



OPEN Enzyme-containing-hydrogel/TiO₂ hybrid photonic crystal for label-free detection of small molecules

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Label-free optical biosensing is a promising key technology in medical diagnosis and biomedical science. The refractive-index (RI) sensitivity of photonic-crystal (PhC)-based biosensors can facilitate rapid, simple, and label-free sensing of biomolecules, with a compact measurement setup. However, the top-down fabrication of highly reproducible PhCs is expensive. Moreover, their RI sensitivity is not sufficient to detect the RI changes caused by small molecules such as glucose. Here, we propose a highly reproducible enzyme-containing-hydrogel/TiO₂ hybrid-PhC-based sensing system for label-free small-molecule detection. Our hybrid PhC can detect small molecules by utilizing the reflection-spectrum responses of TiO₂-based PhCs to the RI changes of the hydrogel on the surface, caused by the shrinking and swelling of the hydrogel in response to a reaction between the immobilized enzyme and target substrate. In this report, we demonstrate the rapid and simple detection of glucose, using a GOx-containing hybrid PhC. Our hybrid PhC can selectively and rapidly detect 1 nM glucose, with a compact setup, from the shrinking of the hydrogel caused by the oxidation of glucose by GOx. Our sensing system is expected to pave the way for the application of PhC-based biosensors in the detection of small molecules.

Keywords Photonic crystals, Nanophotonic sensor, Glucose sensor, Hydrogel, Biosensor

Various optical biosensing technologies have been developed and are being used in various fields such as nutrition, environment, and medicine, to measure specific bio-related substances by mimicking the molecular-recognition functions of the sensory organs in living organisms^{1–5}. Although some optical biosensors have been used practically, various issues exist, such as the complexity, cost, and time required for measurement. Wider application of these biosensors requires the miniaturization of sensing devices, simplification of operation, and speeding up of testing. In addition, the recent social movement toward self-healthcare and telemedicine has created a need for the development of wireless wearable biosensors that can be linked to the internet of things (IoT)^{6–8}. In this context, photonic-crystal (PhC)-based optical biosensors has attracted much attention^{9–12}. PhCs can wavelength-selectively control light in microspaces, such as transmission and reflection. By immobilizing molecular recognizers that specifically react with a target molecule on the PhC surface, they can function as optical biosensors. The sensing principles of PhC-based biosensors are detailed next. The first one is label-free sensing by utilizing the change in the peak wavelength or intensity of reflection or transmission, as an optical signal. This change is produced by the surrounding refractive-index (RI) change due to biomolecular reaction events^{13–16}. Another is labelling-based enhanced sensing by utilizing the spontaneous-emission enhancement of labeled target molecules or probes whose intensities change with biological events^{17,18}. The advantages of using PhCs in biosensors rely on the former label-free method—the measurement setup can be compact, the system operation is simple, and it can be applied in wireless and remote diagnosis by connecting with semiconductor devices. Therefore, PhC-based biosensors are promising for application in widely available and practically usable sensing devices¹⁵.

However, PhC-based label-free biosensors have two challenging issues that must be overcome if they are to be established as a useful sensing technology. One is the high cost of fabrication; PhC is generally fabricated by a top-down process involving several steps by using different types of equipment, which makes it difficult to produce PhC-based biosensors with high throughput and in mass quantities^{18–20}. Another is its low capability to detect small molecules without labeling; the surrounding RI changes caused by small molecules like sucrose or even ions are not high enough to be detected via PhC-based spectrum changes¹⁵. Therefore, the detection of small molecules using PhCs generally needs some labelling of target molecules. However, some types of

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colloidal-particle-based PhCs hybridized with hydrogels have successfully detected small molecules by utilizing the PhC optical-property changes caused by the swelling and shrinking of the hydrogel linker connecting the PhC component particles^{21–26}. However, these types of self-assembly-technique-based PhCs are difficult to fabricate reproducibly; therefore, it is challenging to apply them in practical applications.

