Blooming Hybrid Nanoflowers:

Orchestrating Enzyme-Petals with Metal Ions in Harmony of Immobilization

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Abstract: Natural enzymes possess several drawbacks by operational challenges, to overcome these limitations, enzyme immobilization has emerged as a pivotal technique, offering enhanced stability and reusability. However, challenges arise again, impacting overall efficiency and activity. In response, researchers have explored innovative biocatalytic entities known as "nanozymes." These nanomaterials mimic enzyme catalytic activity and present inherent advantages such as cost-effectiveness, scalability, and tunability. The development of Hybrid Nanoflowers (hNFs), characterized by their flower-like morphology, has gained prominence. The layered structure of hNFs, resembling flower petals, provides an enlarged surface area, resulting in heightened catalytic efficiency, stability, and durability.

1. Introduction:

Enzymes are functionally versatile biological species packed with organized chain of amino acids (or RNA) that catalyse to speed up chemical reactions by decreasing the activation energy without themselves getting altered or getting consumed during chemical reaction in a highly efficient manner. These eco-friendly biocatalysts have extraordinary potential in essence of catalytic power, specificity and regulation. However, as always, there are some potential drawbacks present such as very high sensitivity to surrounding environmental parameters leading to a low operational stability, low reproducibility of desired outcomes, difficult recovery of product and complex purification processes, substrate-product inhibition, high cost of production.

To tackle these limitations, immobilization strategies are deployed in the Research field. The term "*Immobilized Enzyme*" was first recognized at the Enzyme Engineering Conference, Henniker, New Hampshire, USA in 1971. Enzyme immobilization has emerged as a cornerstone of modern biotechnology, offering a powerful tool for various applications. Most of the immobilized enzymes exhibited increased stability attributed to reduced mobility of enzyme strands compared to free enzyme. This technique essentially anchors biomolecules, including enzymes, proteins, and DNA, onto a solid carrier material, effectively restricting their mobility. Enzyme immobilization unlocks several advantages, most notably enhanced stability and reusability. In general, enhancement in enzyme activity and stability is expected with immobilization in order to efficiently use enzymes for given reactions or applications. For instance, immobilized enzymes exhibit greater resilience against harsh conditions such as elevated temperatures, organic solvents, and self-degradation, compared to their free counterparts. This improved stability translates to a longer lifespan for the biomolecules, making them more cost-effective in industrial processes. Furthermore, immobilization simplifies the separation and purification of the biomolecules from the reaction mixture. This ease of separation allows for the biocatalysts to be reused in multiple cycles, significantly reducing waste and production costs.

However, if an improper immobilization takes place, it often comes at the cost of reduced activity. This can happen for a few reasons. During the attachment process, **the enzyme's active site might be partially blocked**, hindering its ability to interact with the target molecule (substrate). Additionally, **the solid support itself can create a barrier**, making it more difficult for the substrate to reach the enzyme, also **mass-transfer limitations between enzymes and substrates can be a potential limit**. Finally, the immobilization process can sometimes cause **slight changes in the enzyme's shape, affecting its overall efficiency**. Studies have shown that enzymes immobilized within these nanomaterials can retain up to 60-90% of their original activity compared to their free counterparts. The dominant approach in enzyme immobilization leverages pre-fabricated carriers. These carriers bind enzymes either through physical adsorption or covalent attachment. Also, this two-step process – carrier synthesis followed by enzyme immobilization – suffers from several limitations. **Firstly, it can lead to a decrease in the overall reaction efficiency, potentially due to factors like partial enzyme deactivation during carrier modification**. **Secondly, the lengthy nature of this approach translates to increased costs associated with production and time investment.**

To win the battle against these limitations of traditional enzyme immobilization, researchers have explored the development of alternative biocatalytic entities. They found Nanomaterials. These can mimic the catalytic activity of enzymes have been designated as "*nanozymes*." This term was first introduced by *Scrimin et al.* in 2004. Compared to natural enzymes, nanozymes offer a compelling alternative due to their inherent advantages, including **cost-effectiveness**, **ease of large-scale production**, **tolerance to harsh conditions**, **exceptional stability**, and **ability to tailor activity**

through size, shape, and composition. Notably, **their high surface area facilitates further modifications**. Researchers can engineer nanozymes via modifications, doping, and functionalization to modulate their catalytic activity, biological functions, and biocompatibility. Notably, nanoparticle coatings applied during preparation or reaction steps enhance biocompatibility and colloidal stability. It is important to note that nanoparticles possess unique physical and chemical properties applicable across diverse fields. Their size and shape significantly influence their characteristic properties, with common nanoparticle morphologies including spheres, stars, rods, and cubes.

