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Label-Free Photonic Crystal-Based β-lactamase Biosensor for β-lactam Antibiotic and β-lactamase Inhibitor

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ABSTRACT: A simple, label-free and visual photonic crystal-based β-lactamase biosensor was developed for β-lactam antibiotic and β-lactamase inhibitor in which the penicillinase (a β-lactamase) was immobilized on the pH-sensitive colloidal crystal hydrogel (CCH) film to form penicillinase colloidal crystal hydrogel (PCCH) biosensing film. The hydrolysis of penicillin G (a β-lactam antibiotic) can be catalyzed by penicillinase to produce penicilloic acid, leading to a pH decrease in the microenvironment of PCCH film, which causes the shrink of pH-sensitive CCH film and triggers a blue-shift of the diffraction wavelength. Upon the addition of β-lactamase inhibitor, the hydrolysis reaction is suppressed and no clear blue-shift is observed. The concentrations of β-lactam antibiotic and β-lactamase inhibitor can be sensitively evaluated by measuring the diffraction shifts. The minimum detectable concentrations for penicillin G and clavulanate potassium (a β-lactamase inhibitor) can reach 1 μM and 0.1 μM, respectively. Furthermore, the proposed method is highly reversible and selective, and it allows determination of penicillin G in fish pond water samples.

The β-lactam antibiotics have been widely used to treat various bacterial infections due to their high effectiveness and relatively cheap price. However, the overuse of antibiotics (e.g. as an additive in livestock and fish feed) has resulted in the antibiotic residues in surface water, food, soil, etc., which brings a great threat to human health and the environment, such as, the evolution of antibiotic-resistant bacteria and disruption of the ecological balance.[1-3] Especially, antibiotic-resistant bacteria can be transferred to humans and cause the failure of clinical treatment. The mechanisms of the β-lactam antibiotic resistance are mainly due to the production of β-lactamases by bacteria.[4,5] The β-lactamases can catalyze the hydrolysis of the β-lactam ring in β-lactam antibiotics to form carboxylic acid, leading to the loss of antibacterial activity of antibiotics.[6] The use of the β-lactamase inhibitors is a common way to enhance the antibacterial activity of β-lactam antibiotics.[5,7]

Over the past decades, some approaches (like LC-MS, electrochemical, and fluorometric methods) have been reported for the determination of β-lactam antibiotics or β-lactamase inhibitors.[8-13] Although various approaches have achieved good results, they often need expensive equipment, trained personnel, and complex signal labels, which limit their application in a certain degree, particularly when the assay has to be performed at sites far away from central laboratories. Therefore, there is still an urgent demand to develop a simple, label-free, portable and cost-effective platform for the analysis of β-lactam antibiotics and β-lactamases inhibitors.

Photonic crystals can be regarded as the structures in which the materials with different dielectric constants have a periodic alignment.[14] The unique and periodic repeating structure leads to the formation of a photonic band gap that restrains the transmission of light with certain wavelength.[15] If the periodic structure is altered by external stimuli, the position of the maximum diffraction wavelength will shift. These distinctive optical properties of photonic crystals combined with appropriate responsive materials could make them ideal for the development of convenient, visual and label-free sensing platform. Recently, photonic crystals combined with responsive hydrogels have attracted more attention in the field of bio/chemical sensors.[16-31] These sensors were constructed by dispersing crystalline colloidal array in responsive hydrogel matrixes to form a kind of so-called colloidal crystal hydrogel (CCH). Environmental stimulus causes a change in the volume of hydrogel, which results in the diffraction wavelength shift or color change of CCH. Thus, it provides a generic sensing approach for the interesting targets by constructing a suitable responsive hydrogel.

