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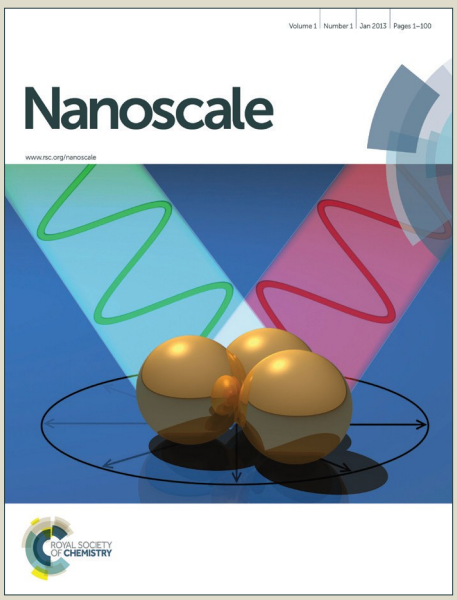

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Label-free and pH-sensitive colorimetric material for the sensing of urea

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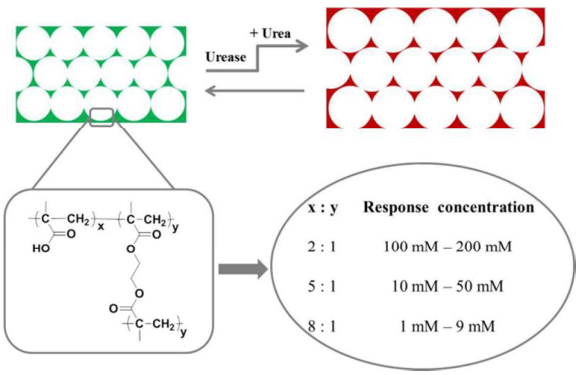
This communication demonstrates a facile method for naked-eye detection of urea based on the structure color change of pH-sensitive photonic crystal. The insertion of urease provides excellent selectivity over other molecules. The detection of urea in different concentration ranges could be realized by changing the molar ratio between the functional monomer and cross-linker.

Urea as a fertilizer of plants and a metabolic product of animals is a very important molecule in biological systems.[1-2] The majority of annual world production of urea is used as fertilizer. Nevertheless, excessive nitrogen fertilizer applications can lead to pest problems by increasing the birth rate, longevity and overall fitness of certain pests.[3] Urea is also used as an animal-feed supplement and has many industrial applications, including the manufacture of glues, resins, solvents and some medicines.[4] In consumer products, urea is found in many detergents, liquid soaps and other cleaning products, and has been extensively used in the treatment of dry skin, both clinically and in cosmetics.[5] Moreover, urea plays a strategic role in the marine nitrogen cycle, including sources of excretion by fish, invertebrates, bacterial decomposition of nitrogenous material and terrestrial drainage.[6-7] Although urea has generally low ecotoxicity to organisms, the indirect, long term consequences of exposure to excessive levels of urea on ecosystems were well documented, such as soil acidification, eutrophication, groundwater pollution and ammonia emissions to air.[4] So the determination of urea is important in a wide range of fields, including clinical diagnostics, environmental monitoring and food science. Many methods are available for urea estimation, such as chromatography[8], near-infrared Raman spectroscopy[9], flow injection analysis[10], nuclear magnetic resonance[11] and electrochemical methods[12-15]. However, these methods, although being precise, are time consuming and mostly laboratory bound. Meanwhile, they suffer from complicated sample pretreatment and are unsuitable for on-site monitoring. Moreover, the use of sophisticated analytical instrumentation is inevitable. Hence, there is an increasing demand for a sensor which has the ability to furnish real time signals in a simple way.



Scheme 1: Schematic illustrations of the detection of urea based on pH-sensitive inverse opal photonic crystal polymer film.

