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# Investigation of Probe DNA Immobilization Method for Label-free DNA Biosensing Using Polymer-based Photonic Crystal

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Polymer-based photonic crystals (PhCs) were fabricated using nanoimprint lithography for use as DNA biosensors by immobilizing the probe DNA. Polymer-based PhCs can be applied to cost-effective and label-free DNA biosensors. Additionally, DNA hybridization can be detected using inexpensive and simple optical systems. In this study, probe DNA was immobilized on the PhC surface using electrostatic adsorption or covalent bonding, and the sensing performance was compared. Consequently, the PhC biosensor prepared using covalent bonding exhibited greater DNA-hybridization-induced reflection intensity changes than that prepared using electrostatic adsorption. Furthermore, discrimination of reflection intensity for target and mismatch DNA was investigated, which revealed that in contrast to the PhC biosensor prepared using the electrostatic adsorption of probe DNA, that prepared using covalent bonding discriminated target DNA at a concentration of 0.1 nM.

## 1. Introduction

The detection of DNA fragments with specific sequences has applications in the diagnosis of infectious diseases(1) and genetic diseases,(2) as well as in precision medicine.(3) In the case of genetic diseases, early diagnosis allows prompt medical treatment before disease progression.(4) Genetic diagnosis has become increasingly important, as it helps to prevent the onset of disease before occurrence. Additionally, genetic diagnosis can be used to identify individual differences in the effects and side effects of drugs that can help determine whether a drug is appropriate for an individual before administration.(5) For this reason, genetic diagnosis has attracted considerable attention in several fields of medicine, health, and life sciences.(6) Thus, it is expected that highly sensitive, simple, and inexpensive genetic diagnostic devices will be indispensable in the future. Currently, widely known genetic diagnostic methods include polymerase chain reaction,(7) DNA microarray,(8) and agarose gel electrophoresis.(9) Although these methods are highly sensitive and selective, they require fluorescent dye labeling and

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expensive detectors for measurement. Therefore, label-free detection of DNA has been studied in recent years using electrochemical,[10] optical,[11] and surface plasmon resonance[12] measurement methods. These detection methods do not require labeling with fluorescent dyes and simplify sample preparation. This feature is advantageous in retaining the function of biological samples and preventing nonspecific binding to target molecules, thus enabling highly selective detection.[13] However, these detection methods require the use of expensive materials, such as gold and silver, for device fabrication.

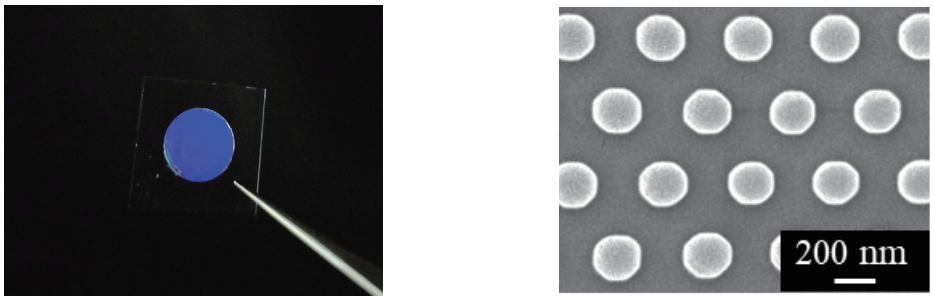
Photonic crystals (PhCs) are optical devices with nano-periodic structures that show optical property changes in response to changes in the surrounding refractive index.[14] Therefore, they have been used in various applications such as optical filters, optical waveguides, optical resonators, and biosensing devices.[15–18] Label-free detection of antigen–antibody reactions and DNA hybridization has been successfully achieved by applying the optical property changes of PhC.[19] However, PhC sensors generally require the use of materials with a high refractive index, such as silicon and titanium dioxide, and the fabrication of PhCs requires the use of an electron beam lithography system.[20] Therefore, mass production is difficult owing to the time and cost required for fabrication; furthermore, the wavelength of the light source used for the measurement is in the infrared region. Notably, nanoimprint lithography (NIL), a technique for transferring nanostructures onto polymers using molds,[21] can be applied to fabricate PhCs inexpensively and easily.[22] We have successfully detected antigen–antibody reactions using NIL polymer-based PhCs;[23] therefore, the application of NIL polymer PhCs for the detection of DNA hybridization is expected to enable the label-free detection of DNA using inexpensive polymer materials. Moreover, detection in the visible-wavelength region makes the measurement device even more inexpensive.

