Advancements in Instrumentation

Development of a Membrane-based Microwave-mediated Electrochemical ELISA Method for TNF-α Detection in Patients with Periodontitis

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The tumor necrosis factor-α (TNF-α) is implicated in periodontal disease, and there was an attempt to quantitate it by a membrane-based microwave-mediated electrochemical enzyme-linked immunosorbent assay (MMeELISA) using *p*-aminophenyl phosphate (pAPP) with an over-all time of 1.5 h. A differential pulse voltammetric signal increased linearly with an increase in the amount of TNF-α with a detection limit of 0.48 pg mL^{-1}. This bio-sensing platform revealed a significant difference in the TNF-α level between GCF samples from periodontal patients and healthy volunteers.

Keywords Membrane-based microwave-mediated electrochemical enzyme-linked immunosorbent assay (MMeELISA), tumor necrosis factor-α (TNF-α), gingival crevicular fluid (GCF), periodontal disease

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Introduction

Periodontitis is a chronic inflammatory disease characterized by a constant interaction between pathogenic bacteria and the host defense mechanisms, which eventually leads to periodontal tissue destruction and tooth loss.[1] More recently, it is now understood through several studies done in gingival crevicular fluid (GCF) samples that the immune and inflammatory responses mediated by pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interferon-γ, interleukin-1β, interleukin-12 and interleukin-6 among others, are critical to the pathogenesis of periodontitis.[2,3] In this regard, increasing attention is now being focused on assessing how the cytokine profile correlates with the extent of periodontal destruction, and helps in the early diagnosis and effective treatment of periodontal disease.

Currently, the antibody-based, enzyme-linked immunosorbent assay (ELISA) represents the gold standard in cytokine detection,[4] because it provides high specificity and high sensitivity *via* signal amplification. However, typical sandwich-type ELISA requires a multistep process of mixing, washing and incubation, the entirety of which is both complicated and time consuming. Over the years, several approaches have been reported in the literature that focuses on improving the ELISA sensitivity, and to shorten the reaction procedure. For example, a variety of solid supports have been tested and developed to enhance the specific capture of target analytes and to lessen the sample volume requirement. Along the same line, several studies have also reported the use of elevated temperature, like polycarbonate plates in thermo cycler, or the use of microwave irradiation on activated polystyrene micro titer plates to shorten the ELISA procedure. Moreover, several detection methods, ranging from fluorescence to electrochemical biosensors, have been integrated to this immunoassay technique that warrants large improvements in the detectability of the enzyme reaction product.[5–7]

In this study, we described an alternative approach to the ELISA technique wherein we demonstrate a practical and ultra-fast membrane-based microwave mediated electrochemical ELISA (MMeELISA) to specifically detect TNF-α, one of the biomarkers involved in periodontitis.

While the literature on the electrochemical immunosensor platform has grown rapidly in recent years, their use as a biochemical analysis tool is still limited to the research field, and to our knowledge no commercial examples of electrochemical immunoassay kits are available at this moment.

Experimental

Materials and Reagents

A mixed cellulose ester membrane filter (MF-membrane

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filter) was purchased from Millipore (Billerica, MA). *p*-Aminophenyl phosphate (pAPP) was synthesized as described previously.[4] Recombinant human TNF-α specific antigen, monoclonal anti-TNF-α and polyclonal anti-TNF-α, biotinylated antibodies were all purchased from R&D Systems (Minneapolis, MN). The neutravidin alkaline phosphatase conjugated product (NA-ALP) was purchased from Thermo Scientific (Somerset, NJ). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Life Technologies (Carlsbad, CA). All reagents were of analytical grade, and used without further purification.

All electrochemical experiments were conducted using an electrochemical analyzer ALS/CH Instrument Model 1222B (CH Instruments, Inc., Austin, TX). The Ag|AgCl reference electrode and Pt counter electrode were purchased from ESA, Inc. (Chelmsford, MA). The Au working electrodes were purchased from Tanaka Kikinzoku Kogyo K.K. (Tokyo, Japan).

Subjects and sample collection

The subjects of this study were fully informed of the protocol. Their written informed consent was obtained according to the Declaration of Helsinki. The experimental protocol for this human study was approved by the ethical committee of Kyushu Dental University (#12-26). GCF samples were collected at the periodontal clinic of the Kyushu Dental University hospital. Patients were then divided in two groups: control (i), formed by those showing healthy sites with probing depths of ≤ 3 mm, no attachment loss and no bleeding on probing (8 subjects); and chronic periodontitis (ii), formed by patients with a tooth with one or more sites with a probing depth of ≥ 4 mm and bone loss (12 subjects). The site of GCF collection was isolated with cotton wool rolls, and saliva was gently removed from the adjacent tooth surface and gingiva using an air syringe. To collect the GCF samples, a sterile paper point was inserted into the gingival crevice for 30 s. The paper points were then preserved in 200 µL of sterilized phosphate-buffered saline or 1×PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4) and kept at –20°C until measurements for the TNF-α level.

