On chip detection of glial cell-derived neurotrophic factor secreted from dopaminergic cells under magnetic stimulation

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Abstract

Glial cell-derived neurotrophic factor (GDNF) is a small protein potently promoting the survival of dopaminergic and motor neurons. GDNF can be secreted from different types of cells including the dopaminergic neural cell line, N27. N27 cells, a rat dopaminergic neural cell line, is regarded as a suitable *in vitro* model for Parkinson's disease (PD) research. For PD treatment, transcranial magnetic stimulation (TMS), a noninvasive therapeutic method, showed beneficial clinical effects, but the mechanism for its benefit is not understood. Because GDNF is a potent neurotrophic factor, it is of great value to evaluate if GDNF secretion from N27 cells can be affected by magnetic stimulation (MS). However, the current methods for detecting GDNF are time-consuming and expensive. In this paper we outline the detection of GDNF secretion from N27 cells by ultrasensitive nanopore thin film sensors (nanosensor) *for the first time*. As low as 2 pg/mL GDNF can be readily detected by the nanosensor. Furthermore, we show that MS can promote GDNF secretion from N27 cells. Specifically, the GDNF concentration in N27 cell-conditioned media under MS treatment shows statistically significant increase up to 2-fold after 5 days *in vitro* in comparison with the control. This nanosensor along with the *in vitro* PD model N27 cells provides a low-cost, easy-to-use, sensitive approach for studying potential cell biological mechanisms of the clinical benefits of MS on PD.

Key words: Dopaminergic cells, transcranial magnetic stimulation, cell secretion, nanopore thin film sensor, neurotrophic factors detection.

1. Introduction

Glial cell line-derived neurotrophic factor (GDNF) has been reported to be a survival promoting molecule for dopamine neurons, and showed its effectiveness for treating subjects with neurodegenerative diseases (Choi-Lundberg et al. 1997). In addition, it has been found that GDNF also has neurotrophic effects on a wide range of neuronal populations such as motor neurons and sympathetic neurons (Pöyhönen et al. 2019). Fundamentally, GDNF supports proliferation, differentiation, maturation, neurite outgrowth and other cell functions (Wang et al. 1997; Schuster, Dambly-Chaudière, and Ghysen 2010). Intriguingly, it has been found that neuronal damage and motor dysfunction can be ameliorated by GDNF, thus it has been regarded as a promising treatment for subjects with neurodegenerative disorders such as Parkinson's disease (PD). Hence, promotion of GNDF secretion is becoming critical and could significantly benefit the treatment of PD. Efforts towards enhancing GNDF secretion have included using some vacuolar ATPase (V-type ATPase) inhibitors such as concanamycin A (ConA) and bafilomycinA1, which enhance GDNF secretion from glioma cells (Nishiguchi et al. 2003). In addition, atypical antipsychotic drugs can also cause GDNF secretion from C6 glioma cells (Shao et al. 2006). GDNF has been detected *in vivo* using confocal microscopy (Ortega-de San Luis and Pascual 2016). It also has been detected *in vitro* by combining an enzyme-linked immunosorbent assay (ELISA) with the light emission from a Renilla luciferase (RUC)-GDNF fusion protein in cerebrospinal fluid (CSF). As demonstrated, RUC can be used as a reporter for GDNF *in vitro* (Liu, Iacono, and Szalay 2001).

One cell line that has been regarded suitable as an *in vitro* model for PD research is the N27 dopaminergic cell line (Gao et al. 2016)**.** Recent experiments have shown that magnetic stimulation (MS) can promote proliferation of N27 dopaminergic cells and affect their proliferation, adhesion, and other cell biological behaviors on nano-textured surfaces (Che et al. 2018). The mRNA transcript of GDNF can be detected using quantitative polymerase chain reaction (qPCR), but this only

measures transcript levels and is an indirect measurement of protein levels or levels of secreted GDNF (Zhong et al. 2018). Consequently, an assay to measure secreted GDNF directly is desirable. Research has found that TMS shows some positive effects for treating PD in clinic trails (Brys et al. 2016; Cohen et al. 2018). However, the mechanism is currently unknown. Hence it is of great value to examine and thus understand if MS can promote GDNF secretion of these dopamine cells. If so, the promotion of the GDNF secretion might be one of reasonable mechanisms for its effectiveness for treating this disease.

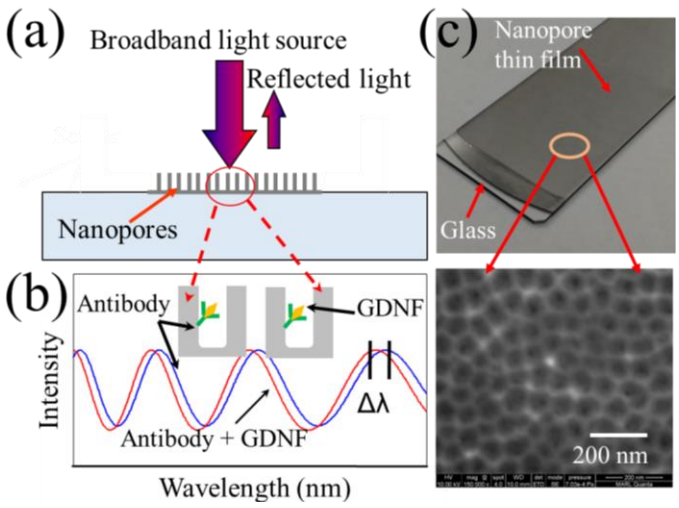

Figure 1. (a) Sketch of the nanopore thin film sensor (nanosensor) and (b) its operational principle: reflected optical interference fringes shift when GDNF is bound to the GDNF antibody, which is immobilized on the sensor surface; (c) A photo of the nanosensor fabricated on a glass substrate and the SEM images showing the nanopores in the nanosensor.

