



Self-assembled protein-enzyme nanoflower-based fluorescent sensing for protein biomarker

Yucheng Liu¹ · Bao Wang¹ · Xinghu Ji¹ · Zhike He¹

Received: 9 July 2018 / Revised: 14 August 2018 / Accepted: 21 September 2018 / Published online: 1 October 2018
© Springer-Verlag GmbH Germany, part of Springer Nature 2018

Abstract

Multi-protein (or enzyme) conjugates play a vital role in biosensing due to the integrated function of each component, such as biological recognition and signal amplification. In this work, a green self-assembled method for the synthesis of multi-functional protein-enzyme nanoflowers has been developed, in which no chemical modification and coupling reaction is needed to fabricate the fluorescent signal probe. The self-assembled protein-enzyme conjugates streptavidin (SA)- β -galactosidase (β -Gal)-CaHPO₄ nanoflowers load sufficient enzymes without damaging their activity, which meets the requirements of signal tags for biosensing. Through integrated multi-function of biorecognition (SA) and signal amplification (β -Gal), the SA- β -Gal-CaHPO₄ hybrid nanoflower-based fluorescent sensor exhibited an ultrasensitive detection of protein biomarker alpha-fetoprotein (AFP), with limits of detection at the fM level. The presented self-assembled strategy can be extensively applied to develop on-demand protein-enzyme conjugates according to the specific requirements in a variety of applications including biosensors, bioimaging, and biomedicine.

Keywords Protein-enzyme conjugate · Self-assembled nanoflower · Signal tag · Fluorescent sensing · Protein biomarker assay

Introduction

Proteins convey a variety of functions in life activities, such as metabolism, heredity, and immunization [1]. Among these, recognition proteins as a kind of bioreporter have been extensively used in bioassay for the high specific interaction with corresponding target analytes, including the specific recognition of antibodies to antigens, the interaction between streptavidin (SA) and biotinylated biomolecules, and the high affinity of concanavalin (Con A) to surface O-antigen [2]. Besides, enzyme as a special kind of protein with excellent activity and substrate specificity has attracted intensive research interest [3]. Many classic enzymic reactions can be applied as diverse amplified signal readout. Accordingly, bioassay techniques, such as the most commonly used Western

blotting and enzyme-linked immunosorbent assay (ELISA), are largely dependent on protein-enzyme conjugates that allow a specific biorecognition of model analytes using recognition probes and then transduce and amplify the signal change of the biorecognition through signal probes [2]. Up to date, many achievements have been made to develop new method for the preparation of protein-enzyme conjugates and to explore ideal nanomaterial for the immobilization of protein-enzyme conjugates [4, 5]. For instance, PEG brushes [6], nanoporous silica [7], nanofibers [8], magnetic nanoparticle [9], and carbon nanotubes/nanowires [10] have already been applied as a new kind of protein/enzyme-embedded materials. However, tedious and complex multi-step synthetic process involved above will sacrifice the function of the protein and enzyme, causing the deactivation of the recognition protein and the activity loss of signal output enzymes. Therefore, to solve this bottleneck, a mild but reliable method should be developed to prepare the protein-enzyme conjugate without damaging the activity of each component.

Recently, an elegant but facile approach for immobilizing enzymes on the Cu₃(PO₄)₂ inorganic component with the morphology of nanoflowers was reported by Zare and his co-workers [11], and then aroused extensive interest by researchers in the field of biocatalysis [12–16], biosensors [17, 18], and bioanalytical devices [19–21]. The flower-like hybrid

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00216-018-1398-7>) contains supplementary material, which is available to authorized users.

✉ Zhike He
zhkhe@whu.edu.cn

¹ Key Laboratory of Analytical Chemistry for Biology and Medicine (Ministry of Education), College of Chemistry and Molecular Sciences, Wuhan University, Wuhan 430072, China

nanomaterials generated by this process are called “organic-inorganic hybrid nanoflower” or “hybrid nanoflower” (NF). Surprisingly, the as-prepared nanoflower exhibited enhanced activity as well as stability. Inspired by this, we present a self-assembled strategy to prepare multi-functional protein-enzyme conjugates integrating the function of the biorecognition of the recognition protein with the signal amplification of the enzyme.

In this work, β -galactosidase attracts our interest because it can specifically catalyze the fluorogenic substrate fluorescein di- β -D-galactopyranoside (FDG) into fluorescein to give the fluorescence signal [22], which can be used as an ideal signal enzyme. Further, we found that with the calcium ions, β -galactosidase (β -Gal) protein molecules can form nanocomplexes which provide templates of nanoparticle self-assembly, acquiring different morphologies as well as with great enzymatic activity. This inspiring result encourages us further to explore multi-functional protein-enzyme conjugates. After systematical study of the mechanism of the self-assembly between the metal ion and the biomolecules, the multi-protein (enzyme) system, SA- β -Gal- CaHPO_4 hybrid nanoflowers were synthesized for the first time, employing new signal enzyme and enzyme-based signal amplification.