Here, we propose a PhC-based biosensor to solve these two challenges. First, a polymer-based nanoimprinted PhC is used as the fundamental sensor substrate, which facilitates mass production and high-throughput fabrication of PhC-based biosensors. Our imprinted PhC can be reproducibly fabricated using one master mold, which solves the problem of low reproducibility that often accompanies low-cost fabricated nanostructures^{27–31}. Second, the hydrogel-based system for detecting label-free small molecules is hybridized with the nanoimprinted PhC surface. The PhC-equipped hydrogel can contain enzymes that react with the target molecules and induce optically detectable RI changes on the PhC surface, owing to the swelling and shrinking of the hydrogel due to the electrostatic-force change accompanying the enzyme reactions^{23,24}. The difference in RI between the PhC-substrate polymer (~ 1.56) and hydrogel (~ 1.4) is too small for the PhC to function as an optical reflector. Therefore, a high-RI TiO_2 layer is coated on the polymer PhC before applying the hydrogel layer, which facilitates the optical detection of the RI change in the hydrogel (Fig. 1).

In this study, we first fabricated a nanoimprinted PhC with TiO_2 -layer coating (TiO_2 PhC), and then fabricated a hydrogel-equipped TiO_2 PhC (Hydrogel/ TiO_2 hybrid PhC). Second, the fundamental optical properties of the hybrid PhC were evaluated, in terms of the optical sensitivity to the swelling and shrinking of the hydrogel. Third, a hybrid PhC equipped with a glucose-oxidase (GOx)-containing hydrogel was fabricated, and label-free specific detection and quantification of glucose was demonstrated as small-molecular detection with a compact optical setup. It was verified that our hybrid PhC facilitated the real-time monitoring and quantification of glucose in a glucose/fructose mixed water solution.

Results and discussion

Fabrication and characterization of hydrogel/ TiO_2 hybrid PhC

TiO_2 -based PhCs (TiO_2 PhCs) were fabricated via liquid-phase deposition (LPD) of nanoimprinted polymer-based PhCs (Fig. 1a). The TiO_2 PhC worked as a wavelength-selectable reflector, and its wavelength and intensity could be controlled by tuning the TiO_2 layer deposition time (Fig. S1). Scanning electron microscope (SEM) observation of the TiO_2 -PhC surface confirmed that the pitch was approximately 420 nm (Fig. S1a). Clacks were observed in the TiO_2 PhC in the SEM images. A thicker TiO_2 layer caused more clacks, which was attributed to its weaker resistance to stress. It should be mentioned that several parts of clacks were generated during the SEM observation; therefore, the actual amount of clacks would be less than that observed using SEM. The reflection peak intensity was the maximum when the LPD time was 90 min, which corresponded to a thickness of approximately 100 nm, according to our previous study (Fig. S1c). Next, a GOx-containing acrylamide hydrogel was hybridized with the TiO_2 PhC by photopolymerization of the prepolymer solution. Figure 2b shows the image of the hydrogel/ TiO_2 hybrid PhC, and Fig. 2c shows its SEM image near the boundary between the TiO_2 layer and hydrogel-coated region. The hybridization of the hydrogel with TiO_2 PhC was also confirmed by the reflection-spectrum shift (Fig. 2d). The RI increase in the surrounding TiO_2 layer by the coated hydrogel caused a red shift of the reflection peak wavelength. Thus, it was concluded that the hydrogel/ TiO_2 hybrid PhC was successfully fabricated.

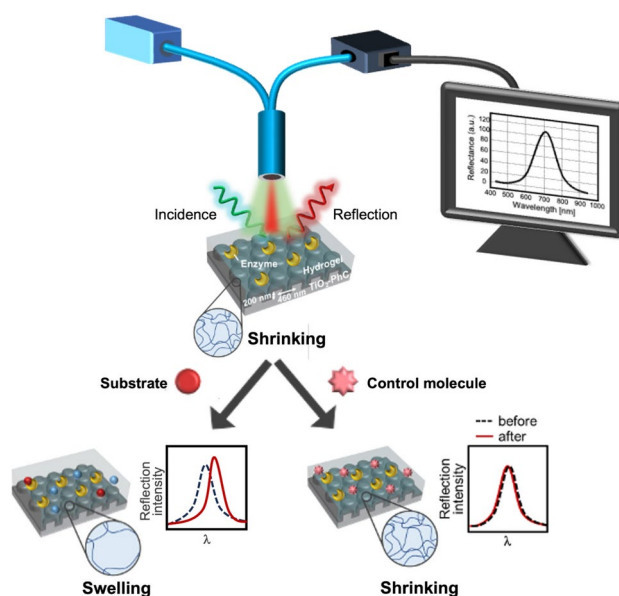


Fig. 1. Graphical concept of hydrogel/ TiO_2 hybrid PhC biosensor with compact optical setup.

