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Review Article

Organic-Inorganic Hybrid Nanoflowers as Potent Materials for Biosensing and Biocatalytic Applications

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Abstract Flower-shaped organic-inorganic hybrid nanostructures, termed nanoflowers, have received considerable recent attention as they possess greatly enhanced activity, stability, durability, and even selectivity of entrapped organic biomolecules, which are much better than those from the conventional methods. They can be synthesized simply via co-incubation of organic and inorganic components in aqueous buffer at room temperature and yield hierarchical nanostructures with large surface-to-volume ratios, allowing for low-cost production by easy scale-up, as well as the high loading capacity of biomolecules without severe mass transfer limitations. Since a pioneering study reported on hybrid nanoflowers prepared with protein and copper sulfate, many other organic and inorganic components, which endow nanoflowers with diverse functionalities, have been employed. Thanks to these features, they have been applied in a diverse range of areas, including biosensors and biocatalysis. To highlight the progress of research on organic-inorganic hybrid nanoflowers, this review discusses their synthetic methods and mechanisms, structural and biological characteristics, as well as recent representative applications. Current challenges and future directions toward the design and development of multi-functional nanoflowers for their widespread utilization in biotechnology are also discussed.

Keywords: Organic-inorganic hybrid nanoflowers, Biosensor, Biocatalysis, Coordination interaction, Enzyme immobilization

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Introduction

There have recently been growing efforts to develop biomolecule-embedded organic-inorganic hybrid structures for potential applications in biocatalysis, drug delivery, and analytical science[1,2]. Among the different types of biomolecules, enzymes have been the most widely studied as organic components for the preparation of hybrid materials with inorganic support materials using approaches including physical adsorption, covalent attachment, and entrapment[3]. This generally referred to as enzyme immobilization and is primarily used to enable recycling, reuse, and to improve stability[4,5]. However, enzyme activity after immobilization is generally reduced compared to their counterpart free enzymes, mainly due to the loss of activity during the immobilization procedures and mass-transfer limitations arising from the solid matrices[6]. To resolve this limitation, uniquely-designed nanostructured materials such as nanoporous materials, electrospun nanofibers, nanotubes, and nanoparticles were utilized as hosts to immobilize enzymes[7,8]. Since they generally provide much larger surface areas than bulk materials, increased apparent activity per unit mass or volume can be observed; however, their preserved activity was generally still lower than that of free enzymes. In this regard, novel *in situ* synthetic methods for enzyme-inorganic hybrid nanomaterials including enzyme-polymer conjugates or enzyme-polymer nanogels based on specific techniques including conjugation, cross-linking, and self-assembly, have garnered special attention due to their ability to effectively retain and stabilize enzyme activity[9,10]. Recently, Zare and his coworkers serendipitously found that organic-inorganic hybrid nanoflowers were formed using copper (II) ions as

the inorganic component and various proteins as the organic component[11]. The hybrid nanoflowers can be simply synthesized but they yield hierarchically-structured flower-like morphology controlled at the nanometer scale which is very beneficial for enhancing the stability, activity, durability, and selectivity of entrapped enzymes compared with those of existing strategies[11].

In conventional, flower-like nanomaterials have attracted attention in various fields, including catalysis, electronics, and analytical sciences, due to their rough surfaces with large surface-to volume ratios[12-14]; however, only inorganic components have been incorporated without any biomolecules because their synthesis generally involves harsh reaction conditions at both high temperatures and pressures with toxic chemical reagents[15]. Thus, there has not yet been any report on "nanoflowers" made from organic components before the aforementioned pioneering study[11]. Novel organic-inorganic hybrid nanoflowers have been synthesized in mild reaction environments at room temperature in aqueous phosphate buffer using proposed synthetic mechanisms, consisting of the initial formation of complexes between nitrogen atoms in amide or amine groups in biomolecules and inorganic metal ions, including copper, via the coordination interaction, which drives the nucleation of primary metal phosphate crystals. Anisotropic growth led to highly-branched flower-like structures. Initially, several proteins, such as bovine serum albumin (BSA), α-lactalbumin, laccase, carbonic anhydrase, and lipase, were used with copper sulfate to prepare protein-copper hybrid nanoflowers[11]. After intensive study, many different organic and inorganic components have been employed to prepare hybrid nanoflowers, allowing for different functionalities and thus extending their potential applications[16]. Moreover, several groups have recently reported on a strategy to incorporate multiple organic or inorganic components within hybrid nanoflowers that allow for unique multi-catalytic or multi-functional reactions[17]. These research advances would lead to greater versatility of hybrid nanoflowers for use in various areas, in particular for biosensors and other biocatalysis-mediated applications.

In this review, we focus on the recent advances in the synthetic methods and the proposed mechanisms, structural characteristics, and corresponding biological properties of organic-inorganic hybrid nanoflowers with representative applications to biosensors and biocatalysis. We also describe current challenges and future prospects for this emerging "nanoflower" technology.