Colloidal photonic crystals have been widely used to develop sensitive polymers with highly ordered structure.[16] Owing to the ordered porous structure, the polymers can generate visual optical signals through Bragg diffraction (bright structure-based colors), i.e. for naked-eye detection. When the polymers suffer from external stimuli, the Bragg diffraction will shift due to a change of the periodic lattice spacing and the refractive index, which can be judged by a visually perceptible change in color. Generally, the polymers can easily achieve a rapid and direct assay by responding to various external stimuli, such as solvent composition, temperature, pH, pressure, electric field and biological molecules.[17-25] In particular, stimuli-responsive inverse opal photonic crystal polymer (IOPP) films as colorimetric materials, allowing facile and smart sensing, have been used for molecules recognition,

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such as glucose[26], stimulant[27-28]and NH_3-HCl[29]. These sensors were based on the molecular imprinting technique or special chemical reactions, which makes them difficult to be widely used due to their self-limitations. These methods showed poor selectivity when they were used in small molecules recognition. The reason is believed to be that the small molecules lack the necessary chemical functional groups and the intermolecular forces such as hydrogen bonding, Van der Waals' force and space steric hindrance are not strong enough when they interact with the corresponding functional monomers. The functional monomers also require complex organic syntheses in some cases. Moreover, these sensors could only complete the whole process in a certain concentration range of phosphate buffer. In this regard, enzymatic catalysis as a mild, highly selective, rapid, environmentally friendly and economically feasible catalyst has expanded the range of application of polymeric photonic crystal in the field of molecules recognition. Enzymatic catalysis can be used as an alternative way to change the chemical conditions such as pH of the solution, which would cause the volume change of the polymer photonic crystal. Some previous works have described immobilization of a variety of enzymes to the polymeric photonic crystal via covalent attachment for the detection of the target molecules or ions, such as creatinine[30], organophosphate nerve agents[31], cholesterol[32] acetylcholinesterase[33] and mercury ions[34].

In this study, a facile method for naked-eye detection of urea with polymeric photonic crystal was developed (Scheme 1,). Selected as a pH modulator, urease has been used for the catalytic pH increase to drive multiple reactions in enzyme immunoassays and enable the controlled release of drugs in pharmaceutical industries.[35-36] Urease is an absolute specificity enzyme and catalyzes the hydrolysis of urea, resulting in a concomitant increase in the pH of the reaction media.[37-38] In previous reports, photonic crystal polymers were shown to be applicable for sensing pH.[39-44] The IOPP film used in this study was fabricated with commercially available monomers and the urease was not immobilized in the polymer (the IOPP film was immersed in urease solution directly). SEM image of the photonic crystal template and IOPP film is shown in Fig. S1 and S2, respectively. Different concentration ranges of urea could be detected by changing the molar ratios between the functional monomer and cross-linker when preparing the polymeric backbone of the photonic crystals. Overall, this method is stable, reusable and it does not require any calibration processes, while generating a readable optical signal directly by responding to the environmental stimulation without labeling.

Urease, catalyses the hydrolysis of urea to produce ammonium and bicarbonate ions, causing a pH increase in the aqueous reaction medium. Considering this reaction, the determination of urea can be indirectly done by using the IOPP film sensing of pH. It was found that when the urease concentration was kept constant (0.2 mg/mL), the pH increase in the solution was solely dependent on the concentration of urea. This indicated that the ability of the IOPP film to respond to the concentration of urea was simply presented by sensing