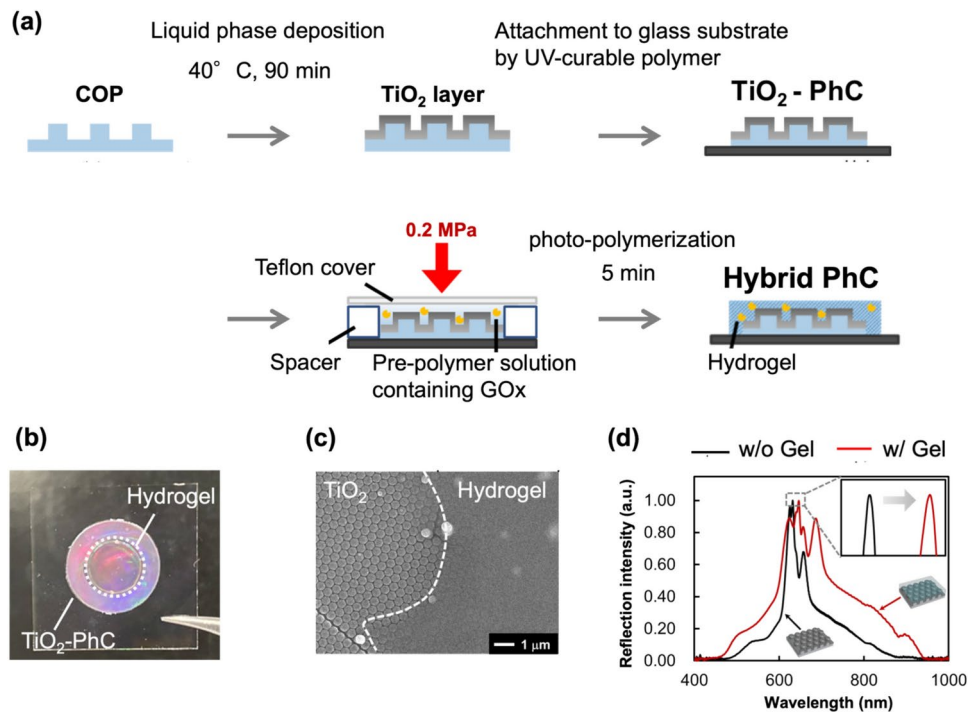


Fig. 2. Fabrication and characterization of the hybrid PhC. **(a)** Fabrication process using LPD with nanoimprinted polymer PhC. **(b)** Image of hybrid PhC. **(c)** SEM image of hybrid PhC around the boundary between the TiO_2 layer and hydrogel-coated region. **(d)** Reflection spectrum of TiO_2 PhC without (black line) and with (red line) hydrogel.

