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Label-free Detection of Protein Released during Platelet Activation by CNT-Enhanced Love Mode SAW Sensors

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Abstract

Platelet-rich plasma (PRP) has been applied in a series of clinical treatments. PRP contains high-concentrated platelets, which, when activated, could secrete a variety of growth factors and cytokines, to promote and/or enhance healing of injured tissues. Non-activated platelets suspension could be prepared by an isolation method of centrifugation and washing currently. However, it is not clear whether platelets, if any, are already activated during this process and there is no simple method to monitor their activation accordingly. Shear-Horizontal Surface Acoustic Wave sensors (SH-SAW, Love Mode) are promising in fundamental biology as well as biomedical engineering, detecting cell behaviors in liquid in a non-invasive, simple and quantitative manner. In this study, Love mode sensors are adopted for the label-free detection of protein secreted by platelets. Carbon nanotube (CNT) is reported as an advisable platform of both non-specific protein adsorption and specific protein binding. For further improvement of Love mode sensor performance, novel CNT-coated parylene-C film is prepared on its surface as both the acoustic-wave-guiding layer and bio-interface layer. The S₂₁ loss curves of Love mode sensors were recorded and the corresponding resonance frequencies were extracted. The results showed that the CNT-enhanced sensor possessed an increased resonance frequency shift when compared to normal sensor with single parylene-C film under identical collagen concentrations.

Then, the modified sensor is used for label-free detection of protein released by various concentrations of platelets. The results revealed high sensitivity and consistency, indicating the potential of CNT-enhanced Love mode sensors in cell-based applications.

Keywords

SH-SAW; CNT; Platelet; Protein

I. Introduction

During the past few years, platelet-rich plasma (PRP) has been applied in a series of clinical treatments, including oral surgery and orthopaedic surgery [1, 2]. Despite the lack of convincing scientific data support, the potential advantages of PRP treatment are extensively addressed in bone regeneration, bleeding reduction and tissue healing [3]. PRP is an autologous concentration of platelets in a small volume of plasma, and a variety of fundamental growth factors are reported to exist in platelets, such as PDGF, VEGF, TGF- β , and so on [3, 4]. Once activated, platelets could release these growth factors, which are generally considered to initiate and accelerate the tissue-repair process. PRP can be prepared simply by centrifugation, a rapid generation way of PRP with a high yield of platelets. However, platelets prepared in this way has a limited stability (no longer than 2 *hour*) since it contains plasma proteins, in particular enzymes, which potentially activate these platelets. In this case, an isolation method by centrifugation and washing was proposed accordingly [5]. Following this method, high-concentration platelet suspension in Tyrode's buffer is obtained without damaging and free from plasma protein. Nevertheless, further problem is concerned that the rest state (unactivated) of platelets in Tyrode's buffer can't be monitored directly. As it is known, when platelets activated in PRP, platelet aggregation occur quickly and fibrin gel forms as an indicator of activation. Without such phenomenon in platelet suspension, the protein released during platelet activation need be detected effectively in a simple and economic way, in order to obtain an affirmative determination on scientific or clinical use.

Shear-horizontal surface acoustic wave sensor (SH-SAW, Love Mode) is considered one of the most promising probing methods in fundamental biology and biomedical engineering, detecting behaviors of cells on their surface in a simple, noninvasive, and quantitative manner [6-8]. Comparing with the traditional bulk acoustic wave sensors (BAW), Rayleigh waves excited by surface electrodes in SH-SAW perform extremely sensitivity to certain surface perturbations as well as favorable inertness to other surrounding factors, especially to the number of bonds formed within the relatively short distance of ~ 50 nm from the surface [8, 9]. As the key part of SH-SAW, acoustic-wave-guiding layer on surface plays a crucial role in improving the sensitivity of device. Different kinds of piezoelectric and nonpiezoelectric materials have been applied as wave-guiding layer in the past years, such as ZnO, SiO₂ and PMMA [10-12]. Among them, parylene-C (poly(2-chloro-*p*-xylylene)), due to its good uniformity, compactness and adhesion to substrate, has been proven as one of the ideal acoustic-wave-guiding layers [13]. More importantly, parylene-C films possess comparable cell and protein compatibility to the standard tissue culture substrates, indicating

them great potentiality as bio-sensitive interface [14]. As a result, SH-SAW sensor is adopted for the detection of released protein in this study, and parylene-C film is deposited on it as both wave-guiding layer and bio-interface layer. However, relatively low shear wave velocity of parylene-C inhibits approaching of the theoretical maximum sensitivity of Love mode sensor, when in such case parylene-C could be quite lossy with its thickness increasing to the corresponding theoretical value [15].

