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Ultrasensitive electrochemical immunoassay for melanoma cells using mesoporous polyaniline

M.U. Anu Prathap^a, Carlos Iván Rodríguez^b, Omer Sadak^c, Jiehao Guan^a, Vijaysaradhi Setaluri^b, and Sundaram Gunasekaran^{a,c}

^aDepartment of Biological Systems Engineering, University of Wisconsin–Madison, 460 H Mall, Madison, WI 53706, USA

^bDepartment of Dermatology, School of Medicine and Public Health, University of Wisconsin–Madison, 1300 University Avenue, B-25, Madison, WI 53706, USA

^cDepartment of Materials Science and Engineering, University of Wisconsin–Madison, 1509 University Avenue, Madison, WI 53706, USA

Abstract

We report the development of antibody (anti-MC1R antibody)-functionalized polyaniline nanofibers modified screen-printed electrode capable of efficient electrochemical detection of melanoma cells at levels (1 cell/mL) not readily achieved by other methods. This immunosensor is highly selective in its detection of melanoma cells over normal human cells.

Graphical abstract

An ultrasensitive electrochemical immunosensor for the detection of melanoma cells was developed using anti-MC1R antibody-functionalized polyaniline nanofibers.

Malignant melanoma, the most aggressive type of skin cancer, arises from melanocytes, the pigment producing cells.¹ When melanoma is localized in the skin (Stages I–II), the five-year survival rate following the excision of the primary tumor is quite high (up to 97%), but the survival rate decreases precipitously as the cancer metastasizes to local and regional lymph nodes (Stage III) and distant organs (as low as 15% for Stage IV).^{1,2} Several tissue-based prognostic molecular markers in melanoma have been investigated.^{3–6} Although these

Conflicts of interest

There are no conflicts to declare.

markers are good indicators of the aggressiveness of the primary lesion, they do not inform whether and how many melanoma cells are dislodged from the primary tumor, entered the blood stream and are poised to seed metastatic lesions.⁷ Recent advances in concepts and methods to identify and enumerate circulating tumor cells (CTC) and isolate and characterize tumor-derived vesicles and macromolecules in the peripheral blood of cancer patients have made 'liquid biopsy' a promising tool in diagnosis, prognosis and management of cancers, including melanoma.^{8,9} The US Food and Drug Administration approved method for CTC detection (CELLSEARCH® CTC Test) is based on immuno-magnetic and fluorescence imaging that detects and enumerates CTC of epithelial origin.^{10,11} This method is time-consuming and expensive, requires primary purification and lacks selectivity.¹² To overcome these limitations, we developed a highly sensitive and selective immuno-electrochemical method for direct, simple, and rapid detection of melanoma cells. Electrochemical sensors are ideal for diagnostic estimations because they are highly sensitive, easy to operate, provide rapid response, and of low cost.^{13,14} Electrochemical methods such as differential pulse voltammetry (DPV), cyclic voltammetry (CV), anodic stripping voltammetry and electrochemiluminescence are employed to monitor cellular processes and detect tumor cells.^{13–15}

To date, noble metals (palladium, silver, osmium, iridium, platinum, and gold) are considered the most efficient electrocatalysts; however, high cost and low abundance preclude their widespread use.¹⁶ Therefore, conducting polymers are attracting significant attention due to their unique properties and promising applications in molecular electronics, chemical sensors, and biomedical science.¹⁷ Among the conducting polymers, polyaniline (PANI) possesses unique electrical and electronic properties, good redox reversibility, and environmental stability.^{17,18} PANI nanofibers (PANI-NFs) are preferred over noble metals since PANI-NFs can be prepared easily with high specific surface area, surface-to-volume ratio, and pore volume. Furthermore, amine nitrogen linkages in PANI facilitate effective binding and immobilization of biomolecules.¹⁹ In addition, PANI differs from other materials because of its sensitivity towards chain conformation changes (i.e., good flexibility), high hole-transporting ability, together with fast charge-separation capability, arising from its π -orbital structure.^{19–21} PANI-NFs are non-cytotoxic and biocompatible, which make them suitable as matrix material in chemical and biological sensors, actuators and microelectronic devices.^{17–21} Since the hybridization of the recognition element and the target analyte can cause perturbations in the chain conformation of PANI, PANI-modified sensors can recognize the biological binding events fast and provide a corresponding electrical signal.^{19–21} Antibody (Ab) loading is an important step in biosensor design. It is reported that PANI can make a covalent couple with Ab and enzyme effectively.^{17,22} Moreover, the biocompatibility of PANI can preserve the Ab activity and cell adhesion.^{17,22}

