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Organic-Inorganic Hybrid Nanoflower Production and Analytical Utilization: Fundamental to Cutting-Edge Technologies

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ABSTRACT

Over the past decade, science has experienced a growing rise in nanotechnology with groundbreaking contributions. Through various laborious technologies, nanomaterials with different architectures from 0 D to 3 D have been synthesized. However, the 3 D flower-like organic-inorganic hybrid nanomaterial with the most direct one-pot green synthesis method has attracted widespread attention and instantly become research hotspot since its first allusion in 2012. Mild synthesis procedure, high surface-to-volume ratio, enhanced enzymatic activity and stability are the main factor for its rapid development. However, its lower mechanical strength, difficulties in recovery from the reaction system, lower loading capacity, poor reusability and accessibility of enzymes are fatal, which hinders its wide application in industry. This review first discusses the selection of non-enzymatic biomolecules for the synthesis of hybrid nanoflowers followed by the innovative advancements made in organic-inorganic hybrid nanoflowers to overcome aforementioned issues and to enhance their extensive downstream applications in transduction technologies. Besides, the role of hybrid nanoflower has been successfully utilized in many fields including, water remediation, biocatalyst, pollutant adsorption and decolourization, nanoreactor, biosensing, cellular uptake and others, accompanied with several quantification technologies, such as ELISA, electrochemical, surface plasmon resonance (SPR), colorimetric, and fluorescence were comprehensively reviewed.

KEYWORDS

biosensor; nanocomposite; nanomaterial; nanostructure; organic-inorganic hybrid nanoflower

Introduction

Nanotechnology is an interdisciplinary science involving physics, chemistry, biology, and engineering to develop its full potential and benefit all mankind. At the beginning of the 3rd millennium, a remarkable upsurge of research activity in nanotechnology is witnessed. In making much smaller and light-weight electronic devices, scientists have reduced the size of a material to nanoscale metrics. At such a low scale, new marvels are observed, leading to the creation of novel devices and promising applications in various domains, from consumer goods to space.[1–8]

Creating intriguing shapes and patterns on the submicron dimension has challenged and inspired researchers. Nanoparticles with porosity and a larger surface area to volume ratio enhance physicochemical properties and have been proved to be useful in different fields including, catalysis, sensing, separation, and storage applications. There are enormous feasible procedures to develop 0 D to 3 D nanocolloids with various shapes, such as nanospheres, nanoplates, nanowires, nanotubes, nanoflowers, nanourchins, nanorods, nanofibers, tetrapods and, to name a few.[9,10] The selection of nanostructure synthesis materials is mainly based on their significance in the studied system and is categorized into organic, inorganic and hybrid. Hybrid materials refer to the integration of multiple materials (organic, inorganic, carbon, and others), which exhibits extraordinary multifunctional performances by combining the intrinsic characteristics of each component. Therefore, it is the most preferable and successfully proven in nanotechnology-based applications.[10–15] The preparation of nanomaterial is often realized through the four types of techniques: 1) Physical, 2) Chemical, 3) Biological, and 4) Hybrid, as depicted in Figure 1.[1,16]

However, these methodologies typically require laborious process steps, sophisticated instrumentation, or involve the use of toxic chemicals, which hampers its wide applicability. Conversely, Zare's group has successfully developed a mild and most direct co-precipitation method to obtain the hybrid nanomaterial with interesting flower-like structures which attracted widespread attention and instantly become a research hotspot since 2012.[17] Since then, various organic elements have been used as crystalline precursors of metal

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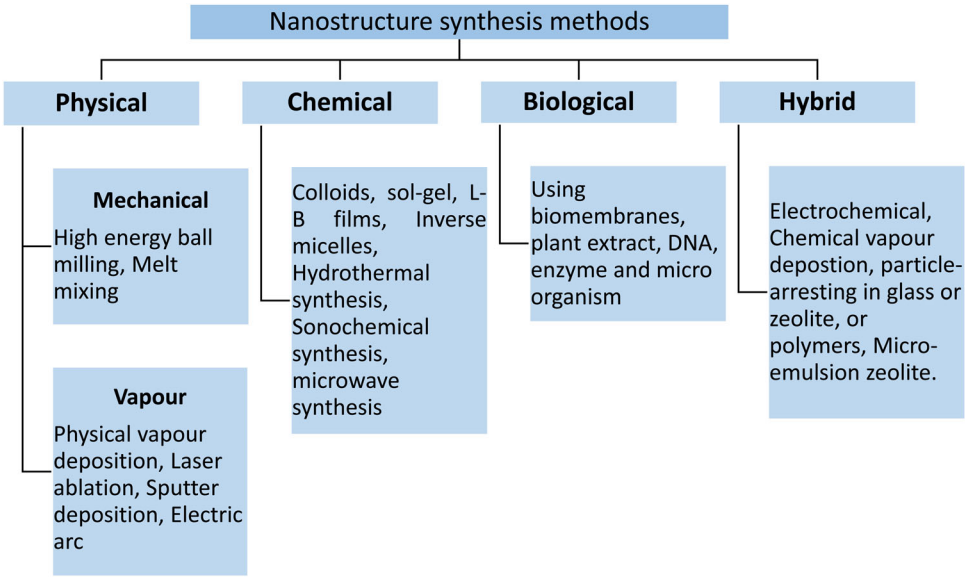

Figure 1. Nanomaterial synthesis technique used commonly; Physical, Chemical, Biological and Hybrid.[1]

ions to form various intriguing flower-like structures, such as rosette, spherical, rhombic, red blood cell-like shape, rod-assembled hollow shuttle-like (Figure 2). The synthesis parameters such as temperature, pH, reaction time, concentration, stirring and sonication procedure are adjusted to achieve the desired morphology and maximum activity of nanoflowers to conform to the system under study.

Different divalent metal ions, for instance, Cu^{2+}, Ca^{2+}, Zn^{2+},[23,24] Mn^{2+},[25] Mg^{2+}, Fe^{2+},[26] Co^{2+},[19,27] and Ag^{+}[28] are commonly used as inorganic elements in co-precipitation method, while the types of organic elements involved are countless. In general, the organic elements mainly vary with the purpose of the experiment, and some systems employed more than a single enzyme to facilitate the cascaded reaction.[29] The hierarchical flower-like morphology with high specific surface area, excellent catalytic activity, high loading efficiency and strong adsorption capacity is interesting factors that have caused the rapid development of the scientific community. Moreover, the high surface area of hybrid nanoflowers could reduce mass transfer due to the flexibility of the substrate and provide a larger contact area with the target.[30] In addition, the presence of metal ions within the nanostructure enhances the enzyme activity.[30] The active sites of enzymes are more exposed in the form of nanoflower,[31] and the natural structure of the biomolecules is better protected in nanostructure than free form. Therefore, their lifespan is extended, and durability, stability, reusability, and enzymatic activity are improved. Table 1 summarizes the enzymatic activity enhancement of various biomolecules in the nanoflower matrix.[50] Nanoflower framework inhibits the aggregation tendency of certain proteins.[51] Also, it is easy to separate the hybrid composite from the reaction mixture after usage. In addition, a cascaded reaction can be realized by co-immobilizing multiple enzymes in a single nanoflower. Apart from that, the inorganic element present in the nanoflower does not affect the enzymatic activity of biomolecules.[52] Through the initial development of the pioneer group, different types of hybrid nanoflowers using various organic and inorganic elements have been generated, and they have been successfully utilized in broad research area, such as hydrogen peroxide detection, protein digestion, enantioselective transesterification, enzyme immobilization, heavy metal adsorption, synthetic dye decolourization, and biosensing disease-related biomarker (e.g., glucose, pathogens) and others. The measurement architecture of transduction technology is another crucial factor that decides the sensitivity of a particular system. For instance, enzyme-linked immunosorbent assay (ELISA) is the most popular immunoassay analysis technique, which can attain a low detection limit (LOD) is in picomolar range.[53] Different types of detection mechanisms and transduction technology has been proposed including, electrochemical, colourimetric, fluorescence, ELISA, and surface plasmon resonance (SPR) in various research area by using the hybrid nanoflower to play a major role.

This overview starts with the synthesis mechanisms of organic-inorganic hybrid nanoflowers and discusses that various amine or amide backbone containing non-enzymatic elements such as DNA, Aptamer, biopolymer, lectin, amino acids, peptide, chitosan, plant extracts, chicken egg white, soy protein isolate, and curcumin to produce hybrid nanoflower. Subsequently, the incorporation of nanoflower with different kinds of nanomaterials was discussed. The nanomaterials effectively enhance the overall performance of nanoflowers to make them suitable for certain specific purposes, such as easier separation, improving mechanical strength, more exposed site for interactions, enhanced acidic resistance, as support for nanoparticle growth, better reusability, moisture resistance and others. The review further focuses on the types of transduction techniques used to evaluate the systems that use hybrid nanoflower as an important element. The techniques used, such as EIS, calorimetry, ELISA, SPR, and fluorescence, was discussed based on how the nanoflowers were integrated with the system under study as part of a performance enhancer.

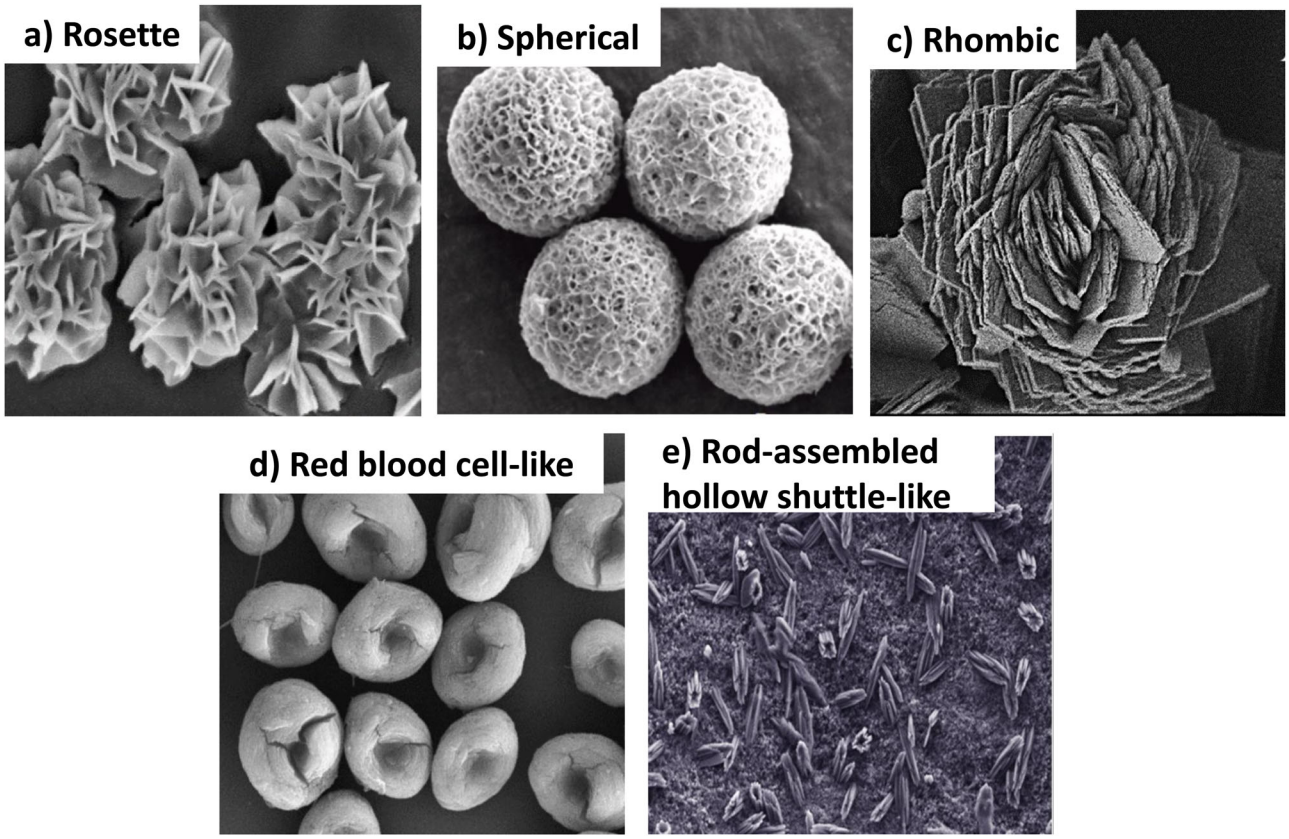

Figure 2. Different morphology of nanoflower a) Rosette, b) Spherical,[18] c) Rhombic, d) Red-blood cell-like, e) Rod-assembled hollow shuttle-like shaped, which was obtained from various types of organic and inorganic components. Permission from.[18–22]

Table 1. Summary of various hybrid nanoflower with specific activity enhancement compared to the free form of biomolecules.

