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Tracking micro-optical resonances for identifying and sensing novel procaspase-3 protein marker released from cell cultures in response to toxins

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Abstract

The response of cells to toxins is commonly investigated by detecting intracellular markers for cell death, such as caspase proteins. This requires the introduction of labels by the permeabilization or complete lysis of cells. Here we introduce a non-invasive tool for monitoring a caspase protein in the extracellular medium. The tool is based on highly sensitive optical micro-devices, referred to as whispering-gallery mode biosensors (WGMBs). WGMBs are functionalized with antibodies for the specific and label-free detection of procaspase-3 released from human embryonic kidney HEK293 and neuroglioma H4 cells after introducing staurosporine and rotenone toxins, respectively. Additional tests show that the extracellular accumulation of procaspase-3 is concomitant with a decrease in cell viability. The hitherto unknown release of procaspase-3 from cells in response to toxins and its accumulation in the medium is further investigated by Western blot, showing that the extracellular detection of procaspase-3 is interrelated with cytotoxicity of alpha-synuclein protein (aSyn) overexpressed in H4 cells. These studies provide evidence for procaspase-3 as a novel extracellular biomarker for cell death, with applications in cytotoxicity tests. Such WGMBs could be applied to further identify as-yet unknown extracellular biomarkers using established antibodies against intracellular antigens.

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Keywords: biosensing, label-free, optical microcavity, cell death, caspase, whispering-gallery mode, procaspase

(Some figures may appear in colour only in the online journal)

1. Introduction

The toxins staurosporine and rotenone can have detrimental effects on cells that result in cell death (Bertrand *et al* 1994, Pan-Montojo *et al* 2010). Mechanisms responsible for cell termination, such as apoptosis (Porter and Jänicke 1999) or necrosis, are routinely investigated by analyzing intracellular components (Lowe and Lin 2000). Examples for this are given by immunocytochemistry and *in vitro* biochemical assays, wherein cells are either permeabilized or completely lysed. Labels are then introduced for the specific detection of apoptotic biomarkers such as caspase proteins or DNA fragmentation (Lowe and Lin 2000). The invariable perturbation of the cell culture, however, may skew quantitative results and further halt continuous monitoring upon harvesting and destroying the cells. Hence, there is a great need for non-invasive and real-time tools capable of detecting cell death (Nowacki *et al* 2015). Detecting a caspase protein extracellularly with a highly sensitive micro-device could thereby enable many applications for lab-on-chip and organ-on-chip assays, potentially evaluating the toxicity of drugs and other chemical compounds.

Optical micro-devices, such as those based on whispering-gallery mode biosensors (WGMBs), have been developed to address the need for a rigorous instrument in biodetection (Fan *et al* 2008, Hunt *et al* 2010, Soteropulos *et al* 2012, Foreman *et al* 2015). Due to their label-free operation WGMBs can be adapted to a variety of biodetection tasks (Washburn *et al* 2009, Luchansky and Bailey 2011, Wilson *et al* 2015), including the detection of several proteins (Gohring *et al* 2010, Luchansky and Bailey 2010, Delezoide *et al* 2012, Wilson *et al* 2012, Huckabay *et al* 2013, Pasquardini *et al* 2013), DNA (Suter *et al* 2008, Wu *et al* 2014), RNA molecules (Scheler *et al* 2012), viruses (Vollmer *et al* 2008, He *et al* 2011, Lu *et al* 2011, Shao *et al* 2013), and even single DNA strands (Baaske *et al* 2014). Exploiting the WGMB detection limits, which have been demonstrated to reach picomolar antigen concentration levels in the above mentioned studies, could advance the field of biomolecular characterization.

