SURFACE-ENHANCED RAMAN SCATTERING-BASED TECHNIQUE FOR DETECTING PERIODONTAL DISEASE

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Surface-enhanced Raman scattering (SERS) technology was combined with nanotechnology to detect the Raman intensity of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in saliva and of three kinds of populations (healthy volunteers, patients with gingivitis, and patients with periodontitis). The Raman intensities of IL-1β and TNF-α in the saliva of the periodontitis group were significantly higher than those of the control and gingivitis groups (P = 0.00). The Raman intensities of the two inflammatory factors in the gingival crevicular fluid (GCF) of the periodontitis group were also significantly higher than those of the other two groups (P = 0.00). In the same group, the Raman intensities of IL-1β and TNF-α showed no significant difference between the saliva and GCF samples, respectively (P > 0.05). Combining SERS technology and nanotechnology can aid in detecting the Raman intensity of inflammatory factors using saliva and GCF more effectively than traditional methods.

Keywords: *surface-enhanced Raman scattering, interleukin-1β, tumor necrosis factor-α, saliva, gingival crevicular fluid.*

МЕТОДИКА НА ОСНОВЕ ПОВЕРХНОСТНО-УСИЛЕННОГО КОМБИНАЦИОННОГО РАССЕЯНИЯ СВЕТА ДЛЯ ОБНАРУЖЕНИЯ ПЕРИОДОНТИТА

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Методика поверхностно-усиленного комбинационного рассеяния света (SERS) объединена с нанотехнологией для определения интенсивности комбинационного рассеяния интерлейкина-1β (IL-1β) и фактора некроза опухоли-α (TNF-α) в исследовании слюны здоровых добровольцев, пациентов с гингивитом и пациентов с периодонтитом. Интенсивность комбинационного рассеяния IL-1β и TNF-α в слюне и десневой жидкости (GCF) у пациентов с периодонтитом значительно выше, чем в контрольной группе и группе с гингивитом (P=0.00). В той же группе интенсивности комбинационного рассеяния IL-1β и TNF-α не показывают существенной разницы между образцами слюны и GCF (P>0.05). Сочетание SERS и нанотехнологий может помочь в определении интенсивности комбинационного рассеяния факторов воспаления с использованием слюны и GCF более эффективно, чем традиционные методы.

Ключевые слова: *поверхностно-усиленное комбинационное рассеяние света, интерлейкин-1β, фактор некроза опухоли, слюна, десневая жидкость.*

Introduction. Saliva is a special body fluid that reflects human health. It is secreted by salivary glands and contains gingival crevicular fluid (GCF) [1]. It also contains proteases, cytokines, hormones, antibodies, and antibacterial ingredients [2]. Changes in the composition of saliva could reflect oral diseases and general health; thus, they can be used for the early prediction and early diagnosis of oral and general diseases [3]. In the past few years, saliva has been used to diagnose cancer, autoimmune diseases, and bacterial/viral infectious diseases (HIV infections) [4, 5]. GCF refers to the fluid that penetrates the gingival sulcus from the gingival connective tissue through the gingival sulcus epithelium and combined epithelium [6]. GCF is mainly derived from serums, adjacent periodontal tissues, and bacteria [7]. The change in the periodontal tissues during disease development can be determined by analyzing the composition of GCF. An increased GCF volume is an early manifestation of periodontal inflammation, which usually precedes the clinical manifestations [8]. Meanwhile, the level of inflammatory mediators in GCF increases [9]. GCF can reflect the local pathological changes in periodontal tissues earlier and more directly than saliva [10]. However, GCF is relatively small in quantity and difficult to obtain during sampling, so it is possible to detect false negatives.

