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Ultrasensitive Detection of Human Liver Hepatocellular Carcinoma Cells Using a Label-Free Aptasensor

Leila Kashefi-Kheyrabadi,[†,‡] Masoud A. Mehrgardi,[*,†,‡] Emilia Wiechec,[§] Anthony P. F. Turner,[†] and Ashutosh Tiwari[*,†]

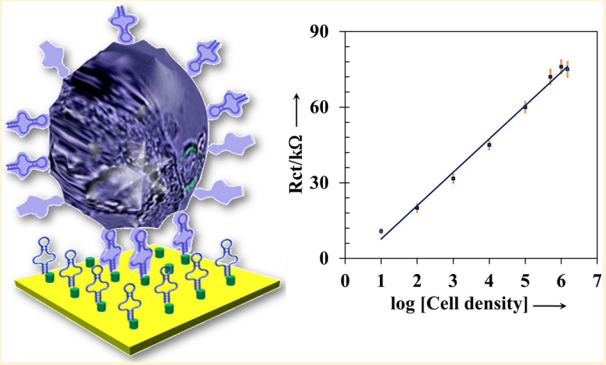
[†]Biosensors and Bioelectronics Centre, Institute of Physics, Chemistry and Biology, Linköping University, Linköping S-58183, Sweden

[‡]Department of Chemistry, University of Isfahan, Isfahan 81746-73441, Iran

[§]Department of Clinical and Experimental Medicine, Linköping University, Linköping S-58185, Sweden

Supporting Information

ABSTRACT: Liver cancer is one of the most common cancers in the world and has no effective cure, especially in later stages. The development of a tangible protocol for early diagnosis of this disease remains a major challenge. In the present manuscript, an aptamer-based, label-free electrochemical biosensor for the sensitive detection of HepG2, a hepatocellular carcinoma cell line, is described. The target cells are captured in a sandwich architecture using TLS11a aptamer covalently attached to a gold surface and a secondary TLS11a aptamer. The application of TLS11a aptamer as a recognition layer resulted in a sensor with high affinity for HepG2 cancer cells in comparison with control cancer cells of human prostate, breast, and colon tumors. The aptasensor delivered a wide linear dynamic range over 1×10^2 to 1×10^6 cells/mL, with a detection limit of 2 cells/mL. This protocol provides a precise method for sensitive detection of liver cancer with significant advantages in terms of simplicity, low cost, and stability.



The International Agency for Research on Cancer (GLOBOCAN 2008) has released worldwide cancer statistics revealing that there were about 12.7 million cancer cases and 7.6 million cancer deaths in 2008.[1] Most cancer-associated deaths are due to the metastasis of the primary cancer tumors.[2] Similarly to many other diseases, early diagnosis is essential for the effective treatment of cancer.[3] Early stage recognition of cancers is a challenge to enable effective treatment, and aptamers are highly promising molecular probes in this field.[4] Two decades ago, they were identified as a new class of recognition molecules, through a process of molecular evolution termed SELEX (systematic evolution of ligands by exponential enrichment)[5] for a broad range of targets from small molecules to intact cells. This approach has since been adopted for whole-cell recognition[6] as an alternative to antibodies.[7]

Typically, antibodies are produced by an in vivo procedure, whereas aptamers are selected by an iterative in vitro evolution procedure. Aptamers are also more thermally stable molecules and preserve their structures at elevated temperatures, while antibodies are easily damaged and lose their tertiary structure at high temperatures. Moreover, aptamers can be chemically modified to enhance their stability and nuclease resistance.[8] Unlike antibodies, aptamers are usually not detected by the human immune system as foreign agents and possess low toxicity.[9] Furthermore, aptamers can be selected for a large variety of targets that cannot be recognized by antibodies, for instance ions or small molecules.[8] In comparison with existing molecular probes for biomarker detection, aptamers are highly specific for the different types of cells.[10] They have also show good prospects for the detection of the molecular characteristics of target cancer cells in the presence of other cells. Hence aptamer-based biosensors have recently attracted considerable attention as a promising approach for clinical diagnostics.