In this study, as basic research for the development of a label-free DNA hybridization detection method using a polymer PhC, we investigated two methods, electrostatic adsorption and covalent bonding immobilization, for immobilizing probe DNA on the surface of a PhC to improve sensor performance. Hybridization detection was implemented with PhC-immobilized probe DNA using each method. DNA hybridization was detected as the changes in the reflection intensity of white light using the polymer PhC and a simple optical system. The differences in the reflection intensity changes between the two immobilization methods were compared.

## 2. Materials and Methods

### 2.1 Materials and chemicals

The cyclo-olefin polymer (COP) PhC (COP-PhC) film (FLH230/200-120) was purchased from Scivax Co., Ltd., Kanagawa, Japan (Fig. 1). Poly allylamine hydrochloride (PAH), poly (sodium 4-styrenesulfonate) (PSS), and ethanolamine hydrochloride (EA) were purchased from Sigma-Aldrich Japan Inc., (Tokyo, Japan). 3-Aminopropyltriethoxysilane (APTES) was purchased from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan. Sodium chloride and a 25% glutaraldehyde (GA) solution were purchased from Wako Pure Chemical Co. (Osaka, Japan). Phosphate-buffered saline (PBS) was purchased from Nacalai Tesque Inc. (Kyoto, Japan). The oligonucleotides used in this study are listed in Table 1 and were purchased from BEX Co., Ltd.




(a) (b)

Fig. 1. (Color online) (a) Photograph of a COP PhC film. (b) SEM image of a COP PhC film surface.

Table 1
Sequences of oligonucleotides used in this investigation.

| Name | | Sequence | |
|---|---|---|---|
| Probe DNA | 5’ | $NH_2$-GGGCAGACTCCTACGGGAGGCAGCAGT | 3’ |
| Target DNA | 5’ | ACTGCTGCCTCCCGTAGGAGTCTG | 3’ |
| Mismatch DNA | 5’ | GCAAACAGGATTAGATACCCTGGTAGTCCACG | 3’ |
| ROX target DNA | 5’ | ROX-ACTGCTGCCTCCCGTAGGAGTCTG | 3’ |
| ROX mismatch DNA | 5’ | ROX-GCAAACAGGATTAGATACCCTGGTAGTCCACG | 3’ |

(Toyota, Japan). Probe DNA was 27 bp in length, with an amino group at the 5’-end. The target DNA and mismatch DNA were fully complementary and non-complementary, respectively, to the probe DNA. ROX target (or mismatch) DNA has the same sequence as the target (or mismatch) DNA, with a fluorescent dye, carboxy-X-rhodamine (ROX), modified at its 5’-end. This sequence is part of the universal sequence of 16S ribosomal RNA.

### 2.2 Immobilization of probe DNA via electrostatic adsorption

The COP-PhC film was treated with plasma (100 W, air 20 sccm, 0.5 Torr, 1 min) to hydrophilize the surface. The PhCs were immersed in a 3 g/L PAH aqueous solution containing 100 mM NaCl for 10 min at room temperature (RT), followed by washing with ultrapure water. Thereafter, to electrostatically immobilize the probe DNA on the PhC surface, the PhC with a positive charge on the surface was immersed in PBS solution (pH 7.0) containing 100 nM probe DNA for 1 h at RT, followed by washing with ultrapure water. The phosphate groups of DNA are negatively charged and can be electrostatically adsorbed onto positively charged surfaces. The PhC-immobilized probe DNA was further immersed in a 3 g/L PSS aqueous solution containing 100 mM NaCl for 10 min at RT to prevent the electrostatic adsorption of the target DNA, followed by washing with ultrapure water.