Tumor necrosis factor-α (TNF-α) is the main inflammatory factor in the development of periodontal inflammation [11]. It also induces and promotes the production of other inflammatory factors, mediates the production of a series of other inflammatory factors, and accelerates the development of periodontitis [12]. Interleukin-1β (IL-1β) is an inflammatory factor that appears in early periodontitis [13]. It is a potential stimulator of osteoclast proliferation, differentiation, and activation and is closely related to the destruction of periodontal tissues [14, 15]. IL-1β and TNF-α have a synergistic effect, which can further promote inflammatory response [16]. These cytokines play an important role in the occurrence and development of periodontal disease, and they are also commonly used as indicators in clinical and scientific research [17]. The emergence of Surface-enhanced Raman scattering (SERS) detection provides a new approach for the spectral detection of saliva. Oral cancer is diagnosed from saliva using electrostatic adsorption to attach Au nanoparticles to a glass substrate. The specific saliva surface enhanced Raman peaks of oral cancer patients were at 670, 1097, and 1627 cm^{-1} [18]. Yan et al. [19] used a surface-enhanced Raman spectrometer based on nanochips to study salivary acquired immune deficiency syndrome detection and obtained a 90.9% accuracy rate through the support vector machine algorithm. Other researchers used SERS active chips to measure saliva drug residues. Only 300 mL saliva samples were used to measure phenobarbital, caffeine, and cocaine in saliva for a total of 10 min. The same research team then used a silver gel glass capillary to separate 5-fluorouracil in saliva and performed a SERS test on this active capillary to detect a significant difference between this drug and its metabolites [20]. This study provided a basis for the choice of drug dosage.

In recent years, SERS has been widely used in biology and biomedicine [21]. Because of its special fingerprint characteristics, it can provide information about large molecules, such as proteins and lipids [22, 23]. At present, there are also reports on the comparison of structural changes in biomolecules after radiation induction [24]. However, few reports are available on the application of SERS technology in the pre-detection of periodontal disease [25]. Based on our previous research, in this study, we used the labeled SERS technology to detect two cytokines in saliva and GCF for the first time and further verified the effectiveness of SERS immune technology in detecting inflammations for the early diagnosis of periodontal disease [26].

Experimental. Saliva and GCF samples were obtained from healthy volunteers with no periodontal history within six months (control group, CTL), patients with gingivitis (gingivitis group, GI), and patients with periodontitis (periodontitis group, P). The inclusion criteria for the GI and P groups were based on Carranza's Clinical Periodontology, 11th Edition. Pregnant women and individuals with a systemic disease were excluded. All participants received oral examinations to exclude evident oral lesions, and they were not allowed to take hyposalivation-inducing drugs or other prescription or non-prescription drugs. Each group comprised 10 subjects. The average age of the participants in the three groups were 32.3 (CTL), 34.5 (GI), and 39.7 (P). The subjects were from West China Hospital of Stomatology. This work was performed with the approval of the hospital ethics committee of West China Hospital of Stomatology, Sichuan University (No. WCHSIRB-OT-2013-055), and the patients signed informed consent. The saliva samples were collected on the morning of the test day; the subjects did not clean their mouths, eat, or drink for 90 min before the samples were collected. More than 3 mL non-irritated saliva samples were centrifuged at 6000*g* for 15 min at 4°C (Thermo Legend Micro 17R, UAS). The supernatant was stored at −80°C. A standard GCF collection filter was placed in a 0.2 mL Eppendorf tube and weighed with a microelectronic balance (Satorius, BT25S, Germany) and recorded. GCF was collected twice from the same site. After the sample collection was completed, the filter paper was removed by shaking for 1 h at a low temperature and centrifuged at 6000*g* for 15 min. The supernatant was stored at −20°C. The silver nanoparticles were synthesized and modified with hydrophilically stabilized molecules, as reported in our previous study [26].

Preparation of Raman antibody probes for IL-1β and TNF-α. The activated product was added to 0.5 μg IL-1β and TNF-α polyclonal antibody, reacted for 1 h at room temperature, and stored at 4°C overnight. A Tris/Tween-20 buffer containing 0.2% bovine serum albumin (BSA) was added to the suspension of the antibody-SERS label conjugate and incubated at room temperature for 15 min with shaking. After the operation, the SERS-labeled antibodies were washed thrice with the Tris/Tween-20 buffer containing 0.2% BSA and centrifuged at 1920*g* for 15 min at 4°C. The precipitate of the SERS-labeled antibodies was re-dispersed in a 500 μL Tris/Tween-20 buffer containing 0.2% BSA and used to bind the functionalized glass surface. The IL-1β and TNF-α monoclonal antibodies were bound on polymer 3-digital glass slides (JingXin, CapitalBio Corporation, Shanghai, China). The glass was incubated with 2 μg/mL monoclonal antibody in 30 μL carbonate buffer I (0.2 M $NaHCO_3$, 0.5 M NaCl; pH 8.5) at room temperature for 1 h and stored at 4°C overnight. The coated substrate was washed with carbonate buffer II buffer (0.1 M $NaHCO_3$ and 0.5 M NaCl; pH 8.0) at first, then with acetate buffer (0.1 M sodium acetate; pH 4.4), and with phosphate buffered saline Tween-20 (PBST) buffer (PBS containing 0.05% Tween-20; pH 7.4) at last. Then, a 2% BSA solution was added on the surface of the substrate and incubated at room temperature for 2 h to reduce nonspecific binding. The blocked substrate was washed thrice with the PBST buffer, shaken, and dried at room temperature.