Herein, we try to report a novel photonic crystal-based β-lactamase biosensor for the colorimetric detection of β-lactam antibiotic and β-lactamase inhibitor. The principles are illustrated in Figure 1. Penicillinase (a β-lactamase) as the specific recognition element is modified into the CCH film to form penicillinase colloidal crystal hydrogel (PCCH) biosensing film. When the PCCH film is immersed into the solution of penicillin G, the penicillinase catalyzes the hydrolysis of penicillin G to produce penicilloic acid, leading to a distinct decrease in the microenvironmental pH of the PCCH film, which increases the protonation degree of carboxyl-rich hydrogel and reduces the amount of charge in hydrogel. Based on the Donnan equilibrium, this enzyme-catalyzed event causes the shrink of hydrogel due to the decreasing osmotic pressure of PCCH film, which narrows the distance between neighboring particles in PCCH film, and thus the diffraction wavelength of PCCH film has a blue-shift.[16] Moreover, if the activity of penicillinase is inhibited by its inhibitors, the hydrolysis reaction will be suppressed and no clear blue-shift occurs. Therefore, the diffraction peak shift can be used as a quantitative signature for the detection of β-lactam antibiotics and β-lactamase inhibitors. More importantly, the target can be easily moni-

tored even by naked eye without any sophisticated instruments since the diffraction lights of PCCH biosensing films are in visible region. The convenient and intuitive features will benefit this photonic crystal biosensing method to be a promising candidate for on-site analysis of antibiotic contamination and screening of β-lactamase inhibitors.

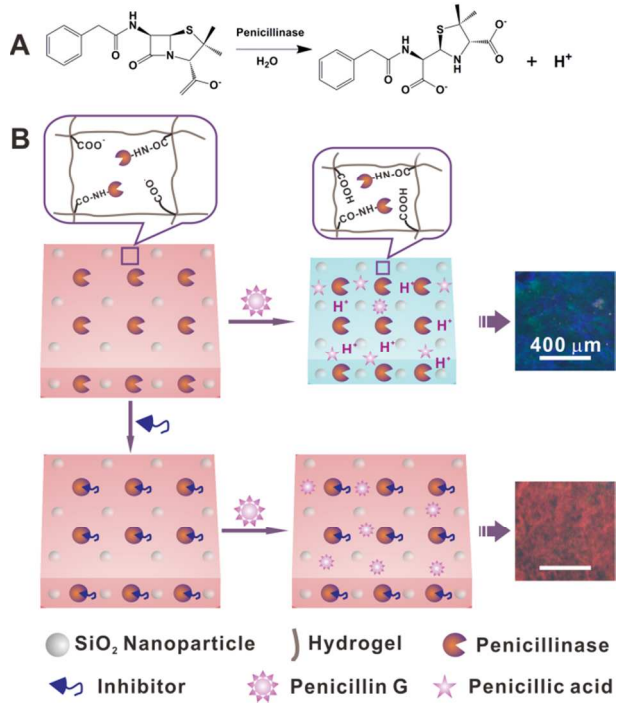

Figure 1. (A) Enzymatic reaction between penicillinase and penicillin G; (B) Schematic illustration of the PCCH sensor for the detection of β-lactam antibiotic and β-lactamase inhibitor.

Experimental Section

Chemicals and materials. Penicillinase from Bacillus cereus, *N,N′*-methylenebisacrylamide (BisAA), acrylamide monomer (AA), *N,N,N′,N′*-Tetramethylethylenediamine (TEMED), poly(ethylene glycol) diacrylate (PEGDA, average M_n=700), 2-hydroxy-2-methylpropiophenone (HMPP), *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC) and clavulanate potassium were procured from Sigma-Aldrich (St Louis, MO, USA). 2-(*N*-morpholino)ethanesulfonic acid (MES) was purchased from Alfa Aesar (MA, USA). Penicillin G, clindamycin hydrochloride, lincomycin hydrochloride, chloramphenicol and fluconazol were obtained from Aladdin (Shanghai, China), Cyanine 5 NHS ester (Cy5) was procured from Lumiprobe (Florida, USA). NaCl, NaOH, Na_2HPO_4 and NaH_2PO_4 were analytical grade. Ultrapure water (18.2 MΩ cm) with high temperature sterilization was used in all solutions.

Preparation of the CCH films. Monodisperse silica spheres (160 nm in diameter) were synthesized by the Stöber method.[32] The suspension of silica nanoparticles was purified by repeated centrifugation and dispersion. The purified particles were stored in ultrapure water and added BioRad ion-exchange resin (AG 501-X8(D)) to remove impurity ions.

