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Research Article

Label-Free Detection of Chondroitin Sulphate Proteoglycan 4 by a Polyaniline/Graphene Nanocomposite Functionalized Impedimetric Immunosensor

JingJing Fu,[1,2] ZhuanZhuan Shi,[1,2] Man Li,[1,2] Yangyang Wang,[3] and Ling Yu[1,2]

[1]*Chongqing Key Laboratory for Advanced Materials and Technologies of Clean Energies, Chongqing 400715, China*
[2]*Institute for Clean Energy & Advanced Materials, Faculty of Materials and Energy, Southwest University, Chongqing 400715, China*
[3]*Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA*

Correspondence should be addressed to Ling Yu; lingyu12@swu.edu.cn

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The chondroitin sulphate proteoglycan 4 (CSPG4), also known as high molecular weight-melanoma associated antigen (HMW-MAA), is a tumor-associated antigen that is expressed in more than 85% of surgically removed melanoma lesions but has restricted distribution in normal tissues. The diagnostic and therapeutic value of CSPG4 drives a need for sensitive and low-cost detection approaches. To this end, we developed a polyaniline/graphene oxide nanocomposite (PANI@GO) that was electrochemically codeposited on indium tin oxide (ITO) electrode. Glutaraldehyde mediated the covalent immobilization of CSPG4 specific antibody mAbD2.8.5 to construct a CSPG4 immunosensor using cell culture media and cell lysate as samples. The fully assembled impedimetric immunosensor was used to detect CSPG4 in CSPG4-positive cell lines M14/CSPG4 and MV3. No impedance signal changes could be observed from CSPG4-negative cell lines M14 and mAbMk2-23 showing the specificity of the CSPG4-impedimetric immunosensor. This low-cost, simple, and label-free analytical method is an alternative to enzyme-linked immunosorbent assay and flow cytometry in screening of CSPG4 in complex biological samples.

1. Introduction

The chondroitin sulphate proteoglycan 4 (CSPG4), also known as high molecular weight-melanoma associated antigen (HMW-MAA), is a membrane-bound tumor antigen that can be recognized by antibodies [1, 2]. It plays an important role in the proliferation, survival, migration, and metastatic potential of cancer cells [3, 4]. This antigen is expressed in melanoma cells, basal breast carcinoma cells, chordoma cells and osteosarcoma cells, glioma cells, and head and neck squamous cell carcinoma cells [4]. It is expressed not only in differentiated tumor cells, but also in tumor initiating cells or cancer stem cells [4, 5]. In addition, clinical data have demonstrated that circulating melanoma cells are a potential source of the HMW-MAA/CSPG4 antigen found in the serum of melanoma patients [6]. Importantly, CSPG4 has a restricted distribution in normal tissues [2, 4]. Because it is found in more than 85% of surgically excised melanoma lesions as well as limited distribution in normal tissue, CSPG4 is an immunotherapy target in patients with advanced melanoma [7–10]. Studies in animal model systems have shown that CSPG4 specific humoral and cellular immunity can reject tumors expressing CSPG4; therefore, this antigen is a tumor rejection antigen [4, 11, 12]. Of note, this effect is not associated with side effects including delayed wound healing most likely because of the very low expression of CSPG4 on resting pericytes in anatomic sites other than the tumor microenvironment [3, 4]. Recently, serum levels of CSPG4 were measured in melanoma patients and the concentrations correlated to clinical outcome [13]. Therefore, CSPG4 might be a useful biomarker to identify malignant cells and an attractive target to apply antibody-based therapy.

In previous studies, enzyme-linked immunosorbent assay (ELISA), the reverse transcription-polymerase chain reaction (RT-PCR) method, flow cytometry, and the immunohistochemical (IHC) staining methods have been used to detect

CSPG4 in melanoma cells and tissues [5, 13]. However, these well-established tools suffer from tedious liquid handling, expensive fluorescent labeling, and large sample consumption and have to be conducted by well-trained specialists. New analytical approaches utilizing bionanotechnology can achieve fast, low-cost, and even label-free testing. Electrochemical sensing in particular is an attractive group of sensing tools that provides increased sensitivities, shorter analysis times, and miniaturized platforms [14, 15]. Electrochemical impedance spectroscopy- (EIS-) based detection is a class of label-free sensors that shows promise in the sensitive measurement of target analytes and has gained interest as a label-free technique [16–20].

The selection of materials is critical in building a sensitive impedance sensor for label-free testing. Chen et al. synthesized polypyrrole/Au nanocomposites for the protein IL-5 with a sensitivity of 1 pg/mL in 1% human serum [18]. Wang et al. used reduced graphene oxide-doped polypyrrole/pyrrolepropylic acid nanocomposite for small molecular aflatoxin B(1) detection [20]. In our previous study, a polyaniline@graphene (PANI@GO) nanocomposite was electrochemically deposited on ITO as a working electrode (WE) to label-free *in situ* sensing of clenbuterol [19]. These studies show that impedance is a good candidate for sensitive analysis of complex samples.