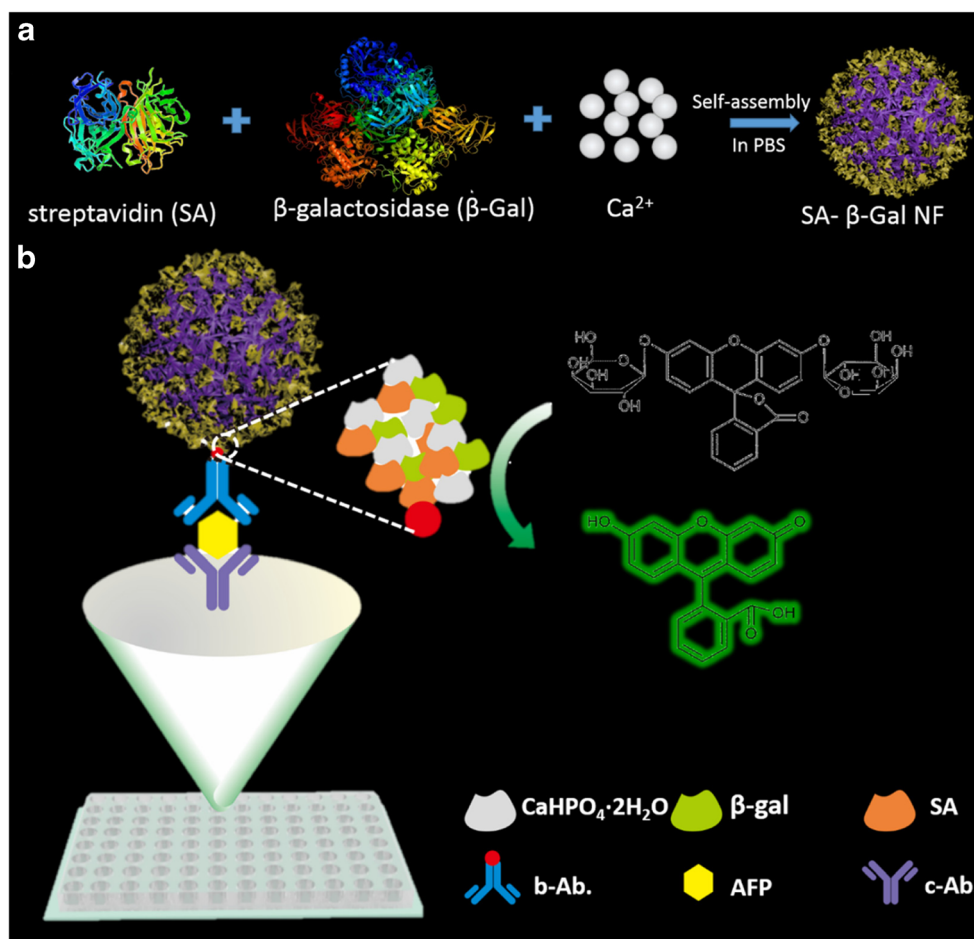
As is illustrated in Scheme 1, the cooperation of SA and β -Gal in the hybrid conveys multi-functions. Specifically, by utilizing the biotin-streptavidin system, SA has the high affinity to bind to biotinylated antibody and serves as the general recognition part to corresponding antigen. Meanwhile, β -Gal can catalyze the fluorogenic substrate FDG into fluorescein to give the fluorescence signal, serving as the signal output component in the hybrid nanoflower. In general, the multi-functional SA- β -Gal- CaHPO_4 hybrid nanoflowers possess the high biorecognition of corresponding analyte and signal amplification by loading abundant enzymes, which can be taken as ideal fluorescent signal probe for sensitive detection of protein biomarkers.

Experimental

Reagents and materials

Anti-human AFP monoclonal antibody (capture Ab), human AFP, and biotin-labeled AFP antibody (biotin-Ab) were purchased from Linc-Bio Science Co., Ltd. (Shanghai, China). Horseradish peroxidase (HRP) and β -galactosidase from

Scheme 1 **A** Self-assembly synthesis of SA- β -Gal- CaHPO_4 hybrid nanoflowers; **B** schematic illustration of multi-functional hybrid nanoflower-based ELISA for protein biomarker AFP detection



Escherichia coli were purchased from Sigma-Aldrich (St. Louis, MO, USA). SA was purchased from Amresco (Solon, OH, USA). Human serum sample was supplied by the Zhongnan Hospital of Wuhan University (Wuhan, China). 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 follows:

oligonucleotide labeled with biotin at one side and FAM at the other side (B-DNA-F): 5'-biotin-AAAAAA-FAM-3'.

oligonucleotide labeled with FAM (DNA-F): 5'-AAAAA-FAM-3'.

Synthesis of β -Gal-CaHPO₄ hybrid nanocomplexes

In a typical synthesis of β -Gal-CaHPO₄ hybrid nanoflower, an aqueous solution of CaCl₂ (200 mM, 20 μ L) was added into 2 mL of phosphate-buffered saline (PBS) solution (5 mM) containing 0.5 mg/mL β -Gal at pH = 6.8. The prepared mixture was mixed with ultrasound, and then left undisturbed for incubation for 24 h at the temperature of 37 °C. After incubation, the self-assembly of biomolecules in this environment was complete and the mixture was centrifuged to obtain a white-colored precipitate. The collected powder was washed three times with DI water and centrifuged at 10,000 rpm for 10 min to remove the unreacted components.

