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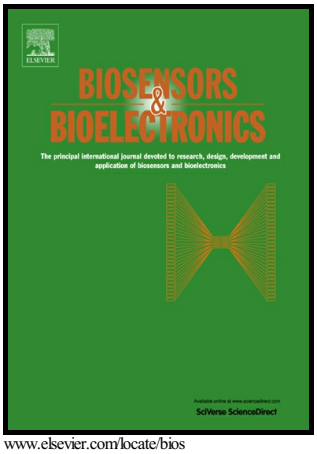

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Recent progress in biosensors based on organic-inorganic hybrid nanoflowers

Junlun Zhu[1], Meiqi Wen[1], Wei Wen[1]*, Dan Du[2], Xiuhua Zhang[1], Shengfu Wang[1]*, Yuehe Lin[2]

[1] Hubei Collaborative Innovation Center for Advanced Organic Chemical Materials, Ministry of Education Key Laboratory for the Synthesis and Application of Organic Functional Molecules, College of Chemistry and Chemical Engineering, Hubei University, Wuhan 430062, PR China,

[2] School of Mechanical and Materials Engineering, Washington State University, Pullman, WA 99164, USA

wenwei@hubu.edu.cn;

wangsf@hubu.edu.cn.

***Corresponding author:** Tel: +86-27-50865309; Fax: +86-27-88663043.

Abstract

Organic-inorganic hybrid nanoflowers (HNFs) are a class of flower-like hybrid materials self-assembled from metal ions and organic components, such as enzymes, antibodies, DNA and amino acids et al. Based on their properties of enhanced enzyme activity, stability, facile synthesis and excellent biocompatibility, HNFs enable them to be a highly versatile platform for latent applications in many realms, such as biological sensing, biomimetic catalyst, dye decolorization and support nanomaterials, etc. Compared with free enzymes, HNFs are potentially advantageous for biological

applications owing to their enhanced catalytic ability and powerful load capacity. Herein, an overview of recent developments achieved in HNFs biosensing, including electrochemical biosensors, colorimetric biosensors, and point-of-care diagnostic devices, is provided. We summarized the preparation of different HNFs based on the type of metal ions and biomolecules used. The signal tags involved in the all-in-one hybrid nanoflowers are particularly emphasized. The challenges, future trends, and prospects associated with HNFs and their related materials for biosensing are also discussed.

Keywords

Organic-inorganic hybrid nanoflowers, biosensor, electrochemistry, colorimetry, point-of-care

1. Introduction

Organic-inorganic hybrid material, a versatile complex, has emerged as an alternative form for obtaining novel materials with desired characteristics. Recent advanced developments in materials science and engineering paved a conclusive headway in the reasonable design and accurate synthesis of organic-inorganic hybrid materials with unique or improved performances (Judeinstein and Sanchez 1996; Sreejith et al. 2015). Further, porous and networked nanomaterials have received increasing attention due to their large specific surface area, strong adsorption capacity, excellent catalytic ability, and efficient loading capability (Yang et al. 2016; Cao et al.

2011; Wang et al. 2016b). Therefore, many flower-shaped inorganic nanomaterials have been broadly developed and applied in larger areas, displaying some key applications in fields with a bright future, such as biosensors (Wang et al. 2016a; Zhang et al. 2016d), wastewater treatment (Mu et al. 2017; Li et al. 2011), adsorption capacity (Lv et al. 2015a; Feng et al. 2013), and catalysis (Amiri et al. 2012; Zhou et al. 2013). Interestingly, starting with the pioneering work of Ge et al. on organic-inorganic hybrid nanoflowers (HNFs), for which they opened the door to new protein-inorganic hybrids in 2012, and the field of HNFs has turned out to be rich and varied (Ge et al. 2012). It is a newly discovered type of porous and flower-like hybrid nanoparticles, containing a large amount of proteins and inorganic salts. With the advent of new immobilization strategy and peculiar nanostructures, efficient use enzyme will be indispensable players in future biosensors development. When the varieties of enzymes and antibodies were being used as the organic part of the HNFs, it exhibits many extraordinary properties, such as higher surface area, enhanced enzymatic activity, with excellent stability compared to the free enzyme, and holds outstanding capture ability compared with unbounded antibody. The great success of HNFs throws a beam of new light for discovering more novel biosensors composed of antibody and enzymes. Compared with the inorganic nanoflowers, the HNFs possess some advantage in biosensing application, such as better biocompatibility, enhanced catalytic activity, and label-free characteristics. In the past five years, variety of HNFs have been synthesized, leading to the application of HNFs based biosensors for disease diagnostics and detection (Seung Woo Lee 2015; Lei et al. 2018; Altinkaynak

et al. 2016a). On account of their performances with high stability, activity and simple synthetic methods, these HNFs raise various potential applications, including biosensors, bioremediation, bioassays, biomedicine, industrial biocatalysis and wastewater treatment (Cui and Jia 2017). Furthermore, a wide range of optimum conditions in the preparation of HNFs offer great opportunities for generating small-size nanoflowers and complex multifunctional nanostructures, which optimize their characters and further develop remarkable performances for diversified application in the early diagnosis and clinical analysis field.

In this review paper, we had classified the recent study developments of novel HNFs and highlighted their significant advanced applications for biosensing, such as electrochemical biosensors, colorimetric biosensors, and point-of-care diagnostic devices. Three biosensing applications that form HNFs through the convergence of different metal ions, enzymes, and antibodies are shown in **Fig. 1**. Initially, we briefly outline the progress of research on HNFs synthesis methods. There are three way to synthesize HNFs based on biomolecules: 1) bio-mineralization, co-precipitation or self-assembly, 2) ultrasonic method and 3) rolling circle replication (RCR). Then, we briefly generalized types of HNFs. In this section, different organic components and inorganic salts forming HNFs have been mentioned, which includes metal ions (Cu^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, etc.), enzymes, antibodies and other biomolecules, compendiously generalize the household of HNFs in recent years. Combining the knowledge we have learned, we roughly summarized the advantages and disadvantages of HNFs. Third, we mainly discussed the multiple functions of all-in-one nanoflowers and their recent

attractive applications in biosensing. According to their detection methodologies, we reviewed numerous HNFs that could replace conventional signal tags for biosensing detection. Beyond a discussion of the recent comment about analytical methods and emerging applications, we modestly addressed the several challenges and future directions with respect to HNFs, as well as the way to devise novel bioassay instruments. With the publication of several academic articles related to HNFs, we might have missed some of the contributions during this period of reference, and we sincerely apologize to the authors for their significant research which may have been failed to mention.

2. Preparation, classification, and characteristics of HNFs

HNFs, a newly discovered type of porous and flower-like hybrid nanomaterials, have drawn wide attraction due to some advantages in the fields of biology, medicine, industry, chemistry, etc. (Altinkaynak et al. 2016a; Cui and Jia 2017). The first discovery of HNFs can date back to 2012 by Ge et al., and the structure of HNFs were displayed the different flower-like and enhance catalytic activity and stability, which referred to different proteins and enzymes (Ge et al. 2012). Due to the outstanding properties of HNFs, plenty of investigations were devoted to the green synthesis, high efficiency, immobilizing biomolecules and so on. It has been demonstrated that the HNFs show better biocompatibility, activity and stability, large specific surface area, and other characteristics than the naked proteins and inorganic salts, which render them more apposite candidates for biosensing. Thus, the HNFs were synthesized by

co-precipitation, self-assembly, bio-mineralization ultrasonic method from different proteins and inorganic salts. In this section, we would discuss the green chemical approaches for the preparation of HNFs and their classification and characteristics for some applications.

2.1 Evolution of the HNFs synthesis method

Compared to a more compact structure such as a solid sphere or a polyhedron, the HNFs have a larger surface area for a given volume, resulting in a higher surface energy. Like other nanostructures with branched morphology, the HNFs must be prepared using kinetics rather than thermodynamic control. The appearance of the final product depends on both the concentration and shape of the primary nanoparticles that are formed in the nucleation step. When the concentration of particles is relatively low, the aggregation process is governed by diffusion and these results in aggregates with a fractal structure characterized by random branching (Lim and Xia 2011; Witten and Sander 1981). When the concentration of particles is relatively high, fast aggregation occurs. In this case, because of the large surface-to-volume ratio and high collision frequency, there is a strong driving force for the particles to aggregate into more compact structures rather than branch out (Zeng and Xia 2012). The synthesis of HNFs can be categorized into three main approaches described in the following subsections. Firstly, the HNFs were synthesized according to the one-step co-precipitation method reported by Zare's lab (Ge et al. 2012). In a typical synthesis, 0.8 mM $CuSO_4$ was added to phosphate buffered saline containing 0.1 mg mL^{-1} bovine serum albumin (BSA) or different

enzymes at pH 7.4 and 25 °C for standing to synthesize HNFs. Second, the all-in-one HNFs were prepared by self-assembly from enzymes, antibodies and inorganic salts. Among them, the synthetic raw materials are similar to the previous ones and the addition of an appropriate amount of antibody as a recognition unit increases the application range (Ye et al. 2016a; Ye et al. 2016a). Although the formation of the HNFs with the different metal ions and the various proteins are easy, green, and have low energy consumption, but it does take a longer time duration for production (3 days). Thus, it is necessary to design a reasonable synthesis strategy to shorten the time for preparing HNFs. Apart from preparing HNFs by self-assembly and co-precipitation, Kim and co-workers developed a novel and efficient process for the preparation of hybrid nanostructures with a three dimensional flower-like nanostructure (Chung et al. 2018; Batule et al. 2015). They applied sonication to speed up the preparation of the HNFs. Using this ultrasonic technique, they prepared the HNFs containing laccase, glucose oxidase and catalase as a model protein and copper phosphate as nucleation centers in 5 minutes at room temperature as well as high activity and stability. These synthetic methods provide more ways to make HNFs, especially using ultrasonic synthesis to obtain uniform and perfect nanoparticles, which will provide a broader stage for subsequent research. Besides, as shown in **Fig. 2**, firstly, the precipitation reaction of zinc ions and phosphate was carried out with the coordination of zinc ions and lipase simultaneously. The products of this step were zinc phosphate crystal nucleus and lipase/Zn^{2+} hybrid materials. Secondly, zinc ions of lipase/Zn^{2+} hybrid materials were converted to zinc phosphate crystal nucleus by

in-situ precipitation. Zinc phosphate crystal nucleus continuously grew and was captured by the lipase. The product of this step was lipase/zinc phosphate plates. Thirdly, self-assembly occurs between the nanoplates thus obtained to form lipase/zinc phosphate precursor particles. The final step is to obtain a fully blended nanoflower with a larger size. The growth mechanism of HNFs has been studied in detail, which can be narrated as crystallization and coordination, *in-situ* precipitation, self-assembly, and size growth by four steps (Zhang et al. 2016c). Consequently, the strategy may be of high influence for offering synthetic method in HNFs.