Here, we used an impedance sensor to measure CSPG4. A polyaniline@graphene oxide (PANI@GO) nanocomposite was codeposited on microfabricated ITO electrode. The CSPG4 specific mAb was covalently immobilized on the electrode to construct the immunosensor. The utility of the fully assembled impedimetric sensor was studied by analyzing culture medium and cell lysate. This shows the considerable potential it has for measuring real world samples.

2. Materials and Methods

2.1. Materials. Graphite, sodium nitrite ($NaNO_2$), sodium hydroxide (NaOH), and glutaraldehyde solution were all purchased from Sigma-Aldrich. Aniline, perchloric acid ($HClO_4$), sodium chloride (NaCl), 30% hydrogen peroxide (H_2O_2), sulfuric acid (H_2SO_4), hydrochloric acid (HCl), phosphate buffered saline (PBS), and potassium permanganate ($KMnO_4$) were from Chongqing Chemical Co. (Chongqing, China). A CSPG4 specific monoclonal antibody (mAb) D2.8.5, isotype control mAbMk2-23 (do not recognize CSPG4 molecule), and melanoma cell lines M14 and M14/CSPG4 were kindly provided by Dr. Soldano Ferrone (Massachusetts General Hospital, Harvard Medical School). The melanoma MV3 cell line was obtained from American Type Culture Collection (ATCC). The MV3 and M14 cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (FCS, Gibco), 100 U mL^{-1} penicillin, and 100 U mL^{-1} streptomycin at 37°C in a humidified 5% CO_2 incubator; the M14/CSPG4 cells were grown in RPMI 1640 medium supplemented with geneticin (G418) (400 μg/mL).

All chemicals were used without further purification unless otherwise indicated. All solutions were prepared with deionized (DI) water produced by PURELAB flex system (ELGA Corporation).

2.2. Fabrication of Electrochemical Sensor for Impendence Measurement

2.2.1. ITO Electrode Fabrication. Following the literature [20], we prepared a 3 × 3 mm^2 working electrode on ITO glass. Briefly, a dry photosensitive film (40 μm) was coated on the ITO glass and patterned following a UV photolithography process. The ITO layer that was not covered by the photosensitive film was dissolved by immersing the chips in etchant solution (37% $HCl : H_2O : FeCl_3 \cdot 6H_2O$ = 3 L : 1 L : 25 g) for 30 min. Finally, the patterned electrodes were recovered by removing the residual photosensitive film.

2.2.2. PANI/GO Nanocomposite Deposition. The ITO working electrode (WE, 3 mm × 3 mm) was coated with a PANI@GO film via electrochemical deposition. First, the electrodes were placed in 1 M $HClO_4$ solution containing 0.3 M aniline and 0.4 mg/mL GO. Next, the polymerization was conducted with a constant current deposition and a current density of 0.6 mA·cm^{-2} for 3 min followed by 0.3 mA·cm^{-2} for 15 min. Finally, after polymerization, the electrode was carefully washed in distilled water and dried at room temperature. The resulting electrode was denoted as PANI@GO/ITO and used for immobilization of CSPG4 specific antibodies.

2.2.3. Immobilization of Probe Antibody. The CSPG4 antibody mAbD2.8.5 was covalently immobilized on PANI@GO nanocomposite film by glutaraldehyde-mediated cross-linking. In brief, 5 μL 0.01 mol/L PBS containing 2.5% glutaraldehyde (v/v) was drop cast onto the PANI@GO/ITO working electrode and incubated for 0.5 h at room temperature (RT) resulting in a glutaraldehyde-modified electrode. Then, 5 μL of antibody solution (100 μg/mL) was added to the glutaraldehyde-modified electrode and incubated at 37°C for 1 h. The excess antibodies were removed with triplicate PBS flushing. The probe antibody on the PANI@GO/ITO electrode was then used for sample testing.

2.2.4. EIS Measurement. A three-electrode system consisting of a nanomaterial-functionalized ITO working electrode, an $Hg/HgCl_2/KCl$ reference electrode, and a platinum wire counter electrode was used for EIS measurements on an electrochemical station (CHI 760D, Chen Hua Instruments Co. Ltd.). In brief, the EIS measurements measured the impedance values before and after reaction with cell samples. The EIS measurements occurred at equilibrium potential without external biasing in the frequency range of 0.5–10^5 Hz with a 5 mV amplitude in 1 M NaCl. After conducting the first run EIS (1st EIS) measurement, a 5 μL cell sample was drop cast onto the Ab decorated PANI@GO/ITO electrode and placed at RT for 30 min and then washed with PBS 3 times. The second EIS run (2nd EIS) measurement was then collected. All experiments were repeated 5 times.

2.3. Preparation of Cell Sample. The human melanoma cell line M14 is one of the few melanoma cell lines with no detectable CSPG4 protein expression; it served as a negative control.