Biomolecule	Metal ion	Enhanced Specific Activity of biomolecule (%)	Application	References
Lactoperoxidase	Cu^{2+}	~360	Detection of dopamine and epinephrine	[32]
Soybean peroxidase	Cu^{2+}	~446	Enzyme purification	[33]
Lipase	Zn^{2+}	147	–	[20]
Lipase	Ca^{2+}	308	Enantioselective transesterification	[34]
Horseradish peroxidase	Fe^{2+}	~710	Enzymatic activity study	[26]
Horseradish peroxidase	Cu^{2+}	~300	Detection of dopamine	[35]
Papain	Cu^{2+}	~4510	–	[36]
Alkaline protease	Cu^{2+}	~927	–	[28]
Alkaline protease	Zn^{2+}	~201	–	[28]
Glucoamylase	Cu^{2+}	204	–	[37]
Laccase	Cu^{2+}	165	Synthesis of viniferin	[38]
Urease	Cu^{2+}	~4000	Hydrolysis of urea	[39]
Papain	Cu^{2+}	~7260	–	[40]
Lipase	Cu^{2+}	~878	–	[40]
Trypsin	Cu^{2+}	~270	Protein digestion	[41]
Turkish black radish peroxidase	Cu^{2+}	~450	Dye decolourization	[42]
Laccase	Cu^{2+}	~180	Synthesis of viniferin	[43]
L-arabinitol 4-dehydrogenase	Cu^{2+}	246	L-xylulose production	[44]
NADH oxidase	Cu^{2+}	144	L-xylulose production	[44]
α-chymotrypsin	Ca^{2+}	266	Protein digestion	[45]
Xylanase	Cu^{2+}	148	Enzyme immobilization	[46]
Lipase ZC12	Ca^{2+}	206	Transesterification reaction of fructose and lauric acid	[47]
Laccase-lysine	Cu^{2+}	~270	Dye decolourization	[48]
Lipase QLM	Cu^{2+}	80.7	Transesterification of sunflower oil	[49]

Formation of hybrid nanoflower and critical parameters

The hybrid nanoflowers can be categorized based on the type of organic and inorganic elements used. Generally, elements containing amide or amine functional groups such as enzymes, protein, amino acids, DNA, peptide, biopolymer, and aptamer are used as organic components. In contrast, divalent metals such as copper, zinc, calcium, manganese, cobalt, iron[4] are considered inorganic components (Figure 3). The synthesis of hybrid nanoflower is simple, green, facile, and consumes lower energy. It can be carried out by mixing organic components with metal ions in the presence of phosphate ions [from Phosphate Buffered Saline (PBS) solution] or directly use metal phosphate. Next, the mixture

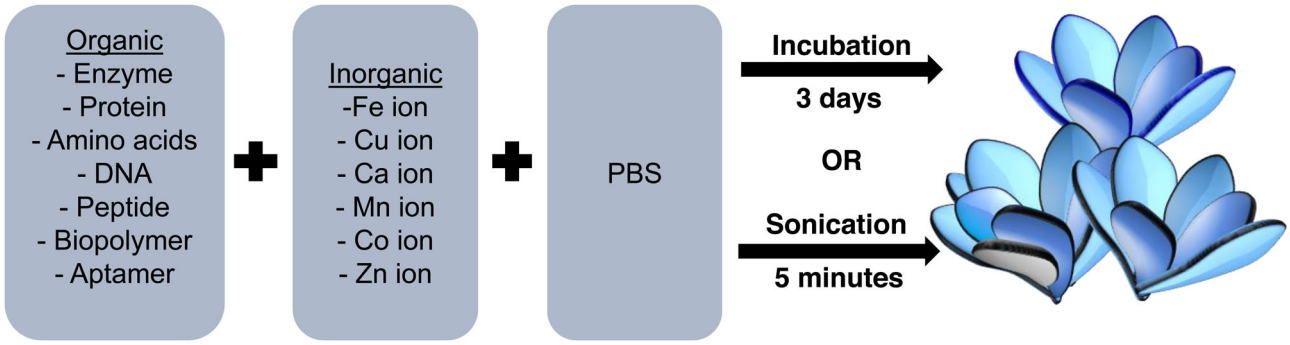

Figure 3. Synthesis of organic-inorganic nanoflower, involving multiple divalent metal ions and biomolecules with incubation for up to three days or ultrasonication in five minutes to obtain a complete flower-like structured nanomaterial.

is incubated for three days at room ambient to obtain fully grown flower-like nanomaterials.[5] However, researchers could also obtain the hybrid nanoflowers within 5 minutes using the sonication method. The formation of beautifully shaped nanoflowers with excellent stability is affected by the incubation period,[1] temperature,[6] pH,[1] organic and inorganic element concentration, and PBS concentration.[6] The formation mechanism of nanoflowers from 0 D to 3 D has been fully understood and explained based on the LaMer model, which involves three stages: nucleation, growth, and completion.[20,25,26,37,39,44,54]

In the nucleation stage, the divalent metal ions (e.g., Cu^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}, Fe^{2+}, Co^{2+}, Ag^{+}) interact with phosphate ions (PO_4^{3-}) to form primary metal phosphate nanocrystals.[55] Organic elements as complexing agents bind with metal phosphate nanocrystal through its amine backbone and forming the coordination interaction. After nucleation is initiated, the primary nanocrystal begins to grow through the assembly of large petals by providing more binding sites for organic molecules. Subsequently, the growth of nanocrystals continued hierarchically and kinetically until reaching the saturation level, at which the 3 D hybrid nanoflower is fully grown. Figure 4A (a) depicts the schematic illustration of Cobalt-BSA nanoflower formation supported by the SEM result in Figure 4A (b–e). The nanoflower's hierarchical growth from 0 D particle-like structure formed in 1 hr evolved to 1 D nanofiber by facilitating the coalescence protein-cobalt phosphate particles in 2 hr. The nanofiber-like structures combine to form irregular multiple branched structures in 3 hr time frame and finally to a complete 3 D nanoflower structure by continuous agglomeration within 24 hrs. The TEM images of the nanoflower and the mapping of O, C and Co element are presented in Figure 4B(a–e) and Figure 4B(f–h), respectively.[56]

Non-enzyme organic material in synthesize of hybrid nanoflowers

In general, nanoflower formation is attributed to the coordination complex interaction between the nitrogen atom of biomolecules and metal ions. Most studies focused on enzymes as organic elements to develop hybrid nanoflowers (Table 1). However, some researchers used other biomolecules such as aptamer, DNA, amino acids, biopolymers, lectin, chitosan, chicken egg white, soy protein lecithin, plant extracts, curcumin as an organic element to construct the self-assembled nanoflower. This is of great significance to their studied system. For instance, He et al. (2016) synthesized nanoflowers comprised of aptamer as an organic element and cobalt as an inorganic element. The synthesized nanoflowers have been successfully used to construct an electrochemical biosensor that can explicitly detect platelet-derived growth factor-BB (PDGF-BB) without needing an additional bioreceptor immobilization procedure. A comparative study between aptamer-cobalt nanoflower and aptamer immobilized on bovine serum albumin (BSA)-cobalt nanoflower was conducted to study aptamer efficiency in detecting PDGF-BB from different frameworks (Figure 5). Aptamer immobilized BSA-cobalt exhibited LOD of 3.7 pg mL^{-1}, while aptamer encapsulated in nanoflower framework exhibited LOD of 61.5 pg mL^{-1}. The LOD decline might be associated with the possible loss of aptamer bioactivity within the nanoflower system.[27]

Besides, biopolymers are also used as organic elements to construct nanoflowers. Biopolymer, i.e., elastin-like polypeptide (ELPs) was used with calcium or copper to synthesize the hybrid nanoflower. Polypeptide provides a binding site for the interaction of active metal ions to form non-covalent complexes and become nucleation sites for primary metal phosphate crystals.[57] Moreover, the chitosan-based nanoflowers synthesized from calcium have mesopores and macropores, thereby showing high adsorption efficiency for Congo red dye, and its catalytic activity was 85% higher than free enzymes.[58]

Ye et al. (2016) prepared the lectin-based nanoflowers, i.e., Concanavalin A (Con A) with invertase as organic and calcium as inorganic compounds. The prepared nanoflowers were used for immediate and sensitive detection of food pathogens (Escherichia coli O157:H7). Firstly, target *E. coli* was recognized by Con A of nanoflower via the specific interaction between Con A and surface O-antigen of *E. coli*. Another enzyme (invertase) acts as a catalyst in the nanoflower, amplifying the signal by converting the added sucrose into glucose readily readable by a personal glucose meter. The inorganic component, calcium ion serves as a promoter by increasing the activity of invertase. A linear relationship between glucose concentrations and target *E. coli* counts were established and attained a point of care detection with a portable glucose meter.[59]

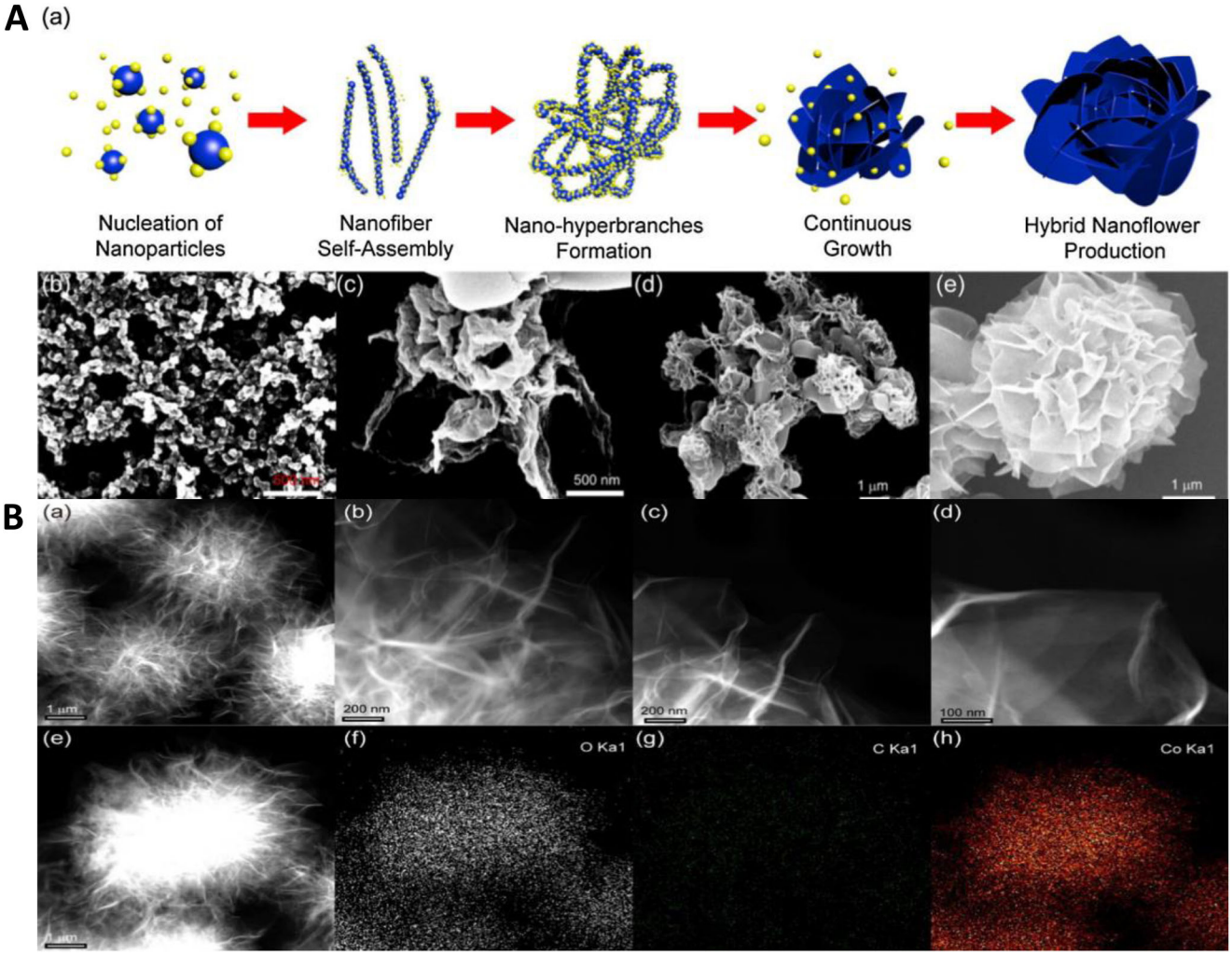

Figure 4. Schematic illustration of the growth process of Co-BSA nanoflower (A), supported by SEM images (B), TEM images (C) and Mapping (D). Permission from.[56]

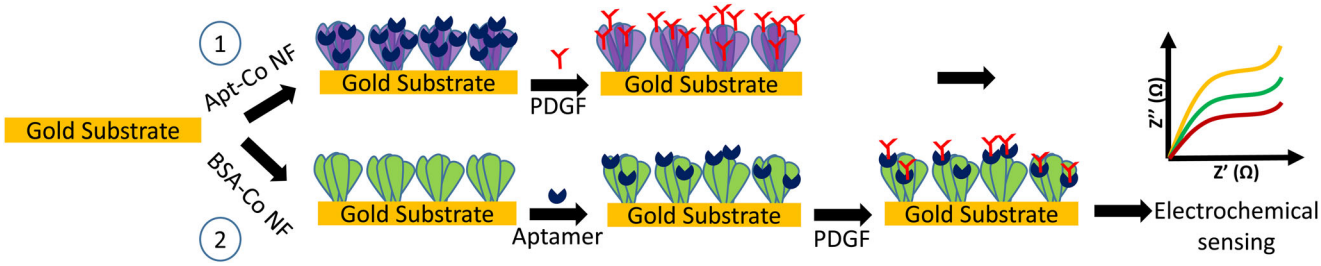

Figure 5. Schematic illustration of comparative electrochemical analysis between (1) aptamer-cobalt nanoflower and (2) aptamer immobilized BSA-cobalt nanoflower to detect PDGF.