SERS immunoassay for IL-1β and TNF-α. The tested sample (saliva or GCF) was dripped onto a solid substrate (10 μL/site, three sites/substrate) coated with IL-1β and TNF-α antibodies and incubated at room temperature for 1 h, and 2% BSA was set as a negative control. After the reaction, the substrate was washed thrice with the PBST buffer, and the labeled AgNP-antibody SERS probe (10 μL/site) was incubated in an antigen antibody recognition site at room temperature for 1 h and stored at 4°C overnight. After the reaction, the substrates were washed thrice with the PBST buffer and dried. IL-1β and TNF-α were labeled with DTNB and 4MBA, respectively.

Raman detection and data analysis. A Raman microspectrometer (LabRAM, Horiba Jobin Yvon, France) was used to perform the Raman detection. With a 10× microscope objective, radiation from the 632.8 nm line of a helium–neon laser was focused on the sample. The SERS spectrum was analyzed using LabSpec 5 and Origin 9 software. The relative area values under the wave peaks were calculated as the relative values of Raman intensity corresponding to the concentration of inflammatory factors, and the average value was taken after three tests for each sample. Data were shown as mean ± SD and analyzed with one-way analysis of variance using SPSS 20 (IBM Inc., Chicago). $P < 0.05$ was considered statistically significant.

Results and discussion. *Comparison of inflammatory indicators in saliva samples.* The peak intensity at 1335 and 1590 cm^{-1} used to characterize the SERS spectra for IL-1β and TNF-α, respectively. Compared with CTL, the levels of IL-1β and TNF-α in GI and P were significantly higher ($P = 0.00$) (Fig. 1a). Moreover, the levels of IL-1β and TNF-α in P were significantly higher than those in GI ($P = 0.00$) (Fig. 1b) (Table 1).

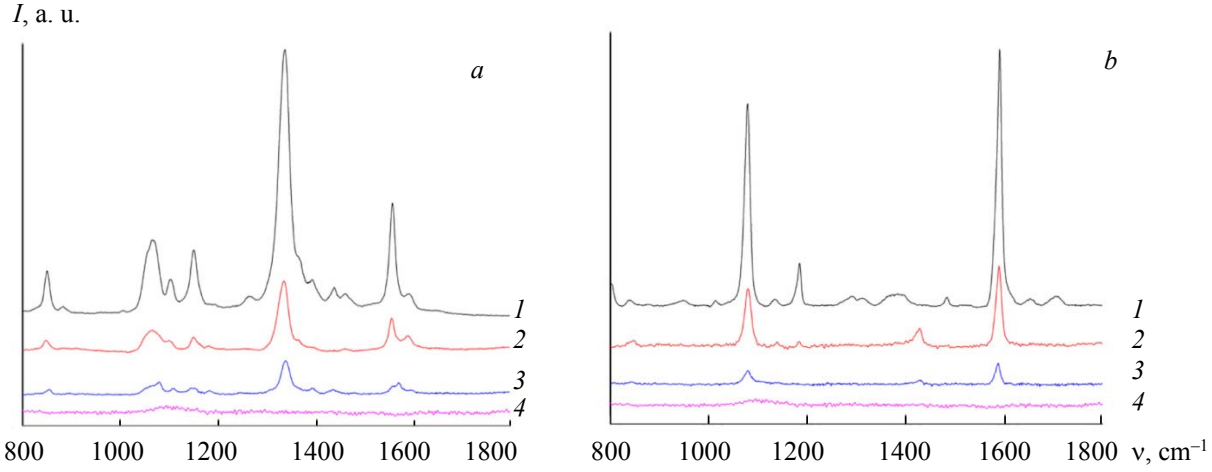

Fig. 1. SERS spectrum of IL-1β (a) and TNF-α (b) in the saliva samples: periodontitis group (1), gingivitis group (2), control group (3), and blank control (4).