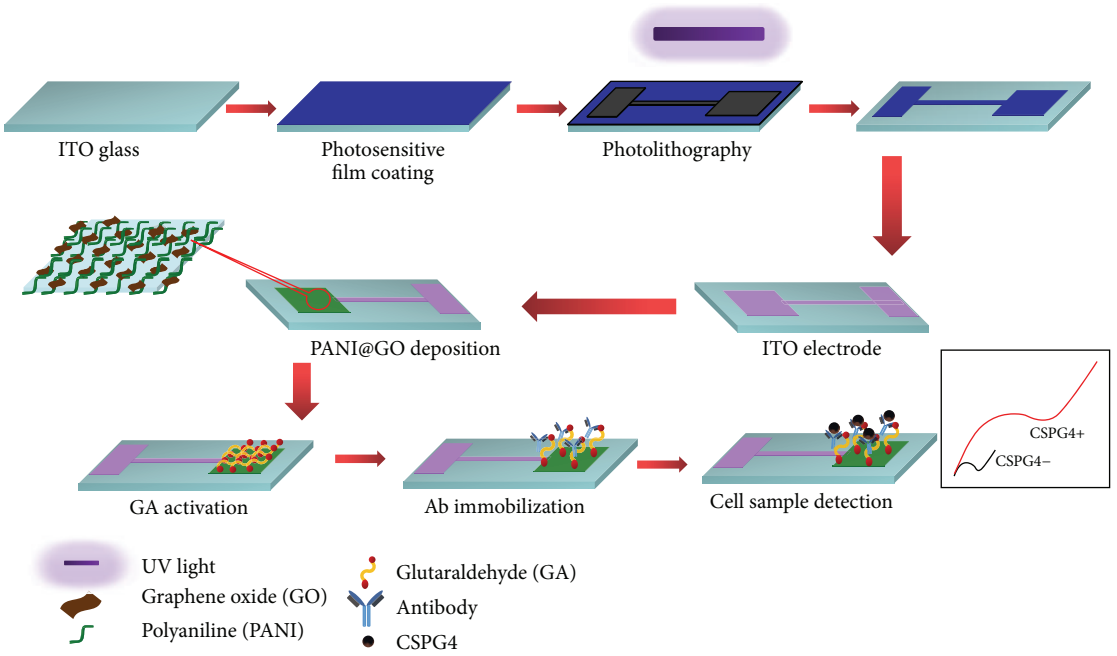

FIGURE 1: Experiment design of the polyaniline@graphene oxide (PANI@GO nanocomposite functionalized electrode for CSPG4 detection). The ITO electrode was fabricated by UV lithography and NaOH etching. The PANI@GO nanocomposite was electrochemically deposited on the ITO electrode. The aldehyde group of the glutaraldehyde reacted with the amine group on the aniline and the protein to covalently immobilize antibodies on the electrode surface. Antibody-coated electrodes were exposed to cell samples for CSGP4 detection.

2.3.1. Supernatant. The M14, M14/CSPG4, and MV3 cells were cultured in RPMI 1640 medium with 10% fetal calf serum at 37°C. A 5 mL cell suspension (5×10^4 cells/mL) was placed in a 25 cm^2 cell culture flask and incubated at 37°C for 3 days in a 5% CO_2 atmosphere. At that time, culture medium was collected and centrifuged at 5 000 ×g at 4°C for 5 minutes. The supernatant was saved for CSPG4 molecular detection.

2.3.2. Cell Membrane Protein. The cell membrane protein was isolated according to the manufacturer's instructions. Briefly, cells were detached by EDTA/PBS buffer and then lysed in lysis buffer (Beyotime Biotechnology, China) with a glass homogenizer on ice. After homogenization, the cell lysate was centrifuged at 700 ×g at 4°C for 10 minutes to remove the nuclei. The supernatant was carefully collected in a new microcentrifuge for a second round of centrifugation (14 000 ×g at 4°C for 30 minutes). Next, the supernatant was collected as the cytoplasm proteins. The membrane protein extraction reagent (Beyotime Biotechnology) was added to suspend the protein pellet in a high-speed vortex. The tube was then placed on ice for 5 minutes. After repeated vortexing and ice bath incubation (two replicates), the lysate was centrifuged at 14 000 ×g at 4°C for 5 minutes. The collected supernatant was the isolated membrane proteins. The protein concentration in the lysates was measured using the Bradford reagent (Beyotime Biotechnology). For CSPG4 molecular detection, a membrane protein with a concentration of 1 mg/mL was used.

3. Results and Discussion

3.1. A PANI@GO/ITO Impedance Sensor Can Measure the Binding of CSPG4 and Its Corresponding Antibody. To construct the electrochemical impedance sensor, codeposition of PANI and GO was used to prepare the PANI@GO film. The polymerization was conducted with a constant current deposition and a current density of 0.6 $mA \cdot cm^{-2}$ for 3 min followed by a current density of 0.3 $mA \cdot cm^{-2}$ for 15 min. The PANI@GO nanocomposites synthesized from PANI mixed with GO were characterized by GO sheets occupying the space between the PANI nanowires (Figure 2(a)). The cyclic voltammogram data of PANI@GO films on the ITO electrode were characterized at 50 mV/s in 1 M NaCl. The two pairs of redox peaks (A/A′, B/B′) refer to the electroactivity of the PANI-based materials. This confirms the presence of aniline in the composite (Figure 2(b)). The principle of impedance detection is that any substance attached to the electrode causes a change in impedance. As illustrated in Figure 1, the CSPG4 antibody and the bound CSPG4 protein could be recognized as a film that affects the sensor impedance. The impedance responses for each step in the stepwise reaction are shown in Figure 2(c).

