

Immobilization and Characterization of Pectinase onto the Cationic Polystyrene Resin

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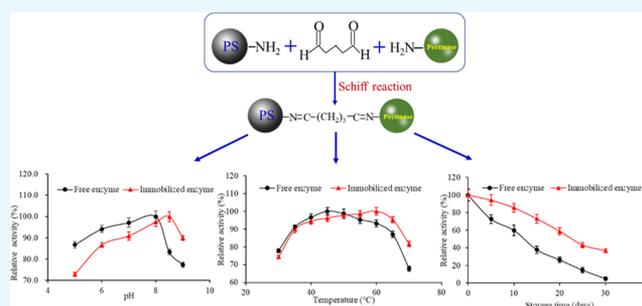
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ABSTRACT: In the present study, the immobilization of free pectinase onto polystyrene resin beads via crosslinking with glutaraldehyde was investigated. The immobilized pectinase was characterized by Fourier transform infrared spectroscopy and confocal laser scanning microscopy. After optimizing the immobilization conditions, the optimum pH of immobilized pectinase shifted from 8.0 to 8.5 and the optimum temperature shifted from 45 to 60 °C, showing its improved stability to temperature and pH compared with the free pectinase. The Michaelis–Menten constant K_m value of free and immobilized pectinase was determined to be 1.95 and 5.36 mM, respectively. The storage stability of immobilized pectinase was demonstrated with 36.8% of the initial activity preserved after 30 days at 25 °C. The reusability of the immobilized pectinase activity was 54.6% of its initial activity after being recycled six times. Therefore, based on the findings mentioned above, it can be inferred that this simple immobilization technique for pectinase appears to be promising for industrial applications.



1. INTRODUCTION

Nowadays, enzymes as green biocatalysts have been widely utilized in different fields such as biomedical production, food chemistry, textiles, wastewater treatment, and pulping and papermaking due to their higher selectivity to substrates, higher catalytic efficiency, and mild reaction conditions.¹ Pectinase, which possesses the ability to catalyze the breakdown of pectic substrates, has been widely applied in some industrial processes, such as plant fiber processing, coffee fermentation, and treatment of industrial wastewater.² In practical applications, usually the enzymes are used in a free form. However, the soluble enzymes have some adverse properties of lower stability, lower activity, lower resistance, and non-reusability or continuous use.³ Improved reusability and storage/operational stability mean reduced cost because most enzymes have a higher price. The defects of free enzymes mentioned above restrict their applications in industrial processes.

In order to overcome the drawbacks of free enzymes, some procedures such as immobilization, chemical modification, and protein engineering have been proposed on both laboratory and industrial scales.⁴ The immobilization technique can overcome most defects of free enzymes, which may offer improved recovery of enzymes for reuse and higher stability, activity, selectivity, and resistance against inhibition.^{2,5} Also, the immobilized enzyme can minimize or eliminate protein

contamination of the resultant product.⁶ There are three typical methods for immobilizing enzymes: binding to a prefabricated support, entrapment in organic or inorganic polymer beads, and cross-linking of enzymes.⁶ The sole and direct immobilization of enzymes without cross-linkers on supports might lead to the loss of enzyme activity due to the lower mechanical stability.⁷ Therefore, cross-linking the free enzyme on a support is adopted for improving the mechanical stability and activity of immobilized enzymes. The cross-linking of soluble enzymes primarily employs the reaction of free amine groups (–NH₂) on the enzyme molecular chain with a bifunctional cross-linker.⁷ Glutaraldehyde (GA) has bifunctional cross-linking aldehyde groups, and it is generally used as the cross-linking agent as it is inexpensive and readily available in commercial quantities. The reaction between free amine groups and aldehyde groups can form a Schiff base.⁸

It should be noted that different supports, for instance, Eupergit C,⁹ diethylaminoethyl (DEAE) cellulose,¹⁰ silica gel,¹¹ molecular sieves,¹² poly(methyl methacrylate)