TABLE 1. Areas under the Main Raman Peaks of IL-1β and TNF-α in Saliva Samples of Each Group

Inflammatory factor	Control group	Gingivitis group	Periodontitis group	*P*
IL-1β	19445.9±2561.7	34630±3475.9	57761±5360.6	0
TNF-α	22043.2±4388.3	33168.1±4715.9	54894.8±4115.6	0

N o t e. The difference between the three groups was statistically significant; $P = 0.00$.

Comparison of the inflammatory indicators of the GCF samples. The levels of IL-1β and TNF-α in the GCF samples were detected and analyzed using the same method. As shown in Fig. 2, in CTL, the levels of IL-1β and TNF-α in the GCF samples were significantly lower than those in GI and P ($P = 0.00$). The levels of the two indicators in P were significantly higher than those in CTL and GI ($P = 0.00$) (Table 2).

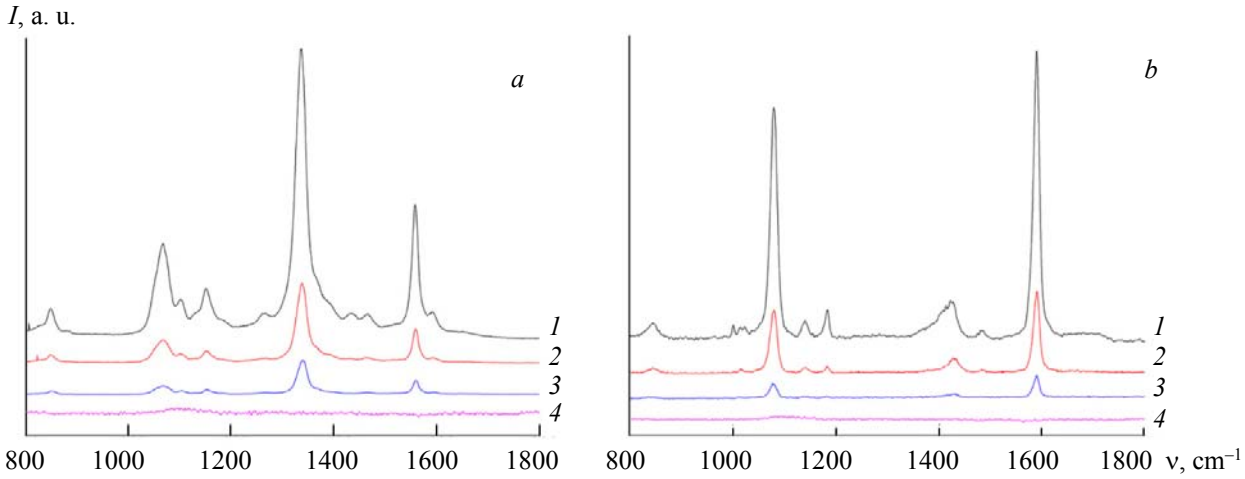

Fig. 2. SERS spectrum of IL-1β (a) and TNF-α (b) in the GCF samples: periodontitis group (1), gingivitis group (2), control group (3), and blank control (4).

TABLE 2. Areas under the Main Raman Peaks of IL-1β and TNF-α in Gingival Crevicular Fluid Samples of Each Group

Inflammatory factor	Control group	Gingivitis group	Periodontitis group	*P*
IL-1β	21174.4±6062.1	31419.2±4846.9	67388.8±6132.5	0
TNF-α	18576.6±4286.6	32999.4±4577.8	66866.8±5862.9	0

N o t e. The difference between the three groups was statistically significant; $P = 0.00$.

Comparison of Raman intensity in the samples. The Raman intensity of IL-1β and TNF-α were further compared between the two samples. As shown in Fig. 3a, in the same group, the Raman intensity of IL-1β showed no significant difference between the saliva and GCF samples ($P > 0.05$). The comparison results of TNF-α were similar to those of IL-1β (Fig. 3b). More than 1000 kinds of proteins have been identified in human saliva, of which 1/3 are also found in blood [27]. Compared with a blood test, a saliva test has more advantages: it does not involve any aggregation or blood cell interference; it also involves a fast, economical, and non-invasive collection and has no risk of blood-borne disease transmission [28, 29]. The current detection methods for saliva include polymerase chain reaction, enzyme-linked immunoassay (ELISA), gel electrophoresis, mass spectrometry, high-performance liquid phase, and microarray [30–33].