This Led to characterization of Nanourchins, Nanospines, Nanopyramids, Nanofilms etc. Similarly, **Hybrid Nanoflowers (hNFs)** derive their remarkable physical properties from their distinctive **flower-like morphology**. (Figure:1,2)

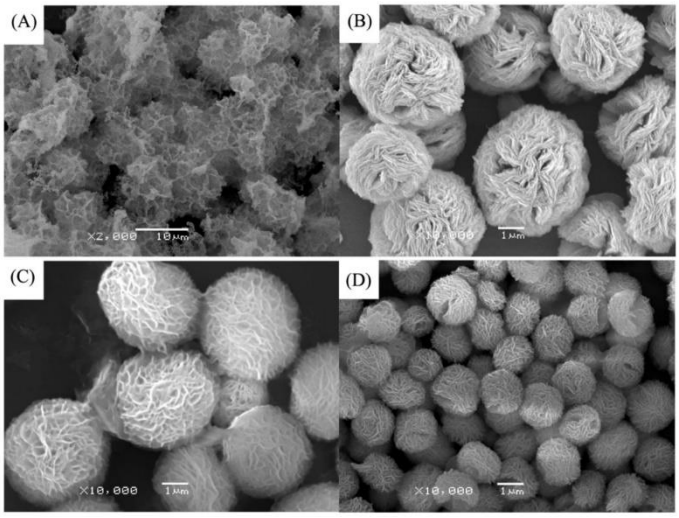

Figure 1: Hybrid Nanoflower Organization

Characterized by a porous structure resembling a flower, hNFs comprise both organic(e.g. enzymes) and inorganic(e.g. metal ions) components meticulously arranged in a layered fashion, akin to flower petals. This unique topography, featuring multiple nanolayers, offers a significant advantage: **a considerably enlarged surface area.** This expanded surface area translates to **enhanced catalytic activity, stability, and durability for hNFs, also these hybrid species hold immense potential due to their high surface-to-volume ratio, high adsorption, and enzyme loading capacity exceeding that of conventional spherical nanoparticles.** This unique characteristic demonstrably translates to enhanced efficiency in surface-based reactions, sparking extensive research into their applications. However, despite this growing interest, synthesizing nanoflowers presents significant challenges. The process often necessitates harsh conditions, including toxic organic solvents, high temperatures, and high pressure. These conditions make it difficult to precisely control the morphological features of the nanoflowers, hindering the ability to tailor their structures for specific applications.

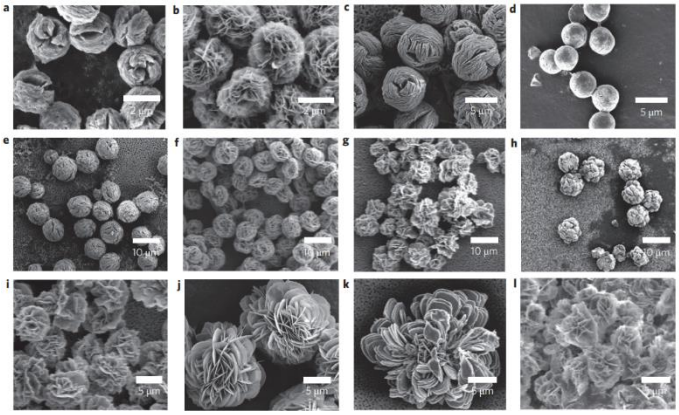

Figure 2: SEM Images of Hybrid Nanoflower

A groundbreaking discovery in **2012 by *Zare et al.*** revealed this novel and fortuitous immobilization technique for enzymes. By *accidentally* introducing copper sulphate into a solution containing phosphate-buffered saline and bovine serum albumin. This typical synthesis protocol commences with the formation of bovine serum albumin (BSA)-incorporated Hybrid Nanoflower. Initially, 20 µL of an aqueous copper sulphate (CuSO4) solution, prepared from a 120 mM stock solution in biological grade water, is added to 3 mL of 10 mM phosphate-buffered saline (PBS) solution of pH 7.4 containing 0.1 mg of BSA at room temperature (25°C). The resulting mixture undergoes vigorous vortexing for 30 seconds and is subsequently left undisturbed for a 3-day incubation period at RT. Following incubation, a blue precipitate forms at the bottom of the reaction tube, signifying the successful synthesis of the hNFs. This unexpected finding led to the development of flower-like organic-inorganic hybrid nanostructures (hNFs) composed of protein and metal ions.