The CCH was prepared by free-radical photopolymerization employing HMPP as a photoinitiator. The pre-polymerization solution was consisted of BisAA (0.3%, w/v), AA (10%, w/v), HMPP (1%, v/v), PEGDA (6%, v/v), and monodisperse silica spheres (55%, w/v). After mixed thoroughly, the solution with 20% ion exchange resin was shaken until its strong structural color presented. Then, the ion exchange resin was removed by centrifugation. The solution was injected into the home-made cell, which comprised of two microscope slides separated by the 125 μm parafilm spacers. The sample cell was irradiated under 365 nm UV light for 1 h to form a CCH film. The film was washed with ultrapure water for several times and stored in ultrapure water.

Preparation of PCCH biosensing films. The CCH film was first hydrolyzed for 3.5 h in a solution containing 10% (v/v) TEMED and 0.1 M NaOH. After this, a lot of carboxyl groups were formed in the matrix of CCH film. The hydrolyzed CCH film was subsequently activated by submersion in a 10 mM MES buffer solution (pH 6.0) with 0.1 M EDC and 0.2 M NHS for 40 min at ambient temperature. Then, the activated CCH was rinsed with 10 mM PBS (pH 7.0), and immersed in the penicillinase solution. After being incubated for 18 h at 4 ˚C, the PCCH biosensing films were obtained.

Preparation of CCH modified with Cy5-labeled penicillinase. Penicillinase was first dissolved in PBS buffer (pH 7.0) to obtain a 500 U/mL solution. Subsequently, 0.1 mg/mL of cyanine 5 NHS ester was added followed by shaking for 3 h at 20 ˚C and storing at 4 ˚C overnight. Then, the above solution was centrifuged and rinsed repeatedly in ultrafiltration tubes (3000 Da MWCO, Millipore Co.) to remove the unreacted cyanine 5 NHS ester until the filtrate had no UV-Vis absorption. After this, the Cy5-labeled penicillinase was then immobilized on CCH with the same aforementioned step.

Detection of penicillin G and clavulanate potassium. For penicillin G, the PCCH film was incubated with different concentrations of penicillin G solution containing 0.1 M NaCl at ambient temperature for 30 min. For clavulanate potassium, the PCCH film was first treated with different concentrations of clavulanate potassium at ambient temperature for 30 min. Then, 5 mM penicillin G was added and incubated at ambient temperature for 30 min. The diffraction spectra of the PCCH films were obtained at room temperature.

Measurements. Diffraction spectra were measured at 90˚ angle by using a fiber spectrometer (NOVA, Ideaoptics) mounted on a microscope (ECLIPSE 50iPOL, Nikon). Optical images were obtained by the microscope (BX51, Olympus) equipped with a digital camera (DP27, Olympus). The UV-Vis spectra were measured by a UV-Vis spectrophotometer (UV-2450, Shimadzu). The fluorescent images of hydrogel were captured by a fluorescence microscope (Eclipse Ti, Nikon) with excitation wavelength at 640 nm. The micro-topography of CCH film was characterized by a field-emission scanning electron microscope (FESEM, Carl-zeiss Sigma HD).

Results and Discussions

The critical step for the construction of the colorimetric photonic crystal biosensor is the preparation of CCH film. To fabricate the CCH film with high-quality, the monodispersed silica spheres with silanol groups (160 nm in diameter) synthesized by Stöber method were well dispersed in the pre-gel solution with a certain concentration.[32] The ionization of silanol groups makes the silica spheres to be negatively charged. After an ion exchange treatment, the impurity ions in the pre-gel were removed, which eliminated the interference of impu-

rity ions on the electric repulsion force between silica spheres. The silica spheres were self-assembled into a highly ordered structure in solution due to the minimum energy configuration, [33,34] and the bright structural color generated. In order to fix the ordered structure, the pre-gel solution was polymerized into a hydrogel film in the home-made cell under UV irradiation. The structure of the CCH film was investigated by scanning electron microscopy (SEM) after vacuum freeze-drying. As seen in Figure 2, The CCH film not only has a periodic structure consisting of silica spheres and hydrogel in the same layer, but also has a good order between layers as suggested by the cross-section of SEM.