Besides, Wu et al. (2016) demonstrated the synthesis of amino acid-based hybrid nanoflowers. Twenty kinds of natural amino acids were used as organic elements, and Cu^{2+} ions as an inorganic compound to synthesize nanoflowers. Each amino acid has different side chains, resulting in a slight change in the nanoflower's morphology. The amino acid-copper nanoflower exhibits peroxidase-like activity, which depends on the Fenton-like reaction with ABTS and Rhodamine B as substrates.[60] Recently, two different hybrid nanoflowers were synthesized using amino acids (l-glutamic and l-cysteine) with copper was immobilized on a pencil graphite electrode to determine calf thymus double-stranded DNA and daunorubicin.[61]

Heme-peptide, i.e., Deuterohemin-b-Ala-His-Thr-Val-Glu-Lys (DhHP-6) with six amino acid residues, was mixed with copper ion to produce nanoflowers. DhHP-6 tends to aggregate in aqueous solutions because Lys shows an intermolecular interaction at the end of the six-amino-acid chain in DhHP-6 with the ferric center of another DhHP-6 through the 6th coordination position. The confinement of DhHP-6 in the nanoflower framework solves the agglomeration issue and renders more accessibility of heme to the

substrates. Compared with the free form DhHP-6, the peroxidase-like activity of DhHP-6 in hybrid nanoflowers attained an excellent enhancement of nearly 300%.[62]

Moreover, plant extracts are also used to form hybrid organic-inorganic nanoflowers. Ildiz et al. (2017) used Viburnum opulus (VO) extract as an organic element and copper as an inorganic element to obtain snowball-like nanoflowers, which can be performed as an active antimicrobial agent toward various types of pathogens. In contrast, no antimicrobial activity was observed by the free VO extract. The VO-copper hybrid nanoflower only exhibits peroxidase-like activity in the presence of copper divalent ion, which depends on the Fenton-like reaction mechanism against guaiacol.[63]

Hybrid nanoflower was also synthesized using DNA as an organic element. Park et al. (2017) synthesized hybrid nanoflower with DNA as an organic component and copper as an inorganic component. DNA is rich in amide and amine groups forms complexes with copper ion to initiate the crystallization. The synthesized hybrid nanoflower has high loading efficiency, low cytotoxicity, and strong nucleases resistance. The DNA confined in the nanoflower matrix is profoundly impervious to endo- and exo-nucleases. Even after 24 hours of a prolonged enzymatic reaction, the image of intact DNA can still be observed via gel electrophoresis. Furthermore, DNA-copper hybrid nanoflowers indicated no cytotoxicity up to 100 μg/mL, recommending their prospective as a nucleic acid delivery agent.[64] Recently, DNA with manganese nanoflowers was prepared via rolling circle amplification reaction for cellular uptake and tumor targeting. Some of Mn^{2+} as co-factor catalyzes the elongation of single-stranded DNA, enhanced the production of many pyrophosphate ions (PPi^{4-}) and the other part of Mn^{2+} reacts with PPi^{4} to form insoluble Mn_2PPi, which acted as a framework of Mn-DNA nanoflowers. The nanoflowers showed morphology collapse and Mn^{2+} release in an acidic environment, thereby enhancing T1-weighted MRI performance at the tumor site.[65]

Apart from that, soy protein isolate (SPI) film and copper were also used as an organic and inorganic component to grow hybrid nanoflowers. The SPI film widely used in the food industry is highly sensitive to water, but mixing with nanoflowers can enhance its moisture resistance. They were also immersed in octadecylamine ethanol to impart hydrophobicity to the films.[66] Furthermore, Altinkaynak et al. (2018) used raw egg white as an organic component with copper for synthetic dye decolorization. The raw egg white was blended to a foamy texture and used directly to synthesize hybrid nanoflowers, which exhibited a polyphenol oxidase activity.[67]

More interestingly, curcumin was also used as organic material to synthesize hybrid nanoflowers with copper metal, and its antimicrobial activity toward bacteria was investigated. The irregular structure of the curcumin-copper hybrid nanoflowers was related to the absence of reactive functional groups such as amine and carboxylic groups on curcumin structure.[68] Recently, calcium and phosphate were extracted from bone waste as a natural source of inorganic components via acidification strategy and formed hybrid nanoflowers with lipase, which was used as a biocatalyst in producing Clindamycin palmitate.[69]

Nanomaterial embedded nanoflowers/modified hybrid nanoflowers

Various improvements have been made to increase the applicability of nanoflowers to meet specific requirements by incorporating different types of nanomaterials and chemical treatments. Lower mechanical strength, difficulty to recover from the reaction system, low loading capacity, poor accessibility and recyclability of an enzyme are the main drawbacks of organic-inorganic hybrid nanoflowers, which hinder its wide-ranging applications in industry. Therefore, the inventive design should be adopted with the conventional synthesis methodology to orchestrate profoundly powerful and recyclable protein-inorganic nanoflowers. Incorporating nanomaterials and chemical treatment into or around the nanoflowers can effectively improve the drawbacks and make them widely used.

For improved mechanical strength and more opulent binding site

Li et al. (2018) successfully synthesized oxidized carbon nanotubes (CNT) embedded lipase - Ca/Fe/Cu nanoflowers. Adding CNTs to hybrid nanoflower can heighten the mechanical strength and provide more accessible adsorption binding sites, thereby effectively enhancing enzyme activity. The activity of the synthesized Cu/CNT/lipase composite was 68 and 51 folds higher than that of free lipase and Cu/lipase, respectively. The synthesized nanoflowers were used effectively as a biocatalyst for the chiral resolution reaction between 1-phenylethanol and vinyl acetate, where 98% of substrate enantiomeric excess (ee_s) was attained in just 10 minutes.[70] Moreover, Li et al. (2017) reported on CNT and graphite oxide (GO) sheet incorporated with laccase-copper nanoflower. The addition of CNT improved the enzyme loading capacity and the accessibility of laccase. CNT acts as an interlayer spacer between the GO sheets to preserve better accessibility of the encapsulated laccase and facilitates direct electron transfer from the active site of laccase. Also, the incorporation of CNT within nanostructure provides a larger surface area, allowing more anchor sites to be loaded with laccase.[71]

For easier separation

The difficulty of separating nanoflowers from the reaction solution limits its wide application, and separation via centrifugation requires additional energy consumptions. Thus, researchers used several techniques to simplify the separation. For instance, Rong et al. (2017) designed laccase-copper nanoflowers on grown nanoflowers over copper foil to solve the problem of recovery and separation of suspended powder samples for wastewater treatment. Firstly, the solution growth technique was used to grow

$Cu_8(PO_3OH)_2(PO_4)_4{\cdot}7H_2O$ nanoflowers (CPN) on the surface of copper foil, and then immerse it in a solution containing laccase to grow laccase-copper nanoflower on the surface of CPN. In other words, CPN was used as an inorganic component. The decolourization efficiency of the nanoflowers against Congo red was almost 3.6 times higher than the free form of laccase, exceeding 95% in three hours.[30]

Apart from that, nanoflower entrapped in microbeads show potential use in the beer brewing industry. α-Acetolactate decarboxylase (ALDC)-calcium nanoflower entrapped in the alginate gel beads solves the problem of the buttery taste of beer and promotes the maturation of beer. Using ALDC alone can prevent the formation of diacetyl, i.e., a by-product formed by α-acetolactate that makes beer taste buttery. However, difficulties in the recovery, stability and reusability of the free form ALDC hamper its extensive industrial applications. Thus, ALDC-calcium nanoflower has been synthesized and encapsulated in alginate gel bead. The alginate bead around the nanoflower prevents the leaching of ALDC upon recycling. Compared with free ALDC, the ALDC encapsulated nanoflower retained 98% of the activity and have higher stability and reusability. Also, the hybrid beads are easily separable from the reaction mixture.[72] Very recently, Feng et al. (2019) developed a magnetically-separable nanoflower with papain-copper-magnetic nanoparticle (PCMN) for enzymatic hydrolysis of cow milk. The magnetic nanoparticle within the nanoflower can be easily separated using a magnet block after applied to hydrolyze the protein *β*-lactoglobulin (*β*-Lg) that present in cow milk. Compared with free papain, the enzymatic activity of the PCMN enhanced to 1556% and the recovery rate maintains at 597.6%.[50] Also, Lee et al. (2019) demonstrated the potential application of magnetic nanoparticle embedded glucose oxidase (GOx)-copper hybrid nanoflower in bacterial decontamination. The antibacterial activity of nanoflowers against *Staphylococcus aureus* and *E. coli* was examined. With the help of an external magnetic block, the magnetic bead-modified hybrid nanoflower can be easily separated from the reaction mixture within 30 seconds, which facilitates its recovery and reuse. Although the bacteria were repeatedly killed eight times, the bacterial activity of the hybrid nanoflower remained at 97%.[73]

For enhanced reusability

Besides, nanoflower is also grown on a nanofiber membrane to form a multilevel surface. Copper-BSA nanoflowers were formed on the nanofiber membrane surface by soaking the Cu^{2+} functionalized nanofiber membrane into a solution containing BSA and PBS. The combination of nanoflower and nanofiber dramatically enhances stability and reusability by reducing enzyme conformational changes. The nanocomposite is expected to open new possibilities in various applications, including biosensors, biocatalysts, bioengineering, and biodevices.[74] Besides, Baek et al. (2019) reported that gold nanoparticle and glucose oxidase-copper nanoflower decorated graphene oxide (GO) nanofiber was used to detect glucose. Gold nanoparticles and hybrid nanoflower modified nanofibers have better electrochemical performance and can effectively detect glucose.[75] Nanoflowers are usually grown on the external surface of the support. However, Luo et al. (2020) grew laccase-copper hybrid nanoflowers on the inner and outer surfaces of a 3 D architecture porous nanofibrous PVA-co-PE membranes (HPNM). The coexistence of small pores and large pores in the nanofiber membrane promoted the penetration of laccase-copper nanoflower and entrapped in the inner structure of the nanofibers, thereby achieving high loading and improved nanoflower distribution. The nanocomposite shows excellent degradation efficiency, about 99.5% toward indigo carmine, and the degradation efficiency only reduced slightly from 99.48% to 98.52% after 14 reuses.[76]

Cross-linking for better robustness

After multiple centrifugations during the collection of nanoflower precipitates, the soft-natured hierarchical shaped petals of hybrid nanoflowers are easily broken, thereby impairing the reusability. For this reason, cross-linking is one of the procedures used to improve the robustness of nanoflower. Consequently, researchers treated hybrid nanoflower with glutaraldehyde (GA), i.e., a strengthening glue to cross-link the entrapped biomolecules. Lee et al. (2017) prepared a lipase-copper hybrid nanoflower by standard co-precipitation method and then incubated it with GA for 1 h. The GA cross-linked nanoflower exhibited high initial activity retention of over 92% after 3 reuses, and over 70% after 4 reuses, while still effectively maintaining its flower-like morphology. The lipase-copper nanoflower without GA treatment cannot be reused because they lost almost 90% of their activity after 4 cycles and their morphology degraded. This may be related to the continuous leaching of lipase from a loosely interlaced nanoflower matrix.[77] Also, Patel et al. (2018) cross-linked the laccase-copper hybrid nanoflowers with GA, which significantly improved the reusability of the nanoflowers and tolerance to solvents and inhibitors. The cross-linked nanoflower can effectively decolour synthetic dyes, including bromophenol blue, Coomassie Brilliant Blue R-250, and xylene cyanol in 48 h with an efficiency of 93.5%.[78]

For acidic resistant

As we have known, hybrid nanoflowers are mainly prepared via *in situ* self-assembly of protein and inorganic crystals. Nevertheless, the inorganic crystal structures, [e.g., $Ca_3(PO_4)_2$ and $Cu_3(PO_4)_2$] would be easily broken in an extremely acidic reaction medium. This property barricades the widespread applications of hybrid nanoflowers under acidic conditions. Liu et al. (2017) prepared Chloroperoxidase (CPO)-calcium hybrid nanoflowers coated with self-repairing material sodium alginate (SA) and tested its feasibility in a solution containing phosphoric acid buffer at pH 2.75. The SA-treated hybrid nanoflowers show excellent acid resistance, maintaining more than 85% catalytic

activity even after 12 cycles. However, the nanoflower without SA loses 70% of its original activity after 5 cycles. The SA around the nanoflower effectively captures the free Ca^{2+} ions and prevents the leaching of CPO at low pH. Moreover, SA-coated nanoflower also exhibit good thermal and storage stability.[79]

Supports the growth of nanoparticles

Hybrid nanoflowers are also used as support for growing nanoparticles. Interestingly, Zhang et al. (2017) used copper and dopamine as inorganic and organic elements, respectively, to produce hybrid nanoflower and infused silver nanoparticles as an antimicrobial agent. The copper ions in solution have catalytic activity and polymerase dopamine into polydopamine (PD). The reductive capability of PD reduces silver nitrate to silver nanoparticles, which are successively attached to the nanoflower surface. In this case, copper plays a major role in polymerizing dopamine into polydopamine and ultimately reduces silver nitrate to silver nanoparticles without using any toxic substances as a reducing agent. The silver/PD/copper hybrid nanoflower effectively inhibits bacterial growth.[17] Hybrid nanoflower was also effectively utilized as carriers for the growth of platinum nanoparticles (PtNPs). PtNPs grew on the surface of $Mn_3(PO_4)_2$@BSA by chemical reduction of H_2PtCl_6 in ethylene glycol. The prepared nanoflower was successfully used as a catalyst for methanol oxidation.[80]

Transduction techniques

Electrochemical technique

The electrochemical technique has received widespread interest because of its quintessential advantages such as affordable cost, simple measurement setup, fast response, and high sensitivity. Cyclic voltammetry (CV), electrical impedance spectroscopy (EIS), and differential pulse voltammetry (DPV) are the most common methods used in electrochemical measurement to study the electrochemical behavior of a biosensor or sensor. These methods are usually performed using a bench-top spectrometry with a three-electrode system, namely the working electrode (the studying electrode), the counter electrode (platinum rod/wire), and the reference electrode (Ag/AgCl electrode). Different kinds of working electrodes, such as gold, glassy carbon electrode, ITO are usually modified with linkers (ex: CDI, APTES, MUA, PEDOT, chitosan) and nanomaterials (nanospheres, nanoplates, nanowires, nanotubes, nanoflowers, nanourchins, nanorods, nanofibers, tetrapods) to aid and enhance the capturing efficiency of desired bioreceptors and target analyte.

