Original Article

Phosphorylated α-synuclein in diluted human serum as a biomarker for Parkinson's disease

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ABSTRACT

Background: Parkinson's disease (PD) is one of the most prevalent neurodegenerative disorders, which characterized by increased pathological marker protein, α-synuclein (α-syn) and phosphorylated-Ser129-α-syn in the extracellular fluids. Current methods of measuring the p-Ser129-α-syn concentration in cerebrospinal fluid for PD are based on ELISA method, however, the amount of area under the curve (AUC) to predict PD is around 0.7-0.8. Higher confidence level of AUC in p-Ser129-α-syn quantification for the early diagnosis of PD would be essential.

Methods: Detection of p-Ser129-α-syn in diluted human serum for diagnosis of PD was investigated by a modified paired surface plasma wave biosensor (PSPWB) using a quarter wave plate for better detection performance. The method combining an immunoassay and non-labeled technique measures the p-Ser129-α-syn level with high sensitivity and specificity. Ten patients with PD at early stage (Hohn & Yahr stage I and II) and 11 age-matched healthy control participants were recruited for measurement of serum p-Ser129-α-syn.

Results: AUC of the p-Ser129-α-syn in diluted human serum was 0.92 and it shows that p-Ser129-α-syn in diluted human serum could be used as a sensitive biomarker for the diagnosis of PD in clinics. Results clearly show that the measured p-Ser129-α-syn concentration in diluted human serum displays a statistical significance between health control subjects and PD patients.

Conclusions: P-Ser129-α-syn has low abundance in human serum, high detection sensitivity and specificity are critical to the success of the diagnosis of PD in clinics. In this study, a

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modified PSPWB was developed that the limit of detection at 1 ng/mL for p-Ser129-α-syn (standard) spiked into diluted human serum of a healthy control was performed. This result shows that the modified PSPWB can be used as a platform for detecting p-Ser129-α-syn in diluted human serum as a potential biomarker for PD.

At a glance commentary

Scientific background

Currently, the diagnosis of PD mostly relies on cardinal motor symptoms. Hence, reliable biomarkers for early PD diagnosis are urgently needed. In this study, a highly sensitive biosensor that was based on the technique of generating a pair of surface plasma waves in optical heterodyne interferometer was developed.

Field of this study

Optical Biosensors, Surface Plasma Wave and Optical Heterodyne Interferometry are the fields added in this study.

Background

Parkinson's disease (PD) is one of the most prevalent neurodegenerative disorders, and currently, its diagnosis mostly relies on cardinal motor symptoms, including rest tremor, bradykinesia, rigidity, and gesture instability [1]. However, this hampers the detection of the earliest progress of the disease. Hence, reliable biomarkers for early PD diagnosis are urgently needed.

In previous studies, α-synuclein (α-syn) and α-syn misfolding species in cerebrospinal fluid (CSF) and blood plasma were highly correlated to the pathophysiology of PD [2]. Both α-syn and α-syn misfolding species play a central role in the development of PD and are suggested to be valid biomarkers of the disease [3]. Genetic mutations and post-translational modifications facilitate α-syn misfolding such that oligomerized α-syn (o-α-syn) and phosphorylated-Ser129-α-synuclein (p-Ser129-α-syn) are generated [4,5]. As a result, the accumulation of misfolded α-syn occurs by intracellular deposition in Lewy bodies (LB), in which p-Ser129-α-syn is a major component (90%) [1,5–8,14].

PD can also be characterized by increased o-α-syn and p-Ser129-α-syn in the extracellular fluids such as CSF and blood plasma [2,9–13]. Recently, the concentration of o-α-syn and p-Ser129-α-syn in the CSF has been measured and treated as a potential biomarker of PD [3]. Clinically, aberrant p-Ser129-α-syn in the CSF is closely associated with PD, and the CSF concentration of p-Ser129-α-syn is higher in PD patients than controls [1,3].

The major phosphorylation site of p-Ser129-α-syn is Ser129, which is located in the negatively charged C-terminal tail of α-syn [10]. However, p-Ser129-α-syn is found at a very low level in the CSF [5] possibly due to CSF is sampled invasively or the sampling method is highly resisted by PD patients. Therefore, only a few studies have demonstrated that CSF p-Ser129-α-syn would be viewed as a PD biomarker while a longitudinal study p-Ser129-α-syn along with disease progress has not been possible.

Current methods of measuring the p-Ser129-α-syn concentration in CSF are based on ELISA. However, analyses have revealed that the area under curve (AUC) of the receiver operating characteristic (ROC) for the amount of p-Ser129-a-syn measured by ELISA to predict PD is around 0.7-0.8 the amount of p-Ser129-α-syn measured by ELISA to predict PD is around 0.7–0.8 of an area under curve (AUC) of the receiver operating characteristic (ROC) [3,5,12,15]. These results need to be improved to a higher confidence level for clinical PD diagnosis. Hence, the motivation for this study is to set up an optical method that allows us to measure p-Ser129-α-syn in diluted human serum sample instead of CSF with high sensitivity and specificity.