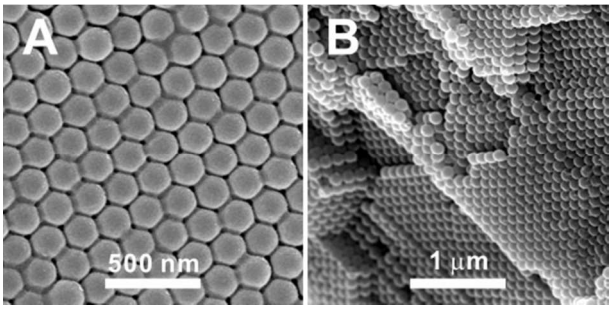

Figure 2. SEM images of (A) the surface of CCH film, and (B) the cross section of CCH film.

The CCH film was then functionalized with penicillinase for specific recognition of penicillin G. The experimental details were presented in the Experimental Section. In brief, the CCH film was first hydrolyzed with NaOH/TEMED solution to transform the amide groups into carboxyl groups. The hydrolysis of CCH film caused an obvious swelling behavior with the charge of carboxyl groups increased the osmotic pressure of the hydrogel. Following this step, the carboxyl groups on hydrolyzed CCH film were activated by EDC/NHS, and the penicillinase molecules were subsequently bonded to the hydrogel matrix through amide covalent bonds between the carboxyl groups in the CCH film and the amine groups in the penicillinase molecules. To confirm the immobilization of penicillinase molecules on the CCH, the penicillinase molecules were labeled with Cy5 fluorescent dye via the ligation reaction between Cy5-NHS and penicillinase, and the UV-Vis spectroscopy proved the conjugation of Cy5 on penicillinase was successful since the Cy5-NHS in PBS presented the main absorption peak at 640 nm and the peak shifted slightly to 643 nm upon conjugation with penicillinase (Figure S1, Supporting Information). After these treatments, the CCH film modified with the Cy5-penicillinase molecules was observed under fluorescence microscopy. A uniform red color micrograph was clearly observed (Figure S2, Supporting Information), indicating that the penicillinase molecules had indeed been bonded to the hydrogel successfully.

To verify the feasibility of this biosensing method, the responses of the PCCH film and activated CCH film to penicillin G were investigated. It can be seen from Figure 3, an obvious blue-shift is found from the PCCH film upon adding penicillin G, whereas there is no significant shift in the absence of penicillin G. In addition, no clear shift is seen with or without penicillin G for control experiments with activated CCH film. These results suggest that the PCCH film can act as a sensing platform for penicillin G detection.

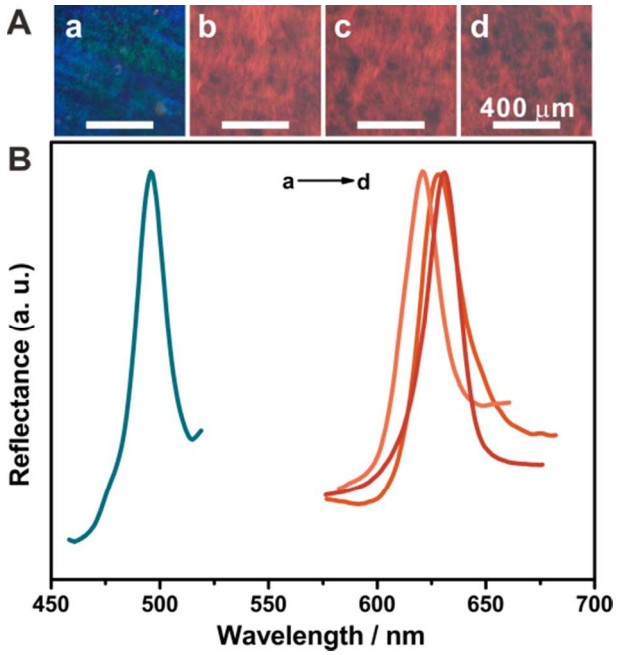

Figure 3. (A) Optical images of (a) PCCH film in the presence of 5 mM penicillin G, (b) PCCH film in the absence of penicillin G, (c) the activated CCH film in the presence of 5 mM penicillin G, and (d) the activated CCH film in the absence of penicillin G. (all solutions contain 0.1 M NaCl). (B) The corresponding diffraction spectra of the above images.

