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Sensitive analysis of α-synuclein by nonlinear laser wave mixing coupled with capillary electrophoresis

Manna F. Iwabuchi, Marcel M. Hetu, William G. Tong*

Department of Chemistry and Biochemistry, San Diego State University, San Diego, CA 92182, USA

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ABSTRACT

Multi-photon nonlinear laser wave-mixing spectroscopy is a novel absorption-based technique that offers excellent detection sensitivity for biomedical applications, including early diagnosis and investigation of neurodegenerative diseases. α-Synuclein is linked to Parkinson's disease (PD), and characterization of its oligomers and quantification of the protein may contribute to understanding PD. The laser wave-mixing signal has a quadratic dependence on analyte concentration, and hence the technique is effective in monitoring small changes in concentration within biofluids. A wide variety of labels can be employed for laser wave-mixing detection due to its ability to detect both chromophores and fluorophores. In this investigation, two fluorophores and a chromophore are studied and used as labels for the detection of α-synuclein. Wave-mixing detection limits of PD-related protein conjugated with fluorescein isothiocyanate, QSY 35 acetic acid, succinimidyl ester, and Chromeo P503 were determined to be 1.4×10^{-13} M, 1.4×10^{-10} M, and 1.9×10^{-13} M, respectively. Based on the laser probe volume used, the corresponding mass detection limits were determined to be 1.1×10^{-23} mol, 1.1×10^{-20} mol, and 1.5×10^{-23} mol. This study also presents molecular-based separation and quantification of α-synuclein by laser wave mixing coupled with capillary electrophoresis.

Parkinson's disease (PD) is the second most predominant neurodegenerative disease in the United States, afflicting 1–2% of the population aged 65 years and over [1]. The disease is clinically characterized by the progressive dysfunction of several motor and nonmotor neurological functions [2]. A series of studies showed that intercellular accumulation of Lewy bodies (LBs), the hallmark of PD, appears 6 years or more before the symptoms emerge [3]. Thus, accurate quantification and detection of LB-related proteins, such as α-synuclein [4], at a sensitive level are key to paving the way to understanding PD.

α-Synuclein is a 14-kDa, 140-residue neural protein highly expressed in central neurons and localized in presynaptic terminals [5]. The protein is a major component of LBs (aggregated proteins), the hallmark pathology of PD and those with dementia [6,7]. The mechanisms and inclusion of α-synuclein in LBs do not explain the pathogenesis of the neurodegenerative disease, and it remains unclear whether LBs are a cause or a symptom [8].

Parkinson's disease was believed to be genetically inherited, because several families with PD history were found to possess a mutation of α-synuclein. Based on this finding, numerous studies developed a hypothesis that both point mutations and multiplications in the α-synuclein gene cause PD [9]. Lee and Trojanowski suggested that β-sheet structure of α-synuclein, readily oligomerized and aggregated, is induced by both pathogenic mutations and elevated levels of α-synuclein [10]. The aggregation of the protein (5–25 μm) [11] is accelerated by various types of post-translational modification such as Ser129 phosphorylation, calpain-mediated cleavage, O-glycosylation, tyrosine nitration, methionine oxidation, and C-terminal truncation [12].

Currently, the toxicity of the oligomerized and protofibril intermediate α-synuclein is widely accepted [13,14]. In addition to the LB hallmark, destruction of dopaminergic neurons is observed during early stages of PD [15–17]. Conway and coworkers reported that the formation of oligomerized α-synuclein can be accelerated

Abbreviations: PD, Parkinson's disease; LB, Lewy body; SNARE, soluble *N*-ethylmaleimide sensitive factor attachment protein receptor; CSF, cerebrospinal fluid; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulfate; CGE, capillary gel electrophoresis; CZE, capillary zone electrophoresis; LIF, laser-induced fluorescence; S/N, signal-to-noise; CE, capillary electrophoresis; UV, ultraviolet; PEG, poly(ethylene glycol); FITC, fluorescein isothiocyanate; CHES, 2-[*N*-cyclohexylamino]ethanesulfonic acid; DMF, *N*,*N*-dimethylformamide; STEM, scanning transmission electron microscopy; PAGE, polyacrylamide gel electrophoresis.

* Corresponding author.
E-mail address: william.tong@sdsu.edu (W.G. Tong).

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by dopamine, although the mechanism remains unclear [18]. Südhof and coworkers suggested that the formation of soluble *N*-ethylmaleimide sensitive factor attachment protein receptor (SNARE) complex, critical to the vesicle fusion leading to dopamine release, is promoted by α-synuclein binding to a vesicular SNARE protein, synaptobrevin-2 [19]. On the other hand, Choi and coworkers reported that large α-synuclein oligomers inhibit neuronal SNARE-meditated vesicle lipid mixing [7].

In addition to cerebrospinal fluid (CSF) analysis, levels of α-synuclein in blood samples have been tested in order to develop less invasive diagnostic methods for PD. Kasuga and coworkers reviewed studies of blood samples from a control group and from PD patients to compare α-synuclein levels. They concluded that, due to the lack of a sensitive detection and quantification method that can differentiate α-synuclein species (truncated, phosphorylated, monomeric, and oligomeric forms) [20], the levels of PD-related proteins are still unclear.

A sensitive detection method is needed in order to develop an early diagnostic tool for Parkinson's disease. Currently, enzyme-linked immunosorbent assay (ELISA) is commonly used for detecting monomeric and oligomeric forms of α-synuclein in CSF and serum; however, ELISA is usually designed specifically for analyzing a single analyte and cannot be coupled to a separation device. ELISA is a time-consuming assay that requires multiple steps to detect and quantify a target protein. The ELISA well is coated with a target protein-specific antibody that binds to the protein when a sample is introduced. The enzyme-linked antibody may react with an unbound site of the protein. Free antibodies that do not react with the protein are removed by washing. In addition, ELISA yields inconsistent results [21–26] for control groups due to background absorption and cross-reactivity interference [27]. The assay may yield false-positive results; that is, a signal might not be generated by α-synuclein antigen–antibody reaction or specify monomeric and oligomeric forms.