Replacing CSF with diluted human serum as a test specimen presents an advantage as a minimally invasive sampling technique and provides the capability to perform longitudinal studies of the disease. Previously, a modified paired surface plasma wave biosensor (PSPWB) was set up [16], and a quarter wave plate associated with an analyzer was used to help improve the alignment of a laser beam propagating in an electro-optic modulator (EOM). This is critical to the quality of the two orthogonal linear polarizations of the laser beam at different temporal frequencies emerging from EOM. The elliptical polarization of the linear polarization laser beam induced by the misalignment of the beam in the EOM produces fluctuation in the polarization angle and increases the intrinsic background noise in the measurement [17].

In this report, the modified PSPWB was integrated with an immunoassay using a non-labeled technique, which enables real-time measurement. In addition, an optical heterodyne interferometer is introduced to a setup with synchronizing beat signal detection at a high signal-to-noise ratio (SNR). This enhances the detection sensitivity significantly and enables biomarker detection in dilute human serum [18–23]. As a result, when coupled with a suitable *p-Ser129-α-syn* antibody, both high sensitivity and specificity are possible and able to quantitatively separate the serum samples from control and PD patients.

Materials and methods

Scientists have successfully used surface plasmon resonance (SPR) biosensors in life science and new drug discovery because of their high detection sensitivity and non-labeled technique for real-time measurement [18–23]. Generally, an SPR biosensor enables monitoring of biomolecular interactions by measuring either the changes in the intensity or the phase shift of a reflected p-polarized (TM wave) laser beam. This is accomplished by using an SPR device (SPRD) at the SPR resonance angle of incidence. The surface plasma

wave (SPW) is excited under attenuated total reflection (ATR) conditions [18]. The SPR resonance angle is critical to the effective refractive index n_{eff}, which is dependent on the mass density and the effective thickness of the active dielectric medium that is adjacent to the gold surface of the SPRD [20]. The high surface quality of gold film coated on a glass plate allows for sensitivity to the localized distribution of the SPW electric field at the gold/dielectric interface.

The detection sensitivity of SPR biosensors was determined by our previous studies [20,21], and the following expression was obtained:

$$\Delta n_{eff} = (\partial n_{eff} / \partial n) \cdot \Delta n + (\partial n_{eff} / \partial d) \cdot \Delta d$$

where Δn and Δd are the changes in the refractive index and effective thickness of the active dielectric medium, respectively. In this setup, a two-frequency p-polarized He–Ne laser beam is used, which presents a pair of highly spatial and temporally correlated p-polarized light waves with different temporal frequencies of ω_1 and ω_2 [21]. As a result, a pair of highly spatially and temporally correlated SPWs is then excited and propagates on the gold/dielectric interface in the SPRD.

The coherence properties of the paired SPWs are sensitive to the surface quality of the gold film [21]. From SPW excitation theory, phase-matching condition is required between the incident p-polarized light wave and SPW [18]. A heterodyne signal of the attenuated p-polarized light waves of the beat frequency at $\Delta\omega = \omega_1 - \omega_2$ is produced and detected.

In this setup, high surface quality of the gold film on the sensor chip is required. Hence, Δn_{eff} with high detection sensitivity is achieved by directly monitoring the change of the amplitude of the beat signal. Then, the interaction among biomolecules can be measured in real time. The concentration of the target molecules bound to the sensor chip is detected.

Fig. 1 illustrates the optical setup of the modified PSPWB. The laser beam is from a single frequency-stabilized linear polarized He–Ne laser (Model 117 A, Spectra Physics Inc.) with a wavelength at 632.8 nm. A half-wave plate is used to provide an azimuth angle of linear polarization of the laser beam of 45° to the x-axis. The laser beam is then transmitted to an EOM (Model 4002, New Focus Inc.), which is driven by a high-voltage amplifier (Model 3211, New Focus Inc.) and a synthesized function generator (DS 345, Stanford Research Systems) that the linear birefringent effect is introduced in the EOM. This results in different phase velocities for p and s polarizations that are traveling in the EOM. A two-frequency orthogonal linear polarized (p and s waves) laser beam with respective temporal frequencies of ω_1 and ω_2 is produced [22]. In the setup, a quarter wave plate (QWP) of the azimuth angle of 45° from the x-axis is located after EOM in order to converts p and s waves of the laser beam into R and L circular polarizations of the temporal frequency at ω_1 and ω_2 respectively. Finally, a polarizer P of its azimuth angle set at 0° selects two p waves (p_1 and p_2) at ω_1 and ω_2 from the R wave and L wave, respectively. Thus, a pair of highly spatially and temporally correlated p waves is generated.