The amount of penicillinase immobilized on CCH is an important factor for obtaining a sensitive PCCH biosensor due to its influence on the microenvironmental pH of PCCH film. To study the effect of the penicillinase concentration, the activated CCH films treated with different concentrations of penicillinase were immersed in 5 mM penicillin G solution. It can be found that the diffraction shift of PCCH film increases gradually with increasing concentrations of penicillinase from 10 U/mL to 500 U/mL, and higher concentrations of penicillinase cause very little change (Figure S3, Supporting Information). The observation indicates that the response signal of PCCH films increases with the penicillinase concentration, and levels off at 500 U/mL. In addition, the incubation time for hydrolysis reaction between penicillinase and penicillin G was also investigated to obtain a quick and accurate result in practical applications. The result shows that the diffraction shift changes gradually from 44.4 ± 2.4 nm to 120.4 ± 1.7 nm with the increase of incubation time from 3 min to 30 min and reaches a plateau after 30 min (Figure S4, Supporting Information). This phenomenon reveals that the response of PCCH film tends to balance after incubating 30 min.

The capability of PCCH biosensing film for penicillin G detection was evaluated. As shown in Figure 4A and B, the PCCH film has a diffraction peak at 623.1 nm and appears an obvious red color in the absence of penicillin G. The diffraction peak gradually shifts to a shorter wavelength along with the increase of penicillin G concentration. Figure 4C further reflects the relationship between the diffraction shifts and different penicillin G concentrations. It can be found that the higher concentration of penicillin G induces the larger diffraction shift and the diffraction shift tends to be stable at concentration higher than 5 mM. The PCCH biosensing film shows a relatively wide detection range from 1 μM to 5 mM and the minimum detectable concentration for penicillin G is 1 μM,

which was comparable to those previously reported pH-sensing assays for β-lactam antibiotic.[35,36] In addition, the diffraction wavelengths of PCCH films are in the visible range throughout the process and the optical signal can be observed by naked eyes, which makes the detection of penicillin G more convenient and intuitive.