In addition, rolling circle amplification technology is an important means of synthesizing nanoparticles. RCR is a recently developed method of constant temperature nucleic acid amplification. DNA can be synthesized by rapid isothermal enzymatic reactions, which have been widely used in academic research and biotechnology (Hu et al. 2014). Tan's group used the RCR technology to produce extended DNA building blocks from the self-assembly preparation of DNA nanoflowers, and as a multivalent CpG nanoagents to effectively protect and deliver the incorporated nucleic acid therapeutic agent to the immune cells (Zhang et al. 2015a). On the basis of the same principle, Kim et al. have developed multiple protein-encapsulated DNA flowers using the well-established rolling circle amplification technique that concedes highly efficient protein loading while maintaining the biological activity of the payloads (Kim et al. 2017). HNFs synthesized buy the above methodologies have been reported to possess many

excellent properties, which are advantageous for biosensing, industrial, medical, environmental applications.

2.2 Classifications and characteristics of HNFs

The use of HNFs can avoid the problems associated with naked proteins and alloy inorganic salts. HNFs can be classified according to the types of metal ions and organic components. Therefore, all the examples of the organic and inorganic parts reported in HNFs systems are listed in **Table 1**. In this table, we have presented different metal ions and biomolecules such as proteins, enzymes, nucleic acids and amino acids, etc. Biosensors associated with HNFs will be discussed in more detail below.

Generally, these HNFs exhibit higher catalytic activity and biological stability owing to combination specificity of enzymes and inorganic salts. To investigate the potential mechanism of HNFs on its enhanced catalytic activity and stability, a reasonable design of calcium hydrogen phosphate-α-amylase nanobiocatalytic system (i.e., nanoflowers, nanoplates and parallel hexahedrons) has been simulated by Zeng's group (Wang et al. 2013). As shown in **Fig. 3A**, Ca^{2+} acts as a switch to regulate the activity of α-amylase catalysis, and allosteric sites topographically separated from functional sites are combined with Ca^{2+}, thereby exciting previously inactivated functional sites. In **Fig. 3B**, when there is a proper amount of α-amylase and $CaHPO_4$, α-amylase is attached to the surface of $CaHPO_4$, exposing a large number of active sites. In **Fig. 3C**, if the $CaHPO_4$ is excessive, a certain amount of α-amylase is embedded in the $CaHPO_4$, which affects the mass transfer efficiency leading to

decreased catalytic activity. It is worthy to mention that, allosteric effects are promising to prove the high stability and catalytic activity in HNFs. Considering the possibility of using different metal ion, Sutti et al. tested onion *burkholderia* bacteria lipase and a series of different valence metal ions: Ag(I), Fe(II), Cu(II), Au(III) synthetic HNFs, to expand on the previous findings (Sharma et al. 2017). When determining the HNFs activity, compared with metal and the coexistence of phosphate ions, the type of metal ions and petals macroscopic arrangement played a secondary role. This strategy shows that with different metal choose HNFs will not influence the enhanced activity of natural enzyme, which opened the way for the future research development. By further studying the enzymatic activity and morphologies, Tian and co-workers found that inorganic salts and protein concentration was not the only factor that affected the HNFs size, enzymatic activity and petal density (Li et al. 2016c). Moreover, temperature and pH were the additional influencing conditions in the preparation of HNFs. Further research revealed, the reason for reduced catalytic activity of lipase nanoflower is that the hydrophobic amino acid chain covers its active site (Manoel et al. 2015). Taking into account the nature of the lipase, Cui et al. developed a reasonable design of the surfactant activation of lipase-inorganic hybrid nanoflowers with higher catalytic activity related to interfacial activation and self-assembly (Cui et al. 2016). Notably, several metal-enzymes have been employed to grow the HNFs. It is confirmed that metal-enzymes incorporated hybrid exhibited much higher catalytic activities and stabilities compared to metal-free enzymes. For example, compared with the solution of the free laccase, the synthesis of laccase (one

kind of oxidase containing copper) HNFs showed increased catalytic activity (in terms of oxidizing syringaldazine, the activity is increased by 6.5 times) (Ge et al. 2012). The catalytic activity and the stability of the enzyme in the HNFs are summarized as follows: 1) Benefiting from the allosteric regulation and morphology of the as-synthesized nanostructures, HNFs exhibited dramatically enhanced enzymatic activity; 2) for metal enzymes, the interactions between metal enzymes and the microenvironment of the HNFs, such as the interactions between laccase and the microenvironment of HNFs, which contains copper ions (copper ions in HNFs may help to enhance laccase activity in a manner similar to when in solution); 3) enzyme molecules embedded in the same HNFs have a certain cooperative effects with each other; 4) the synergistic effect of the enzyme molecule and the inorganic salt forms nanoflower structure that exposed more active sites; 5) the high surface area of the HNFs (namely a much higher surface energy), which does not result in significant blockage of the mass transfer process. Therefore, this strategy may be of great interest for increased metal or free-metal enzymes activity in HNFs. Apart from this, HNFs also exhibit improved catalytic efficiency, excellent biocompatibility, enhanced catalytic activity and stability, and high surface area to volume ratio associated with their particular morphology.

In addition to such exceptional properties, which make them attractive in the field of biomimetic catalyst (Wang et al. 2018; Huang et al. 2015; Lopez-Gallego and Yate 2015; Ocsoy et al. 2015; Song et al. 2017; Li et al. 2016d), enzyme reactor (Lin et al. 2014a; Yin et al. 2015), enriching and screening medicine (Qian et al. 2018;

Kong et al. 2018), decolorization (Patel et al. 2018; Rong et al. 2017; Altinkaynak et al. 2017), support nanomaterials (Luo et al. 2017; Zhang et al. 2015b), and biotransformations (Somturk et al. 2015; Athena A. Papadopoulou 2017), HNFs are also gaining growing interest in the development of biosensors. HNFs is a typical family member of hybrid materials, taking it as an example, several researches have demonstrated the capture activity and catalytic activity of HNFs higher than naked enzymes and antibody. Since then, the all-in-one HNFs have been found to exhibit capture activity and catalytic activity, and they have been successfully applied in biosensing systems.

Furthermore, DNA nanoflowers have the effect of being cleaved by nucleases and have suitable particle size and biocompatibility. Recently, Park et al. synthesized DNA-copper phosphate hybrid nanoflowers via using DNA as the organic part instead of protein (Park et al. 2017). The synthesized of DNA-copper phosphate hybrid nanoflowers has been reported exhibiting high encapsulation yield, low cytotoxicity, and strong resistance against nucleases compare to the single stranded DNA. In addition, Tan's group declared the non-canonical self-assembly of hierarchical DNA nanoflowers with compactly packed DNA using RCR technology (Hu et al. 2014). The DNA nanoflowers not only exhibited high water-solubility, biocompatible, uniform selective and excellent photostability, but also they could be used for the some applications, such as targeted drug delivery, multivalent nanoagents, cancer cell recognition, bioimaging and biomedical.

By investigating the properties of HNFs, we summarized the following conclusions: 1) HNFs can increase the biological activity of enzymes and provide a new direction for industrial enzyme reagents. 2) HNFs have a large specific surface area and can be used as an ideal adsorbent material and support material. 3) HNFs modification recognition unit can be synthesized by self-assembly method without modification. 4) All-in-one HNFs provide excellent catalytic performance and strong capture capabilities, providing a powerful guarantee for signal labeling. 5) DNA nanoflowers have small and uniform particle size and resistant to nuclease cleavage, and they provide a new path for biomedical applications, such as cell bioimaging and nuclear magnetic angiography, etc.

3. Application of biosensor based on HNFs

HNFs, a newly developed type of flower-like hybrid nanoparticles, have attracted wide attention due to its simple synthesis, high efficiency and wonderful stability of the enzyme. In addition, their large surface area offers more active sites, thus providing enhance catalytic performance, capture ability and detection sensitivity. Despite the abundant theoretical and experimental researches of HNFs as a novel material in the field of immobilized enzymes, adsorption of heavy metal ions and remove of dye, etc., significant advances in HNFs based on biosensing cannot be ignored. (Cui and Jia 2017; Altinkaynak et al. 2016a). Compared with common recognition unit-nanomaterial-signal marker unit conjugates, HNFs may be more excellent as signal probes in biosensing application (Du and Guo 2016; Yang and

Jiang 2017). In this section, we will summarize their biosensing applications in electrochemical, colorimetric and point-of-care filed.

3.1. Electrochemical biosensors based on HNFs

Electrochemical biosensor technology is a new technology with long term significance in the field of disease diagnosis due to its characteristics such as the simple operation, low detection cost, good selectivity, high sensitivity and rapid analysis (Fabiana Arduini 2016; Bahadir and Sezginturk 2015). Regarded as an important part of biological testing methods, amperometry, impedance and voltammetry are the most popular and commonly electrochemical methods that is used to a variety of immune responses due to its broad applicability and high sensitivity (Wen et al. 2017; Rotariu et al. 2016). Therefore, the development of more advantageous electrochemical biosensors has been put in place. Over the past two years, high signal-to-noise ratio and signal-amplified detection in electrochemical biosensor has been done with emerging nanomaterials. Due to the advent of HNFs, electrochemical biosensors can detect the target analyze by means of: 1) a resistance mechanism, in which the poor conductivity of HNFs, through the immune response to further prevent the electron transfer, thereby increasing its resistance. 2) a catalytic mechanism, in which HNFs increases the enzyme activity and amplify the electrical signal by enzymatic reactions to detect the target.

In a study by Chandra Mouli and Gajjala, a label-free high-performance electrochemical impedance immunosensor was reported due to the HNFs possess stronger capture ability and the ability to block electronic transmission (Pandey et al.