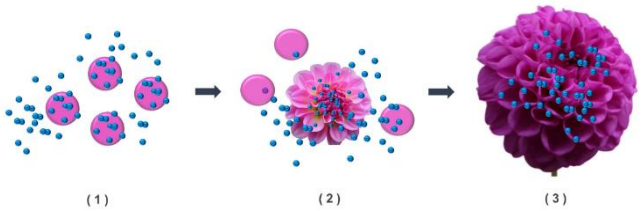

Figure 3: Hybrid nanoflower forming mechanism, where the blue balls are the Cu2+ ions, and pink balls represent the enzyme.

Notably, *Zare's* group reported that these hNFs exhibited considerably **higher enzyme activity and stability compared to both free and conventionally immobilized enzymes**. Their research also delved into the characterization of these protein-organic/inorganic hybrid. **The inorganic component within hNFs plays a crucial role in stabilizing the organic component, consequently enhancing its overall stability and reducing mass transfer resistance.** This ultimately contributes to the increased durability of hNFs.

Interestingly, *Zare et al*. observed a fascinating relationship between enzyme concentration and the size and morphology of the resulting hNFs.

The successful synthesis of blooming nanoflowers hinges on **maintaining a sufficiently low protein concentration during the nucleation step**. Typically, concentrations as low as 0.02 mg ml^{-1} are employed. **Conversely, high protein concentrations (e.g., 0.1 mg ml^{-1}) lead to an abundance of primary particles. This, in turn, diminishes the influence of diffusion and results in the formation of nanoflowers with underdeveloped structures** (petals of enzyme), often resembling underdeveloped buds. The presence of proteins plays a critical role in nanoflower formation. In their absence, the synthesis process yields large crystals instead of the desired nanoflower structures. This highlights the crucial influence of proteins on directing the assembly process. *Zare et al*. capitalized on this discovery by utilizing enzymes as the protein component, leading to the creation of hybrid nanoflowers with significantly enhanced catalytic performance. For instance**, laccase-incorporated nanoflowers exhibited 4.5 to 6.5 times greater activity in oxidizing catecholamine and syringaldazine compared to free laccase.** This remarkable improvement demonstrates the potential of these hybrid structures. Furthermore, the stability of the encapsulated enzymes is significantly enhanced within the nanoflowers**. Laccase-incorporated nanoflowers retained a remarkable 95% of their initial activity after two months, while free laccase lost 50% of its activity within ten days under identical conditions.** These nanoflowers demonstrated reusability, maintaining their activity for at least five consecutive cycles without any observable decline.

Competing Models on Nanoflower Formation: The exact mechanism governing the formation of hybrid nanoflowers remains a topic of ongoing investigation. **Two primary models have emerged to explain this process:**

Three-Stage Model: This model proposes a three-step process consisting of **nucleation, crystal growth, and nanoflower formation**. In the initial stage, copper (Cu2+) ions interact with phosphate ions to form the first crystals. Simultaneously, Cu2+ complexes form with the incorporated enzyme. Subsequent stages involve the growth of these crystals and the eventual assembly of nanoflowers.

Four-Stage Model: An alternative model proposed by *Kim et al.* suggests a four-stage growth process. This model breaks down the process into: **Initial nucleation** and formation of an enzyme-metal nanocomplex**, Self-assembly of nanofibers**, **formation of nano-hyperbranched** segments, and lastly the final **production of mature hybrid** nanoflowers.

