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Rapid Detection of Phenol Using a Membrane Containing Laccase Nanoflowers

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Dedicated to Professor Chunli Bai, President of the Chinese Academy of Sciences, in celebration of his 60th birthday.

With the rapid development of nanoscience and nanotechnology,[1a] nanostructured biocatalysts that take the advantage of nanomaterials in terms of both functional and structural availability have offered new opportunities for improving biological functions of enzymes and expanding applications in areas such as biosensors, bioanalytical devices, and industrial biocatalysis.[1] Recently, we reported a method of preparing protein–inorganic hybrid nanostructures with flower-like shapes,[2] which have shown much greater activities than free enzymes and most of the reported immobilized enzymes.[3] To bring this appealing catalyst into practical use, however, an effective accommodation of these high-performance enzyme catalysts is required. One way is to weakly attach these enzyme nanoflowers to porous materials by physical adsorption. Recently, Krieg et al.[4] reported the fabrication of a supramolecular membrane by noncovalent modification of a commercial membrane, which suggests the possibility of fabricating functional filtration membranes by a simple post-modification procedure, thus enabling many new and interesting applications. It thus came to our mind to fabricate a membrane incorporating enzyme nanoflowers for the rapid detection of hazardous compounds through visualization of the catalyzed product. Owing to their high toxicity even at a low concentration,[5] phenols are listed as major toxic pollutants by the Environmental Protection Agency of the USA and other countries.[6] Sensitive detection of phenolic compounds has been well established using instrumental analysis such as liquid chromatography.[7] However, these methods usually require sophisticated instrumentation and a multistep procedure, making them less convenient for rapid and on-site detection.

The present study started by the fabrication of an enzyme nanoflower incorporated into a membrane. As shown in Figure 1, a suspension of laccase–inorganic hybrid nanoflowers, which have a high activity (ca. 200% that of free laccase) for phenol oxidization, as we observed previously,[2] was injected into a commercial disposable syringe filter equipped with a cellulose acetate membrane (pore size 0.2 μm). This procedure thus deposited enzyme nanoflowers with an average size of 4 μm onto the membrane. Then the aqueous sample containing phenol was mixed with an aqueous solution of 4-aminoantipyrine and was passed through the membrane with incorporated laccase nanoflowers, causing oxidative coupling of phenol with 4-aminoantipyrine to form an antipyrine dye[8] that has an absorption maximum at 495 nm. This procedure allowed rapid analysis by a UV/Vis spectrophotometer or by the naked eye. Finally, pure water was injected into the filter to remove unreacted reagents and the reaction products, followed by drying the membrane in air for the next use.

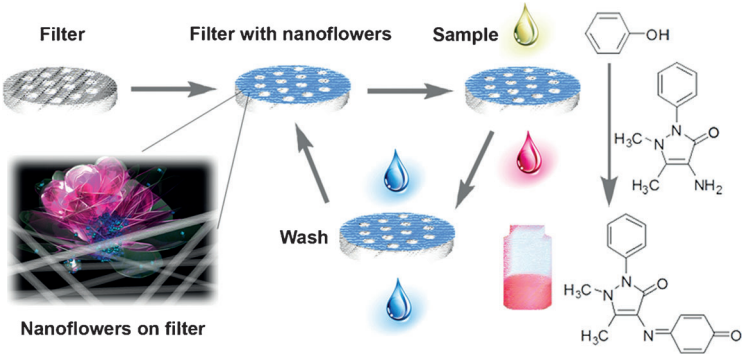

Figure 1. Fabrication, use, washing, and reuse of the membrane with incorporated laccase nanoflowers. Phenol and *ortho*-, *meta*-, and *para*-substituted phenols carrying carboxy, halogen, methoxy, or sulfonic acid groups react with 4-aminoantipyrine to form colored compounds, which can then be readily detected.

For the preparation of laccase–copper phosphate nanoflowers, typically, 0.8 mM aqueous $CuSO_4$ was added to phosphate buffered saline (PBS) containing 0.1 mg mL^{-1} laccase at pH 7.4 and 25 °C. After three days, the precipitate of laccase nanoflowers appeared with porous, flower-like structures. Scanning electron microscopy (SEM) images of the nanoflowers are presented in Figure 2a,b, from which the average diameter of the laccase nanoflowers was determined

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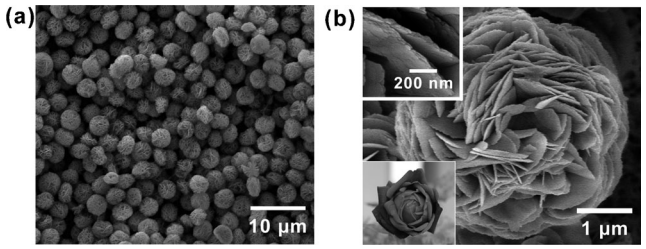