Synthesis and characterization of SA- β -Gal-CaHPO₄ hybrid nanoflower

The SA- β -Gal-CaHPO₄ hybrid nanoflowers were synthesized by self-assembly of protein in the PBS buffer in the presence of CaCl₂ with some modifications. Typically, 20 μ L of CaCl₂ (200 mM) was added into 1 mL PBS (5 mM, pH 6.8, NaCl 37.5 mM) followed by adding 1 mL of β -Gal (1 mg/mL) and 100 μ L of SA (1 mg/mL). The prepared mixture was mixed with ultrasound, and then left undisturbed for incubation for 24 h at the temperature of 37 °C. After incubation, the self-assembly of protein in this environment was complete and the mixture was centrifuged to obtain a white-colored precipitate. The collected SA- β -Gal-CaHPO₄ NF powder was washed three times with DI water and centrifuged at 10,000 rpm for 10 min to remove the unreacted components. The size and morphology of the prepared SA- β -Gal-CaHPO₄ nanoflowers were characterized by scanning electron microscope (SEM, HITACHI S-4800), transmission electron microscope (TEM, JEM-2100F), X-ray diffraction spectrometry (XRD, D8 ADVANCE), and fluorescence microscopy (Kratos Ltd. XSAM-800).

The activity evaluation of β -Gal and SA in SA- β -Gal-CaHPO₄ hybrid nanoflower

Three hundred microliters of FDG (1 μ g/mL) was incubated for 2 h at 37 °C in the presence of free β -Gal, β -Gal-Cu₃(PO₄)₂ NF, β -Gal-CaHPO₄ NF, SA- β -Gal-CaHPO₄ NF, respectively. Then after centrifugation, the supernatant was stored for fluorescence spectrum analysis.

Fifty microliters of 10⁻⁷ M B-DNA-F was incubated with SA nanoflower, β -Gal nanoflower, and SA- β -Gal nanoflower for 2 h, respectively. After centrifugation (10,000 rpm) for 5 min, precipitate was collected and washed with ultrapure water three times for fluorescence microscopy observation.

SA- β -Gal-CaHPO₄ NF-based fluorescent sensing for protein biomarker AFP

The procedures of nanoflower-based sensing for protein biomarker AFP were modified according to the classic ELISA [18]. Specifically, the capture antibody of human AFP (50 μ L, 0.02 mg/mL) was first attached to the bottom of 96-well high-binding ELISA plates. BSA (1%) 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 μ L) were added to the 96-well plates and incubated for 2 h. This step was followed by rinsing with 300 μ L of PBS-Tween buffer three times (all the binding steps described below were followed by a rinsing step). Next, the Biotin-Ab (50 μ L, 0.02 mg/mL) was bound to AFP through an immunoreaction for 2 h, followed by 30 min incubation with the prepared SA- β -Gal-CaHPO₄ hybrid nanoflowers (50 μ L, 0.02 mg/mL). Finally, the FDG was added and incubated in the microplate for 2 h at 37 °C. After that, 100 μ L of Na₂CO₃ aqueous solution (1 M) was added to terminate the reaction in the microplate, and the supernatant was collected and diluted to 500 μ L. And then the fluorescence intensity of the diluted supernatant was measured by using 490 nm for excitation and 515 nm for emission. Fluorescence spectrum analysis using a 1-cm path length quartz cuvette was performed.

Results and discussion

Characterization of the self-assembled nanoflower

Different kinds of β -Gal-CaHPO₄ hybrid nanocomplexes were synthesized through self-assembly. As shown in Electronic Supplementary Material (ESM) Fig. S1, by regulating the concentrations of β -Gal, PBS, and CaCl₂, we accomplished formation of nanostructures with distinctive morphology, and the β -Gal-CaHPO₄ hybrid nanoflower was prepared in the optimized condition. Similarly, SA- β -Gal-CaHPO₄ nanoflowers were

successfully prepared through self-assembly. The morphology and structure of the nanoflower were tentatively characterized. The as-prepared nanoflowers were characterized by scanning electron microscopy (SEM), XRD, energy dispersive X-ray spectroscopy (EDS), and thermogravimetric analysis (TGA). Moreover, the immobilized efficiency of hybrid nanoflowers calculated through Bradford assay. Morphology of the SA- β -Gal-CaHPO₄ hybrid nanoflower was illustrated by scanning electron microscope (SEM) and transmission electron microscope (TEM) images. SEM image showed flower-like nanostructure of as-prepared SA- β -Gal-CaHPO₄ products with high surface-to-volume ratios (Fig. 1A). The TEM image of the SA- β -Gal-CaHPO₄ hybrid nanoflower exhibited that they have hierarchical structures, which seems like they are assembled from hundreds of nanopetals (Fig. 1B). A TEM image of a single nanopetal shows that the complex is formed by the stacked nanocrystals (Fig. 1C). A high-resolution TEM image of the crystal structure of the nanopetal is shown in Fig. 1D, where the lattice spacing values of 0.761 nm correspond to (020) planes further confirming the formation of CaHPO₄. The X-ray powder diffraction (XRD) analysis was performed to demonstrate the formation of CaHPO₄ in the protein-inorganic hybrid nanoflower (Fig. 2A), for the X-ray diffraction pattern of the nanoflower power matched well with that of CaHPO₄ according to the JCPDS card (PDF#72-0713). The EDS of protein-enzyme hybrid nanoflower revealed the distribution of several typical elements including Ca, P, C, O, Na, Cl, and S (ESM Fig. S2) in the SA- β -Gal-CaHPO₄ NF, manifesting the presence of Ca, P, C, O, and S in hybrid nanoflower. And among them Ca and P elements were predominantly distributed in the nanosheets of the nanoflowers. To evaluate the weight percentage of different components in the hybrid system, TGA