Characteristics of Organic-Inorganic Hybrid Nanoflowers

Until now, hybrid nanoflowers using copper (II) ions and proteins have been the most intensively studied and their characteristics are relatively well understood. In addition, other types of biomolecules, including peptides[18], amino acids[19], and DNAs[20] as well as other inorganic ions including calcium[21], zinc[22], cobalt[23], and manganese[24], have been studied for the formation of hybrid nanoflowers, and thus are generally categorized based on the kinds of organic or inorganic components employed. Recently, there have been several studies describing multi-component incorporated organic-inorganic hybrid nanoflowers, which show unprecedented multi-functions for extending their utilization[17]. Thus, in this section, we first present the general characteristics of hybrid nanoflowers made from copper (II) ions and proteins before describing nanoflowers composed of other inorganic and organic components. We also describe the representative characteristics of multi-component incorporated hybrid nanoflowers.

Nanoflowers Using Copper (II) Ions and Proteins

The first organic-inorganic hybrid nanoflowers were synthesized via a self-assembly process using copper sulfate and BSA in phosphate buffered saline (PBS, pH 7.4)[11]. In a typical synthesis, protein molecules such as BSA were added to aqueous PBS solution containing copper sulfate and then incubated at room temperature (RT). After 3 days of incubation, blue-colored precipitates comprised of micrometer-sized particles with nanoscale flower-like morphologies were formed. When different types of enzymes, such as laccase, carbonic anhydrase, trypsin, α-amylase, horseradish peroxidase (HRP), and glucose oxidase (GOx), were used as the protein component, the hybrid nanoflowers exhibited significantly enhanced activity, which was even higher than that of their counterpart free enzymes, as well as a high stability for long-term use[16]. This is attributed to the efficient confinement of enzyme molecules within nanoflower matrices, as well as their large surface area, which makes mass-transfer limitations insignificant[11,21,25].

The step-by-step synthetic mechanism of hybrid nanoflowers is described as follows[11]. At an early stage, primary copper phosphate crystals are formed by the complexation between copper ions and protein molecules, predominantly based on the coordination of amide or amine groups in the protein backbone. These complexes provide a location for the nucleation of the primary copper phosphate crystals. During the second

growth stage, large agglomerates composed of proteins and primary crystals are formed. At the individual copper (II) ions presented on the surface of agglomerates, kinetically controlled growth of copper phosphate crystals is performed, creating separate petals of the nanoflowers. In the last stage, anisotropic growth proceeds and results in the complete formation of a branched flower-like structure. By this mechanism, protein molecules induce the nucleation of copper phosphate crystals to form the scaffold for the petals and serve as a 'glue' to bind the petals together. Thus, collapsed structures were observed after the protein digestion reaction of the hybrid nanoflowers, revealing the presence of protein molecules within the nanoflower matrices[11].

Although the synthesis of hybrid nanoflowers is quite simple to perform, it takes up to three days to complete, which imposes a limit on their practical applications. To solve this problem, we recently reported a sonochemical method that synthesizes hybrid nanoflowers very rapidly (within 5 minutes at RT), presumably due to the sonication approach allowing for the building blocks (copper phosphate) to quickly complete the self-assembly process by uniformly providing high energy to the structure[26]. The resulting sonicated nanoflowers had similar morphology compared to that of nanoflowers prepared over three days, and also exhibited greatly enhanced activity, stability, and reusability. Moreover, Cui *et al*. developed a unique synthetic strategy for lipase-copper hybrid nanoflowers[27]. When lipase was employed with copper sulfate to form general hybrid nanoflowers, their activity was marginally decreased. The authors hypothesized that the interfacial activation of lipase by treatment with appropriate surfactants induced an increase in the catalytic activity of lipase within nanoflower matrices. The resulting surfactant-activated lipase-copper hybrid nanoflowers exhibited 460% and 200% higher activity than that of native lipase and conventionally-prepared lipase-copper nanoflowers, respectively. The reusability and stability of the surfactant-activated lipase-copper nanoflowers was much higher due to their monodispersity as well as mechanical strength, induced by the surfactant[27]. In addition, after the preparation of conventional lipase-copper hybrid nanoflowers, further treatment of glutaraldehyde also resulted in an improvement of the stability as well as the reusability via the additional crosslinking of entrapped enzyme molecules[28]. These new synthetic strategies could facilitate the practical utilization of hybrid nanoflowers in biotechnology.

Enzyme-copper hybrid nanoflowers were also immobilized on other materials to enhance their performance. Zhu *et al*. fabricated laccase-copper hybrid nanoflowers on a cellulose acetate membrane in a syringe filter, which was then efficiently utilized for the detection of phenols[29]. When an aqueous sample containing target phenol and 4-aminoantipyrine (4-AAP) was passed through the filter, the oxidation coupling between phenol and 4-AAP formed a colored antipyrine dye, which could be detected using a spectrophotometer or even by the naked eye. Since the nanoflowers were embedded in the membrane, the reusability and reproducibility of the filter for phenol detection was significantly improved. Li *et al*. also embedded different kinds of protein (BSA, papain, laccase, and HRP)-copper hybrid nanoflowers on fibrous membranes[30]. After immobilization, the hybrid nanoflowers showed enhanced durability and pH stability compared with their counterpart free enzymes. Xie *et al*. grew copper phosphate-based nanoflowers on the surface of a soy protein isolate (SPI) film, which then served as a superhydrophobic and self-cleaning material[31]. The authors demonstrated that the improved stability of the nanoflowers on SPI film was the result of the coordination interaction between SPI and copper ions.