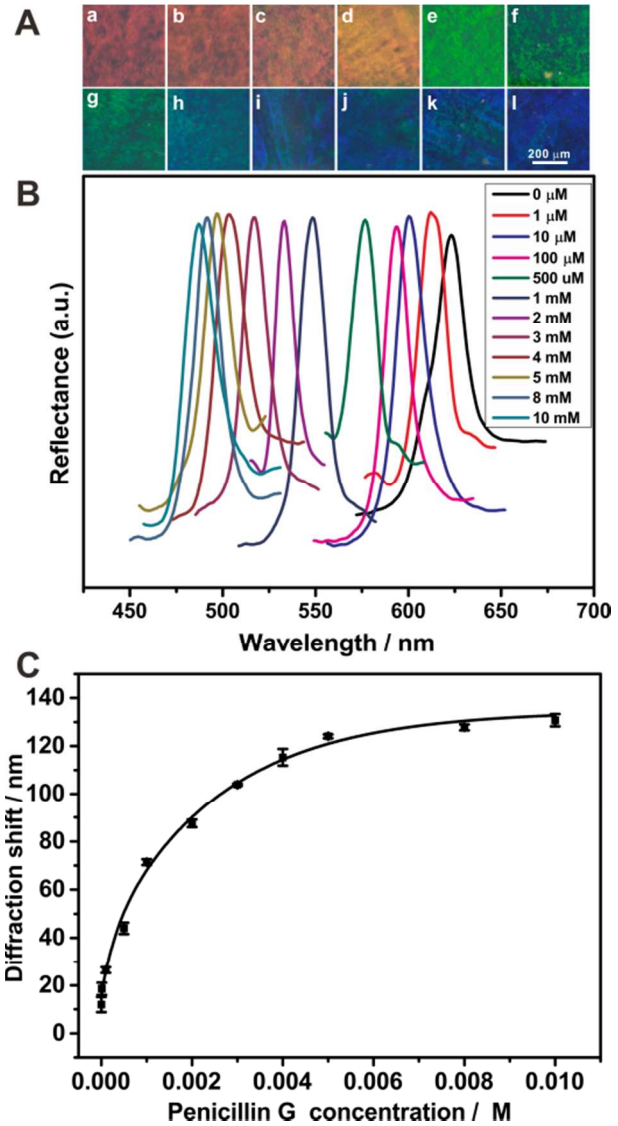

Figure 4. (A) Optical images of PCCH films at different concentrations of penicillin G (a-l: the concentrations of penicillin G were 0 μM, 1 μM, 10 μM, 100 μM, 500 μM, 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, 8 mM, and 10 mM, respectively). (B) Diffraction spectra of PCCH at different concentrations of penicillin G. (C) The corresponding calibration curve of diffraction shifts versus different concentrations of penicillin G. All solutions contain 0.1 M NaCl. The error bars indicate the standard deviation of three assays.

The PCCH biosensing film can also be applied to analyze β-lactamase inhibitors since the hydrolysis reaction between penicillinase and penicillin G can be suppressed by β-lactamase inhibitors. Clavulanate potassium, a representative inhibitor of β-lactamase, was selected to show this application. The diffraction shifts of PCCH films pretreated with different concentrations of clavulanate potassium were recorded after incubated with 5 mM penicillin G. As shown in Figure 5, the diffraction shifts decrease with increasing concentrations of clavulanate potassium, and the minimum detectable concentration of clavulanate potassium reaches 0.1 μM. The results demonstrate that the proposed PCCH biosensing film can serve as a simple tool for the primary screening of β-lactamase inhibitors.

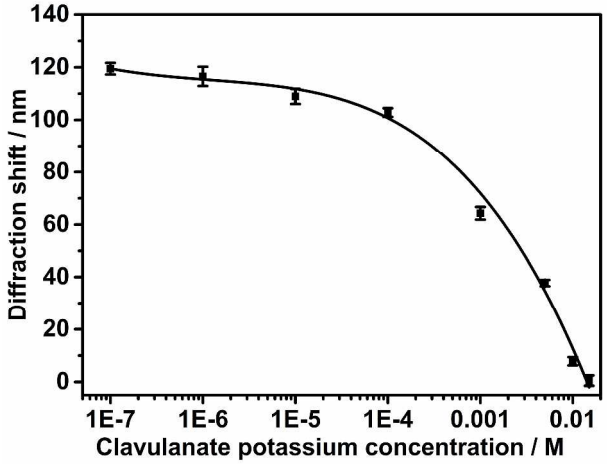

Figure 5. Diffraction shifts of PCCH films pretreated with different concentrations of clavulanate potassium in the presence of 5 mM penicillin G. All solutions contain 0.1 M NaCl. The error bars indicate the standard deviation of three assays.

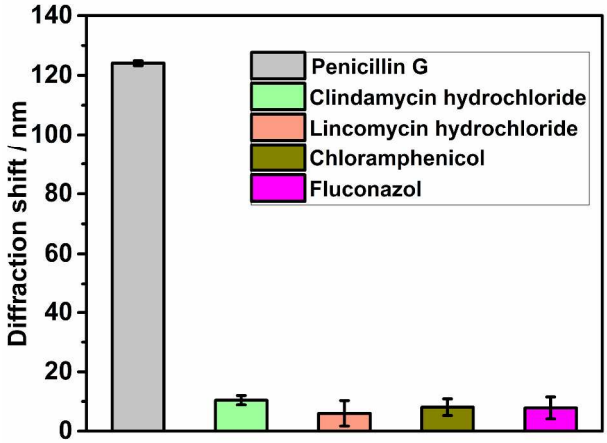

Figure 6. Selectivity of the PCCH system for penicillin G detection (the concentrations of all antibiotics were 5 mM). All solutions contain 0.1 M NaCl. The error bars indicate the standard deviation of three assays.

The specificity of the PCCH biosensing film for β-lactam antibiotic analysis was verified by some randomly selected non-β-lactam antibiotics, including clindamycin hydrochloride, lincomycin hydrochloride, chloramphenicol, and fluconazole. The results show that these non-β-lactam antibiotics do not produce significant diffraction shifts (Figure 6), showing the high selectivity of PCCH film for recognition of β-lactam antibiotics.