Carbon nanotube (CNT) is reported an advisable platform of non-specific protein adsorption, as well as specific protein binding [16-18]. Based on this feature, a variety of CNT-coated biosensors have been developed, including the acoustic wave biosensors [19-22]. In this study, novel CNT-coated parylene-C films were performed as the acoustic-wave-guiding layer for cell-based Love mode biosensors. The modified sensors were applied to non-specific (label-free) detection of protein released during platelet non-selective activation. Results demonstrated a significant increase in mass sensitivity of Love mode sensors, indicating a potential biosensor for label-free protein detection.

II. Experimentally Details and Analysis Methods

Rat blood (freshly collected from 7-9 week old rats) mixed with anticoagulant (1:6) was centrifuged at 2300g to obtain platelet-rich plasma (PRP) in supernatant. Through standard washing procedure, platelets were suspended at various concentrations of 600,000, 1200,000 and 1800,000 / μL in Tyrode's albumin buffer containing 0.02 U/mL apyrase [5]. Washed platelets were activated by adenosine diphosphate (ADP, 20 μM), and corresponding supernatant was collected respectively after 1900g centrifugation.

Fig.1 schematically shows the structure of CNT-enhanced Love mode sensors for protein detection. Cr/Au interdigital-transducer (IDT) electrodes of a dual-delay-line configuration were fabricated on 36° YX- LiTaO_3 surface by photolithography. The period of IDTs is 32 μm , leading to an operating frequency around 131 MHz. Parylene-C films, as the wave-guiding layer, were deposited on these electrodes by thermal deposition system (SCS PDS 2010 Labcoter 2, SCS Equipment, Dallas, TX): the original dimer is sublimated and then undergoes pyrolytic cleavage at 690 °C; the object monomer polymerizes again on LiTaO_3 surface at room temperature in vacuum, forming continuous parylene-C films. The average thickness of parylene-C is 1.8 μm , providing a good combination of both high sensitivity and low energy loss. Subsequently, MWCNT suspension of I-Methyl-2-pyrrolidinone (NMP) (1:10000) was spin coated on the surface of parylene-C. As the volatilization of NMP, MWCNTs were distributed all over the surface as enhanced bio-interface. After coating, one rectangular tube (10 mm $\times$ 12 mm) of poly(dimethylsiloxane) (PDMS) was located around the IDT electrodes, forming a well-like structure for protein solution. In addition, Love mode sensors with parylene-C single layer were also prepared correspondingly. As Fig.2 shows, morphologies of MWCNTs on Au/Cr electrodes were observed by scanning electrical microcopy (XL-30 FEG SEM, FEI/Philips, Japan, accelerating voltage: 5 kV). MWCNTs are distributed randomly around the entire surface, and the average length of CNT is approximately 10 μm .

Prior to measurements, both kinds of Love mode sensors were firstly sterilized by exposing overnight to UV light. A series of collagen type I/phosphate-buffered saline (PBS) solution (0.1, 0.3, 1.0 and 3.0 mg/mL, 600 μ L) were added into each well and incubated at 37°C (100% humidity and 5% CO₂) for 1 hour, covering the entire surface with a absorbed collagen layer. During this process, S21 of Love mode sensors were monitored every minute by an Agilent N5230A PNA Network Analyzer. In parallel, for experimentation of label-free protein detection, the supernatant samples from activated platelet suspension of different concentrations were successively added into wells of CNT -enhanced Love mode sensors. These sensors were maintained in an incubator at 37°C (100% humidity and 5% CO₂) for 1 hour, and S21 in this procedure were recorded.

III. Results and Discussions

Fig. 3 gives the resonance frequency variation of Love mode sensors during collagen type I absorption (3.0 mg/mL): (a) is for normal Love mode sensor with single parylene-C film and (b) is for CNT -enhanced one. The insets are their corresponding S21 loss spectrum sets, where the resonance frequencies are extracted. The spectrum recording endured for 1 hour. As the time goes on, the color of loss spectrums in plots is shifting from pink to black gradually. It is illustrated that with the collagen molecules absorbed to electrode surface, intensity of the loss peak at resonance frequency doesn't present obvious changes. Comparing the S21 loss spectrum of two kinds of sensors, the loss at resonance frequency is slightly decreased from 15 dB ~ 20 dB to 20 dB ~ 25 dB due to the CNT coating. For both the normal and CNT -enhanced Love mode sensors, the resonance frequencies extracted from these loss peaks exhibit similar variation tendency: fast decreasing in the initial 20 min and then tending to be stable. Based on this, the S21 loss spectrums acquired between 40 min and 60 min could be adopted for the following extraction of effective resonance frequency shift.