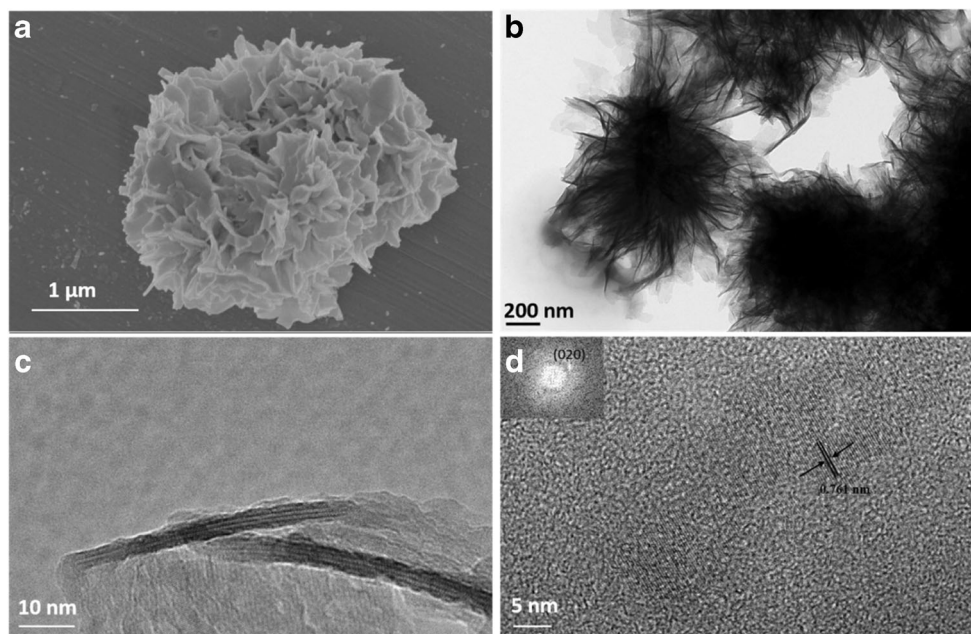
was performed, revealing that the weight percentage of organic component (i.e., SA and β -Gal) of the untreated nanoflowers was 16.32% (Fig. 2B). The immobilization efficiency, with the definition of the ratio of the amount of immobilized protein to the total amount of protein introduced, was determined to be 96.2%, by measuring the free protein in the supernatant using the Bradford protein assay (see ESM and Fig. S3), and the high immobilization efficiency of SA- β -Gal-CaHPO₄ hybrid nanoflower avoids the waste of protein or enzyme.

Activity evaluation of the SA- β -Gal-CaHPO₄ NF

To evaluate the enzymatic activity of β -Gal SA- β -Gal-CaHPO₄ NF and study the effect of different kinds of metal ions to enzymatic activity of β -Gal, free enzyme, β -Gal-CaHPO₄ NF, β -Gal-Cu₃(PO₄)₂ NF, and SA- β -Gal-CaHPO₄ NF were incubated with 300 μ L of FDG (1 μ g/mL) for 2 h at 37 $^{\circ}$ C, respectively. As presented in Fig. 3, from samples b to e, the fluorescence intensity was increasing, demonstrating that β -Gal-CaHPO₄ NF and SA- β -Gal-CaHPO₄ NF exhibited enhanced enzyme activity compared with free β -Gal, while β -Gal-Cu₃(PO₄)₂ NF lost most of its activity, which can be attributed to the fact that Zn²⁺, Ni²⁺, Cu²⁺, and Co²⁺ inactivate wild-type beta-galactosidase while Ca²⁺ binds but has no effect on the activity of wild-type beta-galactosidase [23].

To verify the biorecognition ability of SA in the SA- β -Gal hybrid nanoflower, B-DNA-F in which DNA modified with biotin and FAM at the different end was incubated with different types of nanoflowers. And then after centrifugation, the fluorescence was observed by fluorescence microscope. As shown in the Fig. 4, green fluorescent flowers can be observed in both SA nanoflower (Fig. 4A) and SA- β -Gal-CaHPO₄

Fig. 1 **A** SEM images of SA- β -Gal-CaHPO₄ hybrid nanoflower; **B** TEM images of SA- β -Gal-CaHPO₄ hybrid nanoflower; **C** TEM image of the single nanopetal in SA- β -Gal-CaHPO₄ hybrid nanoflowers; **D** high-resolution TEM image of SA- β -Gal-CaHPO₄ hybrid nanoflowers