Nanoflowers Using Other Inorganic and Organic Components

Until now, copper ions have been primarily used to create organic-inorganic hybrid nanoflowers; however, other inorganic ions have also been investigated as potential candidates in recent years. Wang *et al*. successfully prepared calcium-enzyme hybrid nanoflowers with synthetic methods and mechanisms similar to those used for copper-protein hybrid nanoflowers[32]. In their study, the protein molecules of α-amylase entrapped in nanoflowers also exhibited allosteric effects, where α-amylase changed from its inactive form into its active form by binding with calcium ions. Thus, the hybrid nanoflowers composed of calcium phosphate crystals and α-amylase in close proximity with each other exhibited enhanced activity and stability compared with free enzyme mixed only with free calcium ions, due to the strong interaction between calcium ions and α-amylase within the nanoflower matrices. Calcium ions were also employed with different enzymes, such as α-chymotrypsin[33], lipase[34], GOx[35], or chloroperoxidase[36], to prepare hybrid nanoflowers for diverse catalytic applications. Compared with copper and calcium ions, the reaction rate between zinc ions and phosphate radicals for the preparation of zinc phosphate-based petals of nanoflowers was faster; as a result, hybrid nanoflowers using zinc ions and proteins were prepared more rapidly. Zhang *et al*. re-

ported production of lipase-zinc hybrid nanoflowers using rapid synthetic methods involving growth for only three hours at RT[22]. The resulting nanoflowers showed a ~1.5 fold enhancement in catalytic activity with an excellent operational stability compared with those of free lipase. Other protein molecules, including BSA and papain, were successfully employed with zinc ions to prepare hybrid nanoflowers, which also yielded greatly enhanced catalytic activity, stability, and reusability[37,38]. Cobalt ions were also reported to be successfully incorporated with proteins to prepare hybrid nanoflowers. Yate and coworkers reported on the production of hybrid nanoflowers by the selective mineralization of cobalt phosphate crystals in the presence of His-tagged enzymes[23]. The resulting materials showed higher catalytic activity and reusability than copper phosphate minerals alone without protein. Manganese-protein hybrid nanoflowers were particularly important since they were electrically conductive, whereas other hybrid nanoflowers were not. Based on their conductivity, Zhang and coworkers reported on electrocatalytic biosensors to detect ractopamine[24]. The manganese-BSA hybrid nanoflowers were also utilized as a support material for platinum nanoparticles to catalyze methanol oxidation[39]. Furthermore, Li *et al*. prepared GOx-manganese hybrid nanoflowers on a paper matrix to construct visual microfluidic paper-based biosensors for the detection of glucose[40].

Since any molecules with amide or amine groups could theoretically be used with inorganic ions for the synthesis of hybrid nanoflowers, other types of biomolecules other than proteins were also used as the organic components for the preparation of hybrid nanoflowers. Wu *et al*. successfully employed amino acids with copper sulfate to prepare hybrid nanoflowers[19]. Synthesis involved adding amino acid to copper sulfate solution in PBS (pH 7.4) and incubating at RT for 1 day in order to fully bloom the flower-like structures. The resulting nanoflowers had porous structures dominated by the R groups of the amino acids with high surface-to-volume ratios. Interestingly, owing to the existence of copper phosphate, the amino acid-copper hybrid nanoflowers exhibited peroxidase-like activity as a result of Fenton-like reaction mechanism between copper ions and hydrogen peroxide. Moreover, the same group synthesized hybrid nanoflowers using deuterohemin-peptide (DhHP-6) as the organic component and copper ions as the inorganic component[18]. During synthesis, DhHP-6 peptides were embedded on copper phosphate crystals through the coordination of the end amino acids (lysine) with the copper (II) center, which drove the nucleation of primary nanoparticles for their growth into flowers. Considering the fact that nucleic acids, such as DNA and RNA, contain amide and amine groups in their nucleobases, our group also reported hybrid nanoflowers prepared via 3 days of incubation at RT with DNA molecules and copper ions as the organic components and inorganic components, respectively[20]. Since the resulting nanoflowers showed high DNA loading capacities, strong resistance against nuclease-promoted cleavage, and low cytotoxicities, DNA-copper hybrid nanoflowers have the potential to be applied as a nucleic acid delivery agent. Furthermore, amine-containing organic molecules such as dopamine or chitosan were employed to bloom the nanoflowers. Combining dopamine molecules and copper ions, dopamine-copper hybrid nanoflowers were formed, which were then utilized to reduce $AgNO_3$ to form Ag nanoparticles at the site of dopamine[41]. The Ag-dopamine-copper hybrid nanoflowers exhibited excellent antimicrobial activity towards *Escherichia coli* but negligible effects on co-cultured mammalian cells. Chitosan molecules, which turned into a gel complex after reacting with triphosphate, were employed with calcium ions to form hybrid nanoflowers[42]. The chitosan gel-calcium hybrid nanoflowers were attractive because any further catalytic molecules can be incorporated into chitosan gel through electrostatic interaction, facilitating the generation of the nanoflowers with diverse functionalities.