### 2.3 Immobilization of probe DNA via covalent bonding

The surface of COP-PhC was hydrophilized via plasma treatment for 10 min. The hydrophilic PhCs were silanized by immersion in a 1 wt% APTES ethanol:water (95:5 wt%) solution for 30 min at RT, followed by washing with ethanol. The silanized PhC was then immersed in

1 wt% GA aqueous solution for 1 h at RT, followed by washing with ultrapure water. The probe DNA was immobilized on the PhC surface by incubating the PhC with aldehyde groups in PBS solution (pH 7.0) containing 100 nM probe DNA at RT overnight, followed by washing with ultrapure water. The PhC-immobilized probe DNA was immersed in 100 mM EA aqueous solution for 30 min to block the aldehyde groups and introduce hydroxyl groups.

### 2.4 Confirmation of DNA hybridization

The probe DNA-immobilized PhCs were immersed in 100 nM ROX target (or mismatch) DNA in PBS solution (pH 7.0) containing 1 M NaCl for 1 h at 60 °C, followed by washing with ultrapure water. In this study, PBS was used as the buffer based on previous reports.[24), (25)] Fluorescence images of the PhCs were obtained using a digital microscope (Keyence Multi-Viewer System VB-S20; Keyence Corp., Osaka, Japan) equipped with a charge-coupled device (CCD) camera (VB-7010; Keyence Corp., Osaka, Japan), mercury lamp (120 W), and filter pair (RFP: excitation filter, 540 ± 25 nm; RFP: emission filter, 572 nm high pass) (VB-L11; Keyence Corp., Osaka, Japan). Fluorescence images were converted to numerical fluorescence intensity data using the ImageJ software (NIH, Bethesda, MD, USA).

### 2.5 Label-free detection of DNA hybridization

The probe DNA-immobilized PhC was immersed in a target (or mismatch) DNA (0.1–100 nM) in PBS solution (pH 7.0) containing 1 M NaCl for 1 h at 60 °C. The PhC was then washed with ultrapure water and dried using an air blower before the reflection spectra were measured. Measurements were performed in triplicate using three different PhCs for each experiment. The optical measurements of DNA hybridization were based on reflectometry using equipment that consisted of a white light source (LS-1, Ocean Optics, Dunedin, FL, USA), optical fiber (R400-7, UV-VIS, Ocean Insight, Orlando, FL, USA), spectrophotometer (USB4000, Ocean Insight, Orlando, FL, USA), and software (Ocean View; Ocean Insight, Orlando, FL, USA) (Fig. 2). To measure the reflection spectra of the PhCs, white light was irradiated directly onto their surfaces.

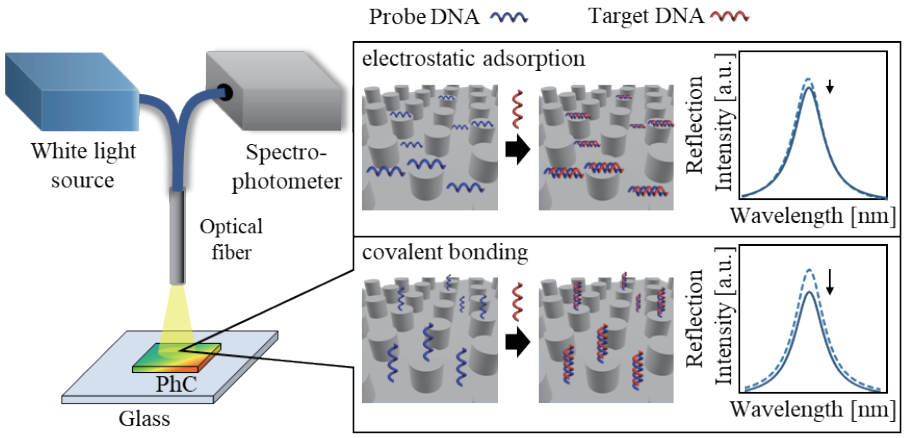



Fig. 2. (Color online) Conceptual illustration for label-free detection of DNA hybridization using the COP-PhC sensor.