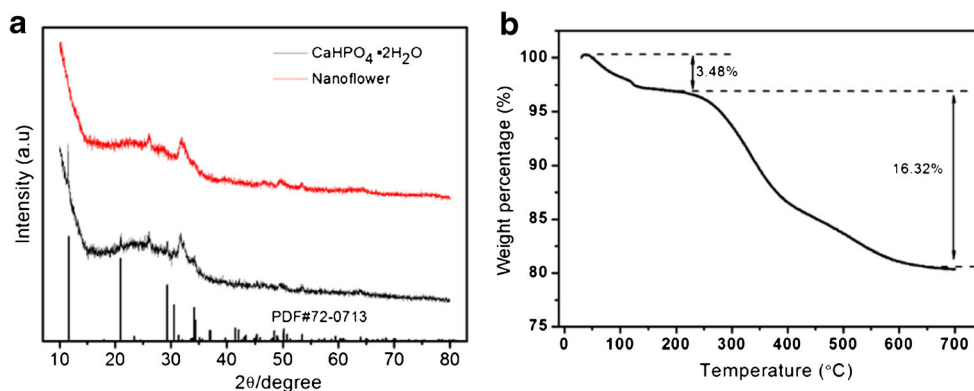


Fig. 2 **A** XRD patterns of hybrid nanoflowers obtained with SA and β -Gal (red line), particles of crystals obtained without protein (black line), and standard $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ (JPCSD PDF#72-0713) (inset). **B** TGA spectrum of the nanoflowers. The weight loss between RT and 200.0 °C

was 3.48%, corresponding to the loss of crystallization water of $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$; with the increase of temperature from 200 to 700 °C, the weight loss was 16.32%, resulting from pyrolytic decomposition of protein

hybrid nanoflower (Fig. 4C) in the dark field, while no green fluorescence can be seen in the β -Gal nanoflower (Fig. 4B). Besides, DNA-F where DNA only fluorescently labeled with FAM was also incubated with SA- β -Gal- CaHPO_4 nanoflower, and no green fluorescence can be observed after centrifugation (Fig. 4D), which demonstrated no nonspecific absorption during the process. As we can see, the SA in SA- β -Gal- CaHPO_4 nanoflower still maintained great biorecognition ability to biotinylated biomolecule during the self-assembly process.

SA- β -Gal- CaHPO_4 NF-based fluorescent sensing for AFP in standard buffer

Integrated recognition unit and signal amplification unit, the self-assembled SA- β -Gal- CaHPO_4 hybrid nanoflower was further used as signal tag to construct the fluorescent sensor

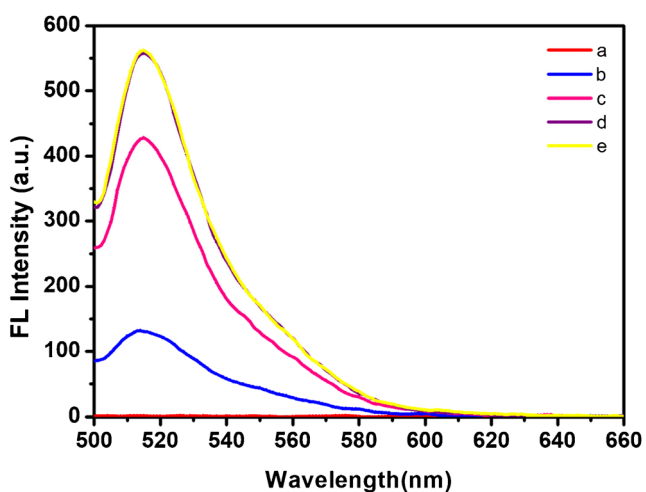


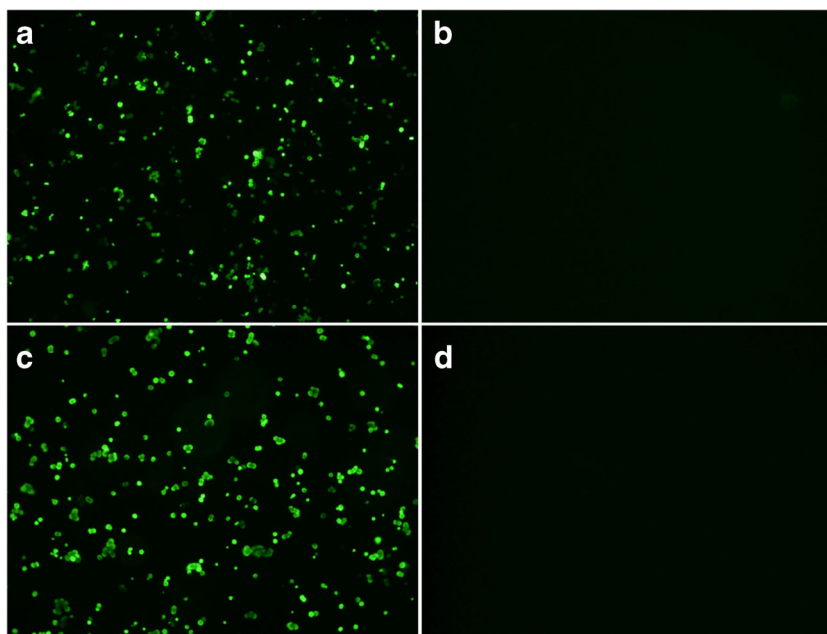
Fig. 3 Fluorescence spectra of the system: (a) FDG only; (b–e) the supernatant of FDG incubated with different kind of systems: (b) β -Gal- $\text{Cu}_3(\text{PO}_4)_2$ NF, (c) free β -Gal, (d) β -Gal- CaHPO_4 hybrid nanoflowers, and (e) SA- β -Gal- CaHPO_4 hybrid nanoflowers