Many different biosensors based on various transducers such as mass-sensitive,[11] electrochemical,[12] and optical transducers[13,14] have been described. Among them, aptamer-based electrochemical biosensors have been extensively considered due to their fabrication simplicity, high sensitivity, cost-effectiveness, and high stability.[15] It is well-known, early stage recognition of cancers is a challenge to enable effective treatment and aptamers are highly promising molecular probes in this field. Aptamer selection for specific recognition of target cancer cells such as B-cell lymphoma,[16] small-cell lung cancers,[17] and liver cancers[18] have already been established.

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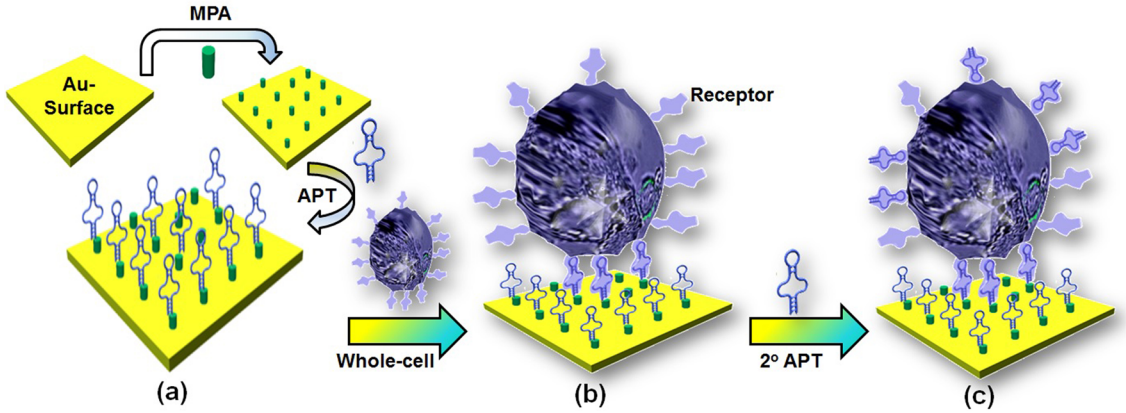
Scheme 1. Schematic Presentation of the Step-Wise Modification Procedure on a Gold Electrode Surface[a]



[a](a) TLS11a Aptamer Conjugated Au Surface via Coupling of Amino-Labeled Aptamer to the MPA–Au Surface; (b) Interaction of HepG2 Cells on Au Surface-Immobilized Aptamer; (c) Binding of Secondary Aptamer to Complete the HepG2 Cells Sandwich Format.

Unfortunately, the majority of hepatocellular carcinomas (HCC) are diagnosed when there are only a few therapeutic possibilities. Measuring the α-fetoprotein (AFP) level in serum as a biomarker is the basis of present monitoring methodologies for liver cancer progress. These diagnostic strategies suffer from lack of specificity due to the increase of the serum level of AFP in many other liver diseases and are also not significantly indicative in the early stage of the disease.[19]

In the present manuscript, we take advantage of the TLS11a aptamer,[18] which specifically binds to the membrane surface of hepatocellular carcinoma cells, to introduce a straightforward strategy for ultrahigh-sensitive detection of liver cancer cells. Conditions were optimized to allow a simple fabrication of the electrochemical biosensor, by immobilization of amino-labeled TLS11a onto the carboxylic acid modified gold electrode via coupling chemistry, and apply it in a sandwich architecture. All steps of modification and characterization of the sensor surface were monitored using electrochemical impedance spectroscopy and cyclic voltammetry techniques.

EXPERIMENTAL SECTION

Materials and Apparatus. Analytical grade magnesium chloride, sodium chloride, potassium chloride, sodium dihydrogen phosphate, disodium hydrogen phosphate, hexaammine ruthenium(III) chloride (RuHex), 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), 3-mercaptopropionic acid (MPA), potassium ferrocyanide, and potassium ferricyanide were purchased from Sigma-Aldrich. All chemicals were used as received.