Herein, we report a highly sensitive electrochemical immunosensing strategy, capable of specifically and rapidly detecting melanoma cells using anti-MC1R Ab-functionalized PANI-NFs. A good linear correlation between the current and the density of target cells from 15 to 7000 cells/5 mL was obtained, with an ultralow limit of quantitation (LOQ) of 1 cell/mL.

Interfacial polymerization of aniline was performed on a heterogeneous biphasic system based on a procedure described by Kaner et al. with modifications (Fig. S1, ESI[†]).²³ X-ray diffraction (XRD) pattern of PANI displayed a sharp peak at 6.4°, indicating a highly ordered metallic conductive state (Fig. 1a).²⁴ Using Bragg's law, interplanar distance of 13.3 Å was calculated. The peaks at 19.2° and 25.5° correspond to periodicities parallel (100) and perpendicular (110) to the PANI chain.^{18,22} The peak at 25.5° indicates highly doped emeraldine salt form of PANI.^{18,22} Figure 1b shows the N₂ adsorption/desorption isotherm of PANI at 77 K, which could be classified as type IV with an H3 hysteresis loop typical of mesoporous materials by IUPAC classification.²² The Brunauer-Emmett-Teller (BET) surface area was 160 m²/g and the pore-size distribution, based on Barrett-Joyner-Halenda (BJH) analysis (Fig. 1b, inset) mainly centered at approximately 7.25 nm. Furthermore, the calculated total pore volume was 0.22 cm³.g⁻¹. Field emission scanning electron microscopy (FESEM) image of PANI shows nanofiber morphology (Fig. 1c), and high-resolution transmission electron microscope (HRTEM) image reveals ~95-nm wide interconnected NFs (Fig. 1d). The FT-IR spectra reveal the vibrational modes of PANI (Fig. S2, ESI[†]). The peaks located at 1586 cm⁻¹ and 1501 cm⁻¹ are due to C=C stretching vibration of the quinoid and benzenoid ring, respectively.^{18,22} The peak observed at 1384 cm⁻¹ can be attributed to the stretching vibration of the -C-N-C- bonds present in polaron.^{22,25} The band at 1304 cm⁻¹ is associated with C-N stretching vibration, and that at 1125 cm⁻¹ is due to the doping effect on the PANI backbone.^{18,22}

The electronic absorption spectra of PANI show three distinct absorption bands at around 356 nm, 438 nm, and 881 nm, corresponding to the π - π^* , the polaron $\rightarrow\pi^*$, and the π $\rightarrow$ polaron band transitions, respectively (Fig. S3, ESI[†]).²² The chemical composition of PANI was investigated with X-ray photoelectron spectroscopy (XPS) (Fig. S4, ESI[†]). The signals from wide-scan spectra of PANI (Figure S4a, ESI[†]) at 284.9, 399.5 and 531.1 eV can be attributed to C1s, N1s, and O1s, respectively.^{26,27} The high resolution N1s spectra of PANI (Fig. S4b, ESI[†]) exhibited peaks at 398.3, 399.5 and 400.2 eV, which correspond to -N=, -NH- and -N⁺- bonds, respectively.^{26,27} The peak at 400.2 eV (-N⁺-) is indicative of the N-doping level of PANI, and the high N⁺/N ratio (23.5%) shows a high proton doping of PANI.^{26,27} The high-resolution spectra of C1s (Figure S4c, ESI[†]) core level was deconvoluted into C-H, C-N, and C=N⁺ at 284.2, 284.9 and 286.1 eV, respectively due to multiple carbon bonding environment in PANI. Furthermore, moisture/water molecules usually present in PANI and the peak at 531.1 eV (O 1s) can be ascribed to the adsorbed and lattice oxygen.²⁷ Overall, the characterization results presented confirm the formation of PANI. Four-probe measurements were also conducted (Fig. S5, ESI[†]). The linear current-voltage (*I*-*V*) curve demonstrated an ohmic response, and the current saturated at a bias voltage less than 0.3 V. It is known that conducting polymers become metal-like (metallic copper) when heavily doped due to the formation of polaronic band.²⁸

