Thesis for the Degree of Master

Fluorogenic Peptide Incorporated Hydrogel Biosensor for Detection of Gingipains in Early Diagnosis of Periodontitis

by

Yul Lee

Department of Chemical and Biological Engineering

The Graduate School

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Fluorogenic Peptide Incorporated Hydrogel Biosensor for Detection of Gingipains in Early Diagnosis of Periodontitis

This certifies that the degree of M.S. in Chemical and Biological Engineering of

Yul Lee is approved by

_____ (Signature)

Chair of Committee

_____ (Signature)

Committee Member

_____ (Signature)

Committee Member

The Graduate School

Sookmyung Women's University

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LIST OF ABBREVIATIONS

AC-PEG-Mal	Acrylic PEG maleimide
AUC	Area under curve
Dabcyl	4-[4-(Dimethylamino)phenylazobenzoic acid]
Darocur 1173	2-hydroxy-2-methylpropio-phenone
DCM	Dichloromethane
DIPEA	*N,N*-Diisopropylethylamine
DMF	*N,N*-Dimethylformamide
DW	Distilled water
FITC	Fluorescein-isothiocyanate
FRET	Fluorescence resonance energy transfer
LOD	Limit of detection
PBS	Phosphate-buffered saline
PCR	Real-time polymerase chain reaction
PEG	Poly(ethylene) glycol
PEGDA	Poly(ethylene) glycol diacrylate
PI	Photo-initiator
POC	Point-of-care
RFU	Relative fluorescence unit
ROC	Receiver operator characteristic
TMSPMA	3-(trimethoxysilyl)propyl methacrylate
UV	Ultraviolet

ABSTRACT

Fluorogenic Peptide Incorporated Hydrogel Biosensor for Detection of Gingipains in Early Diagnosis of Periodontitis

Yul Lee

Department of Chemical and Biological Engineering

The Graduate School

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Periodontitis, a chronic oral inflammatory disease, affects more than 1 billion people worldwide. Untreated periodontitis has been demonstrated to facilitate the dissemination of oral bacteria throughout the body via the bloodstream, thereby increasing the risk of systemic complications, including cardiovascular disease, stroke, Alzheimer's disease, and rheumatoid arthritis. The early detection of periodontitis and its causative pathogens has emerged as a critical component of oral health and overall physical health. The primary pathogen *Porphyromonas gingivalis* secretes two cysteine proteases, Lysine-gingipain (Kgp) and Arginine-gingipains (RgpA and RgpB), which have the ability to degrade periodontal tissue and modulate the immune response. Conventional diagnostic methods,

such as polymerase chain reaction (PCR), are costly and necessitate specialized personnel. In addition, pH-based techniques provide a limited amount of information.

In this study, we developed a hydrogel-based biosensor for the sensitive and selective detection of Kgp and Rgp. The fluorescence resonance energy transfer (FRET)-based peptide incorporated within the hydrogel emits fluorescence upon cleavage by the target protease. Subsequent to exposure to their respective target proteases, the Kgp, RgpA, and RgpB substrate hydrogels exhibited significant fluorescence responses in a concentration-dependent manner, thereby confirming their substrate specificity. Subsequent evaluation aimed at confirming substrate selectivity revealed that Kgp and RgpB substrate hydrogels, devoid of cross-reactivity, were deemed suitable for utilization as detection sensors. The clinical applicability of the developed hydrogel sensor was further validated using saliva samples from patients with periodontitis and normal individuals.

In summary, the fluorinated peptide substrate hydrogel sensors developed herein provide a rapid and sensitive method for detecting Kgp and RgpB in saliva. These hydrogels offer notable potential as a non-invasive diagnostic tool for the early detection and monitoring of periodontitis.

Keywords: Periodontitis, Gingipains (Kgp, RgpA, RgpB), Hydrogel, Fluorogenic peptide substrate, Biosensor.

I. INTRODUCTION

Periodontitis is among the most prevalent inflammatory diseases in humans, and a chronic inflammatory disease of the oral cavity that affects nearly 50% of the global population [1, 2]. This disease stems from an imbalance in the oral microbiome, known as dysbiosis, and affects individuals of all ages [3]. It manifests in symptoms such as swollen gums, bad breath, painful chewing, deepened periodontal pockets, and tooth loss [4]. Untreated periodontitis can lead to the dissemination of oral bacteria throughout the body via the bloodstream, which has been associated with an increased risk of complications, including cardiovascular disease, stroke, Alzheimer's disease, and rheumatoid arthritis [5, 6]. Early detection of periodontitis and its causative pathogens has become a critical aspect of both oral health and overall physical health. Periodontitis is caused by a variety of bacteria. These bacteria are classified into various complexes based on their degree of pathogenicity in the disease process, including the Aa complex, green complex, yellow complex, orange complex, and red complex [7]. The Red Complex, the most detrimental strain, consists of *Porphyromonas gingivalis, Tannerella forsythia, and Treponema denticola* [8]. Among them, *P. gingivalis*, a Gram-negative, anaerobic rod-shaped bacterium, has been identified as a keystone pathogen of periodontal disease [9, 10]. *P. gingivalis* has been detected in 85% of patients with chronic periodontitis, and it evades the immune response and causes chronic periodontitis through a sustained inflammatory response [10]. Furthermore, *P. gingivalis* has been demonstrated to produce virulence factors, known as gingipain, which is a group of cysteine proteases that can cause tissue destruction by themselves or trigger inflammation through other mediators [11, 12]. Gingipains are classified into two types: lysine-specific (Kgp) and arginine-specific (RgpA

and RgpB) [13]. Kgp is the most potent fibrinogen/fibrin inhibitor, recognizing and specifically cleaving Lys residues, while Rgp recognizes and specifically cleaves Arg residues [14]. Its role in the pathogenesis of periodontitis is pivotal, as it directly cleaves host proteins, such as immunoglobulins and complement factors [15]. In addition to being a evident virulence factor in periodontal disease, gingipains have also been associated with the development of systemic diseases, including Alzheimer's disease (AD) [16]. Recent studies have identified the presence of gingipains in the brain tissue of AD patients, suggesting the potential for a neurodegenerative mechanism resulting from chronic oral infection [16, 17]. These findings underscore the significance of early detection of gingipains, not only for oral health but also for systemic health in a broader sense.