The amino-labeled TLS11a aptamer was synthesized and purified by Integrated DNA Technologies (Coralville, IA) with the following sequence: 5′-$(CH_2)_6$–NH_2–ACA GCA TCC CCA TGT GAA CAA TCG CAT TGT GAT TGT TAC GGT TTC CGC CTC ATG GAC GTG CTG-3′

The aptamer solution was prepared from its lyophilized powder using phosphate buffer saline (PBS 1× at pH 7.4) and kept at −20 °C. All solutions were prepared using Milli-Q water (18.2 MΩ·cm). Electrochemical experiments were carried out with an Autolab PGSTAT30 (Ecochemie, The Netherlands) driven by GPES 4.9 software provided by the manufacturer. A conventional three-electrode system, consisting of a gold disk electrode as a working electrode, a platinum wire as an auxiliary electrode, and a Ag/AgCl/3.0 M KCl as a reference electrode, was used for electrochemical experiments. Electrochemical impedance spectroscopy (EIS) experiments were performed in equimolar $K_3[Fe(CN)_6]/K_4[Fe(CN)_6$ (1:1, 0.5 mM) solution containing 0.10 M KCl. The spectra were recorded by applying an open circuit (OCP) and ac potential of 5.0 mV over the frequency range of 10 kHz to 0.1 Hz. Cyclic voltammograms were recorded at a scan rate of 100 mV s^{-1}. The coverage of aptamers on the electrode surface was quantified using chronocoulometry in the presence of 50 mM RuHex.[20] Chronocoulometry experiments were performed using a pulse period of 500 ms and an applied pulse width of 500 mV. A computer-interfaced quartz crystal microbalance (QCM) (Q-Sense E4, Q-sense, Sweden) equipped with an AT-cut 4.95 ± 0.05 MHz piezoelectric quartz crystal (14 mm diameter) was used for QCM experiments. All measurements were performed at room temperature in an enclosed and grounded Faraday cage.

Preparation of a Biorecognition Surface. Prior to the electrode modification, gold disk electrodes with a geometric area of 0.03 cm^2 (CH Instrumental Inc., Austin, TX) were polished with a 0.3 and 0.05 μm alumina slurry successively, rinsed in ultrapure water, and sonicated in water for a couple of minutes, to remove bound particulates. Subsequently, the electrodes were electrochemically cleaned by potential cycling over the range of 0–1.5 V in 0.5 M H_2SO_4 solution, until reproducible voltammograms were observed.[21] By assuming 482 μC cm^{-2} charge for reduction of one monolayer of AuO to Au(111),[22] the actual electroactive surface area for each electrode was calculated as 0.08 ± 0.01 cm^2. The fabrication steps for the label-free electrochemical aptasensor are shown in Scheme 1.

A layer of MPA was self-assembled onto the surface of a gold electrode by incubating it in 1 mL of 50 mM aqueous solution of MPA for 18 h. The MPA-modified electrode was then washed using Milli-Q water, and the carboxylic groups on the electrode surface were activated using 20 mM EDC and 30 mM NHS solutions in PBS 1× at pH 5.5 for 2 h. The electrode was washed again with Milli-Q water and dried, followed by the addition of a 10 μL drop of TLS11a aptamer to the electrode surface for 2 h. The tip of the electrode was covered with a plastic cap to prevent evaporation. In this step, a carbodiimide bond forms between the activated carboxylic groups of MPA

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and the amino-labeled aptamers. Finally, the aptamer-modified electrode was rinsed using Milli-Q water and incubated in PBS 1× at pH 7.4 before use.

Modification of the QCM Crystal. The QCM gold-coated quartz crystal was cleaned using the TL1 procedure.[23] Briefly, the QCM sensors were heated in a 5:1:1 mixture of H_2O/NH_3 (25%, v/v)/H_2O_2 (30%, v/v) at 85 °C for 10 min and then rinsed thoroughly with water. Next, the aptamer was self-assembled onto the sensor using a similar procedure to that described for the gold electrode modification.

Cell Culture and Immobilization. MCF-7 (human breast cancer cells), HCT116 (human colon carcinoma cells), and PC3 (human prostate cancer cells) were cultured using Roswell Park Memorial Institute (RPMI 1640) medium. HepG2 (human liver cancer cells) were grown in Dulbecco's minimal essential (DMEM) medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin (100 U/mL) under a 5% CO_2-humidified chamber at 37 °C. The cells were collected and separated from the medium after culturing for 72 h, by centrifugation at 1200 rpm for 3 min. The cells were then rinsed using a sterile Dulbecco's phosphate-buffered saline, DPBS (pH 7.4), containing calcium chloride (10 mM) and magnesium chloride (5 mM). The cell sediment was resuspended in the DPBS (pH 7.4) to obtain a homogeneous cell suspension. Cells were counted using a Beckman cell counter. The aptamer-modified electrode was then incubated in various counts of HepG2 cells suspension for ∼2 h to attain cell immobilization (GE/MPA/APT/Cell). The electrode was rinsed using PBS 1× at pH 7.4 to remove nonspecifically adsorbed cells and then dried. Finally, another 10 μL aliquot of 4.0 μM amino-labeled aptamer solution was dropped on the electrode surface to complete the sandwich layout format (GE/MPA/APT/Cell/APT). Finally, the modified electrode was rinsed using Milli-Q water.

