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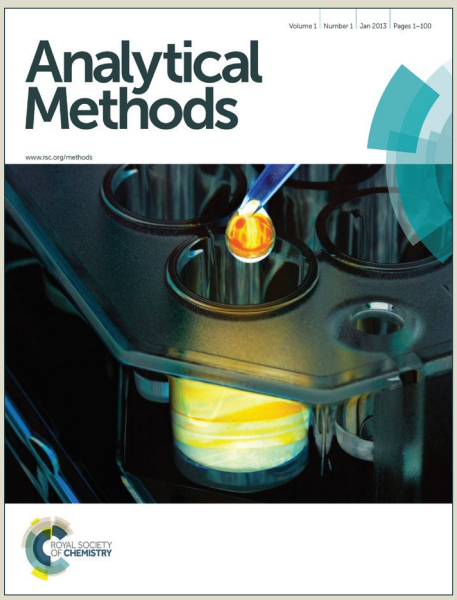

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Immunoblot-based optical biosensor for screening of osteoarthritis using a smartphone-embedded illuminometer

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Yoo Min Park[a], Yong Duk Han[a], Ka Ram Kim[a], Cunqiang Zhang[a] and Hyun C. Yoon[a]*

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 the 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.[1-5] 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

[a] Department of Molecular Science and Technology, Ajou University, Suwon, 443-749, South Korea.
* E-mail: hcyoon@ajou.ac.kr
† Electronic Supplementary Information (ESI) available. See DOI: 10.1039/x0xx00000x

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).[6] 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. The insoluble precipitate, which was produced during the immunoblotting assay reaction, blocks the penetration of light in a wide range of 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 of mobile software.

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

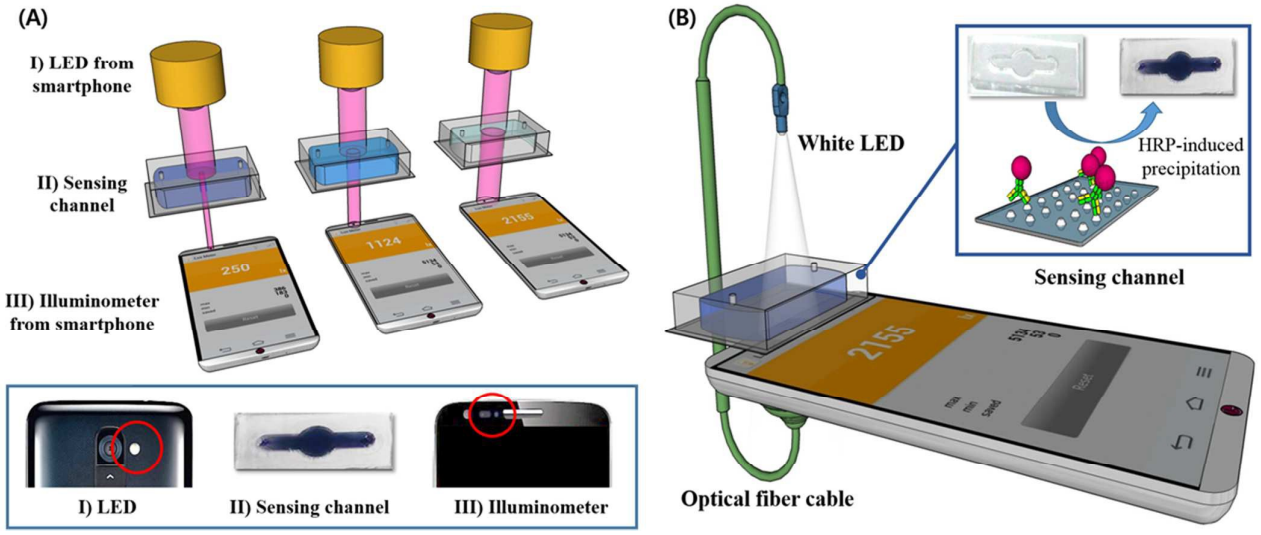

Figure 1 (A) Schematic illustration of the smartphone-based illumination sensor. (I) LED flash from the smartphone. (II) Sensing channel for uCTX-II analysis. (III) Smartphone illuminometer. (B) Configuration of the illumination sensor. The optical fiber was connected to the front of the smartphone to provide the LED flash. The LED was turned ON/OFF by using the mobile application. The intensity of the LED light was analysed by using lux meter application.

