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Integration and synergy in protein-nanomaterial hybrids for biosensing: Strategies and in-field detection applications

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ABSTRACT

Biosensors contribute a lot to the reliable and sensitive detection in various fields, especially emerging trends of in-field and real-time detection for point-of-care diagnosis, food safety and environmental monitoring. The signal amplification that improves the analytical performance and the compact integration of various biosensing components with/in miniaturized and portable devices are essential but still challenging. Integrating the merits of bio-active proteins (enzyme, antibody, etc.) and nanomaterials (nanoparticles, nanotubes, nanosheets, nanoflowers etc.) with abundant physicochemical properties, numerous protein-nanomaterial hybrids (PN hybrids) have been designed and applied for biosensing in recent years. PN hybrids can serve as not only sensitive probes for analyte recognition and signal generation/amplification thereby enhancing analytical performance, but also miniaturized and full-functional sensing components that are easily combined with other devices, greatly simplifying the construction and assay procedures. In this review, the state-of-art strategies of PN hybrids for biosensing are summarized from the view of the role of nanomaterial components, i.e. immobilization matrix, catalyst, and label. Recent advances for the emerging in-field detection applications of PN hybrids with the incorporation of portable hand-held readers and miniaturized devices are then surveyed. The features of PN hybrids for the construction of these miniaturized biosensors are focused. The integration and synergy between proteins and nanomaterials for biosensing is emphasized and discussed.

1. Introduction

Due to high accuracy, specificity, reliability and rapid response, biosensors have been widely applied in clinical diagnosis, environmental monitoring, and food safety surveillance (Lawal, 2018; Wongkaew et al., 2019). Improving the analytical performance is one of the unfailing goals of this field, since ultra-sensitive detection, even for single molecule detection, is favorable for timely diagnose while the limit of detection (LOD) of most current biosensors are above the concentrations of targets in many application scenarios. Effective target recognition, signal generation and amplification are vital for improving the performance of biosensors (Liu et al., 2019; Shandilya et al., 2019), which significantly depend on the full realization of the functions of recognition biomolecules and signal labels. On the other hand, the application of biosensors for in-field and real-time detection is also an urgent trend in some important fields that require immediate information acquirement, such as point-of-care diagnosis, food safety, and environmental monitoring (Brothers et al., 2019; Mayer and Baeumner, 2019; Wen et al., 2017). In this case, the miniaturization and portability of biosensor are essential, which generates significant challenges on the integration of biosensing elements. All above together, advanced strategies and materials that can efficiently assemble biosensing elements and integrate functions are urgent for the development of biosensors.

Integration and synergy is an universal idea that creating a whole in which the individuals cooperate to contribute respective properties and functions. Through the cooperation, the obtained multi-function-integrated complex can accomplish tasks with multiple requirements that individual component cannot able to achieve, or complete tasks synergistically with higher efficiency (Corning, 2010). With the help of rapid-developed nanotechnology, the integration and synergy between proteins and nanomaterials (protein-nanomaterial hybrids, PN hybrids) provides a promising chance to integrate various physicochemical properties of nanomaterials to append functions to proteins and/or to enhance the performance, having shown significant advantages and

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potentials in various applications, such as biomedicine (Zhao et al., 2019), bio-catalysis (Doonan et al., 2017) and biosensing (Kempahanumakkagari et al., 2018). In PN-hybrids-based biosensors, protein and nanomaterial components cooperatively accomplish the biosensing process from analyte recognition to signal generation in a function-enhancing (synergy) way. Proteins recognize targets with high affinity and specificity, while nanomaterials can assist and facilitate the event: the high surface area and/or porous interiors of nanomaterials provide abundant immobilization sites for the load of proteins or the separation/concentration of analytes; the good biocompatibility benefits to remain the activity of proteins; and nanomaterial components around proteins can protect fragile proteins from denaturation against external perturbation, enhancing the stability and reproducibility (Zhang et al., 2019b). Meanwhile, nanomaterials with diverse physicochemical properties can serve as direct labels or indirect immobilization matrices/catalysts for signal generation, greatly amplifying the signal and enriching the variety of signals (Ju, 2018). Besides, due to the diverse surface chemistry of both protein and nanomaterial, they can be conjugated together in a diverse fashion under facile conditions, endowing PN hybrids with highly-integrated characteristics and diversified functions in a confined space. Obviously, this is favorable for the integration/assembly of multiple elements to construct performance-enhanced and miniaturized biosensors. They can be easily combined with portable readers and/or integrated in miniaturized substrates (devices) for in-field and easy-operation detection.

As a result, numerous PN hybrids have been designed and have contributed significantly to the development of biosensors, in particular, in enhancing the analytical performance of biosensors, and applications with the cooperation of portable and miniaturized devices for in-field detection. A series of important progress have been achieved on PN hybrids containing metallic nanomaterials (Nakhjavani et al., 2019; Samadi Pakchin et al., 2018; Wang et al., 2018b), carbon nanomaterials (Eguilaz et al., 2018; Heidari et al., 2019; Nie et al., 2018), graphene analogues (e.g. transition metal dichalcogenides (Dalila et al., 2019) and 2D metal carbide and nitrides (MXene) (Wu et al., 2018)), porous frameworks (metal-organic frameworks (MOFs) (Wang et al., 2017) and covalent organic frameworks (COFs) (Zhang et al., 2018b)). Herein, this article reviews the advances of PN hybrids for biosensing in past three years, paying close attention to the integration and synergy between proteins and nanomaterials (Fig. 1). Initially, the general state-of-art strategies of PN hybrids for biosensing are systematically introduced form the view of the role of nanomaterial components. Electrochemical and optical biosensors are focused. Following this discussion, the emerging in-field detection applications of PN hybrids with the incorporation of miniaturized devices are presented, including combination with portable hand-held readers (smartphone and metric analyzers) and integration in miniaturized platforms (test strips and microfluidic paper-based analytical devices). The features of PN hybrids for the construction of these miniaturized biosensors are emphasized. Finally, conclusions, current challenges and future perspectives of this rapidly-developing field are given, especially the fundamental knowledge of the interaction between proteins and nanomaterials and commercialized prospect of PN-hybrids-based biosensors.

2. State-of-art strategies of PN hybrids for biosensing

Inheriting the properties and functions of both proteins and nanomaterials, PN hybrids not only possess high and stable load of proteins for sensitive biological recognition, but also provide abundant choices for signal generation/amplification with the presence of lots of nanomaterial components. Generally, the biosensing strategy of PN hybrids can be categorized into three main types by the role of nanomaterial components: immobilization matrices of recognition elements and labels, catalysts towards signal generation reaction, and electrochemical/optical labels. In this section, we will deliver a recent survey to summarize the general state-of-art strategies of PN hybrids according to above category, emphasizing the integration and synergy between proteins and nanomaterials for biosensing.