As such, the organic-inorganic hybrid nanoflower was deposited on the working electrode of biosensor and mainly treated as biocompatible support for the immobilization of bioreceptors (antibody, DNA, aptamer, lectin, phage) targeting various biomarkers (protein, cell, enzyme, DNA). The hybrid nanoflower with 3 D porous structure provides larger specific surface area that allows the loading of more bioreceptors. Moreover, the metal present within the nanoflower framework enhances the electrical conductivity thereby improving the overall electrochemical signal. Various biosensors such as immunosensor, cytosensor, aptasensor and genosensor were reported excellent performance using the hybrid nanoflower in their biorecognition site.

For Instance, Cao et al. (2015) reported nanoflowers made of BSA-silver were used as biocompatible support to immobilize lectin (Sambucus nigra agglutinin, SNA) to specifically target glycoprotein (sialic acid). The nanoporous structure and larger surface area of BSA-silver hybrid nanoflower enable cells to be highly loaded on the substrate. Besides, BSA embedded in nanoflower has excellent biocompatibility and provides a multifunctional interface for the immobilization of SNA. The excellent electrical conductivity of Ag amplifies the electrochemical signal and improve sensing capability. When BSA-Ag nanoflowers modified the surface of glassy carbon electrode (GCE), almost a straight line was observed in the Nyquist plot (lower than the bare GCE), which indicates diffusion-limited electron transfer and significant signal magnification. They reported that an electrochemical cytosensor using a GCE as a working electrode has an excellent LOD of 40 cell mL^{-1}.[81]

Moreover, a cascade effect of the three redox reactions catalyzed by organic and inorganic elements in the nanoflower framework significantly amplifies the electrochemical signal. For this, Li et al. (2017) developed an electrochemical biosensor using GOx-HRP-copper nanoflowers and enhanced it with gold nanoparticles and thionine, was used as support to immobilize T4 phage targets live E. coli in the urine (Figure 6). The three types of molecules (GOx, HRP, thionine) within nanoflower realized three cascaded redox reactions, which has an immense effect in amplifying the electrochemical signal. These strategies allow detection of live E. coli ranges from $15–1.5 \times 10^8$ CFU mL^{-1}, with a meager LOD of 1 CFU mL^{-1}.[82]

The nanoflowers are also used as a support material for platinum nanoparticles in methanol oxidation. For instance, Zhang et al. (2015) reported the use of precursor H_2PtCl_6 to grow platinum nanoparticles (PtNPs) on the surface of BSA-manganese nanoflowers by chemical reduction and used as an electrocatalyst for methanol oxidation. The hybrid nanoflowers supported PtNPs exhibited higher stability and superior electrocatalysis for methanol oxidation with electrochemically active surface area (ECSA) value of 106 m^2 g^{-1}.[80] Likewise, Song et al. (2017) prepared BSA-zinc nanoflowers as a support for platinum nanoparticles (PtNPs) and applied it for methanol oxidation with ECSA value was 149.11 m^2 g^{-1}.[83]

To further enhance the detection sensitivity and reduce fabrication procedure, most of the researchers directly co-precipitated the bioreceptor itself (such as antibody) as organic element with metal ion, forming a hybrid nanoflower-based bioreceptor. The enhanced stability of bioreceptors within the nanoflower framework enables it to exert the highest performance and improves detection efficiency. For instance, Gao et al. (2020) prepared hemoglobin-manganese nanoflower for electrochemical detection of H_2O_2. The

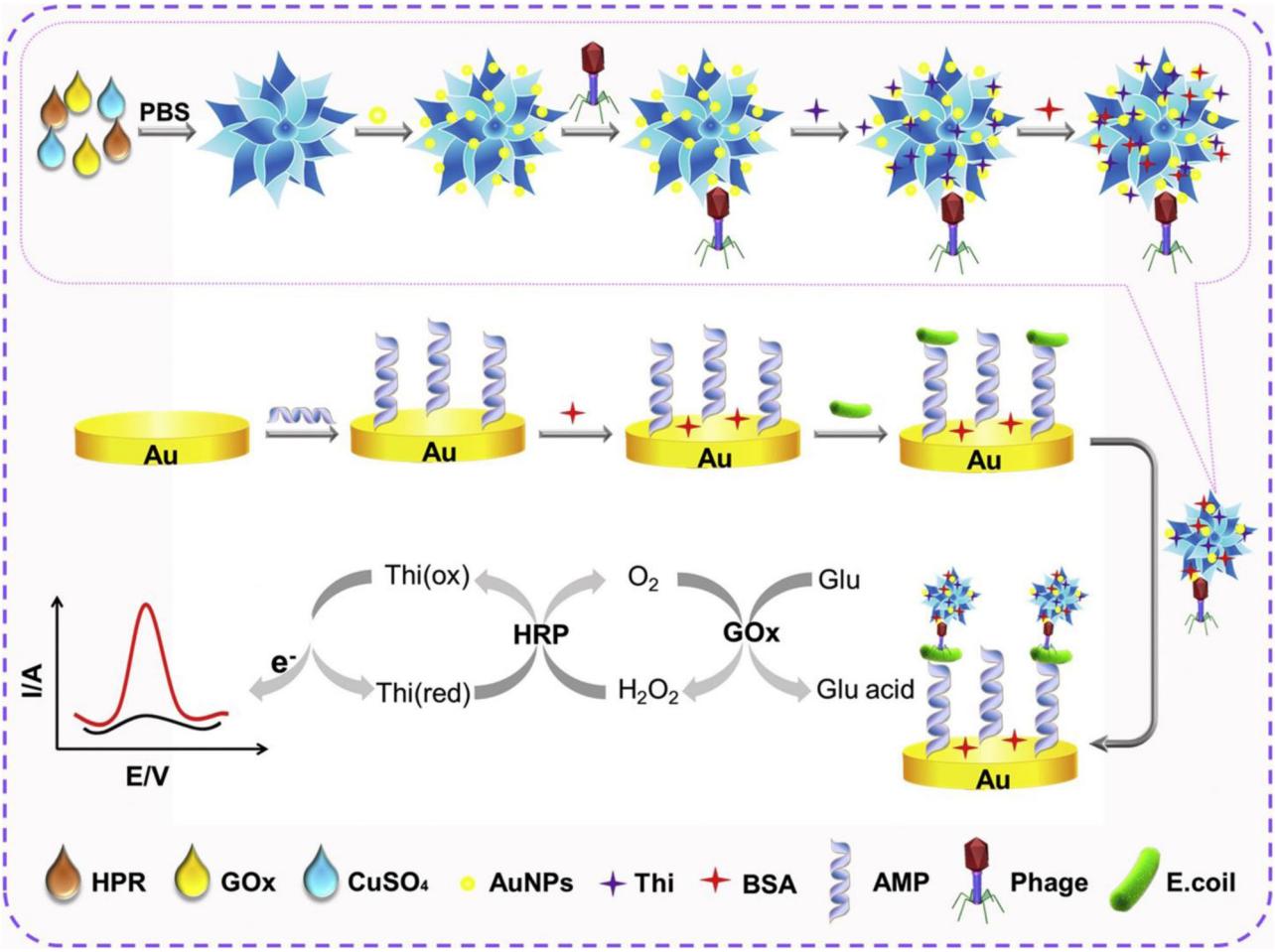

Figure 6. Schematic illustration of gold electrode surface modification with AMP magainin I and electrochemical detection of phage by the live *E.Coli*; the detection was amplified by three cascaded redox reactions from organic-inorganic hybrid nanoflower. Permission from.[82]

three-dimensional hemoglobin structure was unfolded in the nanoflower framework, exposing more electroactive centers to improve electrochemical performance. The sensor attained a lower LOD of 7 nM with a linear range from 20 nM to 3.6 μM.[84]

In a study by Tang et al. (2019), an antibody was co-precipitated with BSA-copper to form the hybrid nanoflower as a biorecognition unit to detect the C-reactive protein. The heavy phosphate ions present in the nanoflower framework was effectively exploited with molybdates (Mo) to generate redox-active molybdophosphate, which was eventually precipitated under acidic condition and generate redox electric current. In the CV graph, two pairs of redox peaks of molybdophosphate were observed at 0.34 V and 0.14 V, which was associated with the electron transfer of Mo in the molybdophosphate. Therefore, the signal enhancement mainly depends on the presence of plentiful phosphate ions in the nanoflowers, which reacts with molybdate to generate a large amount of electrochemically active molybdophosphate. The inorganic component of the nanoflower has been efficiently utilized to transduce the electrochemical readout. Also, an increment trend was observed in the Nyquist curve upon each modification. This is due to the successful anchoring of biomolecules on the electrode surface, which hinders the electrons transfer from $[Fe(CN)_6]^{3-/4-}$ to the electrode surface. The immunoassay presented an excellent analytical performance with a linear range of 5 pg mL^{-1} to 1 ng mL^{-1}, and the LOD was 1.26 pg mL^{-1}.[18]

The electrochemical performance of the biosensors was further enhanced by tuning the hybrid nanoflower with additional nanomaterial forming multi-nanocomposite. Xu et al. (2019) created Streptavidin-copper nanoflower integrated with graphene oxide (GO) and was immobilized with aptamer for electrochemical sensing of Interferon-gamma (IFN-ɣ). A comprehensive detection ranges from 0.1 pg mL^{-1} to 500 ng mL^{-1} was achieved with a lower LOD of 19 fg mL^{-1}, which gives an idea of the role of GO-modified nanoflower in the transduction.[85] Apart from that, Baek et al. (2019) demonstrated the electrochemical detection of glucose with the aid of GOx-HRP-Cu nanoflower. In work, a gold chip was first modified with gold nanoparticles decorated GO nanofiber, then further modified with GOx-HRP-Cu-nanoflower, and finally subjected to different concentrations of glucose. The sensor achieved a more comprehensive linear range of 0.001–0.1 mM, with a LOD of 0.018 μM.[75] Most recently, Zhang et al. (2020) reported that hybrid nanoflowers integrated with multi-elements could enhance its electrochemical immunosensing performance against carcinoembryonic antigen (CEA). BSA-Ag nanoflowers were first synthesized by doping carboxylated single-walled

Table 2. Summary of electrochemical transduction used for the system uses hybrid nanoflower.