Sandwich immunoassay

The chemical reactions forming the basic concept of MMeELISA are as follows (Fig. 1). Disks (6 mm diameter) were punched from a MF-membrane filter, which has a 0.45-µm pore size. It has a 150 µg cm^{-2} protein binding capacity. To carry out the sandwich-type ELISA on the disk membrane, the following steps were performed. A 10-µL portion of 10 µg mL^{-1} TNF-α monoclonal antibody in 1×PBS was dropped on a disc membrane. Then, for the immobilization process, the discs were properly placed in a solid support, and were exposed for 30 s at 500 W (optimized time and power output), ordinary microwave range according to the modified method of Nahar *et al.*[7] The effect of microwave irradiation is that it enhances the movement of molecules, and thus shortens the chemical reaction processes, leading to accelerated physical adsorption of the antibody with the membrane surface. After which, the membrane discs were placed in a 1.5-mL microcentrifuge tube and 800 µL of buffer solution 1 (100 mM ammonium acetate in 1×PBS) was added to each tube. The disks were allowed to stand for 4 min at room temperature. Then, the membrane disks were removed from the tube and were quickly dried by placing them on a blotting paper, and immediately placed in a new tube with 800 µL of 0.5% casein in 1×PBS. The tubes were tightly capped and properly sealed using parafilm before placing them in a microwave oven for 30 s at 500 W. This step shortened the incubation time for the blocking process. The tubes were then allowed to cool down at room temperature for 10 min. After this, the membrane discs were removed from the blocking solution, and were placed in a new microcentrifuge tube with 800 µL of buffer solution 2 (100 mM ammonium acetate, 0.05% Tween-20) for the next 20 min. The discs were quickly dried for 1 min at a 500 W optimized microwave setting. After this, 10 µL of authentic TNF-α or the one contained in the sample was drop onto the membrane disk and was allowed to bind to the antibody through short microwave irradiation (30 s at 500 W). Then, 15 µL of combined biotinylated TNF-α antibody (2.5 µg mL^{-1}) and neutravidin-conjugated alkaline phosphatase, 1.0 µg mL^{-1} in solution (100 µg mL^{-1} BSA, 10 mM HEPES

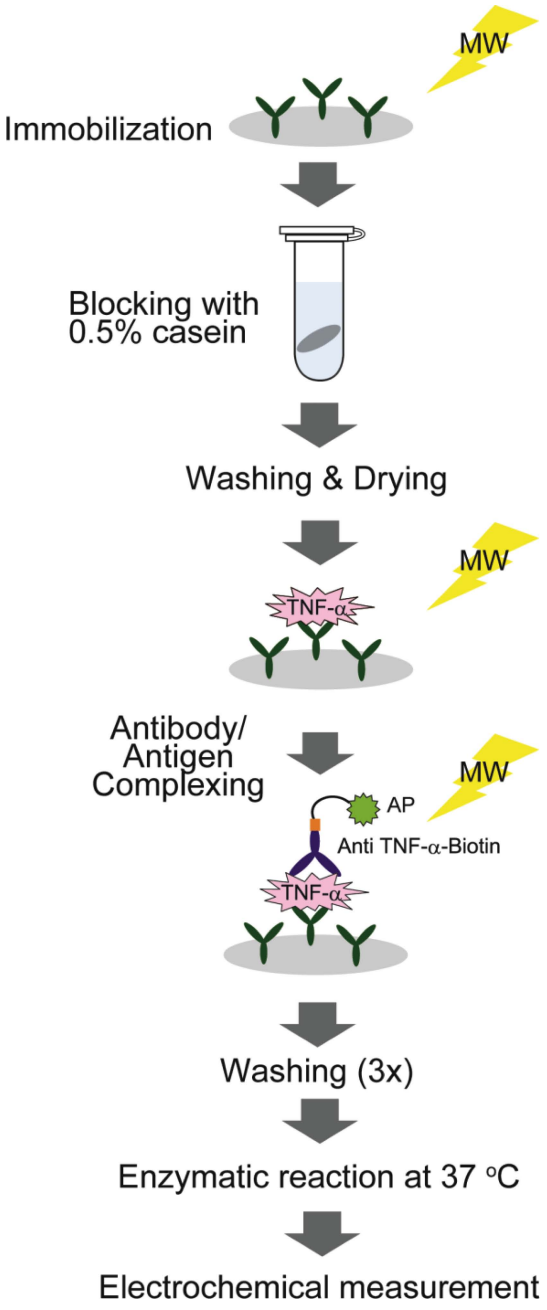

Fig. 1 Schematic diagram of the MMeELISA procedure. The technique utilizes the sandwich-type ELISA format with microwave mediation, and the use of the substrate pAPP for a redox reaction to take place that results in a current signal based on *p*-aminophenol.

