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Immunoblot-based optical biosensor for screening of osteoarthritis using a smartphone-embedded illuminometer

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We report a new smartphone-based immunosensing system that integrates an immunoblotting assay and a built-in illumination sensor to assay an osteoarthritis marker. The simple optical biosensing system developed in this study effectively uses smartphone-embedded components such as white light-emitting diode and illumination sensor as light source and optical receiver, respectively. In contrast to the conventional optical sensors, which utilize a specific spectrum and focused wavelength, the illumination sensor sensitively responds to the variations in external light intensity over a wide range of wavelengths. This functionality of the illumination sensor in the smartphone was employed as a signal transducer in the optical system. The immunoblotting technique, which uniformly changes the intensity of light because of the precipitation reaction, was introduced to the developed optical system. The horseradish peroxidase-induced insoluble precipitate interferes with the penetration of incident light, thereby facilitating the variation of applied light intensity. Subsequently, the quantity of light passing through the biosensing channel was immediately analysed using the lux meter in the mobile application. Herein, the urinary C-terminal telopeptide fragment of type II collagen (uCTX-II) was selected as an osteoarthritis biomarker and analysed to demonstrate the feasibility of the developed illumination sensor. Results indicate an obvious change in the lux value in accordance with the uCTX-II concentration ranging from 0 to 10 ng/mL. The results were highly reproducible and sensitive to the variations in the concentration of the analyte. This suggests the potential use of the developed illumination sensor as a promising tool for the quantitative diagnosis of target analyte and point-of-care testing.

Introduction

Development of rapid, accurate and sensitive diagnostic device for point-of-care-testing (POCT) is the major issue currently faced by the clinical and commercial fields. To this end, several studies have investigated and proposed various approaches to construct an ideal POCT device that can facilitate convenient diagnosis without constraints in time and space. Recently, smartphones have attracted considerable attention in diagnostics as a promising platform for the development of POCT device owing to their functionalities, such as embedded physical sensors and related mobile programs. Thus far, several studies have reported various types of smartphone-based biosensing techniques that take advantage of the functionalities of smartphone. However, most of these studies have utilised only the imaging functions of smartphone using the embedded camera. Besides, the images thus obtained from the smartphone were transferred to a computer and were analysed using external image analysis software. In simple terms, the smartphone was essentially utilized like a digital camera in the biosensing system. However, current smartphones contain high-end technical components, such as infrared, gyroscope and illumination sensors. Considering their high accuracy and sensitivity, there is a great potential for using these smartphone-embedded sensors as a signal transducer in the biosensing system. Besides, data acquisition from those sensors can be operated and controlled using related mobile software without the need of any external programs. Among the different sensors embedded in the smart phone, in this study, we used the smartphone illumination sensor as an optical transducer for the fabrication of biosensing system. The smartphone considered in this study uses an embedded illumination sensor that modifies the brightness of the mobile display depending on the variations in ambient light intensity. This feature of the smartphone can be used as an optical receiver in the optical biosensor.

Conventional optical sensing devices consist of a light source and a receiver set according to the specific absorption wavelength of the target analyte. Although these optical systems enable accurate and specific analysis, they necessarily present limitations in terms of portability due to the complex arrangement of the optical components. In conventional optical systems, the emission spectrum of the light source should be adjusted according to the spectral characteristics of the target analytes. The monochromator, which is often used as a light source in the spectrometer, is composed of a complicated array of elements, such as halogen lamp, mirror, filter, grating or...
prism, in order to carry out the selection of light of desired wavelength from a wide range of wavelengths. Current optical biosensing systems, however, use cheap and small light sources, such as light-emitting diodes (LEDs). Nevertheless, the increase in the numbers of devices introduces unnecessary complexity of the system. Overall, the conventional optical systems are not sufficiently capable of analysing a diverse range of wavelengths. To overcome this limitation, we have made an attempt to simplify the complexity of the optical system using a simple smart IT device.