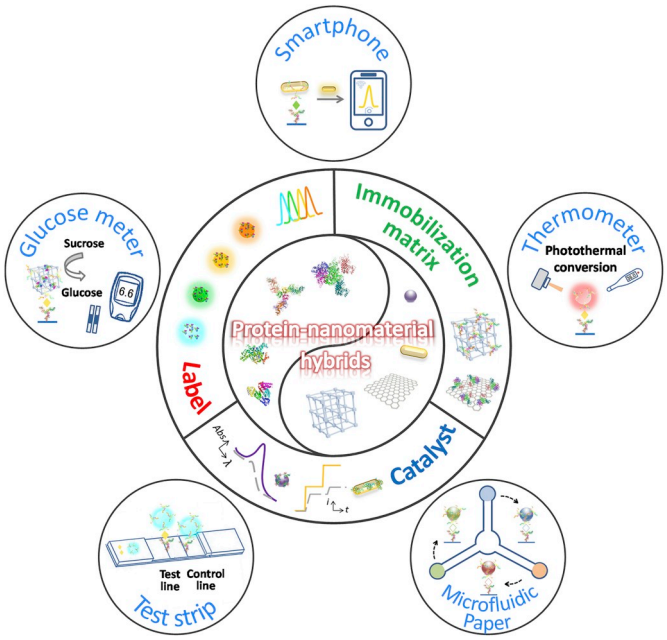

Fig. 1. Schematic illustration of the biosensing strategies of PN hybrids and their combination with portable and miniaturized devices for in-field detection.

2.1. PN hybrids with the immobilization of recognition elements and labels

Biological recognition elements (e.g. enzymes, antibodies) and labels (e.g. redox and luminescent molecules) are two key elements in biosensors, and the reliable enrichments of them are beneficial to biosensing performance, such as wide detection range, high sensitivity and rapid response. Due to the superior capabilities of nanomaterial components to bind other materials, PN hybrids with high loading of proteins and/or labels are widely applied as sensitive and stable sensing components in the construction of biosensors.

PN hybrids containing 0D/1D/2D nanomaterials (e.g. metal and metal oxides (Nakhjavani et al., 2019; Samadi Pakchin et al., 2018; Shaikh et al., 2019; Tang et al., 2018; Wang et al., 2018b), carbon nanomaterials (da Silva et al., 2018; Li et al., 2019b; Zhang et al., 2019c; Zhang et al., 2019d), and graphene analogues (Guo et al., 2017; Wu et al., 2018)) as the immobilization matrices have been studied extensively for biosensing. These nanomaterial components possess favorable characteristics for immobilization, including high surface area and surface-to-volume ratio, good stability and biocompatibility, leading to enhanced amount of loaded proteins and signal labels. Besides, they can be readily conjugated with proteins and labels in diverse routes, either by chemical/biological linkage or physical interactions under facile conditions, which are beneficial to the retention of protein activity during the fabrication of PN hybrids (Zhang et al., 2019b). For example, rod-like AuPt bimetallic nanoparticles were employed to load second antibodies (Ab_2) against Galectin-3 and redox-active methylene blue (MB) labels to prepare Ab_2-AuPt-MB hybrids, which served as a new kind of nanoprobes for signal generation and amplification (Tang et al., 2018). Benefiting from the synergistic effect between individual metallic components, the hybrids possessed much higher current response than monometallic counterparts, and the immunosensor exhibited a linear detection range (LDR) from 100 fg mL^{-1} to 50 ng mL^{-1} and a low LOD of 33.33 fg mL^{-1} for Galectin-3 in spiked serum. Tyrosinase-MXene--Ti_3C_2-chitosan hybrids modified electrodes exhibited 1.5 time enhancement of sensitivity for phenol detection (Wu et al., 2018).

Furthermore, protein orientation, in particular, antibody orientation in PN hybrids has also attracted ever-growing concern, since the conformation and orientation of antibody in the recognition elements determine the efficiency of antibody-antigen interaction and thus affect the analytical performance of the biosensor (Shen et al., 2019; Tripathi and Driskell, 2018; Yang et al., 2018a). Fc-binding proteins (e.g. protein A/protein G), amine acid and functionalized phenyl film were utilized as the intervening layers between nanomaterial and antibody components to guide the orientation of antibody to improve the immunoreaction efficiency, thereby improving the sensitivity and selectivity of the biosensor (Li et al., 2018b; Matysiak-Brynda et al., 2018; Raghav and Srivastava, 2016). Redesigning of protein mutants was applied to achieve protein orientation on graphene surface, presenting potentials in optimizing protein function on 2D nanomaterials for biosensing (Wei et al., 2019).

To further improve the stability of proteins/labels against leakage or denaturation during the long-term storage and recycling, PN hybrids containing porous MOFs or COFs grab highly progressive attention owing to their significant capabilities for efficient immobilization (Fig. 2) (Yang et al., 2018b; Zhang et al. 2017a, 2018b; Zhong et al., 2018). For example, comparing with native state, Cyt c in Cyt c@ZIF-8 hybrids displayed increased apparent substrate affinity (Michaelis constant reduced by ~50%), 1.28-fold increased enzymatic activity, and 1.4-fold increased sensitivity for H_2O_2 detection (Zhang et al., 2017a).

2.2. PN hybrids with catalytic activity for signal amplification

Besides increasing the amount of recognition elements and labels in the sensing system for signal amplification, tremendous attentions have been devoted to PN hybrids containing nanomaterial components with catalytic activity towards signal generation reaction. In this strategy, the sensitivity can be improved dramatically.

For conventional electrochemical (EC) biosensors, PN hybrids with electro-catalytic activity to redox reactions were widely applied, including noble metal nanoparticles (NPs), metal oxides and carbon nanomaterials involved PN hybrids (Eguilaz et al., 2018; Farka et al., 2018; Li et al., 2019b; Yang et al., 2018c). For example, due to the synergy effect of Au, Ag and Cu_2O, novel Ab_2-Au@Ag–Cu_2O hybrids exhibited excellent electrocatalytic activity towards the reduction of H_2O_2 to detect prostate specific antigen (PSA) with a low LOD of 0.003 pg mL^{-1} (Yang et al., 2018c). Notably, if the nanomaterial components were also highly conductive (electrochemically active), they can synergistically amplify signals via facilitating the electron transfer between electrodes and redox molecules (da Silva et al., 2018).