## 3. Results and Discussion

### 3.1 Confirmation of hybridization with fluorescence-labeled DNA

ROX target DNA or ROX mismatch DNA was hybridized to probe the DNA-immobilized COP-PhC surface via electrostatic adsorption, and each fluorescence image was acquired using a digital microscope [Figs. 3(a) and 3(b)]. These fluorescence images were converted to fluorescence intensity and normalized to the fluorescence intensity of the PhC that reacted with the ROX target DNA. The normalized fluorescence intensities (NFIs) obtained were compared, and the results showed that the fluorescence intensity of the PhC reacting with the ROX target DNA was greater than that reacting with the ROX mismatch DNA [Fig. 3(c)]. This confirmed that the probe DNA on the PhC surface and the ROX target DNA hybridized specifically. The ROX target DNA or ROX mismatch DNA was also reacted with the probe

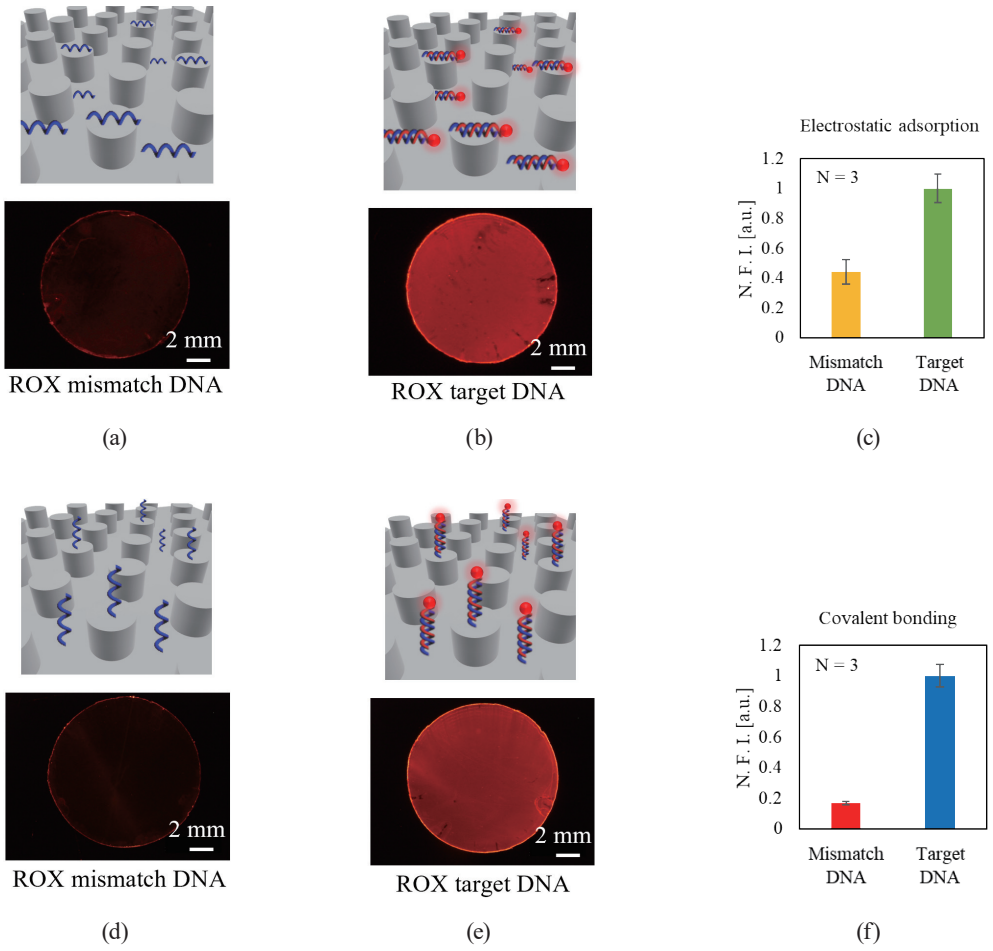



Fig. 3. (Color online) Illustration and fluorescence image of hybridization of probe DNA immobilized via electrostatic adsorption with (a) ROX mismatch DNA or (b) ROX target DNA. (c) Comparison of normalized fluorescence intensity (NFI) between (a) and (b). Illustration and fluorescence image of hybridization of probe DNA immobilized via covalent bonding with (d) ROX mismatch DNA or (e) ROX target DNA. (f) Comparison of NFIs between (d) and (e).