The main differences between Fig.3 (a) and (b) are the magnitude of frequency variation. With S21 loss spectrum sets recorded between 40 min and 60 min, the effective resonance frequency shift for both kinds of sensors under the collagen concentration of 3.0 mg/mL are obtained. The results reveal that the resonance frequency shift of CNT -enhanced Love mode sensors is significantly increased as 11266 ± 264 Hz, compared with that of the normal sensor (5363 ± 297 Hz). Generally speaking, the mass sensitivity S_m , as one of the most important criteria for mass sensors, is defined as follows [12],

$$S_m = \lim_{\Delta m \rightarrow 0} \frac{1}{\Delta m} \frac{\Delta f}{f_0} \quad (1)$$

where m is the uniformly distributed mass per unit area added to the sensor surface, f_0 is the unperturbed resonance frequency and f is the change in operational frequency due to mass loading. As it is discussed previously, CNT has been proven great potential of non-specific protein adsorption [16]. Therefore, attributed to the excellent performance of CNT bio-interface, the mass sensitivity of novel Love mode sensors has been improved up to two folds.

Sending all loss spectrum sets acquired between 40 *min* and 60 *min* to the analyzer, a series of resonance frequency shifts for both normal and CNT -enhanced Love mode sensors are extracted. Fig.4 shows the effective resonance frequency shifts after 40 *min* for both kinds of sensors. It is illustrated that the resonance frequency shifts of both possess the quite similar variation tendency with the changing of platelet concentration. As the platelet concentration is rising, resonance frequency shift continues increasing. The resonance frequency shift of the normal and CNT -enhanced sensors increases from $1022 \pm 178 \text{ Hz}$ to $5363 \pm 297 \text{ Hz}$ and from $3468 \pm 149 \text{ Hz}$ to $11266 \pm 264 \text{ Hz}$, respectively, when collagen concentration increasing from 0.1 *mg/mL* to 3.0 *mg/mL*. These results confirm further that the performance of CNT -enhanced Love mode sensors in nonspecific protein detection are improved successfully compared with the normal ones, indicating their potential for detection of protein released during platelet activation in the following paragraphs. In addition, it is also deduced that when the collagen concentration is limited in the range of 0.1 *mg/mL* ~ 1.0 *mg/mL*, the resonance frequency shifts of both sensors present a nearly-linear variation with the ascension of collagen concentration. However, as concentration of collagen solution is increasing further, the variation of frequency shift is deviated from the near-linear relation.

The S21 loss spectrum sets of CNT -enhanced Love mode sensors with the supernatant from activated platelet suspension were recorded. Subsequently, the corresponding resonance frequency variation for different platelets concentrations were obtained as Fig.5 shows. During the recording time, resonance frequency of Love mode sensors express the same changes as that in collagen absorption. The frequency shifts are tending to be stable gradually only after 20 *min*, presenting the similar fast absorption procedure of protein. With the concentration of platelet suspension increasing from 600,000 to 1800,000/ μL , resonance frequency shift is increased from $3802 \pm 126 \text{ Hz}$ to $8870 \pm 99 \text{ Hz}$ as well. This range is considered as the near-linear range as discussed before. As a result, the near-linear relation between the resonance frequency shift and the activated platelet concentration (number) could be established. Although above relation is not strictly linear but show small variations, this novel Love mode sensors provide one simple and quantitative way for the quick examination of activated platelets. Both mass sensitivity and consistency are improved in label-free detection of protein, indicating satisfying potential of CNT -enhanced Love mode sensors for future cell-based applications.

IV. Conclusions

In this study, two kinds of acoustic-wave-guiding layer, single parylene-C film and novel CNT -coated parylene-C film, were prepared respectively on the surface of electrodes of Love mode biosensors. The S21 loss spectrum of Love mode sensors were recorded and corresponding resonance frequencies were extracted. The results illustrate that for the collagen solution of same concentrations, the resonance frequency shifts of CNT-enhanced Love mode sensors are significantly increased compared with the normal ones with only parylene-C film. This novel CNT-enhanced sensor was then applied in label-free detection of protein released by platelet suspension of different concentrations. The relation between resonance frequency shifts and platelet concentrations in the measuring range is nearly linear with only small variations. This novel Love mode sensors possess both improved mass sensitivity and consistency, presenting their great potential for future cell-based

applications. This technology holds great promise in fundamental biology as well as biomedical engineering.

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Figure 1. Schematic diagram of CNT-enhanced Love mode sensor for label-free protein detection

Figure 2. SEM photos of MWCNT on Au/Cr electrodes

Figure 3. S21 loss spectrum sets and corresponding resonance frequency shift of Love mode devices with collagen absorption

Figure 4. Resonance frequency shift of Love mode sensors with different-concentration collagen solution

Figure 5. Resonance frequency shift of CNT-enhanced Love mode devices for label-free detection of protein released by different-concentration platelet suspension