Figure 2. Scanning electron microscopy (SEM) images of a) laccase–copper phosphate nanoflowers; b) a laccase–copper phosphate nanoflower at high resolution. Inset image shows the nanostructure of the petals, inset photo shows a flower in nature.

to be 4 μm. As discussed previously,[2] in the solution containing protein and Cu^{II}, aggregates were formed between protein molecules and Cu^{II} mainly from coordination reactions between protein and Cu^{II}. These aggregates provide the sites for nucleation of the copper phosphate ($Cu_3(PO_4)_2{\cdot}3H_2O$) nanocrystals. The flower-like copper phosphate nanocrystals gradually grow from these nucleation sites. In the formation of nanoflowers, the protein serves as a "glue" to bind together the petals. By measuring the concentration of unencapsulated protein in solution, the encapsulation yield and weight percentage of laccase in the nanoflower were calculated to be 72% and 10%, respectively.

The aqueous suspension of laccase nanoflowers (0.06 mg mL^{-1} protein, 0.2 mL) was injected into the commercial syringe filter with cellulose acetate membrane (pore size: 0.2 μm) to deposit the nanoflower onto the membrane surface. The membrane was then dried in the air at room temperature. Figure 3a gives the SEM image of the membrane with incorporated nanoflowers, which suggested a satisfactory distribution of the nanoflowers on the membrane. Because the average size of a nanoflower is much larger than the pore size of membrane, most of the nanoflowers are deposited onto the membrane. For comparison, free laccase solution was injected into the filter and dried inside the filter by the same procedure, allowing the dried protein to be adsorbed onto the membrane. In a typical experiment for detecting phenol in water, aqueous phenol (1 mL in 50 mM pH 6.0 phosphate buffer) was mixed with 4-aminoantipyrine water solution (1 mL, 1 mg mL^{-1}). Then, 300 μL of the mixture was injected into the nanoflower-coated membrane filter and kept in the filter chamber for five minutes. The high activity of laccase nanoflowers allowed the fast oxidative coupling of phenol with 4-aminoantipyrine to form antipyrine dyes.[8] Afterwards, the solution was pushed out and collected for measurement by naked-eye visualization or by using a UV/Vis spectrophotometer at 495 nm. Figure 3b shows analysis of the samples with different concentrations (from 50 to 0.4 μg mL^{-1} in 50 mM pH 6.0 phosphate buffer). A linear relationship between the absorption intensity at 495 nm and the concentration of phenol could be established (Figure 3c), which served as a calibration. Phenols are employed as raw materials for drugs, pesticides, and dyes and are discharged from industrial plants to environmental water. Phenols in water are toxic to fish and other aquatic organisms and thus have been listed as high-priority pollutants. Gas chromatography (GC) and liquid chromatography (LC) have been used to determine phenolic compounds. GC is mainly used for the determination of individual phenolic compounds but requires their derivatization, which is time-consuming.[9] The method developed in this study has a detection range of phenol from 0.4 to 50 μg mL^{-1} in water, which meets the requirements of detection of phenols in environmental water, although the sensitivity of this method is lower than that of GC or LC analysis.[8] The main advantages of this method are that the analytical procedure only requires five minutes, and the laccase-catalyzed coupling reaction is specific to phenolic compounds,[10] which is very suitable for on-site and fast detection of phenols in environmental water.

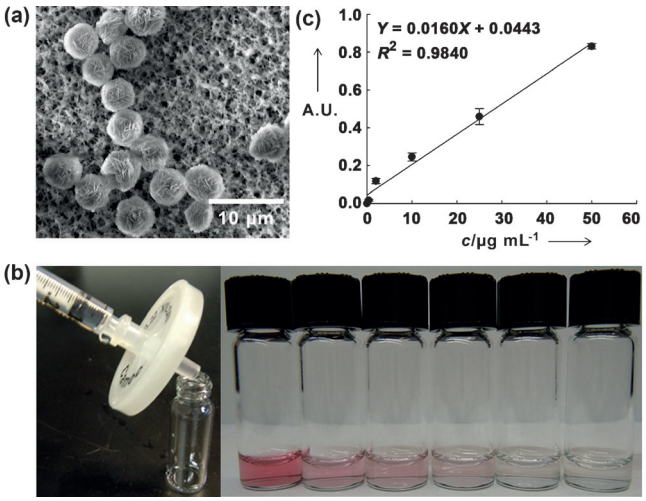

Figure 3. a) SEM images of laccase–copper phosphate nanoflowers on a filter membrane. b) Photos showing the analysis of phenols in water by the nanoflower-coated filter membrane and visual comparison of the nanoflower-catalyzed detection of phenol in aqueous solution (50 mM, pH 6.0 phosphate buffer) with different concentrations (from left to right: 50, 25, 10, 2, 0.4, and 0 μg mL^{-1}). c) UV/Vis absorption at 495 nm of the nanoflower-catalyzed detection of phenol in aqueous solution (50 mM, pH 6.0 phosphate buffer) as a function of concentration.