MTT Assay. The cell viability upon TLS11a aptamer treatment was measured using the methylthiazoletetrazolium (MTT) assay protocol.[24] Briefly, HepG2 cells (1×10^4 in 100 mL) were placed in 96-well plates and grown overnight at 37 °C. The cells were treated with various concentrations of the aptamers for 24 h or left untreated in the control experiments. Twenty-four hours post-treatment, 10 μL of MTT (5 mg/mL) was added to each well of 96-well plate, and the cells were further incubated for 5 h at 37 °C. Next, the culture medium containing the MTT was discarded and 150 μL of ethanol/DMSO at 1:1 ratio per well was added for 30 min incubation with gentle shaking. Absorbance was measured at λ = 570 nm (with λ = 630 nm as the reference wavelength) using an ELISA microplate reader (Bio-Rad, U.S.A.). All the measurements were carried out in the triplicates, and the data are shown as a mean of ± SD.

RESULTS AND DISCUSSION

The characteristics of the developed aptasensor were investigated by electrochemical and mass-sensitive techniques. Electrochemical impedance spectroscopy is a nondestructive electrochemical method because only a very narrow range of small potentials is applied.[25] The changes in capacitance or resistance properties of the solid support−electrolyte interface by the immobilization of biomaterial films can be followed using EIS.[26] The presence of biomaterials on the electrode surface, which is dependent on the molecular and immobilization characteristics of the recognition layer, decreases the double layer capacitance and restricts the interfacial electron-transfer kinetics. Both aptamers and cancer cells can affect the electron-transfer resistance in EIS due to blocking of access of the redox probe to the electrode surface and the difference in dielectric constants of biological materials compared to water molecules. The electrostatic repulsion between the anionic probe and negatively charge phosphate backbone of the aptamer could hinder access to the surface due to the stereoconfiguration of the aptamer and cells and would also be affect electron-transfer resistance. EIS is one of the most powerful electrochemical techniques for investigation of living cells because of their excellent insulating properties at low frequencies and dielectric features at high frequencies.[26]

Whole-Cell Aptasensing. Using EIS, each modification step can be readily monitored by following the changes in the electron-transfer resistance (R_{et}). The effects of different modification steps on the sensing interface were characterized by cyclic voltammetry (CV) and EIS as illustrated in Figure 1.

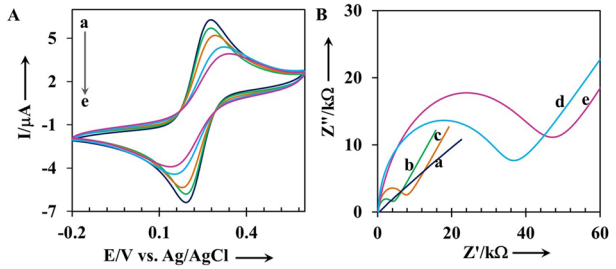

Figure 1. Characterization of different modification steps of the sensor surface by (A) cyclic voltammetry in 5×10^{-4} M $[Fe(CN)_6]^{3-}$ and 0.1 M KCl solution at a scan rate of 100 mV s^{-1}, (a) bare GE, (b) GE/MPA, (c) GE/MPA/APT, (d) GE/MPA/APT/HepG2 cell, and (e) GE/MPA/APT/HepG2/APT, and (B) EIS in 5×10^{-4} M $[Fe(CN)_6]^{3-/4-}$ (1:1) solution at a constant dc potential of +0.23 V (cell density, 1×10^6 cells/mL).