for ultrasensitive detection of protein biomarker AFP. As indicated in Scheme 1B, the detection of AFP was based on the sandwich immunoassay. On the one hand, the function of recognition was realized by the specific binding between the SA in SA- β -Gal- CaHPO_4 hybrid nanoflower and the corresponding biotinylated antibody. On the other hand, the function of signal reporter was achieved by β -Gal in SA- β -Gal- CaHPO_4 nanoflower, which can catalyze the fluorogenic substrate FDG into fluorescein producing a fluorescence signal. Besides, the inorganic component of CaHPO_4 in hybrid nanoflower played the role of immobilizing massive SA and β -Gal, which has the effect of signal amplification. Therefore, when AFP was recognized by corresponding biotinylated antibody which was further bound to SA in SA- β -Gal- CaHPO_4 hybrid nanoflower, the β -Gal in SA- β -Gal- CaHPO_4 hybrid nanoflower can catalyze FDG into fluorescein giving a fluorescence signal. In this way, it can determine the concentration of the target protein simply by monitoring the fluorescence intensities under the UV lamp. In the absence of target, the biotin-Ab and the SA- β -Gal- CaHPO_4 nanoflowers were washed away, resulting in a low background signal.

In the assay, the performance of the fluorescent sensor was affected by the employed nanoflowers. As shown in the ESM Fig. S4, several different kinds of hybrid nanoflower were employed as signal probes, including SA- CaHPO_4 NF, SA- β -Gal- CaHPO_4 NF with SA- β -Gal at the ratio of 1:1, SA- β -Gal- CaHPO_4 NF with SA- β -Gal at the ratio of 1:10, and β -Gal- CaHPO_4 NF. The lack of biorecognition unit (SA) or signal output unit (β -Gal) led to a low fluorescent intensity, while SA- β -Gal- CaHPO_4 NF with the ratio of 1:1 and 1:10 exhibited an obvious fluorescent increase, which indicates that the combination of the biorecognition unit and signal output unit ensures the signal tag function of the SA- β -Gal- CaHPO_4 NF hybrid nanoflower.

To detect AFP by this proposed method, different concentrations of AFP in PBS buffer were added. An obvious increase

Fig. 4 Fluorescence microscopy images of different hybrid nanoflowers. **A–C** Dark-field images of SA-CaHPO₄, β -Gal-CaHPO₄, and SA- β -Gal-CaHPO₄ nanoflowers with B-DNA-F, respectively; and **D** SA- β -Gal-CaHPO₄ nanoflowers with DNA-F



in the FL peak at 515 nm was clearly observed with the increase of AFP concentration. In addition, the fluorescent color change of the product solutions induced by 1 ng/mL of AFP can be detected with naked-eye readout under the UV lamp (ESM Fig. S5). Furthermore, quantitative analysis was conducted by comparing the FL at 515 nm in the presence of different concentrations of AFP. As presented in Fig. 5, SA- β -Gal-CaHPO₄ NF-based fluorescent sensor exhibited excellent performance for AFP assay in a linear range of 0.1 to

10 ng/mL, with the detection of 13 pg/mL (the LOD was calculated according to the method described by Demchenko, as illustrated in the ESM), while for the SA-HRP-CaHPO₄-based ELISA and SA-HRP-Cu₃(PO₄)₂-based ELISA, the LOD were calculated to be 0.17 ng/mL (ESM Fig. S6) and 78 pg/mL [18], respectively, indicating the improvement of sensitivity in SA- β -Gal-CaHPO₄ NF-based ELISA can be contributed to the change of signal output enzyme from colorimetric to fluorescent.

Fig. 5 **A** Fluorescence spectra of the sensing system in response to the different concentrations of AFP: 0, 0.1, 0.5, 1.0, 2.5, 5.0, 10 ng mL⁻¹; **B** relationship between the fluorescence intensity and AFP concentration in standard buffer. Inset: corresponding calibration equation for AFP detection; **C** relationship between the fluorescence intensity and AFP concentration in human serum. Inset: corresponding calibration equation for AFP detection; **D** specificity of the assay for AFP detection with different nontarget protein. Experimental conditions: 0.02 mg/mL capture antibody, 1 ng/mL different protein, 0.02 mg/mL SA- β -Gal-CaHPO₄ nanoflowers