In this paper, we employ a WGMB to identify and detect a novel protein marker at low concentration in the cell culture medium. Our study thereby focuses on detecting proteins in the context of signaling cell death, i.e. release measurements from human embryonic kidney (HEK293) and neuroglioma (H4) cells, by surface functionalization with antibodies. Quantifying the release of procaspase-3 from H4 cells and their alpha-synuclein overexpressed variant (H4 WTS) may point to a method for assessing the cytotoxicity of proteins, such as extracellular alpha-synuclein (aSyn), as well as that of other toxic substances. This study is motivated by the fact that aSyn is known to reduce neuronal cell viability in response to rotenone (Xiang *et al* 2013) and presumed to play a role in Parkinson's disease (PD) (Cookson 2009). Through tracking micro-optical resonances of WGMBs with antibodies in a HEK293 cell culture medium, accurate, label-less sensing of procaspase-3 can take place following triggered cell death with the toxin staurosporine (a kinase inhibitor). Accumulation of procaspase-3 in the medium is further investigated by Western blot, while cell viability is quantified by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assays (Denizot and Lang 1986). The extracellular detection of procaspase-3 as a result of cytotoxicity in neuroglioma cells is further investigated. More specifically, release of procaspase-3 from H4 and H4 WTS cells in response to rotenone is quantified.

2. Materials and methods

2.1. System diagram

A schematic of the WGMB is shown in figure 1(A). Samples of cell culture medium post-toxin treatment are analyzed by injection into the WGMB sample cell (gray dashed arrow). The replaceable sensor consists of a ~400 μm silica microsphere functionalized with antibodies. Introduction of a micro-magnet for rapid mixing throughout the measurement is justified in the absence of a microfluidic approach. Micro-optical WGMB resonances are tracked by recording resonance wavelength shifts in units of picometers, primarily originating from proteins binding at the sensor surface. This shift is converted into units of mass loading or ng mm^{-2} (figure 1(B)), given that it is linear and quantitative with respect to mass loading (whose conversion scheme is described in the supplement). We achieve specific procaspase-3 detection by immobilizing a biotinylated anti-procaspase-3 antibody (BioLegend, 622704). A calibration curve for the sensor response with respect to procaspase-3 concentration is obtained with recombinant procaspase-3 protein diluted in Tris buffer, wherein the WGM sensor signal is normalized by the highest concentration that we could achieve by dilution of stock solution in our sample cell (figure 1(C)).

The resulting nonlinear binding isotherm is later used to estimate the unknown concentration of procaspase-3 from the sensor response recorded in the cell culture medium. A calibration curve for the binding rate is then obtained by averaging over the first 25 min of each WGMB response (figure 1(D)). A linear response is expected in this instance since the initial binding rate is often proportional to procaspase-3 concentration.

2.2. Surface modification of the WGMB microsphere

The ~400 μm diameter glass microsphere sensor is coated with a sandwich structure composed of biotin-dextran (Enzo Life Sciences GmbH, MGT-M0788-M010) and streptavidin-biotinylated-antibody complex to immobilize a rabbit anti-procaspase-3 antibody (BioLegend, 622704) at the sensor surface. Streptavidin (New England Biolabs, N7021S) binds to pre-physisorbed biotin-dextran at the sensor interface, also binding to the biotinylated antibody to thereby tether the procaspase-3 specific antibody to this surface. In order to coat the microsphere, the sensor is first exposed to a biotin-dextran solution (10 mg ml^{-1}) to physisorb the first biotin-dextran