Herein, we report the studies of MS effect on the GDNF secretion from N27 cells. Due to the extremely small amount of GDNF secreted from N27 cells in the culture media, a nanopore thin film sensor (nanosensor) providing ultrasensitivity was used for the measurements. Similar to the surface Plasmon resonance (SPR) sensor (Homola 2008) and biolayer interferometry (BLI) sensor (Naik et al. 2013), the nanosensor is also a label-free optical sensor, which utilizes different

operational principles of electrochemical nanopore array sensors (Santos, Kumeria, and Losic 2013a). The nanosensor consists of a layer of Au-coated nanopore thin film on a glass substrate as schematically shown in **Fig. 1a**. Its operational principle is illustrated in **Fig. 1b**. Its transducing signals are the reflected optical signals (optical interference fringes) from the nanopore thin film (Zhang et al. 2010, Santos, Kumeria, and Losic 2013a, 2013b; Santos et al. 2012). The optical signal shift (OSS) results from the changes of the effective optical thickness when the GDNF is bound to the GDNF antibody. A photo of the sensor chip and one SEM image of anodic aluminium oxide (AAO) nanopore thin film are shown in **Fig. 1c**.

2. Materials and Methods

2.1. Chemicals and materials

11-Mecaptoundecanoic acid ($HSC_{10}COOH$, 99%), 8-mercapto-1-Octanol (HSC_8OH, 98%), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), ethanolamine (EA) were purchased from Sigma. Deionized (DI) water was obtained from a DI water purification system (Millipore, FRANCE). Two fluorophores, calcein AM (*Sigma-Aldrich*), and propidium iodide (*Sigma-Aldrich*) were purchased from Sigma-Aldrich. Transforming growth factor β1 (TGF- β1) was purchased from Thermo Fisher Scientific, Inc. Alpha-synuclein (α-syn) was purchased from Thermo Fisher Scientific, Inc.

2.2 N27 cell subculture and seeding

N27 neural cells (Millipore Sigma), which were immortalized rat mesencephalic cells (1RB3AN27), were sub-cultured for this study. The N27 cells were growing in a T25 flask with RPMI-1640 medium (Sigma-Aldrich), 10% fetal bovine serum (FBS) (Sigma-Aldrich), 1% L-glutamine (Sigma-Aldrich), penicillin (100 U/mL), and streptomycin (100 U/mL) and maintained at 37 °C in 5% CO_2 atmosphere.

For the purpose of cell seeding, the N27 cells in the T25 flask were transferred to a 96-well plate (flat bottom). Briefly, after the culture medium was completely removed from the T25 flask, 1 mL trypsin-EDTA (0.25%) was added in the flask. The N27 cells would detach from the flask bottom within 5 min. The solution with suspending cells was put in a conical tube and subsequently centrifuged at 200 x g for 5 min. The supernatant was removed, leaving a cell pellet at the bottom of the conical tube. Then, the pellet was resuspended in culture media with 10% FBS. N27 cells were added to the 96-well plate at a cell density of ~800 cells/well and ~8,000 cells/mL culture media in each well. The plate was then kept in the incubator, maintained at 37 °C 5% CO_2.

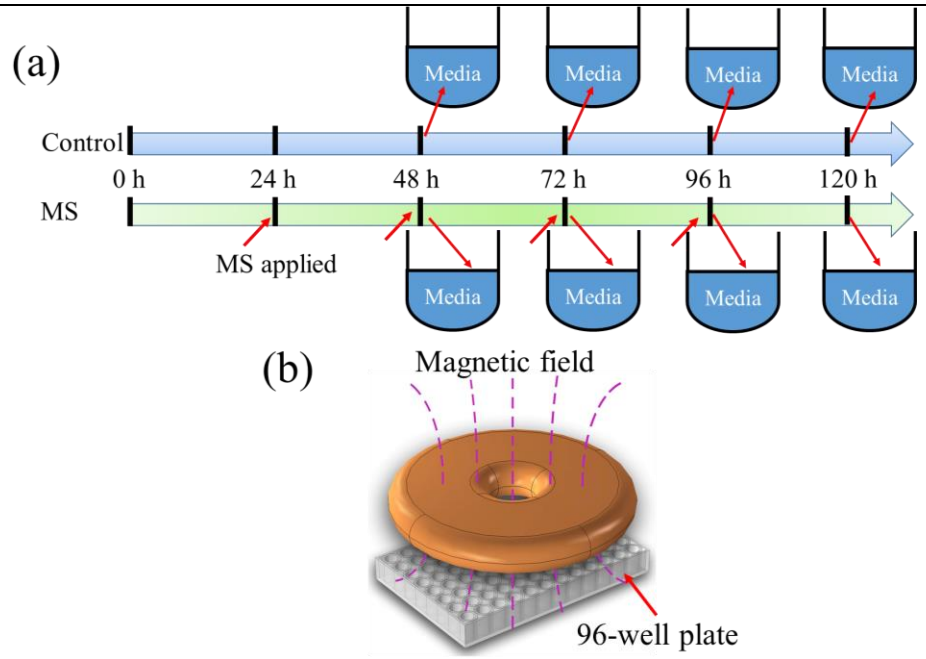

Figure 2. (a) Time line for the TMS treatment on experimental samples and the collection of the N27 cell conditioned media from experimental samples and control samples; (b) Sketch showing the TMS treatment on N27 cells cultured in 96-well plate.