As shown in Fig. 1, a lateral displacement beam splitter (LDBS) is located after the P polarizer in the laser beam to generate two parallel laser beams with waves $p_1 + p_2$ in each beam. Simultaneously, two beat signals at photodetectors D_1 and D_2 are generated. One beam is called the signal channel, and the other beam is called the reference channel. Both
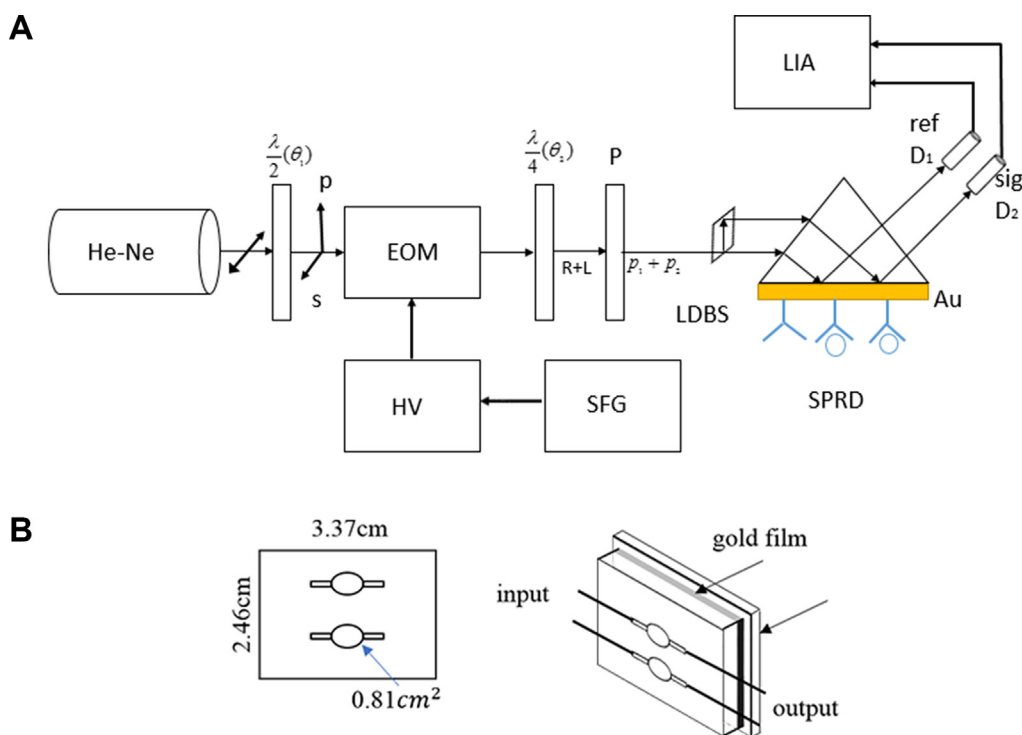
A

B

Fig. 1 (A) Optical setup of the modified PSPWB where HV is high voltage amplifier and SFG is synthesized function generator (B) Design of the flow system.

beams are incident to the SPRD at the same incidence angle simultaneously in Fig. 1(A). Meanwhile the design of flow system made by PDMS is shown in Fig. 1(B). The amplitudes of beat signals in the signal channel and the reference channel are measured simultaneously in real time using lock-in amplifiers (LIAs). These are expressed as follows:

$$I_{p_1+p_2}^{sig}(\Delta\omega t) = \frac{1}{2}{A'_{p_1}}^2 + \frac{1}{2}{A'_{p_2}}^2 + A'_{p_1}A'_{p_2}\cos(\Delta\omega t) \quad (1)$$

$$I_{p_1+p_2}^{ref}(\Delta\omega t) = \frac{1}{2}{A_{p_1}}^2 + \frac{1}{2}{A_{p_2}}^2 + A_{p_1}A_{p_2}\cos(\Delta\omega t) \quad (2)$$

$$\chi = \frac{A'_{p_1}A'_{p_2}}{A_{p_1}A_{p_2}} \quad (3)$$

where χ is the normalized attenuated amplitude of the signal channel normalized by the detected amplitude in the reference channel. The excess noise which is caused by laser intensity fluctuation and environmental disturbances in the flow system of SPRD, is then reduced effectively [16,20,21].

Preparation of the sensor chip

The SPR sensor chip used in this study is a BK7 glass slide coated with laminated layers of Au/Cr (50/2 nm). A mixed self-assembly monolayer (SAM) of dithiols was assembled on an Au/Cr substrate at 26 °C overnight. The SAM consists of 90% SH-PEG-COOH and 10% $HS(CH_2)_6OH$ as a diluent at a mixing ratio of 9:1. The binding matrix was optimized, and a captured antibody monolayer was generated using the amine-coupling protocol.

Rabbit monoclonal anti-α-syn (phospho-S129) antibody (anti-p-S129-α-syn; Abcam, Cambridge, MA, USA) was covalently immobilized on SAM surface of the SPR sensor chip as follows:

1. The SPR sensor chip was activated by injecting the mixed solutions of 0.4 M of EDC (1-Ethyl-3-carbodiimide) and 0.1 M of NHS (N-hydroxysuccinimide) into the reaction chamber in SPRD (Fig. 1(A)) at 22 °C for 30 min. A washing step was then taken by injecting PBS (phosphate-buffered saline) into the flow system to clean up EDC and NHS in the reaction chamber for 10 min. The flow speed was adjusted at 0.4 μL/s.
2. Anti-p-S129-α-syn antibody at a concentration of 5 μg/mL was injected into the SPRD reaction chamber for 2 h at 22 °C and the flow speed was arranged at 0.4 μL/s. Anti-p-S129-α-syn antibody was then immobilized onto the activated SAM surface. Finally, PBS was injected into the flow system in order to remove the unbound anti-p-S129-α-syn antibody from the reaction chamber.
3. ETH (2-Hydroxyethylammonium chloride) was then injected into the reaction chamber to block the unbound binding sites on SAM surface. It was done for 7 min at 22 °C in the reaction chamber and then followed by a washing step involving the injection of PBS into the flow system.
4. Anti-p-S129-α-syn sensor chip was then ready for detecting p-Ser129-α-syn either in PBS or diluted human serum.