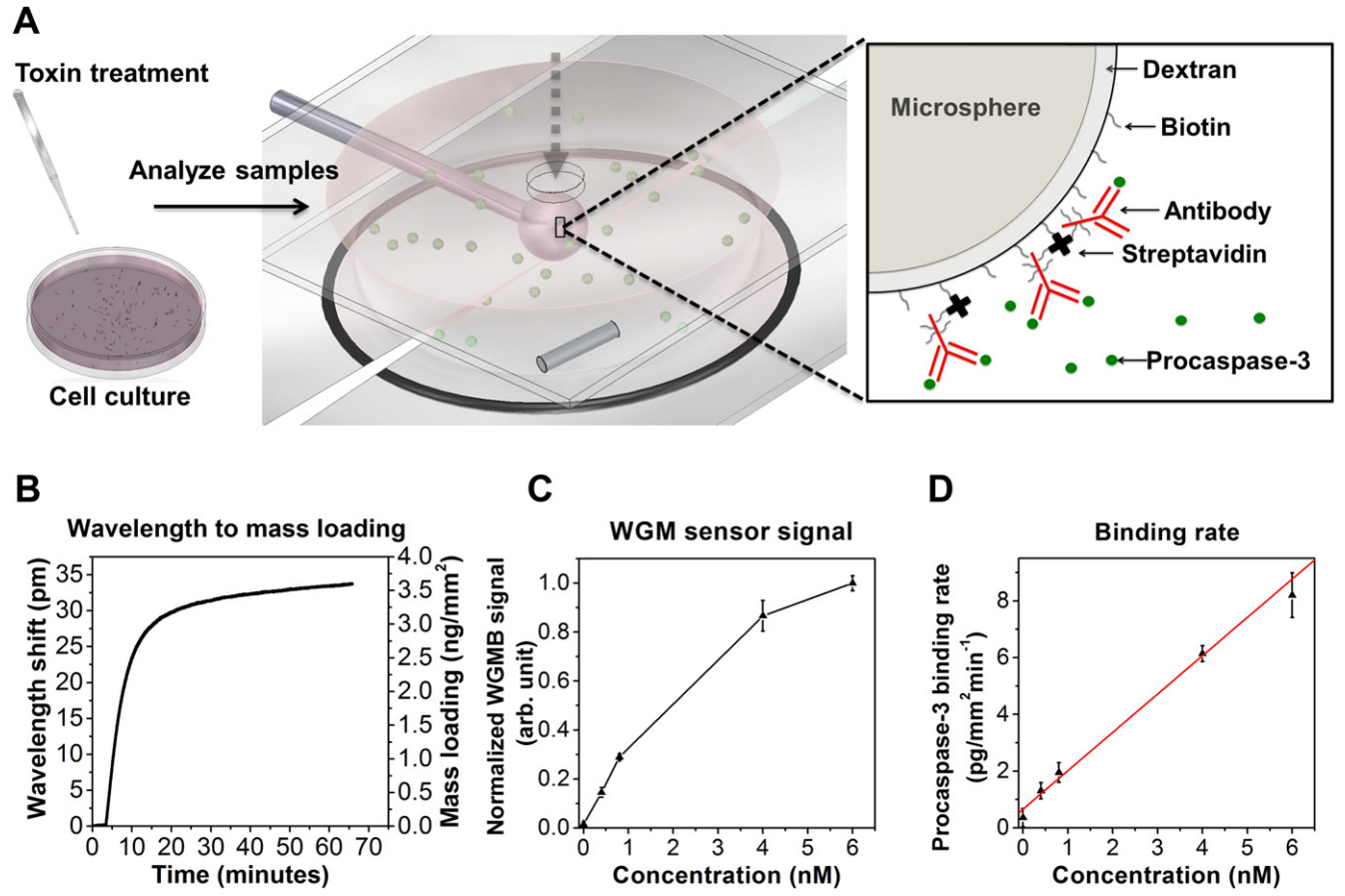

Figure 1. WGMB extracellular sensing device and calibration. (A) The detection and identification of procaspace-3 proteins in a cell culture medium via tracking optical resonances of an antibody surface-modified, glass microsphere. (B) Example of a sensor response due to protein binding. The resonance wavelength shift is converted to mass loading (the relationship for which is shown in the supplement). (C) Langmuir-like isotherm for equilibrium binding of recombinant procaspase-3 proteins to anti-procaspase-3 antibodies on the WGMB, where lines are added to help guide the eye. (D) Binding rate of recombinant procaspase-3 protein on the WGMB as determined by averaging over the first 25 min of the sensor response. The red line is a linear fit, while the error bars denote the standard deviations from three separate measurements.

layer onto the glass surface (Wu *et al* 2014). After a ~20 min incubation period, the microsphere sensor is briefly dipped in distilled water to remove any unbound biotin-dextran. Afterwards, the biotin-dextran coated microsphere is exposed to a pre-mixture solution of streptavidin-biotinylated-antibody complex mixed at an optimized molar ratio of 10:1, respectively.