For optical biosensors, there are different modes for PN hybrids to exert the catalytic capability to luminescence reactions for signal amplification. Typically, PN hybrids containing nanomaterials with peroxidase-like activity/electro-catalytic activity can be utilized as catalysts for H_2O_2-based luminescence systems, such as 3,3′,5,5′-tetramethylbenzidine (TMB)-H_2O_2 and luminol-H_2O_2 systems with chemical stability and high emission yields (Farka et al., 2018; Guo et al., 2019). In this case, the sensitivity of optical biosensors is improved directly with the enhanced luminescence efficiency. For instance, capture antibody (Ab_1)-PBNPs (Prussian blue nanoparticles) hybrids were developed as catalytic probes in a sandwich nanozyme-linked immunosorbent assay for human serum albumin (HSA) and *Salmonella typhimurium*, in which PBNPs possessed peroxidase-like activity to catalyze the oxidation of colorless TMB in the presence of H_2O_2 to form intensely blue product (Farka et al., 2018).

Moreover, the peroxidase-like activity of nanomaterial components can be applied to cooperate with oxidases in PN hybrids to develop tandem reactions for novel biosensing methods (Batule et al., 2019; Guo et al., 2019; Hu et al., 2017; Lin et al., 2018; Wang et al., 2017). Briefly, the hybridized oxidases catalyze the oxidation of targets to yield H_2O_2 firstly, and then the nanomaterial components catalyze H_2O_2 to generate various detectable optical signals. In this cascade mode, the biosensing processes are highly integrated in PN hybrids whereby protein and nanomaterial components cooperatively complete the entire sensing process ranging from initial recognition to final signal output, bringing unique advantages for the proposed biosensors. For example, for the biosensor based on glucose oxidase (GOx)@ZIF-8(NiPd) nanoflower hybrids, glucose molecules were catalytically oxidized by GOx to generate H_2O_2, which were then catalyzed by ZIF-8(NiPd) to trigger the oxidation of co-substrate *o*-phenylenediamine and produce yellow products. Therefore, colorimetric detection of glucose was readily achieved in one-step (Wang et al., 2017). Moreover, other properties of PN hybrids can be applied together with peroxidase-like activity for direct signal output, such as surface-enhanced Raman spectroscopy (SERS) property. Owing to the peroxidase-like activity of gold nanoparticles (AuNPs)@MIL-101 that can oxidize Raman-inactive reporter malachite green into the active malachite green in the presence of H_2O_2, GOx@AuNPs@MIL-101 hybrids and lactate oxidase (LOx)@AuNPs@MIL-101 hybrids were used to detect glucose and lactate in living tissues via SERS. The hybrids not only served as enzyme mimics, but also acted as SERS substrates that enhance the Raman signals of the generated malachite green (Fig. 3) (Hu et al., 2017).

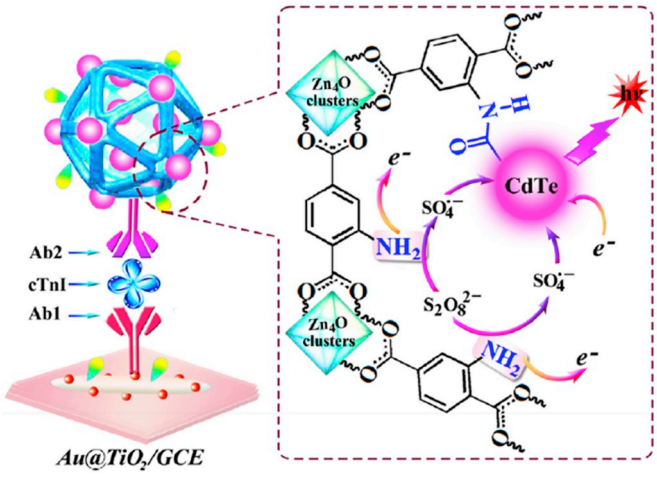

Fig. 2. Schematic illustration of Ab_2-CdTe@IRMOF-3@CdTe-hybrids-based ECL biosensors, in which IRMOF-3 served as not only immobilization matrices for Ab_2 and CdTe QDs (labels) via the encapsulating effect and internal/external decoration, but also co-reactant accelerators to boost the ECL emission of CdTe. Adapted with permission from Ref. (Yang et al., 2018b). Copyright (2018) American Chemical Society.

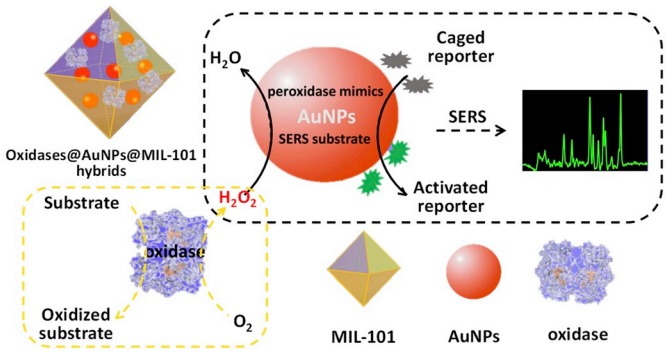

Fig. 3. Schematic illustration of GOx@AuNPs@MIL-101-hybrids-based SERS biosensors. Adapted with permission from Ref. (Hu et al., 2017). Copyright (2017) American Chemical Society.

2.3. PN hybrids containing nanomaterials as direct labels

In addition to above two roles for signal amplification, PN hybrids were also widely applied as direct probes for signal generation. The diverse properties and functions of nanomaterial components, such as redox activity, optical and thermal properties, provide diverse routes for signal generation, while a large number of nanomaterials in PN hybrids can amplify the signal simultaneously. Moreover, due to the robustness of nanomaterials comparing with conventional molecules, this kind of PN hybrids endows the biosensor with improved stability and reusability.

PN hybrids containing nanomaterials with redox activity can be used as labels in EC biosensors (Liu et al., 2016; Zhang et al. 2017b, 2018a). The nanomaterial components can generate electrical signals directly. For example, Ab_2-DNA-NMOFs (nanoscale zirconium-porphyrin MOFs) hybrids were reported to serve as photoelectrochemical signal nano-probes for ultrasensitive PSA immunoassay with a LOD of 0.2 pg mL^{-1} (Zhang et al., 2018a). The NMOFs in PN hybrids significantly enhanced photocurrent response in the presence of dopamine under oxygen-containing aqueous media at −0.3 V.