Optical characterization of hydrogel/ TiO_2 hybrid PhC

This section presents an evaluation of the optical response of the hybrid PhC to the swelling and shrinking of the hydrogel. The expected working principle of the hybrid PhC is as follows: the swelling and shrinking of the hydrogel because of a chemical-environment change in the hydrogel will cause an RI change in the hydrogel, which will be optically or spectrally detected by the TiO_2 PhC. Therefore, before the evaluation of the hybrid PhC, the RI sensitivity of the TiO_2 PhC was measured by using sucrose aqueous solutions with various RIs. Fig. S2a, b show the reflection spectra of the TiO_2 PhC in each sucrose aqueous solution, and Fig. S2c, d present the plots of peak wavelength and intensity corresponding to each RI of the sucrose solutions. The RI sensitivity of the TiO_2 PhC was confirmed based on the wavelength shift of 79.3 nm/RIU, where RIU is an RI unit, whereas the peak intensity barely changed with the bulk RI change. Next, the optical properties of the hybrid PhC were investigated by measuring the response to acetone-concentration change in a water/acetone mixed solution. The hydrophilic interaction increased as the hydrophobicity of the medium solution increased, which led to the shrinking of the hydrogel. Therefore, by changing the acetone concentration of the mixed solution, the swelling degree of the hydrogel could be controlled. To evaluate the effect of the hydrogel on the PhC's response to the sample solution, we compared the optical responses of TiO_2 PhCs with and without hydrogels. Figure 3a shows the plots of the peak-wavelength shift ($\Delta\lambda_{\text{peak}}$) corresponding to each acetone concentration, and no clear difference was observed in the peak-wavelength shifts of the samples with and without the hydrogel. The red shift of the peak wavelength could be attributed to the bulk RI increase due to the acetone concentration increase. The peak intensity of the hybrid PhC clearly decreased when the acetone concentration increased beyond 40 vol%, whereas that of TiO_2 PhC increased slightly. Figure 3c presents the plots of peak intensity for an acetone concentration range from 0 to 40 vol%. It was observed that the peak intensity of the hybrid PhC differed from that of TiO_2 PhC at an acetone concentration around 30 vol%, which agreed well with the hydrogel swelling caused by acetone/water mixed solution. Therefore, the peak intensity decrease for the hybrid PhC could be attributed to the shrinking of the hydrogel. However, against the expectation from the RI sensitivity of the TiO_2 PhC shown in Fig. S2, the peak wavelength shifts did not differ from each other though the shrinking of the hydrogel was expected to cause an RI increase on the TiO_2 PhC surface. In view of the above results, it was considered that the shrinking of the hydrogel could increase the scattering of the TiO_2 PhC's reflection mode. However, the surrounding RI increase was not sufficient to be detected by the TiO_2 PhC because of the low RI sensitivity, which is generally true in the case of PhC sensors (Fig. 3d). The scattering of the hybrid PhC clearly increased after incubation into 70 vol% acetone aqueous solution. Thus, our hybrid PhC worked as a wavelength-selectable reflector that could detect the shrinking and swelling of the hydrogel caused by scattering change.

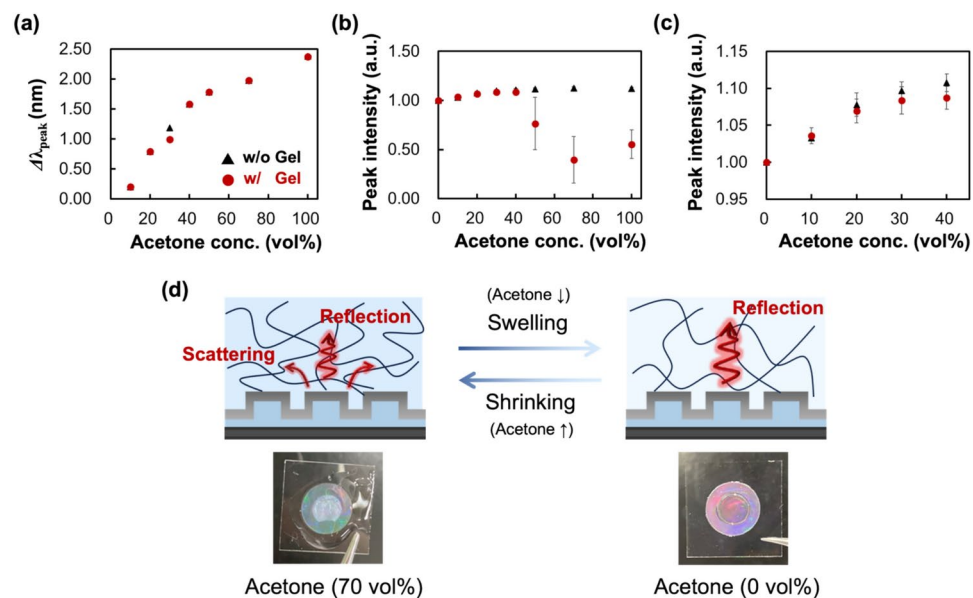


Fig. 3. Optical characterization of the hybrid PhC. (a) Plots of peak shift corresponding to each acetone concentration. (b) Plots of peak intensity corresponding to each acetone concentration in the range from 0 to 100 vol% and (c) plots for the range from 0 to 40 vol%. The plots are the average values of the triplicate measurement and the error bars indicate the standard deviation. (d) Working principle of the hybrid PhC based on swelling and shrinking of hydrogel. Insets display the image of the hybrid PhC before and after incubation into 70 vol% acetone/water mixed solution for 60 min.

