Ultrafast sonochemical synthesis of protein-inorganic nanoflowers

**Abstract:** We developed a simple but efficient method to synthesize protein-inorganic hybrid nanostructures with a flower-like shape (nanoflowers), which relies on sonication to facilitate the synthesis of the nanoflowers. With this technique, we synthesized nanoflowers containing laccase as a model protein and copper phosphate within 5 minutes at room temperature. The resulting laccase nanoflowers yielded greatly enhanced activity, stability, and reusability, and their usefulness was successfully demonstrated by applying them in the colorimetric detection of epinephrine. The strategy developed could be used to rapidly synthesize nanoflowers for various applications in biosensor and enzyme catalysis and would expand the utilization of nanoflowers in diverse fields of biotechnology.

**Keywords:** copper phosphate, laccase, epinephrine, hybrid nanomaterials

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**Introduction**

Nanobiocatalysis, which utilizes enzymes incorporated within nanostructured materials for improving the performance of natural enzymes, has created new opportunities in enzymatic applications including biosensors and biocatalysis. To achieve an efficient nanobiocatalysis system, enzymes have been generally immobilized within various nanostructured materials by employing approaches such as physical adsorption, covalent attachment, and entrapment. Physical adsorption is very simple to perform and effective at retaining enzyme activity during adsorption, but stability is poor due to the leaching of adsorbed enzymes during the operation. On the other hand, covalent attachment can achieve fairly good stabilization of enzyme activity, but requires tedious procedures to covalently attach the enzyme molecules to the surface of the materials. Entrapment of enzyme molecules within a polymeric network could efficiently retain and stabilize enzymes, however, its practical use is generally restricted by mass transfer limitation through the solid support. Therefore, there has been a continuous need to develop a novel immobilization method that effectively retains and stabilizes enzyme activity by overcoming the mentioned limitations.

Along this line, several groups have reported different innovative strategies to synthesize enzyme-inorganic hybrid nanostructures having flower-like shapes (nanoflowers) from enzyme and copper or calcium phosphate, which are more stable and active than the conventionally immobilized enzymes. The nanoflowers made from laccase and copper phosphate were also successfully applied to detect phenol in an aqueous solution with the enhanced activity and stability. Furthermore, several groups have utilized enzyme-inorganic hybrid nanoflowers for different applications such as detection of hydrogen peroxide, glucose, and protein digestion. Although the enzyme-inorganic hybrid nanoflowers resulted in greatly enhanced activity and stability, their synthesis was so slow that they had to be incubated in the reaction mixture for up to 3 days at room temperature.
Scanning electron microscopy (SEM) images were obtained for characterization of nanoflowers. Aqueous solutions were prepared using UltraPure™ DNase/RNase-Free Distilled Water (Thermo Fisher Scientific). The conventional method used to prepare nanoflowers involves 20 μL of aqueous CuSO₄ solution (120 mM) being added to 3 mL of PBS (pH 7.4) at 25°C containing proteins at different concentrations, followed by incubation at RT for 3 days. In the study reported here, we synthesized nanoflowers following the same procedure but sonicated the solution for different time intervals instead of incubating at RT. For sonication, we utilized a Branson (1510) bath sonicator (length 15.2 cm, breadth 14 cm, height 10.2 cm, operating capacity 1.9 L) at a frequency of 40 kHz and power of 70 W.

The storage stability of the epinephrine sensing system was checked in aqueous buffer (PBS, pH 7.4) under static conditions at 25°C. First, the initial activity was determined by measuring the fluorescence emission intensity at 506 nm with the excitation at 375 nm during the typical reaction that involved sonicated laccase nanoflowers (30 μg-mL⁻¹) or free laccase (30 μg-mL⁻¹) with epinephrine as a substrate (50 μg-mL⁻¹) in PBS (pH 7.4) at 25°C for 20 minutes. The residual activity of each sample was then determined at predetermined time. Reusability was assessed after repeated cycles involving the typical reaction using sonicated laccase nanoflowers (0.1 mg mL⁻¹) and epinephrine (50 μg-mL⁻¹) in PBS (pH 7.4) at 25°C for 20 minutes, separation by centrifugation
at 11,000 rpm for 2 minutes, and excessive washing (three times) with PBS. The excessively washed sample was reused for the measurement of the residual activity. The relative activity (%) was determined by calculating the ratio of the residual activity to the initial activity. Fluorescence intensities were measured using the Tecan Infinite M200 PRO microplate reader with a black 384-well plate.

**Results and discussion**

The overall scheme for the sonochemical synthesis of protein-inorganic nanoflowers is described in Figure 1. To synthesize the sonicated nanoflowers, aqueous PBS solution containing copper(II) sulfate and protein (BSA or laccase) was simply sonicated for 5 minutes, which resulted in the formation of protein-inorganic nanoflowers with flower-like nanostructures, as obtained using the conventional method.\(^1\) We expected that the nanoflowers rapidly prepared by using this simple sonication treatment would exhibit enhanced catalytic activity and stability like those prepared by the conventional method, and thus, this method could be employed to very conveniently synthesize protein-inorganic nanoflowers.

