be an alternative of colorimetric detection. In addition, due to the general SA-biotin binding system, the constructed strategy has the potential to provide a wide range of approaches for medical diagnosis and biotechnology through the utilization of different recognition elements such as aptamers and peptides.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2017.02.004.

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