Moreover, in addition to the inner redox activity of nanomaterials in PN hybrids for direct signal generation, more hybrids were devoted as redox labels through self-sacrificial transformation methods (Liu et al., 2016; Zhang et al., 2017b; Zhong et al., 2018). PN Hybrids can be destructed through appropriate chemical/physical treatments, and the released "products" (usually, metal ions) are applied to generate signals based on redox reaction. This strategy subtly takes advantage of the high abundance of metal elements in PN hybrids themselves to achieve signal amplification in a facile and cost-effective way. For example, PN hybrids containing quantum dots (QDs) can be readily dissolved by H_2O_2 or acid to release metal ions, realizing sensitive detection through collecting the EC reduction current of metal ions (Zhong et al., 2018). In addition, PN hybrids containing MOFs are novel metal sources of self-sacrificial redox probes. The first example using MOFs as direct redox probes for EC biosensor is Ab_2-Au-HKUST-1 hybrids with large amount of Cu^{2+} in the 3D infinite extend skeleton of HKUST-1. When negative potential was applied, the topological structure of HKUST-1 in PN hybrids collapsed to release Cu^{2+} to generate signals (Liu et al., 2016). Moreover, released metal ions can also be converted into redox-active materials to generate signals. Our group proposed an EC conversion method, in which iron ions from superparamagnetic Con A-MNPs (concanavalin A-magnetic nanoparticles) hybrids were converted to electrochemically active ferrous ferrocyanide (Prussian white, PW, analogues of PB) to generate current signal. Taking full advantages of the high abundance of iron in MNPs and interesting surface effects from EC conversion, this PN hybrid-based sandwich biosensor exhibited high sensitivity and the lowest LOD among analogues (down to 0.0022 hemagglutination units) for rapid detection of avian influenza virus H5N1 (Fig. 4) (Zhang et al., 2017b) (see Table 1).

As for optical biosensors, nanomaterials with optical properties can serve as luminophores for signal generation and amplification (Babamiri et al. 2018a, 2018b; Karimzadeh et al., 2018; Li et al., 2017; Nie et al., 2018; Yang et al., 2018b). Due to the distinctive merits of nanomaterial components such as high stability, tunable optical property and high emission efficiency, PN hybrids become powerful candidates as direct luminescent labels in optical biosensors.

Among numerous luminescent nanomaterial components in PN hybrids, QDs have been widely used in fluorescence (FL) and electrochemiluminescence (ECL) biosensors due to their high quantum yield, controlled optical properties, broad absorption, narrow emission, and large surface area (Li et al., 2017; Moro et al., 2017). A series of PN hybrids containing QDs have been synthesized and applied for optical biosensors (Benítez-Martínez and Valcárcel, 2015; Karimzadeh et al., 2018; Li et al., 2017; Ng, 2019), such as ZnS, CdTe, CdS, carbon QDs (CQDs), graphene QDs (GQDs), and N-doped graphene QDs (N-GQDs). PN Hybrids containing CdTe and CdTe@CdS QDs are popularly used as metal semiconductor luminescent labels (Babamiri et al. 2018a, 2018b; Moro et al., 2017). Very recently, Ab_2-CdTe@IRMOF-3@CdTe hybrids were reported as ECL signal probes for the detection of biomarker . In conventional co-reaction accelerator system, QDs and co-reaction accelerators are spatially separated, which leads to decreased efficiency. In this work, the luminophore and co-reaction accelerator were integrated in PN hybrids. IRMOF-3 in hybrids not only allowed for the loading of a large amount of Ab_2 and CdTe, but also contributed organic ligands (2-amino terephthalic acid) as the accelerator to promote the conversion of co-reactant $S_2O_8^{2-}$ into $SO_4\bullet^-$. Both of them drastically enhanced the ECL intensity of CdTe. As a result, the proposed sandwich biosensor for cardiac troponin-I antigen presented a very low LOD of 0.46 fg mL^{-1} (Yang et al., 2018b). In addition to CdTe, CQDs have also been extensively studied in PN-hybrids-based biosensors due to high photo-stability, feasibility for surface modification, tunable excitation, and good biocompatibility. Recently, Ab_2-AuNPs/CQDs-polyetherimide-GO hybrids were applied as ECL signal probes for carcinoembryonic antigen (CEA) detection with a LOD of 1.67 pg mL^{-1} (Li et al., 2017).

Graphene-based PN hybrids were also explored for optical biosensing. As the new type of promising nanomaterials in carbon family, GQDs have attracted lots of attention due to their attractive merits including low cost, large specific surface area, unique electro-optical properties, and good biocompatibility (Benítez-Martínez and Valcárcel, 2015). Ab_2-GQDs@AuNPs hybrids with improved electron transfer capability and stable ECL intensity were used as ECL probes for CEA detection with a LOD of 3.78 fg mL^{-1} (Nie et al., 2018). Moreover, PN hybrids based on N-GQDs with more active sites and improved quantum yields than pure GQDs were developed (Karimzadeh et al., 2018).

3. PN-hybrids-based biosensors for in-field detection

Besides the above conventional laboratory-based biosensors, in-field detection applications, being characterized by the advantages of small

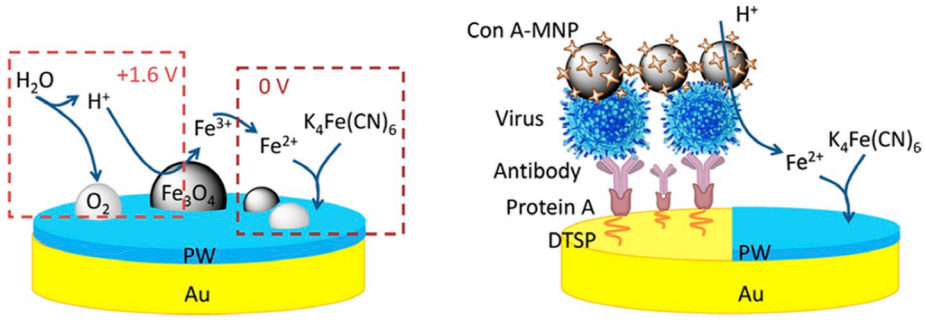

Fig. 4. Schematic illustration of Con A-MNPs-hybrids-based immunosensors using MNPs as labels via EC conversion. Adapted with permission from Ref. (Zhang et al., 2017b). Copyright (2017) American Chemical Society.

Table 1
Representative strategies of PN hybrids for biosensing.