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(PMMA) nanofibers,¹³ nano-Fe₃O₄ particles,¹⁴ Fe₃O₄ nanoparticles–PMMA films,¹⁵ ultra-thin 2D CuO nanosheets,¹⁶ calcium alginate-coated polypyrrole/silver nanocomposites,¹⁷ and hydroxyapatite-decorated ZrO₂,¹⁸ have various influences on the properties of different immobilized enzymes. The polystyrene (PS) resin, which has the distinct advantages of high strength, high specific surface area, high stability against heat and chemicals, low cost, and easy functional modification, has been successfully utilized as a solid support in enzyme immobilization with a high retention of activity.^{19–21} The enzyme can be immobilized on the cationic PS resin with amine groups using GA as the crosslinker through the Schiff reaction. Due to the less content of free –NH₂ groups on the traditional cationic PS resin, the amount of immobilized enzyme was very lower. In the present work, the PS resin grafted with poly(methyl acryloyloxyethyl trimethylammonium) chloride via atom transfer radical polymerization (ATRP) was employed as the support to immobilize the free pectinase. The ATRP reaction can provide more amine groups on the PS resin and thus more cross-linking sites for GA. As a result, the PS resin with a large particle size and high strength was loaded with more pectinase molecules, and it can be utilized as a column packing material in a continuous flow system for separation or purification operations.

2. RESULTS AND DISCUSSION

2.1. Optimization of the Pectinase Immobilization Procedure.

In the present study, the effect of GA concentration, immobilization time, and immobilization temperature on the immobilization efficiency was investigated based on the activity of the immobilized enzyme. As shown in Figure 1, the GA concentration in the immobilization process

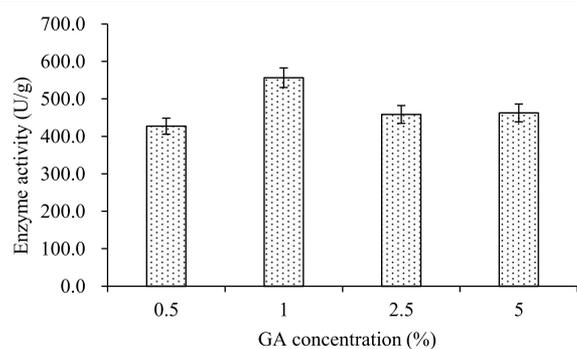


Figure 1. Effect of GA concentration on the activity of immobilized pectinase (the reaction between GA and cationic PS resin at 30 °C for 3 h, and the reaction between GA and the enzyme at 45 °C for 2 h).

had a great impact on the activity of the immobilized enzyme. The optimum GA concentration was found to be 1.0% under the current conditions in terms of the enzyme activity. The lower enzyme activity at a lower concentration (0.5%) was mainly due to the fewer bonds between GA and the cationic PS resin and subsequently between GA and the enzyme. However, the higher GA concentrations led to the decrease of the enzyme activity. As is known to all, the formation of Schiff bases between GA and the enzyme is primarily responsible for the immobilization behavior.⁸ A study had found that higher GA concentrations would result in the introduction of pendant-GA-related molecules rather than crosslinks, which led to the lower enzymatic activity.⁸ The electrostatic bonds between the amine groups of cationic PS and the carboxyl

groups of the enzyme were also formed during the simple adsorption besides the bonds between GA and the enzyme.²² These enzymes via electrostatic bonds might be covered by the excessive GA molecules causing the decrease of the enzyme activity.

Figure 2 shows that the highest value of enzyme activity was obtained at the immobilization time of 2 h under the present

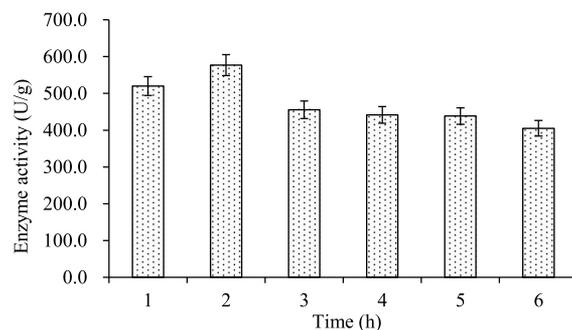


Figure 2. Effect of the immobilization time on the activity of immobilized pectinase (the reaction between GA and the enzyme at 45 °C).

conditions. The enzyme activity was lower at a shorter contact time (1 h), induced by the partial release of the immobilized enzyme into the solution. At longer contact times (>2 h), the decrease of the enzyme activity was mainly caused by the enzymatic deactivation due to the conformational and surface modifications resulting from GA.^{8,22}