Our wave-mixing detection sensitivity is orders of magnitude better than those of conventional absorption techniques and is comparable to or better than those of ELISA and fluorescence methods. Laser wave mixing also provides shorter analysis times and better chemical specificity without the use of expensive antibody. Sodium dodecyl sulfate (SDS)–capillary gel electrophoresis (CGE) or capillary zone electrophoresis (CZE) can distinguish monomeric and oligomeric forms. Unlike laser-induced fluorescence (LIF), laser wave mixing can detect both fluorophores and chromophores. Moreover, the wave-mixing signal is a coherent laser-like beam and can be detected conveniently with high collection efficiency and high signal-to-noise (S/N) ratios.

The wave-mixing signal exhibits excellent properties, including quadratic dependence on analyte extinction coefficient (i.e., concentration) and cubic dependence on intensity of the excitation laser source. The wave-mixing signal can be described with the following equation [28]:

$$I_3 = \left(\frac{b}{8\pi}\right)^2 {I_1}^2 I_2 \frac{\lambda}{\sin^4(\theta/2)} \left(\frac{dn}{dT}\right)^2 \frac{\alpha^2}{\kappa^2}. \quad (1)$$

The intensity of the wave-mixing signal (I_3) produced by the probe and pump beams depends on the cross section of the pathlength of a laser beam (b), the intensities of the excitation laser source (I_1 and I_2), the wavelength of the laser source (λ), the angle between the probe and pump beams (θ), a derivative of the refractive index with respect to solvent temperature change (dn/dT), the extinction coefficient (α), and thermal conductivity (κ). Eq. (1) indicates that the wave-mixing signal has a quadratic dependence on analyte concentration, and hence it allows more effective measurement of small changes in analyte concentration as compared with conventional absorption and fluorescence methods. Both sensitivity and selectivity levels are enhanced when wave mixing is coupled with capillary electrophoresis (CE). Excellent separation resolutions, zeptomole-level sensitivity, fast analysis, and small sample requirements [29,30] are some of the advantages of wave-mixing CE. Taking advantage of these unique features of laser wave mixing, concentration detection limits of PD-related protein conjugated with fluorescein isothiocyanate, QSY 35 acetic acid, succinimidyl ester, and Chromeo P503 were determined to be 1.4×10^{-13} M, 1.4×10^{-10} M, and 1.9×10^{-13} M, respectively. Based on the small probe volume (79 pl) used, corresponding mass detection limits of 1.1×10^{-23} mol, 1.1×10^{-20} mol, and 1.5×10^{-23} mol were determined.

Materials and methods

Forward-scattering laser wave-mixing experimental setup

Fig. 1 shows a schematic diagram of the forward-scattering wave-mixing optical setup interfaced to a CE system. A continuous-wave 488-nm blue laser with adjustable power tuned to 40 mW (Coherent, Santa Clara, CA, USA) and a 50-mW 473-nm diode laser (CNI, Changchun, China) are used for visible-wavelength wave-mixing detection. A 266-nm ultraviolet (UV) pulsed laser (20 mW, 7 kHz, CNI) is used for UV wave-mixing detection of α-synuclein. The laser output is first split by a beam splitter (R:T, 30:70) to create two input beams. The weaker (reflected) input beam is modulated by an optical chopper (SR541, Stanford Research Systems, Sunnyvale, CA, USA) at 200 Hz. In Fig. 1, the solid line and dotted line represent unmodulated and modulated beams, respectively. The two input laser beams travel the same distances and then cross at an angle of 0.95° (488-nm laser) or 1.5° (473- and 266-nm lasers). The excitation lasers yield beam diameters of 1.70 mm (488 nm), 1.20 mm (473 nm), and 1.10 mm (266 nm) and yield probe volumes of 78 pl (488 nm), 58 pl (473 nm), and 55 pl (266 nm). A 75-μm inner diameter fused-silica capillary (Molex, Lisle, IL, USA) is used as the analyte cell into which the analyte is loaded by electrokinetic injection. A small portion of the capillary coating is removed by a butane flame so that the input laser beams can propagate through the capillary analyte cell. The wave-mixing signal is collected by a simple photodetector (PDA25K, Thorlabs, Newton, NJ, USA). The optical chopper, the photodetector, and the computer are interfaced to a lock-in amplifier (SR810 DSP, Stanford Research Systems).

Chemicals

Solutions used for this study were prepared with distilled water from a compact water distillation system. Borax, Tris base, SDS, sodium bicarbonate and poly(ethylene glycol) (PEG, 10,000) were purchased from Sigma–Aldrich. Fluorescein isothiocyanate (FITC), CHES (2-[*N*-cyclohexylamino]ethanesulfonic acid) buffer, hydrochloric acid, sodium hydroxide, unstained protein ladder, *N,N*-dimethylformamide (DMF), and dialysis tubing (MW_{CO} 12–14 kDa) were purchased from Thermo Fisher Scientific. The chromophore label, QSY 35 acetic acid, succinimidyl ester, was obtained from Life Technologies. The capillary was coated with Ultratrol LN (Target Discovery) by flowing through the solution for 2–5 min, and a sieving matrix was prepared by flowing PEG in the sample cell. Recombinant α-synuclein, a DNA sequence encoding the human α-synuclein sequence, was expressed in *Escherichia coli* (rPeptide). Fluorophore protein label, Chromeo P503, was purchased from Active Motif.