PN hybrids	Strategies	Detection principle	Target	Performance(LDR, and LOD)	References
Ab_2-polystyrene/Ag	Conductive Ag nanoshells for the oriented immobilization of Ab_2	Electrochemical impedance spectroscopy	Microalbuminuria	30–300 μg mL^{-1}	Shaikh et al. (2019)
Ab_2-AuNPs-LOx	AuNPs immobilized Ab_2 and LOx	Chronoamperometry (CA)	Carcinoma antigen 125	0.01–0.5 and 0.5–100 U mL^{-1}, and 0.002 U mL^{-1}	(Samadi Pakchin et al., 2018)
Ab_2-Co_3O_4@MnO_2-thionine	Co_3O_4@MnO_2 immobilized Ab_2 and thionine	Differential pulse voltammetry (DPV)	α-fetoprotein	0.001–100 ng mL^{-1}, and 0.33 pg mL^{-1}	Wang et al. (2018b)
Tyrosinase-MXene-Ti_3C_2-chitosan	MXene-Ti_3C_2 immobilized tyrosinase	CA	Phenol	0.05–15.5 μM, and 12 nM	Wu et al. (2018)
Ab_2-Ru@GO	GO immobilized Ab_2 and Ru–NH_2	ECL	Anti-carbohydrate antigen 125	0.001–100 U mL^{-1}, and 0.4 mU mL^{-1}	Guo et al. (2017)
Ab_2- toluidine blue-Au-COFs	Au-COFs immobilized Ab_2 and toluidine blue	Square wave voltammetry	Cardiac troponin I	0.5–10000 pg mL^{-1}, and 0.17 pg mL^{-1}	Zhang et al. (2018b)
Ab_2-PBNPs	PBNPs immobilized Ab_2 and catalyzed H_2O_2-TMB colorimetric system	Colorimetric method	HSA and *Salmonella typhimurium*	[a]	Farka et al. (2018)
Ab_2-$ZnCo_2O_4$/N-CNT[b]	[c]	DPV and CA	B-type natriuretic peptide	0.01–1000 pg mL^{-1}, and 3.4 fg mL^{-1}	Li et al. (2019b)
GOx-$Fe_3(PO_4)_2{\cdot}8H_2O$ nanoflowers	$Fe_3(PO_4)_2{\cdot}8H_2O$ immobilized GOx and catalyzed H_2O_2-TMB colorimetric system	Colorimetric method	Glucose	0.01–20 mM	Guo et al. (2019)
Ab_2-AuNPs/g-C_3N_4[d]	g-C_3N_4 generated ECL signal and AuNPs immobilized Ab_2	ECL	Squamous cell carcinoma antigen	0.001–100 ng mL^{-1}, and 0.33 pg mL^{-1}	Guo et al. (2017)
Ab_2-AuNPs/CQDs-polyetherimide-GO	[e]	ECL	CEA	0.005–500 ng mL^{-1}, and 1.67 pg mL^{-1}	Li et al. (2017)
GOx-MIL-53(Fe)	MIL-53(Fe) immobilized GOx and generated FL signal within H_2O_2	FL	Glucose	0.5–27 μM, and 8.44 nM	Lin et al. (2018)

[a] HSA: up to 1 μg mL^{-1}, and 1.2 ng mL^{-1}, *Salmonella typhimurium*: up to 10^6 CFU mL^{-1}, and 6×10^3 CFU mL^{-1}.
[b] N-CNT: N-doped carbon nanotubes.
[c] Conductive N-CNT immobilized Ab_2 and improved the stability and electrocatalytic activity of $ZnCo_2O_4$, and $ZnCo_2O_4$ QDs catalyzed H_2O_2 reduction.
[d] g-C_3N_4: graphitic-phase carbon nitride nanosheet.
[e] CQDs generated ECL signal, AuNPs and polyetherimide-GO immobilized Ab_2 and amplified ECL signal.

size, low cost, real-time reading and good portability, have been undergoing rapid development in recent years, providing alternative ways to laboratory-based biosensing technologies in home-health care, food quality control and environmental monitoring (Kanchi et al., 2018; Kumar et al., 2019; Nasseri et al., 2018; Wen et al., 2017). To achieve the remarkable characterization and fulfill the "ASSURED" criteria for an ideal rapid test: **A**ffordable, **S**ensitive, **S**pecific, **U**ser-friendly, **R**apid and Robust, **E**quipment-free, **D**eliverable to end-users, many efforts have been devoted to using portable hand-held instruments for the transduction and output of signal, e.g. smartphone and wireless system (Kanchi et al., 2018), and metric analyzers (Fu et al., 2018; Zhu et al., 2018b), as well as the integration/assembly of the whole biosensing elements into miniaturized platforms (devices), such as paper-based analytical devices (PADs) (Chinnadayyala et al., 2019) and microfluidic system (Nasseri et al., 2018). Unfortunately, these changes for portability and miniaturization would lead to compromise of analytical performance, since these devices possess low resolution and high background noise, frangibility, poor stability and reproducibility, comparing with large-scale precise equipment. Therefore, major challenges in this filed nowadays are to amplify the sensing signal greatly, improve the stability and reproducibility of both signal and devices, and to fabricate full-functional sensing elements and elaborate miniaturized devices with high integration level (Liu et al., 2019; Zhu et al., 2018b).

Clearly, PN hybrids with highly-integrated structure and functions are perfect candidates as biosensing components to combine with miniaturized devices for in-field detection. At first, the sensing process from target recognition to signal generation can be achieved in one hybrid, greatly simplifying the construction of biosensor and assay procedures for in-filed detection. While the abundant surface properties of PN hybrids make them easily to combine with various substrates (devices), benefiting for the integration and miniaturization of biosensors. Moreover, the above PN-hybrids-based signal amplification strategies can be applied to improve the analytical performance, ensuring reliable and sensitive detection results in in-field detection. The robust nanomaterial components in PN hybrids provide robust protection towards fragile proteins whereby improving the stability and reproducibility of the biosensor. Therefore, PN hybrids have stirred a wide range of concerns and have contributed significantly to in-field detection applications (Shandilya et al., 2019). Here, we focus on the recent research about the combination of PN hybrids with portable hand-held readers including commercialized smartphone, and metric analyzers (e.g. pH meter, glucose meter and thermometer), and the integration of PN-hybrids-based biosensors in miniaturized platforms (devices), mainly in PADs. The integration and synergy between PN hybrids and various miniaturized devices are discussed.

3.1. Combination with portable hand-held readers

Coupled with sensitive biosensing components, the application of portable hand-held readers reduces the requirement for large-scale expensive instruments for signal readout and trained personnel for professional operation, significantly improving the portability and practicability of biosensors, especially for home-health care and resource-limit settings (Li et al., 2019a; Roda et al., 2019; Zhang et al., 2019a). The key of this combination is to convert and amplify the biomolecule recognition event into a measurable numeric signal by a simple reader. In this case, PN hybrids exhibit promising potentials in this conversion. When combined with portable hand-held readers, PN hybrids take charge of target recognition and signal generation, and then the reader recognizes/transduces the signal for output, cooperating successively to accomplish the whole in-field detection in a highly-efficient (synergy) way.