Metal	Protein	Bioreceptor	Target	Application	Performance	References
Copper	Cysteine	Antibody	*E. coli* O157:H7	Biosensor	10 CFU ml^{-1}	[88]
Silver	BSA	lectin	Sialic acid	Biosensor	40 cell mL^{-1}	[81]
Copper	BSA	DNA	TB DNA	Biosensor	60 pM	[21]
Copper	BSA	Aptamer	Hepatitis B virus DNA	Biosensor	100 copies mL^{-1}	[89]
Copper	GOx-HRP	T4 phage	*E. coli*	Biosensor	1 CFU mL^{-1}	[82]
Manganese	Immunoglobulin G (IgG),	Antibody	Ractopamine	Biosensor	9.32 pg mL^{-1}	[90]
Manganese	BSA	Antibody	Ractopamine	Biosensor	26 pg mL^{-1}	[90]
Manganese	Ractopamine antibody (RACanti)		Ractopamine	Biosensor	4.6 pg mL^{-1}	[90]
Manganese	BSA			Methanol oxidation	106 m^2 g^{-1}	[80]
Zinc	BSA			Methanol oxidation	149.11 m^2 g^{-1}	[91]
Calcium	Polyclonal antibodies		*Salmonella*	Biosensor	28 colony-forming units mL^{-1}	[92]
Copper	Antibody-BSA		C-reactive protein	Biosensor	1.26 pg mL^{-1}	[18]
Copper	Streptavidin		Interferon-gamma	Biosensor	19 fg mL^{-1}	[85]
Copper	GOx-HRP		Glucose	Biosensor	0.018 μM	[75]
Manganese	Hemoglobin		H_2O_2	Gas sensor	7 nM	[84]
Silver	BSA		Carcinoembryonic antigen	Biosensor	0.0001 ng mL^{-1}	[86]
Copper	Amino acids 1) l-cysteine 2) L-glutamic		Calf thymus double-stranded DNA (ctdsDNA)	Biosensor	1) 0.93 μg mL^{-1} 2) 2.37 μg mL^{-1}	[61]
Copper	Amino acids 1) l-cysteine 2) L-glutamic		Daunorubicin (DNR)	Biosensor	1) 2.93 μM 2) 2.06 μM	[61]
Calcium	BSA, Glucose oxidase, Anti-Salmonella polyclonal antibodies		Salmonella	Biosensor	10^1 CFU mL^{-1}	[93]
Copper	Glucose oxidase (GOx), Laccase, Catalase			Glucose biofuel cell	200 μWcm^{-2}	[87]

carbon nanotubes, reducing GO, and poly (3,4-ethylenedioxythiophene) to form multi-nanocomposite. Then, the nanocomposite was deposited on the gold electrode and immobilized the antibody (anti-CEA) to identify the target CEA at different concentrations. The sensor able to achieve a more comprehensive linear range from 0.002 to 50 ng mL^{-1} with a considerably lower LOD of 0.0001 ng mL^{-1}.[86]

Hybrid nanoflower is also compressed with carbon nanotubes to form bioelectrodes in the glucose biofuel system. Chung et al. (2018) prepared three types of hybrid nanoflowers to construct a glucose biofuel cell. They applied glucose oxidase (GOx) nanoflower as an anode and laccase nanoflower as a cathode. Besides, catalase nanoflower was applied additionally at the anode, which helps to scavenge the unwanted hydrogen peroxide generated via GOx catalysis. The prepared nanoflowers were mixed and compressed with carbon nanotubes to improve the enzyme wiring efficiency and electron transfer rate. CV analysis was implemented to examine the properties of bio-electrodes, and the current density was studied to understand the electrochemical reaction. The prepared mediatorless nanoflower-based biofuel achieved a high power density of 200 μWcm^{-2}, which was 80% higher than that of biofuel using free-form enzymes.[87] Table 2 summarizes the electrochemical quantification technique reported using the hybrid nanoflower. Biosensors are mostly reported with these techniques, and the capturing of the target analyte was mainly carried out either by immobilizing the target analyte on surface of hybrid nanoflower or directly embedded into nanoflower during synthesis procedure.

Colourimetric/visual detection

The colourimetric technique falls under optical-based detection method is mainly based on color changes. The basic form of colourimetric assays is to evaluate the changes in absorbance or reflection intensity of analyte-reagent complexes. These changes are commonly caused by the structural shifts or plasmon resonance phenomena, which typically result in a shift in the optical properties of the sample over a wide range of wavelengths.[94] Colourimetric is one of the most popular evaluation methods because of its simplicity, low-cost, faster response and convenient features, making it suitable for environments with limited facilities. Most importantly, it can easily visualize the target molecule with a naked eye without the need for detection instruments. Chromogenic dyes [e.g., 3,3′,5,5′-tetramethylbenzidine (TMB), 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), o-phenylenediamine dihydrochloride (OPD), guaiacol] principally used to recognize the target and generate the color signal. The intensity of color change indicates the presence of the target, and a table-top spectrophotometer is commonly used to analyze the absorbance value of colourimetric assays. A conventional colourimetric assay based on free enzymes suffers drawbacks such as limited stability, reproducibility and recovery.[95] Thus, researchers immobilized the enzymes on a nanomaterial, but its enzymatic activity was hampered compared with the free form. Thereby, hybrid nanoflower-assisted colourimetry was extensively used to detect various targets, including hydrogen peroxide, phenol, disease-related biomarkers (e.g., glucose, epinephrine, cholesterol, catecholamine and foodborne pathogens), synthetic dyes, and heavy metals.

Detection of hydrogen peroxide and phenol

Phenol is one of the major toxic pollutants even at low concentrations, indicating the significance of proper management. Zhu et al. (2013) prepared laccase-copper nanoflowers and incorporated it into cellulose acetate membrane for judicious phenol detection. In the detection procedure, the nanoflower suspension was initially injected into a commercial disposable syringe filter to deposit on the surface of the equipped cellulose acetate membrane. Then, an aqueous mixture of phenol and 4-aminoantipyrine solution was passed through the syringe, causing the oxidative coupling of the mixture to form an antipyrine dye with maximum absorption at 495 nm. The enhanced enzymatic activity of laccase in the nanoflower framework allows faster coupling of phenol and 4-aminoantipyrine to form a dye. The assay showed a linear relationship between absorption intensity and phenol concentration. A hybrid nanoflower-treated membrane with an absorption intensity at 495 nm is reproducible in 30 consecutive cycles within a month, whereas free laccase continuously reduced to less than 20% of its initial value within the first five days.[96]

Following this germinal research, Lin et al. (2014) developed HRP-Copper nanoflowers for the detection of phenol and hydrogen peroxide. HRP catalyzes the oxidation of phenol and OPD in the presence of H_2O_2. As the concentration of H_2O_2 increases, the color of the reaction solution changed from colorless to pink and the absorption peak at 505 nm increased higher. The hybrid nanoflower system completed the oxidation reaction within 5 minutes, while the free form of HRP-containing system took a longer reaction time. This phenomenon was also observed for phenol detection. Visualized by naked eyes, the LOD for H_2O_2 and phenol was 0.5 μM and 1 μM, respectively. Such results meet the requirements of clinical diagnosis and detection of two analyte in environmental water.[97]

Apart from that, Thawari et al. (2016) developed hybrid nanoflowers from β-LG and copper for phenol detection. The hybrid nanoflowers were added in a mixture of PBS containing ABTS and H_2O_2, and the oxidized $ABTS^+$ was investigated using spectroscopy. A strong absorption was observed at 415 nm, and as the phenol concentration increased, the color of the reaction solution changed from colorless to pink.[98] Also, Zhang et al. (2019) reported the colourimetric detection of H_2O_2 using catalase-copper hybrid nanoflower synthesized by sonication methods. In a detection test under room ambient, different concentrations of H_2O_2 were added to PBS solutions containing the hybrid nanoflowers, excess phenol, and 4-aminoantipyrine (AAP). As the concentration of H_2O_2 increases, the color of the solution changed from light red to brownish red and the colourimetric assay showed a linear absorbance response toward H_2O_2 in the concentration range of 1 μM to 1 mM, and the LOD was 1 μM.[95]

Dye decolourization and heavy metal detection

Colourimetric-based quantification is mainly devoted to study the efficiency of hybrid nanoflowers as adsorbents toward synthetic dyes and heavy metals. Different types of biomolecules (e.g., BSA, laccase, egg white protein, vegetable drum stick seed protein, Turkish black radish peroxidase) have been used for this purpose.

For instance, Wang et al. (2014) reported a chitosan-calcium hybrid nanoflower for Congo red decolourization.[58] Zhang et al. (2016) prepared BSA-zinc nanoflowers for Cu^{2+} adsorption. The nanoflowers exhibited an adsorption efficiency of 86.33% and 98.9% in 5 and 30 mins, respectively.[99] Interestingly, Altinkaynak et al. (2017) prepared hybrid nanoflowers from copper and Turkish black radish peroxidase to decolourize Victoria blue. The decolourization efficiency reaches more than 90% at pH 7.0 within 1 hour. The activity of hybrid nanoflower was ~450% higher than free Turkish black peroxidase, which was determined using guaiacol as a substrate. The reusability of the nanoflower was also tested, and it showed 77% efficiency even after the tenth cycle.[42]

Moreover, Altinkaynak et al. (2018) developed hybrid nanoflowers composed of crude egg white and copper, and tested for decolourization of different textile dyes. In the presence of H_2O_2, the hybrid nanoflowers showed substantial polyphenol oxidase-like activity and enhanced dye coloring efficiency. All dyes have been tested with and without H_2O_2, and the discoloration range was about 34 to 80%.[67] Besides, Polepalli et al. (2018) prepared hybrid nanoflowers from copper and Moringa Oleifera proteins, which were isolated from vegetable drum stick seed. Six different types of dyes were tested, three of which were anionic, and the remaining three were cationic. The hybrid nanoflowers with cationic nature preferentially adsorbs anionic dyes (congo red, eosine, rose bengal) and the color changed was observed within 5 minutes, while only marginal adsorption toward cationic dyes (rhodamine b base, rhodamine 6 G, methylene blue). Moreover, complete decolourization was observed for the dye-containing solution with the presence of catalytic copper salt and H_2O_2. Also, these NFs can adsorb heavy metal ions (e.g., Pb^{2+}, Cd^{2+} and Hg^{2+}), but have a higher selectivity (>99%) for Pb^{2+}.[100] Recently, Alhayali et al. (2020) synthesized nanoflowers composed of inorganic iron and copper with catalase as organic elements. The nanoflowers were used to reduce nitrobenzene and organic dyes, showing excellent reduction of nitroarenes to aminoarenes within 30 minutes.[101]

The laccase-copper hybrid nanoflower was used majorly for decolourization of various dyes. For Instance, Li et al. (2017) developed laccase-copper hybrid nanoflowers integrated with carbon nanotubes and GO to remove reactive dye (Crystal Violet and Neutral Red).[71] Moreover, Rong et al. (2017) also applied the laccase-copper nanoflower for Congo red decolourization. Compared with the free laccase on Congo red dye solution, the hybrid nanoflower showed higher decolouration efficiency (over 95%) and decolouration rate (nearly 3.6 times) within 3 hours.[30] Patel et al. (2018) cross-linked the laccase-copper nanoflowers with glutaraldehyde and applied it to decolourize highly concentrated synthetic dyes. The decolourization efficiency of the cross-linked hybrid nanoflowers reached 93.5% within 48 hours and was 120% higher than free laccase.[77]

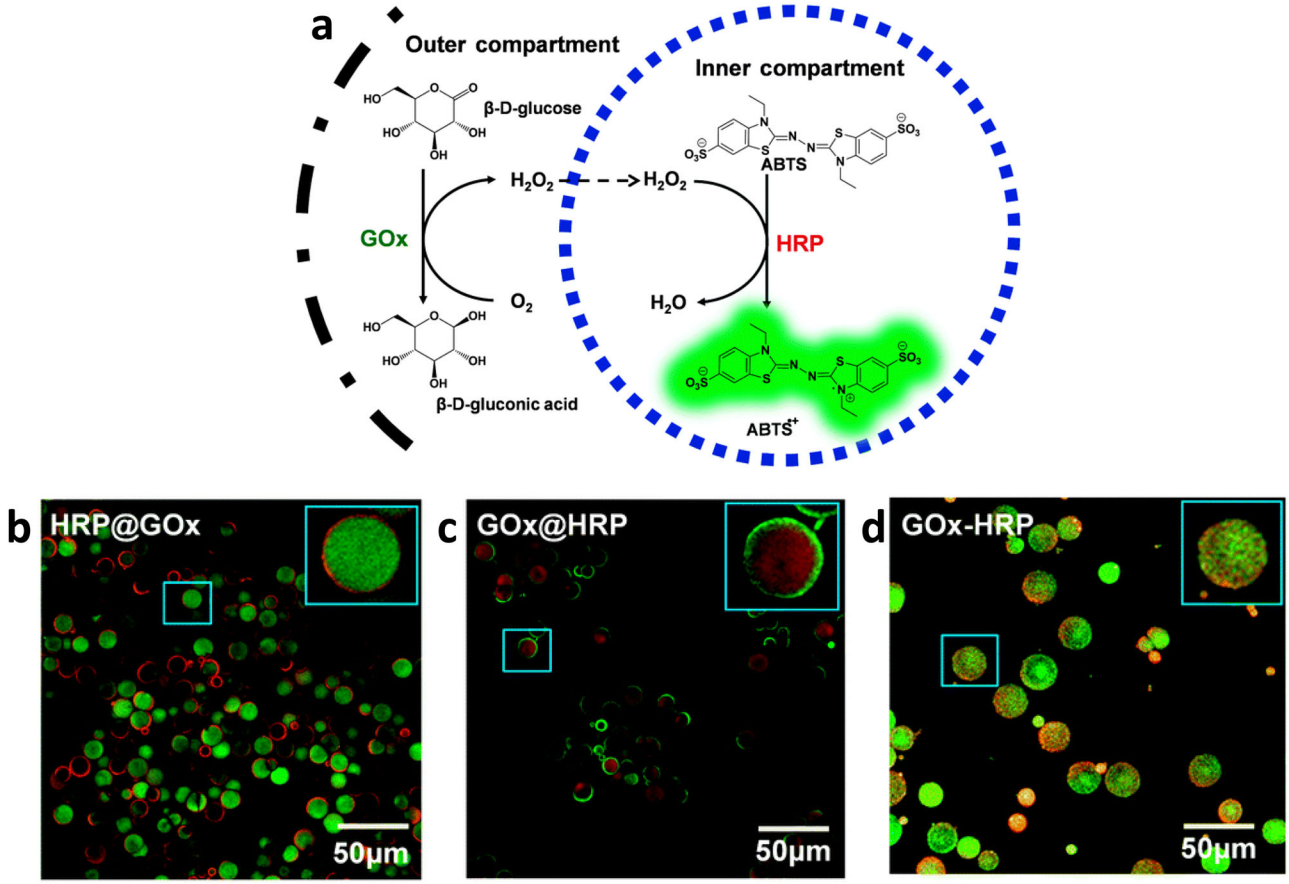

Figure 7. Schematic illustration of the compartmentalized GOx-HRP hybrid nanoflower (a) and confocal microscopy images (b, c, d) of different compartmentalized GOx-HRP nanoflower. Three types of nanoflowers are showing the distribution of biomolecules at inner and outer parts of the nanoflower. Permission from.[103]

Similarly, laccase-lysine-copper hybrid nanoflowers exhibited the decolourization efficiency of 89% within 1 hour, which is 30% higher than free laccase.[41]

Glucose detection

Generally, the conventional glucose colourimetric sensor operates in a separate dual system. At first, glucose was oxidized by reacting with Glucose oxidase (GOx) to produce gluconic acid and H_2O_2. Then, the produced H_2O_2 reacts with horseradish peroxidase (HRP) or its mimics added with a chromogenic substrate to generate a color signal. Sensitivity reduction and multiple steps are the drawbacks of this method, and hybrid nanoflowers are an effective alternative. Almost all reported glucose detection strategies with hybrid nanoflowers employed dual enzymes, i.e., GOx and HRP as organic and copper as inorganic components. A cascaded reaction can be achieved in one step by placing both enzymes closely in a single nanoflower framework. The glucose detection mechanisms using dual enzyme are almost similar for every reported assay.