2.3. Cell lines and sample preparation

H4 neuroglioma cells of human origin (ATCC, HTB-148) are maintained in an Opti-MEM + GlutaMAX medium (Invitrogen, 51985-4042) and supplemented with 10% fetal calf serum (FCS; Invitrogen, 10270-106) at 37 °C. To generate H4 cells stably overexpressing aSyn, H4 cells are transduced by lentiviral delivery of aSyn-IRES-GFP or IRES-GFP constructs. Post-transduction, cells are grown in standard conditions and cloned by a single-cell colony culture using a limited dilution of stably transduced cells (H4 WTS cells). One clone of each transgenic cell line is used for the experiments. H4 and H4 WTS cell cultures are treated by 1 μM rotenone (Sigma, R8875-1G) for 2 h in Opti-MEM + GlutaMAX (Invitrogen, 51985-4042), followed by collection of the media and brief centrifugation at 1500 rpm for 5 min.

The HEK293 cells are placed in DMEM medium at 37 °C for 2 days, then transposed to a serum-free DMEM medium and incubated for another 8 h to starve the cells. Subsequently, these cells are treated with 1 μM staurosporine (Sigma-Aldrich, S6942-200UL) for 0, 2, 4, and 8 h. Gathered cell culture media are briefly centrifuged at 1500 rpm for 5 min to separate the cells from the medium.

2.4. MTS assays

HEK 293 cells are plated on 96-well plates at an average density of ~4 $\times$ 10^4 cells per well for 48 h and transferred to a serum-free medium for 12 h, then treated with 1 μM staurosporine and incubated at 37 °C for 2, 4, and 8 h. As for the H4 and H4 WTS cells, with the exception of an altered average density of ~8 $\times$ 10^3 cells per well, they are plated in an identical fashion to that of the HEK 293 cells. The H4 cells and H4 WTS cells are later treated with 1 μM rotenone and incubated at 37 °C for 2 h. Viability of these cells is measured

by using an MTS assay (Promega), where a 20 μl MTS solution is added into each well and they are incubated at 37 °C for 30 min. Normalizing by the absorption of untreated cells, the viability of treated cells is eventually compared to that of untreated cells.

2.5. Western blot

Western blot detection of procaspase-3 in a cell culture medium requires a certain minimum concentration. Before the concentration step, salt is diluted in the medium by dialysis in a dialysis tube with 3.5–5 k MWCO (Spectrum lab, 131201, USA). This process takes place four times (i.e. for 4, 4, 12, and 4 h) in 5 l of 20 mM Tris buffer. Finally, the dialyzed samples are lyophilized and reconstituted with 100 μl of PBS to effectively produce a 200-fold concentration for the Western blot analysis.

Cell culture media are mixed with one volume of SDS sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol), and separated on 15% SDS-PAGE. The gels are blotted onto nitrocellulose membranes (Millipore) and, afterwards, the membranes are probed with a non-biotinylated primary rabbit anti-procaspase-3 antibody (1:1000, Abcam, ab32150, Germany). A secondary goat anti-mouse antibody is coupled to horseradish peroxidase (1:10 000, Dianova, Hamburg, Germany), wherein the proteins are visualized with a Femto Chemiluminescent Substrate (Thermo Scientific).

3. Results

Specificity of detection is particularly important for sensing in complex environments. The medium contains a variety of proteins that may generate WGMB signals due to non-specific binding. To mitigate a non-specific sensor response as well as additional shifts arising from matrix effects in the medium, we first immerse the antibody-modified microspheres in a fresh cell culture medium. After ~25 min of incubation time, we observe no further shifts in resonance wavelength, indicative of efficient blockage of non-specific binding sites. The microsphere sensors are next used to test the medium of HEK293 cell culture, the data for which is displayed in figure 2. Before testing the medium for procaspase-3, the HEK293 cells are incubated for the specified time after adding 1 μM staurosporine. WGMB analysis of the medium shows a significant procaspase-3 protein concentration after 2 h of treatment with staurosporine, in that the sensor signal corresponds to over 108.5 pg mm^{-2} of mass loading. This threshold is set by a control experiment where 1 μM BSA solution was added to fresh cell culture medium. After the WGMB response to the BSA-spiked medium reaches equilibrium, we average over 5 min of the equilibrium sensor response plus the standard deviation to determine the threshold of ~108.5 pg mm^{-2}.