2. Superiority of hNFs Over Other Structures:

Storage Stability of Enzymes in hNFs: There are enhanced storage stability of enzymes encapsulated within hybrid nanoflowers (hNFs). Study investigated the storage stability of laccase within magnetic hNFs. At room temperature, free laccase rapidly lost activity, retaining only 23% and 10% after 30 and 60 days, respectively. Conversely, **laccase immobilized within hNFs exhibited significantly improved stability, maintaining 60% activity after 30 days and 45% after 60 days**. Both free and hNF-bound laccase displayed good activity at or above 4°C over the 60-day period. The authors attributed the activity loss in free laccase to conformational changes. Notably, electron microscopy (SEM) analysis revealed **no change in the size or hierarchical structure of the hNFs throughout the storage period**, indicating their excellent morphological stability. Additionally, minimal protein leaching was observed from the hNFs at both room temperature and 4°C, suggesting the strong interaction between the enzyme and the hNF structure.

Stability in pH Spectrum: Another study explored the impact of pH during hNF synthesis on enzyme stability using urease as a model enzyme. Regardless of the synthesis pH (6, 7.4, 8, or 9), hNFs consistently offered superior storage stability compared to the free enzyme. After 30 days at 4°C, hNFs **exhibited significantly lower activity loss (ranging from 3.7% to 22.55%) compared to the free enzyme (73.55% activity loss).** Similar trends were observed at room temperature, with hNFs demonstrating improved stability across all pH values. Notably, **hNFs synthesized at pH 7.4 displayed the most remarkable storage stability**, highlighting the potential for optimizing hNF synthesis conditions for enhanced enzyme preservation.
These findings collectively demonstrate that hNFs not only offer a protective environment for enzymes, leading to improved storage stability, but also suggest the possibility of tailoring hNF synthesis parameters to further enhance enzyme functionality.

High Surface Area Advantage: The **high surface area to volume ratio** of nanoflowers significantly enhances surface adsorption, ultimately accelerating reaction kinetics. This three-dimensional structure amplifies the effect of reactions occurring on the surface by providing a greater number of adsorption sites. This characteristic notably improves the efficiency of Surface Enhanced Raman Scattering (SERS) sensing.

Superior Enzymatic Potential: Recent studies have revealed that protein-inorganic hybrid frameworks, such as $Cu_3(PO_4)\cdot 3H_2O$ nanoflowers, exhibit **intrinsic peroxidase-like activity**. Researchers have successfully synthesized nanoflowers with augmented **catalytic efficacy** for enhanced protein digestion. For instance,

alpha-chymotrypsin-$Cu_3(PO_4)_2$ nanoflowers can digest protein **in just one minute**, whereas trypsin, a conventional enzyme, **requires nearly 12 hours to complete the same reaction.** This remarkable improvement can be attributed to the **266%** increase in enzymatic activity observed upon immobilization of alpha-chymotrypsin on the nanoflowers.

Amplified Reusability: hNFs demonstrate remarkable reusability, retaining activity for multiple reaction cycles before significant deactivation. Additionally, they offer the advantageous ability to be recycled by incorporating fresh enzymes.
Yn et al. established **a method for hNF reblooming**. **The process involves: (a)**Dissolving the original hNFs using acetic or phosphoric acid (0.2 mL). **(b)**Heating the solution at 100°C for 10 minutes to denature all enzymes. **(c)**Removing the denatured enzymes through filtration or centrifugation. **(d)**Adjusting the solution pH to 6.7 using $Ca(OH)_2$. **(e)**Reblooming the hNFs by adding fresh enzymes and inducing co-crystallization with $Ca(PO_4)_2$ at 4°C for 24 hours. **(f)**Separating the regenerated nanoflowers for reuse. The researchers evaluated both the activity of these "dual-cycle" hNFs and the recovery rate of $Ca(PO_4)_2$ for six enzyme models. The results **revealed no significant difference** in activity between the **original and the re-used hNFs**, indicating that residual molecules like amino acids do not impede catalysis by the regenerated structures.

3. Decoding The Impact of Environmental Factors on hNFs:

Beyond the selection of organic and inorganic components, the final structure and performance of hNFs are significantly influenced by the synthesis procedure and environmental factors. Three key process-related Criterion – **pH, temperature, and incubation time** – can dramatically affect hNF formation and the enzymatic activity.