2.3 MS operation for the experiments

Magstim 200[2] monophasic stimulator with a coil (90 mm remote control stimulating coil 3192-00),

which can provide a magnetic field suitable for medical treatment, was used to magnetically stimulate the N27 cells. The cells were treated with one session (100 pulses, 10 s interval and 100% machine output) at 24 hrs (1 day *in vitro* (DIV)), 48 hrs (2 DIV), 72 hrs (3 DIV), and 96 hrs (4 DIV) after cell seeding, respectively. The conditioned media was collected from 2 DIV to 5 DIV for the measurements (**Fig. 2a**). The coil was held directly above the 96-well plate (**Fig. 2b**). The control samples were placed far away (>2 m) from the coil, while the counterpart samples were undergoing stimulation.

2.4 Magnetic field measurements

The magnetic field strength (*H*) generated by the stimulator (Magstim 200^2 monophasic) and coil (Magstim 90 mm circular coil) was measured using a Gaussmeter (Lakeshore 475) with an axial Hall probe (Lakeshore HMMA-1904). The Hall probe was placed orthogonal to the plane of the coil at a distance of 17 mm. The placement of the Hall probe was in the same orientation and at the same distance between the coil and the N27 cells when magnetic stimulation was applied. The stimulator was set to 100% output power. The Gaussmeter was set to record the peak *H* during a single pulse cycle. The pulse rise time was ~100 μs with a period of 1 ms (1 kHz). Measurements were made in 1 mm increments across the diameter of the coil. Multiple sets of measurements were recorded, then averaged. This method was repeated for three different paths across the center of the coil.

2.5 ELISA kit for GDNF measurement

In order to detect GDNF secreted by N27 neural cells (Millipore Sigma), which were immortalized ***rat*** mesencephalic cells (1RB3AN27), a *rat* GDNF enzyme-linked immunosorbent assay (ELISA) kit was purchased from Boster Biological Technology (catalogue number: EK0363). The kit was designed to measure GDNF with a 96-well strip plate that was pre-coated with antibody specific for GDNF. The detection antibody was a biotinylated antibody specific for GDNF. The capture antibody was a mouse monoclonal antibody, and the detection antibody was a goat polyclonal antibody.

To measure the GDNF in the culture media, the samples were loaded to the wells (100 μL per well), incubated at 37 °C for 90 min. After completely removing the sample solution, the 1× biotinylated anti-rat GDNF antibody (100 μL) was added to each well, incubated at 37 °C for 60 min. After rigorous washing with phosphate buffered saline (PBS) solution, 1× avidin-biotin-peroxidase complex (100 μL) was added into each well, incubated at 37 °C for 30 min. After rigorous washing, a colour developing reagent (90 μL) was added to each well, incubated at 37 °C for 40 min in darkness. Then, the ELISA stop solution (100 μL) was added to each well. The colour in the well changed to yellow. Within 30 min, the media in each well on plate was measured by a micro plate reader (Molecular Devices, LLC) at a wavelength of 450 nm to obtain their absorbance data. Using a calibration curve, the GDNF concentrations can be determined.

2.6 Cell labeling and viability test

N27 cells were labeled with fluorophores calcein AM and propidium iodide at a concentration of 2 μM for the live/dead assay and evaluating the cell viability (Gong et al. 2011). The fluorescence images of N27 cells were taken with a fluorescence microscope (Olympus, Inc.). Green (calcein AM) indicates live cells and red (propidium iodide: PI) indicates dead cells (Penmetsa et al. 2010), respectively.

2.7 Fabrication of the nanosensor

Briefly, the nanosensor was fabricated using the following steps. *First*, a layer of Ti (10 nm) was deposited on a rigorously cleaned glass substrate by electron-beam evaporation. The Ti layer was used as an adhesion layer. Then a layer of aluminum (2-3 μm) was deposited on the Ti-coated glass substrate by electron-beam evaporation. *Second*, the nanopore thin film, anodic aluminum oxide (AAO), was formed by anodizing the aluminum thin film on a glass substrate. Typical thickness of the nanopore thin film was 2-3 μm and the nanopore size was 80-90 nm. *Third*, a layer of Cr thin film (5Å) was deposited on the nanopore thin film as an adhesion layer, followed by depositing a

layer of Au thin film (100 Å) (Fig. 1d). As a result, the nanosensor was fabricated. Basically its fabrication process is the same as that in our previous work (Song et al. 2020). The steps are schematically illustrated in Fig. S1 in the supplementary document.

2.8 Nanosensor surface functionalization

Using a similar surface functionalization process in our previous work (Alzghoul et al. 2016), the GDNF antibody was immobilized on the nanosensor surface using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/N-hydroxysulfosuccinimide (NHS) chemistry. The steps are schematically illustrated in Fig. S2a in the supplementary document. Briefly, a solution of 0.1 mM (1:9) $HSC_{10}COOH/HSC_8OH$ was applied to the nanosensor surface for 30-minute incubation, followed by a rigorous wash with ethanol. After the surface was dried naturally, a solution of NHS and EDC (NHS 0.2 M, EDC 0.05 M) was applied to the sensor surface for 30-minute incubation, followed by rigorous rinse by PBS buffer. Then the nanosensor surface was immersed in the 5 μM GDNF antibody solution from the rat ELISA kit overnight. The next step was to apply 100 μL 1 M ethanolamine (EA) to the nanosensor surface to react with the non-occupied sites activated by the EDC/NHS, followed by rigorous rinse with the buffer to remove non-specifically adsorbed proteins. Thereafter, the nanosensor can be used to detect GDNF in buffer or conditioned media (i.e. the media harvested from cultured N27 cells).