It can be found from Figure 3 that the enzyme activity increased with increasing immobilization temperature within

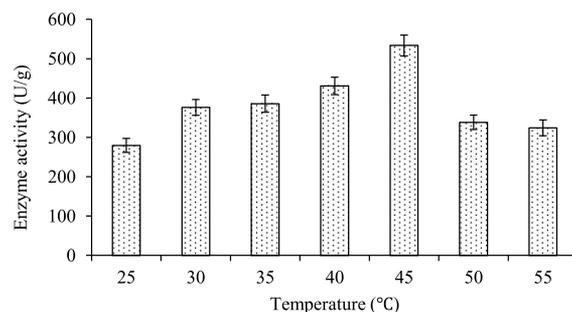


Figure 3. Effect of the immobilization temperature on the activity of immobilized pectinase (the reaction for 2 h between GA and the enzyme).

25–45 °C. A further increase of the temperature above 45 °C resulted in the decline in the immobilized pectinase activity. The lower enzyme activity was mainly induced by the inactivity of enzyme molecules at lower temperatures. However, the higher temperature above 45 °C would lead to the deactivation of the free enzyme resulting in the lower activity of immobilized pectinase. Consequently, 45 °C was chosen as the optimal temperature for immobilizing pectinase onto the cationic PS resin within the tested conditions.

2.2. Characterization of Free and Immobilized Pectinase. **2.2.1. Fourier Transform Infrared Analysis of the Cationic PS Resin and Free and Immobilized Pectinase.** The Fourier transform infrared (FTIR) spectra of the cationic PS resin, free pectinase, and immobilized pectinase are shown in Figure 4. Significant structural changes were observed from

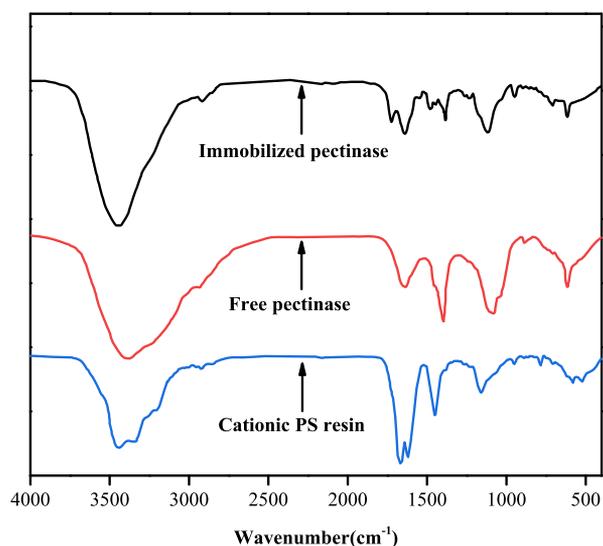


Figure 4. FTIR spectrum of the cationic PS resin and free and immobilized pectinase.

the FTIR spectra. In the FTIR spectrum of the cationic PS resin, the peak around 1603 cm^{-1} , the peak at 1668 cm^{-1} , and the peak at 1152 cm^{-1} were, respectively, ascribed to the aromatic C–C stretching vibration band, the O=C–O ester bond stretching vibration band, and the C–N stretching vibration band. The main component of an enzyme is an amino acid, and the characteristic groups of pectinase are $-\text{NH}_2$ and $-\text{COOH}$. An amine N–H stretching peak was found around 3500 cm^{-1} . The $-\text{OH}$ and $-\text{C}=\text{O}$ stretching vibration bands of carboxyl groups were found around 2900 and 1637 cm^{-1} , respectively. Schiff bases with the C=N bond can be formed by the cross-linking reaction between GA and the enzyme or cationic PS resin with amine groups.⁸ The stretching vibration band of C=N at 1647 cm^{-1} was found in the FTIR spectrum of immobilized pectinase. The peaks of C–N and N–H in addition to C=N were also found in the spectrum of the immobilized enzyme. The conclusion can be drawn from the FTIR analysis that the free pectinase was successfully immobilized onto the cationic PS resin.

2.2.2. Confocal Laser Scanning Microscopy Analysis. The thiocarbamide of isothiocyanate can react with the amine group of the enzyme protein forming a fluorescent complex. As shown in Figure 5, the green fluorescence around the PS resin microsphere surface was distinctly found after fluorescence staining with isothiocyanate. This illustrates that the pectinase was successfully immobilized onto the surface of the PS resin.