The increase in WGMB sensor signal from the HEK293 medium with toxin treatment duration implies a stable accumulation of procaspase-3. In parallel experiments, staurosporine-induced cell death is quantified by determining

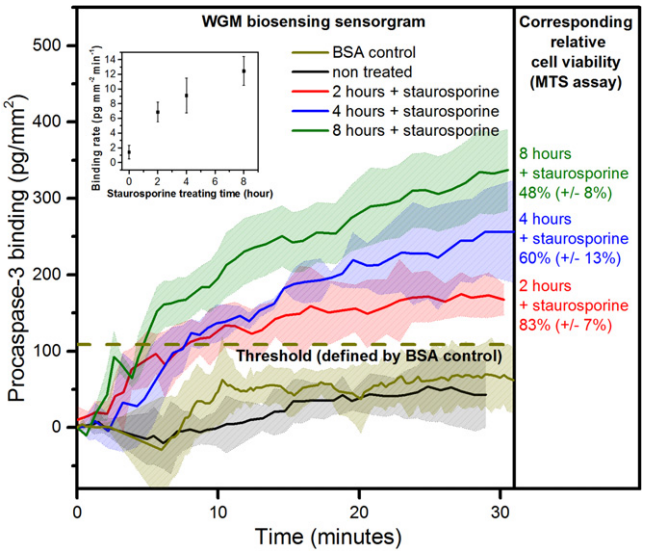

Figure 2. Testing of the HEK293 culture medium for procaspase-3 protein. The mass loading signals for the binding to anti-procaspase-3 antibody functionalized WGMBs is plotted versus time. The solid curve is the mean of three measurements and the shadow plots represent error bars (equivalent to standard deviations). Without staurosporine treatment (black solid curve), no significant sensor signal (i.e. below the 108.5 pg mm^{-2} threshold line) is observed. The supernatant of the HEK293 cells 2 h post-staurosporine treatment shows a significant sensor response, indicating pM levels of extracellular procaspase-3 protein concentrations (red solid curve). This corresponds to ~83% relative cell viability in the MTS assay. As for the supernatant of the cells 4 h post-staurosporine treatment, the sensor response relates to ~nM concentration levels of procaspase-3 (blue solid curve) and ~60% relative cell viability. Eight hours post-staurosporine treatment, there is a strong sensor response indicating ~nM concentration levels of procaspase-3 in the medium (green solid curve) and, in turn, ~48% relative cell viability. Control (yellow curve): 1 μM BSA solution added to a DMEM cell culture medium with no significant WGM sensor signal (i.e. $<$108.5 pg mm^{-2}). Inset: binding rates as determined by averaging over the first 25 min of the WGMB responses.

the relative cell viability in MTS assays (figure 2). We find that the equilibrium WGMB response as well as the binding rate is in proportion to the reduction in relative cell viability. This data suggests that the extracellular procaspase-3 concentration is interrelated with cell viability. We note that a significant WGMB response could already be determined within 2 h of toxin treatment at a time point when relative cell viability was still measured above ~83%. Such behavior reveals that procaspase-3 may be released early during necrosis or apoptosis, possibly by active mechanisms such as vesicle secretion. Evaluating the sensor response using the recombinant procaspase-3 calibration curves (figures 1(C) and (D)), we find that procaspase-3 can reach up to ~1 nM concentration for ~2.5 $\times$ 10^5 HEK293 cells cultured in 2 ml of cell culture medium after 8 h of treatment with 1 μM staurosporine. This result signifies a remarkable, prolonged stability of the procaspase-3 protein in the extracellular medium, which stands in stark contrast to the commonly held notion that caspase-3 rapidly degrades (Tawa *et al* 2004, Milam and Clark 2009). By comparing figure 2 to 1, one can see that the standard deviation of the sensor signal is expectedly larger in