2.9 Detection procedure

The nanosensor was put under an optical fiber probe connecting with a white light source (Ocean Optics, Inc.), allowing the light delivered from the fiber probe to be perpendicular to the sensor surface. The reflected light signal from the sensor was collected by the same optical fiber probe and recorded by a miniature spectrometer (Ocean Optics, Inc.). The optical signal from a fresh nanosensor without any surface chemical treatment was measured as a reference. Thereafter, the optical signal from the nanosensor after each step of surface chemical treatment (Fig. S2b in the

supplementary document) was measured until the GDNF antibody was functionalized on the nanosensor surface. Then the nanosensor was applied with the sample solution (buffer with spiked GDNF or the N27 cell conditioned media) and incubated at room temperature for 30-40 min before the measurement. Care should be taken for collecting the conditioned media for both control samples and experimental samples. The wells should be shaken or the media should be stirred gently so that the secreted GDNF from N27 cells can be uniformly distributed in the culture media. In this way, accurate concentrations of the secreted GDNF can be measured after applying the collected samples on the nanosensors. Unless specifically mentioned otherwise, each sample (buffer with spiked GDNF or the N27 cell conditioned media) was detected eight times in the measurements.

2.10 Data acquisition, calibration, and statistical analysis

The transducing signal (i.e. optical signal shift) was obtained by averaging the shifts of the peaks of the interference fringes of the optical signal by comparing the nanosensor spectra before and after the sample solution was applied on the nanosensor. It is worth noting that the blank culture media without GDNF can still cause spectral shift in the optical signal due to nonspecific binding between nanosensor surface and the chemicals in the blank culture media. The shift was considered as background shift, which was subtracted from all the measured results.

The analysis technique of the optical signal shift (OSS) was used as outlined in (Hao et al. 2014; Sheng et al. 2006) to measure shifts due to GDNF binding. The measured transducing signal (OSS) vs. the known GDNF concentration curve can be plotted against the logarithm of concentration of GDNF through a five-parameter logistic model in equation (1), which is suitable to calibrate the relationship between the OSS of the nanosensor and the GDNF concentrations.

$$OSS = \frac{A_1 - A_2}{1 + (x/x_0)^p} + A_2 \quad (1)$$

Where x is the GDNF concentration, x_0 is the GDNF concentration at the midpoint or

inflection point, p is the slope of tangent at inflection, A_1 and A_2 are the minimum and maximum OSS, respectively.

There were 3 independent trials in these experiments. In each trial, 4 independent samples were in the control or the TMS-treated group. The OSS of each sample on 5 DIV was measured for one-way analysis of variance (ANOVA). Statistical analysis was performed in OriginLab 8.1. Statistical significance was set at p-value less than 0.05.

3. Results and Discussion

3.1 ELISA measurement of GDNF in culture media and in conditioned media

Using the rat ELISA kit, a series of known amounts of GDNF spiked in culture media, resulting in a series of different concentrations, was detected. As shown in **Fig. 3a**, the lowest concentration of GDNF in culture media detected was 32 pg/mL for this type of rat ELISA kit. When the concentration of GDNF is at or below 16 pg/mL, it is not distinguishable from the GDNF concentration of 0 pg/mL, the culture media, which is consistent with datasheet of this ELISA kit.

The conditioned media of N27 cells without and with magnetic stimulation after different days *in vitro* (DIV) have been collected for the measurements. In these experiments, four control samples and four experimental samples were prepared, respectively. The four control samples were cultured under the same conditions as the experimental samples, but not subjected to the magnetic stimulation. The procedure for magnetic stimulation on the experimental samples is illustrated in **Fig. 2a.** After the N27 cells were cultured in media for 1 DIV, the experimental samples were subjected to the one MS treatment per day from 1 DIV to 4 DIV. Then the conditioned media were collected on 2 DIV, 3 DIV, 4 DIV, and 5 DIV, and consequently the GDNF in the conditioned media was detected using this ELSA kit. As shown in **Fig. 3 b**, no signal was observed for either control samples or experimental samples collected, indicating the concentrations of the secreted GDNF in the media are lower than its detection limit (~32 pg/mL).