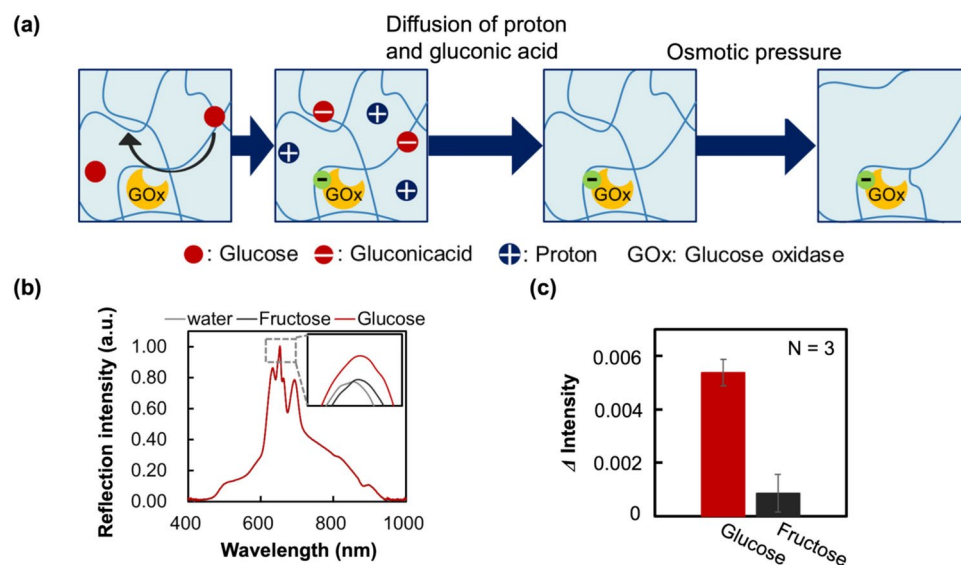


Fig. 4. Selective detection of glucose. (a) Working principle of the hybrid PhC to detect glucose based on swell of hydrogel caused by enzyme reaction. (b) Reflection spectrum of the hybrid PhC after water conditioning (grey line), and after incubation into 1 nM glucose (red line) and fructose (black line) for 60 min. (c) Peak-intensity change after incubation into each sample solution. The average values of triplicate measurements are displayed; the error bars indicate the standard deviation.

Label-free glucose sensing with hydrogel/ TiO_2 hybrid PhC

In this section, the selective label-free detection of glucose using a hybrid PhC is explained. The working principle is as follows. Glucose is oxidized by GOx, producing proton and gluconic acid. Next, the proton and gluconic acid diffuses into the bulk solution while the reduced GOx remaining in the hydrogel. Osmotic pressure is generated and the hydrogel swells (Fig. 4a). The swelling of the hydrogel is optically detected by the reflection intensity change. First, the selectivity to glucose is evaluated by comparing the responsivity to glucose and fructose (1 nM). Figure 4b shows the reflection spectrum of the hybrid PhC after conditioning in

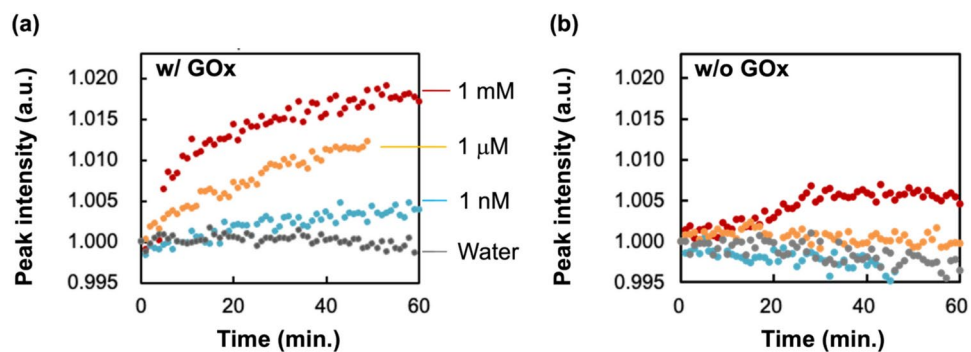


Fig. 5. Real-time measurement of glucose. (a) Plots of the peak intensity of the hybrid PhC with and (b) that without GOx, to time-course of glucose aqueous solution injection (10 µL/min) per min in the case of 0 (grey), 1 nM (sky blue), 1 µM (orange), and 1 mM (red).