The effect of sonication time on the synthesis of the nanoflowers was first investigated by sonicating the solution containing model protein BSA and copper(II) sulfate in an ordinary ultrasonic bath under a 40 kHz operating frequency for 1 minute, 5, and 7 minutes at RT (Figure 2A). After 1 minute’s sonication, small sized microspheres (average size of ~2 μm) with relatively smooth surfaces were observed, indicating that 1 minute’s sonication was not long enough to bloom flowers, which is required for the formation of nanoflowers. On the other hand, after 5 minutes’ sonication, BSA-copper nanoflowers having average size of ~8 μm were clearly formed and no further blooming of the flowers or growth of flower-like structures was observed during prolonged sonication, as evidenced by comparing the nanoflowers with those synthesized from 7 minutes’ sonication. On the basis of encapsulation yield for the nanoflowers prepared by three different sonication times, 5 minutes was selected as an optimal sonication time showing the highest encapsulation yield, 82% (Figure 2B).

We also investigated the effect of BSA concentration on the morphology of sonicated nanoflowers and encapsulation yield. The investigation proved that the flower-like morphology of sonicated nanoflowers was not significantly affected by the concentration of BSA, whereas a negative control sample without any protein molecules did not result in flower-like structures (Figure 2C). When we used 0.02 mg·mL\(^{-1}\) BSA, we observed the formation of nanoflowers. The encapsulation yield was found to be 82% (Figure 2D).
BSA in the sonochemical synthesis of nanoflowers, the encapsulation yield and weight percentage of BSA in the nanoflowers were determined to be the highest ones, 82% and 7%, respectively (Figure 2D).

Next, we employed laccase, one of the important enzymes in biosensing applications to detect diverse kinds of catecholamines, in the sonochemical synthesis of nanoflowers to demonstrate the real applicability of this method in biosensing fields. As illustrated in Figure 3, sonochemical treatment for 5 minutes successfully synthesized flower-like nanostructures similar to the BSA-based nanoflowers. Among the tested concentrations of laccase, 0.1 mg·mL⁻¹ was chosen as the optimal one because it resulted in highly elaborate flower-like morphology as well as the highest enzyme encapsulation yield (Figure 3A, B). High-magnification SEM images of the sonicated laccase nanoflowers clearly revealed their hierarchical structures with high surface-to-volume ratios (Figure 3C). Also, XRD patterns obtained from the sonicated laccase nanoflowers synthesized under the optimal reaction condition fit well with those of Cu₃(PO₄)₂•3H₂O (JCPDS 00-022-0548), indicating copper was incorporated in the nanoflowers (Figure 4).¹¹

The catalytic activity of the sonicated laccase nanoflowers synthesized under the optimal condition was then evaluated. In a typical activity assay using syringaldazine as a substrate, the sonicated laccase nanoflowers showed ~150% activity compared to the free laccase (Figure 5A), confirming that the synthesizing of laccase nanoflowers by simple sonication also effectively enhanced their catalytic activity like the conventional method.¹¹ The enhanced catalytic activity of sonicated laccase nanoflowers could be a very distinctive advantage considering that the immobilization of enzymes generally leads to lowered activity.²³ We also confirmed that the sonication treatment of free laccase for 5 minutes did not deteriorate the enzyme activity (Figure 5A).

Finally, we demonstrated the applicability of rapidly synthesized, sonicated laccase nanoflowers for the detection of epinephrine, which should be monitored for the appropriate treatment of anaphylactic shock, bronchial asthma, and organic heart disease.²⁴ ²⁵ In a typical colorimetric reaction to employ epinephrine as a substrate, the laccase nanoflowers yielded much denser and greater color changes than those yielded by free laccase, confirming their higher activity.
Ultrafast sonochemical synthesis of protein-inorganic nanoflowers

in detecting epinephrine (Figure 5B). Weak reddish color intensities were produced by the laccase nanoflowers at a low substrate concentration of epinephrine (below 5 µg mL−1); these can be attributed to the fact that the green-colored nanoflowers may have critically attenuated the reddish color because the photograph was obtained without removing the nanoflower pellet from the reaction tube. Next, we investigated the stability and reusability of our sonicated laccase nanoflowers – two very important factors to evaluate in determining the performance of the enzymatic system. As presented in Figure 5C, the laccase nanoflowers showed remarkable stability, preserving their initial activity over 95% for 1 month, whereas free laccase lost ~50% of initial activity within 10 days during the incubation at RT (Figure 5C). Moreover, when the laccase nanoflowers were recycled and reused after simple centrifugation for 2 minutes, there was no significant loss of activity observed in detecting epinephrine for up to four cycles (Figure 5D). These vividly enhanced stability and reusability characteristics could enable the nanoflowers to be widely employed in various applications for biosensor and enzyme catalysis.

Conclusion

Overall, in this study, we discovered that a simple sonication treatment can significantly reduce the synthesis time for organic–inorganic hybrid nanoflowers, potentially enabling scaled-up production within a significantly short period of time of 5 minutes at RT. The resulting nanoflowers yielded highly enhanced activity, stability, and reusability, and thus, this strategy should be a powerful strategy in the preparation of the nanoflowers. Furthermore, the method developed in this study may be applied to rapidly synthesize multi-enzyme co-embedded nanoflowers for potential application in biosensor, biocatalysis, and biofuel cells. Future research will range from the development of new types of organic–inorganic hybrid nanoflowers with certified synthetic mechanism...
to more diverse applications in theranostics (therapy and diagnosis) and other bio-related devices.

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References