2014). Based on this finding, HNFs were employed in a pathogens sensing platform. As a typical example, the novel flower and palmleaf like three-dimensional cystine microstructures were large scale prepared based on a simple and reproducible method (through a facile aqueous solution route as a function of pH and concentration). The novel three-dimensional cystine microstructures were used for the reliable determination of *E. coli (O157:H7)* with a linear range of 10 to 3×10^9 CFU mL^{-1}. In this work, monoclonal antibody-modified three-dimensional cystine microstructures were used as the biosensing platform with the enhanced sensitivity and stability, and it always exhibits higher surface coverage for the immobilization of antibodies and providing excellent electronic/ionic conductivity toward the three-dimensional cystine microstructures. Similar to the several of developed electrochemical impedance spectroscope bioassays, the faradaic biosensors depends almost entirely on measuring charge transfer resistance as a parameter in the electrolyte solution. By integrating the electrodes with HNFs, the electrochemical signals can be potentially enhanced due to the high chemical activity and good electrochemical performance of HNFs. Zhang and co-workers have realized the specific function of porous HNFs together with gold nanoparticles and different proteins or recognition unit for the detection of ractopamine(Zhang et al. 2015c). In a typical setup, they designed an electrochemical biosensor based on three kinds of HNFs: immunoglobulin-$Mn_3(PO_4)_2$ HNFs, ractopamine antibody-$Mn_3(PO_4)_2$ HNFs, and BSA-$Mn_3(PO_4)_2$-Au nanoparticles HNFs. After the three kinds of nanocomposites were dropped onto the gold electrode, the amount of ractopamine was evaluated by monitoring changes of electron-transfer

resistances through specific recognition of the antigen and antibody. Compared with the conventional method, the three kinds HNFs based electrochemical signals can be enhanced due to the HNFs possess a large specific surface area and poor electrical conductivity. This electrochemical sensor can detect ractopamine at extremely low concentrations down to 9.32, 4.6 and 26 pg mL^{-1}, respectively. Among them, the meaningful work showed two advantages compared to other types of electrochemical biosensors. The first advantage was that a large amount of ractopamine antibody loaded on the HNFs structure can enhance direct capturing of ractopamine. Another advantage was that the flower-like nanostructure of nanocomposites with excellent biocompatibility and high specific surface area provides bionic carrier and sufficient active sites for the attachment of ractopamine. To address concerns related to the detection of target by biosensor, research has focused on the use of aptamers as recognition units to augment the type of detection target and reduce inspection costs. As an example, a novel three-dimensional porous architectures $Co_3(PO_4)_2$-based nanocomposites were synthesized via a manageable self-assembly approach assisted by BSA and aptamers (He et al. 2016). A promising electrochemical impedance aptasensor for the sensitive platelet-derived growth factor-BB (PDGF-BB) detection was developed using the immobilization of PDGF-BB targeted aptamer by BSA-templated $Co_3(PO_4)_2$ nanoflowers as the sensitive layer. The use of targeted aptamer to identify the target molecule results in a change in the electrical signal to quantitatively detect the target, which is the beginning of the use of HNFs for electrochemical aptasensors. The 3D-nanostructured $Co_3(PO_4)_2$@BSA and

$Co_3(PO_4)_2$@Apt nanocomposites, known for their large surface active sites, good biocompatibility, and strong practicality, exhibit superior performance for the fabricated aptasensor which could detect PDGF-BB concentrations as low as 3.7 pg mL^{-1} and 61.5 pg mL^{-1}, respectively. The developed biosensors are also more sensitive than previously reported EIS sensors because of the large surface area of the 3D nanostructure, good biocompatibility, and strong practicality of the $Co_3(PO_4)_2$-based nanomaterials and the high affinity between the aptamer and nanocomposite. The observed difference was attributed to the loss of biological activity of the aptamer in the $Co_3(PO_4)_2$@Apt nanocomposite. In addition, $Co_3(PO_4)_2$@Apt was used not only as an adsorption matrix but also as a protein affinity enhancer. More significantly, the $Co_3(PO_4)_2$ HNF is a promising modified-electrode nanomaterial that can generate high-performance electrochemical impedance responses and enhance the detection signal for the designed assay in the future work. Simultaneously, Kong and co-workers reported the synthesis of a novel protein-inorganic hybrid nanocomposites, namely BSA incorporated Ag nanoflowers, as biomimetic three-dimensional nanoporous biosensing interfaces for electrochemical measurements of tumor cells and sialic acid on the DLD-1 cells surface (Cao et al. 2015). The attachment of DLD-1 cells to the BSA-incorporated Ag nanoflowers decorated glassy carbon electrode increased the electrochemical signals, electron-transfer resistance and stability, with a good correlation with the logarithm of the concentration from 1.35×10^2 to 1.35×10^7 cells mL^{-1}, and with a lower detection limit of 40 cells mL^{-1}. The BSA-incorporated Ag nanoflowers have a larger surface

area and an open nanoporous structure, providing an electrochemical sensing matrix that is better suited for cell sensor fabrication. First, a 3D porous structure promotes cell anchoring and increases the number of cells that adhere to the substrate. Second, the incorporated BSA molecule improves water solubility and maintains the biological activity of the biomolecule and reduces non-specific interactions, and provides a multifunctional interface for conjugation of the targeting molecule. Finally, Ag has excellent electrical conductivity, which can amplify electrochemical signals and improve cell biosensing ability. Because of these advantages, BSA-incorporated Ag nanoflowers are expected to provide an effective sensing platform for monitoring sialic acid changes on the surface of tumor cells. Then, this proposed electrochemical cytosensor reflected that it has the great white hope applications for the early monitoring of tumor cells and convenient evaluation of sialic acid on living cells. Inspired by this setup, researchers will design multi-component HNFs to detect multiple targets by surface-enhanced Raman signals.

In addition to the above applications, the coupling of enzyme-based amplification with electrochemical biosensors enables the convenient detection of target biomolecule through the combination of rapid, selective and high specific catalytic enzyme reactions and the sensitivity of electrochemical transducers. Since natural enzyme molecules are usually used as potential candidates for signal labels, this strategy is the most common and efficient method in the field of electrochemical biosensors. Due to high surface-to-volume ratio, not covalently modified, good biocompatibility, and powerful capture ability, the HNFs have become another

commonly used carrier for electrochemical biosensors. Very recently, Chen's group fabricated an electrochemical biosensor for the accurate and rapid detection of *Escherichia coli* in urine using horseradish peroxidase (HRP)-glucose oxidase (GOx)-$Cu_3(PO_4)_2$ HNFs as a label and achieved a large determination range from 15 to 1.5×10^8 CFU mL^{-1} with a considerably low detection limit of 1 CFU mL^{-1} (Li et al. 2018b). In the detection system that was designed, as shown in **Fig. 4**, the HNFs, Au NPs, T4 phage, thionine, and BSA were prepared for signal amplification by self-assembly. Then the AMP magainin I was immobilized on electrode surface and sealed with BSA through Au-S bonding interaction. After the immobilization of electrochemical labels, this platform could generate strong and stable electrochemical signal. Upon the specific recognition of T4 phages and the cascade effect of GOx-HRP-thionine-gold electrode electronic transfer system, the electrochemical signal increased thus the concentration of *Escherichia coli* could be determined. In this research, this biosensor offered several advantages, as follows: 1) The same concentration of antibody and AMP magainin I were immobilized on the electrodes of the presented biosensor, respectively, and the biosensor with AMP magainin I had higher current response. 2) The HNFs loaded a large amount of GOx and HRP and embedded thionine, which can catalyse two cascade redox reactions and a third redox reaction. Therefore, the electrochemical signal could be amplified obviously and remarkably. With these merits, the HNFs effectively amplified electrochemical signals, and the proposed electrochemical biosensor has momentous promising application in diagnosing and monitoring urinary tract infection in clinical settings. In

future studies, development of biosensors can be replaced with new recognition elements, such as antibodies or aptamers, so that novel HNFs-based sensing platforms can be used to detect corresponding targets, including toxins, cells, and tumor markers. In addition, researchers will attempt to construct a sensor set to detect multiple pathogens in the same sample with different specific recognition units. The use of novel HNFs for the construction of electrochemical biosensors will promote a new era of bioassays and medical analysis.

HNFs have great interest about their integration into electrochemical biosensor devices since they can improve the detection sensitivity and limit of detection values. They are proffered due to their easy synthesis and easy integration to any type of sensors. For example, metal nanoclusters or quantum dots are synthesized by proteins, which are used as precursors for the preparation of HNFs. HNFs will have good biocompatibility and electrochemiluminescent properties and will be used to construct electrochemiluminescent biosensors for the detection of disease markers. This will be the frontier of the development of electrochemical biosensors. In addition to these, integrating HNFs into different electrochemical biosensors using different signal amplification strategies will be an important advancement in analytical testing.

3.2. Colorimetric biosensors based on HNFs

The colorimetric method is an analytical method that is used to determine the concentration of colored compounds. The concentration of the compound in the solution can be estimated by measuring its absorbance at the appropriate wavelength using a colorimeter or a standard spectrophotometer. When there is a target, the

colorimetric sensor allows direct observation of the color change (Gopinath et al. 2014; Du and Dong 2016). Compared to other detection methods, colorimetric biosensors holds several superior advantages: (1) the colorimetric sensor has high sensitivity, good stability, and is easy to use; (2) they can minimize or even eliminate dependence on the analytical instrument; and (3) the price of colorimetric sensor is inexpensive, and a small number of objects cause visible color changes (Ma et al. 2016; Li et al. 2016a). Therefore, with the gradual discovery of nanomaterials and novel method, more and more colorimetric biosensors are used to detect metal ions, small molecules, pathogens and other substances (Kim et al. 2012; Wu et al. 2016a). For successful development of user-friendly colorimetric assays, development of signal probe is very important because colorimetric assays are basically designed for visual inspection. Since the discovery of HNFs, the new colorimetric biosensors used in detection of biological target molecules have been constructed.