Nanoflowers Incorporating Multiple Inorganic or Organic Components

Recently, several groups have reported a strategy for the incorporation of multiple organic components within hybrid nanoflowers which allow for the creation of unique multi-functions. Representatively, HRP as well as GOx were employed as two different enzyme molecules with copper ions to form multi-enzyme incorporated organic-inorganic hybrid nanoflowers for the detection of target glucose via the entrapped GOx-HRP mediated cascade reaction[43]. Similarly, both cholesterol oxidase (ChOx) and HRP were recently employed with copper ions to form hybrid nanoflowers, which served as a colorimetric cholesterol biosensor[44]. The synthetic procedures of multi-enzyme incorporated nanoflowers were similar to those of generally-prepared single-enzyme incorporated nanoflowers; however, they were formed from two kinds of enzymes with different ratios, which determines the level of cascade reaction activity. Interestingly, Li *et al*. recently demonstrated that the cascade reaction activity of GOx-HRP incorporated nanoflowers could be also determined via the spatial co-localization of GOx and HRP within nanoflower matrices[45]. The spatial-

ly co-localized GOs and HRP within the nanoflowers were simply prepared by a bloom of the flowers with HRP in the first round, followed by second round of blooming with GOx. This sequential formation of hybrid nanoflowers consequently determined the places where HRP (inside) and GOx (outside) were located throughout the nanoflowers, thereby mimicking *in vivo* compartmentalization to facilitate substrate transport and thus enhancing the catalytic activity of the cascade enzyme reaction. Furthermore, other types of organic components, such as antibody[46], streptavidin[47], or concanavalin A (Con A)[35], all of which have a high affinity to their corresponding target molecules, were additionally employed with the generally-used enzyme molecules and inorganic components, to develop triple component-incorporated hybrid nanoflowers. These nanoflowers had multi-functionalities composed of biorecognition and signal amplification, which were generated from the entrapped biomolecules, including antibody, streptavidin, or Con A, and signaling enzymes, respectively.

Compared with organic biomolecules, multiple inorganic components have rarely been incorporated within hybrid nanoflowers, possibly due to their intrinsic insufficiency of amine and amide groups, which act as essential blooming sites for nanoflower formation. Zhang *et al*. reported that Pt NPs were additionally embedded on BSA-manganese hybrid nanoflowers, revealing their excellent electrocatalytic activity due to the creation of a more electrochemically active surface area after the incorporation of Pt NPs[24]. Graphene oxides and carbon nanotubes, which are carbon-based nanostructures, were also reported to be successfully incorporated within laccase-copper hybrid nanoflowers through the self-assembly after 2 days of incubation at RT[48]. The resulting nanoflowers showed excellent electrical conductivity and dye removal efficiency, which were resulted from entrapped nanocarbons and laccase, respectively. These studies demonstrate the potential of nanoflowers for incorporating multiple kinds of organic or inorganic components in order to increase their fields of application.

Recent Applications

Until now, many hybrid nanoflowers with improved catalytic performances have been developed for applications in biotechnology, ranging from biosensing to other catalytic applications, including pollutant removal, protein digestion, biofuel cell, and even industrial biocatalysis. In this section, we briefly summarize some of the recently reported and representative applications of nanoflowers.

Biosensing Applications

Due to the significance of the determination of glucose in biological fluids, hybrid nanoflowers entrapping both GOx and HRP were studied for the development of efficient glucose biosensors. In the presence of glucose, GOx entrapped within the nanoflowers catalyzed the oxidation of glucose to produce H_2O_2, which induces the oxidation of employed substrates, such as 3,3′,5,5′-Tetramethylbenzidine (TMB) or 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), to produce colorimetric signals by the entrapped HRP. Compared with the conventional glucose assay mediated by free mixtures of GOx and HRP, nanoflowers entrapping both enzymes are advantageous as GOx and HRP are located within close proximity in nanoflower matrices which may facilitate substrate transfer between them as well as relieving the degradation of H_2O_2 during the cascade reaction. In this regard, Sun *et al*. first reported GOx-HRP co-incorporated hybrid nanoflowers, which allow for the very sensitive detection of glucose to levels as low as 0.2 μM, one of the lowest values among those of previously reported colorimetric glucose biosensors[43]. Through spatial co-localization of GOx and HRP within the nanoflowers, an enhanced catalytic performance for the sensitive glucose detection was observed, due to the facilitated substrate transfer via compartmentalized GOx and HRP within the nanoflowers[45]. Furthermore, GOx-HRP nanoflowers were grown in the presence of cellulose filter paper, successfully yielding nanoflowers supported on cellulose networks, thus resulting in 3D microfluidic paper-based colorimetric biosensors for the convenient determination of glucose (Figure 1)[49]. Using a digital camera, the colorimetric responses from the oxidation of TMB were successfully converted to numerical intensities, providing a potent approach for glucose detection with many advantages, including low-cost, speed, disposability, and low-sample consumption, and could thus be recognized as a ready-to-use analytical platform for glucose detection.