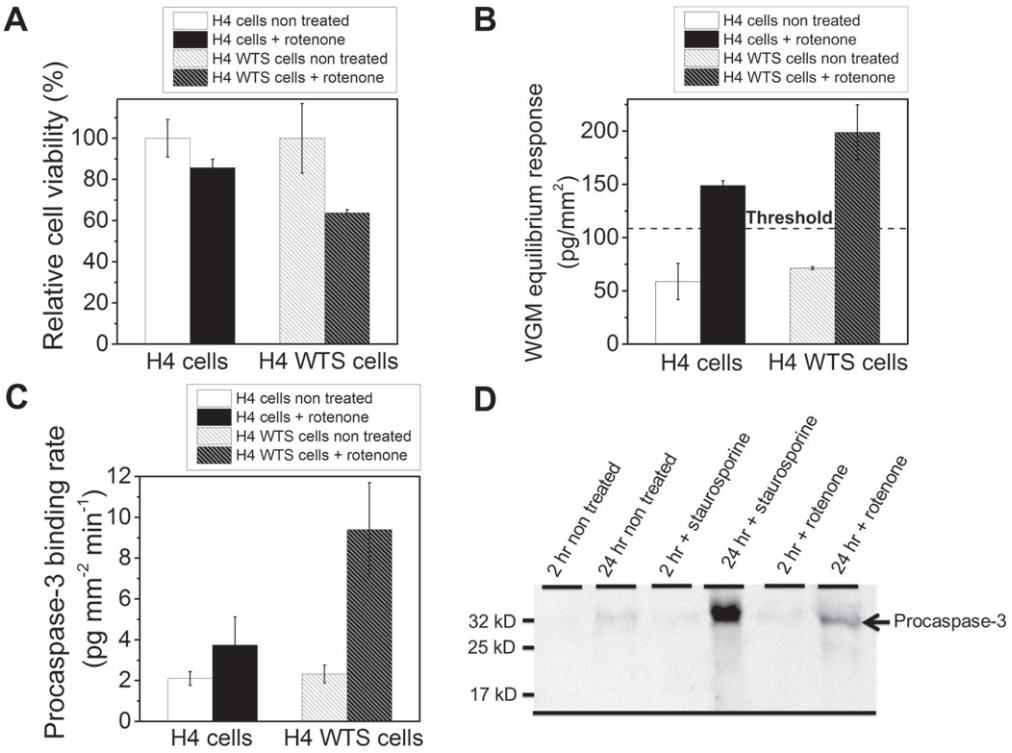

Figure 3. Cell toxicity test via MTS and WGMB assay of H4 WTS and H4 cells 2 h post-treatment with 1 μM rotenone. (A) H4 WTS and H4 cells respectively display a ~63% and ~85% relative cell viability. (B) WGMB procaspase-3 equilibrium sensor response. The procaspase-3 concentration in the H4 WTS cell media is significantly higher in respect to that of the H4 cells. (C) WGMB procaspase-3 binding rate, wherein the supernatant from H4 WTS cells shows a higher procaspase-3 binding rate than that of the H4 cells. (D) Western blot detection of procaspase-3. The H4 cell culture medium treated with 1 μM staurosporine and 1 μM rotenone is collected, concentrated, and probed with an anti-procaspase-3 antibody. Once again, error bars are standard deviations obtained from three measurements.

the complex medium as compared to the calibration measurements performed with recombinant protein in plain buffer solution. Note that error bars reported throughout this study always refer to standard deviation, where the standard deviation is the square root of the variance.

In an effort to verify if WGMB-based, extracellular detection of procaspase-3 is interrelated with aSyn mediated cytotoxicity in neuroglioma H4 cells, we consider H4 WTS cells. aSyn is a crucial protein in the pathogenesis of PD—one of the most common neurodegenerative disorders. Duplication and triplication of the aSyn gene (SNCA) has been identified as a genetic cause of familial PD (Singleton *et al* 2003, Chartier-Harlin *et al* 2004) and, ergo, such findings lead to the hypothesis that overexpression of aSyn is an important mechanism underlying PD-associated neurodegeneration. Indeed, many studies show that an increase of intracellular aSyn reduces cell viability (Cookson 2009).