HNFs have been used as a platform for immobilized enzymes in the past, because of the catalytic activity of enzymes that can produce the chromogenic substrates leading to significant changes in color for the detection of target. For instance, Zare's group designed a cellulose acetate membrane containing a suspension of laccase-$Cu_3(PO_4)_2$ $3H_2O$ HNFs for rapid and on-site detection of phenol, showing a linear relationship at a sample concentration of 0.4 to 50 μg mL^{-1}(**Fig. 5**) (Zhu et al. 2013b). The detection procedure is based on a film containing HNFs to cause fast oxidative coupling of phenol with 4-aminoantipyrine to form an antipyrine dye that has an UV/Vis absorption maximum at 495 nm. As a novel setup, laccase-inorganic

hybrid nanoflowers were first injected into a commercial disposable syringe filter equipped with a cellulose acetate membrane for phenol oxidation. This procedure thus deposited enzyme nanoflowers with an average size of 4 μm onto the membrane. Then the aqueous sample containing phenol was mixed with an aqueous solution of 4-aminoantipyrine and was passed through the membrane with incorporated HNFs, causing oxidative coupling of phenol with 4-aminoantipyrine to form an antipyrine dye that has an absorption maximum at 495 nm. The HNFs are deposited on the membrane by a filter injection method and presented a simple and effective detection method. This strategy utilizes a high concentration of bioactive enzyme porous membrane to provide a centralized signal interface that is beneficial to improve the detection sensitivity. In short, the proposed sensor shows us a series of advantages. First, the deposition of the HNFs on the membrane provided a simple but effective method to adapt to the repeated measurement of the HNFs. Next, a thin and porous membrane with a high concentration of active enzyme provided a concentrated signal transmissive interface for high sensitivity detection. Finally, the stability of laccase in HNFs is enhanced compared to natural counterparts in natural forms, ensuring high repeatability. In addition, the HNFs achieved the outstanding signal transduction and amplification. As hydrogen peroxide can be produced by GOx-biocatalyzed oxidation of glucose yields or depleted by HRP oxidation reaction, the hydrogen peroxide-related biocatalytic reaction could be monitored by colorimetric sensing system. For example, Ge et al. demonstrated the synthesis of a space-based multi-enzyme system based on inorganic nanocrystal-protein hybrid nanocomposites

by a simple co-precipitation method (Li et al. 2014). The HNFs were prepared by HRP and GOx as a model enzyme for the cascade reaction to construct colorimetric sensors. In this colorimetric biosensor format, glucose and oxygen molecules first migrate to GOx on the shell, while GOx-catalyzed reaction product hydrogen peroxide was then directed to HRP in the core of the nanocomposites. The substrate and intermediate concentration gradients assist drive and promote the cascade of GOx-HRP to produce highly effective colored products. The ease of operation and rapidity of analysis makes this method available for the production of a multi-enzyme system with restrictive spatial distribution of enzymes, thus making it possible to improve the performance of biosensors in diagnostics, and biomedical devices with promised applications. Related to a similar scheme, Lin et al. developed a colorimetric platform based on enzyme-inorganic hybrid nanoflowers for the detection of hydrogen peroxide and phenol and explored several parameters that affect the formation of HNFs (Lin et al. 2014b). Using HRP as a signal label, the target can convert a weakly absorbed phenol and hydrogen peroxide to a highly absorbed quinone imine, which leads to a high signal-to-noise ratio for the colorimetric determination. In this typical experiment, HNFs not only have a large surface area to immobilize more enzymes and improve the catalytic performance and stability of the enzyme, but also have rapid response and reusability. When the target is detected, the response speed and sensitivity are improved and have greater potential in biomedical and environmental chemistry. In another work, Wang's group prepared HRP and GOx co-embedded enzyme-inorganic hybrid nanoflowers for glucose

detection by the coinstantaneous catalysis of multi-step cascade enzymatic reactions (Sun et al. 2014). The multi-enzymes co-embedded cascade enzymatic effect shortened the detection time and improved the utilization of the enzymatic hydrolysis product. In this catalytic detail, the GOx component in the HNFs first oxidized glucose to generate hydrogen peroxide, and then the generated hydrogen peroxide was immediately catalyzed by the adjacent HRP to oxidize 3, 3', 5, 5'-tetramethylbenzidine (TMB), resulting in an apparent color change from clear to blue. In view of closed proximity of the two enzyme components in single HNFs, this new colorimetric sensor greatly reduced the diffusion and decomposition of hydrogen peroxide and significantly improves the sensitivity of glucose detection. With the increase of glucose concentration, the absorption peak at 652 nm was significantly increased by UV-Vis spectroscopy. When the glucose concentration was linear in the range of 0-50 μM (R = 0.993), and the detection limit was 0.2 μM. The homogeneous solution colorimetric method mainly uses the color change to generate the signal. The key is to design the clever color changing principle and the appropriate color changing reagent. HNF is an excellent carrier for enzymes, has a large specific surface area to provide a large number of active sites and can improve the activity and stability of the enzyme, which will lead to more development directions in the future than the colorimetric method.

Vibrantly enzymatic nanomaterials have been indicated to be significant elements in the analytical performance of enzyme linked immunosorbent assay (ELISA). Lin's group devised an ultrasensitive colorimetric immunosensing platform

on the basis of antibody-enzyme-inorganic hybrid nanoflowers using HRP as signal amplifiers (Wei et al. 2016). In this detection system, HRP possessed the enzyme activity to specifically catalyze TMB to generate blue substance in the presence of hydrogen peroxide, which was employed as an efficient signal transduction vectors in the ELISA platform. Antibody-HRP-inorganic hybrid nanoflowers and TMB are integrated into the colorimetric immunosensor through the sandwich immunoreaction and the TMB-catalyzed blue substrate could be used for UV/Vis detection. Due to the appropriate color signal differentiation, this judicious approach achieved the determination of *E. coli O157:H7* in tap water and lake water, which represents a potential application in the detection of food pathogens. In addition, this approach inspired us to select different assay modes based on the enzyme chosen, rather than a colorimetric assay that resides on the HRP-based HNFs. It will be very meaningful to open up new applications based on HNFs. It is worth noting that, antibody-enzyme-inorganic nanoflowers could potentially replace the common existing enzyme-labeled antibody method in ELISA and will have meaningful prospects in the practical detection of other pathogenic bacteria or disease markers. Using the analogous strategy, Liu's et al. synthesized a kind of dual-functional streptavidin-HRP-inorganic hybrid nanoflowers for the ultrasensitive detection of alpha-fetoprotein (Liu et al. 2017a). The HNFs integrated the functions of biological recognition and signal amplification, namely, streptavidin was selected as the recognition unit and HRP serves as the signal amplification unit. As shown in **Fig. 6**, the construction of the colorimetric sensor platform is first to raise the capture

antibody in a 96-well high-binding ELISA plates, then capture different concentrations of the target, next incubate the biotin-labeled secondary antibody, and finally form antibody-enzyme-$Cu_3(PO_4)_2$ flower-like structure, which was used as signal amplifier label. The bifunctional HNFs were prepared via a self-assembly method and the biological substances (streptavidin and HRP) employed in the synthesis suffer from less manipulation compared with other conventional methods to maintain the activity of the immobilized protein. Among them, HRP, a ferriporphyrin-containing biological enzyme, which serves as the core of the signal amplification unit, could effectively catalyze the oxidation of TMB to a blue-colored product in the presence of hydrogen peroxide. Streptavidin，a biomolecule with the ability to specifically bind biotin, is an important linker, which provides new research ideas for HNFs as signal labels. The synthesized streptavidin-HRP-$Cu_3(PO_4)_2$ HNFs loaded abundant streptavidin and HRP exhibited specific binding force and perfect signal tag. Furthermore, dual functional HNFs simultaneously illustrated enhanced catalytic activity, outstanding stability and durability compared to free enzymes, which fits greatly well with the requirement of signal tag for bioassay. This strategy immobilized a large number of HRP for signal amplification by the specific recognition of antigen-antibodies and the binding of biotin-streptavidin, which was used for the detection of alpha-fetoprotein with an excellent performance. Under the best experimental conditions, this detection method demonstrated a very low detection limit of 78 pg mL^{-1}, which is far superior to commercial ELISA kits. Moreover, the bifunctional streptavidin-HRP-$Cu_3(PO_4)_2$ HNFs preparation strategy

described in this work can be extended to many other hybrid systems. In addition to HRP, other types of enzymes can be used as signal amplification units for hybrid systems. By changing the enzyme, different signal outputs will be an alternative to colorimetric detection. Furthermore, based on the general streptavidin-biotin binding system, the strategy constructed has the potential to provide a wide range of methods for medical diagnosis and biotechnology by utilizing different recognition elements such as aptamers and peptides.

In summary, there are two main types of HNFs-based colorimetric sensors: homogeneous solution systems and sandwich structure interface systems. Some important issues and challenges should be addressed. First, stable preparation of high quality of HNFs in large area for applications in industrial-scale is still challenging. Besides, HNFs-based colorimetric sensors still a lack of stable and reliable signal output for multi-component detection. But there are some classic methods, such as enzyme-induced metallization, enzyme-induced metal mineralization, etc., that can be combined with HNFs to build a new colorimetric sensor. This will greatly expand the development direction of colorimetric biosensors.

3.3. Point-of-care diagnostic devices based on HNFs

Over the past few years, with the development of digital technology and nanomaterials, the preparation of biosensors based on point-of-care has attracted the interest of engineers and researchers. Portable devices such as smart glasses, 3D printers, paper machines, wearables sensors, smartphones, pH meters, and glucose

meters provide real-time readings and use multiplexing measurements using equipment to quantify test results. Point-of-care (POC) equipment is cheap, easy to use, fast and independent of the instrument, and for clinical biomarkers and other analytes to provide a powerful, portable, reliable, fast, cheap and simple detection (Petryayeva and Algar 2015; Zarei 2017; Justino et al. 2013). POC testing have many advantages over laboratory-based standard diagnostics: such as greater availability in resource-constrained areas, and evolving technological advances that make it possible for new POC testing instruments (Nayak et al. 2017; Zhang et al. 2016e). Now, the main challenge is to amplify and convert the biomolecule recognition signal into a readable digital signal by a simple biometric analyzer. To address this issue, a new type of HNFs has been explored to build novel biosensors for POC testing that have stirred a wide range of concerns. Thus, an inexpensive diagnostic platform for converting to digital signals using portable devices for reliable measurements includes pH meters, glucose meters, smart phone and microfluidic system. Here, we focus on the recent research on POC testing platforms based on HNFs.

pH Meter.