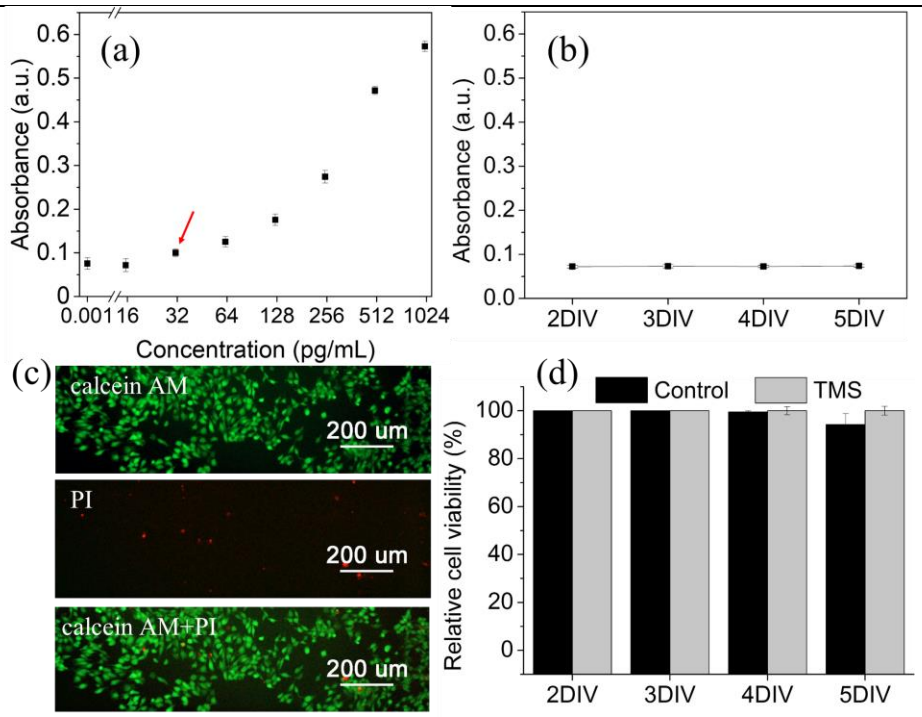

Figure 3. ELISA measurement: (a) the measured optical absorbance of the samples spiked with GDNF of a series of known concentrations in culture media; (b) the measured optical absorbance of the N27 cell conditioned media with and without TMS treatment; (c) representative fluorescence images of N27 cells labeled with calcein AM and PI on 5 DIV; (d) 97-100% viability of N27 cells cultured on 96-well plate under MS from 2 DIV to 5 DIV. Error bars were obtained by calculating standard deviation values.

3.2 Viability of N27 cells under magnetic stimulation

In these experiments, the N27 cells in the T25 flask were transferred to a 96-well plate (flat bottom). MS was then applied after the cells grew in the wells for 24 hrs (1DIV)), 48 hrs (2 DIV), 72 hrs (3 DIV), and 96 hrs (4 DIV) after cell seeding, respectively. Then the fluorescence images were taken on 2 DIV, 3 DIV, 4 DIV and 5 DIV, respectively. Representative fluorescence images of cells on 5 DIV are shown in **Fig. 3c**. The viability quantification of the cells based on the fluorescence images from 2 DIV to 5 DIV is shown in **Fig. 3d**. As expected, MS has a negligible cytotoxic effect on cells (Che et al. 2018).

3.3 Nanosensor measurement of the GDNF in culture media

In order to detect GDNF secreted in culture media at lower concentrations than 32 pg/mL, the nanosensor with improved sensitivity functionalized with GDNF antibody was used for the measurements. First we calibrated the nanosensor to establish the relationship between the transducing signals (OSS) and the GDNF concentrations in culture media. Again, the known amounts of GDNF were spiked in culture media, resulting in the known concentrations from 0 pg/mL to 128 pg/mL, for the measurements. The measured interference fringes of the optical transducing signals for different GDNF concentrations are shown in **Fig. 4a,** the shift of the peak of interference fringes is clearly observed with the increased concentration of GDNF. One representative real-time measurement of the OSS at one concentration of GDNF (8 pg/mL) has been carried out on the nanosensor. As shown in **Fig. 4b,** it took at least 30 min for the OSS to become saturated after the GDNF sample was applied to the nanosensor, suggesting that the time required for GDNF to diffuse to and bind GDNF antibody is at least 30 min (Chen et al. 2019). Hence, all the measurements for different GDNF samples were taken with 30-40 minute incubation after GDNF samples were applied to the nanosensor. The plot of the OSS ($\Delta\lambda$) and the GDNF concentrations is displayed in **Fig. 4c**. Note that the background signals, due to the non-specific binding of chemicals in culture media with the nanosensors, has been subtracted using a reference nanosensor (**Fig. 4c)**. When the GDNF concentrations increase from 0 pg/mL to 128 pg/mL, the $\Delta\lambda$ increases up to 2.5 nm. As shown, the nanosensors can readily detect GDNF of a concentration as low as 1 pg/mL in culture media with high specificity, much lower than that (32 pg/mL) of the rat ELISA kit. The relative standard deviation values for the detection of GDNF are ~6.5 %, suggesting good repeatability of the nanosensors. The limit-of-detection (LOD) of this nanosensor for GDNF is 2 pg/mL, at which the OSS is greater than the average OSS from zero concentration of GDNF + 3σ (σ is the standard deviation of OSS from zero concentration). The relationship between the OSS and the GDNF

concentration has been fitted (**Fig. 4c**) based on these measurements using the data analysis method described in **Section 2.10**. This calibration curve was then used to assess the unknown GDNF concentrations secreted from N27 cells in conditioned media.