Characteristic EIS curves that include a semicircle and a straight line can be observed. According to impedance theory, a semicircle part at a high frequency region corresponds to an electron transfer limited process, and the linear part at lower frequencies refers to the diffusion-limiting step of the electrochemical process. The diameter of the semicircle

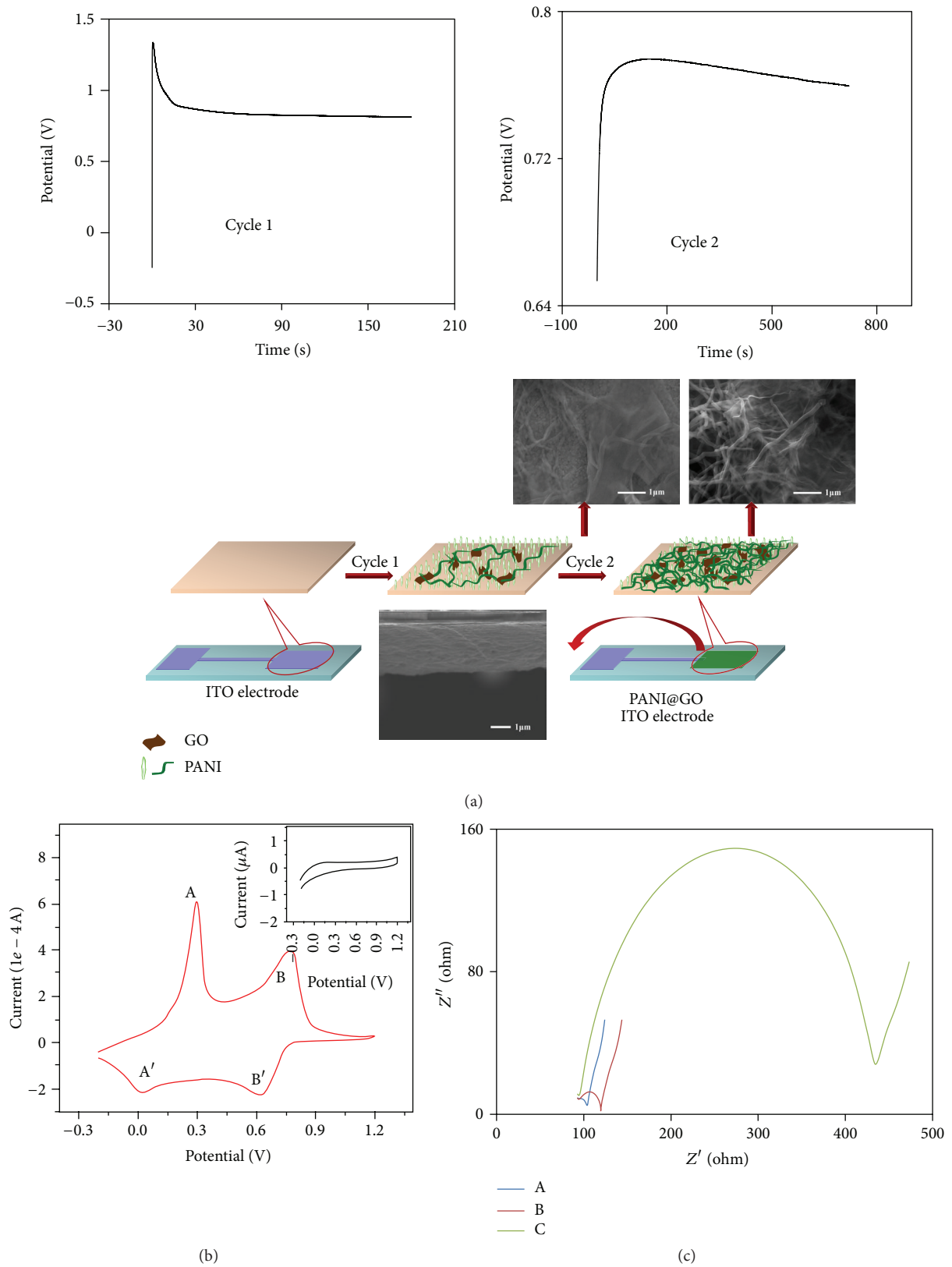

FIGURE 2: Fabrication and characterization of polyaniline@graphene oxide (PANI@GO) immunosensor. (a) Electrochemical codeposition of PANI@GO on ITO electrode: cycle 1: current density of 0.6 mA·cm^{-2} for 3 min; cycle 2: a current density 0.3 mA·cm^{-2} for 15 min. The SEM morphology of the PANI@GO nanocomposite at each deposition circle. (b) The cyclic voltammograms of PANI@GO nanocomposite and bare ITO electrode (insert) scanning in 1 M NaCl with a scan rate of 50 mV/s. (c) Electrochemical impedance spectra of PANI@GO/ITO (A), glutaraldehyde activated PANI@GO/ITO (B), and CSPG4 specific mAbD2.8.5 immobilized PANI@GO/ITO.