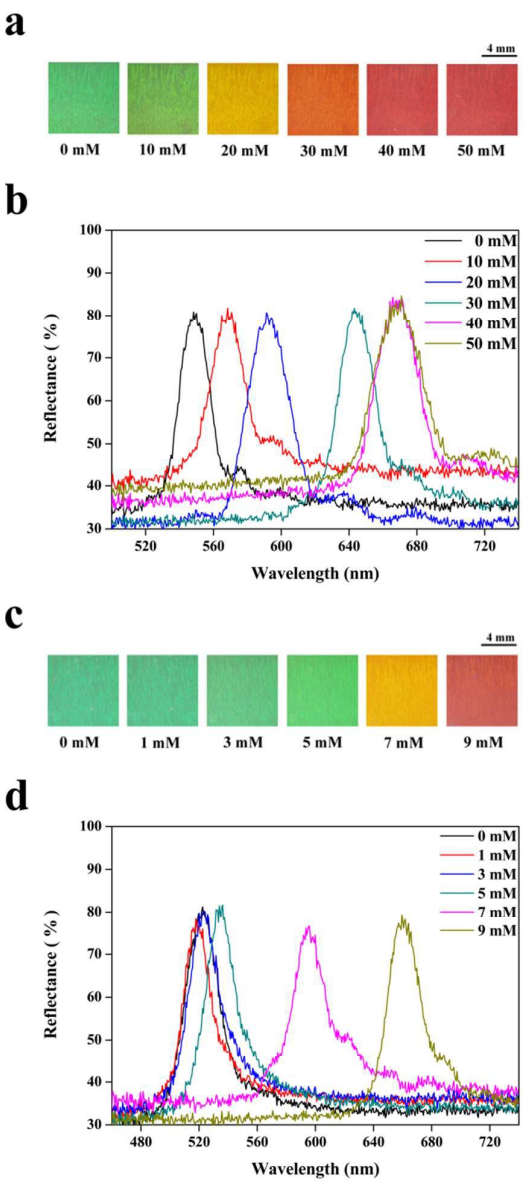

Fig. 1 (a) Color change of the IOPP film fabricated at 5:1 molar ratio of MAA and EGDMA. (b) Optical response of the IOPP film, MAA: EGDMA = 5:1. (c) Color change of the IOPP film fabricated at 8:1 molar ratio of MAA and EGDMA. (d) Optical response of the IOPP film, MAA: EGDMA = 8:1.

of the pH in the solution. During the urease-catalyzed hydrolysis of urea, hydroxyl ions were released, enabling an increase of solution basicity. Then the IOPP film swelled and the degree of swelling increased with the increase of pH value within a certain range. The -COOH group on the polymer dissociated gradient of free ions in the alkaline solution, which produced the osmotic pressure and electrostatic repulsion force that mainly contributed to the swelling of the IOPP film.[45] The swelling of the IOPP film is also affected by the imbalance of the molecular forces in the polymer such as Van der Waals' force, hydrophobic-hydrophobic attraction, hydrogen bonding, and static interactions.[46] It was found that