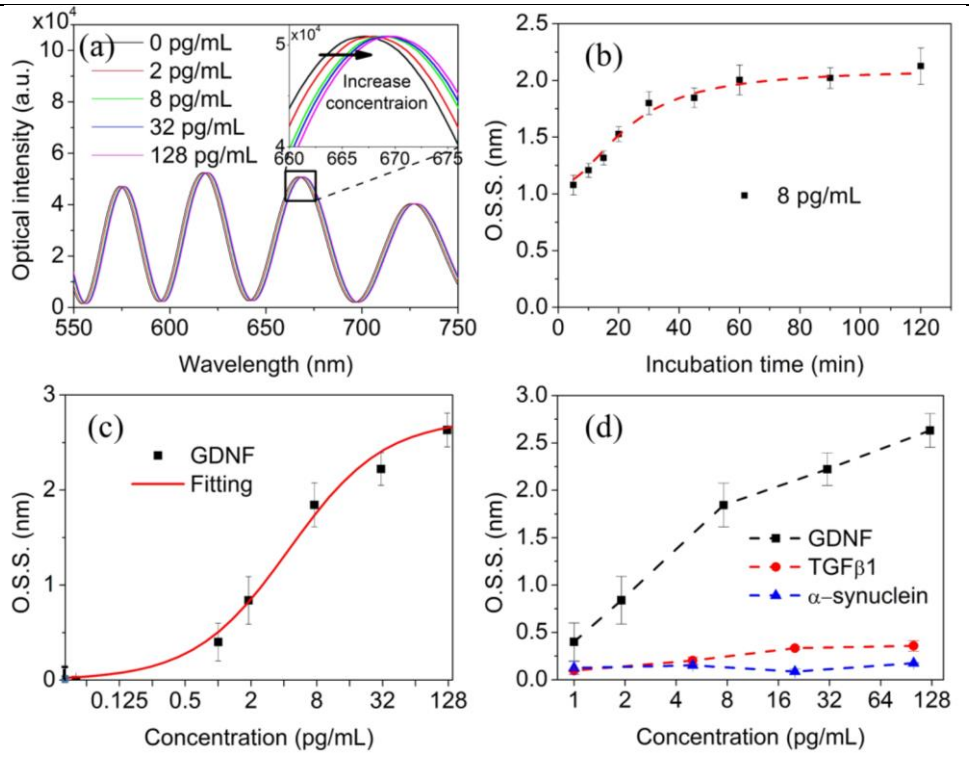

Figure 4. (a) The measured optical interference fringes from the nanosensors for the culture media spiked with GDNF of a series of known concentrations; (b) real-time measurement of the optical signal shift (OSS) with GDNF at a concentration (8 pg/mL); (c) the measured (OSS) and the fitting curve for the OSS vs. the GNDF concentrations based on a five-parameter logistic model; (d) selectivity measurement: the measured OSS of the culture media samples spiked with α-syn and TGF-β1, respectively in comparison with the culture media samples spiked with GDNF. Error bars were obtained by calculating standard deviation values.

In order to measure GDNF in conditioned media, the selectivity of the nanosensor need to be evaluated. As such, two PD related biomolecules (α-syn and TGF-β1) spiked in culture media have been detected by the nanosensors. α-syn is a synuclein protein, which is widely regarded as the

biomarker for the PD (Song et al. 2020), while TGF-β1 is a multifunctional secreted protein, which is a potential factor for slowing neurodegeneration and reducing neuroinflammation in both animal and human PD models (Spittau 2015). As shown in **Fig. 4d**, the OSS for GDNF samples are much larger than those for the samples of α–syn and TGF-β1 spiked in culture media with a series of concentrations in a range from 1 pg/mL to 128 pg/mL, indicating very good selectivity of the nanosensors.

3.4 Proliferation quantification of cultured N27 cells with and without MS

The effect of magnetic stimulation on the proliferation of N27 cells has been evaluated. Representative fluorescence images of N27 cells on 2 DIV and 5 DIV without and with MS stimulation are shown in **Fig. 5a-b**. As shown clearly, the numbers of N27 cells increase from 2DIV to 5 DIV for both control and with magnetic stimulation. Based on these images, it has been found that the numbers of N27 cells increased about 13.8% with magnetic stimulation (**Fig. 5c**) over the control on 5 DIV, which is consistent with previous work (Zhong et al. 2018).