In order to calibrate the detection sensitivity of the modified PSPWB, we first prepared the anti-IgG (immunoglobulin) sensor chip for measuring IgG at different concentrations in PBS: 0 (pure PBS), 600 fg/mL, 6 pg/mL, 60 pg/mL, 600 pg/mL, 6 ng/mL, and 60 ng/mL. The attenuated amplitude of the signal detected was normalized by the detected amplitude in the reference channel. For this experiment, mouse IgG and goat anti-mouse IgG antibody were purchased from Sigma–Aldrich Co (St. Louis, MO, USA).

Patients and controls

Ten PD patients and 11 age-matched healthy controls were recruited from outpatient clinics in neurology, Chang Gung Memorial Hospital-Linkou Medical Center, Taiwan (see Table 1). The recruited patients had a diagnosis of PD which was confirmed by neurologists (Dr. C-M Chen and Dr. Y-R Wu) who specialize in movement disorders using the UK PD Society Brain Bank clinical criteria [24]. Gender- and age-matched healthy controls (HC) were recruited via convenient sampling of individuals from outpatient clinics in neurology. The participants did not have any co-morbidities including renal, cardiac or liver dysfunctions, systemic infections, autoimmune diseases, malignancy, and neurological diseases based on the past history and routine blood tests. In addition, this study was conducted according to the protocol approved by the Institutional Review Boards of Chang Gung Memorial Hospital (Ethical license No: 201600265B0) and all examinations were performed with a written informed consent from each participant.

Serum preparation

Serum samples were prepared after collecting the whole blood and then allowed the blood to clot by leaving them undisturbed at room temperature for 15–30 min. The clot was removed by centrifuging at 1500×g for 15 min at room temperature. The resulting supernatant is designated serum. Following centrifugation, the serum samples were immediately transferred into a clean polypropylene tube and stored at −80 °C or lower. Samples which are hemolyzed, icteric or lipemic were not used.

Statistical analysis

All statistical analyses were performed using Prism 6 (GraphPad) and results are expressed as mean ± SD. For comparison of means of p-Ser129-α-syn levels between HC and PD groups, a two-tailed unpaired t-test was used to

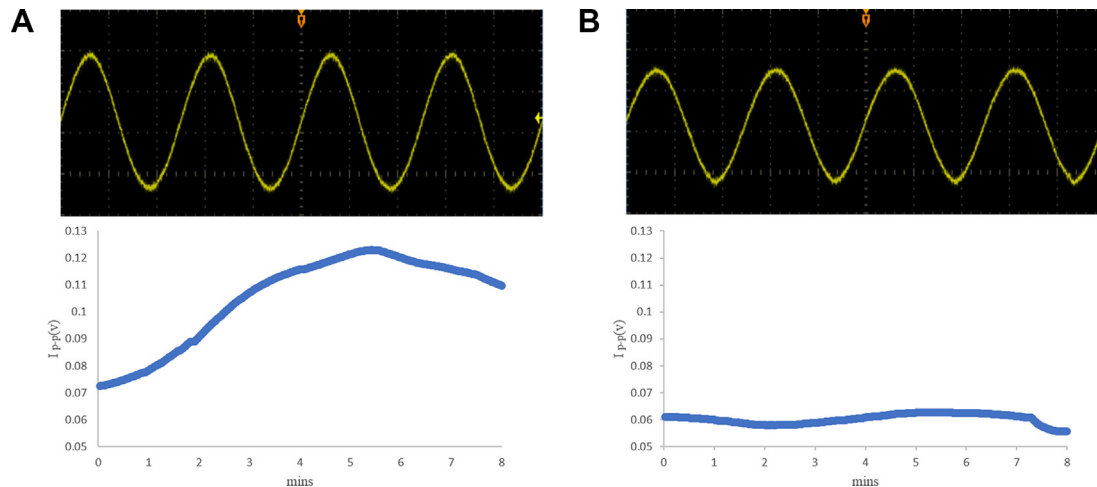| Table 1 Clinical characteristics of Parkinson's disease (PD) patients and controls.			
	PD (%)	Controls (%)	p value
Number	10	11	
Age (years)	61.8 ± 9.26 years	61 ± 5.28 years	0.84
Gender (Female/male)	4/6	8/3	0.13
Hohn & Yahr stage			
I	6 (60%)		
II	4 (40%)		

Fig. 2 The heterodyne signal and long term stability of the peak to peak voltage in the setup of (A) by using a single polarizer (B) by the combination of quarter wave plate and polarizer.

compare data from two independent group and assumed Gaussian distribution. P values < 0.05 were regarded as statistically significant. For correlation analyses, Pearson's correlation coefficients were calculated with IgG concentrations as independent variable and normalized PSPWB amplitude as dependent variable.