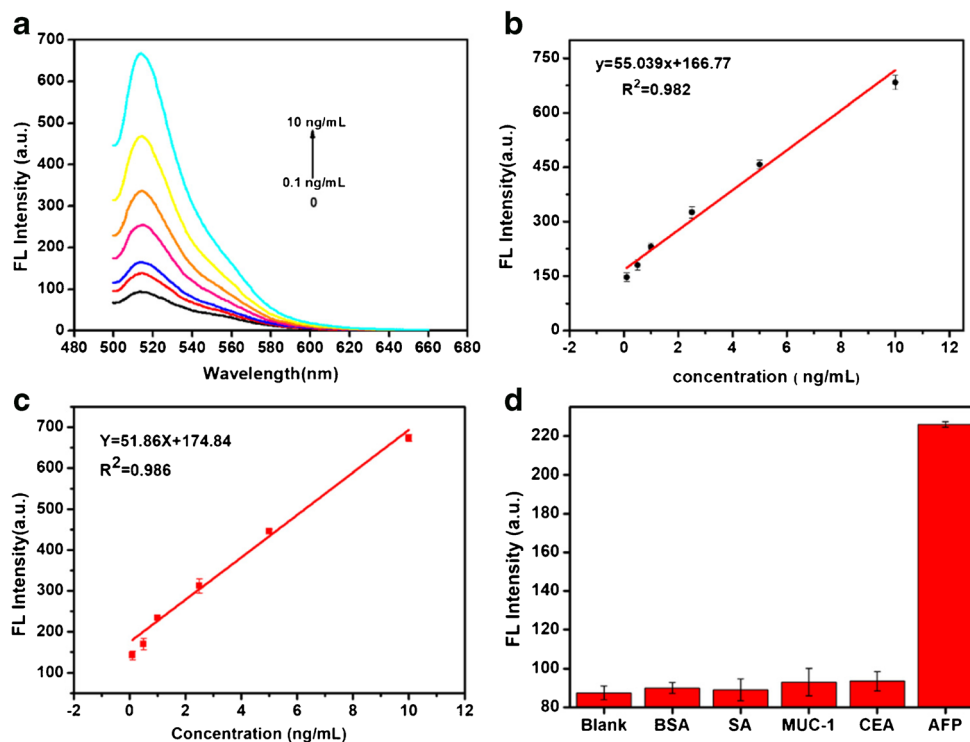


Table 1 Recovery of AFP in healthy human serum with this proposed method

| Sample number | Added (ng/mL) | Found (ng/mL) | Recovery (%) | RSD (%) ($n=3$) |
|---------------|---------------|---------------|--------------|-------------------|
| 1 | 0.1 | 0.109 ± 0.08 | 109.0 | 4.1 |
| 2 | 2.5 | 2.47 ± 0.73 | 98.8 | 2.7 |
| 3 | 5.0 | 5.22 ± 0.75 | 104.4 | 3.8 |
| 4 | 10.0 | 9.13 ± 0.77 | 91.3 | 6.1 |

SA- β -Gal-CaHPO₄ NF-based fluorescent sensing for AFP in human serum samples

To estimate the performance of this proposed method in real sample analysis, the developed SA- β -Gal-CaHPO₄ NF-based sensor was used to detect AFP in human serum samples. After the addition of AFP in human serum, the reaction exhibited an obvious increase of fluorescent intensity, exhibiting a linear to the concentration of AFP throughout the range of 0.10–10.0 ng/mL (Fig. 5C). To further demonstrate this method is free from the complex sample matrix effect of human serum, 10% healthy human serum was adopted as model matrix and recovery tests were conducted. Different concentrations of AFP were separately spiked into the 10% diluted human serum, and then the samples were tested. Fifty microliters spiked AFP samples were loaded on the ELISA 96-well plate for analysis. The human serum without spiked AFP used as the control. The concentration of AFP in the human serum was determined by the obtained standard curve. Recoveries of AFP were ranging from 91.3 ± 6.1 to 109.0 ± 4.1% (Table 1). These results successfully proved the feasibility and reliability of this proposed method, which is applicable for the determination of AFP in real sample.

The specificity of the SA- β -Gal-CaHPO₄ NF-based fluorescent sensor was evaluated by replacing the target AFP with BSA, SA, MUC-1, and CEA. As depicted in Fig. 5D, obvious fluorescent increase was observed in the presence of target AFP, whereas BSA, SA, MUC-1, and CEA led to slight increase of fluorescent intensity. It can be concluded that the high specific binding of the antigen and antibody ensures the specificity of SA- β -Gal-CaHPO₄ NF-based fluorescent sensor.

Conclusion

In summary, an innovatively self-assembled method has been presented for the facile and green synthesis of protein-enzyme conjugates integrating the functions of biorecognition and signal amplification. Besides, no chemical modification and coupling reaction is necessary to fabricate the signal probe. By adopting the as-prepared SA- β -Gal-CaHPO₄ hybrid nanoflowers as fluorescent signal probe, a simple but potentially powerful amplification biosensor with excellent simplicity, sensitivity, and adaptability has been constructed for protein biomarker AFP assay. By altering the different signal output enzyme, fluorescent decoction would be an alternative

to conventional colorimetric immunosensor, thus further improving the sensitivity of nanoflower-based sensor. On the other hand, due to the general SA-biotin binding system, SA- β -Gal-CaHPO₄ hybrid nanoflowers have the potential to detect a wide range of protein biomarkers by varying the corresponding of the biotinylated antibody. Therefore, this self-assembled approach can be generally employed to prepare a variety of protein or enzyme conjugates for the various applications, including biocatalysis, biosensors, and drug delivery.

Funding information This work was supported by the National Natural Science Foundation of China (21475101, 21675119).

Compliance with ethical standards

Ethical standards and informed consent The study was approved by the Ethical Committee of Wuhan University. Human fluid samples used in this study do not have any identifying information about all the participants that provided written informed consent.

Conflict of interest The authors declare that they have no conflict of interest.