In contrast to the optical system in a conventional optical sensor, the illumination sensor embedded in the smartphone responds and also reflects the integrated light intensity in a wide range of wavelengths. This makes it possible to apply various types of light sources, such as monochromatic and polychromatic light sources, to the illumination sensor. To utilize the illumination sensor as an optical transducer in biosensing system, it is necessary to use certain biochemical assay methods that could uniformly reduce the intensity of incident light over wide range of wavelengths. To this end, we integrated the immunoblotting assay principle to the optical sensing system with the white LED light of a smartphone exhibiting a wide range of emission wavelengths. As a result, the intensity of a broad spectrum of LED light is altered based on the target analyte concentration. When light is passed through the target sample undergoing a precipitation reaction, the sample blocks the light, thereby reducing its intensity. The target analyte can be quantified by measuring the alterations in light intensity through the lux meter application. To conduct smartphone-based competitive immunoassay for uCTX-II analysis, PEG$_4$-EKGPDP was immobilized onto the surface of the sensing channel. Subsequently, the prepared uCTX-II samples were treated with horseradish peroxidase (HRP)-conjugated uCTX-II antibody. Upon completion of the uCTX-II assay, the biosensing channel was applied to the prepared illumination sensor. The changes in the intensity of LED light passing through the sensing channel were numerically quantified using lux meter application. In this study, uCTX-II was immediately analysed by introducing the illumination sensor-based assay. Details are reported herein.

### Experimental

#### Chemical and apparatus

Dopamine hydrochloride was purchased from Sigma-Aldrich, 3,3′-Dithiobis[ sulfo-succinimidylpropionate] (DTSSP) was obtained from Pierce and 3,3′,5,5′-tetramethylbenzidine membrane (TMBM) substrate solution was obtained from SurModix. HRP was purchased from Toyobo Enzyme. The peptide fragments, namely, EKGPDP and PEG$_4$-EKGPDP, were provided by Peptron Inc. The uCTX-II ELISA kit, which included HRP-labelled monoclonal anti-uCTX-II antibody, was obtained from Immunodiagnostic Systems Inc. The Sylgard® 184 silicone elastomer kit was purchased from Dow Corning. Double distilled and deionized water (DDW) with a specific resistance greater than 18 MΩ cm was used in all the experiments.

#### Surface modification of the biosensing channel

To conduct smartphone-based competitive immunoassay for uCTX-II analysis, PEG$_4$-EKGPDP was immobilized onto the surface of the sensing channel. Subsequently, the prepared uCTX-II samples were treated with horseradish peroxidase (HRP)-conjugated uCTX-II antibody. Upon completion of the uCTX-II assay, the biosensing channel was applied to the prepared illumination sensor. The changes in the intensity of LED light passing through the sensing channel were numerically quantified using lux meter application. In this study, uCTX-II was immediately analysed by introducing the illumination sensor-based assay. Details are reported herein.
The biosensing channel was prepared using polydimethylsiloxane (PDMS) and one-side-adhesive polyethylene terephthalate (PET) film. The mold used for casting of PDMS were composed of acrylic resin. In the typical process, the PDMS monomer and its curing agent (at a volume ratio of 10:1) was cured in an acrylic substrate mold at 80°C for 60 min in a vacuum oven. Subsequently, the solidified PDMS substrate was detached from the acrylic resin mold. The fabricated PDMS channels included one inlet and one outlet hole (20 mm in length x 5 mm in width x 2 mm in depth). The volume of channel was 200 µL. (Fig. S1, in the supplementary information). Furthermore, the biosensing layer was fabricated using the following procedure: dopamine hydrochloride (2 mg/mL) was added to 10 mM Tris-HCl buffer solution (pH 8.5). The resulting solution was applied to the prepared channel for 16 h at room temperature. The polydopamine-coated reaction channels were rinsed and filled with DDW.

**Fabrication of biosensing layer for uCTX-II competitive immunoassay**

The biosensing layer utilized for competitive uCTX-II immunoassay was fabricated via peptide immobilization using self-assembled monolayer technique. In the typical process, 5 mM DTSSP solution in PBS was injected into the polydopamine-coated sensing channel for 3 h. Subsequently, after washing, 20 µg/mL of PEG₂-EKGPDP peptide was supplied to the channel for 1 h. This resulted in the covalent immobilization of PEG₂-EKGPDP onto the channel surface through amide bond formation. In this study, EKGPDP hexapeptide was used as a target analyte, as the EKGPDP sequence is the epitope of uCTX-II. Furthermore, 20 mM ethanolamine was loaded into the channel for 15 min, in order to block the unreacted functional group of polydopamine and DTSSP.