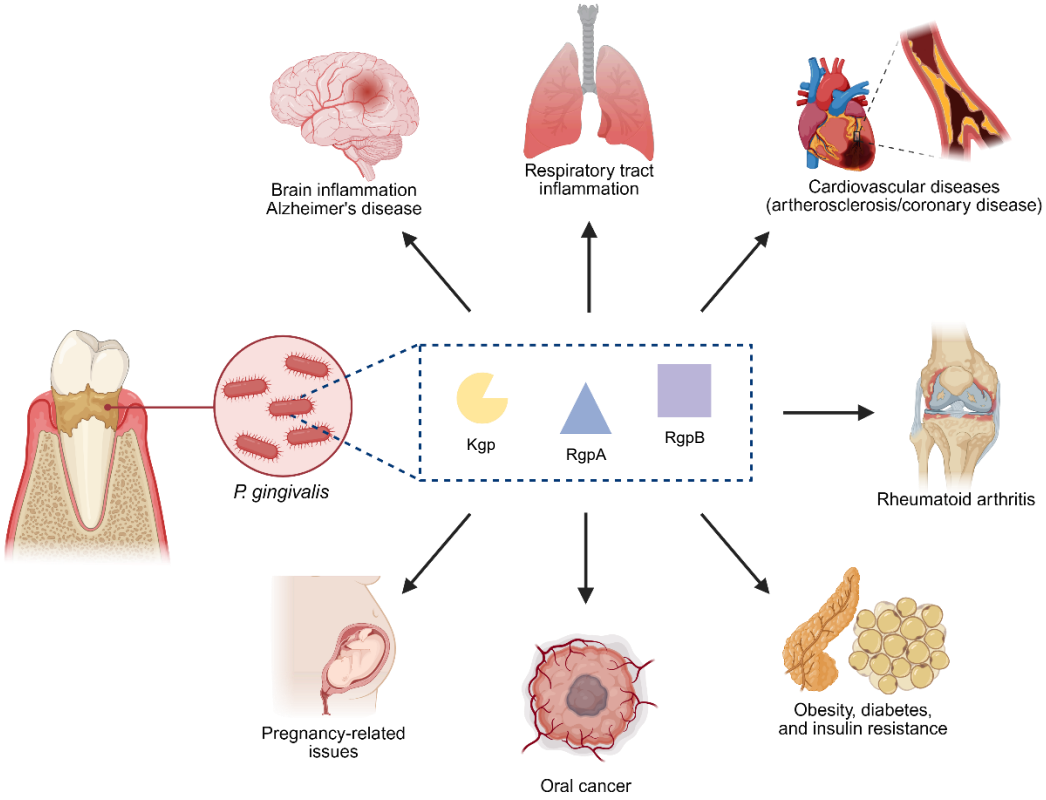
The conventional diagnosis of periodontitis is based on clinical assessments, including periodontal pocket depth, bleeding on probing, clinical attachment level, periodontal index, and gingival index, as well as methods such as radiographic examination, polymerase chain reaction (PCR) testing, and pH-based assays [18, 19]. However, these conventional methods have limitations such as being costly or requiring specialized personnel, reflecting only past evidence of inflammatory changes and not indicating whether they will progress or abate in the future, and lacking the sensitivity to detect disease at an early stage [20]. Consequently, there is an increasing demand for sensors capable of rapidly and accurately diagnosing periodontitis at the point-of-care (POC).

Peptide is promising probes for the *in situ* detection of proteases because their enzymatic cleavage immediately generates a detectable signal [21]. The fluorogenic substrates were designed as peptides containing sequences known to specifically react with Kgp, RgpA, and RgpB, respectively [22, 23]. The peptide substrates were also designed to incorporate a fluorescence resonance energy transfer (FRET) pair using 4-[4-(dimethylamino)phenylazo]benzoic acid (Dabcyl) as the C-terminal quencher and fluorescein isothiocyanate (FITC) as the N-terminal fluorophore to generate a real-time

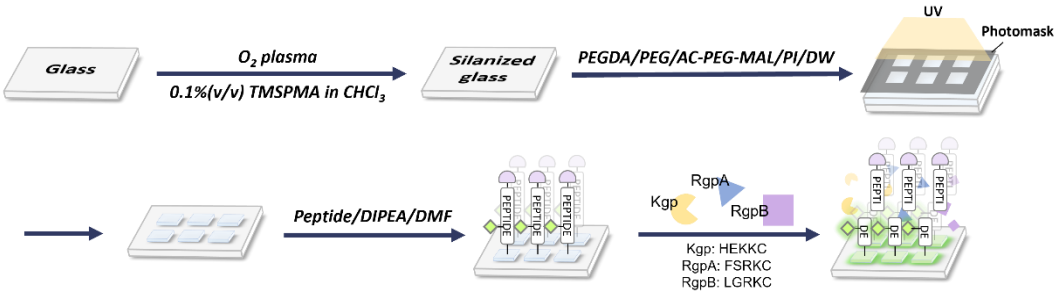
fluorescence signal upon protease cleavage. In the quenched state, fluorophores exhibit negligible fluorescence emission due to energy transfer to the quencher [24, 25]. However, during enzymatic hydrolysis, the spatial separation between the fluorophore and quencher leads to a significant increase in fluorescence intensity [25]. This fluorescence change indicates successful proteolytic cleavage of the peptide. When these fluorogenic peptides are incorporated into a hydrogel matrix, they form a functional biosensing platform capable of detecting enzyme activity in a spatially confined and controlled environment. [26, 27]. Hydrogels are three-dimensional polymeric networks characterized by high water retention and tissue-like physical properties, which contribute to their excellent biocompatibility [28, 29]. In addition, they provide an ideal platform for the effective immobilization of biomolecules, such as peptides and antibodies, thereby enabling the sensitive and selective detection of specific analytes [29, 30]. These properties have led to the hydrogels a widely used materials in biosensor development and tissue engineering applications. Among these, polyethylene glycol (PEG)-based hydrogels have garnered significant attention due to their exceptional biocompatibility and capacity to diminish non-specific protein adsorption [31-34]. Furthermore, due to their remarkable mechanical and chemical stability, a variety of functional groups can be incorporated at the PEG terminus to bind peptides, fluorophores, and other molecules [32]. Their substantial water content is advantageous in preserving the activity rate of enzymes or cells under conditions that mimic the biological environment [33]. PEG hydrogels are characterized by high sensitivity and specificity in detecting proteases, rendering them suitable for POC diagnostics [34].