Other nanoflowers-based biosensors for the detection of important small molecules in clinical and environmental fields have also been reported. Zare's group deposited laccase-copper hybrid nanoflowers onto membrane filters to achieve the sensitive on-site detection of phenols, which met the requirements for the detection of phenol levels in environmental water[29]. Lin's group synthesized HRP-copper hybrid nanoflowers as a colorimetric platform for the rapid and sensitive visual detection of H_2O_2 and phenol[50]. The catalytic ac-

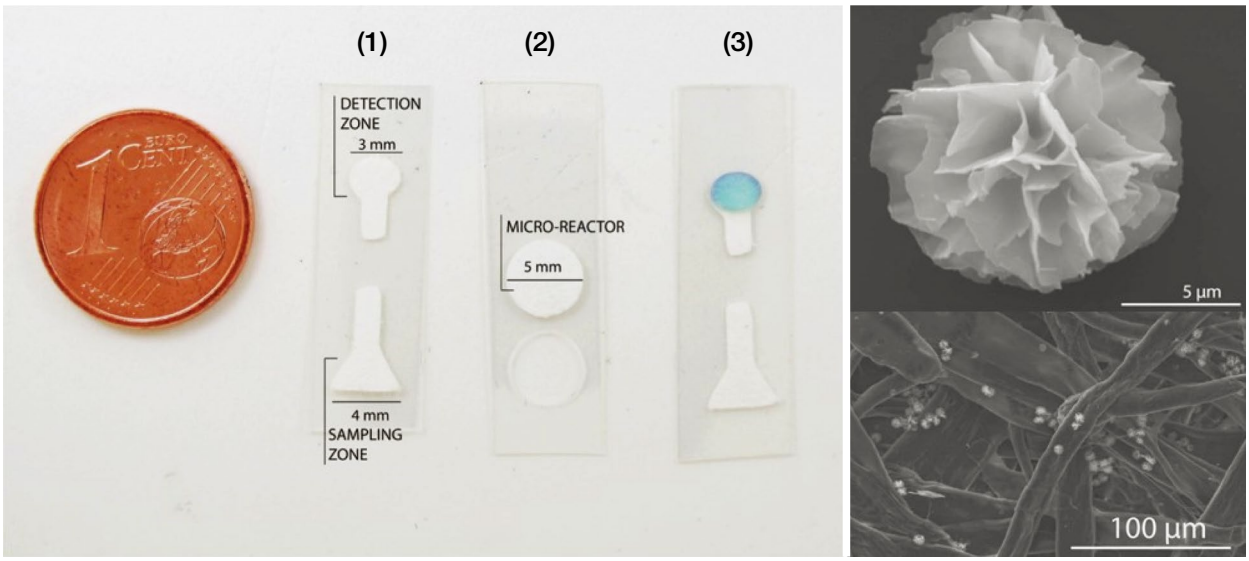

Figure 1. Pictures of the 3D µPAD device showing the two layers (left) and SEM images showing embedded GOx-HRP-copper hybrid nanoflowers and paper membrane surface after depositing the hybrid nanoflowers (right). (1) Disposable layer showing the two separate zones for detecting and sampling, (2) Reusable second layer made with nanoflowers, and (3) Disposable layer after reaction with glucose. Reproduced with permission from Elsevier[49].

tivity of embedded HRP within the hybrid nanoflowers was over 5-fold higher that of free HRP, and thus the limits of detection (LODs) for H_2O_2 and phenol were very low, down to 0.5 µM and 1.0 µM, respectively. Lactoperoxidase-copper hybrid nanoflowers were also reported to exhibit enhanced catalytic activity, up to 160% and 360% higher at pH 6 and pH 8, respectively, compared with their counterpart free enzymes[51]. Based on this enhanced activity, the hybrid nanoflowers were applied for the sensitive detection of dopamine and epinephrine at levels as low as 10 µg/mL, revealing the potential use of nanosensors for the determination of biologically important small molecules.

Organic-inorganic hybrid nanoflowers were also used as multi-functional probes for the affinity-based detection of important biomarkers, including proteins, cells, and small molecules. Wei *et al*. first incorporated multiple organic components consisting of HRP and antibodies toward *E. coli* O157:H7 with copper ions to form hybrid nanoflowers, which exhibited dual functions in conventional ELISA assays[46]. The first function of the above-mentioned nanoflowers was the specific recognition of target pathogens by entrapped antibody molecules and the second was signal amplification by HRP-mediated catalysis. Since the entrapped HRP showed highly enhanced activity compared to free enzymes, HRP-antibody-copper hybrid nanoflowers could detect target *E. coli* O157:H7 very sensitively, at levels as low as 60 CFU L^{-1}, a much greater sensitivity than that of commercial ELISA systems. The colorimetric signals from the immunological and enzymatic reactions by HRP-antibody-calcium hybrid nanoflowers were also read using a commercial smart phone[52], yielding rapid and convenient signal output at low LOD levels of 1 CFU mL^{-1}. Liu *et al*. developed streptavidin-HRP-copper hybrid nanoflowers for the detection of an important biomarker protein, α-fetoprotein, based on the affinity between entrapped streptavidin and a biotinylated target antibody with efficient catalytic signaling by entrapped HRP[47]. Ye *et al*. also reported concanavalin A (Con A)-GOx-calcium hybrid nanoflowers for the easy-to-use detection of pathogenic *E. coli* O157:H7 (Figure 2)[35]. Entrapped Con A allowed for the specific recognition of *E. coli* O157:H7 among many other pathogens due to its high binding affinity with lipopolysaccharides O-antigen of *E. coli*. Entrapped GOx catalyzed the oxidation of glucose to produce gluconic acid, which led to a decrease in pH, detected using a portable pH meter or pH strips. Using this simple strategy, levels as low as 10 CFU mL^{-1} could be detected for 1 month without losing activity, much lower than those detectable using conventional methods. The same group also reported another sensitive pathogenic bacteria detection strategy using Con A-invertase-calcium hybrid nanoflowers, where entrapped invertase rather than GOx was used as a signal amplification unit[53]. In addition, platelet-derived growth factor-BB (PDGF-BB) targeted aptamer was