**Results and discussion**

**Signal transducing principle of designed illumination sensor**

This study focuses on the development of an optical biosensing system that utilises a smartphone-embedded illumination sensor as an optical transducer, in conjunction with a horseradish peroxidase (HRP)-induced precipitation assay. During the biochemical assay, HRP digests H₂O₂ and converts the colourless chromogenic substrate into a coloured precipitate. Here, HRP is used as a detection molecule, as it is produced in the precipitate reaction in proportion to the analyte concentration and could disturb the penetration of light. Based on this, it is possible to quantify the analyte by estimating the intensity of penetrated light, as shown in Fig. 1(A). In this study, the illumination sensor and white LED on a smartphone were employed as the light source and optical receiver, respectively, in order to produce an optical biosensing system.

The LED light from the back of the smartphone was used to provide a certain amount of light to the illuminometer on the smartphone. Using an optical cable, the LED flash was connected to the front of the smartphone, as shown in Fig. 1(B).

**Verification of developed optical sensing principle**

To verify the accuracy and applicability of the developed biosensing system, the HRP and 4-chloro-1-naphthol (4-CN)-induced precipitation method was used as the proof of concept (Fig. 2). In the typical process, 4-CN substrate was oxidized by HRP in the presence of H₂O₂ and converted to a blue-coloured precipitate. The HRP concentration was varied from 0 to 1,000 µg/mL, and a blue-coloured precipitate was observed. The HRP concentration was determined by using a lux meter freeware application developed by ‘NotQuiteThem’. As a result, the lux value of the applied light was found to display spontaneously on the smartphone without the use of any external software. As shown in Fig. 2(A), the extent of development of the purple-coloured precipitate increased in accordance with the HRP concentration.
The lux value of the sample treated with 0 µg/mL of HRP (1,765 lux) was similar to that of plain LED light (1,780 lux). However, we observed a gradual decrease in the lux value in accordance with the increase in HRP concentration reacted. To register the change of lux value from the assay result, absolute value of lux intensity change was calculated by the following equation.

\[
\Delta \text{Light intensity} = |\text{lux intensity at 0 µg/mL of HRP} - \text{lux intensity at each conc. of HRP}|
\]

The calibration curve of Δlight intensity based on the assay result is shown in Fig. 2(B). As the concentration of applied HRP increased, a parabolic increase in signal was registered. The observed deviation from linearity is assumed from a rapid saturation of HRP activity by using a relatively high concentration of 4-CN, especially when the HRP concentration is low. The limit of detection (LOD) of the HRP assay, calculated as three times the standard deviation of the background signal, was 1.4 µg/mL. This result indicated that the penetration of LED light is interrupted as a result of the HRP-mediated formation of precipitates in the sensing channel. Consequently, the reduced light intensity reached the illuminometer on the smartphone, as intended. Therefore, the designed illumination sensor, together with a precipitation-induced sensing system, appears to be sufficient to apply for the HRP-mediated immunoblot assay.

Comparison of HRP precipitating substrate

The abovementioned test validates the feasibility of the proposed biosensing system. Nevertheless, the precipitation reaction must be improved in order to precisely analyse even small variations in the amount of analyte. Therefore, for practical applications of the developed system to the immunoassay, we assessed and compared the reactivity of the precipitating substrates, such as TMBM and 4-CN, against HRP. During the reaction, HRP oxidized TMBM and produced a blue-coloured precipitate, similar to 4-CN. To gain deeper insights on this process, HRP of concentration ranging from 0.01 to 1,000 µg/mL was reacted with TMBM and 4-CN at a volume ratio of 1:1 in a microwell plate. As shown in Fig. S2 (in the supplementary information), the reaction between HRP
and 4-CN resulted in colour change of the reaction mixture for
HRP concentrations ranging from 1 to 1,000 µg/mL. Conversely, in the case of TMBM, the colour change of the
reaction mixture was obvious for HRP concentrations ranging
from 0.01 to 1,000 µg/mL.
When HRP is used as a signalling probe in the immunoassay,
a very low concentration of HRP will remain on the sensing
surface of the channel after the completion of the assay.
Therefore, the TMBM reagent, which changes the colour of the
reaction mixture even at low concentrations of HRP, is
considered more efficient than 4-CN in the analysis of uCTX-II
using an immune-based sensing system.