In this study, we developed a FRET-peptide incorporated hydrogel biosensor that targets gingipains secreted by *P. gingivalis* for the early detection of periodontitis. The sensor was fabricated by conjugating the maleimide end group of the PEG hydrogel with the cysteine residue of a fluorogenic peptide substrate [35]. The property of FRET-peptide to emit fluorescence upon proteolysis by proteases allows for sensitive and specific

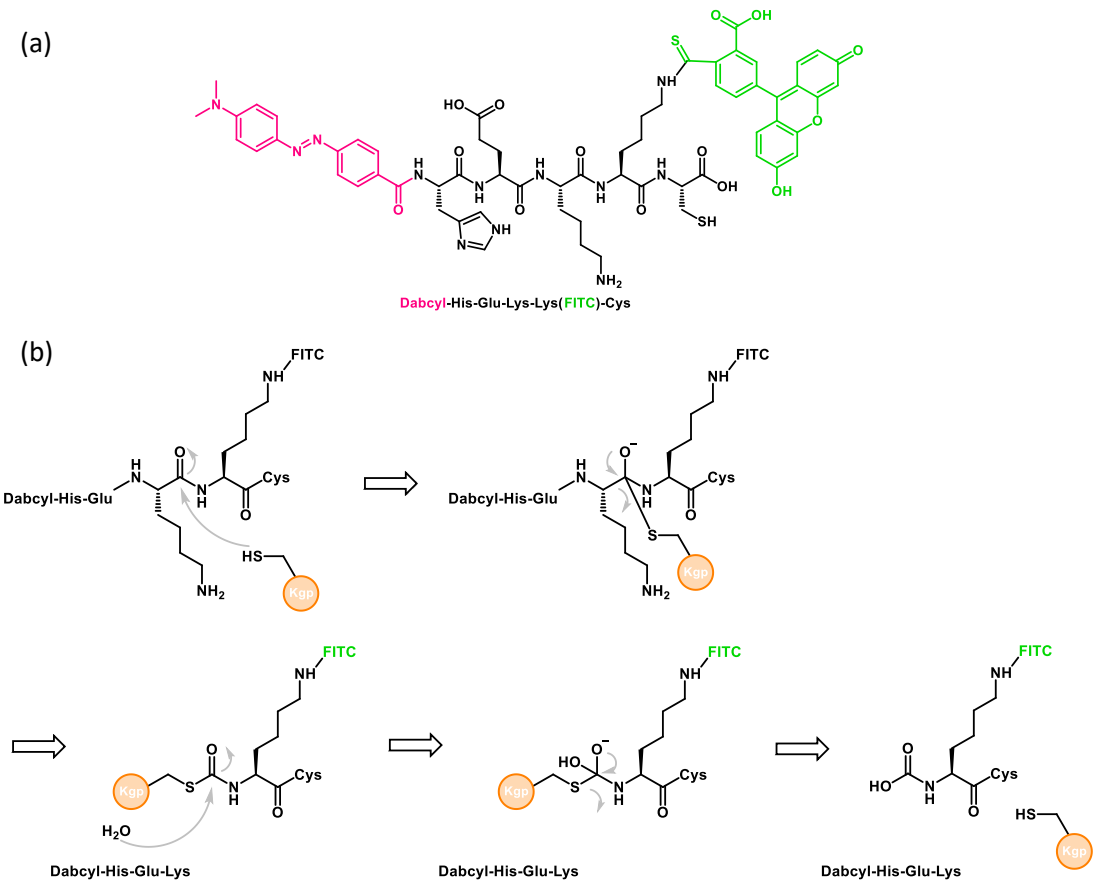
detection of the target protease [36]. Compared to previous solution-phase reactions, immobilizing peptides onto the hydrogel matrix has been shown to increase the available surface area, thereby enhancing enzyme–substrate interactions and enabling more rapid and sensitive detection [37]. Furthermore, the immobilization of peptides within the hydrogel matrix has been demonstrated to enhance their chemical stability [38]. This is achieved by reducing degradation and non-specific interactions, thereby rendering them suitable for extended storage and POC applications. To evaluate the sensitivity and enzymatic kinetics of the sensor, various concentrations of gingipains (Kgp, RgpA, and RgpB) were applied onto the hydrogels embedded with each fluorogenic peptide substrate, and the fluorescence intensity was measured over time. Substrate specificity was assessed by exposing each substrate to non-targeting gingipains, and clinical validation was also performed using saliva samples from 15 patients diagnosed with periodontitis and 15 healthy individuals. In this study, we propose a novel FRET-peptide incorporated hydrogel biosensor for the early diagnosis of periodontitis by targeting gingipains. This sensor is expected to serve as a sensitive and reliable platform for non-invasive detection in clinical applications.



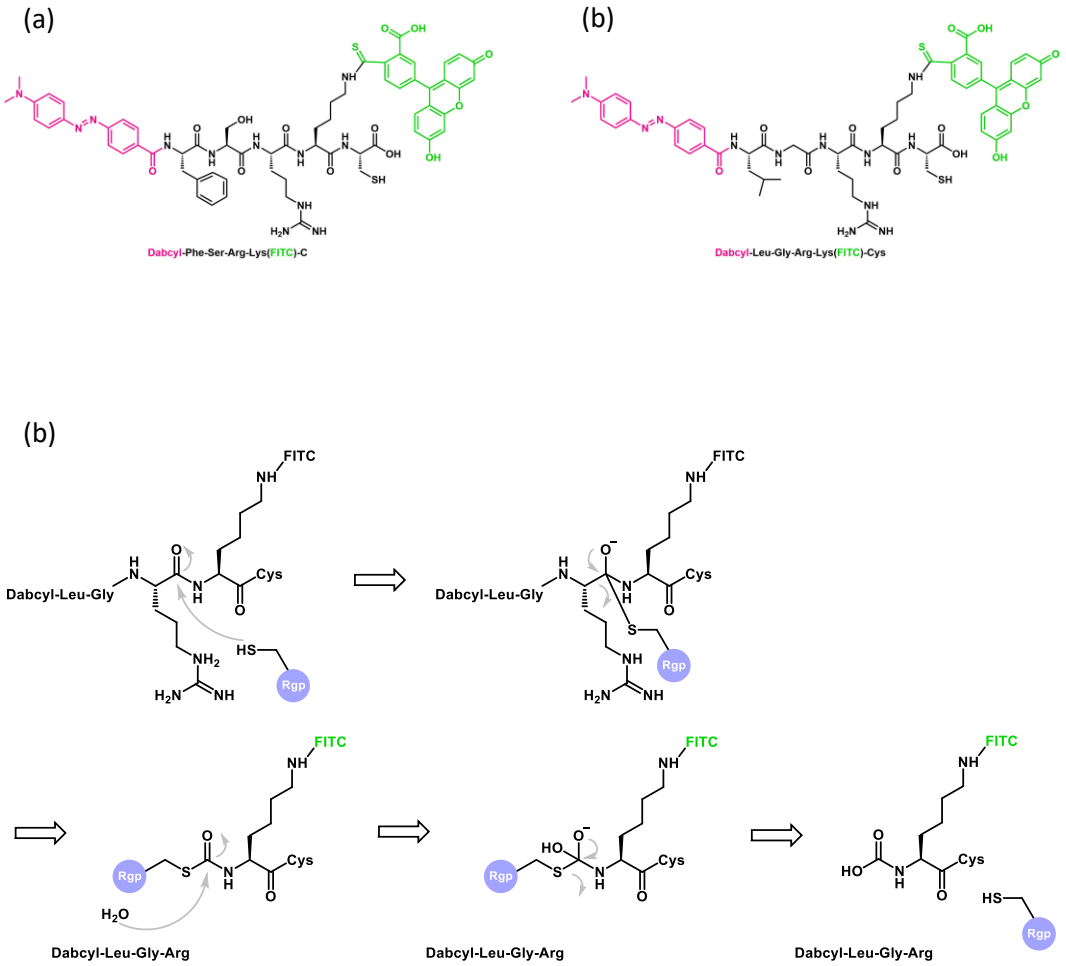
Scheme 1. Overview of *P. gingivalis* derived proteases and their roles in systemic diseases