So far, the pH meter has been an excellent device for POC testing. It has the characteristics of low cost, simple operation and portability. In a pH meter-based biosensor, the key signal transduction of sensor was the conversion of the target concentration into hydrogen ion concentration response signal, including glucose to gluconic acid, acetylcholine to acetic acid, urea to ammonia, etc., followed by the utilization of a pH meter, pH paper strips and litmus dyes to provide a reliable

numeric readout or a diacritical color change. The GOx, acetylcholinesterase and urease acted as powerful tags as they could efficiently catalyze the substrate to generate alkaline or acidic substance that could elicit the response of litmus dyes, pH meter or pH paper strips in the fabrication of a biosensor. Thus, the analytical performance of the biosensor was in connection with the activity of the enzyme and the sensitivity of the litmus dyes, pH meter or pH paper strips, even pH-responsive probes. Recently, Lin's group constructed a biosensor by combining concanavalin A (Con A)-GOx-$CaHPO_4$ hybrid nanoflowers with a pH meter or pH indicator, which integrated bio-recognition unit (namely Con A), signal amplification unit (namely GOx), and carrier unit within a self-assembly procedure for the rapid and sensitive analysis of food pathogens (Ye et al. 2016a). As indicated in **Fig. 7**, the principle of detection was to construct a sandwich-structured biosensor that used HNFs as a signal tag to catalyze glucose to produce gluconic acid to cause a change in pH, thereby performing signal transduction for accurate determination of *Escherichia coli O157:H7*. In this system, firstly, the HNFs were prepared via a bio-mineralization process by GOx, Con A and $CaHPO_4$ solution. Next, in order to investigate that both glucose oxidase and Con A were immobilized on HNFs, GOx and Con A were fluorescently labeled with cyanine 5 and fluorescein isothiocyanate, respectively, to prepare the HNFs. At last, the readout signal was generated by the resulting decrease in the pH originating from the GOx-catalysis of glucose into gluconic acid after the sandwich immunoreaction of *E. coli O157:H7*. Conspicuously, Con A was selected as bio-recognition unit for its high affinity to lipopolysaccharides O-antigen of

Escherichia coli O157:H7. GOx was elected to signal amplification unit owing to the catalytic activity to efficiently convert glucose to gluconic acid resulting in a decrease in pH, which is easily measured with pH meter or pH indicator strip. All experimental results revealed successful fixation of GOx and Con A in HNFs and also confirmed that the immobilized Con A molecule indirectly has binding sites exposed on the HNFs surface. Their large amount of work showed us the self-assembly procedure for the clearing of HNFs, which provided a profound guiding significance for further exploration in the future. But beyond that, under the optimal conditions, the proposed biosensors retained an excellent detection performance of *E. coli O157:H7* with the linear range of 10 to 10^6 CFU mL^{-1}, and a detection limit of 10 CFU mL^{-1}. Employed the as-prepared all-in-one hybrid nanoflowers as signal tags, a simple but potentially powerful amplification biosensing technology with excellent simplicity, portability, sensitivity, and adaptability has been achieved. However, one of the challenges in the assay is that the hydrogen peroxide produced will inhibit the activity of GOx. To solve this problem, hydrogen peroxide can be removed by adding catalase (or nanozyme) or increasing the appropriate reaction time. This is a highly significant study that may increase the systematic understanding and technological development of the bio-mineralization process for the synthesis of extremely innovative integrated HNFs, and further up-build signal amplification principles for a variety of bioassay applications in field of food, medicine, and laboratory science.

Glucose Meter.

In a glucose meter-based biosensor, the key to adapting the glucose meter to measure a wide range of targets is to incorporate an intermediate step linking the binding of either recognition units (such as, antibody, aptamer, nanobody, etc.) to its target with the production of glucose using invertase, which is capable of hydrolyzing carbohydrate to glucose. Another key challenge in this endeavor is to find a feasible way to translate recognition of many targets into an easily measurable glucose signal using a glucose meter. In another design, invertase was used to replace the glucose oxidase in Con A-GOx-$CaHPO_4$ hybrid nanoflowers. As a result, a one-pot green method for bioinspired synthesis Con A-enzyme-$CaHPO_4$ nanoflowers has been developed by Lin's group (Ye et al. 2016b). The Con A-invertase-$CaHPO_4$ hybrid nanoflowers integrate both essential functions of biological recognition (namely, Con A specific recognition of *E. coli*) and signal amplification (namely, invertase converts the substrate to glucose), and they are used as model signal labels for sensitive real-time detection of *E. coli O157: H7* in the milk. In the research works, invertase serves as the signal output component in the HNFs correlation of the biorecognized *E. coli O157:H7* with the concentration of hydrolyzed glucose, read by a portable personal glucose meter. The proposed biosensors exhibit excellent performance for detecting *E. coli O157:H7*, with the sensitivity detection limit of 10 CFU mL^{-1} and the linear range of 10 to 10^5 CFU mL^{-1}. Since majority of commercially available enzymatic assay kits utilize GOx in their detection, this discovery will allow the transformation of almost all of these clinical lab tests into POC tests that use a glucose

meter. Thus, the simplicity, portability, sensitivity and low cost of presented work can be generally employed to prepare a variety of enzyme-antibody hybrid materials for various applications, which will make good contributions for pushing the frontier of current research forward in molecular recognition, analytical sciences, material science and engineering, and biomedical engineering areas. As expected, this approach can be generally employed to prepare a variety of protein-protein conjugates for various applications, which will make good contributions for pushing the frontier of current research forward in areas.

Smartphone.

With the widespread use of smartphones in many of our modern times, researchers have attempted to develop miniaturized biosensors for smartphones, and reduce the cost of the system with simplified the electronic design. Biosensors based on smartphones will also enable real-time monitoring and messaging of digital networks. Zeinhom et al. established a highly sensitive novel type of magnetic nano-biosensor for the visual and quantitative detection of *Salmonella enteritidis* from milk, cheese and water (Zeinhom et al. 2018). The samples inoculated with different concentrations of *Salmonella* were tested by using streptavidin magnetic beads labeled with a biotin-labeled antibody as a capture platform and coupled with antibody-HRP-inorganic hybrid nanoflowers. This method was based on signal amplification of HRP-HNFs and produced a visual color that is easily detectable at very low concentrations by smart phone devices. The enhanced signal of the smartphone has the low detection limit of 1.0 CFU $mL^{-1}$and 1.0 CFU g^{-1}. In addition

to the use of magnetic antibody-based system capture antibodies for *Salmonella* target detection, for validate the detection method, we took advantage of smartphone based on 3D printing for optical detection. Compared with the normal microplate reader, smartphone device gives similar accuracy and sensitivity in the detection of *Salmonella*. This study provides a convenient and promising tool for on-the-spot detection of food-borne pathogens without the need for costly micropulture readings, providing a good future for development.

Microfluidic system.

As a widely applicable approach, microfluidic platforms possess a variety of characters, including low-cost, lightweight, portable, time-dependent and energy efficient, making them ideal for analysis and testing in low-resource environments worldwide. Owing to the sensitivity of enzymes to environmental conditions, it is also an important issue to immobilize the enzyme into the fabrication process of the system (Mross et al. 2015; Xia et al. 2016). In view of these advantages and challenges, microfluidic paper-based analytical device based on HNFs have emerged. For example, Salinas-Castillo's group investigated a 3D microfluidic paper-based analytical device for the accurate determination of glucose using the multi-enzyme HNFs to immobilize the HRP and GOx (Ariza-Avidad et al. 2016). This biosensor is based on bi-enzyme HNFs supported on cellulose paper coupled to 3, 3', 5, 5'-tetramethylbenzidine as the colorimetric probe in the detection zone and used the S-coordinate of the HSV color space of the digital camera as the analytical parameter for the quantitative analysis of glucose with an acceptable detection limit of limit of

15.6 μM. The co-immobilization system retains its activity compared to the free enzyme, making it reusable and stable for 75 days under conventional storage conditions. All of these possible applications for biosensors, as well as new strategies to improve reusability, long-term stability and low cost (estimated cost of 20 cents per unit, considering reuse), will be the subject of future work. This research opened a new platform for the application of HNFs. Inspired by the microfluidic paper-based analytical device, a novel biosensor was developed by Yu's group for sensitive and visualized detection of glucose in the concentration range of 0.1 to 10 mM with a detection limit of 25 μM (Zhu et al. 2017). In a more facile approach, this biosensor would be constructed from the HNFs consisting of GOx, HRP and $Cu_3(PO_4)_2$ by self-assembly using the wax printing technology in which the HNFs are deposited in the detection zones. The microfluidic paper-based measuring device containing GOx-HRP-$Cu_3(PO_4)_2$ hybrid nanoflowers retained the excellent properties of the HNFs and improved the detection performance in the field of research and diagnosis. In addition, the design of microfluidic paper-based devices provided very low cost and contrast as well as colorimetric and visual detection. Thanks to these advantages, the composed method may have a bright future for the low-cost and sensitive monitoring of glucose in real-world clinical point-of-care tests. The combination of HNFs and microfluidic paper-based devices with better performance has drawn enhanced attention owing to its stability and catalytic activity and its simple fabrication process. The huge varieties of HNFs mentioned above can serve for many different functions to enable biosensing. Encouragingly, Li et al. developed

enzyme-inorganic hybrid nanoflowers based paper strips that were constructed with a visual colorimetric microfluidic paper-based analytical device for glucose detection (Li et al. 2018a). The biosensor was constructed following the in situ growth of enzyme-inorganic hybrid nanoflowers on the paper matrix. In situ grown GOx-$Mn_3(PO_4)_2$ hybrid nanoflowers on the paper strip specifically detected glucose in complex biological samples, thus demonstrating its potential for achieving rapid on-site glucose detection. The ability of natural and artificial enzyme hybrids to intercalate μPAD was verified by a glucose test based on colorimetric detection. The efficient growth of enzyme-inorganic hybrids on paper is an innovative method for efficiently loading enzymes on the biosensing surface. The different kind of enzyme-inorganic hybrid nanoflower growth on paper strip will provide a new idea for the timely monitoring of other small molecules such as glucose, uric acid, etc.