GCF plays a vital role in the study of periodontal disease, and its various components and content changes can reflect the occurrence, development, and prognosis of the disease [34]. Researchers suggested that more than 65 components in GCF can be used as markers for the preliminary detection of periodontal disease [35]. GCF is the first choice for detecting the susceptibility of patients to periodontal disease. Howe-

ver, in actual clinical practice, the content of GCF at each single site is very limited [36]. Gamonal et al. [37] found that the GCF volume at a single site in patients with periodontitis is 0.11 to 1.54 μL, and that in healthy people, it is 0.11 to 0.48 μL. At present, the method used to detect GCF is mainly ELISA, and using ELISA to detect an object requires ~50–100 μL GCF [38]. In the ideal detection using GCF, multiple components of a single site of GCF can be simultaneously detected and analyzed. To improve the sensitivity of GCF detection under trace components, a more sensitive detection method is needed. As a trace and high-sensitivity spectral detection method, SERS provides the possibility for the micro-detection of GCF and parallel detection of multiple components. In the past, the detection limit of cytokines by SERS had reached 1 pg/mL [39, 40].

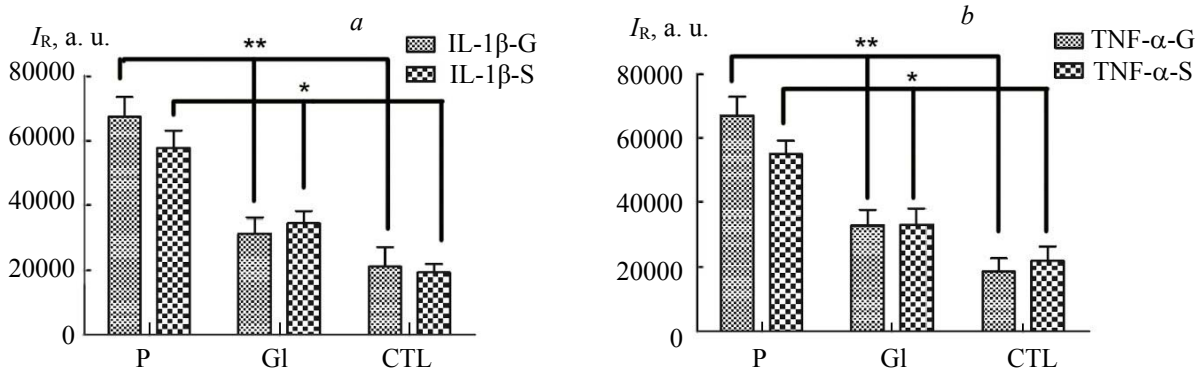

Fig. 3. Comparison of Raman intensity between the saliva and GCF samples. a — comparison of Raman intensity of IL-1β in the saliva and GCF samples, b — comparison of Raman intensity of TNF-α in the saliva and GCF samples; IL-1β-S and IL-1β-G represent the Raman intensity of IL-1β in the saliva and GCF samples, respectively; TNF-α-S and TNF-α-G represent the Raman intensity of TNF-α in the saliva and GCF samples, respectively.

We used the SERS immunoassay method for the first time to detect two periodontitis-related inflammatory factors in saliva and GCF trace samples from different populations. The results indicated that the content of IL-1β and TNF-α in saliva and GCF of CTL were significantly lower than those in GI and P ($P = 0.00$). The contents of the two inflammatory factors in saliva and GCF of P were significantly higher than those of GI ($P = 0.00$). However, the results of this study also show that in the two clinical samples of saliva and GCF, there was no statistical difference in the detection rate of the two inflammatory factors from different body fluid sources in the same group ($P > 0.05$). The possible reasons for these results are as follows. The self-assembled silver nanosol substrates have an uncontrollable nanoparticle size and uniformity, which affect the stability and repeatability of the experimental results. The detection method we have established for protein standard limits reached 1 pg/mL, and the high sensitivity may affect the specificity and cause false-positive results.

Conclusions. Based on the high-sensitivity trace detection of SERS and the immunological detection characteristics of SERS markers, we have, for the first time, detected two factors (IL-1β and TNF-α) in body fluid samples from patients with gingivitis and early periodontitis. This method is indeed more economical, simple, and effective compared with traditional detection methods.

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