**Illumination sensor-based urinary CTX-II analysis**

One of the most important biomarkers for osteoarthritis (OA)
analysis, namely, uCTX-II was chosen to test the application of
the illumination sensor for detecting disease conditions.7,8
During the progression of OA, cartilage components, such as
collagen, are degraded by collagenase and protease.9 Among
them, CTX-II is a one of the degraded collagen products. In
general, CTX-II initially accumulates in the synovial fluid, and
is subsequently secreted into the serum and urine.9,13 While
serum CTX-II has a cross-linked dimeric epitope (EKGPDP),
the HRP concentrations ranging from 0 to 10 ng/mL in PBS buffer and HRP-conjugated
anti-CTX-II antibody (from the uCTX-II ELISA kit) was
applied to the prepared sensing channel. According to the
principles of competitive immunoassay, the uCTX-II antibody
competitively reacts with the immobilized PEG2-EKGPDP
concentration, the spectral variations in the LED light intensity and the precipitation reaction as a function
of uCTX-II concentration, the spectral variations in the
intensity of LED light passing through the sensing channel
were analysed using a modular spectrometer. Results indicated
an increase in the intensity of LED light proportional to the
increase in uCTX-II concentration (Fig. 3(C)). This clearly
showed that the penetration of LED light was blocked by the
enzymatic product as a result of the disturbance caused by the
precipitate in the sensing channel.
The conventional optical sensing system utilized in the
quantification of target analytes evaluates the changes in the
specific absorption spectrum of the target analyte. In contrast,
the immunoblotting-based optical sensing system showed a
complete change for a wide range of wavelengths, resulting
from the blockage in penetration of light by the precipitate, as
clearly seen in Fig. 3(C). The white LED light of a smartphone
exhibits two major spectral peaks in the visible wavelength
range. The immunoassay induced an alteration in the intensity
of LED light along the whole wavelength range of the visible
light region. This obviously indicated that the insoluble
precipitate interrupts the penetration of light over a wide
spectrum of wavelengths, as intended. By using this, the light
intensity could be stably analysed by integrating the same
within the whole spectrum of wavelengths. Therefore, it
appears reasonable to conclude that the developed optical
analysis system, which employs only the LED light and
illumination sensor from a smartphone without additional
installation of specific optical equipment and external analysis
program, is suitable for the quantification of uCTX-II.

After locating the biosensing channel on the illumination
sensor of the smartphone (as shown in Fig. 2(A)), the LED
intensity was analysed using the lux meter application. The biosensing channel contained the HRP-TMBM-induced
precipitation reaction solution for uCTX-II analysis; all
measurements were performed in the dark to prevent the
external light and provide consistent light intensity from the
smartphone-embedded LED. The lux value of the straight LED
light, as registered by the illumination sensor, was 4,000 lux,
while 0 lux was detected without LED light. Subsequently, the
uCTX-II assayed biosensing channel was sequentially applied.
Results indicated an increase in the lux value proportional to
the decrease in precipitation reaction. Also, as shown in Fig. S3
(in the supplementary information), the lux value increased
proportionally in accordance with the increase in uCTX-II
concentration. This result clearly validated the success
functioning of the competitive immunoassay for uCTX-II
analysis. A calibration curve was constructed by calculating the
ratio between the maximum signal and signal for each test
result based on the immunoassay, as shown in Fig. 3(D). The
lux value was increased with the increase in uCTX-II
concentration from 0.3 to 10 ng/mL. To confirm the accuracy
of the illuminometer-based immunoassay, the uCTX-II samples
were repetitively assayed for at least three times. Accordingly,
the coefficient of variation and R² value for the uCTX-II assay
was found to be approximately 5% and 0.98, respectively,
demonstrating the suitability of the developed illumination sensor
for use in OA diagnosis. The limit of detection (LOD) of the
developed assay, calculated as three times the standard
development of the background signal from 0 ng/mL of uCTX-II,
was 0.3 ng/mL. The detection range for uCTX-II assay, as
calculated using the LOD value, was within the clinical range
for OA diagnosis (1–10 ng/mL of uCTX-II).17,18 Therefore, this
study successfully established an accurate and reliable assay for
uCTX-II analysis by using a simple smartphone-embedded illumination sensor.

Conclusions
This study demonstrates a novel application of the smartphone-embedded sensor, in particular the illuminometer, as an optical transducing system. The optical system developed in this study used a unique immuno-precipitation reaction to vary the light intensity over a wide range of wavelengths. Using this phenomenon, uCTX-II was rapidly detected without the use of professional devices or software. Furthermore, the entire biosensing process, including LED light generation and analysis of light intensity, was performed solely using the functionalities of a smartphone. The developed illumination sensor will open up more opportunities in the biosensing field, given the fact that the HRP is a commonly used signalling probe in biochemical assays. The encouraging results obtained in this study show great promise for the use of this biosensing system as a POCT device and as an effective tool for OA diagnosis.

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