4. Conclusions

This paper provides an up-to-date review on HNFs for biosensing application. Since the research on HNFs started in 2012, the research on this topic has engendered much interest for investigating various types of protein and anime acid nanoflower composites. With the emergence of novel excellent properties of HNFs, as well as various new signal amplification methods, all-in-one hybrid nanoflowers have paved the way for advancement of highly sensitive bioassays. Especially, the outstanding properties of HNFs have shown enormous potential for the development of biosensors with attractive and bright performance in term of capture ability, excellent catalytic activity and reliability. The introduction of enzyme into the HNFs allows the

enhancing of the properties of antibody and enzyme, thus providing high performance new materials with advanced novel function. Furthermore, the applications of HNFs have been spread to bioassays, nanoreactors, biosensors and diagnostics due to the behaviors of energetic capture ability, enhanced catalytic performance and splendiferous stability.

Here, we have discussed HNFs that have been designed and successfully applied in biosensors. Based on the literature discussed in this review, the novel HNFs can exhibit the following advantages: (1) The integration of proteins (antibody, enzyme) into the HNFs have greatly improved the specific surface area of the novel materials, which results in higher accessibility to the recognition sites, larger binding ability and faster catalytic rate. (2) In terms of protein-protein linkage, the effect of the modified protein on its activity is avoided. (3) Compared with the free form of enzyme, the HNFs have enhanced enzymatic activity and stability due to its unique hierarchical nanostructure. In addition, in the sandwich system, HNFs as a novel and fascinating nanomaterial have many remarkable properties. The detection signals can be amplified by the catalytic function of the enzyme to improve the detection sensitivity. The all-in-one hybrid nanoflowers have multiple functions: (1) HNFs enhance the catalytic activity, durability and stability of the enzyme, which produced an amplified signal. (2) In the double enzyme system, the HNFs make the double enzyme close to reduce the diffusion and decomposition of the substrate, greatly improves the reaction efficiency. (3) HNFs have a huge surface area that could load more enzymes and antibodies. (4) Using all-in-one nanoflowers as enzyme-labeled antibodies, the effect

of modified enzymes and antibodies on their capture ability and activity was avoided and the HNFs part of the antibody retains and enhances the specific ability to capture the corresponding antigen. Therefore, HNFs have a strong prospect for biosensing application.

5. Outlooks and perspectives

With the development of life science and biomedical science, the detection of low-abundance proteins and the acquisition of ultra-weak biological signals have been become a bottleneck of these fields. We predict a bright future for HNFs-based biosensors owing to their unique physcial and chemical properties. By introducing functionalized HNFs, new technologies, and target DNA recycling amplification, it has been used to amplify electrochemical, optical and visual signals. In interface systems, HNFs can be used as excellent signal probes to amplify signals. The high catalytic activity of HNFs enables the determination of trace amounts of target and greatly contributes to signal amplification. For example, ELISA, an immunodiagnostic technique, uses the enzyme-labeled antibody as one of its critical components. The enzyme-antibody-inorganic three-in-one nanoflower showed advantages over the commercial enzyme-labeled antibody. First, it is much easier to prepare with no need for any complex covalent modification. Second, it is an aggregate of numbers of antibodies and enzymes, so it has fairly high captive ability and catalytic activity. The methodology proposed here could potentially replace the common existing enzyme-labeled antibody method in ELISA and will have

significant prospects in the practical detection of clinically relevant molecules. In addition to ELISA, the other detection techniques (such as electrochemistry, surface enhanced Raman spectroscopy, and surface plasmon resonance, etc.) can also be applied to biosensors. In homogeneous solutions, HNFs can be used as highly efficient catalysts for the detection of biological samples. For example, laccase is capable of oxidizing catecholamines-including epinephrine, norepinephrine and dopamine-to coloured quinone type products detectable by colorimetric or fluorescent methods. Due to the higher catalytic activity of HNFs, a visual comparion of free laccase- and HNFs-catalysed detection of epinephrine shows a higher sensitivity when using HNFs. Moreover, phenols are common water pollutants, most being produced from the wastewater streams of various industries. In another application, the oxidative coupling of phenols with 4-aminoantipyrine to form antipyine-dyes was used to evaluate the catalytic activity of the HNFs for the detection of phenolic compounds or phenol derivatives (such as phenol phosphate, phenolgalactoside, etc). Taking advantage of the excellent molecular biological properties provided by all-in-one hybrid nanoflowers, prominent immunoassay instruments (such as wearable chemical sensors, test strip and microfluidic chips etc.) can be further developed and integrated into smartphone, hand-held devices and mini-computers for real-time monitoring in near future. Furthermore, HNFs allow us to develop small biosensing systems that can be used to simultaneously detect multiple analytes in clinical diagnostics, environmental monitoring and food safety. Portable meters are used for glucose testing, but for other electronic technologies, small instruments need to be

developed that can be easily operated by non-experts. Therefore, there is still a long way to go to address the miniaturization of biosensors and integrate them into the lab to achieve real-time online detection. We predict that with the development of nanomaterials and the revolution in sensor technology, HNFs-based biosensor devices, including sensitivity, long-term stability, selectivity, reproducibility, operability and micro-analysis will be further improved, thus opening up new method for efficiently and reliably detecting biomarkers in clinical diagnosis.

Even though HNFs based biosensors have a desirable advantages in analyte detection, there are also some rigorous challenges to be determined in future research. In particular, most of the biosensors remain at the level of exploring proof-of-concept and can't be applied in real-world detections and diagnostics. Although considerable efforts have been made to develop promising HNFs-based sensors, their practical application in actual samples is still in its infancy. Some important issues and challenges should be considered regarding this issue. The first issue is to explore the interaction between organic and inorganic components, which may provide some principles in designing HNF with highly maintained biological activity. The second factor to be noted is a nucleation event that does not quantify inorganic components in the presence of enzymes and antibodies at different concentrations. Most of research papers are prodigiously focused on the stabilizing and catalyzing effect brought by HNFs, which is mainly due to the scarcity of the corresponding HNFs in the formation mechanism. The third factor is how to synthesize HNFs with a relatively narrow size distribution because any inhomogeneous distribution of the signal tags

will reduce the accuracy of the analysis. The fourth problem is how to control the performance of each batch of synthesized nanoparticles with similar properties, such as size, activity, stability, etc. There remain issues to be addressed before the full potential of such HNFs for biosensing applications can be achieved. Moreover, another compelling research direction is the preparation of HNFs with embedded different carbon materials (graphene, g-C_3N_4, CNT, etc.) and emerging materials (nanozyme, black phosphorus, etc.). Peptides, nucleic acids and other organic components combined with different metal ions to form the new functional HNFs are also a promising direction. In addition to research of new compositions and biological activity, attempts to further improve the analytical performance of multifunctional HNFs at the current juncture. In a word, we really believe that the study of HNFs has entered a new era where the molecular recognition ability of the new recognition elements, including artificial antibodies, nanobodies, peptide and protein receptors, etc., permit designing unique biosensors with unprecedented analytical performances. To sum up, we envision that HNFs will certainly play significant roles in future biosensing.

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Conflict of interest

Authors report no conflict of interest on their parts.

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Fig. 1: Schematic illustration for biosensing applications based on organic-inorganic hybrid nanoflowers.

Fig. 2: Schematic diagram of the formation mechanism of lipase/$Zn_3(PO_4)_2$ hybrid nanoflowers, reprinted with permission from Zhang *et al.*, copyright (2016) Elsevier.

Fig. 3: Schematic synthesis of the activated α-amylase-$CaHPO_4$ hybrid nanoflowers preparation. (A) Ca^{2+} binds to allosteric site in inactive α-amylase and generates active α-amylase. (B,C) α-amylase immobilized in thinner or thicker $CaHPO_4$ nanocrystals, reprinted with permission from Wang *et al.*, copyright (2013) American Chemical Society.

Fig. 4: Schematic diagram of the preparation of the hybrid nanoflowers and the electrochemical detection of *Escherichia coli*, reprinted with permission from Li *et al.*, copyright (2018), Elsevier.

Fig. 5: Fabrication of the colorimetric biosensor and the reaction mechanism, reprinted with permission from Zhu *et al.*, copyright (2013) Nature Publishing Group.

Fig. 6: (a) Synthesis process of SA-HRP hybrid nanoflowers. (b) Schematic illustration of ultrasensitive ELISA for AFP detection based on the dual-functional hybrid nanoflowesrs, reprinted with permission from Liu *et al.*, copyright (2017) Elsevier.

Fig. 7: Illustration of the synthetic process for Con A-GOx-CaHPO4 organic-inorganic hybrid nanoflowers and the corresponding scheme for immunoassay of *E. coli O157:H7*, reprinted with permission from Ye *et al.,* copyright (2016) Wiley-VCH.

Table 1. Organic-inorganic hybrid nanoflowers based on the types of metal ion and organic components used.

Metal ion	Organic component	References
Cu^{2+}	Protein (BSA; Egg white; β-Lactoglobulin, α-Lactalbumin; Soy protein isolate; Antibody)	(Altinkaynak et al. 2018; Huang et al. 2015; Ge et al. 2012; Lang et al. 2017; Luo et al. 2017; Wang et al. 2018; Wei et al. 2016; Xie et al. 2016)
	Enzyme (Laccase; Lipase; Carbonic anhydrase;	(An et al. 2016; Altinkaynak et al. 2016b; Altinkaynak et al. 2017; Ariza-Avidad et al. 2016; Athena A.