References

- Somturk B, Hancer M, Ocsoy I, Ozdemir N. Synthesis of copper ion incorporated horseradish peroxidase-based hybrid nanoflowers for enhanced catalytic activity and stability. *Dalton Trans.* 2015;44:13845–52.
- Mittal S, Kaur H, Gautam N, Mantha AK. Biosensors for breast cancer diagnosis: a review of bioreceptors, biotransducers and signal amplification strategies. *Biosens Bioelectron.* 2017;88:217–31.
- Zhou Z, Hartmann M. Progress in enzyme immobilization in ordered mesoporous materials and related applications. *Chem Soc Rev.* 2013;42:3894–912.
- Ming L, Youzeng F, Tingwei W, Jianguo G. Micro-/nanorobots at work in active drug delivery. *Adv Funct Mater.* 2018. <https://doi.org/10.1002/adfm.201706100>.
- Spicer CD, Jumeaux C, Gupta B, Stevens MM. Peptide and protein nanoparticle conjugates: versatile platforms for biomedical applications. *Chem Soc Rev.* 2018;47:3574–620.
- Fang Y, Xu W, Wu J, Xu Z-K. Enzymatic transglycosylation of PEG brushes by β -galactosidase. *Chem Commun.* 2012;48:11208–10.
- Tang F, Li L, Chen D. Mesoporous silica nanoparticles: synthesis, biocompatibility and drug delivery. *Adv Mater.* 2012;24:1504–34.
- Jia H. Enzyme-carrying electrospun nanofibers. In: Wang P, editor. *Nanoscale biocatalysis: methods and protocols*. Totowa: Humana Press; 2011. p. 205–12.
- Kalska-Szostko B, Rogowska M, Satuła D. Organophosphorous functionalization of magnetite nanoparticles. *Colloids Surf B.* 2013;111:656–62.

10. Wang Q, Zhou L, Jiang Y, Gao J. Improved stability of the carbon nanotubes–enzyme bioconjugates by biomimetic silicification. *Enzym Microb Technol.* 2011;49:11–6.
11. Ge J, Lei J, Zare RN. Protein–inorganic hybrid nanoflowers. *Nat Nanotechnol.* 2012;7:428–32.
12. Wang X, Shi J, Li Z, Zhang S, Wu H, Jiang Z. Facile one-pot preparation of chitosan/calcium pyrophosphate hybrid microflowers. *ACS Appl Mater Interfaces.* 2014;6:14522–32.
13. Hua X, Xing Y, Zhang X. Enhanced promiscuity of lipase–inorganic nanocrystal composites in the epoxidation of fatty acids in organic media. *ACS Appl Mater Interfaces.* 2016;8:16257–61.
14. Thawari AG, Rao CP. Peroxidase-like catalytic activity of copper-mediated protein–inorganic hybrid nanoflowers and nanofibers of β -lactoglobulin and α -lactalbumin: synthesis, spectral characterization, microscopic features, and catalytic activity. *ACS Appl Mater Interfaces.* 2016;8:10392–402.
15. Cui J, Zhao Y, Liu R, Zhong C, Jia S. Surfactant-activated lipase hybrid nanoflowers with enhanced enzymatic performance. *Sci Rep.* 2016;6:27928.
16. Li M, Luo M, Li F, Wang W, Liu K, Liu Q, et al. Biomimetic copper-based inorganic–protein nanoflower assembly constructed on the nanoscale fibrous membrane with enhanced stability and durability. *J Phys Chem C.* 2016;120:17348–56.
17. Zhu X, Huang J, Liu J, Zhang H, Jiang J, Yu R. A dual enzyme–inorganic hybrid nanoflower incorporated microfluidic paper-based analytic device (μ PAD) biosensor for sensitive visualized detection of glucose. *Nanoscale.* 2017;9:5658–63.
18. Liu Y, Chen J, Du M, Wang X, Ji X, He Z. The preparation of dual-functional hybrid nanoflower and its application in the ultrasensitive detection of disease-related biomarker. *Biosens Bioelectron.* 2017;92:68–73.
19. Ariza-Avidad M, Salinas-Castillo A, Capitán-Vallvey LF. A 3D μ PAD based on a multi-enzyme organic–inorganic hybrid nanoflower reactor. *Biosens Bioelectron.* 2016;77:51–5.
20. Li W, Lu S, Bao S, Shi Z, Lu Z, Li C, et al. Efficient in situ growth of enzyme–inorganic hybrids on paper strips for the visual detection of glucose. *Biosens Bioelectron.* 2018;99:603–11.
21. Sun J, Ge J, Liu W, Lan M, Zhang H, Wang P, et al. Multi-enzyme co-embedded organic–inorganic hybrid nanoflowers: synthesis and application as a colorimetric sensor. *Nanoscale.* 2014;6:255–62.
22. Guan Z, Zou Y, Zhang M, Lv J, Shen H, Yang P, et al. A highly parallel microfluidic droplet method enabling single-molecule counting for digital enzyme detection. *Biomicrofluidics.* 2014;8(1):014110.
23. Martinez-Bilbao M, Gaunt MT, Huber RE. E461H- β -galactosidase (*Escherichia coli*): altered divalent metal specificity and slow but reversible metal inactivation. *Biochemistry.* 1995;34:13437–42.



Bao Wang is a graduate of the College of Chemistry and Molecular Sciences of Wuhan University. He is devoted to the field of materials chemistry.



Xinghu Ji received his Ph.D. degree in analytical chemistry from Wuhan University in 2007. In 2014, he worked at the University of Kansas as a visiting scholar. He is currently Associate Professor at Wuhan University. His research interest focuses on microfluidics and applications in analytical chemistry for biology and medicine.



Zhike He is Outstanding Professor of Chemistry College of Chemistry and Molecular Science, Wuhan University. He received his Ph.D. degree in analytical chemistry from Wuhan University in 1995. He was also a Humboldt Research Fellow. Currently, his research interest covers nano-bio-analytical chemistry and applications in biology and medicine.



Yucheng Liu is working for her Ph.D. degree from the College of Chemistry and Molecular Sciences of Wuhan University. She works on the synthesis of bioinspired nanomaterials and application in biosensing.