incorporated with BSA and inorganic cobalt ions to prepare aptamer-incorporated hybrid nanoflowers[54]. PDGF-BB targeted aptamer not only played a role as a scaffold for the nanoflowers, but also improved the target detecting capability via its specific interactions, consequently yielding high efficiency, selectivity, and stability for PDGF-BB detection.

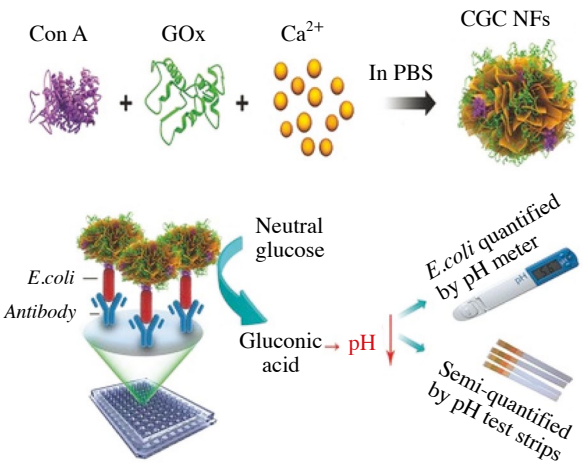

Figure 2. Illustration of the synthetic process for Con A-GOx-calcium organic-inorganic hybrid nanoflowers and the corresponding scheme for immunoassay of *E. coli* O157:H7. Reproduced with permission from Wiley[35].

In general, entrapped enzymes played a role in catalyzing signal amplification. Peng *et al*. incorporated fluorescent proteins within the nanoflowers to develop fluorescent immunoassay (Figure 3)[55]. The authors incorporated protein modified fluorescent gold nanoclusters with calcium ions to prepare hybrid nanoflowers that performed the dual functions of biological recognition and fluorescent signal output for the detection of target clenbuterol (Clen). Through immunomagnetic separation, 0.167 μg L^{-1} of Clen, which is well suited for food safety monitoring, was detected with excellent precision in real samples.

Other Catalytic Applications

Although the primary applications of hybrid nanoflowers mainly focus on biosensing, other catalytic applications in diverse biotechnological areas have also been widely studied based on the versatile functions of entrapped enzymes. Lin *et al*. reported on trypsin-copper hybrid nanoflowers and their application in proteome analysis[56]. The entrapped trypsin within the nanoflow-