Scheme 2. Preparation of FRET-peptide incorporated hydrogel patterns and sensing mechanism of gingipains.



Scheme 3. Mechanism of peptide cleavage by Kgp. (a) General substrate of Kgp. (b) Specific cleavage of Lys-Xaa peptide in the presence of Kgp.



Scheme 4. Mechanism of peptide cleavage by Rgp. (a) General substrate of RgpA. (b) General substrate of RgpB. (c) Specific cleavage of Arg-Xaa peptide in the presence of RgpB.

II. MATERIALS AND METHODS

1. Materials and Instruments

CUSABIO (Wuhan, China) supplied the fluorogenic peptide Dabcyl-His-Glu-Lys-Lys (FITC)-Cys-OH (Dabcyl-HEKK(FITC)C), Dabcyl-Phe-Ser-Arg-Lys (FITC)-Cys-OH (Dabcyl-FSRK(FITC)C) and Dabcyl-Leu-Gly-Arg-Lys (FITC)-Cys-OH (Dabycl-LGRK(FITC)C). The phosphate-buffered saline (PBS) was obtained from BIOWEST (Nuaillé, France). MyBioSource (San Diego, CA, USA) provided the Lys-gingipain (Kgp) recombinant protein (MW = 56.6 kDa), Gingipain R1 (RgpA) recombinant protein (MW = 56 kDa), and Gingipain R2 (RgpB) recombinant protein (MW = 43.3 kDa). Poly(ethylene glycol) diacrylate (PEGDA, MW 700), PEG (MW 200), N,N-diisopropylethylamine (DIPEA), dichloromethane (DCM), N,N-dimethylformamide (DMF), 3-(trimethoxysilyl)propyl methacrylate, chloroform, and the photoinitiator 2-hydroxy-2-methylpropiophenone (Darocur 1173) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acrylic PEG maleimide (AC-PEG-Mal, MW 3400) was supplied by Laysan Bio (Arab, AL, USA). The glass slides were purchased from Herenz (Hamburg, Germany). Sterile syringe filters (25CS45AS) were provided by ADVANTEC (Tokyo, Japan). Fluorescence signal was measured using a fluorescence microscope (Axio Observer 7, Carl Zeiss, Jena, Germany).

2. Fabrication of micropatterned functional hydrogels

The glass slides were treated for 6 min at 100 W to an oxygen plasma chamber (CUTE,

Gyeonggi-do, Korea) and followed by incubation in 0.1% (v/v) 3-(trimethoxysilyl) propyl methacrylate in chloroform for 2 h to modify the surface. The acrylic silane-modified slides were washed with DCM and dried in nitrogen gas with nitrogen for immediate utilization or stored in a desiccator until use. The micropatterned hydrogels were fabricated photolithographically via UV-initiated polymerization of PEGDA and AC-PEG-MAL on acrylic silaned glass slides. The precursor solution for hydrogel patterning was prepared by dissolving 20% (v/v) PEGDA (MW 700), 40% (v/v) PEG (MW 200), 10% (v/v) 20 mM AC-PEG-Mal, and 1% (v/v) Darocur 1173 in 3' Distilled water (DW). The precursor solution was pipetted onto the glass substrates and covered with a cover glass (18 mm × 18 mm). A photomask with dimensions of 50 μm × 50 μm for each pattern was placed on the glass substrates and exposed to UV light (4500 mW/cm², LC8 (L9566), Hamamatsu, Shizuoka, Japan) at a wavelength of 365 nanometers for 0.5 sec. The glass substrates were then washed with DW and dried under nitrogen gas.

3. Preparation of FRET-peptide incorporated hydrogels

Peptide substrates specific to Kgp, RgpA, and RgpB were dissolved in DMF to prepare 4 mM stock solutions. To enhance the efficacy of the conjugation reaction of the peptide solution, 2 mM of DIPEA was added to the peptide solution. Subsequently, the hydrogel micropatterns were incubated with the peptide solution for a period of two hours at ambient temperature. During this process, covalent bonding was established between the thiol group of the cysteine residue and the maleimide group functionalized on the hydrogel network, enabling stable immobilization of the peptides onto the hydrogel surface [39]. After the incubation, the surface of hydrogels was rinsed with PBS and DW to eliminate unbound peptides.

4. Measurement of fluorescence intensity through proteolytic cleavage

The FRET-peptide sequence Dabcyl-His-Glu-Lys-Lys (FITC)-Cys could be cleaved between Lys and Lys by Kgp, which reacts specifically with Lys. Furthermore, Dabcyl-Phe-Ser -Arg-Lys (FITC)-Cys and Dabcyl- Leu-Gly-Arg -Lys (FITC)-Cys could be cleaved between Arg and Lys by Rgp, which reacts specifically with Arg. Upon cleavage of the FRET-peptide by a protease, the fluorescence of the hydrogel is observed to increase. Recombinant Kgp, RgpA, and RgpB were serially diluted in PBS to create protease solutions at various concentrations (0.3125, 0.625, 1.25, 2.5, and 5 μg/mL). The hydrogels were dropped with 10uL of a protease solution that specifically reacts with the FRET-peptide conjugated hydrogels. Subsequently, the hydrogels were covered with a cover glass, and the cleavage reaction of the peptides immobilized in the hydrogel was observed using a fluorescence microscope. The fluorescence of the sensor was measured using a FITC excitation/emission filter at 469 ± 35 nm/525 ± 39 nm. Fluorescence images were acquired using a Gen Pro software (Carl Zeiss, Jena, Germany) and subsequently analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The relative fluorescence intensity (RFU) was derived by subtracting the background fluorescence intensity from the fluorescence intensity of the hydrogel.