The biosensing channel was prepared using polydimethyl siloxane (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 PEG_4-EKGPDP peptide was supplied to the channel for 1 h. This resulted in the covalent immobilization of PEG_4-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 conjugation with a horseradish peroxidase (HRP)-induced precipitation assay. During the biochemical assay, HRP digests H_2O_2 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 flash 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).

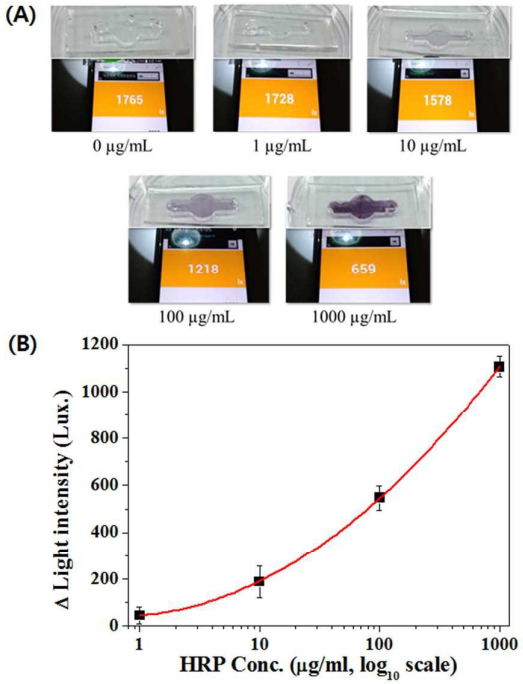

Figure 2 (A) Images obtained from illumination sensor for HRP concentration ranging from 0 to 1,000 µg/mL. (B) Corresponding quantitative analysis of the samples. The assay was performed in triplicate and the error bars indicate the standard deviations.

The ON/OFF function of the LED flash was controlled via operating the flash application, and the variations in light intensity was analysed as a numerical value using the lux meter application. In principle, the intensity of LED light depends on the distance between the light source and the optical receiver. Therefore, to maintain a constant LED light intensity, the optical cable was fixed using common office supplies. As shown in Fig. 1(B), the optical cable connected to the LED flash was installed at a fixed position. The fabricated illumination sensor showed stable LED intensity, and was used for the detection of analytes.

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 H_2O_2 and converted to a blue-coloured precipitate. The HRP concentration was varied from 1 to 1,000 µg/mL and 4-CN solutions were pre-reacted and injected into the sensing channel. Following that, the sensing channel was placed between the LED light and the illumination sensor of a smartphone (model LG-F320L), and the LED light intensity 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.