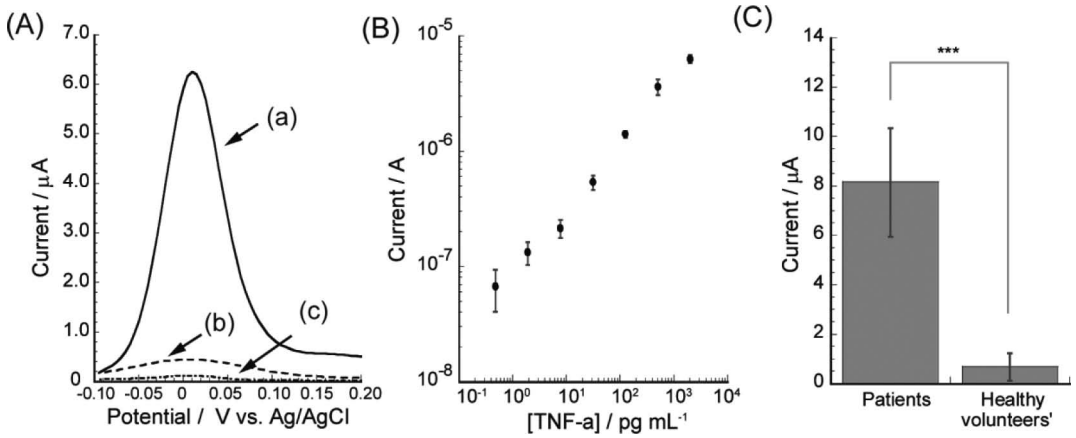

Fig. 2 (A) Differential pulse voltammograms under 2000 pg mL^{-1} (a), 0.48 pg mL^{-1} (b), and 0 pg mL^{-1} of TNF-α (c). (B) Standardization curve between the peak current and the concentration of TNF-α antigen using the MMeELISA system. All experiments were carried out 3 times. The data represent the mean and standard errors. (C) Current responses based on the amount of TNF-α detected in GCF samples for both patients and healthy volunteers. The bar graph represents the mean ± SD (n = 3). The statistical significance of the data was assessed using the Student's t-test.

(pH 7.4), 10 mM NaCl, 0.05% Tween-20) was then applied to the disk to form a ternary antibody-antigen-antibody complex through short microwave irradiation. For the final washing, the discs were then placed in new tubes, and were washed twice with 1×PBS and 0.05% Tween-20 for 5 min. The tubes were placed in shaker rotator to thoroughly wash the discs. The final washed was in a 1×buffer containing 50 mM Tris–HCl (pH 9.0), 10 mM KCl, and 10 mM $MgCl_2$ for 5 min. Finally, the discs were then placed in each well of a 96-well micro titer plate. Then, a 200-μL portion of 50 mM Tris–HCl buffer (pH 9.0) containing 10 mM KCl, 10 mM $MgCl_2$, and 0.5 mM pAPP was placed in each well, and the plate was incubated at 37°C for 30 min to allow the enzyme reaction to proceed to generate electrochemically active *p*-aminophenol, which was then quantified by differential pulse voltammetry (DPV).

Electrochemical measurements

Electrochemical measurements were carried out with a three-electrode configuration consisting of a Ag|AgCl reference electrode, a Pt counter electrode and a disposable Au electrode (theoretical surface area is 1 mm^2) as a working electrode. The conditions for DPV measurements were the following: initial potential, –0.4 V; final potential, 0.3 V; scan rate, 50 mV/s; pulse amplitude, 0.05 V; sample width, 8.33 ms; pulse period, 0.05 s; quiet time, 2 s.

Results and Discussion

The principle of our membrane based microwave mediated electrochemical ELISA (MMeELISA) is illustrated schematically in Fig. 1.