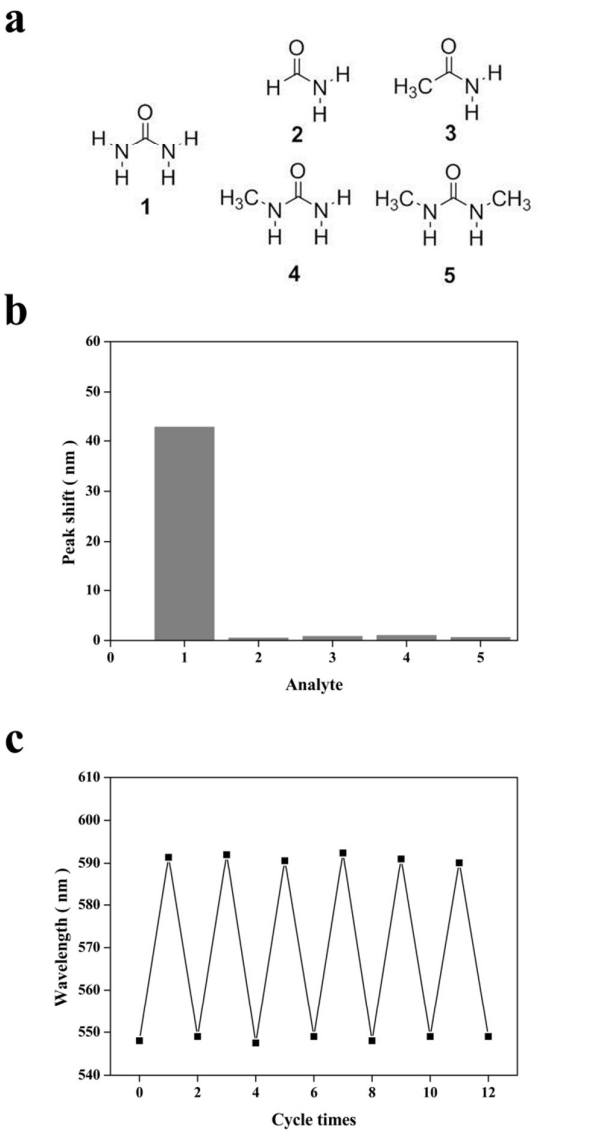

Fig. 2: (a) Chemical structures of the analytes were used: (1) Urea; (2) Formamid; (3) Acetamide; (4) Methylurea; (5) 1,3-Dimethylurea. (b) Peak shift of the IOPP film to 20 mM of analytes: (1) Urea; (2) Formamid; (3) methylurea; (4) Methylurea; (5) 1,3-Dimethylurea. (c) Recoverability of the IOPP film immersed in urea solution and recovered by immersing in acetic acid (5%) and followed by rinsing with deionized water.

the IOPP film showed different sensing abilities to pH when it was fabricated with different ratios of monomer and cross-linker. To obtain the optimal desired sensors for detecting different concentration ranges of urea, the molar ratio between the monomer and cross-linker were exploited. Generally, when the IOPP film was fabricated with a lower ratio of cross-linker to monomer, it would be more sensitive to the analyte. However, below a certain concentration level of cross-linker, it is hard for the IOPP film to sustain the framework, and results in disappearance of the structure color (without the highly ordered structure). Contrarily, when the IOPP film was fabricated with a higher ratio of cross-linker to monomer, the 3D-ordered structure with higher integrity was obtained, yet the less sensitive it is to the analyte. Thus, the IOPP film fabricated with different molar ratios of the monomer as methacrylic acid (MAA) to the cross-linker as ethylene glycol dimethylacrylate (EGDMA) were utilized when detecting different concentration ranges of urea in different systems.

Fig. S3 shows the IOPP film fabricated at 2:1 molar ratio of MAA and EGDMA responding to the urea concentration from 100 mM to 200 mM. The IOPP film displayed about 36 nm diffraction red-shifts in 200 mM urea solution. When keeping the IOPP film immersed in higher concentration of urea solution, the IOPP film separated from the PMMA substrate, curled and resolved eventually. For sensing of lower urea concentration, the IOPP film was fabricated with 5:1 molar ratio of MAA and EGDMA. The sensing behavior of this IOPP film responding to the urea concentrations from 10 to 50 mM is shown in Fig. 1b. The original Bragg diffraction wavelength of the IOPP film in deionized water was 548 nm, and red shifted to 570, 591, 644 and 668 nm when the urea concentration increased to 10, 20, 30 and 40 mM, respectively. However, no further shift of the diffraction peak was observed with the increase of the concentration of urea (50 mM), which suggests that the IOPP film reached the maximum limit of swelling. Meanwhile, as seen in Fig. 1a, along with the peak shift of the Bragg diffraction, the color of the IOPP film was also changed, which covered the range from green to red. Therefore, it could be directly assessed by the naked eye for semi-quantitative detection of urea.

The normal concentration range of urea in blood is 2.5 to 7.5 mM, depending on the build and relative health of the body.[47-48] A person will suffer from renal deficiency and the kidneys fail to excrete the excess nitrogen successfully when the concentration of urea above 7.5 mM. To detect urea at this concentration level, the IOPP film fabricated with 8:1 molar ratio of MAA and EGDMA was used. As shown in Fig. 1d, this IOPP film gives sensitive optical behavior corresponding to the urea concentrations from 1 to 9 mM. There was almost no peak shifts when immersing the IOPP film in 1 mM and 3 mM urea solution. Only about 15 nm diffraction red-shifts were observed when the concentration of urea solution increased to 5 mM. However, the Bragg peak red-shifted about 72 nm and 141 nm, respectively, when the IOPP film was immersed in the urea solution with concentration of 7 and 9 mM. The color of the IOPP film also changed to orange and red (Fig. 1c), which indicated a potential application of monitoring the level of urea to determine the health of kidney in human body. In such a case, a sensor that signaled these concentrations through distinctive colors would be useful in diagnosis. Practically, the IOPP film displayed a yellow color when the urea level was at borderline concentration. The color of the IOPP film turned red in higher urea concentrations associated with a clear-cut diagnosis of renal deficiency. For green, yellow, and red are used internationally in traffic signals, we introduced this color variation of the IOPP film in an easy way to understand.