Results

System setup

In the setup, the elliptical polarization of two orthogonal linear polarizations in the emerging beam from EOM was induced by the misalignment of the laser beam in EOM. It enlarges the fluctuation of the polarization angle that increases the intrinsic background noise in the measurement. In addition, the temperature instability of high voltage amplifier applied on EOM causes the instability of heterodyne signal as well. In order to reduce the elliptical polarization of the laser beam in Fig. 1, polarizer P was rotated during alignment while QWP was slightly adjusted until the minimum variation of the heterodyne signal being reached. Fig. 2(A) shows the heterodyne signal and its long term stability of the peak to peak voltage where a single P polarizer of the azimuth angle set at 45° was used. This is for generating a pair of parallel linear polarized light waves of their polarization angles along 45° from x-axis. In Fig. 2(B), the heterodyne signal and its long term stability of the peak to peak voltage are shown where the QWP and polarizer P work together in measurement. QWP was adjusted properly and the azimuth angle of P was set at 0° for $p_1 + p_2$ wave selection. It is clearly seen that the heterodyne signals in both setups perform similar on SNR. They are 18 in Fig. 2(A) and 15 in Fig. 2 (B). The SNR is defined by V_{p-p}/σ. V_{p-p} and σ are the peak to peak voltage and noise level of the heterodyne signal respectively. However, the performance of long term stability of the peak to peak voltage in Fig. 2 (B) is superior to Fig. 2 (A). This is due to less temperature sensitivity in the setup of Fig. 2 (B) than one of Fig. 2 (A). The long term

stability of the heterodyne signal is critical to the measurement in this setup particularly for the biomarker detection at very low concentration. In the experiment, total 60 min was taken in each measurement in Fig. 5.

Biomolecular detection

Fig. 3 illustrates the calibrated linear response of the normalized attenuated amplitude versus the IgG concentration in PBS on a logarithmical scale. The negative slope of the response curve was caused by the inverted proportional relationship between the normalized attenuated amplitude χ and concentration. The standard error in each measurement is too small to be shown by use of error bar following the scale on vertical axis in Fig. 3. The limit of detection (LOD) was 6 pg/mL. During the measurement, we used single sensor chip for all different concentration measurements wherein no blocking step was taken in each measurement in order to keep
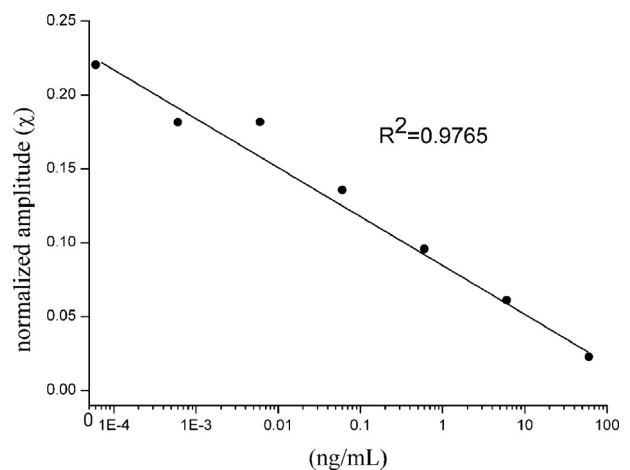

Fig. 3 Calibration of the detection sensitivity of modified PSPWB using different IgG concentrations in PBS.
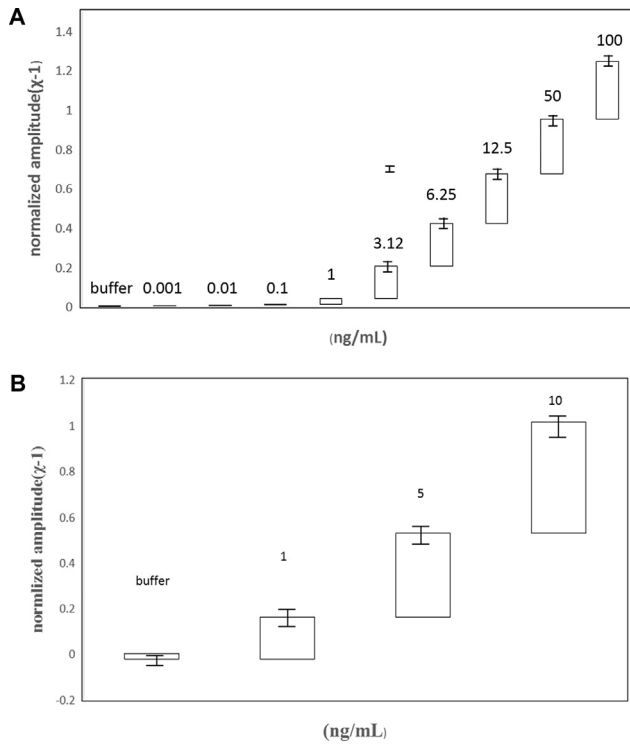
Fig. 4 The calibration responses of (A) the detection p-Ser129-α-syn (standard) at different concentrations in PBS and (B) the detection of p-Ser129-α-syn (standard) at different concentrations, spiked into the serum sample of a healthy control.