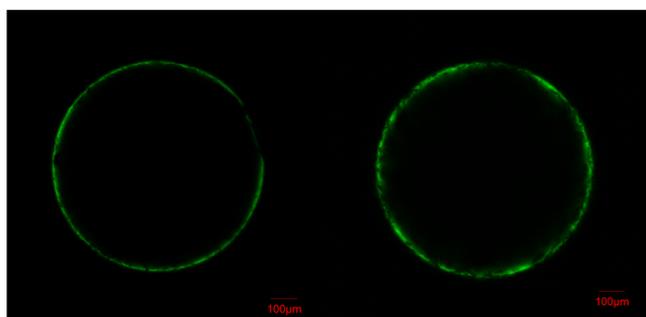


Figure 5. Confocal laser scanning microscopy (CLSM) images of immobilized pectinase.

Furthermore, it can also be found that the pectinase was uniformly distributed on the surface of the PS resin through the fluorescence intensity.

2.2.3. Effect of pH on Free and Immobilized Pectinase. The effect of pH on the relative activity of free and immobilized enzymes was assayed in the range of pH from 5.0 to 9.0. As shown in Figure 6, it was found that the optimum

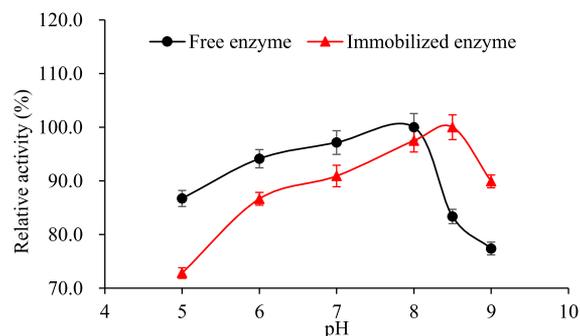


Figure 6. Effect of pH on the relative activity of free and immobilized pectinase.

pH for free pectinase was 8.0. However, the cross-linked pectinase showed an optimum pH of 8.5. Afterward, the relative activity of both pectinases showed a gradual decreasing trend. The relative activity of the immobilized enzyme was also higher than that of the free enzyme at higher pH values, suggesting that the immobilized enzyme has good alkaline resistance. The slight change in the optimum pH of the immobilized enzyme through cross-linking has been also observed.^{22,23} The results may be attributed to the micro-environmental phenomenon that the pectinase was bound to a polycation support.²⁴

2.2.4. Effect of Temperature on Free and Immobilized Pectinase. As shown in Figure 7, the optimum temperature for

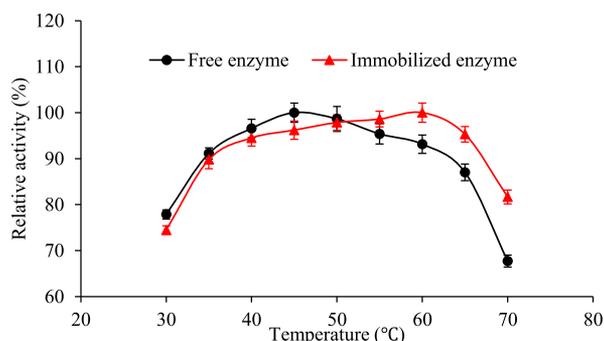


Figure 7. Effect of temperature on the relative activity of free and immobilized pectinase.

free pectinase and immobilized pectinase was 45 and $60\text{ }^{\circ}\text{C}$, respectively. The increased temperature generally induces an increased rate of enzymatic reactions. However, when the temperature exceeds a certain value, the protein would denature and subsequently decrease the reaction rate and the enzyme activity. These results suggest that the immobilized pectinase had improved activity at higher temperatures. This finding is considerably significant because it implies that the immobilized enzyme can be utilized under high-temperature conditions.