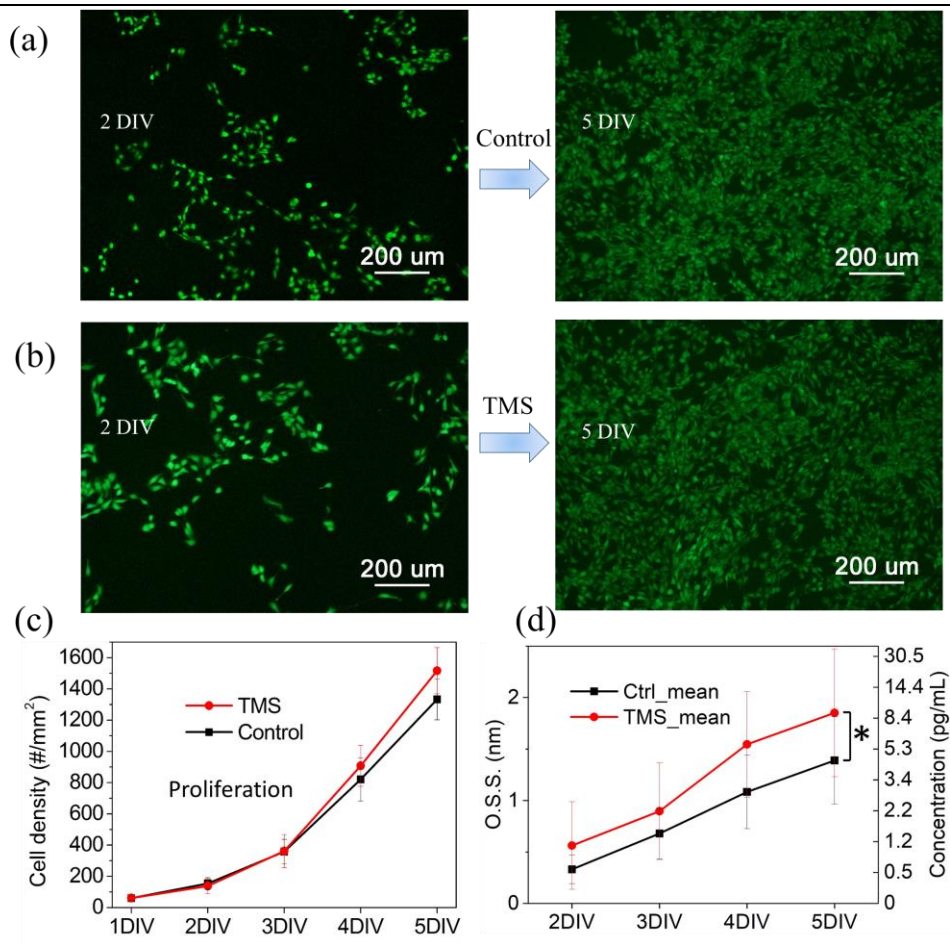

Figure 5. Representative fluorescence images of N27 cells on 2 DIV and 5 DIV (a)without magnetic stimulation; (b) with magnetic stimulation; (c) Measured areal density of the N27 cells without and with MS; (d) the measured OSS of the N27 cell conditioned media with and without MS treatment and the calculated corresponding GDNF concentrations. The asterisk represents significant difference between the control and MS-treated samples ($p < 0.05$). Error bars were obtained by calculating standard deviation values.

3.5 Measurements of GDNF in conditioned media

GDNF secretion of cultured N27 cells with and without magnetic stimulation: The effect of magnetic stimulation on the GDNF secretion of N27 cells has been evaluated using the nanosensors. The measured OSS for both control and experimental samples and their corresponding GDNF concentrations, which are calculated based on the calibration curve in **Fig. 4c**, are displayed in **Fig.**

5d. For the control samples, the GDNF concentration increases from 0.6 pg/mL to 4.5 pg/mL with the culture time from 2 DIV to 5 DIV. This statistically significant increase was undetectable using traditional ELISA assays. Increases in GDNF are seen because cells continuously release GDNF and the number of cells increase due to proliferation. As a result, increased amount of GDNF was secreted from N27 cells into the conditioned media over time. A similar trend was also found for the MS-stimulated samples. In comparison with the control samples at the same culture time (2 DIV, 3 DIV, 4 DIV and 5 DIV), clearly increased GDNF was secreted from N27 cells under MS (magnetic field strength of 0.41-0.73 MA/m). As shown in **Fig. 5c**, MS can promote the proliferation of N27 cells, typically the number of cells can increase ~14% compared to the control without MS. In contrast, as shown in **Fig. 5d**, the secreted GDNF concentration increases up to 2-fold compared to that of the corresponding control samples, suggesting the increased GDNF concentrations mostly result from the MS effect and not proliferation. Namely, MS can enhance GDNF secretion from N27 cells. This trend is consistent with the results indicating that GDNF transcript is enhanced after MS (Zhong et al. 2018). For the *first time* the concentration levels of the secreted GDNF from N27 cells in conditioned media without and with MS is measured.