water, and after incubation in 1 nM glucose or fructose aqueous solutions for 60 min. The peak intensity change (Δ Intensity) in the case of glucose is higher than that in the case of fructose, proving that the hybrid PhC exhibits selectivity to glucose (Fig. 4c). The peak intensity response to glucose is expected to be attributed to the swelling of the hydrogel and increase in scattering near the TiO_2 PhC surface. Fig. S3 represents the comparison of peak intensity change between glucose and water, providing that the signal to fructose is slightly higher than that to pure water but much less than that to glucose. This difference in signals suggested that this hybrid PhC is affected little by nonspecific molecular interactions. Fig. S4 presents the peak-wavelength shifts ($\Delta\lambda_{\text{peak}}$) corresponding to glucose and fructose. The peak-wavelength shifts are comparable to each other, which agrees with the evaluation using acetone/water mixed solution. However, red shift less than 0.2 nm is too small to recognize it as signal due to limitation in spectrometer resolution, therefore this hybrid PhC could detect target molecules based on the signal intensity change. Differences in peak intensity arise from variations in scattering behaviors at the hydrogel- TiO_2 interface and the bulk in hydrogel layer, influenced by the distinct swelling and shrinking responses and molecular interactions of each analyte. The refractive index change induced by shrinking of hydrogel could not be detected by the wavelength shift. Next, the real-time measurement of glucose using the hybrid PhC with and without GOx was investigated. The peak intensity was plotted per minute for up to 60 min after conditioning the hybrid PhC in water for 60 min. The glucose-containing sample solution (0, 1 nM, 1 µM, 1 mM) was fluxed into a flow cell at a rate of 10 µL/min. In the case of the hybrid PhC with GOx, the peak intensity gradually increased, and the increase in peak intensity was higher as the glucose concentration increased (Fig. 5a). On the contrary, the hybrid PhC without GOx could not detect 1 nM and 1 µM glucose, which proved that the existence of GOx enhanced the optical signal of the peak-intensity change because it caused the hydrogel to swell more, based on the expected working principle. As shown in Fig. 3b, the peak intensity increased as the RI of the sample solution increased, which reflects the results in Fig. 5b (that the peak intensity increased with time in the case of 1 mM glucose). In addition, the hybrid PhC with GOx could identify 1 µM and 1 mM glucose at approximately 5 min, and 1 nM glucose at approximately 20 min. Therefore, it could be concluded that our hybrid PhC could rapidly detect label-free glucose with a compact optical setup. In terms of the optical response, the pH condition was not controlled and only a water solution sample was used in this experiment. However, the response of the GOx-containing hydrogel to glucose could be improved by finely controlling the pH to maximize the shrinking and swelling of the hydrogel. Another possible factor that could enhance the optical response was the GOx concentration. An optimized GOx concentration is expected to exist for a certain glucose concentration; thus, the GOx concentration should be carefully determined according to the target glucose concentration range. When considering the measurement speed, a thinner hydrogel layer could reduce the response time because the diffusion of chemical components including glucose occurred faster. In this experiment, the thickness of the hydrogel layer was not investigated, and its thickness was approximately 10 µm. To realize faster detection of glucose, the thickness of hydrogel should be lesser; however, this could reduce the optical response to the shrinking of the hydrogel. Thus, an optimized thickness and GOx concentration could offer the maximum optical response with the fastest detection time.

Conclusions

Here, we proposed a PhC-based biosensing system to rapidly and simply detect label-free small molecules using a hybrid enzyme-containing hydrogel, which could cause RI changes in accordance with the shrinking and swelling of the hydrogel upon reaction with an enzyme, and nanoimprinted TiO_2 PhC, which detected the RI change in the hydrogel. First, a TiO_2 PhC was fabricated via LPD on a nanoimprinted polymer-based PhC. Then, GOx-containing hydrogel was coated on the TiO_2 PhC surface. The fabricated hybrid PhC was characterized by observing its SEM image and measuring the reflection-spectrum shift. Next, the TiO_2 PhC and hydrogel/ TiO_2 hybrid PhC were optically characterized. TiO_2 PhC exhibited an RI sensitivity with a peak wavelength shift of 79.30 nm / RIU, whereas the peak intensity barely changed with the bulk RI change. On the contrary, the hybrid PhC optically responded to the shrinking of the hydrogel with the reflection-peak intensity change, which could be attributed to the scattering increase near the TiO_2 PhC surface owing to the shrinking of the hydrogel, leading to a reflection-intensity decrease. Finally, the selective detection and real-time measurement of glucose was