Table 1: Comparison of impedimetric immunosensor and ELISA.

	Reagent consumption	Labeled antibodies	Assay time	Point-of-care potential
Impedimetric immunosensor	~5 μL	No	~2 h	High
ELISA	100–200 μL	Yes	6~8 h	Less

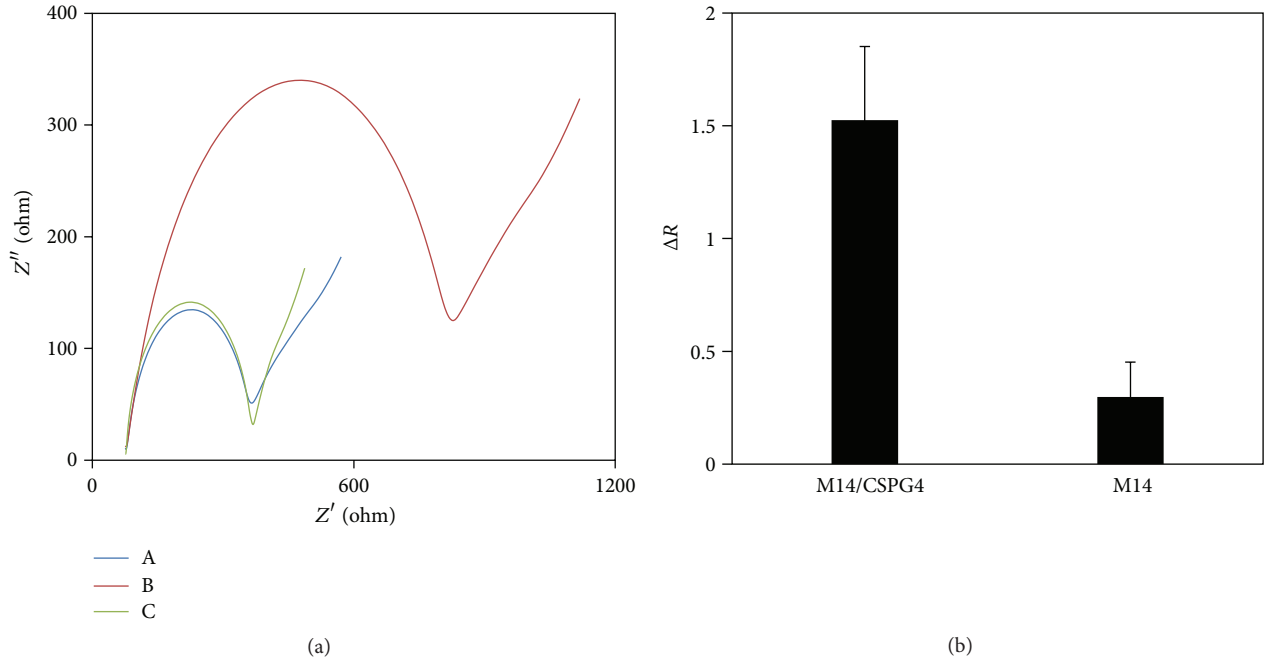

Figure 3: Analysis of cell culture media by impedimetric immunosensor. (a) Impedance spectra of (A) CSPG4 specific mAbD2.8.5 immobilized electrode, (B) CSPG4 mAbD2.8.5-coated electrode reacted with culture medium of M14 cells, and (C) CSPG4 mAbD2.8.5-coated electrode reacted with culture medium of M14/CSPG4 cells. (b) Histogram of normalized impedance signal changes.

equals the electron transfer resistance, and the increased value corresponds to blocking behavior and is due to assembly on the electrode surface. In Figure 2(c), PANI@GO/ITO and glutaraldehyde activated PANI@GO/ITO were characterized by impedance spectra with a small semicircle. The glutaraldehyde-cross-linked, CSPG4 specific mAb showed a sharply increased impedance curve. The increase in charge transfer resistance is due to the generation of an insulating protein layer on the electrode.

3.2. CSPG4 Can Be Detected in Supernatant of CSPG4-Positive Cell Line Culture Medium. To evaluate the interaction between CSPG4 antibody and antigen, the D2.8.5 antibody functionalized PANI@GO/ITO electrodes were exposed to cell culture supernatant that was pretreated by centrifugation (5 000 ×g at 4°C for 5 minutes). The corresponding plots of impedance spectra are shown in Figure 3(a). It is clear that reaction with cell culture medium from the positive cell line M14/CSPG4 significantly changes the diameter of the semicircle, but incubation with CSPG4-negative M14 medium causes little change. To quantify the protein-induced impedance change and eliminate the variation rooted in the electrodes, the changes in R were normalized according to a previous report [18]:

$$\Delta R = \frac{(R_2 - R_1)}{R_1}. \quad (1)$$

Here, R_1 and R_2 are the resistance values measured at the beginning and after incubation with cell samples, respectively. The results show that ΔR for M14/CSPG and M14 media are 1.5 and 0.3, respectively. This is due to the probing and binding of CSPG4 molecules in the cell supernatant, which leads to an increased charge transfer resistance. The same cell culture supernatant was analyzed by a direct ELISA and a sandwich ELSIA (Supplementary information; see Supplementary Material available online at http://dx.doi.org/10.1155/2016/7834657). The result of direct ELISA shows that a higher absorbance signal can be obtained from coating of M14/CSPG4 supernatant (Figure 4(a)). At the same time, CSPG4 molecule can be specifically detected by a sandwich format ELISA, where two types of anti-CSPG4 mAb were used (Figure 4(b)). The ELISA result is well in agreement with what is given by our impedance measurement. As the experiment protocols are detailed in supplementary information, the assay time of ELISA is normally 6–8 hours. For each testing, the sample consumption is few hundred microliters. In addition, peroxide-conjugated antibody is a must. Table 1 lists the comparison of this impedance sensor and standard ELSIA. In contrast to ELISA, this impedance sensor offers a simple reaction scheme because only one antibody is required. In addition, the total assay time is significantly reduced. Considering the importance of CSPG4

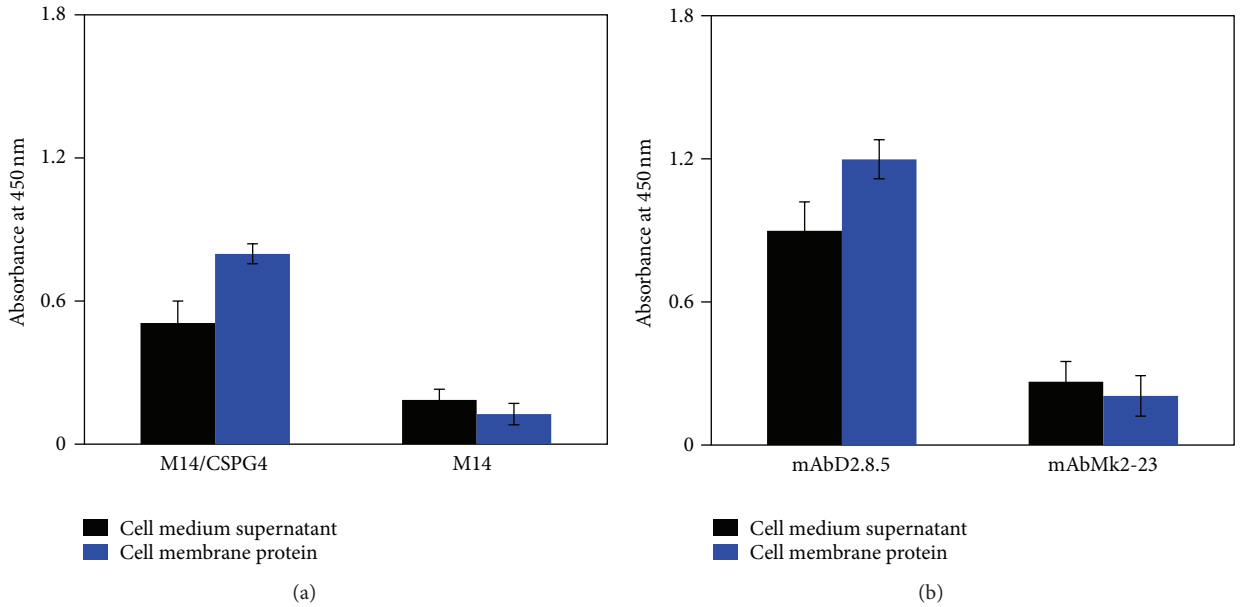

Figure 4: Analysis of cell samples by ELISA (enzyme-linked immunosorbent assay). (a) Direct ELISA: cell samples (M14/CSPG4 cells and M14 cells) were coated on 96-well microplate; then anti-CSPG4 specific mAbD2.8.5 was added to specifically detect CSPG4. (b) CSPG4/14 sample was analyzed by sandwich ELISA: CSPG4 specific mAbD2.8.5 and isotype control mAbMk2-23 were coated on 96-well microplate; the cell samples were added to the Ab-coated microplate; another CSPG4-specific mAb 763.74 was added to probe the CSPG4.