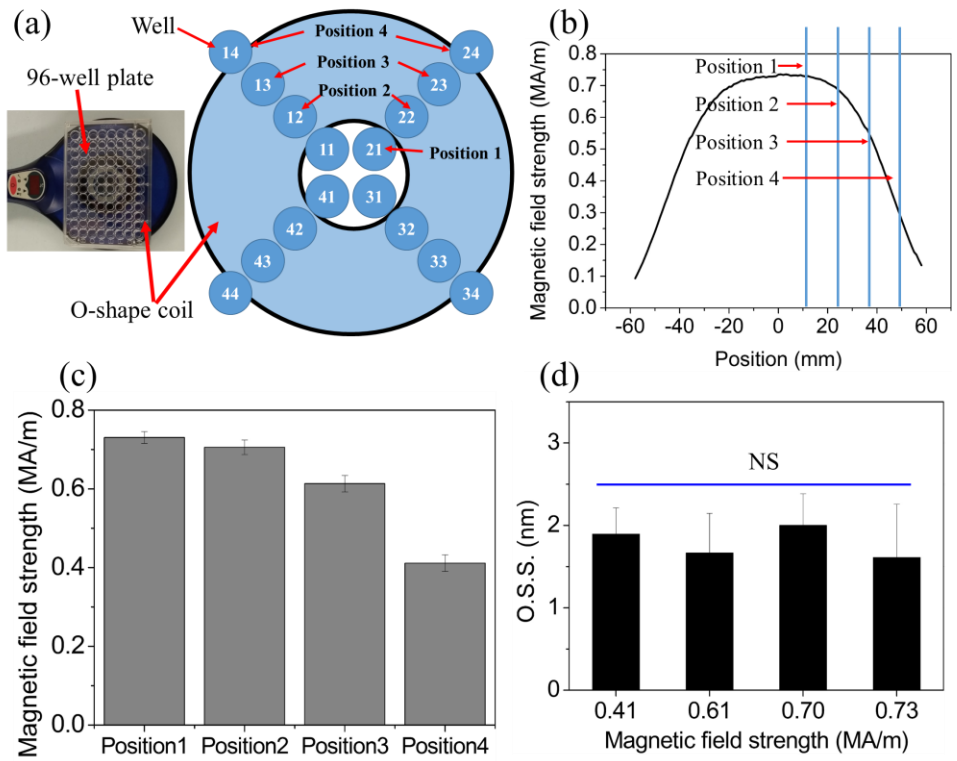

Figure 6. (a) Photo showing a magnetic simulator (O-shape coil) to stimulate the N27 cells cultured inside the wells on a 96-well plate, and the corresponding positions (Position 1, Position 2, Position 3 and Position 4) of the wells under different MF strength; (b) the measured MF strength profile along the radial direction from the center to the edge of the O-shape coil; (c) the measured the local MF strength in the wells in Position 1 to 4; (d) the measured OSS of the N27 cell conditioned media under MS treatment of different magnetic field strength. NS denotes no statistically significant difference. Error bars were obtained by calculating standard deviation values.

The effect of different strength of the magnetic field on GDNF secretion: GDNF secretion from N27 cells under different strengths of magnetic field (MF) was also evaluated. *First*, the specific strength of the magnetic field applied on each well on the 96-well plate was determined by measuring the strength of the magnetic field from the center of the *O-shape coil magnetic stimulator* to the edge along the radial direction (**Fig. 6a-c**). The measurement procedure is illustrated in **section 2.4.** *Second*, in these experiments, sixteen samples of the same concentrations of N27 cells were prepared. Then each of the four strength of the magnetic field of 0.73 MA/m, 0.70 MA/m, 0.61 MA/m and

0.41 MA/m were used to treat the four samples. The GDNF secretion from N27 cells under different strengths of magnetic field was evaluated. The MS treatment and the media collection procedure was the same as that illustrated in **Fig. 2a**. The measured transducing signals (i.e. OSS) from the conditioned media at 5 DIV are shown in **Fig. 6d**. It was found that the GDNF secreted from N27 cells shows no statistically significant difference under MS of different field strengths.

For comparison with the nanosensor used in this study, some other mainly reported methods to measure GDNF level include ELISA(Lin and Tseng 2015), western blot (Lin and Tseng 2015), immunostaining(Ortega-de San Luis and Pascual 2016), luciferase assay (Liu, Iacono, and Szalay 2001), and polymerase chain reaction (PCR) (Chermenina et al. 2014). More specifically, several GDNF ELISA kits are commercially available with LOD from 3 pg/mL to 32 pg/mL. The LOD of ***rat*** GDNF ELISA kit is ~32 pg/ml. Western blot is a traditional method to measure the protein concentration. But its LOD is much higher than either ELISA (32 pg/mL) or our developed nanosensor (2 pg/mL). The LOD of western blot depends on the protein of interest, but a reasonable estimate is on the order of 3-30 ng/mL (Bakalova et al. 2005). The immunostaining includes immunocytochemistry and immunohistochemistry. The cells expressing GDNF can be immunolabeled with biomarkers and therefore yield fluorescence signals under proper excitation. However, this method cannot be used for quantitative analysis. In luciferase assay, renilla luciferase (RUC) cDNA and GDNF cDNA can be fused together and then genetically engineered into glial cell lines. RUC is expressed concurrently with GDNF. The GDNF concentration correlates with the RUC bioluminescence intensity. The Luciferase assay is complicated, laborious and expensive. PCR can amplify the mRNA of the GDNF synthesis. It gives evidence for GDNF gene expression. However the cell secretion and mRNA levels are not necessarily correlated, and consequently the best output to measure is GDNF secretion but not mRNA transcript level (Chermenina et al. 2014).

While our developed nanosensor overcomes some issues of these reported methods, it is only

suitable for *in vitro* detection of GDNF level, and unsuitable for *in vivo* detection. In addition, the current version of the nanosensor can only detect one type of molecule (i.e. GDNF) without multiplexed detection capability.

4. Conclusions

In this paper, the effect of MS on the secretion of GDNF from N27 cells has been studied by using nanosensors. This type of nanosensor offers a much improved LOD for GDNF (2 pg/mL) over that of a rat ELISA kit (32 pg/mL). The GDNF concentrations in N27 cell conditioned media have been measured. It has been found that the concentration of GDNF in conditioned media samples increases up to 2-fold compared to the control on 5 DIV after one MS treatment on 1 DIV, 2 DIV, 3 DIV and 4 DIV, respectively. However, the different strength of the magnetic field from 0.41 to 0.73 MA/m of MS does not show a statistically different effect on the GDNF secretion. As a survival promoting factor for dopamine-producing neural cells, the enhanced secretion of GDNF from N27 cells under MS treatment might be one of possible reasons to explain why the MS shows some benefits for treating PD. Given its simple-to-use, low-cost, and ultrasensitivity, this type of nanosensor is a particularly suitable technique to analyze *in vitro* GDNF secretion of a PD cell model under MS treatment, and also could easily be used for other systems where secretion drives the biological response. In the future, a new chip with arrayed nanosensors interfaced with a microfluidic network will be developed for multiplexed detection and applications so that the GDNF and other molecules secreted from N27 cells can be monitored *in real time* with and without MS, facilitating the possible comprehensive studies of MS effect on the neural cells.

Acknowledgements

We would like to thank the W. M. Keck Metabolomics Research Laboratory Iowa State University for assistance with ELISA measurement and analysis. This research was funded in part by NSF Award ECCS 1461841, ECCS 1610967, and ECCS 2024797.

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