This involves the use of a modified mixed cellulose ester membrane as a solid-phase support, which is a porous hydrophilic paper-like material that has a uniform pore size and high protein binding capacity, which makes it suitable for protein immobilization. The use of paper-based devices for ELISA has already been reported in several studies.[8,9] However, it required multiple steps of coating and fabrication of the cellulose paper, which is time consuming since it involves several steps of preparation prior to use. In our technique, the modified mixed cellulose ester membrane was use as it was. In addition, we carried out our assay using microwave irradiation with 500 W as the power output in a domestic microwave oven, since it has been reported in many studies that microwave-mediation increases the reaction yields, and reduces the incubation time for most enzymatic reactions.[7,10,11] Finally, we used pAPP as a substrate to generate electrochemically active *p*-aminophenol, which is quantitated by DPV as one of the techniques to reduce the background current.[12] Figure 2A shows representative DPV data of the highest to lowest TNF-α concentrations, 2000 (a) to 0.48 pg mL^{-1} (b) with 0 pg mL^{-1} as the background noise (c). The magnitude of the peak current signal at 0.02 V (a) was found to be proportional to the concentration of the authentic TNF-α antigen. The results were statistically significant at p = 0.05, as revealed by a t-test. The assay reproducibility was also quite good at taking into account the lowest-to-highest point determination with a mean % coefficient of variance of 22 and 8% for 0.48 and 2000 pg mL^{-1}, respectively. In Fig. 2B, data show several points of the peak current relevant to the various concentrations of the authentic TNF-α antigen, wherein the highest point represents the concentration at 2000 pg mL^{-1} and the lowest point is the 0.48 pg mL^{-1} concentration. To determine the smallest detectable analyte (TNF-α antigen) concentration in our assay, the signal-to-noise ratio (S/N) in our system was measured. The detection limit in our assay was 0.48 pg mL^{-1} with 67.3 ± 26.4 nA as the peak current.

To point out the advantages of the MMeELISA technique from the conventional ELISA system, we compared MMeELISA with the specification of one commercially available ELISA kit for TNF-α.[13] The total assay time for MMeELISA was 1.5 h, while the conventional ELISA requires 21.5 h. This system also requires less sample volume of about 10 μL, as compared to 200 μL for the conventional ELISA. The technique is also more feasible to use, since electrochemical devices are less expensive compared to spectrophotometric apparatus.

The simplicity and sensitivity of this MMeELISA procedure gains an upper hand as an alternative tool to the more expensive spectrophotometric-based conventional ELISA system in TNF-α detection.

To further evaluate the applicability of our system, we used

Table 1 Clinical characteristics of periodontitis and control groups (mean ± SD)

	Periodontitis group (n = 12)	Control group (n = 8)
Age (years)	62.67 ± 3.03	35.75 ± 4.82
% Males	66.67	25.00
% Females	33.33	75.00
Mean probing depth/mm	5.58 ± 0.26	2.00 ± 0.01
% Sites with bleeding on probing	100.00	0

GCF samples from both adult patients with periodontitis and from control subjects with clinically healthy gingiva. In the present study, periodontitis was diagnosed almost entirely on the basis of an array of clinical measurements, including the probing depth (PD) and percent of sites with bleeding on probing (BOP), as shown in Table 1. The PD and BOP measurements provide information about past periodontal tissue destruction, but do not expound on the current state of disease activity or prognosis. Therefore, it is interesting to know the level of inflammatory mediators that might give clues as to the progression of periodontal disease. The data in Fig. 2C show average electrochemical readings of TNF-α in GCF samples from both patients and healthy groups. Based from the electrochemical reaction, the lowest levels of TNF-α were found in the healthy group (0.67 ± 0.56 μA), whereas the highest levels of this mediator were found in subjects clinically diagnosed to suffer from periodontitis (8.15 ± 2.20 μA). Using t-test analysis, the difference in the electrochemical measurements in groups with chronic periodontitis were significantly higher compared to those in the healthy groups, statistically significant at $p = 0.001$. This data that we obtained is in accordance to a previously published report that mentions the link between elevated TNF-α and the severity of periondontitis.[13] The MMeELISA was able to differentiate the level of TNF-α in the two periodontal samples by showing significant differences in their current signals, which shows the sensitivity and applicability of the technique.

Conclusions

The MMeELISA technique has several practical advantages over conventional ELISA techniques presented in previous works, primarily because of its simple preparation and sensitivity in measuring TNF-α, even at a very low concentration. The sensitivity of detection for this method is 0.48 pg mL^{-1}. Moreover, this method enabled us to detect TNF-α at ultra-low concentration in GCF and in the shortest time possible. The method developed here could further be used in the detection of other biomarkers, and may have the potential to be a commercialized form as an electrochemical kit in the near future.

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