demonstrated. The hybrid PhC exhibited more selectivity to glucose, than to fructose, and successfully detected 1 nM glucose within 20 min. In addition, 1 μ M glucose was detected within 5 min. The response to glucose was attributed to the existence of GOx, from the results of the comparison with the results when using a hybrid PhC without GOx. In the future, we plan to optimize the pH condition, GOx concentration, and hydrogel layer thickness to maximize the response and minimize the detection time. Although reusability is a critical aspect for practical applications, it was beyond the scope of the current study. Future research will evaluate the reversibility and stability of sensor responses. Comprehensive studies evaluating sensor selectivity and potential interference from various substances are necessary and will be addressed in future investigations. Our sensing system can be applied to other target small molecules by adjusting the enzyme immobilized in the hydrogel. Thus, we expect this work to pave the way for the rapid and simple sensing of small molecules using PhCs.

Methods

Fabrication of the TiO₂ PhC and hydrogel/TiO₂ hybrid PhC

TiO₂-based photonic crystals (TiO₂ PhCs) were fabricated via LPD on a nanoimprinted polymer-base PhC. A cycloolefin polymer (COP)-based nanopillar array structure film, which was used as the mold, was washed with ethanol and ultrapure water and its surface was then modified by atmospheric plasma treatment. The COP mold was filled with the LPD solution (diammonium hexafluorotitanate [0.15 M, (NH₄)₂TiF₆], boric acid (0.45 M, H₃BO₃), and hydrochloric acid, with a solution Ph of approximately 3.0) at 40 °C for 90 min. The TiO₂-PhC-deposited mold was washed with water. After TiO₂ deposition, the TiO₂ PhC was attached to the glass substrate using a photocurable resin (Fig. 2a). Next, a GOx-containing hydrogel was hybridized with the TiO₂ PhC. The prepolymer solution containing 30 wt% acrylamide aqueous solution (167.0 μ L), ultrapure water (833.0 μ L), 2-hydroxy-2-methylpropiophenone (HOMPP) (10 μ L), and GOx (180 unit/mg, 5.0 mg) was prepared and applied onto the TiO₂ PhC by photopolymerization of the prepolymer with a spacer (10 μ m). Then, the hybrid PhC was obtained. The COP mold (FLP230 = 200–120, pillar diameter and distance: 230 nm, height: 200 nm) was purchased from Scivax Co. Ltd. (Kanagawa, Japan). Diammonium hexafluorotitanate, ethanol, boric acid, and GOx were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). The photocurable polymer (NOA81) was purchased from Norland Products Inc. (Cranbury, USA).

Optical characterization of the hybrid PhC

The experimental optical characterization of the PhC was performed by measuring its reflection spectrum using our home-made compact setup composed of a tungsten halogen lamp, fiber probe, collimated lens (Sigma Koki Co., Ltd., Hidaka, Japan), and spectrometer (Ocean Optics, Tokyo, Japan). The reflection spectrum was normalized as follows: R/R_{max} , where R and R_{max} were the reflection intensity at each wavelength and maximum intensity, respectively. In the evaluation of the RI sensitivity of the TiO₂ PhC, aqueous solutions (0–20 wt%) of sucrose (Wako Pure Chem. Co., Osaka, Japan) were prepared, and their refractive indices were measured ($n = 1.333$ – 1.361) and used in the measurement of the RI sensitivity of the TiO₂ PhC. A mixed solution of water and acetone was used to evaluate the sensitivity of the hybrid PhC toward the swelling and shrinking of the hydrogel. The peak intensity and wavelength change was measured before and after incubation of the hybrid PhC in the mixed solution for 60 min. Before the measurement, the hybrid PhC was conditioned by incubating it in water for 60 min, after which the reflection spectrum of the hybrid PhC became stable.

Label-free glucose sensing

1 nM, 1 μ M, and 1 mM aqueous solutions (600 μ L) were used to evaluate the sensitivity to glucose, and the peak intensity change was monitored per minute for 60 min via flow-cell-based measurements. 1 mM glucose and fructose aqueous solutions were used to evaluate the selectivity to glucose, and the peak intensity and wavelength change were measured before and after incubation of the hybrid PhC in the solution. All experiments utilized D-glucose obtained from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan).

Data availability

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

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Author contributions

D. K. designed and performed all the experiments. D. K. and T. E. wrote the paper.

Declaration

Competing interests

The authors declare no competing interests.

Additional information

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