Smartphone, a multifunctional platform with advanced computing capability, good memory system and high-quality camera, now not only serves as a ubiquitous communication tool, but also acts as a sensing platform for screen display, data analysis, signal detection, transduction and readout, or an interface device via Wi-Fi, Bluetooth and microUSB with analytical instruments (Kanchi et al., 2018). Generally, in a

PN-hybrids-smartphone-based biosensor, PN hybrids served as immobilization matrices to load antibodies for capturing and concentrating targets (Zeinhom et al., 2018b), to load labels for signal amplification (Roda et al., 2019), and/or the direct labels for signal generation (Hosu et al., 2017; Wang et al., 2018a; Zeinhom et al., 2018a; Zhang et al., 2019a).

For instance, two signal amplification strategies based on PN hybrids and smartphone were combined to develop a capillary biosensor for in-field concentration, separation and detection of foodborne bacteria (Fig. 5) (Zhang et al., 2019a). Ab_1-MNPs hybrids in capillary can separate *Salmonella typhimurium* with a high separation efficiency of 83%, and Ab_2-Fe-nanoclusters binding on Ab_1-MNPs-bacteria complex can be dissolved by HCl to release lots of iron ions, which reacted with potassium hexacyanoferrate to form the PBNPs. Blue PBNPs were finally measured and analyzed using the Hue-Saturation-Lightness color space based smartphone APP for the determination of the target bacteria. The enhanced signal of the smartphone exhibited a wide LDR with a low LOD of 14 CFU mL^{-1}, and the mean recovery in spiked chicken samples was ~105.0%, indicating the applicability and potential for in-field detection of foodborne pathogens.

In addition to optical biosensors combined with smartphone for signal readout, EC signal can also be conducted with smartphone (Wen et al., 2017). A reusable glucose biosensing system that features integration of electrodes directly onto the smartphone platform was established (Bandodkar et al., 2018). This system included a controllable stylus loading with GOx-nanomaterial hybrid pellets, sensor instrumentation circuits, a smartphone case that housed a permanent bare sensor strip and custom-designed Android-based software for the easy and clear display of measured glucose concentration. With the introduction of glucose samples into this system, the selective oxidation of glucose occurred and produced gluconic acid and H_2O_2, which was then catalytically oxidized by the nanomaterial components in the pellets to generate EC signal. And then, the electronic module acquired and wirelessly transmitted the data to the application software and displayed the result on the screen. Notably, the loaded GOx in the hybrid pellets were stable for up to 8 months at ambient conditions, and can generate reproducible sensor signals.

Besides smartphone, metric analyzers, e.g. pH meter, glucose meter, thermometer and pressure meter, are other popular hand-held readers in in-field detection owing to the ubiquity, easy-operation, portability and inexpensiveness (Fu et al., 2018; Huang et al., 2018b; Ji et al., 2016; Zhang et al., 2016). When metric analyzers are applied in in-field detection, they need a medium transferring the substrate into acidic/alkaline substance, glucose, temperature or gas that can be measured by specific meters. Imaginably, PN hybrids consist of enzyme and catalytic nanomaterials are perfect candidates for the transfer, as a result, many attempts have been made to the combination of metric analyzers with PN hybrids.

pH meter is an early-explored metric analyzer measuring the concentration of hydrogen ions. Reactions causing the acidity change in microenvironment can be detected and read by pH meter, such as the GOx-based catalytically oxidation of glucose. As a result, the detection result was related to the acidity change and the sensitivity of the pH meter. In this case, PN hybrids with high and stable loading of catalytic enzymes were favorable for the detection (Ye et al., 2016). The pH-meter-based biosensor exhibited a 14-fold enhancement of the sensitivity in comparison with conventional enzyme-linked immunosorbent assay for the quantification of human oncogenic protein (Zhang et al., 2016). Similarly, glucose meter is another widely applied reader in household scenarios because of its ease of use. In this case, the key is to link the target recognition event with the change of glucose concentration, followed by response of a glucose meter to provide a reliable signal. The concentration change can be triggered by the direct release of glucose in the probes or the production via the hydrolysis of carbohydrate via invertase (Tang et al., 2016; Zhu et al., 2019). Hence, the analytical performance is determined by the loaded amount of glucose or invertase in the probes, obviously, PN hybrids with high immobilization capacity were acclaimed. Recently, biosensors based on PN hybrids with glucose meter reader were designed for the sensitive detection of biomolecules and foodborne pathogens (Huang et al. 2018a, 2018b; Zhao et al., 2017; Zhu et al., 2019).

Besides, not limited to serve as physical immobilization matrices for substrates or enzymes, the chemical properties of nanomaterial components in PN hybrids were also applied to assist the response of signal, such as the catalytic activity and photothermal effect (Fu et al. 2016, 2018; Li et al., 2019a). For example, in the Ab_1-iron oxide NPs hybrids-mediated (catalyzed) TMB-H_2O_2 colorimetric system, the oxidized TMB exhibited not only traditional color changes, but also a strong NIR laser-driven photothermal effect. The unique effect can convert the immunoassay event into heat and then read through a

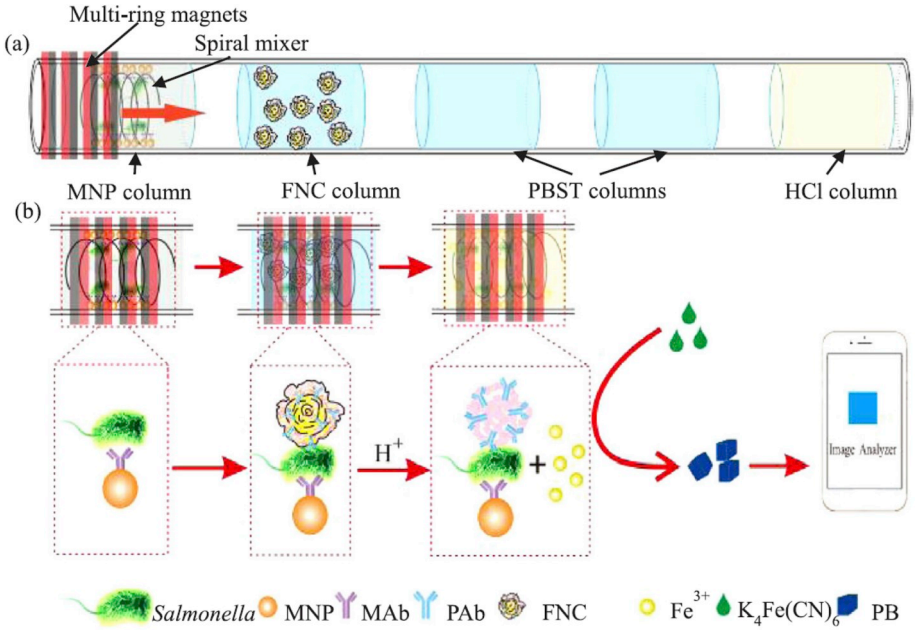

Fig. 5. Schematic illustration of PN hybrids-based capillary biosensor using a smartphone for readout. Reproduction from Ref. (Zhang et al., 2019a).

thermometer for output (Fig. 6). The as-proposed sandwich-type biosensor exhibited excellent performance for visual quantitative biomolecule detection. The LOD of PSA detection was 1.0 ng mL^{-1}, being lower than the clinical diagnostic threshold (Fu et al., 2018).