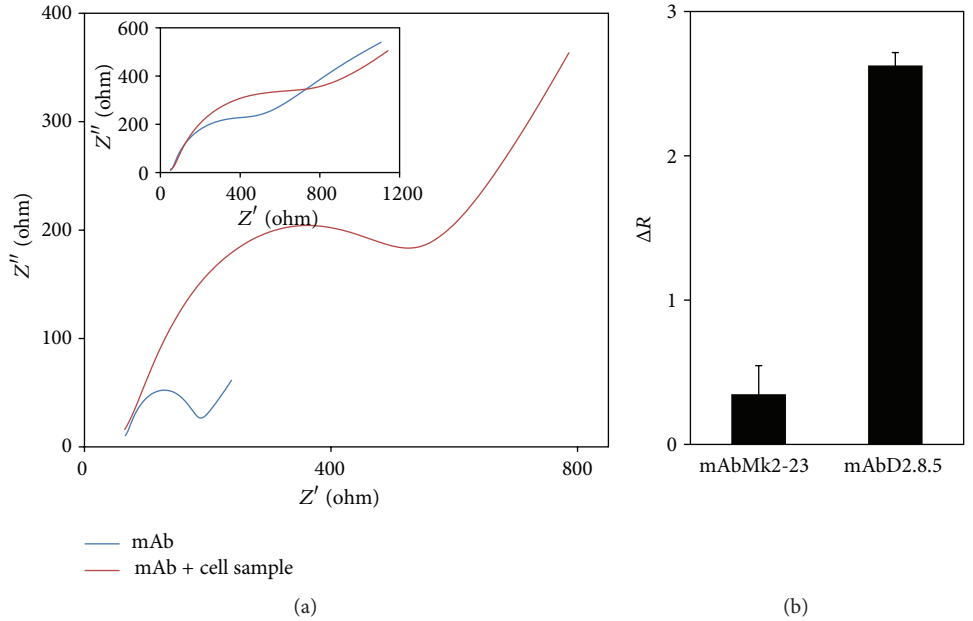

Figure 5: Analysis of cell membrane protein isolated from CSPG4-positive line MV3 by impedimetric immunosensor. (a) Impedance spectra of CSPG4 specific mAbD2.8.5 immobilized electrode before and after reaction with cell membrane protein; inset: impedance spectra of isotype mAbMk2-23 (does not recognize CSPG4) immobilized electrode before and after reaction with cell membrane proteins. (b) Histogram of normalized impedance signal changes.

in tumors, especially melanoma, the diagnosis, therapy, and prediction of clinical outcomes via CSPG4 testing would offer important information in disease screening and therapy.

3.3. The Impedance Sensor Can Specifically Test for CSGP4 in Cell Samples. Cell membrane proteins were isolated from CSPG4-positive melanoma MV3 cells to confirm the utility of the impedance sensor for cell lysate proteins. The CSGP4-specific mAbD2.8.5 and its isotype control mAbMK2-23 were placed on PANI@GO/ITO electrodes and were exposed to the lysate of MV3 cell. The results show that CSPG4 specific antibody can recognize and bind to the CSPG4

protein in the lysate. The binding event is characterized by a sharp increase in the semicircle. In contrast, incubation with isotype control mAbMk2-23—an anti-idiotypic monoclonal antibody of CSPG4—does not change the impedance spectra signal (Figure 5(a)). The histogram of ΔR clearly shows thatthe PANI@GO/ITO impedance immunosensor is feasible for analysis of cell lysate. In addition, the direct and sandwich ELISA were conducted to analyze the same cell membrane protein lysate. The results support the information given by impedance measurement. In previous studies, the surface expression of CSPG4 and intracellular expression of CSPG4 were evaluated by flow cytometry [5], and cell membrane proteins could be studied with ELISA and flow cytometry. As listed in Table 1, ELISA in general requires a longer assay time and larger sample volume, while flow cytometry also has to be done with fluorescent labeled antibodies. Moreover this advanced cell analysis technique relies on expensive equipment and has to be conducted by a specialist. Considering the sensitive, cost-effective, and label-free testing offered by this approach, it is an alternative solution for clinical CSPG4 screening, therapy prediction, and monitoring response.

4. Conclusion

In this study, a label-free impedimetric immunosensor based on a PANI@GO composite film was developed for the first time to detect CSPG4—a tumor associated antigen expressed in malignant cells. The CSPG4-positive cell lines M14/CSPG4 and MV3 were studied. The feasibility of the impedimetric immunosensor for real world sample testing was validated by analyzing CSPG4 molecules secreted in cell culture medium and cell lysate. The results showed that the impedance signal increases and corresponds to expression of CSPG4—both in the culture medium and in the lysate protein. This confirms the potential of the sensor to quickly measure CPSG4. This low-cost, simple, and label-free method is an alternative to ELSIA and flow cytometry.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References

[1] P. C. Maciag, M. M. Seavey, Z.-K. Pan, S. Ferrone, and Y. Paterson, "Cancer immunotherapy targeting the high molecular weight melanoma-associated antigen protein results in a broad antitumor response and reduction of pericytes in the tumor vasculature," *Cancer Research*, vol. 68, no. 19, pp. 8066–8075, 2008.

[2] M. R. Campoli, C.-C. Chang, T. Kageshita, X. Wang, J. B. McCarthy, and S. Ferrone, "Human high molecular weight-melanoma-associated antigen (HMW-MAA): a melanoma cell surface chondroitin sulfate proteoglycan (MSCP) with biological and clinical significance," *Critical Reviews in Immunology*, vol. 24, no. 4, pp. 267–296, 2004.

[3] X. Wang, T. Osada, Y. Wang et al., "CSPG4 protein as a new target for the antibody-based immunotherapy of triple-negative breast cancer," *Journal of the National Cancer Institute*, vol. 102, no. 19, pp. 1496–1512, 2010.