5. Evaluation of the specificity of FRET-peptide hydrogel

The RFU was measured by dropping low concentrations (0.3125, 0.625, and 1.25 μg/mL) of Kgp onto the Kgp substrate hydrogels. In addition, PBS was utilized as a negative control to ensure that any observed fluorescence was not due to non-specific interactions. To investigate potential cross-reactivity, 5 μg/mL of RgpA and RgpB were dropped onto the Kgp substrate hydrogels, respectively. Furthermore, the cross-reactivity of RgpA and RgpB substrate hydrogels was thoroughly assessed using the same method in

the Kgp substrate hydrogels experiment.

6. Detection of gingipain activity in human saliva samples using FRET-peptide hydrogels

Human saliva samples were obtained from 30 individuals, including 15 patients with periodontitis and 15 healthy controls. These samples were stored in a deep freezer until use. The sample was filtered using a 0.22 μm sterile syringe filter to effectively remove cell debris and other particulates. A volume of 10 μL of the filtered samples was dropped to the Kgp and RgpB substrate hydrogels, respectively. Subsequently, the RFU was measured at 5 min intervals for a duration of 30 min.

III. RESULTS AND DISCUSSION

1. Fluorescence response of Kgp substrate hydrogels through proteolytic cleavage

The detection ability of the FRET-peptide hydrogel biosensor for Kgp was quantitatively evaluated by acquiring temporal fluorescence images of the micropatterned hydrogels. To this end, a series of fluorescence images of the hydrogel were obtained over time using an automated fluorescence microscopy apparatus. Subsequently, a quantitative fluorescence analysis was performed on the acquired images. In this experiment, the concentration of the protease and the reaction time were systematically set as the primary variables that can affect the fluorescence intensity. A thorough analysis was conducted to examine the impact of both variables on the response of the biosensor.

As demonstrated in Fig. 1A, the examination revealed a swift escalation in RFU within the initial 5 min for all concentration categories. Subsequent to this initial observation, a period of stable fluorescence intensity ensued. This rapid saturation pattern suggests that the Kgp-specific peptides in the hydrogels were efficiently and rapidly cleaved by Kgp, and that most of the enzymatic reaction was completed early, regardless of the concentration used. The fluorescence reactivity with increasing concentrations of Kgp was also evident. As the concentration of Kgp increased, RFU values emitted from the hydrogels exhibited a tendency to increase as well, indicating that the Kgp-specific peptide is cleaved by the enzyme and the fluorescence signal it emits is directly proportional to the amount of Kgp present. However, it was also observed that with increasing concentration, the difference in fluorescence intensity between concentrations was progressively relaxed

due to the saturation effect of the enzyme and substrate. The findings indicate that the hydrogel-based sensor exhibits the capacity to detect Kgp over a substantial concentration range, suggesting that it exhibits enhanced sensitivity, particularly at low concentrations.

To quantitatively assess this, a concentration-RFU curve was derived based on RFU values measured after incubating the hydrogels with different concentrations of Kgp for 10 min (Fig. 1C). An excellent linear correlation ($R^2 = 0.9651$) was identified in the low concentration range of 0.3125-1.25 μg/mL, indicating the biosensor's sensitivity to changes in Kgp concentration within this range and its capacity to provide stable and reliable detection results. These results demonstrated the potential for the sensor to assess recombinant Kgp concentrations.

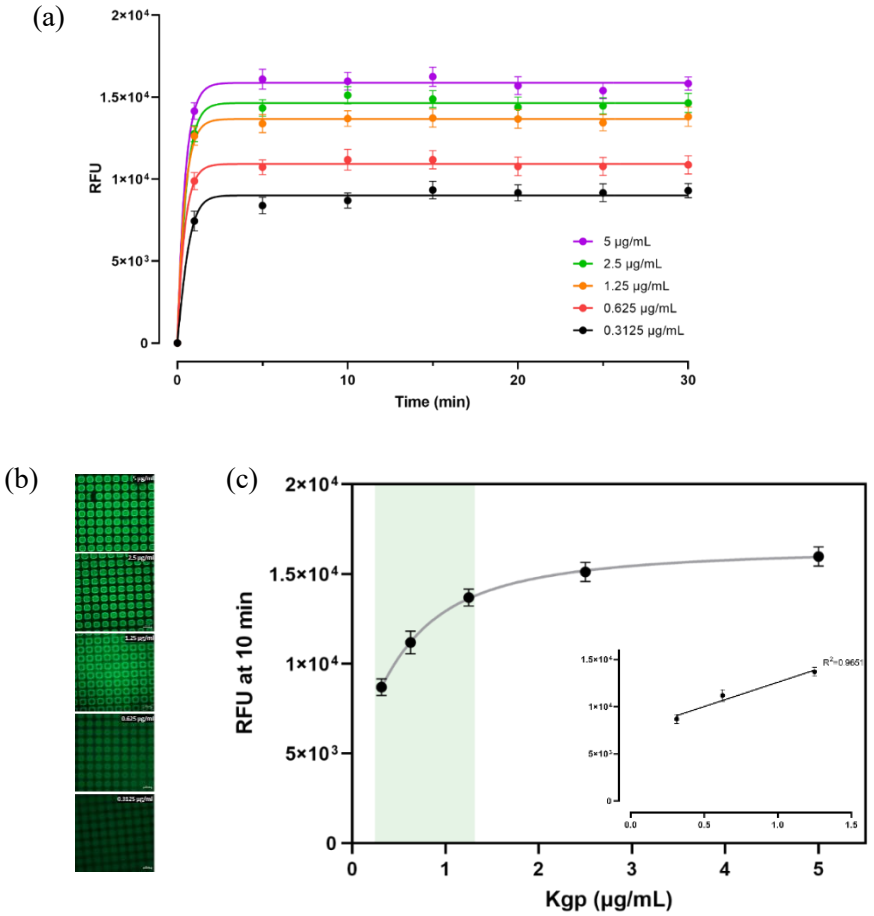


Figure 1. Fluorescence response of Kgp substrate hydrogels exposed to Kgp. (a) Fluorescence intensity over time. (b) Fluorescence images acquired after 10 min incubation with Kgp (each spot: 50 μm × 50 μm). (c) RFU values of the Kgp substrate hydrogels after 10 min incubation with Kgp.