2.2.5. Calculation of Kinetic Parameters. The kinetic parameters of free and immobilized pectinase were calculated from the Michaelis–Menten equation. The Michaelis–Menten constant K_m usually reflects the effective characteristics of the enzyme and is affected by both the partitioning and diffusional effects.^{24,25} In the current investigation, it is found that the K_m value of the immobilized enzyme increased from 1.95 to 5.36 mM in respect of the free form. Similar results were obtained in the study by Lei and Bi, in which the pectinase was successfully immobilized onto the amphiphilic block copolymer poly(styrene-*b*-acrylic acid).²⁴ The increased K_m value indicates that the affinity of immobilized pectinase for its substrate was lower than that of free pectinase. This may be induced by the substrate diffusional limits, steric hindrance of the active sites by the support and GA, or the loss of pectinase flexibility necessary for substrate binding.^{26,27}

2.3. Storage Stability and Reusability of Immobilized Pectinase. Storage stability is very important for enzymes in practical applications. The enzyme is usually not stable when it is stored in a free form which is expressed as the decrease of the retained activity. As shown in Figure 8, the relative enzyme

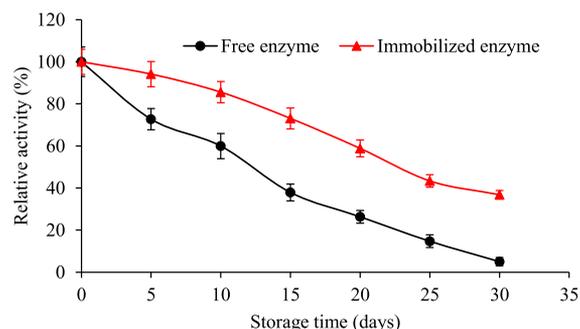


Figure 8. Storage stability of free and immobilized pectinase.

activity of both free and immobilized enzymes decreased with the increase of storage time. The relative activity of the free enzyme reduced more than that of the immobilized enzyme after 7 days of storage at 25 °C and optimal storage pH. The immobilized enzyme retained more than 70% activity compared to 37% of the free enzyme after 15 days of storage at 25 °C. The result suggests that the immobilization protocol used in the current study can improve the enzymatic endurance of high temperatures. Similar results were also observed by a previous study, in which the pectinase was immobilized onto an amphiphilic block copolymer poly(styrene-*b*-acrylic acid) and stored at 4 and 25 °C.²⁴ However, a lower enzymatic activity was observed at the high storage temperature of 25 °C in respect of the storage temperature of 4 °C.

As is well known, it is difficult for traditional free enzymes to carry out continuous operation in industrial practice. The reusability of the immobilized enzyme is very significant in the economic aspect and gives the immobilized enzyme more advantage than its free form. The residual activity of immobilized pectinase after seven cycles is illustrated in Figure 9. It was observed that the immobilized enzyme retained more than 54% of the original activity after the sixth cycle. The decrease of the relative enzyme activity after recycling may be due to the direct weak interactions between the enzyme and the cationic PS resin through the adsorption mechanism, so that some of the adsorbed pectinase was released during the

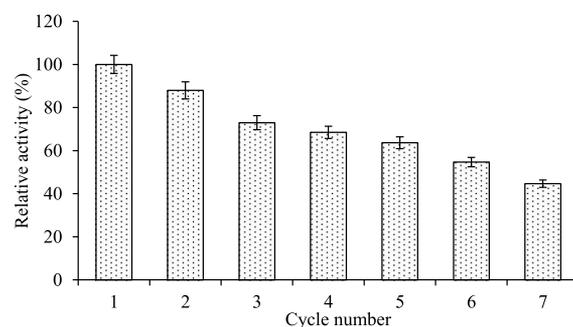


Figure 9. Reusability of immobilized pectinase.

washing procedure.^{28–30} Besides, the cross-linked pectinase may also be released during the washing procedure.

3. CONCLUSIONS

Immobilized enzymes have some advantages compared to free enzymes in industrial practice. The PS resin with high cationization was utilized as beads for immobilizing pectinase via cross-linking GA. The large amount of cationic amine groups within the PS resin provided more cross-linking sites and thereby led to an increase in the activity of immobilized pectinase. The immobilized pectinase was found to be more tolerant to conditions of a higher pH and temperature. An improvement of the storage stability and reusability was found compared to the free form. The results show the practical significance of using the immobilized pectinase due to the large particle size and high strength of the PS resin.