	α-glycosidase; Esterase; L-arabinitol 4-dehydrogenase, NADH oxidase; HRP; Catalase; GOx; Urease; Soybean peroxidase; Papain; Trypsin; L-arabinose isomerase; Glucoamylase; Lactoperoxidase; Turkish black radish peroxidase; Lysozyme)	Papadopoulou 2017; Batule et al. 2015; Chung et al. 2018; Cui et al. 2016; Duan et al. 2015b; Ge et al. 2012; He et al. 2015; Hua et al. 2016; Jiang et al. 2018; Kong et al. 2018; Lang et al. 2017; Lee et al. 2017; Li et al. 2014; Li et al. 2016b; Li et al. 2016c; Li et al. 2016d; Li et al. 2017; Li et al. 2018b; Liang et al. 2015; Lin et al. 2014a; Lin et al. 2014b; Liu et al. 2017a; Luo et al. 2017; Nadar et al. 2016; Patel et al. 2017; Patel et al. 2018, Qian et al. 2018; Rong et al. 2017; Somturk et al. 2015; Somturk et al. 2016; Sun et al. 2014; Thawari and Rao 2016; Wang et al. 2018; Wei et al. 2016; Wu et al. 2017; Xu et al. 2016; Yu et al. 2015; Zhu et al. 2013b; Zhu et al. 2017)
	Others (Amino acids; DNA; Green tea(caffeine, catechin); Cytochrome; (Poly)dopamine; Streptavidin; Biosurfactant)	(Baldemir et al. 2017; Cui et al. 2016; Duan et al. 2015a; He et al. 2017; Jiao et al. 2017; Li et al. 2016d; Liu et al. 2017a; Park et al. 2017; Wu et al. 2016b; Zhang et al. 2017b)
Ca^{2+}	Enzyme (α-amylase; GOx; Chloroperoxidase;	(Liu et al. 2017b; Ye et al. 2016a; Ye et al. 2016b; Yin et al. 2015;

	α-acetolactate decarboxylase; α-chymotrypsin)	Zhao et al. 2017)
	Protein (Antibody)	(Ye et al. 2016a; Ye et al. 2016b)
	Others (Gelatin; Chitosan; Peptide)	(Ghosh et al. 2014; Tian et al. 2017; Wang et al. 2014; Zhao et al. 2016)
Mn^{2+}	Protein (IgG, antibody; BSA	(Zhang et al. 2015b; Zhang et al. 2015c)
	Enzyme (GOx)	(Li et al. 2018a)
Co^{2+}	Protein (BSA; His-tagged proteins)	(He et al. 2016; Kim et al. 2016; Lopez-Gallego and Yate 2015)
	Enzyme (Nitrile hydratase)	(Song et al. 2017)
Zn^{2+}	Protein (BSA)	(Zhang et al. 2016b)
	Enzyme (Papain; Lipase)	(Zhang et al. 2016a; Zhang et al. 2016c)
Fe^{2+}	Enzyme (HRP)	(Ocsoy et al. 2015)
Cu^{2+}, Fe^{2+}, Ag^{+}, $HAuCl_4$	Enzyme (Lipase)	(Sharma et al. 2017)
Ca^{2+},Cu^{2+},Zn^{2+}	Enzyme (Lipase)	(Escobar et al. 2017)
Ca^{2+},Cu^{2+}	Elastin-like polypeptides	(Ghosh et al. 2014)
Others inorganic	DNA, RNA, BSA, dopamine, etc.	(Cao et al. 2015; Guo et al. 2017; Hu et al. 2014; Kim et al. 2017; Lv et al. 2015b; Mei et al. 2015; Pandey et al. 2014; Zhang et al. 2015a; Zhang et al. 2017a; Zhu et al. 2013a)

Highlights:

1. Organic-inorganic hybrid nanoflowers type, characteristics and application has been discussed comprehensively.
2. A novel method of tagged for biosensor designing incorporating organic-inorganic hybrid nanoflowers has been described in detail.
3. Biosensing of various target molecules by using organic-inorganic hybrid nanoflowers based electrochemical biosensor, colorimetric biosensor, and point-of-care diagnostic device has been discussed.
4. Comparative analysis of organic-inorganic hybrid nanoflowers based biosensor performance and perspective has been discussed briefly.