DNA immobilized on the COP-PhC surface via covalent bonding, and NFIs were compared [Figs. 3(d)–3(f)]. These results also indicate that the probe DNA and target DNA hybridized specifically. Furthermore, the NFIs for the ROX mismatch DNA in the two immobilization methods were compared, and it was found that the NFI for electrostatic adsorption was greater, suggesting an increase in the nonspecific adsorption of mismatch DNA. This was due to insufficient blocking by PSS, and the mismatch DNA was electrostatically adsorbed on the PhC surface.[26] In the case of using BSA as a blocking agent, the difference of NFI between ROX target DNA and ROX mismatch DNA was very small and DNA hybridization could not be confirmed. Thus, the covalent immobilization method was more specific for this measurement.

### 3.2 Comparison of sensor performance between two immobilization methods

Reflection spectra upon hybridization of the target DNA (0–100 nM) with the probe DNA immobilized onto the COP-PhC via electrostatic adsorption are shown in Fig. 4(a). Each reflection intensity was normalized to that of 0 nM target DNA. Figure 4(a) shows that the normalized reflection intensity (*NRI*) decreased as the concentration of the target DNA increased. This was caused by the hybridization of the target DNA with the probe DNA immobilized on the COP-PhC surface, which disrupted the periodic refractive index distribution on the surface and reduced the diffraction intensity. Moreover, as a control experiment, mismatch DNA (0–100 nM) was used instead of the target DNA, and the reflection spectra were measured; the results are shown in Fig. 4(b). These spectra were normalized in the same way as for the measurement of the target DNA. Figure 4 shows that *NRI* decreased as the concentration of the mismatch DNA increased; nevertheless, the amount of change was smaller than that obtained for the target DNA. As the mismatch DNA does not hybridize with the probe DNA, we expected that no change in *NRI* would occur. However, as described in Sect. 3.1, the mismatch DNA was electrostatically adsorbed on a COP-PhC surface, and this was the cause of the decrease in *NRI* with increasing mismatch DNA concentration. We also measured the reflection spectra of COP-PhCs immobilized with the probe DNA via covalent bonding when the target DNA and mismatch DNA reacted. Figures 4(d) and 4(e) show that as the target and mismatch DNA concentrations increased, respectively, *NRI* for the target DNA decreased, while the change in *NRI* for the mismatch DNA was very small. This suggested that the effect of the nonspecific adsorption of the mismatch DNA on the COP-PhC surface was negligible. The changes in the *NRI* for the target and mismatch DNA using the two immobilization methods were compared. Figures 4(c) and 4(f) show the difference in *NRI* from that of the 0 nM target or mismatch DNA, respectively. In the case of electrostatic adsorption, the difference in $\Delta NRI$ between the mismatch DNA and the target DNA was small, satisfying $p < 0.05$, for only 100 nM. In contrast, when using covalent bonding, the range above 1 nM showed $p < 0.05$, confirming the difference in $\Delta NRI$ between the mismatch DNA and the target DNA. The differences between the two immobilization methods can be explained as follows: The first is the nonspecific adsorption effect. As previously mentioned, in the immobilization method using electrostatic adsorption, the mismatch DNA is electrostatically adsorbed; thus, *NRI* decreased when the