The reversibility of the PCCH sensor for penicillin G analysis was investigated by immersing the PCCH in 3 mM penicillin G solution and washing with 0.1 M NaCl solution repeatedly. After being washed with 0.1 M NaCl solution, the PCCH film can recover its initial microenvironment and the diffraction wavelength of PCCH film return to the original position. The reversible changes in the diffraction peaks show that the PCCH biosensor possesses good reversibility for penicillin G detection during five cycles (Figure 7), which indicates that

the PCCH film holds great potential in practical application due to its good stability and reversibility.

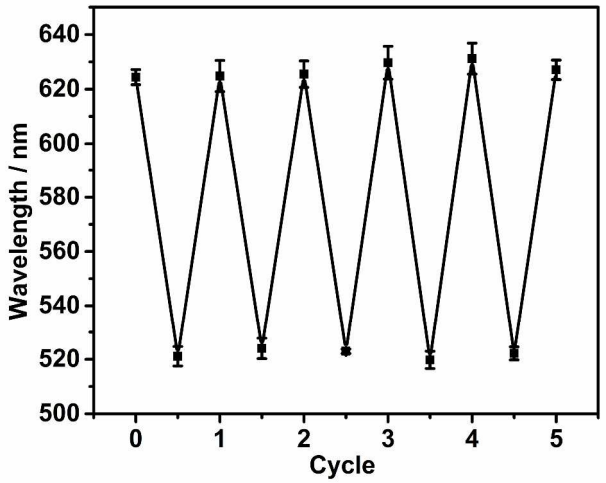

Figure 7. Reversibility of the PCCH film to 3 mM penicillin G solution. The error bars indicate the standard deviation of three assays.

To evaluate the capability of the PCCH biosensing film for the analysis of penicillin G in environmental water samples, the diffraction wavelength shifts of PCCH films were investigated by spiking different concentrations of penicillin G in fish pond water samples. The water samples were first filtered to remove sediments with 0.1 M NaCl added to control the ionic strength before detection. The results were basically consistent with those detected in ultrapure water (Figure 8), suggesting that such a device can be employed to monitor the β-lactam antibiotics contamination in real water samples.

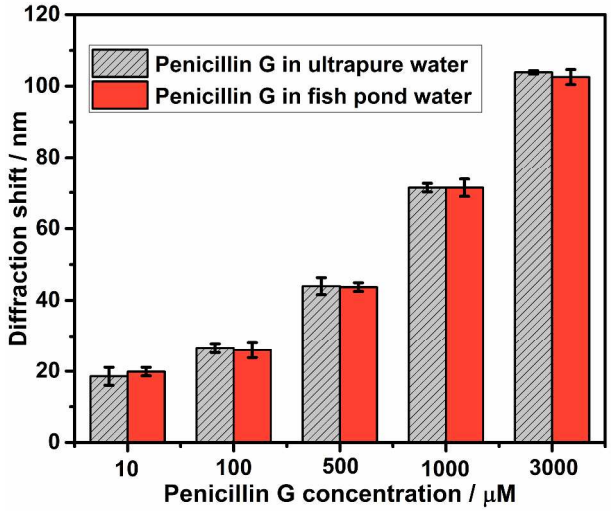

Figure 8. Capability of the PCCH film for the analysis of penicillin G in environmental water samples. The error bars indicate the standard deviation of three assays.

Conclusions

In conclusion, we have developed a novel photonic crystal-based β-lactamase biosensor for the detection of β-lactam antibiotic and β-lactamase inhibitor utilizing the pH-sensitive PCCH film responding to the pH change caused by the hydrolysis reaction between β-lactamase and β-lactam antibiotic. Compared with other β-lactamase biosensors, this sensor can directly employ the diffraction wavelength shift or the color change of the PCCH film as output signal, which greatly simplifies the operation of the detection process. Moreover, the good reversibility and selectivity of the PCCH film provide a stable and reliable result for practical application. The proposed photonic crystal biosensor demonstrates a simple, visual and label-free sensing platform for the analysis of β-lactam antibiotics and β-lactamase inhibitors, and it may become a potential alternative technique for point-of-care testing in resource-limited settings.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.
Additional four figures as noted in the text.

AUTHOR INFORMATION

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Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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