same background level at different concentration. In senor chip preparation, 5 μg/mL of the captured antibody was immobilized onto the activated SAM layer in order for a proper dynamical range detection of the analytes. Generally, different physical parameters of the biochip such as the
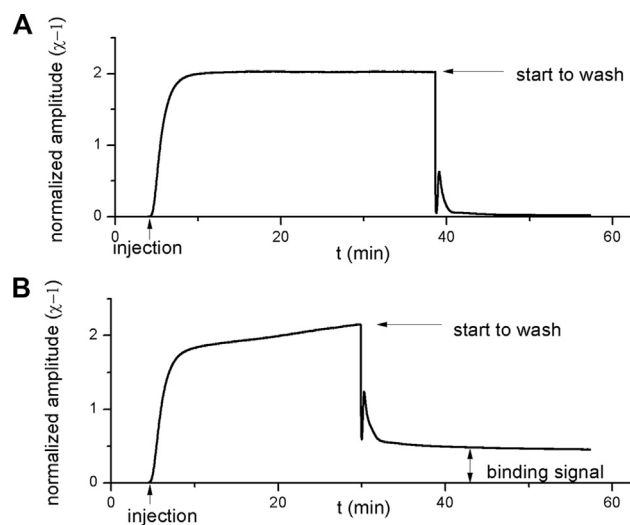

Fig. 5 Sensorgram to detect p-Ser129-α-syn: (A) in diluted serum from a subject in the healthy control group and (B) in diluted serum from a PD patient.

thickness and surface roughness of gold film would cause different response in the measurement. This is due to the spatial correlation between the paired surface plasma waves excited on the gold film/dielectric medium interface in SPRD. The propagation properties of the paired surface plasma waves are highly sensitive to the surface roughness of the gold film. Meanwhile the thickness of gold film determines the resonance angle of SPW too. From our previous research, the arrangement of using single chip for a series of different concentration detection enables to calibrate the detection sensitivity of the setup precisely [21,23]. In the statistical analysis, it was performed using Prism 6 (GraphPad) and results are expressed as mean ± SD.

The detection of p-Ser129-α-syn in PBS on the modified PSPWB sensitivity was assessed, of which p-Ser129-α-syn in a human phosphorylated alpha-synuclein ELISA kit (purchased from MyBioSource Co., Vancouver, Canada) was used as a standard. Fig. 4 shows the measurement of the detected signal versus different p-Ser129-α-syn concentrations of 1 pg/mL, 10 pg/mL, 100 pg/mL, 1 ng/mL, 3.12 ng/mL, 6.25 ng/mL, 12.5 ng/mL, 50 ng/mL, and 100 ng/mL in PBS. To keep the same condition, one biochip was used in the measurement for all different concentrations of p-Ser129-α-syn. They were injected into the flow system in SPRD sequentially. The procedure was done by following the steps described in the sample preparation, but the blocking step by ETH was omitted. Therefore, an increment of the detected signal at different concentrations was added onto the previous result, as shown in Fig. 4(A). The error bar in Fig. 4(A) indicates the standard deviation of the measurement of the same sample within a period of time (2 min) after the washing step being taken. The LOD for p-Ser129-α-syn in PBS measured is 100 pg/mL.

In Fig. 5(A), diluted human serum (1:1000) from a subject in the healthy control group was tested by injecting it into the SPRD. No binding event was observed in the final stage after the sensor chip was washed with PBS (Fig. 5(A)). Furthermore, when diluted human serum sample from a PD patient was injected into the SPRD, a significant difference was observed in the normalized amplitude detected at the final stage after being washed with PBS (Fig. 5(B)). This was caused by the binding of p-Ser129-α-syn from the tested human serum to the sensor chip during measurement.

To test the detection sensitivity of the modified PSPWB, different concentrations of p-Ser129-α-syn (standard) were diluted with PBS at 2 ng/mL, 10 ng/mL, and 20 ng/mL at 500 μl. They were mixed into the diluted human serum (1:1000 by PBS) from a healthy control subject at 500 μl for measurement. Thus, 1 ng/mL, 5 ng/mL and 10 ng/mL of p-Ser129-α-syn in 1000 μl of diluted human serum samples were tested. In order to maintain the same measurement conditions, single biochip was used in the measurement whereas the same steps were taken as shown in Fig. 4(A). The Fig. 4(B) shows the response of p-Ser129-α-syn (standard) detection at different concentrations in the human serum of healthy control group. The error bar indicates the uncertainty of each measurement at different concentrations of p-Ser129-α-syn. The LOD was 1 ng/mL in this experiment. It is consistent with 1 ng/mL in LOD of the p-Ser129-α-syn detection sensitivity noticed by vender using the human phosphorylated alpha-synuclein ELISA kit
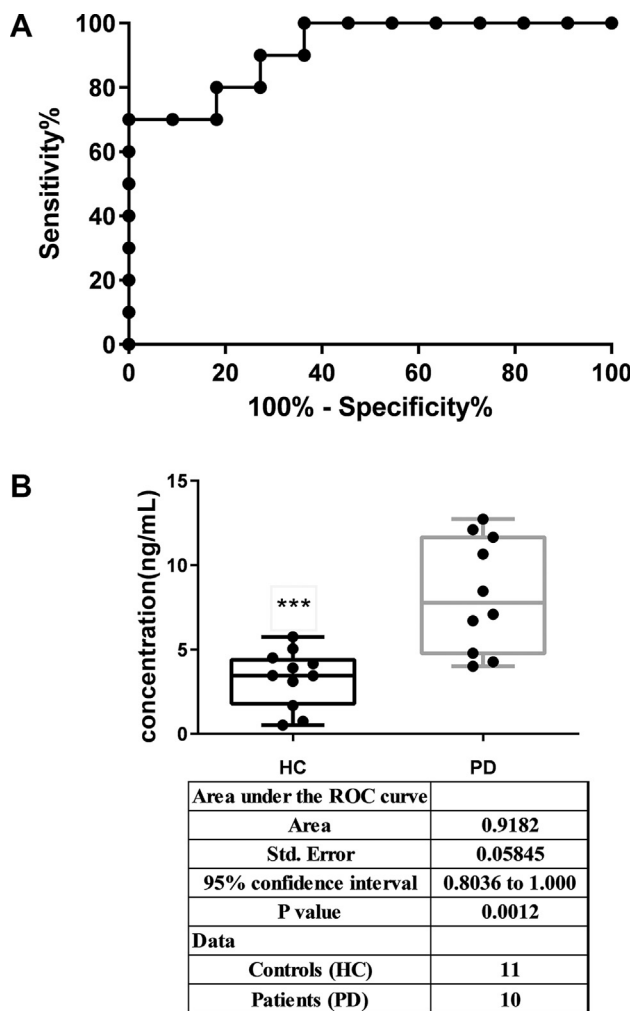
Fig. 6 ROC curve (A) and box diagram (B) of p-Ser129-α-syn detected in diluted human serum samples from 10 PD patients and 11 healthy controls using modified PSPWB according to unpaired t-test (***: $p \sim 0.001$).