Impact of pH: The surface charge of the enzyme component, which dictates its interaction with metal phosphate nanocrystals, is highly pH-dependent. As a result, the pH of the synthesis solution has a profound effect on the morphology of the resulting hNFs.
At pH values lower than the isoelectric point (pI), repulsion between positively charged biomolecules and metal phosphates hinders hNF formation. Conversely, at pH values exceeding the pI, repulsion between negatively charged biomolecules also inhibits hNF formation. Choosing the appropriate synthesis pH is therefore crucial for desired hybrid Nanoflower production. A recent study investigated the effect of pH (ranging from 5 to 9) on the formation and catalytic activity of L-Asparaginase (ASNase)@$Cu_3(PO_4)_2$ hNFs. No flower-shaped structures were observed at pH 5, while successful hNF formation occurred at a pH range of 6-9. Both the morphology and activity of the hNFs depended on the synthesis mixture's pH. ASNase@$Cu_3(PO_4)_2$ hNFs prepared at pH 8 exhibited the highest activity compared to hNFs formed at other pH values.

Influence of Temperature: The temperature during the incubation period is another variable affecting hNF morphology and catalytic activity. For instance, magnetic catalase (CAT)@$Cu_3(PO_4)_2$ hNFs formed at 20°C displayed a well-developed bloomed structure with a diameter of 23.1 μm. Under identical conditions, magnetic CAT hNFs incubated at 4°C exhibited a spherical morphology with a significantly smaller diameter of 7.8 μm. hNFs prepared at pH 9 exhibited an irregular morphology due to the partial dissolution of copper phosphate crystals and repulsion between negatively charged CAT molecules. Tri-enzyme-based hNFs containing cellobiohydrolase (CBH), endoglucanase (EG), and β-glucosidase (BG) were developed for efficient glucose production from cellulose. These tri-enzyme hNFs were prepared at temperatures ranging from 4 to 55°C. Interestingly, scattered structures formed at temperatures exceeding 35°C, while hNFs prepared at 4, 15, and 25°C exhibited more organized and well-developed flower-like morphology. The glucose production of the tri-enzyme hNFs prepared at lower temperatures (4, 15, and 25°C) was significantly higher than those obtained at 35-55°C. This might be attributed to the alteration of enzyme 3D structure at elevated temperatures, potentially affecting their activity and incorporation within the hNF structure.

The Impact of Incubation Time on hNFs: Several studies have explored the impact of synthesis duration on the morphology of hybrid nanoflowers, aiming to control their growth and elucidate their formation mechanism. Researchers investigated the evolution of cytochrome P450 enzyme-copper phosphate hybrid nanoflowers with varying incubation times (0.1 to 72 hours). At the earliest stage (0.1 h), only **irregular nano-plates were observed. Flower-like nanocrystals** emerged with **extended incubation (6 h),** and **fully formed hybrid nanoflowers were achieved after 24 hours**. Similar observations were reported by *Zhu et al.*, where nanoflowers exhibited progressive growth and diverse morphologies with increasing reaction time, culminating in compact flower-like structures after 36 hours. *Sun et al*. delved into the formation mechanism of multi-enzyme co-embedded hybrid nanoflowers. They discovered that protein molecules **primarily interacted with metal ions via coordination with amide groups in the protein backbone within 2 hours.** Subsequently**, protein-metal ion crystals aggregated into larger structures, forming the initial flower petals between 8 and 36 hours.** As the reaction progressed, the nanoflowers continued to grow, ultimately resulting in complete,

multi-layered flower-like formations. *He et al.* observed that although the diameters of metalloporphyrins-copper phosphate hybrid nanoflowers remained similar across various incubation times, their morphologies differed. At the **initial stage (0.1 h), scattered petals and rudimentary flowers were present.** With extended incubation, the **flower-like shapes became more defined and compact**, and the number of petals per flower increased significantly (**from dozens at 0.1 h and 6 h to hundreds at 24 h and 72 h**). These findings suggest that hybrid nanoflower formation involves a rapid nucleation stage followed by a slower growth process, aligning with previous reports.

4. Current Scopes and Applications:

Nanoflowers holds immense application and tremendous future promises as robust biocatalyst and has wide range of application in biotechnology, with distinct operational fields such as biosensors, industrial biotechnology, enzymology, bioanalysis, waste-water treatment, therapeutics and diagnosis.