To test the reusability of the filter with the deposited nanoflowers and the reproducibility of the detection, a water solution with 50 μg mL^{-1} phenol was used as the sample. Each time after analysis the filter was washed with 1.5 mL pure water, exposed to air to dry, and then subjected to the next run. The above-mentioned analysis was conducted once a day for a period of one month. Figure 4 shows that the membrane filter treated with laccase nanoflowers gives reproducible absorption intensity at 495 nm within the 30 cycles conducted consecutively in one month. In contrast, the absorption intensity obtained with membrane filter treated with laccase reduced continuously to less than 20% of its initial value within the first five days. The well preserved analytical ability of the filter is a result of the high stability of laccase nanoflower on the membrane at room

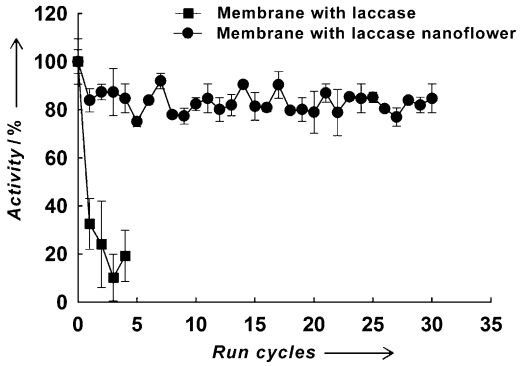

Figure 4. Reusability of the laccase-nanoflower-coated filter membrane in the analysis of phenol in water. In the period of one month, one cycle of use was performed every day.

temperature, which is consistent with the high stability of laccase nanoflowers in aqueous solution.[2]

In summary, a new and rapid detection of aqueous phenol solutions was demonstrated using enzyme–inorganic hybrid nanoflowers of laccase as a catalyst that generates visible products convenient for either quantitative measurement or direct observation. The deposition of the enzyme nanoflower onto a membrane presented a simple but effective way to accommodate enzyme nanoflowers for repeated measurements. Moreover, the thin and porous membrane with a high concentration of active enzyme provides a concentrated signaling interface and thus favors high sensitivity. The strengthened stability of laccase in nanoflowers, in comparison to its native counterpart in free form, ensures high reproducibility. All these factors cause the laccase-nanoflower-coated membrane filter to be able to achieve rapid, on-site detection of phenols.

Experimental Section

Laccase from *Trametes versicolor* and copper (II) sulfate pentahydrate were purchased from Sigma–Aldrich and used as received. Phosphate buffered saline (PBS, 1X, pH 7.4) was from Invitrogen. For the preparation of laccase nanoflowers, 0.8 mM $CuSO_4$ water solution was added to phosphate buffered saline (PBS) containing 0.1 mg mL^{-1} laccase at pH 7.4 and 25 °C. After three days, the laccase nanoflowers were obtained as precipitates. The precipitates were dried and subjected to SEM and XRD analysis. The suspension of the nanoflowers (0.06 mg mL^{-1} protein, 0.2 mL) in PBS (1X, pH 7.4) was injected into a syringe filter (Whatman Puradisc 30, diameter 30 mm, pore size 0.2 μm). Subsequently, the filter was dried at room temperature. In a typical analysis experiment, aqueous phenol (1 mL in 50 mM pH 6.0 phosphate buffer) was mixed with aqueous 4-aminoantipyrine (1 mL, 1 mg mL^{-1}). Then the mixture (300 μL) was injected into the nanoflower-coated filter and kept in the filter chamber for five minutes. Subsequently, air (1 mL) was injected into the filter to push out the solution, the color of which is detectable by the naked eye or by use of a UV/Vis spectrophotometer (Shimadzu UV2550) at 495 nm. After analysis, the membrane was rinsed in deionized water (1.5 mL) to wash the filter. Then, the filter was dried and stored at room temperature for the next cycle of analysis.

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Keywords: analytical methods · enzyme immobilization · enzyme catalysis · nanostructures

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