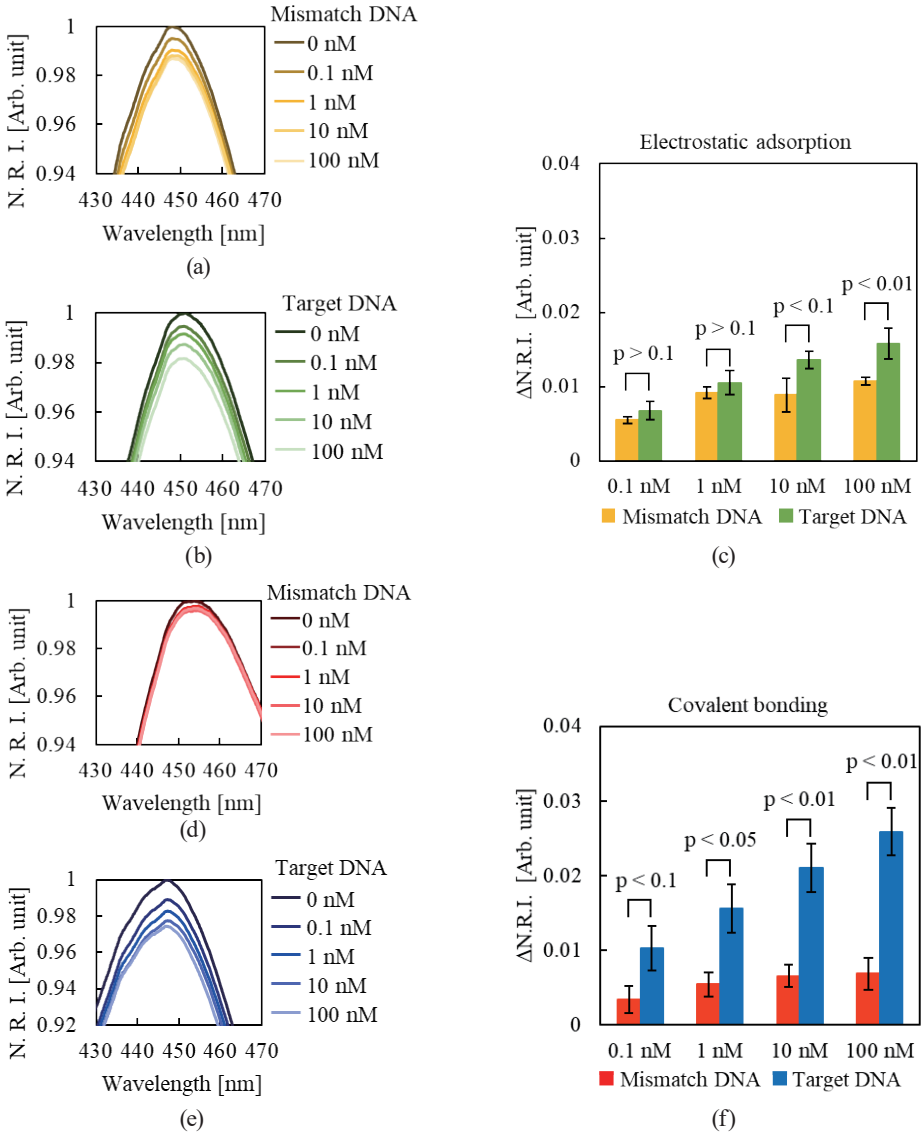



Fig. 4. (Color online) Reflection spectra of COP-PhCs with probe DNA immobilized via electrostatic adsorption after hybridization of (a) mismatch DNA or (b) target DNA. Reflection spectra of COP-PhCs with probe DNA immobilized via covalent bonding after hybridization of (d) mismatch DNA or (e) target DNA. Comparison of $\Delta NRI$ of mismatch DNA and target DNA in the immobilization methods via (c) electrostatic adsorption and (f) covalent bonding.

mismatch DNA was reacted. Therefore, there is little difference in $\Delta NRI$ between the mismatch DNA and the target DNA. The second reason is the difference in hybridization efficiency. In the case of electrostatic adsorption, the surface of COP-PhC is considered to be negatively charged by the PSS used for blocking, which can repel the negatively charged target DNA and inhibit hybridization. Consequently, the COP-PhC device using covalent bonding exhibited higher sensor performance.

## 4. Conclusions

In this study, as fundamental research for the development of a label-free detection method for DNA hybridization using COP-PhCs, we compared two methods of immobilizing probe DNA on the surface of COP-PhCs. First, we performed an experiment using fluorescent-dye-labeled DNA to confirm whether DNA hybridization occurred on the surface of the COP-PhCs in each immobilization method. The results suggest that in both immobilization methods, the probe DNA was immobilized on the COP-PhC surface hybridized with the target DNA. However, nonspecific adsorption of mismatch DNA occurs in the case of electrostatic adsorption. Next, reflection intensities were measured using the two immobilization methods to compare the performance of the label-free detection of DNA hybridization. When electrostatic adsorption was used, there was no difference ($p > 0.1$) in the change in reflection intensity between the mismatch DNA and the target DNA at concentrations below 1 nM. In contrast, when covalent bonding was used, there was a clear difference ($p < 0.1$) in the reflection intensity change between the mismatch DNA and target DNA at a DNA concentration of 0.1 nM. Conclusively, covalent immobilization of the probe DNA improved the sensor performance. Because COP-PhC was fabricated by NIL, it can be mass-produced. Additionally, DNA hybridization can be detected using inexpensive and simple optical systems. Therefore, the COP-PhC has the potential for application in cost-effective and simple measurement systems.

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