Application in Biocatalysis: Nanoflowers offer a promising approach to creating biocatalysts. These tiny structures are both straightforward to produce and remarkably stable. They also improve how efficiently materials move within the catalyst, leading to better overall activity. As a result, researchers are exploring their use in various biocatalysis applications.

Facilitating the Enzymatic Production of Chemical Compounds: Enzymes offer a versatile tool for various chemical reactions, and their ability to synthesize esters finds applications in diverse fields, from cosmetics to biofuels. Researchers have explored immobilizing enzymes within nanoflower structures to improve their performance. Studies by *Zhang et al*. (2013) demonstrated that lipase ZC12 immobilized on calcium-based nanoflowers exhibited a significant increase (206%) in catalytic activity compared to the free enzyme. Additionally, these nanoflowers displayed improved selectivity at lower temperatures and maintained their activity after repeated use. Their effectiveness was further confirmed by a higher conversion rate (57%) for lauric acid to a fructose ester compared to the free enzyme under similar conditions. Another study by *Jiang et al*. (2011) explored the use of porcine pancreas lipase (PPL) immobilized on copper phosphate nanoflowers for biodiesel production. These hybrids displayed enhanced stability at high temperatures, leading to more efficient reactions. The immobilized enzymes also showed improved storage stability, retaining over 93% activity after one week at room temperature, while the free enzyme lost activity within two days. Notably, the nanoflowers exhibited better resistance to methanol, a common biodiesel production challenge, and maintained a high conversion rate even after multiple cycles. The unique structure of nanoflowers allows for the simultaneous immobilization of multiple enzymes. This creates "multifunctional biocatalysts" that can perform complex, multi-step reactions or eliminate unwanted byproducts. *Zhang et al.* (2014) successfully created nanoflowers containing both glucose oxidase and lipase for styrene epoxidation. They tested use of the combined enzymes in the free form, immobilized separately and immobilized in the same structure for the epoxidation of styrene, with 51%, 65%, and 89% conversions, respectively. These studies highlight the potential of enzyme-embedded nanoflowers as a powerful tool for enzymatic synthesis. They offer improved stability, activity, and reusability, making them a promising approach for various biocatalytic applications.

Nanoflower for Biodegradation: Enzymes play a crucial role in bioremediation, the process of cleaning up pollutants using natural biological processes. They excel at breaking down complex molecules, including drugs, dyes, and proteins, under mild conditions, making them eco-friendly tools. The diversity of dyes used in various industries necessitates a wide range of enzymes for effective decolorization. Notably, enzymes can target both synthetic and natural dyes. Studies have shown promising results with enzyme-embedded nanoflowers for dye removal. *Rong et al*. (2017) demonstrated copper-laccase nanoflowers that achieved a remarkable 95% removal of Congo Red dye, exceeding the free enzyme's removal rate by 3.6 times. *Patel et al*. (2017) further enhanced the performance of copper-laccase nanoflowers by cross-linking them with glutaraldehyde. This treatment improved the enzyme's catalytic activity, storage stability, and reusability compared to the untreated nanoflowers. This resulted in efficient removal of various dyes like Bromophenol Blue, Coomassie Blue, and Xylene Cyanol. *Luo et al*. (2018) explored a novel approach by growing laccase and copper together on a 3D nanofiber membrane. This method not only improved the mechanical strength of the nanoflowers but also enhanced their dye degradation efficiency. The supported structures effectively degraded various dyes, including reactive blue 2, acid blue 25, acid yellow 76, and indigo carmine, with removal rates exceeding 83%.These studies showcase the immense potential of enzymes and enzyme-immobilized nanoflowers for bioremediation applications. Their ability to efficiently break down pollutants under mild conditions makes them a valuable tool for a cleaner environment.

Hydrolysis and Digestion of Protein: Researchers are exploring the use of enzyme-embedded nanoflowers for protein digestion, demonstrating significant improvements compared to free enzymes. *Lin et al.* (2017) developed copper phosphate nanoflowers containing trypsin for protein degradation. While the free enzyme required 24 hours to achieve specific