(MyBioSource Co., Vancouver, Canada). All statistical analyses in Fig. 4 were performed using Prism 6 (GraphPad).

A total of 21 subjects, including 10 patients with PD in Hohn & Yahr stage I and II, and 11 control subjects, were recruited (Table 1). The mean age of PD patients was 61.8 ± 9.26 years, and that of control subjects was 61 ± 5.28 years. There is no significant difference in age between PD patients and the controls, but the proportion of female is higher in controls compared to that of the PD patients, although not statistically significant.

In the setup for patients and healthy control sample testing, different sensor chip was prepared for each specimen. This arrangement is for the measurement of p-Ser129-α-syn in diluted human serum that would be able to cover whole dynamic range of p-Ser129-α-syn in different concentration. Diluted human serum samples from PD patient or healthy control subjects were injected into the SPRD flow system individually at a flow speed adjusted to 0.4 μL/s and at 22 °C to

form the antigen/antibody immuno-reaction on the sensor chip. From experimental results, the concentration of p-Ser129-α-syn in diluted human serum of the healthy control ranges from 0.5 ng/mL to 5 ng/mL while the range from 4 ng/mL to 12 ng/mL is noticed for PD patients. They are calibrated by the normalized amplitude in Fig. 4(B) that measured p-Ser129-α-syn (standard) in a diluted serum sample of a control group subject. The normalized attenuated amplitude χ measurement is independent of the laser intensity in each measurement. Finally, the normalized attenuated amplitudes of the beat signal were analyzed by using Prism GraphPad software.

Fig. 6(A) shows the ROC curve result, and an associated box diagram is shown in Fig. 6(B) where the Gaussian distribution of the healthy control group in box diagram indicates the confidence of this test. The AUC was 0.92 (p-value 0.001; 95% confidence interval (CI), 0.80−1.03). These results clearly show that the measured p-Ser129-α-syn concentration in diluted human serum displays a statistical significance between HC subjects and PD patients, hence could be potentially considered as an effective biomarker for PD.

The detection sensitivity of the modified PSPWB is comparable with those of ELISA-based methods for p-Ser129-α-syn detection in CSF. In Ref. [15], the mean level of PD is 756.8 ± 2419.9 ng/ml (mean ± SD) and controls is 143.4 ± 531.8 ng/ml, suggesting a high variance within each group and the P value is 0.012, which is also higher than the P value in our study. In addition, the minimally invasive sampling in diluted human serum provides high accessibility to the PD patients as well. This method is favorable to patients for not only the early diagnosis but also monitoring the disease progression.

Discussion

In this study, a modified PSPWB was proposed and demonstrated. A QWP coupled with a rotating polarizer was arranged to improve laser beam alignment in the EOM. This is crucial to the performance of the long term peak to peak voltage stability of the heterodyne signal particularly in an immunoassay detection method at very low concentration for early stage biomarker detection. Because p-Ser129-α-syn has low abundance in human serum, high detection sensitivity and specificity are critical to the success of the diagnosis of PD. In this study, the LOD was 6 pg/mL for IgG in PBS and 1 ng/mL for p-Ser129-α-syn (standard) spiked into diluted human serum of a control. These results show that the modified PSPWB can be used as a platform for detecting p-Ser129-α-syn in diluted human serum as a potential biomarker for PD. In addition, this method is featured by paired highly spatial and temporal correlated SPWs of different temporal frequency which propagating on the interface of gold and dielectric medium. Optical heterodyne interferometer and synchronized detection technique are involved and high detection sensitivity is resulted. Meanwhile, non-labeled technique and real time measurement are capable too. Thus, binding kinetics of

biomolecular interaction becomes applicable in this setup. In addition, a larger dynamic range of the change of effective refractive index based on the amplitude-sensitive detection of the heterodyne signal is performed which is compatible with light intensity detection in conventional ELISA methods. These features of the modified PSPWB ensure the capability of measuring p-Ser129-α-syn in diluted human serum for PD both in diagnosis and prognosis.