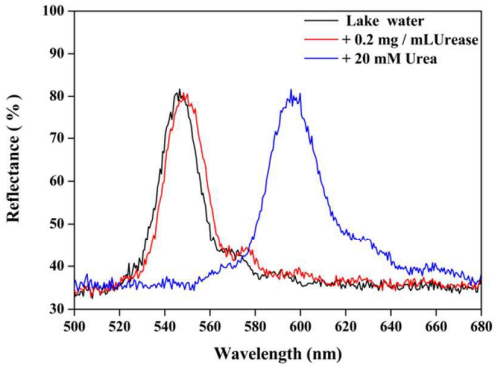

Fig. 3 Optical response of the IOPP film in natural lake water, MAA:EGDMA = 5:1.

Selectivity is also an important parameter for a sensor. For comparative study, several other molecules including formamid, acetamide, methylurea and 1,3-Dimethylurea, which are structurally similar to urea, were used as reference compounds in this work (Fig. 2a). It can be seen that only urea induced an obvious red shift of 43 nm, and the other molecules almost did not cause any peak shifts (all in 20 mM solution, Fig. 2b). A significant shift of the diffraction peak was observed only in the presence of urea, which indicated the excellent selectivity of the IOPP film over other molecules. To test the selectivity against other molecules, same concentration of formamid and methylurea were added into 20 mM of urea aqueous solution separately. As shown in Fig. S4, there is no obvious change in the optical response comparing with the urea aqueous solution. The reason is that urease is an absolute specificity enzyme, which can only catalyze hydrolysis of urea and resulting in the increase of solution basicity which makes the IOPP film swell.

Because the IOPP film has a high surface-to-volume ratio 3D ordered interconnected structure, it provides a better site accessibility and lower mass-transfer resistance. The sensing property of the IOPP film was tested by immersing into solutions of the analytes one after another from low to high concentrations. Generally, the signal was measured when the IOPP film reached a swelling equilibrium after the catalytic reaction finished in the solution. The whole process could be accomplished in about 20 minutes. The IOPP film needed less time to reach swelling equilibrium in the high concentration than that in the low concentration. The reason is that higher concentration gave higher mass transfer velocity. As the IOPP film is of a highly cross-linked polymeric nature, it also shows good physical stability and chemical inertness. This IOPP film can be easily recovered by immersing in acetic acid solution (5%) followed by rinsing with deionized water to restore the neutral blank state. Fig. 2c shows the recoverability of the IOPP film over several cycles. The standard error is within 5 %, which indicates good reproducibility.

In real applications, numerous potential competitive interferents could be exist. In order to test the applicability of the developed method, the detection of urea in natural lake water using IOPP film was performed. As shown in Fig. 3, there is almost no peak shifts when the urease was added to the natural lake water, meaning that no urea was measured in the sample. After adding urea (the total concentration of the solution was 20 mM), the original Bragg diffraction wavelength of the IOPP film red-shifted and the total red-shifts were about 45 nm, indicating the good applicability. In this system, the IOPP film can maintain relative good practicability and accuracy as long as the urease is kept active.

In summary, based on a special enzyme catalytic reaction and the unique optical property of the pH sensitive IOPP film, a novel label-free urea sensor has been successfully fabricated. The IOPP film can detect different concentration ranges of urea in different environmental system by changing the molar ratio between the functional monomer and cross-linker when preparing the polymeric backbone of the photonic crystals. Due to the hierarchical inverse opal structure, the IOPP film directly generate visually perceptible optical signals (change in color) to report events, which is easily observable by the naked eye. Meanwhile, this sensor is stable, reusable and it showed different structure colors as a response to varying concentrations of urea under ambient conditions with a swift detecting velocity and even semi-quantitative determination on the spot. Because urease is an absolute specificity enzyme, the IOPP film showed excellent selectivity over other molecules including formamid, acetamide, methylurea and 1,3-Dimethylurea, which are structurally similar to urea. We believe this work will provide a useful way in the design of sensors for the detection of urea in clinical measurements and environmental systems.

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