For instance, Sun et al. (2014) reported a colourimetric glucose sensor based on GOx-HRP-copper hybrid nanoflowers. Firstly, the GOx of nanoflowers catalyzes the oxidation of glucose to generate gluconic acid and H_2O_2. The generated H_2O_2 immediately reacts with the adjacent HRP entrapped in the nanoflowers and oxidizes the chromogenic substrates, resulting in a noticeable color change. The closely encapsulated enzymes in a close frame dramatically enhance the sensor's sensitivity by reducing the drawback of diffusion and H_2O_2 decomposition.[102] Similarly, Li et al. (2014) reported GOx-HRP-copper hybrid nanoflower for glucose detection. They synthesized three types of hybrid nanoflowers and studied their spatial distribution effects and related activities. The first type of nanoflower denoted as GOx@HRP. It was produced by mixing HRP with copper and incubated for 24 hr to develop HRP nanoflower and then incubated it with GOx for another 24 hr to deposit on the outside of HRP nanoflower. The same procedure was repeated to synthesize HRP@GOx, in which GOx was firstly used to form the core followed by HRP forming outside layer. The third type of nanoflower was synthesized by adding both enzymes simultaneously and denoted as GOx-HRP. The confocal microscopy image indicates that the distribution of enzymes depends on the synthesis procedure (Figure 7). The target glucose and chromogenic substrate ABTS were used to estimate the overall enzymatic activity of three types of hybrid nanoflowers (i.e., GOx@HRP, HRP@GOx, and GOx–HRP) with the same total protein content. An increment in the overall enzymatic activity of all three types was observed. However, GOx@HRP attains the highest performance due to its ordered substrate transport and consistency with the enzymatic cascade reaction. For GOx@HRP, glucose and oxygen molecules first react with the GOx on the outer surface to form H_2O_2 and gluconic acid. Then, the H_2O_2 traveled to the core reacts with HRP, resulting in UV-

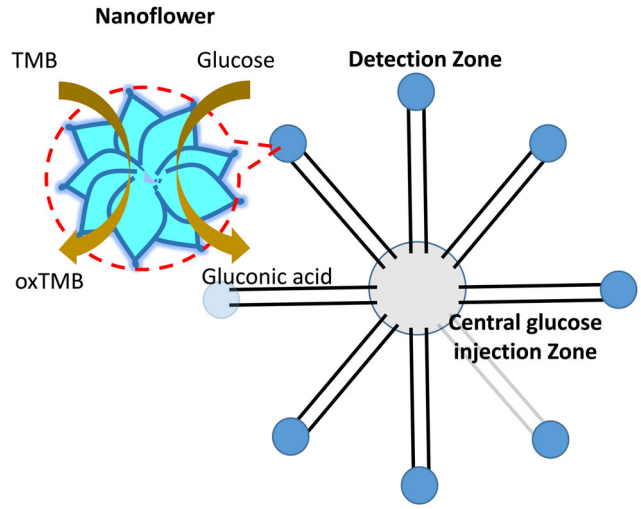

Figure 8. Schematic illustration of microfluidic channels with a circular central zone for sample injection and surrounded by eight detection zones modified with hybrid nanoflower. Reproduced with permission from.[105]

Vis to detect ABTS$\bullet^{+}$ with color change, and the measured LOD was 0.3 mM.[103]

Researchers reported a glucose detection method based on hybrid nanoflowers with the help of a paper-based 3 D microfluidic device (μPAD). Microfluidics technology is a powerful tool with many advantages, including quicker response time, less sample intake, superior sensitivity, small size, short diffusion distance and high surface tension, making it ideal for resource-limited analytical practice.[104] Remarkably, the white paper of μPADs can highly contrast with the color indicator, making colourimetric assays useful for qualitative and quantitative analyses of different target analytes.[105] The use of μPAD for glucose detection was successfully performed through hybrid nanoflowers.

Ariza-Avidad et al. (2016) applied the similar cascade reaction principle to develop a colourimetric glucose sensor using GOx-HRP-copper hybrid nanoflowers in a 3 D μPAD and the LOD was 15.6 μM.[106] Likewise, Zhu et al. (2016) prepared GOx-HRP-copper hybrid nanoflowers with wax-printed paper-based μPADs for visual detection of glucose. The μPAD has eight circular detection zones with microfluidic channels, and a central zone was designated for sample injection (Figure 8). A solution containing glucose was injected at the central zone and diffused into the detection zones modified with hybrid nanoflowers through the microfluidic channels. The sensor is promising to be applied in a complex whole blood sample because the paper can filter cells and only direct glucose to the detection zone.[105]

It is well known that HRP is commonly used for glucose detection because it has peroxidase-like activity and can transform a chromogenic substrate from colorless to blue by reacting with H_2O_2, thereby improving the visual signal in the cascade reaction. However, HRP role was replaced by manganese and ferrous phosphates in the following study. Both materials exhibit peroxidase-like activity to react H_2O_2 and convert the chromogenic substrate from colorless to blue.

For instance, Li et al. (2018) reported a microfluidic paper-based device integrated with the *in-situ* synthesis of GOx-Manganese nanoflowers for visual detection of glucose. GOx-Manganese was synthesized by drop-casting manganese sulfate ($MnSO_4$) and GOx containing solution onto cellulose paper, and the growth procedure was completed within 5 to 10 minutes. As standard, GOx catalyzes the oxidation of glucose to produce gluconic acid and H_2O_2. Then, H_2O_2 reacts with manganese and exhibits peroxidase-like activity, turning TMB from colorless into blue. The assay exhibits LOD of 0.01 mM. The *in-situ* synthesis of nanoflowers on a paper matrix eliminates the centrifugation and drying process involved in the typical synthesis procedure in a solution environment.[107]

Whereas, Guo et al. (2019) developed a new GOx-ferrous phosphate hybrid nanoflowers for the glucose detection. The by-product gluconic acid helps lower the pH of the reaction solution to 4. This acidic condition is favorable for ferrous to exhibit its peroxidase-mimicking activity reacts *in-situ* with formed H_2O_2 and oxidizes the chromogenic substrate, TMB to give a color change.[108]

Other disease biomarkers

Hybrid nanoflowers are also applied to detect other disease-related biomarkers, such as epinephrine, cholesterol, catecholamine and foodborne pathogens. For instance, Batule et al. (2015) developed Laccase-Copper nanoflowers within 5 mins via the sonication method and applied it for colourimetric detection of epinephrine. Compared with free laccase, the prepared laccase hybrid nanoflowers produced a denser and more significant color change, confirming its effectiveness in detecting epinephrine.[109]

Chung et al. (2018) developed cholesterol oxidase (ChOx) and horseradish peroxidase (HRP)-copper hybrid nanoflowers for colourimetric detection of cholesterol in blood samples. Cholesterol in the sample triggers a cascade reaction in which ChOx catalyzes the production of H_2O_2, which subsequently activates the adjacent HRP, thereby changing the TMB into a blue signal and reducing H_2O_2 to H_2O. This strategy is a judicious alternative for conventional dual-step cholesterol detection.[110] Also, HRP-copper nanoflowers were used to detect dopamine by oxidation in the presence of H_2O_2. Compared with the free form of HRP, the HRP in the nanoflowers effectively catalyze the oxidation of dopamine to orange color quinone-like product.[35]

Besides, Wang et al. (2018) reported a new hemin-concanavalin A (Con A)-copper hybrid nanoflowers for the sandwich-type colourimetric immunoassay of foodborne pathogenic bacteria, *E. coli* O157:H7. In the sandwich-type assay, hybrid nanoflowers were used as detection antibodies while magnetic nanobeads modified polyclonal antibodies were used as capture antibodies. Upon addition of target, *E. coli*, the polyclonal antibody of magnetic nanobeads and Con A of the nanoflowers interact with the target to form a sandwich complex. The captured nanoflowers exhibited peroxidase-mimicking activity catalyzes the oxidation of ABTS substrate to a green-coloured product in the presence of H_2O_2 and has an optimum absorption peak at 414 nm. The sensor showed a linear range from 10^1 to 10^6 CFU mL^{-1}, and the LOD of *E. coli* O157:H7 was 4.1 CFU mL^{-1},

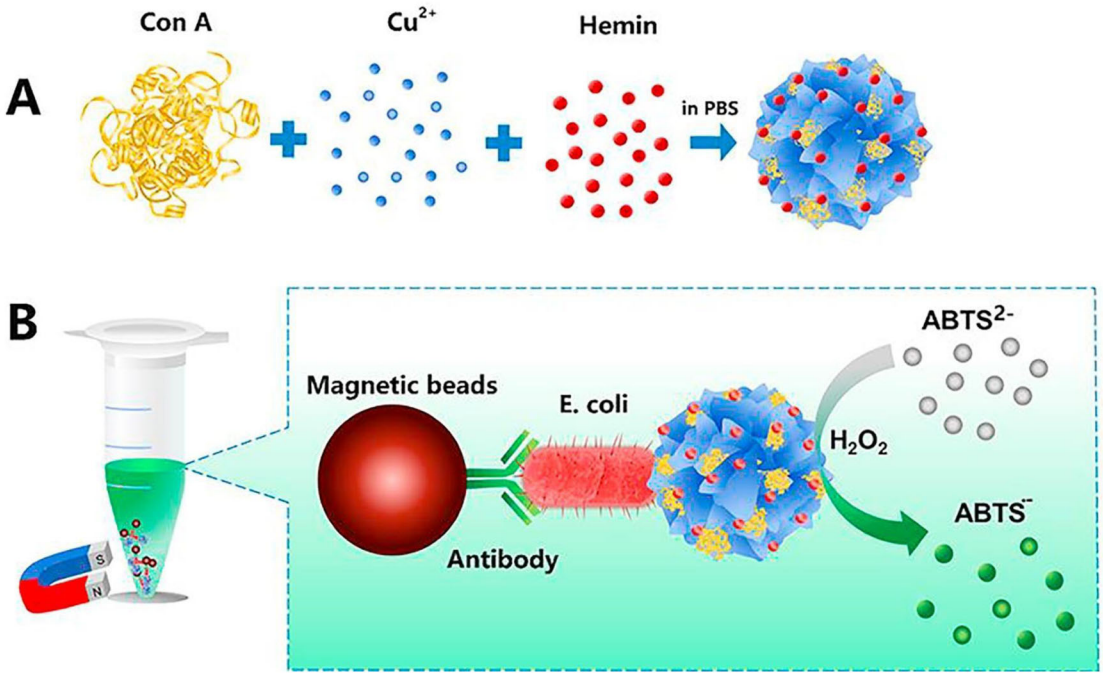

Figure 9. Schematic illustration of colourimetric immunoassay based on hybrid nanoflower. Permission from.[111]

indicating its potential application in the detection of many pathogens. The schematic illustration of colourimetric immunoassay based on hybrid nanoflowers is depicted in Figure 9.[111]

Enzyme-linked immunosorbent assay (ELISA)

ELISA is a popular plate-based immunoassay that utilizes antibodies and an enzyme-mediated color shift of chromogenic substrate to detect the presence of targets in a given sample. The presence of an antigen is revealed as the chromogenic substrate reacts with an enzyme labeled antibody and produces a visible color change or fluorescence. This color change can be measured quantitatively or qualitatively to detect the presence of a specific concentration of the antigen. ELISA uses the strong affinity interaction between antigen and antibody to detect ultra-low amounts of antigen (target) with high sensitivity and selectivity. The robustness and simplicity of ELISA make it widely used as a measurement tool in many fields.