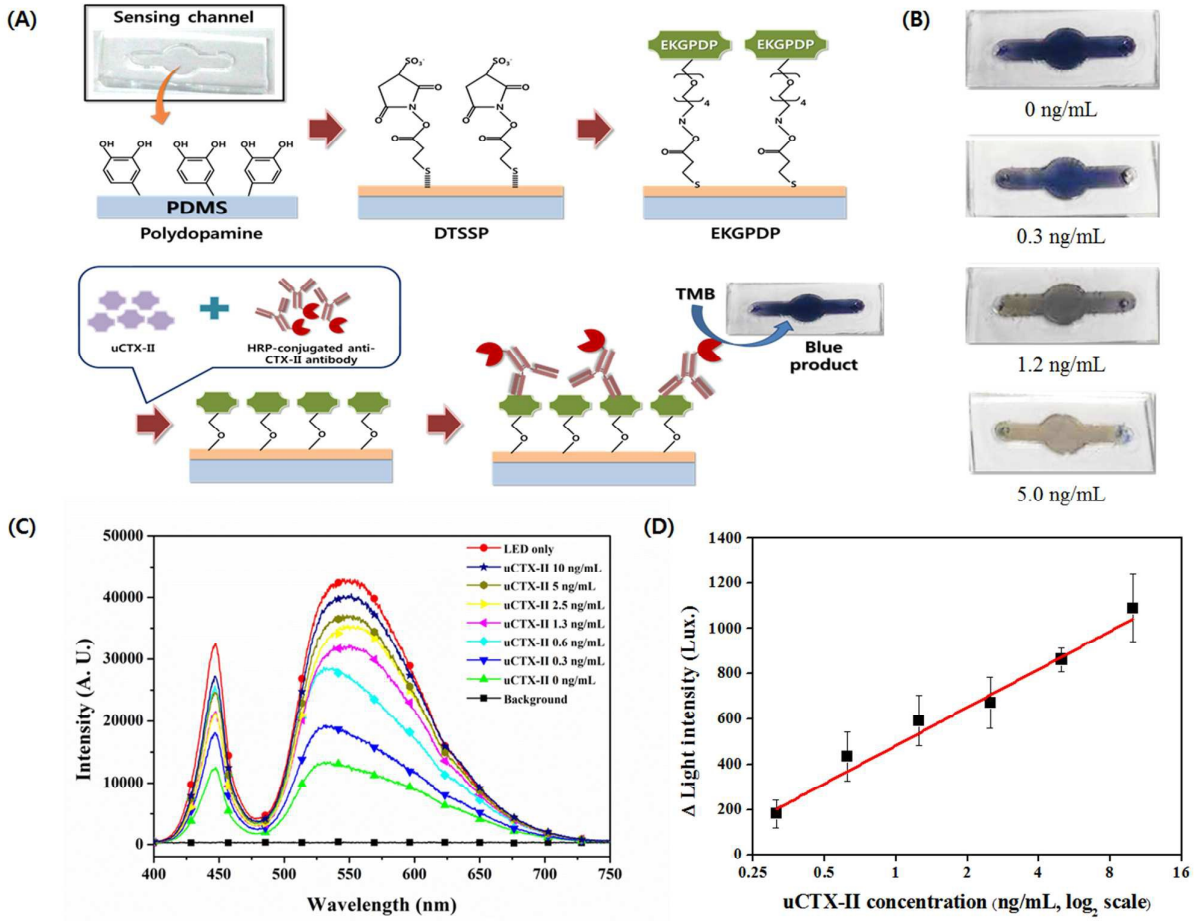

Figure 3 (A) Schematic illustration of the construction of the biosensing channel for uCTX-II competitive immunoassay. The uCTX-II-containing sample was pre-reacted with a solution containing the HRP-conjugated anti-CTX-II antibody; this was applied to the biosensing channel. (B) Photographs obtained for uCTX-II concentration ranging from 0 to 10 ng/mL. (C) Corresponding spectral intensity for the samples. (D) Calibration curve constructed for the uCTX-II assay employing the illumination sensor. Each data point represents the average and standard deviation of independent triplicate assays.

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.

$$\Delta\ Light\ intensity = |lux\ intensity\ \ at\ 0\ \mu g/mL\ of\ HRP - lux\ intensity\ at\ each\ conc.\ of\ HRP|$$

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), uCTX-II contains monomeric or a variant monomeric epitope (EKGPDP).[13,14] Due to its unique structural properties, uCTX-II can be precisely quantified by using competitive immunoassay method. To facilitate this process, PEG_4-EKGPDP, an uCTX-II analogue molecule, was immobilized into the biosensing channel by using polydopamine-mediated surface modification technique.[6,15,16] The pre-reacted solution containing synthesized uCTX-II (EKGPDP) of concentration 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 PEG_4-EKGPDP present on the sensing surface of the channel, in the presence of the target uCTX-II. In this assay, the unreacted uCTX-II antibody was bound to the PEG_4-EKGPDP on the sensing surface. Then, reacted uCTX-II antibody and uCTX-II were washed out from the sensing channel. Upon introducing the TMBM solution, the HRP-induced precipitation reaction is initiated. Fig. 3(A) shows the detailed graphical sketch of the assay scheme.

According to the principle of competitive immunoassay, the quantity of surface-bound antibodies would be inversely proportional to the concentration of uCTX-II. Therefore, we observed a decrease in the formation of blue-coloured precipitate with increase in uCTX-II concentration, as shown in Fig. 3(B). To verify the correlation between the variations in 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 R^2 value for the uCTX-II assay was found to be approximately 5% and 0.98, respectively, indicating 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 deviation 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.

Acknowledgements

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