2. Specificity of Kgp substrate hydrogels against other gingipains

To assess the specificity of the developed the Kgp-specific peptide hydrogel biosensor for Kgp detection, its fluorescence response was compared with those of other gingipains. The Kgp substrate hydrogels were incubated with low concentrations (0.3125–1.25 μg/mL) of Kgp and 5 μg/mL of RgpA and RgpB, respectively, followed by measurement of fluorescence intensity changes after 15 min (Fig. 2). A substantial increase in RFU was observed exclusively in the Kgp treated group, whereas the RgpA and RgpB treated groups exhibited negligible fluorescence signals, comparable to the enzyme-free control. These results indicate that the Kgp substrate is specifically cleaved by Kgp, leading to fluorescence expression. Moreover, RFU values in the RgpA and RgpB treated groups were approximately 4.6- and 5.3-fold lower than those in the Kgp treated group at 1.25 μg/mL, respectively, suggesting structural resistance of the Kgp substrate hydrogels to nonspecific cleavage by Rgp. This high selectivity demonstrates the hydrogel's ability to sensitively detect Kgp activity alone and supports its potential for selective detection of Kgp in complex biological samples containing multiple proteases.

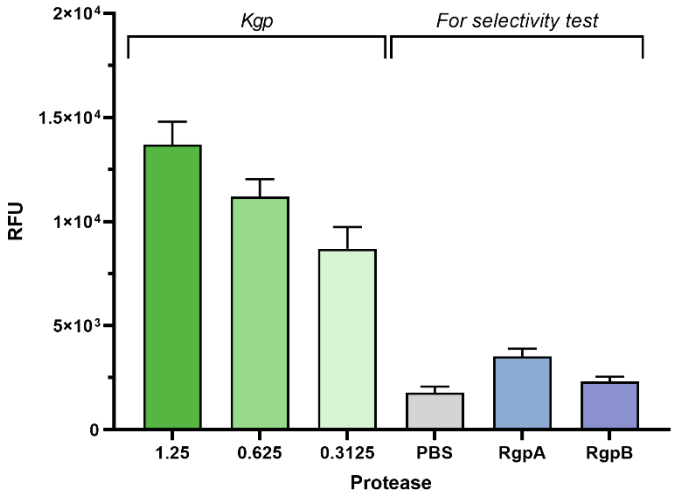


Figure 2. Evaluation of protease specificity of Kgp substrate hydrogels after 15 min incubation.

3. Fluorescence response of Rgp substrate hydrogels through proteolytic cleavage

Peptide sequences specific to RgpA and RgpB were incorporated into hydrogels, and the response characteristics and suitability of each sensor as a diagnostic target were evaluated by comparing the fluorescence expression as a function of time and concentration.

To evaluate the protease concentration-dependent fluorescence intensity of the RgpA and RgpB substrate hydrogels, each hydrogel was exposed to 0.3125–5 μg/mL of protease, and the RFU change over time was measured. The experimental protocols, including measurement times and intervals, were consistent with those previously described for Kgp.

An increase in the fluorescence intensity of the RgpA substrate hydrogels was observed at the 10 min reaction time point, which was concentration-dependent (Fig. 3A). The low concentration range (0.3125-1.25 μg/mL) exhibited discernible variations in fluorescence intensity, but the slope of signal amplification between concentrations was modest.

For the RgpB substrate hydrogels, a concentration-dependent increase in RFU was observed at the 15 min reaction time point (Fig. 3B). The RgpB substrate hydrogels exhibited sensitivity in the low concentration range comparable to that of the RgpA substrate hydrogels. The response patterns of both hydrogels converged to a saturation curve at higher concentrations (2.5–5 μg/mL), contrasting with their behavior at relatively low concentrations. The enzyme-substrate reaction of the Rgp substrate is hypothesized to proceed rapidly, analogous to that of the Kgp substrate, indicating the potential utility of this sensor in clinical settings where rapid detection is critical.

A quantitative sensitivity analysis was conducted, which entailed deriving concentration-response curves. These curves were derived based on RFU values at the 10

min and 15 min response time points for RgpA and RgpB, respectively. The linearity of both substrates was observed within the low concentration range of 0.3125-1.25 μg/mL. The RgpA sensor showed relatively good linearity, with an R^2 value of 0.9517. However, the RgpB sensor demonstrated a higher degree of correlation, with an R^2 value of 0.9959. The results suggest that the RgpB sensor may serve as a more precise indicator of alterations in fluorescence signal intensity. In addition, the RgpB substrate hydrogels exhibited a higher overall fluorescence intensity in comparison with the RgpA substrate hydrogels, which is consistent with the previously reported high activity and substrate affinity properties of RgpB. These findings support the conclusion that the RgpB sensor is more suitable for accurate quantification in biosensing applications.

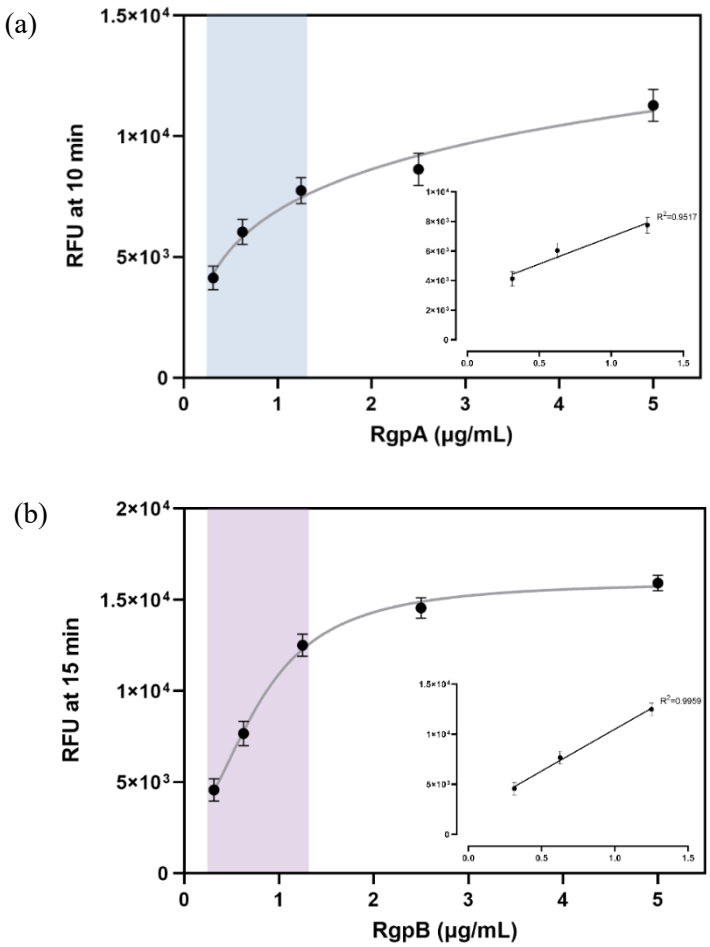


Figure 3. Fluorescence response of Rgp substrate hydrogels exposed to target proteases. (a) RFU of the RgpA substrate hydrogels after 10 min incubation with RgpA. (b) RFU of the RgpB substrate hydrogels after 15 min incubation with RgpB.