results, the nanoflowers accomplished the same feat in just one minute, highlighting a dramatic increase in digestion efficiency. *Memon et al*. (2018) investigated calcium-based nanoflowers containing the enzyme alkalase to hydrolyse soy protein. The resulting soy protein hydrolysate displayed enhanced functionalities, including improved solubility and free radical scavenging activity. Compared to the free enzyme, the nanoflower-produced hydrolysate exhibited a 70% radical-scavenging capacity and maintained high activity even after repeated use. *Feng et al.* (2017) explored magnetic papain-copper phosphate nanoflowers for degrading allergenic proteins in cow's milk. These nanoflowers displayed an impressive 1556% increase in activity compared to free papain. Additionally, their magnetic properties facilitated easy separation from the reaction mixture, eliminating the need for additional protein separation steps during milk allergen hydrolysis. These studies demonstrate the remarkable potential of enzyme-embedded nanoflowers for protein digestion. Their ability to achieve faster digestion rates, enhance product functionality, and offer easier separation methods makes them a promising advancement in this field.

Optimizing Nanoflowers for Lactose Breakdown: The effectiveness of enzyme-embedded nanoflowers for lactose hydrolysis depends heavily on the metal ion incorporated within the structure. *Talens-Perales et al*. (2018) investigated this by creating nanoflowers with different metal ions (Cu^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, and Ca^{2+}) and the enzyme β-galactosidase. As expected, the metal type influenced not only the nanoflower morphology but also the enzyme activity. Interestingly, while structures containing Mn^{2+}, Co^{2+}, and Ca^{2+} exhibited activity comparable to the free enzyme, those with Zn^{2+} and Cu^{2+} showed significantly lower activity, with Cu^{2+} causing a tenfold decrease. This finding is crucial because copper, despite being commonly used in nanoflower synthesis, can inhibit the specific enzyme used in this study. By analysing activity, stability, and reusability, the researchers demonstrated that calcium-containing nanoflowers outperformed those with other tested metal ions for lactose hydrolysis. This study highlights the importance of considering metal ion selection when designing enzyme-embedded nanoflowers for specific applications. By carefully choosing the right metal component, researchers can optimize these structures for enhanced performance.

Blooming with Potential for Biosensors: Nanoflowers have emerged as a promising tool for developing biosensors, particularly for detecting harmful environmental pollutants. This section explores their application in detecting phenol and hydrogen peroxide.

Fast and Easy Phenol Detection: Zhu et al. presented a simple and efficient method for phenol detection using enzyme-embedded nanoflowers. These hybrids, made with copper ions and laccase, were immobilized in a syringe filter. When a sample containing phenol passes through the filter, it reacts with 4-aminoantipyrine to form a colored compound (quinoneimine). This color change allows for easy quantification of phenol in the sample. Notably, these nanoflowers exhibited an activity level 200% higher than the free enzyme for phenol oxidation.

Detecting Hydrogen Peroxide: The concept of using enzyme-based nanoflowers for biosensing can be extended beyond phenol detection. *Lin et al.* developed copper phosphate nanoflowers containing horseradish peroxidase (HRP) for simultaneous detection of phenol and hydrogen peroxide. These nanoflowers displayed a remarkable 506% increase in activity compared to the free HRP enzyme solution. *Zhang et al.* explored the use of nanoflowers for hydrogen peroxide detection specifically. They created hybrids containing catalase and copper phosphate using sonication, a rapid synthesis method. These nanoflowers demonstrated significantly improved stability compared to the free enzyme. While the free catalase lost 62% of its activity within 28 days, the nanoflowers retained 90% of their initial activity during the same period. These studies showcase the versatility and effectiveness of enzyme-embedded nanoflowers for developing biosensors for environmental pollutants. Their ability to enhance enzyme activity and stability makes them a valuable tool for environmental monitoring applications.

Nanoflowers as Early Warning System for Pathogen Detection: Rapid and accurate detection of pathogens in food and water is crucial for ensuring public health and reducing healthcare costs associated with foodborne illnesses. Biosensor technology offers promising solutions, and enzyme-embedded nanoflowers are emerging as a powerful tool in this field.