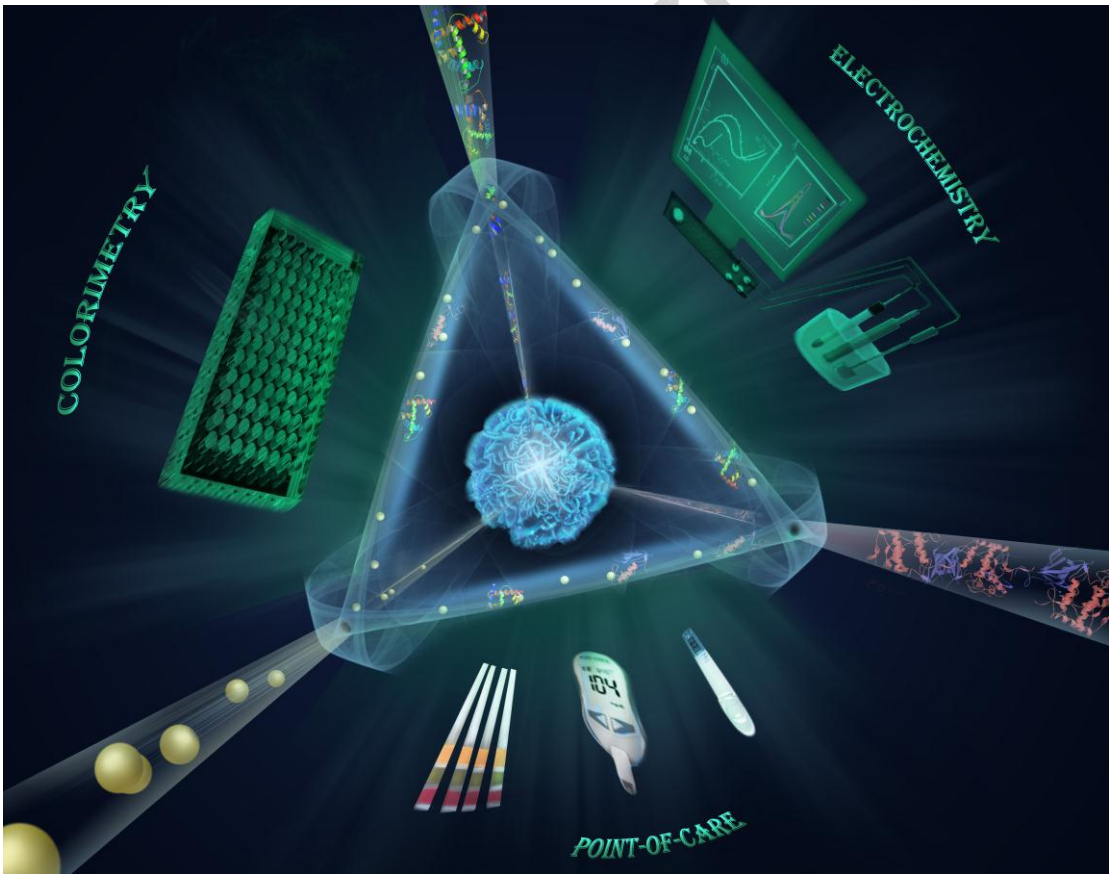

Fig. 1

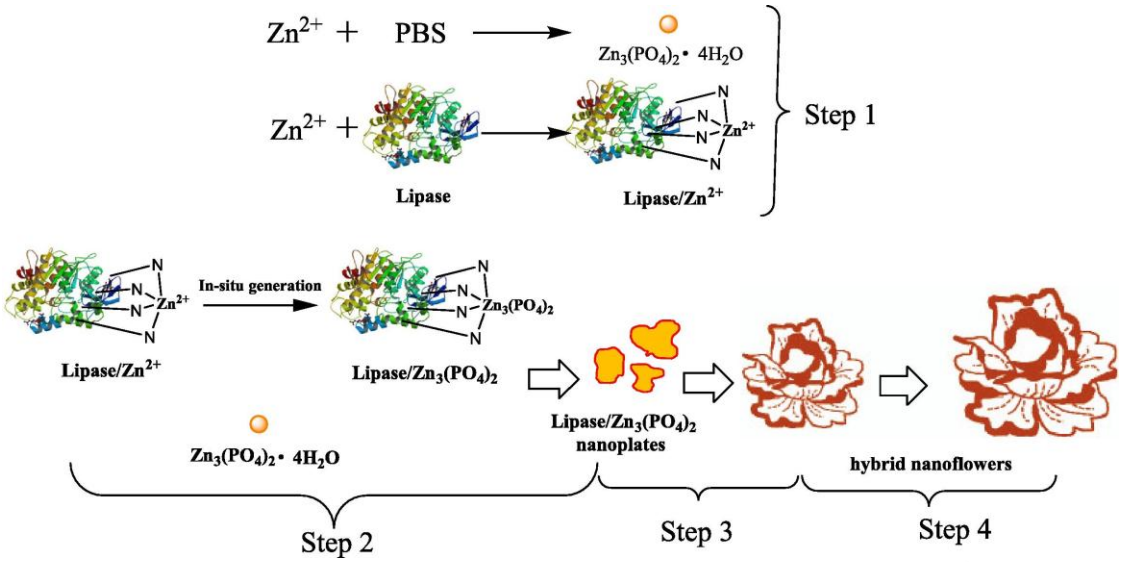

Fig. 2

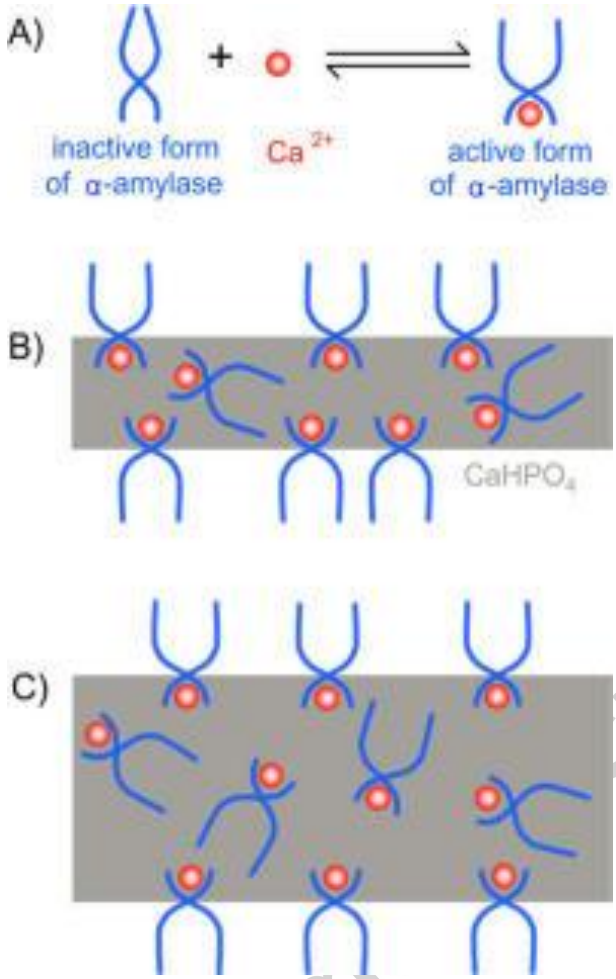

Fig. 3

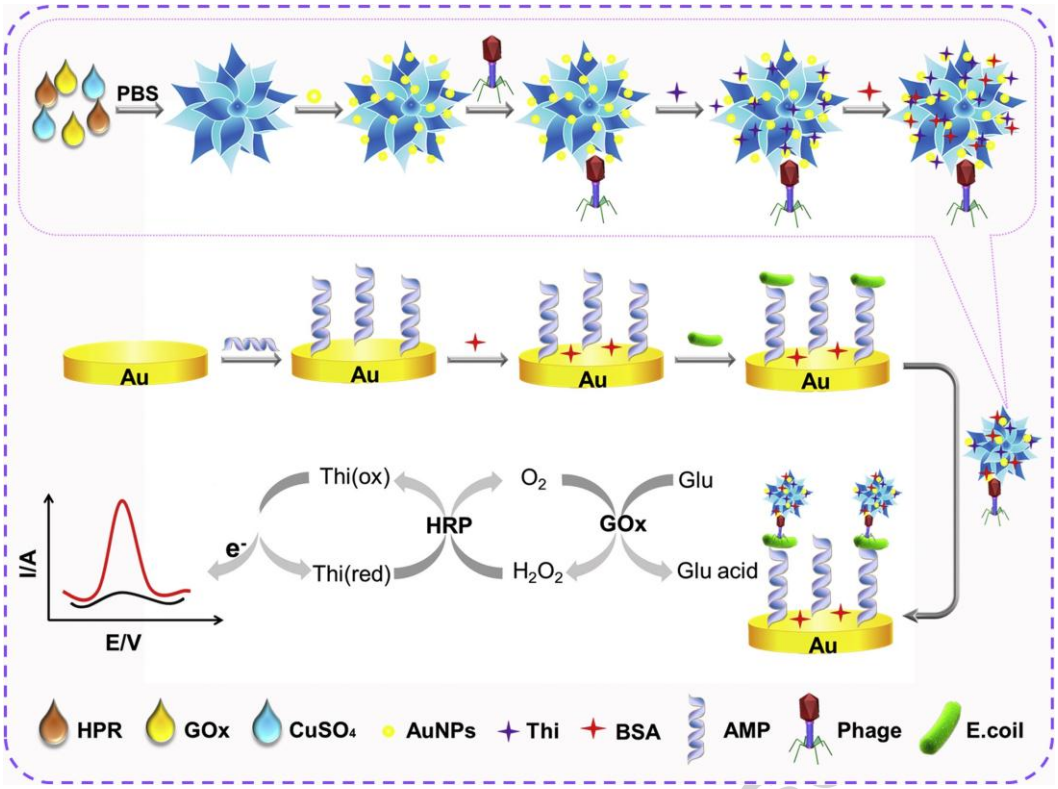
PBS
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Fig. 4

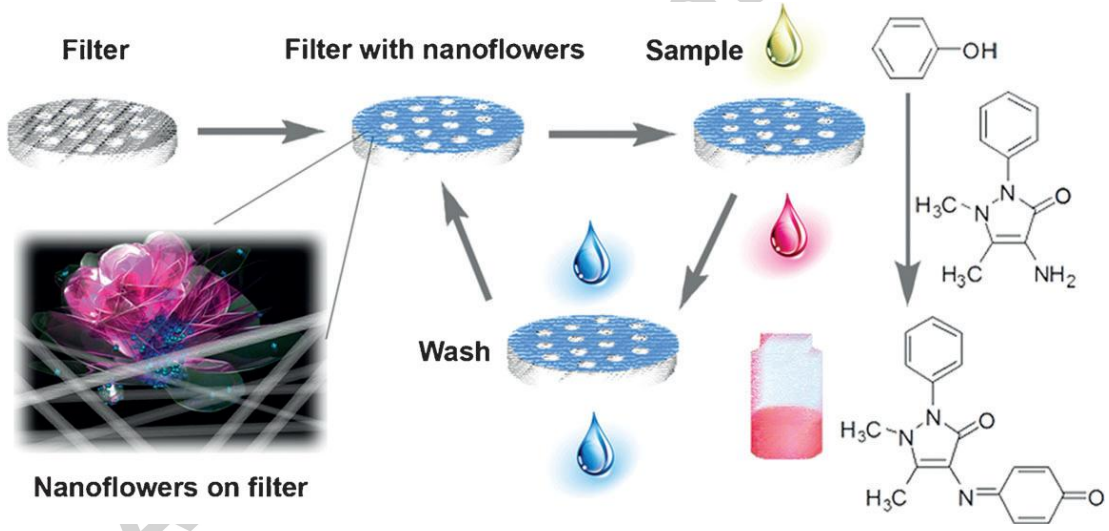
Filter
Filter with nanoflowers
Sample
OH
Wash
Nanoflowers on filter

Fig. 5

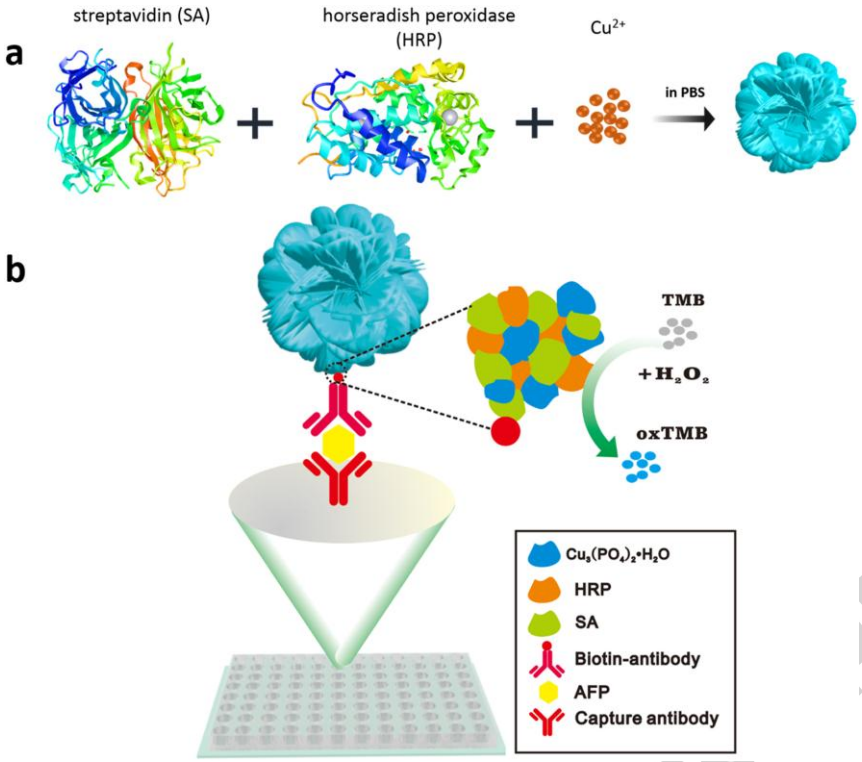
a
streptavidin (SA)
horseradish peroxidase (HRP)
Cu2+
in PBS
b
TMB
+H2O2
oxTMB
Cu3(PO4)2·H2O
HRP
SA
Biotin-antibody
AFP
Capture antibody

Fig. 6

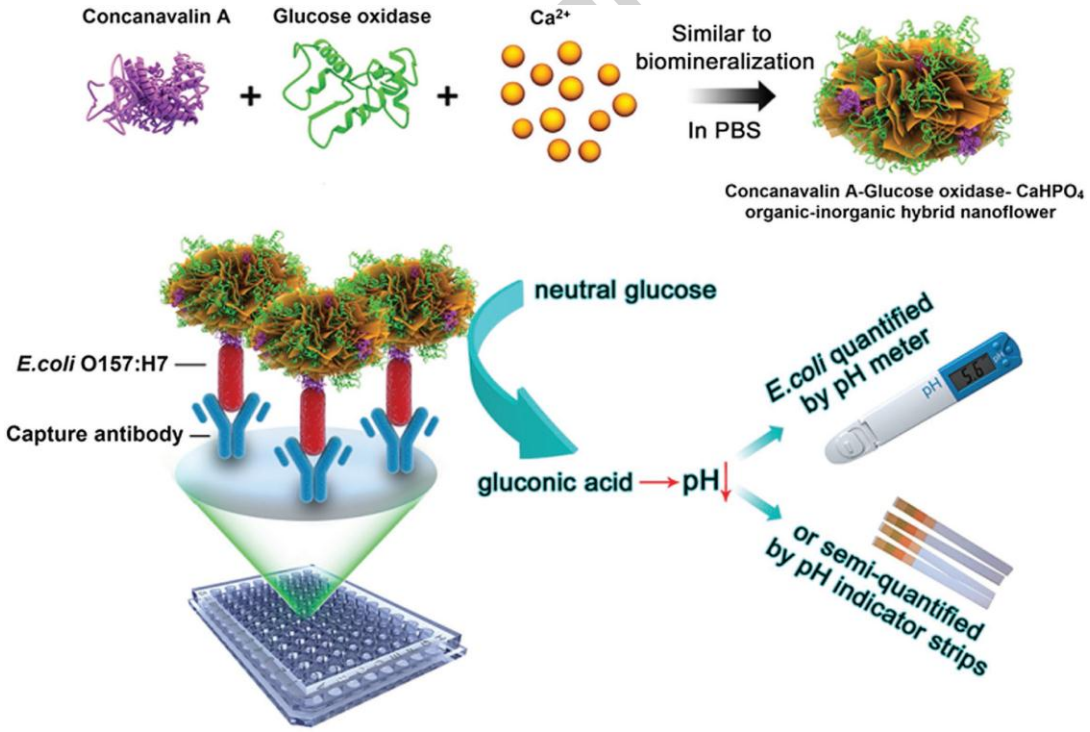
Concanavalin A
Glucose oxidase
Ca2+
Similar to biomineralization
In PBS
Concanavalin A-Glucose oxidase- CaHPO4 organic-inorganic hybrid nanoflower
E.coli O157:H7
Capture antibody
neutral glucose
gluconic acid → pH
E.coli quantified by pH meter
or semi-quantified by pH indicator strips

Fig. 7