4. Specificity and sensor suitability of RgpA and RgpB substrate hydrogels

The performance of the RgpA and RgpB specific peptide hydrogel sensor was determined by evaluating the selectivity of substrates for target proteases. Low concentrations (0.3125- 1.25 μg/mL) of RgpA and 5 μg/mL of Kgp and RgpB, respectively, were added to the RgpA-specific peptide incorporated hydrogels. The change in fluorescence intensity was measured after 10 min. When the RgpA substrate hydrogels reacted with low concentrations of RgpA, a modest increase in RFU was observed with increasing protease concentration (Fig. 4A). The Kgp treated group exhibited a comparable level of fluorescence signal to the control group without protease. Conversely, the group exposed to RgpB exhibited nonspecific fluorescence levels more than twofold higher than those demonstrated in the group exposed to 1.25 μg/mL of RgpA. This suggests that the RgpA substrate reacts with both RgpB and RgpA, resulting in a cross-reactivity.

In accordance with the preceding experiment, low concentrations (0.3125–1.25 μg/mL) of RgpB, as well as 5 μg/mL Kgp and RgpA added into the RgpB-specific peptide incorporated hydrogel. Subsequently, the alterations in the RFU were measured after a 15 min incubation. The RgpB substrate hydrogels exhibited a distinct increase in RFU values that was proportional to the RgpB concentration (Fig. 4B). In contrast, the Kgp and RgpA treated groups demonstrated fluorescence signals that were comparable to the control group without protease. Despite the protease concentration in the RgpB treated group being more than fourfold lower than that in the Kgp and RgpA treated groups, the resulting fluorescence intensity was over threefold higher. These results suggest that the Rgp substrate exhibits distinct specificity for the target protease and can effectively distinguish the activity of RgpB from that of other gingipains.

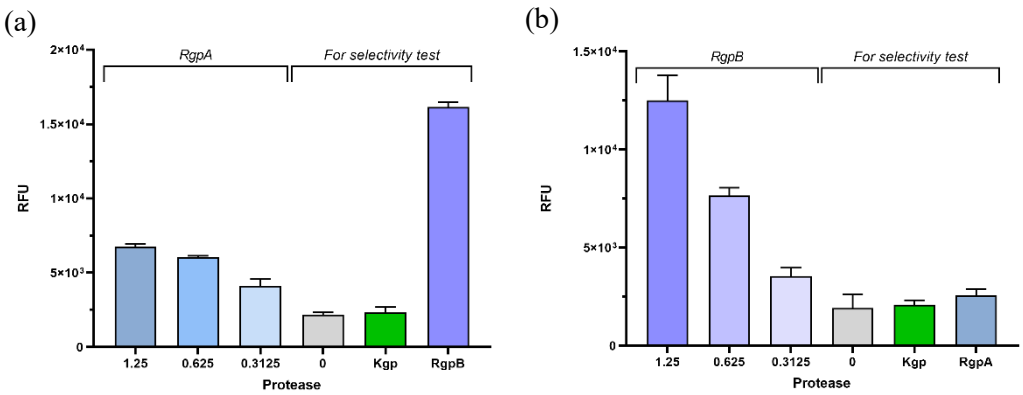


Figure 4. Evaluation of protease specificity of Rgp substrate hydrogels after 15 min incubation. (a) RFU of the RgpA substrate hydrogels. (b) RFU of the RgpB substrate hydrogels.

5. Clinical evaluation of FRET-peptide hydrogels using saliva samples

The clinical applicability of the developed FRET-peptide hydrogel sensor was determined by applying saliva samples from 15 subjects diagnosed with periodontitis and 15 normal subjects. The objective of this study was to validate the diagnostic potential of the sensor in distinguishing between periodontitis patients and normal controls.

Each saliva sample was filtered using a filtered syringe, and the supernatant was extracted. Subsequently, 10 μL of the sample solution was dropped onto each of the Kgp and RgpB substrate hydrogels, and the fluorescence was subsequently measured after a 15 min incubation. For the Kgp substrate hydrogels, a higher fluorescence response was observed in patient subjects compared to normal subjects (Fig. 5A). The Kgp-specific peptide incorporated hydrogels enabled the detection of Kgp in saliva samples containing various proteases, demonstrating their selectivity toward the target protease.

Consistent with the result for the Kgp substrate hydrogels, a disparity between the groups was evident for the RgpB substrate hydrogels (Fig. 5B). The patient group demonstrated a substantial increase in fluorescence signal, suggesting consistent RgpB activity with a relatively uniform distribution. These findings suggest that the Rgp-specific peptide substrate hydrogel also exhibited high RgpB selectivity in real saliva samples.

Receiver Operating Characteristic (ROC) curve analysis was conducted to quantitatively evaluate the diagnostic performance of the FRET-peptide incorporated hydrogels, with the area under the curve (AUC) used as a quantitative measure of diagnostic accuracy. Clinical assay results demonstrated excellent diagnostic performance, with AUC values of 0.9867 and 0.9911 for the Kgp and RgpB substrate hydrogels, respectively (Fig. 6). Both AUC values exceeded 0.65, and their p-values were less than 0.05. The findings suggest that both the Kgp and RgpB substrate hydrogel sensors exhibit

considerable efficacy as diagnostic tools, facilitating the differentiation of periodontitis cases from normal controls.

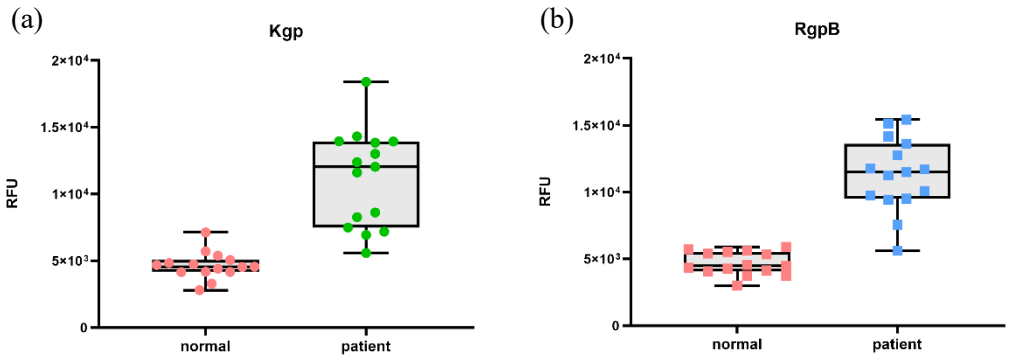


Figure 5. Box and whisker plot of RFU values from saliva samples of the periodontitis group (n = 15) and the normal group (n = 15). (a) RFU of the Kgp substrate hydrogels. (b) RFU of the RgpB substrate hydrogels.