3.2. Integration in miniaturized platforms (devices)

The integration of the whole biosensing elements into miniaturized platforms (devices) can not only overcome the limitations of conventional approaches, including the consumption of large volumes of reagents and the requirement for long detection time, but also decrease the cost and increase the portability further (Wen et al., 2017). Unfortunately, these platforms suffer from the relatively low signal intensity, poor quantitative discrimination and limited robustness when they are applied for real-life applications (Liu et al., 2019). The introduction of interesting PN hybrids as novel integrated sensing components in these biosensors not only simplifies the construction and assay procedures, more importantly, the above signal amplification strategies are favorable for the improvement of performance (Bhardwaj et al., 2017).

Among various miniaturized platforms, PADs (e.g. test strips, and microfluidic PADs (μPADs)) have been demonstrated to be well suitable candidates owing to their evident advantages of ubiquity, light-weight, flexibility, disposability and eco-friendliness (Akyazi et al., 2018; Li et al., 2018c). In general, there are three steps in the construction of paper-based biosensors: fabrication of paper testing substrate, integration of various sensing components on the substrate, and deposition of samples (Li et al., 2018c). A large number of interesting PN hybrids were designed as sensitive sensing components on the paper to improve the performance in classic lateral-flow assays: Ab_1-MNPs hybrids were widely applied for capturing/concentrating targets (Fang et al., 2019; Yan et al., 2018), while various PN hybrids with novel optical-active nanomaterial components acted as direct labels for simultaneous signal generation and amplification, such as PN hybrids containing upconversion NPs (Gong et al., 2019; Ji et al., 2019), brightly colored silica NPs (Zhu et al., 2018a), gold nanoflowers with different sizes (Zhang et al., 2019e), iridium oxide NPs (Quesada-Gonzalez et al., 2019), and QDs (Hassan et al., 2019; Shao et al., 2018; Zamora-Galvez et al., 2018).

Similarly, PN hybrids were also designed to combine with μPADs to improve the analytical performance of μPADs for real-life applications, and highlight the remarkable advantages of μPADs of low cost, miniaturization, high integration level and portability further. The high immobilization capacity of PN hybrids was utilized to enhance the amount of bio-active enzymes in μPADs, which enhanced the sensitivity, durability and reusability of μPADs-based biosensors (Zhu et al., 2017). For example, the biosensor based on GOx-HRP-$Cu_3(PO_4)_2{\cdot}3H_2O$ nanoflower hybrids incubated on 3D μPADs exhibited excellent performance for glucose detection (a LOD of 15.6 μM) (Ariza-Avidad et al., 2016). More importantly, this biosensor was reusable (6 times cycles with no loss of activity) and remained stable for 75 days in conventional storage conditions, while the activity of free GOx-HRP bi-enzymatic system was below to 20% after 4th cycle. Moreover, beyond the simple immobilization, the peroxidase-like catalytic activity of nanomaterial components in PN hybrids can be utilized alone or combined with the immobilized oxidases for synergistic signal generation and amplification, greatly improving the performance of μPADs-based biosensors (Ilacas et al., 2019; Li et al., 2018a). Recently, PN hybrids consist of metal NPs and antibody were designed as robust optical labels for multiplex cardiac biomarker simultaneous detection (Fig. 7) (Lim et al., 2019). The combination of differently colored PN hybrids with μPADs containing integrated multiplex channels enables the identification of multiplex biomarker release profiles with improved specificity, sensitivity, and visual judgment (see Table 2).

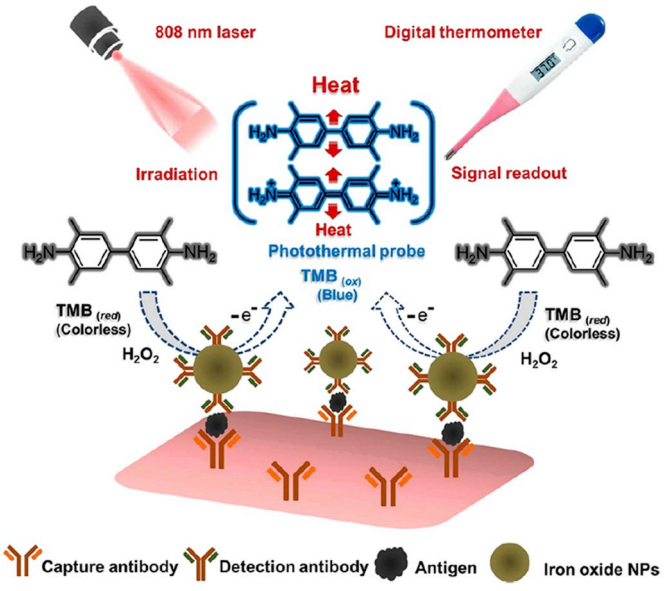

Fig. 6. Schematic illustration of Ab_1-iron oxide NPs hybrids-mediated TMB-H_2O_2 colorimetric system with photothermal effect. A digital thermometer was applied for signal readout. Adapted with permission from Ref. (Fu et al., 2018). Copyright (2018) American Chemical Society.

4. Conclusions, current challenges and future perspectives

In conclusion, this review summarizes and discusses the general state-of-art biosensing strategies and recent in-field detection applications of PN hybrids, focusing on the integration and synergybetween proteins and emerging nanomaterials. Inheriting the diversified properties of both proteins and nanomaterials, PN hybrids act as robust immobilization matrices for proteins and labels, highly-active catalysts for signal amplification, or direct probes containing nanomaterial-labels for simultaneous signal generation and amplification. Following these synergistic routes, PN hybrids not only enrich/enhance signal generation and amplification ways/efficiencies, but also improve the stability and reusability of fragile proteins, together significantly promoting the overall analytical performance of conventional biosensors. Meanwhile, benefiting from the highly-integrated structure and full functions for biosensing requirement, PN hybrids exhibit unique advantages and potentials in in-field detection applications, coupling with portable and miniaturized devices, such as smartphone, metric analyzers for pH/glucose/temperature/gas, test strips and microfluidic papers.