Generally, the enzyme-coupled antibody is used as a secondary antibody in a sandwich-based ELISA detection system as illustrated in Figure 10(A). Scientists strive to advance the conventional enzyme-labeled antibody method to attain high sensitivity. Antibody labeled with the enzyme-loaded nanomaterial has been used to achieve excellent signal amplification; however, this strategy involves complex covalent immobilization, resulting in decreased enzyme activity.[112] To solve this problem, researchers have recently used organic-inorganic hybrid nanoflowers as an alternative for nanomaterial loaded enzyme. The secondary antibody or/and enzyme is treated as an organic element to synthesize the hybrid nanoflowers. These biomolecules in a 3 D nanoflower architecture are well preserved, leads to stable and sensitive detection.

The streptavidin (SA)-Horse Raddish Peroxidase (HRP)-Copper hybrid nanoflowers prepared by Liu et al. (2017) was applied in the sandwich-based ELISA system to detect alpha-fetoprotein. The SA in the hybrid nanoflower specifically binds to the biotinylated secondary antibody through SA-biotin interaction. Meanwhile, the enzyme, i.e., HRP as a signal enhancer, catalyzes the oxidation of TMB liquid substrate from colorless to blue in the presence of H_2O_2. The schematic diagram of the hybrid nanoflowers in ELISA is elucidated in Figure 10(B). The multi-biomolecule (SA and HRP) co-immobilized hybrid nanoflower poses a dual function. That is the specific binding affinity to the biotinylated antibody and amplified signal with improved enzymatic activity. Compared with conventional ELISA, this ELISA reaches a superior LOD of 78 pg mL^{-1}.[113] Moreover, Ye et al. (2021) recently reported a similar ELISA construction, in which SA and HRP co-immobilized with posnjakite (inorganic mineral, $Cu_4(SO_4)(OH)_6H_2O$) to capture Cry1Ab protein and attained LOD of 63 pg mL^{-1}.[22]

Interestingly, Wei et al. (2016) has co-precipitated both the secondary antibody and enzyme with copper to synthesize nanoflowers and directly applied as an enzyme-labeled antibody (in nanoflower format) for sandwich-based ELISA to determine *E. coli* O157:H7, which was achieved LOD of 60 CFU L^{-1}. The detection format is represented in Figure 10(C). Antibody (anti-*E. coli* O157:H7)-enzyme (HRP)-copper nanoflower used as an enzyme-labeled antibody in ELISA proffered several advantages: 1) simple preparation without involving laborious covalent modification, 2) a large number of enzyme and antibody aggregates in nanoflower and enhances the captive and catalytic ability, 3) stability of antibody and enzyme is significantly improved compared to its free form. Furthermore, the processing steps were reduced since nanoflowers were directly used as a secondary antibody, which is different from the previously reported

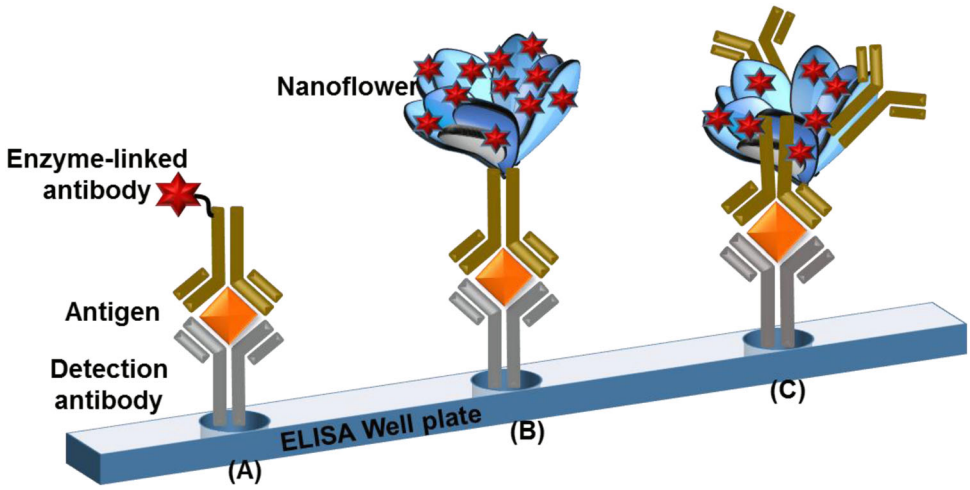

Figure 10. Schematic illustration for three types of sandwich ELISA; A) Conventional ELISA, B) Enzyme embedded nanoflower based-ELISA, C) Enzyme and secondary antibody embedded nanoflower based-ELISA.

procedure, i.e., nanoflowers were only used after the secondary antibody recognition.[112] Likewise, another group has also reported the synthesis of nanoflowers made from antibody (monoclonal anti-*S. enteritidis*)-enzyme(HRP)-Calcium to detect *Salmonella* in tap water, milk and cheese, with a LOD of 1 CFU mL^{-1} and 1 CFU/g, respectively.[114]

Thus, the use of nanoflower as enzyme coupled secondary antibody in ELISA proffered several benefits and advanced strategy, for instance, more biomolecule confinement, enhanced enzymatic activity and stability, as well as reducing ELISA procedure and the complex enzyme linking procedure paving way for high performance ELISA.

Surface plasmon resonance (SPR)

Surface Plasmon is a P-polarized light wave has a magnetic vector vertical to the plane of incidence, which strikes a conductive layer between two media with different refractive indices. The photon energy is directly absorbed by the electrons of the conductive film and converted into an evanescent field with a certain incidence angle. SPR technique in biosensing was first reported in 1983 as a crucial label-free technique that allows the detection of molecular interaction in real sample with high accuracy.[115,116] SPR technology mainly relies on the change of the refractive index of the sensor chip surface, which is associated with the interaction between the immobilized bioreceptor and the target on the sensor chip.[104]

However, Kong et al. (2018) developed a new plasmonic sensing strategy using nanoflower as a nanoreactor. A cascaded reaction using multi-enzyme nanoflower mediated plasmonic sensing to screen α-glucosidase (GAA) inhibitors, i.e., an anti-diabetic drug, was achieved. Glucose oxidase (GOx) and GAA were co-embedded into a single nanoflower, which acts as a nanoreactor and reacts with maltose as a substrate. The maltose was converted into glucose through GAA catalysis, and the adjacent GOx immediately uses it to generate H_2O_2 and gluconic acid. The generated H_2O_2 etched the triangle-shaped silver nanoplates into a disk, causing a blue shift in the SPR signal. The schematic illustration and process flow are represented in Figure 11(A and B). The SEM (C1&C2) and TEM (D1&D2) images in Figure 11 represent the hybrid nanoflowers and silver nanoplates, respectively. Figure 11 (D1 and D2) represents the TEM image of silver nanoplates before and after etching by H_2O_2. The enzymatic activity of GAA was suppressed upon the addition of an inhibitor (e.g., Acarbose). As a result, the production of glucose and H_2O_2 was hindered and causing SPR blue shift inhibition. Also, other traditional Chinese herbs were tested to study their inhibitory performance on GAA. The enzymes entrapped in the nanoflower system exhibited a nearly 148% increase in activity than the free enzyme system and attained a lower LOD of 5 nM, suggesting the reliability of nanoflower in providing an opulent microenvironment for enzymes that could potentially be applied in plasmonic sensing.[117]

Fluorescence-based detection

Fluorescence is one of the leading technology used by many researchers in countless disciplines, such as flow cytometry, medical diagnostics, forensics, genetic analysis, DNA sequencing, and to name a few. Some typical fluorescence substance or fluorophores commonly used are Rhodamine B, Quinine, Fluorescein, POPOP, Pyridine 1, and Acridine orange.[118] The fluorophores are usually linked to the bioreceptors that capture a particular analyte, thereby transducing a measurable fluorescent signal. In nanoflower-based detection, fluorophores are either embedded into nanoflower with the desired biomolecules or placed in the reaction mixture and catalyzed by biomolecules in the nanoflower.

Based on this philosophy, Peng et al. (2018) developed a newfangled fluorescence hybrid nanoflowers to be utilized in fluorescence immunoassay combined with immunomagnetic separation for the detection of Clenbuterol (Clen), i.e., veterinary drug. For the detection, the anti-Clen antibody (mAb) was conjugated on magnetic nanoparticles (MNP) via biotin-streptavidin interaction and used as a capture probe (mAb-MNP) to capture and separate the Clen in swine urine. The fluorescence hybrid nanoflower was prepared

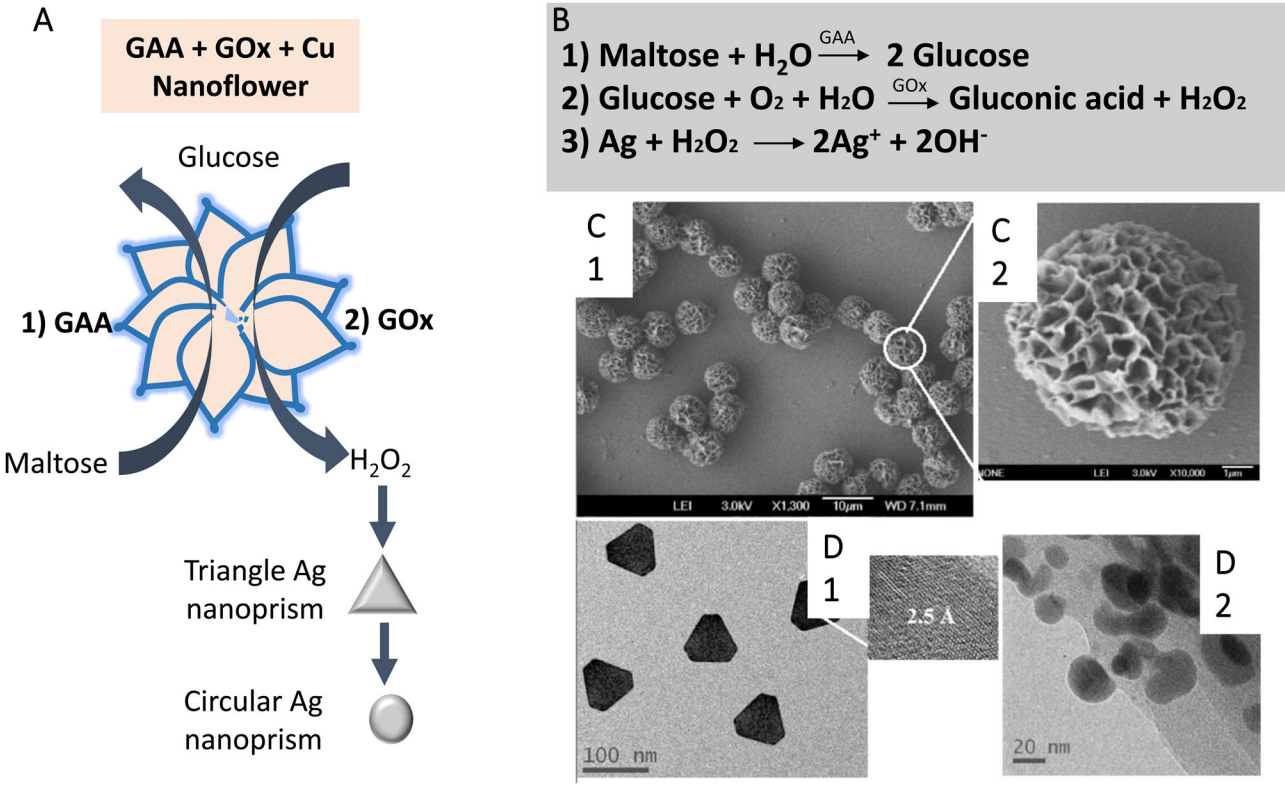

Figure 11. Schematic illustration of plasmonic sensing with hybrid nanoflower as nanoreactor (A), and reaction process flow (B), SEM image of nanoflower (C1&C2), TEM image of triangle-shaped silver nanoplate (D1) and after etching by hybrid nanoflower (D2). Permission from.[117]

using calcium as inorganic and BSA-gold nanoclusters (BSA-AuNCs)-(Clen-BSA) as organic components. The AuNCs acts as a fluorescence nanomaterial. Next, the prepared nanoflowers were immersed and coupled to the remaining binding site of magnetically separated mAb-MNP-Clen. The stepwise detection is depicted in Figure 12. The fluorescence intensity of the nanoflowers was slightly enhanced compared with BSA-AuNCs, which may be due to aggregation-induced emission. It was found that after two immunomagnetic separations, the red fluorescence of the supernatant (measured at an excitation/emission wavelength of 360/640 nm) was directly proportional to the concentration of Clen. The immunoassay exhibits a LOD of 0.167 $\mu g\ L^{-1}$ with a linear range from 0.5 $\mu g\ L^{-1}$ to 40 $\mu g\ L^{-1}$.[119]

In a recent study, Gao et al. (2018) reported the use of fluorescence biosensors to detect hydrogen peroxide in a biocatalytic system containing multiple substances (HAc-NaA, Rh6G, KI, and H_2O_2) by using hemoglobin (Hb)-copper hybrid nanoflowers. In the reaction system, firstly, Hb-Copper hybrid nanoflower decomposes H_2O_2 to produce hydroxyl radical (•OH). Then, the excess I^- was oxidized to I^{3-} by •OH and combined with the fluorescence dye Rhodamine 6 G (Rh6G) to form (Rh6G ~ I_3)n, i.e., a hydrophobic complex, which causes fluorescence quenching. As the concentration of H_2O_2 increases, the emission intensity of fluorescence gradually decreases due to the formation of more (Rh6G ~ I3)n as a quencher. The prepared biosensors display a wide linear range of 2-10 ppb and a low LOD of 0.01 ppb.[31]

Apart from that, Liu et al. (2018) used calcium and dual biomolecules, i.e., streptavidin (SA) and β-galactosidase (β-Gal) to prepare hybrid nanoflowers for fluorescence sensing of alpha-fetoprotein (AFP). The β-Gal enzyme was utilized as a fluorescent signal probe because of its specificity in catalyzing the fluorogenic substrate fluorescein di-β-D-galactopyranoside to fluorescein to produce a fluorescence output signal. Meanwhile, the SA can precisely capture the biotinylated secondary antibodies in ELISA-based detection mechanisms. The multi-biomolecules in nanoflower have integrated functions, which helps to show the ultralow LOD at fM level and can be used for fluorescent detection of AFP.[120]

Much recently, Batule et al. (2018) reported GOx-copper nanoflowers for fluorescence-based glucose detection. The GOx in the hybrid nanoflowers catalyzes target glucose to produce H_2O_2, then induces peroxidase-like activity and oxidizes the substrate, i.e., Amplex UltraRed, to a highly fluorescent product. Consequently, the target glucose was reliably determined with high selectivity, which exhibits a LOD down to 3.5 μM.[121] Moreover, Yu et al. (2019) reported an exciting method that utilizes a smaller-sized hybrid nanoflower made up of Y-shaped DNA with loop structure and copper to perform fluorescence-based imaging of tumor-related mRNA in living cells. The Y-DNA was linked with fluorescence (Cy3) and quencher (Black Hole Quencher 2) and used as a fluorescence probe. In the absence of a target, the Cy3 was quenched by tightly positioned BHQ-2. The Y-DNA-Cu nanoflowers disassembled once they entered the cancer cell and released Y-DNA probes. Then, the target TK1 mRNA in the cell hybridized with the loop of Y-DNA.