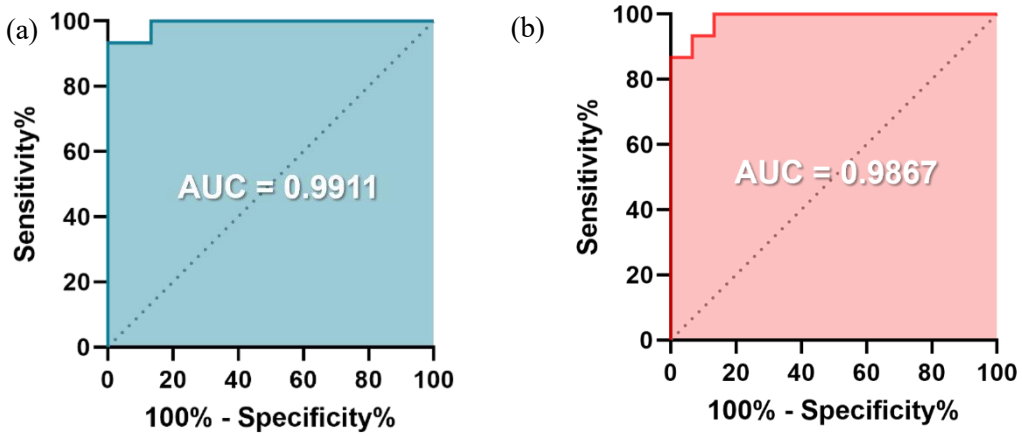


Figure 6. ROC curves of FRET-peptide hydrogels using saliva samples. (a) Kgp substrate hydrogels. (b) RgpB substrate hydrogels.

IV. CONCLUSION

In this study, we developed and evaluated a fluorogenic peptide substrate hydrogel-based biosensor platform targeting gingipains, major proteases secreted by *P. gingivalis*, for the early and non-invasive diagnosis of periodontitis. This hydrogel sensor utilized FRET-peptide hydrogels that generated a fluorescence signal upon proteolysis by gingipains, enabling a simple, rapid, and sensitive detection strategy.

Hydrogels incorporating Kgp, RgpA, and RgpB substrates exhibited distinct concentration-dependent fluorescence responses upon exposure to their respective target proteases, thereby confirming their substrate specificity. Furthermore, it was observed that the reaction occurred promptly in all hydrogels, reaching saturation within 10-15 min. The findings suggest that the sensor is suitable for POC applications. In an evaluation to confirm substrate selectivity, the RgpA substrate hydrogel was found to react not only with RgpA but also with RgpB. In contrast, the Kgp and RgpB substrate hydrogels exhibited no cross-reactivity with other gingipains and selectively reacted with the target protease, thereby confirming the substrate's selectivity. Consequently, it was determined that Kgp- and RgpB-specific hydrogels are suitable for the development of biosensors intended for the expeditious detection of Kgp and RgpB. The clinical applicability of the developed hydrogel sensor was further validated using saliva samples from patients with periodontitis and normal subjects. Both the Kgp-specific and RgpB-specific hydrogels successfully distinguished patient subjects from normal controls. ROC curve analyses confirmed the high diagnostic performance of both hydrogel sensors, achieving an AUC of 1.0, indicative of perfect classification accuracy.

In conclusion, the fluorogenic peptide substrate hydrogel developed in this study provides a rapid and sensitive method for detecting Kgp and RgpB in saliva, offering significant potential as a non-invasive diagnostic tool for early detection and monitoring of periodontitis.

V. REFERENCES

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ABSTRACT IN KOREAN

치주염 조기 진단을 위한 진지페인 검출용 형광 펩타이드 하이드로겔 바이오센서

이율

숙명여자대학교 대학원 화공생명공학과 화공생명공학 전공

만성 구강 염증성 질환인 치주염은 전 세계적으로 10 억 명 이상이 앓고 있으며, 치료하지 않고 방치할 경우 구강 내 세균이 혈류를 통해 전신으로 확산되어 심혈관 질환, 뇌졸중, 알츠하이머병 등 다양한 전신 질환의 발병 위험을 높이는 것으로 알려져 있다. 주요 원인균인 *포르피로모나스 진지발리스(Porphyromonas gingivalis)*는 치주 조직을 분해하고 면역 반응을 조절하는 단백질 분해효소인 라이신-진지페인 (Kgp), 아르기닌-진지페인 (RgpA, RgpB)를 분비한다. 이러한 진지페인(gingipain)의 민감하고 특이적인 검출은 치주염의 조기 진단과 치료에 핵심적이다. 그러나 기존의 치주염 진단 방법 중 PCR 기반 진단법은 고가이며 숙련된 인력이 필요하고, pH 기반 기술은 제한된 정보를 제공한다는 한계가 있다. 본 연구에서는 형광 공명 에너지 전달(FRET) 기반 펩타이드를 하이드로겔 지지체에 고정화하여,

Kgp 및 Rgp 의 신속하고 선택적인 검출을 구현하고자 하였다. 각각의 진지페인 특이적 하이드로겔 센서는 표적 진지페인에 노출되었을 때 농도 의존적으로 형광 반응을 보여 기질 특이성을 확인하였다. 기질 선택성을 확인하기 위한 평가에서 교차 반응이 없는 Kgp 와 RgpB 특이적 하이드로겔이 진지페인 검출 센서로 적합하다는 것을 확인하였다. 또한, 실제 치주염 환자 및 건강한 대조군의 타액 샘플을 사용한 임상 실험을 통해, 개발된 센서의 진단적 유효성과 적용 가능성을 추가로 검증하였다.

본 연구의 하이드로겔 기반 바이오센서는 특정 단백질 분해효소에 의해 절단된 펩타이드로부터 발생하는 형광 신호를 분석함으로써, 치주염의 초기 단계를 감지하고, 더 나아가 관련 합병증 예방에 기여할 수 있을 것으로 기대된다.

주제어: 치주염, 진지페인 (Kgp, RgpA, RgpB), 하이드로겔, 형광 펩타이드 기질, 바이오센서.