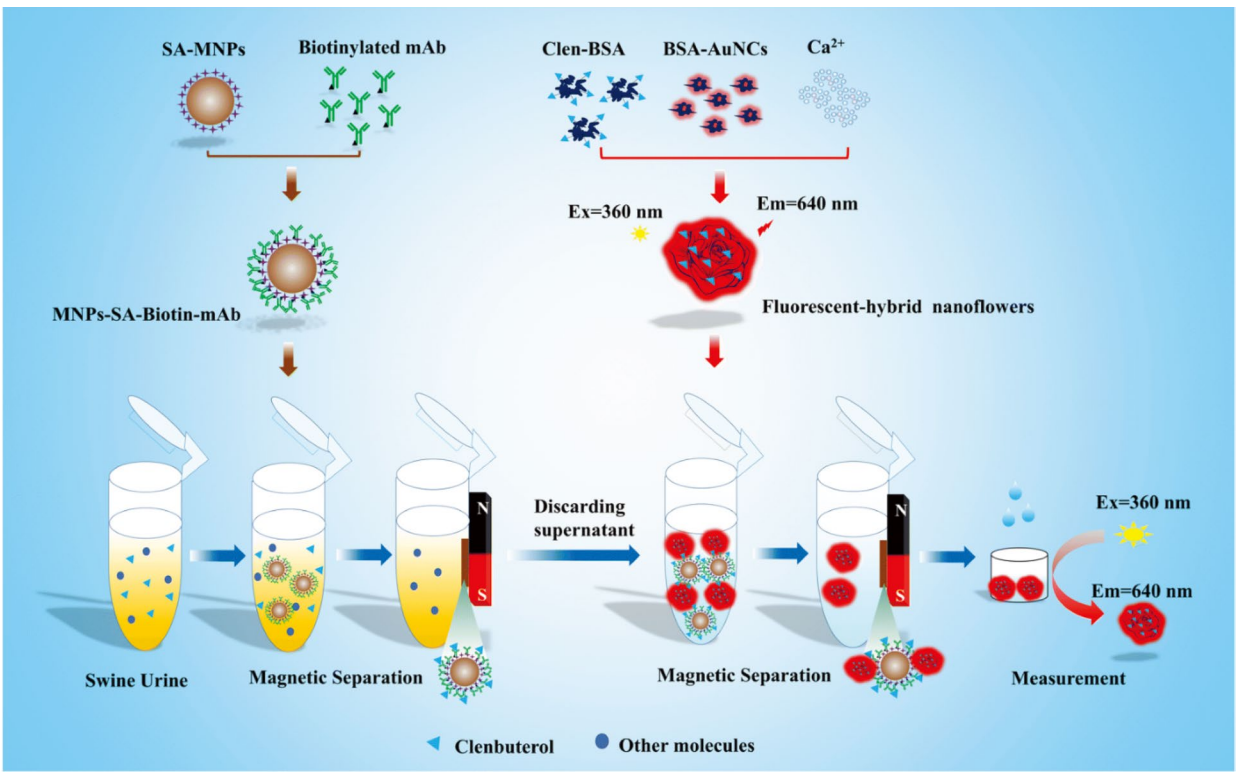

Figure 3. Schematic illustration of the fluorometric immunoassay based on fluorescent hybrid nanoflowers and immunomagnetic separation. Reproduced with permission from Springer Nature[55].

ers was very efficient, taking only 1 minute for protein digestion, compared to free trypsin which took at least 12 hours. Another proteolytic enzyme, α-chymotrypsin, was also mixed with calcium ions to prepare hybrid nanoflowers, showing highly enhanced proteolytic activities for the digestion of BSA and human serum albumin, which were 48% and 34% higher than those from free enzymes, respectively[33]. Xu *et al*. prepared L-arabinose isomerase-copper hybrid nanoflowers which were successfully utilized to produce valuable L-ribulose from L-arabinose with a conversion rate greater than 60% in only 2 days[57]. Ke *et al*. prepared lipase-calcium hybrid nanoflowers for the chiral resolution of (R,S)-2-pentanol with vinyl acetate as an acyl donor[34]. The results of this study indicated that a high enantioselectivity of over 90% was achieved under optimal conditions, revealing the great potential of nanoflowers in industrial biocatalytic applications. In addition, metalloporphyrin-copper hybrid nanoflowers with enhanced catalytic activity were reported to catalyze the cyclohexene epoxidation reaction[58]. Yan *et al*. developed a collagen sponge reinforced with chitosan-calcium hybrid nanoflowers for rapid hemostasis[59]. Based on distinct advantages, such as rapid water absorption, positive surface charge, and large surface area, the nanoflowers-incorporated sponge obtained efficiently induced hemocyte and platelet adherence, thus promoting blood clotting and hemorrhage control *in vitro* and *in vivo*. These applications based on the unique catalytic behaviors of entrapped biomolecules demonstrated the promising potential of hybrid nanoflowers for use in diverse biotechnological applications.

In particular, hybrid nanoflowers have garnered attention for use in an environmental pollutant treatment. Huang *et al*. applied BSA-copper hybrid nanoflowers as peroxidase mimetics to decompose organic pollutants such as Rhodamine B with a high rate of efficiency rate, where up to 97% of pollutants were removed in 4 hours[60]. BSA-copper hybrid nanoflowers were also applied as effective adsorbents for cadmium and lead ions in water, hair, food, and even cigarette samples without any significant interferences[61]. Red blood cell-like BSA-zinc hybrid nanoflowers were prepared and used for the adsorption of copper ions[38]. During 30 min of adsorption, 98.9% of the copper ions were adsorbed, indicating their potential for rapid and efficient removal of copper ions. Furthermore, Li *et al*. synthesized laccase-copper-nanocarbon (graphite oxide or carbon nanotube) hybrid nanoflowers via self-assembly, and applied them to dye removal, whereby around 70% of crystal violet and 45% of neutral red dyes were degraded after 8 days of treatment[47]. These nanoflower-based strategies provide significant opportunities for applications in the treatment and removal of environmental pollutants.