4. MATERIALS AND METHODS

4.1. Materials. Pectinase (liquid, activity of 55 U/mL) was provided by KDN Biotech Co., Ltd., Shanghai, China. The cationic PS resin (average particle size, about 300 μm; specific surface area, 16 m²/g; and content of free amine group, 2.7 mmol/g) was prepared in the laboratory through ATRP. Analytical grade GA (1.0 wt %), pectin, phenol, anhydrous sodium sulfate, potassium sodium tartrate, 3,5-dinitrosalicylic acid (DNS), and D-galacturonic acid were purchased from Innochem Co., Ltd., China.

4.2. Immobilization of Pectinase onto a Cationic PS Resin. 0.1 g of cationic PS resin was added to 10 mL of GA solution with different concentrations. The cross-linking reaction between the cationic PS resin and GA was continued for 3 h at the stirring rate of 120 rpm and 30 °C. Thereafter, the cationic PS resin cross-linked with GA was washed with ultrapure water to remove the excess GA. Immobilization of pectinase on the cationic PS resin was subsequently conducted as follows: 5 mL of free pectinase was mixed enough with the cationic PS resin loaded with GA, and the cross-linking reaction was continued for different times at different temperatures at a stirring rate of 120 rpm. Finally, the PS resin immobilized with pectinase was washed with ultrapure water and filtered to remove unbound pectinase.

4.3. Activity Assay for Free and Immobilized Pectinase. The activity of free/immobilized pectinase was determined by measuring the amount of D-galacturonic acid liberated from citrus pectin. 1 mL of free pectinase or 0.1 g of immobilized pectinase was added to the reaction mixture containing 5 mL 0.5% (w/v) pectin and 4 mL phosphate buffer, 0.2 mol/L with pH 8.0 or pH 8.5. The reaction mixture was incubated for 30 min under optimized conditions. One

unit (1 U) of enzyme activity is expressed as the amount of enzyme required to release 1 μmol of galacturonic acid per milliliter or per gram under the assay conditions, as quantified by the DNS method at 520 nm.^{31,32}

4.4. Optimization of the pH and Temperature of Free and Immobilized Pectinase. The activity was assayed according to Section 4.3 at an optimum temperature in the buffer with different pH values in order to optimize the pH. To optimize the temperature, the activity was assayed according to Section 4.3 at different temperatures in the buffer with an optimum pH value. The relative activity is defined as the ratio of the retained activity to the maximal activity of the enzyme.

4.5. Determination of Kinetic Parameters. Kinetic parameters of free and immobilized pectinase were determined through reaction with different concentrations of pectin (2.5–20 and 5–25 g/L) at optimum pH values and temperatures. The Michaelis–Menten constant (K_m) values of free and immobilized enzymes were calculated using the double reciprocal plot of Lineweaver–Burk plots according to the Michaelis–Menten equation.³³

4.6. Storage Stability of Free and Immobilized Pectinase. The storage stability of free and immobilized pectinase was investigated by determining the relative activity periodically. Both the enzyme preparations were stored at 25 °C for 30 days. The relative enzyme activity is expressed as the ratio of the retained activity with respect to the activity of the first day.

4.7. Reusability of Immobilized Pectinase. In order to assess the reusability, the immobilized pectinase was utilized to hydrolyze pectin in the buffer. The conditions used were the same as those used for the pectinase activity assay. The immobilized pectinase was washed with buffer and filtered after each cycle. The relative enzyme activity is expressed as the ratio of the retained activity after each cycle to the activity of the original immobilized enzyme.

4.8. Characterization of the Cationic PS Resin and Free and Immobilized Pectinase. The solution of free enzyme was first freeze-dried. All the samples of freeze-dried free enzyme, cationic PS resin, and immobilized enzyme were vacuum-dried at 40 °C for 24 h. Thereafter, the dried samples were each crushed with potassium bromide to obtain a fine powder. Then, the chemical characterization of all the samples was performed via FTIR spectroscopy using a Bruker VERTEX 80 spectrometer (Saarbrücken, Germany).

The PS resin immobilized with pectinase was first fluorescent stained with fluorescein isothiocyanate at 37 °C for 30 min. Then, the resin microspheres were washed with deionized water three times. Finally, the resin sample was suspended in 1 mL deionized water and used for CLSM observation. The excitation wavelength was 488 nm, and the wavelength range of received light was 500–600 nm.

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Notes

The authors declare no competing financial interest.

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