As to examine the relative cell viability of H4 and H4 WTS cells in response to a toxin, we challenge both cell cultures with rotenone. The choice of rotenone toxin is motivated by its ability to reproduce pathological staging as found in PD patients (Pan-Montojo *et al* 2010). Accordingly, both cell cultures are exposed to 2 h of 1 μM rotenone treatment and analyzed by MTS and WGMB assays. The MTS assay shows that H4 WTS cells exhibit less relative cell viability as compared to H4 cells (figure 3(A)). Following this we use the WGMB method to test the H4 and H4 WTS cell culture media for procaspase-3 protein. Both cell lines show significant WGMB sensor signals after toxin treatment, corresponding to values greater than 108.5 pg mm^{-2} of procaspase-3 mass loading (figure 3(B)). The threshold, like before, is the maximum signal obtained for a nonspecific sensor response in media that were spiked with 1 μM of BSA (see figure 2). Evaluating this data with the aid of the calibration curves, there is an indication of sub-nM levels of procaspase-3 protein concentration in the cell culture medium for ~10^4 H4 or H4 WTS cells and culturing for 2 h with rotenone in 2 ml of Opti-MEM + GlutaMAX. A higher concentration of procaspase-3 protein is found in the H4 WTS cell medium as compared to that of the H4 cells. Therefore, there is an interrelation between the increase in procaspase-3 release and the reduction of cell viability in H4 WTS cells as compared to H4 cells. Similarly, H4 WTS media show a larger WGMB procaspase-3 binding rate as compared to H4 cells (figure 3(C), mass loading rate determined by the first 25 min of the WGMB response). Both MTS and WGMB procaspase-3 assays exhibit a lower resistivity of H4 WTS cells towards the rotenone toxin as compared to H4 cells. Note that all experiments are performed for the same average number of total cells, as outlined in the Materials and methods section.

Next, we apply the Western blot technique to validate the procaspase-3 protein accumulation in the cell culture medium of H4 cells. Western blot is in itself not sensitive enough to

directly detect the low concentration of procaspase-3 in the supernatant, so the cell culture medium was highly concentrated in this case (i.e. 200 fold, view the provided Material and methods information). Figure 3(D) illustrates the Western blot result for a supernatant taken from H4 cell culture after 2 and 24 h of treatment with 1 μM staurosporine and 1 μM rotenone, respectively. The procaspase-3 band at 32 kDa (Li *et al* 2000) is clearly visible on the film and, with longer treatment times, the procaspase-3 signal becomes stronger. Such an outcome confirms that procaspase-3 accumulates in the cell culture medium of H4 cells undergoing cell death in response to toxin treatments. Furthermore, the procaspase-3 protein remains stable in the medium even after several hours of toxin treatment.

4. Discussion

We have introduced a non-invasive WGMB tool for identification and monitoring of the novel protein marker procaspase-3 that is released from cells in response to toxins. We observed that this protein accumulates in HEK293 and H4 cellmedia in response to triggered cell death by the toxins staurosporine and rotenone, respectively. A concomitant reduction in relative cell viability was confirmed by a MTS test, while accumulation of procaspase-3 in the concentrated cell culture medium was also seen via Western blot. In the case of neuroglioma cells overexpressing aSyn protein, the extracellular marker procaspase-3 was monitored and a relative increase in its release over that of the naive H4 cells, together with a reduction in relative cell viability, was observed. This clarifies that procaspase-3 release is interrelated with cell viability in neuroglioma cells expressing aSyn. These results point to procaspase-3 as a potential extracellular biomarker in cytotoxicity tests, suggesting a role for aSyn in promoting neurodegeneration related to PD.

We emphasize that a WGMB tool enables detection of procaspase-3 after only 2 h of incubation with the toxins, thereby detecting ~pM concentrations of extracellular protein at a time point at which most of the cells still remain viable and no Western blot signals are observable without concentrating the sample. The procaspase-3 concentration in the medium could thus be used to quantify the susceptibility of HEK293, H4, and other cell types to a particular toxin. The potency of a toxin could be evaluated from the magnitude of the WGMB procaspase-3 signal above the threshold set by control experiments that evaluate non-specific binding signals. This method could have applications in rapid and highly sensitive cell-based toxicity tests. Such tests could be administered on lab-on-chip and organ-on-chip devices, culminating to the first commercial method for non-invasive, rapid, real-time, and extracellular detection of cell death by procaspase-3 markers.

In summary, this study introduces WGMB as a tool for discovering and inspecting novel extracellular biomarkers using established antibodies against intracellular antigens. This study also opens the way towards a completely new research direction—that of investigating extracellular procaspase-3's role in apoptosis. This topic should prove interesting in terms of how the protein is released from cells and what *in vivo* function it might have as a secreted protein.

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