Targeting E. coli with Nanoflowers: Researchers like *Ye et al*. have developed biosensors using hybrid nanoflowers for detecting E. coli O157:H7, a harmful bacteria strain. These nanoflowers combine concanavalin A (a molecule that binds to specific structures on E. coli), glucose oxidase (an enzyme), and calcium ions. The biosensor works by exploiting the binding interaction between concanavalin A and E. coli. When bacteria are present, the enzyme converts glucose into gluconic acid, increasing the acidity of the surrounding environment. By measuring the pH change, the sensor can detect and quantify the number of bacteria in the sample. Another study by *Wang et al*. explored a different approach using hemin (an iron-containing molecule) and concanavalin A embedded in copper phosphate nanoflowers. This sensor displayed peroxidase-like activity, allowing detection through a colorimetric

method. The biosensor achieved a detection limit of up to 4.1 colony-forming units per millilitre (CFU/mL) for E. coli, demonstrating its sensitivity. These studies highlight the potential of enzyme-embedded nanoflowers for developing highly sensitive and specific biosensors for pathogen detection in food and water samples. This technology could revolutionize food safety protocols and contribute to a healthier future.

Nanoflower Offer Hope for Parkinson's Disease: Parkinson's disease is a neurodegenerative disorder characterized by the loss of dopamine-producing neurons. Researchers are exploring the potential of nanomaterials to combat this condition. *Manganese Oxide Nanoflowers: Multi-Enzyme Mimics:* Manganese oxide nanoflowers (Mn_3O_4) have shown promise as potential therapeutic agents. These nanostructures exhibit the activity of three crucial antioxidant enzymes: superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). These enzymes work together to maintain a healthy balance of reactive oxygen species (ROS) within cells, protecting cells from oxidative damage. Mn3O4 nanoflowers can also effectively scavenge harmful hydroxyl radicals. Studies suggest that the large surface area, mixed oxidation states of manganese, high stability, and good porosity of these nanoflowers contribute to their potent multi-enzyme activity. Additionally, they can integrate into human cells, offering protection in models of Parkinson's disease. *Cerium Oxide Hybrid Nanoflowers: Targeting Different ROS Sources:* Another approach involves cerium oxide (CeO2) nanoflowers. *Kwon et al*. reported three types of CeO2 nanoparticles designed to target different ROS sources within cells. These nanoparticles can clear ROS from the mitochondria (the cell's energy centers) and the surrounding cytoplasm. They also suppress lipid peroxidation and microglial activation, mimicking the activity of natural antioxidant enzymes like SOD and catalase.

5. The Future of Hybrid Nanoflowers: Overcoming Challenges and Unlocking Potential:

While nanozymes and hybrid nanoflowers have demonstrated remarkable potential in various fields, there are still hurdles to overcome for their continued development. This section explores these challenges and future directions for this exciting research area.

Nanozymes with Fine-Tuning Performance: Specificity and Dispersion: Current nanozymes often lack the specificity and good dispersibility of natural enzymes. However, researchers are making progress. By modifying nanozymes with materials like cysteamine or histidine, scientists can improve surface chemistry, control the microenvironment around the active center, and enhance dispersion.

Reaction Diversity: Majority nanozymes currently mimic oxidoreductases, limiting their reaction types. However, recent studies suggest tailoring nanozyme composition can regulate activity. Additionally, researchers are investigating the use of pH and light to modulate nanozyme function. Further exploration is needed to identify more factors that govern and influence nanozyme activity.

Hybrid Nanoflowers: Unveiling the Mysteries Organic-Inorganic Interaction: A deeper understanding of the interaction between enzymes and metal ions within hybrid nanoflowers is crucial. This knowledge will guide the design of nanoflowers with optimal biological activity, controlled morphology, and tailored properties. It will also pave the way for the development of new, application-specific hybrid nanoflowers.

Multi-Enzyme Systems: More research is needed on hybrid nanoflowers containing multiple enzymes or dual enzyme systems. These systems have the potential to mimic even more complex biological processes.

Organic Media Synthesis: Most reported hybrid nanoflowers are synthesized in aqueous media. Developing methods for organic media synthesis would be beneficial for industrial biocatalysis applications.

Industrial Applications: The potential applications of hybrid nanoflowers extend beyond biosensing and biomedicine. Exploring their use in energy applications like fuel cell fabrication and biodiesel production is a promising future direction.

Looking Ahead to Brighter Future with handful of Hybrid Nanoflowers: By addressing these challenges and continuing research efforts, nanozymes and hybrid nanoflowers hold immense promise for various fields. Their potential applications range from improved disease diagnosis and treatment to environmental remediation and industrial biocatalysis. As the understanding of these materials deepens, we can expect even more innovative and groundbreaking applications in the years to come.

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