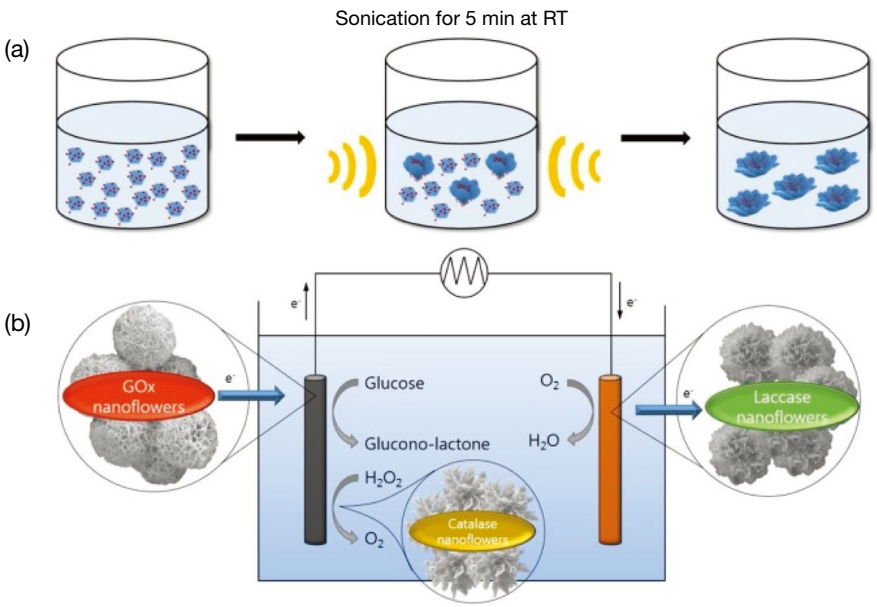

Figure 4. Schematic illustration of sonicated enzyme nanoflower-based biofuel cell. (a) Facile synthesis of enzyme-copper hybrid nanoflowers using ultra-rapid sonication method. (b) Setup of the enzyme nanoflower-based biofuel cell. At the anode, glucose is oxidized to glucono-lactone, where the electrons are transferred from GOx nanoflowers to employed carbon nanotubes. Catalase nanoflowers catalyzed the decomposition of hydrogen peroxide into oxygen and water. At the cathode, electrons are transferred from carbon nanotubes to laccase nanoflowers where dioxygen is reduced to water. Reproduced with permission from Elsevier[62].

Since the entrapped enzymes maintain the high activity and stability of the hybrid nanoflowers, our group recently developed new biofuel cell systems prepared from hybrid nanoflowers mixed with multi-walled carbon nanotubes (Figure 4)[62]. With glucose as the biofuel, GOx and laccase nanoflowers were used to form the enzyme anode and cathode, respectively, and catalase nanoflowers were applied to the anode to catalyze the decomposition of H_2O_2, which is known to be deleterious to GOx, into oxygen and water. Without any mediator involved, the nanoflowers-based biofuel cell successfully generated electricity and had a high power density of up to 200 $\mu W\ cm^{-2}$. Additionally, many research groups have recently reported on the high electrocatalytic activity of hybrid nanoflowers, which could be further applied for the development of novel biofuel cell systems.

Conclusions and Future Prospects

Nanoflowers are recently emerging as one of the most efficient organic-inorganic hybrid nanostructures, showing unprecedented opportunities for significantly improving the biological functions of entrapped biomolecules and creating new applications in diverse biotechnological areas, including biosensing, pollutant treatment, biofuel cells, and other industrial catalysis processes (Table 1). When enzymes are incorporated with inorganic ions within hybrid nanoflowers, their activity, stability, durability, and even selectivity are enhanced majorly due to the large surface area of the nanoflower matrices as well as the efficient confinement of enzyme molecules with little to no mass transfer limitations. For the widespread practical application of nanoflowers, there are certain challenges that will need to be resolved in future research.

First, the synthetic mechanisms and molecular fundamentals regarding reaction-diffusion phenomena and confinement environments need to be elucidated in order to optimize the performances of hybrid nanoflowers. As a result, the design and development of novel hybrid nanoflowers for specific application purposes would be possible. As for applications in industrial biocatalysis, a strategy for the use of hybrid nanoflowers in organic media is required since many substrates

Table 1. Various application studies based on hybrid nanoflowers.

Inorganic component	Organic component	Details of application	Ref.
Copper	HRP	H_2O_2 and phenol detection	50
	GOx and HRP	Glucose detection	43, 45
	GOx and HRP	Microfluidic paper-based glucose detection	49
	Laccase	Syringe filter-based phenol detection	29
	Lactoperoxidase	Dopamine and epinephrine detection	51
	HRP and antibody	*E. coli* O157:H7 detection	46
	Streptavidin and HRP	α-fetoprotein detection	47
	Trypsin	Protein digestion	56
	L-arabinose isomerase	L-ribulose production	57
	Metalloporphyrin	Cyclohexene epoxidation	58
	BSA	Rhodamine B decomposition and Cd and Pb adsorption	60, 61
	Laccase	Dye removal (nanocarbons were incorporated)	48
	GOx, laccase, and catalase	Glucose fuel cell	62
Calcium	Con A and GOx	*E. coli* O157:H7 detection	35
	Con A and invertase	*E. coli* O157:H7 detection	53
	BSA and antigen	Clenbuterol detection (fluorescent gold nanoclusters were incorporated)	55
	HRP & antibody	*Salmonella* species detection with smart phone	52
	α-chymotrypsin	Protein digestion	33
	Lipase	Chiral resolution	34
	Chitosan	Hemostasis	59
Zinc	BSA	Copper ion detection	38
Cobalt	BSA & aptamer	PDGF-BB detection	54
Manganese	IgG, BSA, and ractopamine antibody	Ractopamine detection	24
	BSA	Electrocatalytic methanol oxidation	39
	GOx	Glucose detection	40

in industrial processes have limited solubility in aqueous media. Until now, enzyme-inorganic hybrid nanoflowers have rarely been used in organic media and thus the development of highly-dispersed or even solubilized nanoflowers in organic media has significant incentives. Multi-component incorporated organic-inorganic hybrid nanoflowers have a great potential that is yet to be fully explored. As such, the development of novel strategies for obtaining nanoflowers that can facilitate multi-catalytic functions via compartmentalization should be the topic of prospective research. In addition to the above mentioned studies, we expect organic-inorganic hybrid nanoflowers to be widely employed in a wide range of applications in the near future.

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