Even though PN-hybrids-based biosensors have been undergoing unprecedented development in recent years, there are still some challenges. 1) Mutual interference of properties between protein and nanomaterial components is exist without delicate design and optimization of PN hybrids, sometimes, neutralization effects rather than synergistic effects dominate the integration. For example, excess and random distribution of insulative protein molecules would compromise the high conductivity/electrochemical activity of nanomaterials for electron transfer in EC biosensors. While the conformational changes/denaturation, and randomization rather than orientation of proteins occur generally during the fabrication of PN hybrids. All of these suppressed the analytical performance. In this case, fundamental knowledge about the integration mechanism between proteins and nanomaterials is favorable but still limited. Efforts are continuously needed to investigate the specific interactions and the conformation/property matching between proteins and nanomaterials. The understanding is one of the most essential factors for the ingenious design, controllable preparation and appropriate application of PN hybrids. 2) Nowadays, various PN-hybrids-based biosensors were utilized for single analyte detection, simultaneous detection of multiple analytes is preferred but rare, especially for bio-medical diagnosis. Meanwhile, accurate detection in practical samples is hard due to the limited

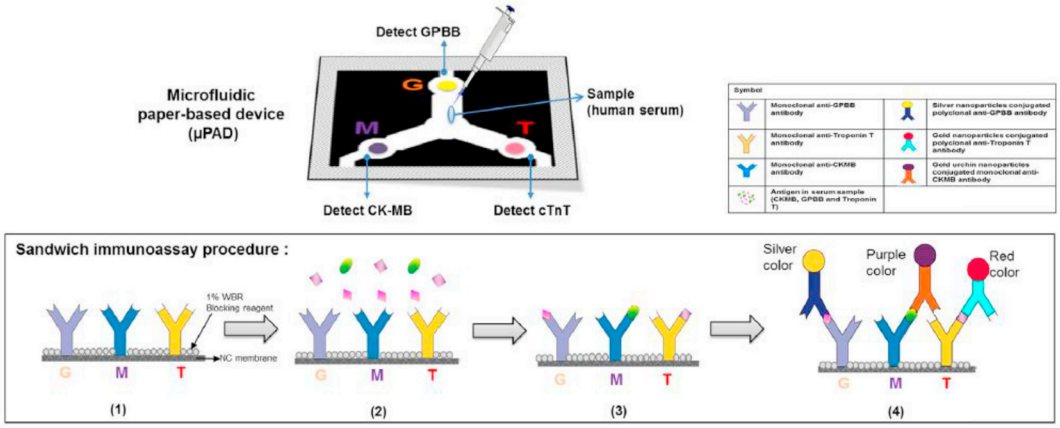

Fig. 7. Schematic illustration of μPADs-based immunosensors for multiplex cardiac biomarker simultaneous detection using PN hybrids with different colors as the labels. GPBB, CK-MB and cTnT were specific cardiac biomarkers for acute myocardial infarction diagnosis. Reproduction from Ref. (Lim et al., 2019).

Table 2
Typical examples of in-field detection based on PN hybrids coupled with miniaturized and portable devices.

PN hybrids	Miniaturized devices	Detection principles	Target	Performance (LDR, LOD)	References
Ab_1-MNPs, Ab-HRP-$Ca_3(PO_4)_2$	Smartphone	Colorimetric method	*Salmonella* in milk, cheese and water	LOD of 1.0 CFU mL^{-1} and 1.0 CFU g^{-1}	Zeinhom et al. (2018a)
Ab_2- PbS	Smartphone	Resonance light scattering	Alpha-fetoprotein	LOD of 0.1 pg mL^{-1}	Wang et al. (2018a)
Con A-GOx-$CaHPO_4$	pH meter	Acidity measurement	*E. coli* O157:H7	10–10^6 CFU mL^{-1}, 10 CFU mL^{-1}	Ye et al. (2016)
Invertase-Ab-AuNPs	Glucose meter	Glucose measurement	8-OHdG[a] and PSA	[b]	Zhu et al. (2019)
Ab_2-iron oxide NPs	Thermometer	Temperature measurement	PSA	2–64 ng mL^{-1}, 1.0 ng mL^{-1}	Fu et al. (2016)
Ab_2-PtNPs	Pressure meter	Pressure measurement	C-reactive protein	0.25–25 ng mL^{-1}, 0.2 ng mL^{-1}	Ji et al. (2016)
Ab_2-IrO_2 NPs	Test strip and smartphone	Colorimetric method	Human immunoglobulin	LOD of 0.07 μg mL^{-1}	Quesada-Gonzalez et al. (2019)
Ab_1-SiO_2 and Ab_2-QDs	Test strip	Photoluminescence	Human immunoglobulin G	LOD of 1.35 ng mL^{-1}	Zamora-Galvez et al. (2018)
GOx@Zr–PCN-222(Fe)	μPADs	Colorimetric method	Glucose	LOD of 250 μM	Ilacas et al. (2019)
GOx@$Mn_3(PO_4)_2$	μPADs	Colorimetric method	Glucose	LOD of 10 μM	Li et al. (2018a)

c Accurate on-body tear glucose monitoring before and after meal.
[a] 8-OHdG: 8-hydroxy-2′-deoxyguanosine.
[b] 8-OHdG: 0.1–100 ng mL^{-1}, 0.23 ng mL^{-1}; PSA: 1–100 ng mL^{-1}, 1.26 ng mL^{-1}.

specificity and weak anti-interference ability towards complex interferences. Systematic approaches for full exploration of the diversified functions of PN hybrids may address these challenges, for example, using other fantastic and robust properties of nanomaterials to develop more reliable biosensing strategies. 3) Current fabrication process of PN hybrids and construction of biosensors are still complicated with tedious procedures and time-consuming in most cases, which may cause deactivation of both protein and nanomaterial components before application. On the other hand, current methods are not ready for cost-effective and mass production. More efficient methods with simplified procedures and low cost are always worthy of attempt. 4) The selection of miniaturized platform/device is still limited, more efforts are needed to explore more types of devices to make this strategy more applicable. 5) PN-based biosensors are powerful in acquiring information in field and real time, the incorporation of these data with other information tools should benefit down-streaming steps, such as modeling and decision-making.

We strongly believe that PN hybrids would contribute a lot to biosensing with the fast development in a near future. Taking solid improvements to PN hybrids, biosensors should benefit much more to our life. New PN hybrids with intriguing merits not only endow biosensors with solidly improved analytical performance, but also gift biosensors with more suitable abilities for our daily life and production, such as point-of-care testing for health purpose, in-field detection/monitoring for food safety and environment treatment. Furthermore, because information acquisition has been long-regarded as a crucial role but a bottleneck for down-streaming steps of a system, such as an entire production chain. The realization of sensitive, rapid, in-field and real-time detection will make the results much more informative to reveal more accurate and timelier situation. Incorporating with other information tools, such as big data and machine learning, biosensors with powerful abilities in information acquisition will greatly benefit the monitoring of processes and the final decision-making, in turn making themselves more and more important contributors in relevant systems.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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