To investigate the redox properties of PANI, CV was performed (Fig. S6, ESI[†]). Two sets of redox current peaks were obtained corresponding to leucoemeraldine/emeraldine couple and emeraldine/pernigraniline couple structural conversions.²⁹ Anti-MC1R Ab-functionalized

[†]Footnotes relating to the title and/or authors should appear here.

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PANI-NFs-modified screen-printed electrode (SPE) (MC1R-Ab-PANI/SPE) constituted our sensor platform (Scheme 1).

Electrochemical impedance spectroscopy (EIS) is an effective and informative method to measure electrical properties of the electrode/solution interface.^{30,31} Some parameters such as solution resistance (R_s), charge transfer resistance (R_{CT}) and double layer capacitance (C_{dl}), can be determined from EIS measurements.³⁰ We applied EIS to characterize MC1R-Ab-PANI/SPE (Fig. S7, ESI[†]). The EIS parameters were determined at various stages of electrode surface modification (Table S1, ESI[†]). The value of R_{CT} (i.e., the diameter of semicircle) signifies the resistance to electron transfer at the electrode/electrolyte interface. Warburg element (W) was insert in model which accounts for the Nernstian diffusion (Fig. S7, ESI[†]). The change in R_{CT} of the bare SPE from about 19 k Ω to 0.24 k Ω after coating with PANI is indicative of the substantial effect of PANI in improving electrical conductivity and hence speed up the electron transfer at the interface of the PANI-modified surface and the redox probe $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (Table S1, ESI[†]). When PANI/SPE electrode was activated with the 0.5 M carbonyldiimidazole (CDI) for 3 h, the R_{CT} value increased to 0.71 k Ω , reflecting the insulating nature of CDI (Table S1, ESI[†]). EIS was also conducted to study the performance of the sensor and quantify its immunogenic affinity and specificity against HEK293 (normal human embryonic kidney cells) and SKMEL-2 melanoma cell lines (Figure S8, ESI[†]). The Nyquist plots of the impedance spectra confirm that our electrode responds specifically to the presence of SKMEL-2 cells, and not to HEK293 cells (Figs. 2a,b). The impedance of the sensor electrode prior to binding cells can be represented by an equivalent circuit shown in Figure 2c. Here, the impedance of the electrode/solution interface is represented by R_s , which is in series with diffuse layer capacitor C_E .^{31–33} Ideally, Ab-coated electrode is predicted to have another capacitor in series with C_E .^{31–33} Using parallel combination of a constant phase element (CPE_{AB}) and a resistance (R_{AB}) to account for Ab can yield good-fitting result.^{31–33} Upon cells bind to the sensor, the system can be represented by adding a constant phase element of the cell (CPE_{Cell}) to the equivalent circuit of the electrode (Fig. 2d). The best-fit values for the circuit elements are given in Table S2, ESI[†]. On evaluating the values of different circuit elements after each subsequent immunoreaction with the added SKMEL-2 cells, the changes in R_{AB} value is more prominent than those of other components due to Ab-antigen interaction. (Table S2, ESI[†]). A small decrease in CPE_{CELL} values was observed indicating a lowering in the capacitive behavior of the bioelectrode (Table S2, ESI[†]). Consequently, we are able to describe a facile method not only to detect cell adhesion, but also to investigate the Ab-antigen interaction. The Nyquist diagram exhibits two semicircles (Fig. 2b); the smaller semicircle in the higher frequency range is attributed to R_{CT} of the depletion layer,^{34,35} and the bigger semicircle in the lower frequency range to R_{CT} of the Helmholtz layer.^{34,35} Electrochemical single frequency impedance (SFI) profiling was performed with 1000 SKMEL-2 cells to further confirm the specificity of the immunogenic interaction.³⁶ For the SFI experiments, the potentiostat was fixed at a frequency of 70 Hz. This working frequency was selected by consulting the Bode plot, as it includes important frequency information of the system (Fig. 2e). Bode response was studied during the incubation period. Maximum shift in phase angle and the total impedance was observed at 70 Hz. (Fig. 2e). In the Bode-phase diagram (Fig.