PD-specific molecules, particularly in body fluids such as blood, could be used as biomarkers for detecting the disease at preclinical or early stages, indicating disease progression, and monitoring the efficacy of future therapeutic strategies. As the need for biomarkers of PD is growing, the useful PD biomarkers remain to be identified. Misfolded α-syn has been shown to be the main culprit for PD [25] and is one of the major constituents of cytoplasmic Lewy bodies [27]. While α-syn is abundantly expressed in the human brain, its presence in extracellular biological fluids, such as CSF, plasma, and serum has been shown by previous studies [26—30]. Since peripheral blood is practically more accessible than CSF, biochemical biomarkers in blood are favorably searched. However, the reported data for α-syn in blood of PD patients have been inconclusive, showing evidence of a non-changed, lower, or higher level of total α-syn in PD patients when compared with controls [28,30—34]. The inconsistent results possibly come from different detection methods used and easily contaminated with red blood cells which contain a high amount of α-syn. Therefore, the total α-syn in blood may not be a good biomarker for PD. Hyperphosphorylated forms of α-syn protein at S129 (p-Ser129-α-syn) accounts for 90% of α-syn in Lewy bodies in the brains of PD patients, highlighting the important role played by phosphorylated α-syn in pathogenesis of PD [7]. The p-Ser129-α-syn promotes aggregation and increases neurotoxicity in a transgenic *Drosophila* model [34,35], and deemed to be a candidate as a biomarker for PD. However, there are only a few studies investigating p-Ser129-α-syn levels in extracellular biological fluids of PD patients. CSF p-Ser129-α-syn level has been shown to be elevated in PD compared with controls [3].Wang and colleagues demonstrated that CSF p-Ser129-α-syn concentrations correlated weakly with PD severity and, when combined with total α-synuclein CSF concentrations, was able to distinguish PD from other parkinsonism (multiple system atrophy and progressive supranuclear palsy) [36]. Up to date, there are only two studies examining the plasma levels of p-Ser129-α-syn in PD patients [15,37]. Using a sandwich immunoassay, Foulds and colleagues found that plasma p-Ser129-α-syn was higher in the PD patients compared to the controls, but the ability of p-Ser129-α-syn (AUC of 0.717) to discriminate PD patients from healthy controls needs to be improved for clinical application [15]. Lin and colleagues using a more sensitive immunomagnetic reduction (IMR)-based immunoassay showed that with an AUC of 0.94, p-Ser129-α-syn could distinguish PD patients from the controls [37]. They also showed a positive correlation of p-Ser129-α-syn levels with unified Parkinson's disease rating scale (UPDRS) part III motor scores, suggesting a potential role of p-Ser129-α-syn for index of disease severity. In accordance with the results reported by Lin et al. our results demonstrate an AUC of 0.92

for serum p-Ser129-α-syn to predict early stage of PD. It should be addressed that our method needs to be tested in a larger cohort of PD patients with different Hohn & Yahr stages before it is applied to clinical practice. The mean concentration in plasma or serum of PD patients is 756.8 ng/mL, 12.9 fg/mL, and 8.2 ng/mL, respectively, from Foulds et al. Lin et al., and our group. It is noted that the mean concentrations of p-Ser129-α-syn in PD with different assays show a significant discrepancy, the reason for which should be further investigated. The huge difference makes the comparisons between studies difficult. Nevertheless, these three studies agree on the suggestion that p-Ser129-α-syn in blood may serve as a potential biomarker to detect the PD.

There are some limitations in the current study. First, the size of our samples only including 10 PD patients and 11 controls were collected. To test if the sample size would be enough for a proper calculation, we applied G $\times$ power analysis and randomly selected N = 10,10 and the result showed that power value of 0.8504. Further calculation using 'SPSS ROC Curve Analysis' indicates minimum of N = 7,7 could still reach statistical significance of ROC = 0.816 and $p = 0.048$. These analyses suggesting the numbers of current sample set (N = 11,10) is appropriate to perform the statistical comparison. However, due to the small sample size, it is difficult to correlate the p-Ser129-α-syn levels with different disease stages. Second, the use of one sensor chip for measurement of a series of diluted concentrations of p-Ser129-α-syn and IgG standards system calibration might produce small uncertainty in measurement due to lack of blocking process in the sample preparation. As a result, the non-specific binding could occur during the measurement. However in this setup, 1000x dilution of the specimen was prepared. This implies that the level of non-specific binding becomes much lower than it is in the method of using non-diluted plasma or serum as the specimen for measurement. Nevertheless, although our cohort is small, we still have the 0.92 AUC to detect early stage of PD, since our PD patients were averagely at the stage II of Hohn & Yahr stage.

In conclusion, using the present method, we have shown that serum p-Ser129-α-syn is a potential good biomarker in predicting early stage of PD. This minimally invasive method of serum sampling instead of invasive sampling of CSF could offer the capability to conduct longitudinal studies in the PD progression. Clinically, a large cohort study is highly recommended to determine if serum p-Ser129-α-syn measured by using our technique is a valid biomarker of PD.

Conflicts of interest

The authors have no financial or ethical conflicts of interest to report.

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