[4] X. Wang, Y. Wang, L. Yu et al., "CSPG4 in cancer: multiple roles," *Current Molecular Medicine*, vol. 10, no. 4, pp. 419–429, 2010.

[5] Y. Wang, F. Sabbatino, X. Wang, and S. Ferrone, "Detection of chondroitin sulfate proteoglycan 4 (CSPG4) in melanoma," *Methods in Molecular Biology*, vol. 1102, pp. 523–535, 2014.

[6] A. Ulmer and G. Fierlbeck, "Circulating tumor cells and detection of the melanoma-associated antigen HMW-MAA in the serum of melanoma patients," *The Journal of Investigative Dermatology.*, vol. 126, no. 4, pp. 914–916, 2006.

[7] F. Riccardo, S. Iussich, L. Maniscalco et al., "CSPG4-specific immunity and survival prolongation in dogs with oral malignant melanoma immunized with human CSPG4 DNA," *Clinical Cancer Research*, vol. 20, no. 14, pp. 3753–3762, 2014.

[8] H. Brehm, J. Niesen, R. Mladenov et al., "A CSPG4-specific immunotoxin kills rhabdomyosarcoma cells and binds to primary tumor tissues," *Cancer Letters*, vol. 352, no. 2, pp. 228–235, 2014.

[9] M. Van Sinderen, C. Cuman, A. Winship, E. Menkhorst, and E. Dimitriadis, "The chrondroitin sulfate proteoglycan (CSPG4) regulates human trophoblast function," *Placenta*, vol. 34, no. 10, pp. 907–912, 2013.

[10] A. Poli, J. Wang, O. Domingues et al., "Targeting glioblastoma with NK cells and mAb against NG2/CSPG4 prolongs animal survival," *Oncotarget*, vol. 4, no. 9, pp. 1527–1546, 2013.

[11] M. Kusama, T. Kageshita, Z. J. Chen, and S. Ferrone, "Characterization of syngeneic antiidiotypic monoclonal antibodies to murine anti-human high molecular weight melanoma-associated antigen monoclonal antibodies," *The Journal of Immunology*, vol. 143, no. 11, pp. 3844–3852, 1989.

[12] A. Mittelman, G. Z. J. Chen, G. Y. Wong, C. Liu, S. Hirai, and S. Ferrone, "Human high molecular weight-melanoma associated antigen mimicry by mouse anti-idiotypic monoclonal antibody MK2-23: modulation of the immunogenicity in patients with malignant melanoma," *Clinical Cancer Research*, vol. 1, no. 7, pp. 705–713, 1995.

[13] F. Morandi, M. V. Corrias, I. Levreri et al., "Serum levels of cytoplasmic melanoma-associated antigen at diagnosis may predict clinical relapse in neuroblastoma patients," *Cancer Immunology, Immunotherapy*, vol. 60, no. 10, pp. 1485–1495, 2011.

[14] L. Yu, Y. Tian, A. Gao, Z. Shi, Y. Liu, and C. Li, "Bi-module sensing device to in situ quantitatively detect hydrogen peroxide released from migrating tumor cells," *PLoS ONE*, vol. 10, no. 6, Article ID e0127610, 2015.

[15] L. Yu, L. X. Gao, X. Q. Ma, F. X. Hu, C. M. Li, and Z. Lu, "Involvement of superoxide and nitric oxide in BRAFV600E inhibitor PLX4032-induced growth inhibition of melanoma cells," *Integrative Biology*, vol. 6, no. 12, pp. 1211–1217, 2014.

[16] L. T. N. Truong, M. Chikae, Y. Ukita, and Y. Takamura, "Labelless impedance immunosensor based on polypyrrole-pyrolecarboxylic acid copolymer for hCG detection," *Talanta*, vol. 85, no. 5, pp. 2576–2580, 2011.

[17] R. Li, D. Wu, H. Li et al., "Label-free amperometric immunosensor for the detection of human serum chorionic gonadotropin based on nanoporous gold and graphene," *Analytical Biochemistry*, vol. 414, no. 2, pp. 196–201, 2011.

[18] W. Chen, Z. Lu, and C. M. Li, "Sensitive human interleukin 5 impedimetric sensor based on polypyrrole-pyrrolepropylic acid-gold nanocomposite," *Analytical Chemistry*, vol. 80, no. 22, pp. 8485–8492, 2008.

[19] Z. Z. Shi, Y. L. Tian, X. S. Wu, C. M. Li, and L. Yu, "A one-piece lateral flow impedimetric test strip for label-free clenbuterol detection," *Analytical Methods*, vol. 7, no. 12, pp. 4957–4964, 2015.

[20] D. Wang, W. Hu, Y. Xiong, Y. Xu, and C. M. Li, "Multifunctionalized reduced graphene oxide-doped polypyrrole/pyrrolepropylic acid nanocomposite impedimetric immunosensor to ultrasensitively detect small molecular aflatoxin B_1," *Biosensors and Bioelectronics*, vol. 63, pp. 185–189, 2015.