2e, red line), two phase angles can be distinguished, one at high frequency corresponds to the depletion layer, and another at low frequency relates to the Helmholtz layer.^{31,34,35}

SFI data help examine frequency-dependent impedance (Z) and phase angle (Φ) represented by the equation $Z' = Ze^{i\Phi}$ (Fig. 2f).³⁶ The binding between the antibody and the melanoma cells causes a change in Φ of the circuit. Significant changes in impedance ($Z = 80 \Omega$ in 1000 s) and phase angle ($\Phi = 1.4^\circ$ in 1000 s) were due to the strong binding affinity of SKMEL-2 cells toward anti-MC1R antibody (Fig. 2f). The chronocoulometric response curves in the absence and presence of 1000 SKMEL-2 cells were also recorded (Fig. 2g). The linear part of the plot was extrapolated back to time zero to obtain the intercept value of the charge (Q), which decreased when the cell density was increased from 0 to 1000 cells/5 mL, which indicates high binding affinity towards the target SKMEL-2 cells with high sensitivity (Fig. 2g).³⁷ The above coulometric studies prove that our sensor can recognize SKMEL-2 tumor cells with excellent specificity. The Ab loading efficiency at PANI-modified electrode was also evaluated. The current value did not show a good response with low Ab loading (100 ng) due to the hindered electron transfer pathway (Fig. S9, ESI[†]). DPV curves of samples with different SKMEL-2 cell densities (at 100 ng Ab) exhibit a shift in peak potential, which is due to oversaturation of SKMEL-2 cells at the electrode surface resulting in overpotentials (Fig. S9, ESI[†]). Thus, 200 ng Ab was used in subsequent experiments. DPV curves for different concentrations of HEK293 cells starting from 0 to 5000 cells/5mL at our sensor (MC1R-Ab-PANI/SPE) was found insensitive (Fig. S10, ESI[†]).

The effectiveness of our electrochemical immunoassay is primarily believed due to the modification of the SPE working electrode with PANI, which after MC1R-Ab immobilization, served as the sensor platform. The amine groups in PANI help to bind MC1R-Abs onto the modified PANI/SPE.³⁸ After treating with bovine serum albumin (BSA) to block any nonspecific binding, our sensor becomes highly selective toward MC1R antigens on the SKMEL-2 cell surface. The binding of SKMEL-2 cells to the electrode surface blocks the $[\text{Fe}(\text{CN})_6]^{4-/3-}$ redox probe from accessing the electrode surface, which increases the impedance response in proportion to the number of SKMEL-2 cells that bind.^{39–41} The selectivity of MC1R-Abs confers the sensor the ability to target only the tumor (SKMEL-2) cells, and not other normal human cells.

We investigated the relationship between peak current responses for various SKMEL-2 cell densities by DPV (Fig. 3a). The cathodic ratio of signal intensity $(I_{\text{cells}} - I_B)/(I_{7000 \text{ cells}} - I_B)$ in the presence and absence of cells were recorded (Fig. 3b-d). I_B and I_{cells} were the DPV peak currents in the absence and presence of cells. $I_{7000 \text{ cells}}$ is the maximum peak current after the sensor was saturated by 7000 cells/5 mL. The two calibration plots show excellent linear relationships between the peak current and the logarithmic value of SKMEL-2 cell density over the ranges of 15–600 cells/5 mL (Fig. 3c) and 600–7000 cells/5 mL (Fig. 3d). The LOQ of 1 cell/mL was calculated based on three consecutive DPV measurements. The LOQ is the lowest analyte concentration necessary to generate a characteristic signal at least 10-fold higher than the background noise (Fig. S11, ESI[†]).^{42,43} The sensitivity and LOQ of the present electrochemical immunosensor is superior to those of previously reported immunosensors (Table S3, ESI[†]). The low LOQ we obtained is attributed to the use of