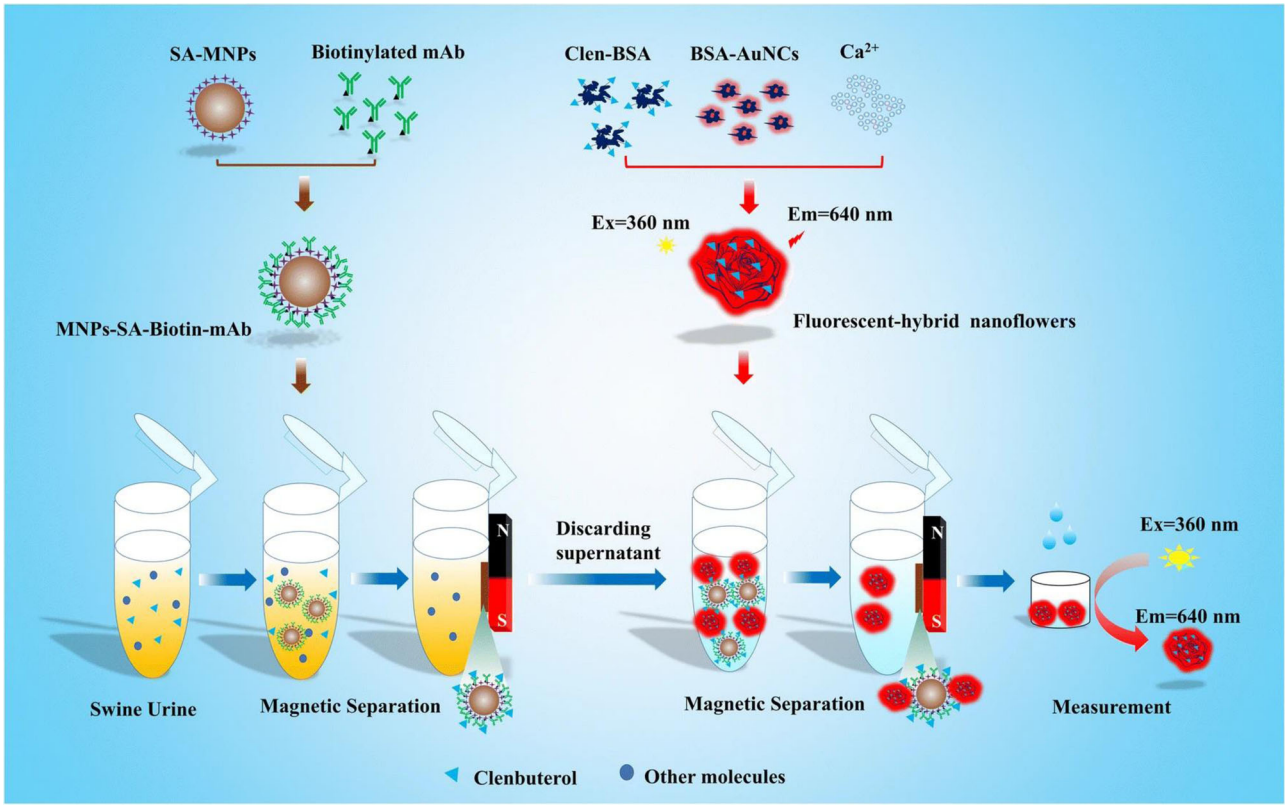

Figure 12. Illustration of fluorescence immunoassay using AuNCs encompassed hybrid nanoflower and immuno-magnetic separation. Permission from.[119]

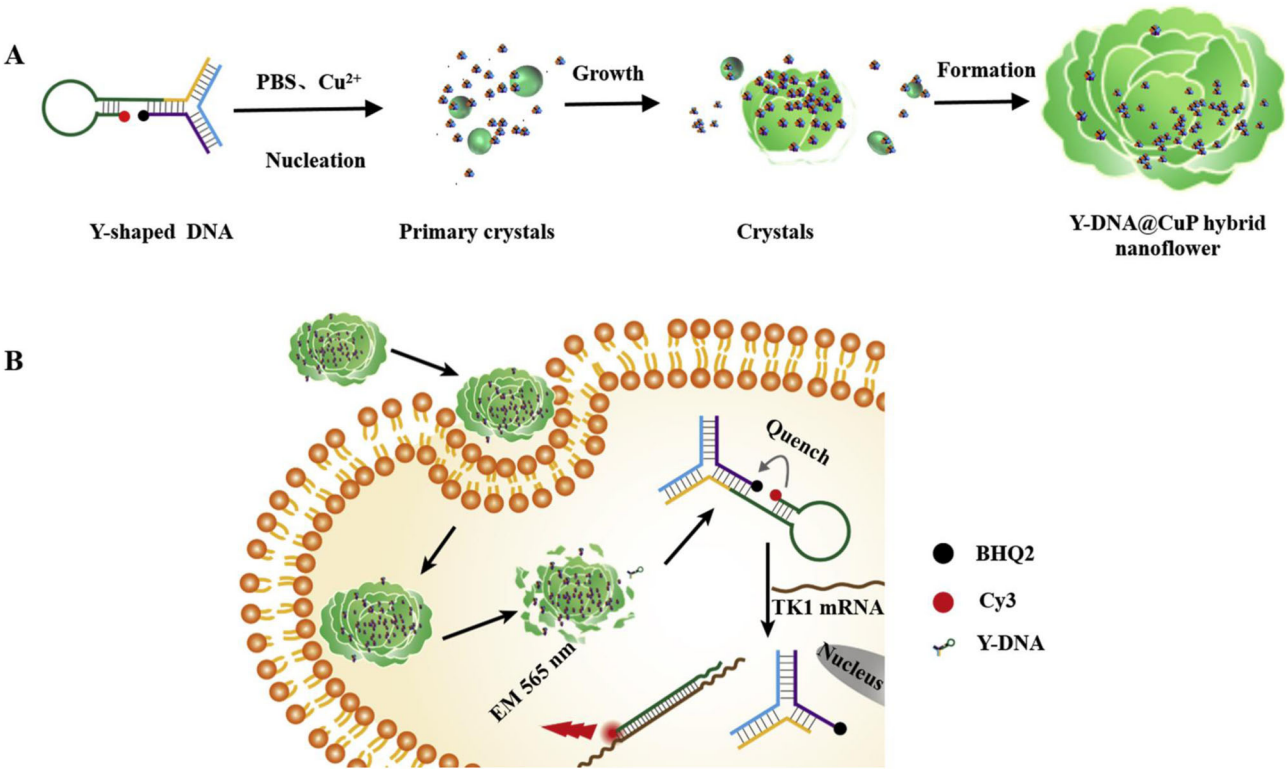

Figure 13. (A) Synthesis of Y-DNA-Cu hybrid nanoflower, (B) Schematic illustration of intracellular imaging. Permission from.[122]

Subsequently, the loop detaches from Y-DNA framework and turns on the fluorescence of Cy3 (Figure 13). The technique attained a linear response ranging from 2 to 150 nM, with a LOD of 0.56 nM, suggesting the practicability of nanoflower in intracellular application.[122]

Conclusions and prospects

The nano-scale materials with intriguing structures have boosted-up the performance of various mechanisms in several application domains. In this regard, organic-inorganic

hybrid nanoflower has proved to be a superior new technology due to their three-dimensional architecture, protected and enhanced confinement of biomolecule, green synthesis methodology, tunable physical, chemical and biological properties. The robustness, stability and reusability of the designed nanoflowers can be further improved through chemical treatment and integration of different nanomaterials, including carbon nanotubes, magnetic nanobeads, and nanofibers and to name a few. Various transduction techniques have been used for many purposes to study systems that use hybrid nanoflowers.

Even though the hybrid nanoflower attains the expected performance, there is still room for improvement. So far, several researchers have developed hybrid nanoflowers, which contain more than one organic element and can accomplish cascaded reactions in a single frame. However, there are no or very few reports on nanoflowers synthesized from multi inorganic components. The contribution of inorganic components in the working system must be further explored to benefit from every hybrid nanoflower fragment. Also, there are numerous types of organic elements have been explored, but only a few common inorganic elements were used to synthesize hybrid nanoflowers. Therefore, the selection of inorganic elements should be more diversified and explored to expand their feasibility and applicability in many fields. Moreover, the centrifugation process after three days of incubation involves collecting and washing hybrid nanoflowers, resulting in leaching of biomolecules from the nanostructure. Also, the supernatant containing the leftover biomolecules should be reused to avoid wastage. Thus, appropriate procedures should be developed to utilize the complete portion of biomolecules for synthesis.

Authors' contributions

Indra Gandi Subramani: Conceptualization, Data curation, Investigation, Writing original draft preparation, Writing-Reviewing and Editing.

Veeradasan Perumal: Conceptualization, Methodology, Data curation, Investigation, Validation, Visualization, Supervision, Writing-Reviewing and Editing.

Subash C.B. Gopinath: Data curation, Validation, Writing-Reviewing and Editing.

Khor Shing Fhan: Validation, Writing-Reviewing and Editing.

Norani Muti Mohamed: Validation, Writing-Reviewing and Editing.

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Disclosure statement

No potential conflict of interest was reported by the authors.

List of abbreviations

ELISA	Enzyme-linked immunosorbent assay
SPR	Surface plasmon resonance
PBS	Phosphate buffered saline
BSA	Bovine serum albumin
PDGF-BB	Platelet-derived growth factor-BB
LOD	Limit of detection
Con A	Concanavalin A
E. coli.	Escherichia coli O157:H7
ABTS	2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt
DhHP-6	Deuterohemin-b-Ala-His-Thr-Val-Glu-Lys
VO	Viburnum opulus
SPI	Soy protein isolate
CNT	Carbon nanotubes
GO	Graphite oxide
CPN	$Cu_8(PO_3OH)_2(PO_4)_4{\cdot}7H_2O$ nanoflowers
ALDC	α-Acetolactate decarboxylase
PCMN	Papain-copper-magnetic nanoparticle
GOx	Glucose oxidase
HPNM	PVA-co-PE membranes
GA	Glutaraldehyde
CPO	Chloroperoxidase
SA	Sodium alginate
PD	Polydopamine
PtNPs	Platinum nanoparticles
CV	Cyclic voltammetry
EIS	Electrical impedance spectroscopy
DPV	Differential pulse voltammetry
R_{ct}	Electron transfer resistance
IgG	Immunoglobulin G
RACanti	Ractopamine antibody
AuNP	Gold nanoparticles
ECSA	Electrochemically active surface area
PtNPs	Platinum nanoparticles
MWCT	Multi-walled carbon nanotube
GCE	Glassy carbon electrode
Ag	Silver
SNA	*Sambucus nigra* agglutinin
IFN-ɤ	Interferon-gamma
CEA	Carcinoembryonic antigen
TMB	3,3′,5,5′-tetramethylbenzidine
ABTS	2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt
OPD	O-phenylenediamine dihydrochloride
β-Lg	*β*-lactoglobulin
HRP	Horseradish peroxidase
3D μPAD	3D microfluidic device
ChOx	Cholesterol oxidase
SA	Streptavidin
GAA	α-glucosidase
Clen	Clenbuterol
mAb	Anti-Clen antibody
MNP	Magnetic nanoparticles
AuNCs	Gold nanoclusters
Rh6G	Rhodamine 6 G
AFP	Alpha-fetoprotein
β-Gal	*β*-galactosidase

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