PANI-NFs, whose large surface area and mesoporosity facilitated binding of large amount of Ab on the electrode surface, and hence the substantially greater detection effectiveness. The reproducibility of our biosensor was investigated by recording the impedance of the three separately prepared electrodes ($n = 3$). The relative standard deviation (RSD) of the impedance responses of the electrodes for detecting 1000 cells/5 mL did not exceed 6.4%, exhibiting good reproducibility. Stability of the sensor was determined by measuring charge transfer resistance with 400 cells/5 mL, using the same electrode at three-day intervals over nine days. Three measurements were made each time, washing the electrode with buffer after each measurement and storing the electrode at 5 °C when not in use. Results show no change in the impedance signals, which can be attributed to the long-term stability of antibody attachment to the electrode and the retention of its immunogenic affinity to SKMEL-2 cells (Fig. S12, ESI[†]). The facile preparation and ultrahigh sensitivity make the proposed immunosensor suitable as a potential low-cost device for routine detection of cancer cells, which could be established in any laboratory.

Conclusions

We have systematically studied the interaction between anti-MC1R Ab-functionalized PANI-NFs and SKMEL-2 melanoma tumor cells by various electrochemical methods. The experimental parameters of the immunosensor (MC1R-Ab-PANI/SPE) were optimized, and an LOQ of 1 cell/mL was established with a relatively wide linear range from 15 to 7000 cells/5 mL owing to the use of PANI-NFs which, because of its high surface area, afforded a large number of reaction sites. The immunosensor was non-responsive to HEK293 cells (normal human kidney cell), confirming high specificity towards the target melanoma cells. The ultralow LOQ of this immunosensor makes it a promising device for detecting melanoma and other cancer cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Characterization of PANI-NFs (a) XRD pattern, (b) N_2 -adsorption isotherm (the inset shows pore size distribution), (c) FESEM image, and (d) HRTEM image.

Fig. 2.

Nyquist diagrams for MC1R-Ab-PANI/SPE electrode recorded in a frequency range of 0.1 Hz to 10 kHz at a standard potential of +0.15 V (E_{app}), using a sinusoidal potential perturbation with an amplitude of 5 mV in 0.1 M KCl solution containing 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$, with different concentrations of (a) HEK293 cells and (b) SKMEL-2 cells. The equivalent circuits of system impedance before (c) and after (d) binding of SKMEL-2 cells. R_s : solution resistance, R_{AB} : antibody resistance, C_E : diffuse layer capacitance, CPE_{AB} : constant phase element for antibody, CPE_{CELL} : constant phase element for SKMEL-2CELL. (e) Bode plot for the electrode recorded in a frequency range of 0.1 Hz to 10 kHz at a standard potential of +0.15 V (E_{app}), using a sinusoidal potential perturbation with an amplitude of 5 mV in 0.1 M KCl solution containing 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$. (f) SFI profiling results of 1000 SKMEL-2 cells (red line, variation in phase angle; blue line, variation in impedance). (g) Plot of charge (Q) versus time curves in the absence and presence of 1000 SKMEL-2 cells in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and 0.1 M KCl solution (5 mL). The dotted lines represent linear fit to determine the intercept at $t = 0$. Q is the charge difference in the absence and presence of 1000 SKMEL-2 cells.

Fig. 3.

(a) DPV curves with different SKMEL-2 cell densities from 0 to 7000 cells/5 mL at MC1R-Ab-PANI/SPE (200 ng Ab loading). (b) Peak current ratio versus SKMEL-2 cell concentration. Calibration curves of peak current ratio versus the logarithmic value of SKMEL-2 cell concentration: (c) 15-600 cells/5 mL and (d) 600-7000 cells/5 mL.

Scheme 1.

Illustration of PANI-NFs-modified SPE electrochemical immunoassay for melanoma (SKMEL-2) cell detection.