The bare gold electrode (GE) showed a very small electron-transfer resistance, suggesting a fast electron-transfer process at such electrodes (Figure 1, curve a). As a result of the formation of MPA self-assembled monolayer (SAM) on the electrode surface, a stack of negative charges is spread on the electrode surface at neutral pHs. The self-assembly of the negatively charged MPA on the electrode surface efficiently repels the anionic redox indicator $[Fe(CN)_6]^{3-/4-}$ and thus leads to an increase in electron-transfer resistance (Figure 1, curve b). To optimize the immobilization of aptamer, the surface of the MPA-modified electrode was treated using various concentrations of TLS11a aptamer (APT) (2−10 μM) after activation of MPA carboxylic groups by EDC/NHS. EIS spectra demonstrated that the immobilization of the aptamer increased with increasing concentration up to 4.0 μM (Supporting Information Figure S1), and this optimum concentration was therefore used for further experiments (Figure 1, curve c). By increasing the aptamer concentration above 4 μM, the possibility of intermolecular hybridization of the complementary sections of the aptamer might be increased, which decreases the aptamer immobilization on surface of the electrode. In the next step, the treatment of the aptamer-modified electrode using HepG2 cells dramatically increased the interfacial electron-transfer resistance, because of the blocking of direct access of $[Fe(CN)6]^{3-/4-}$ ions to the electrode surface (Figure 1, curve d). The effect of incubation time HepG2 cells on the sensor surface was also investigated.

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EIS results showed that the optimum incubation time to capture more cells onto sensor surface and achieve the maximum electron-transfer resistance was about 2 h. During this time the maximum loading of cells occurred, and longer incubation times (up to 3 h) did not further improve cell capture. Therefore, an incubation time of 2 h was selected for the detection of HepG2 cells (Supporting Information Figure S2). The aptasensor showed a reasonable response time at this stage. Finally, in the last step, the captured cells on the surface were treated with more aptamers to form a sandwich format. This causes further accumulation of aptamers and increases the negative charges due to the phosphate backbone of the aptamer on the surface, therefore increasing the electron-transfer resistance (Figure 1, curve e). Different modification steps of the sensor surface were also characterized using a QCM (Supporting Information Figure S3). The QCM results were consistent with the electrochemical results.

Analytical Performances of the Aptasensor. The efficiency of this label-free electrochemical aptasensor for detection of liver cancer cells at very low concentrations and its reproducibility and stability were demonstrated. The aptasensor was treated with various concentrations of HepG2 cells, and cyclic voltammograms and electrochemical impedance spectra were recorded. As Supporting Information Figure S4 shows, increasing the concentration of cells up to 1.5×10^6 cells/mL resulted in significantly decreased currents while the peak potential separations (ΔE_p) increased. Furthermore, Nyquist plots in Figure 2A show that R_{et} values increase with

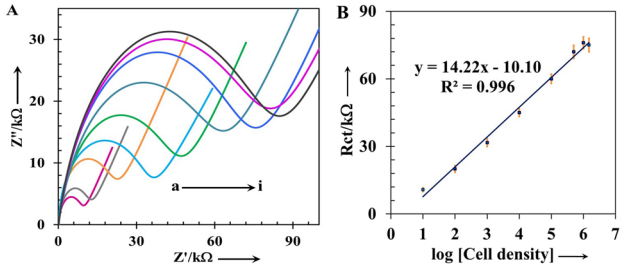

Figure 2. (A) Nyquist plots in the same conditions of Figure 1, (a–i) GE/MPA/APT/cell/APT (0, 10, 1×10^2, 1×10^3, 1×10^4, 1×10^5, 5×10^5, 1×10^6, and 1.5×10^6 HepG2 cells/mL), and (B) calibration curve of the aptasensor for HepG2 cells detection; the data points and error bars correspond to the average and standard deviations from three independent measurements.

increasing concentration of HepG2 cells, implying a higher content of HepG2 cells captured onto the sensor surface. One of the most important features of this biosensor is its potential to detect the cancer cells over a wide linear dynamic range with an impressively low detection limit. As the calibration curve (Figure 2B) shows, R_{et} is linearly proportional to the logarithmic value of HepG2 cell concentrations over the range of 1.0×10^2 to 1.0×10^6 cells/mL. The limit of detection was calculated as 2 cells/mL, based on three times the standard deviation of the blank (R_{et} was obtained when the sensor was incubated in fresh culture medium without cells.). The analytical performance of this aptasensor was compared to previously reported biosensors for the detection of the cancer cells using aptamers as recognition layer and different transduction systems in Supporting Information Table S1. The calculated detection limit is significantly smaller than those previously reported label-free assays.

Control Experiments. The presented aptasensor also exhibited an excellent selectivity for the detection of HepG2 cells, as confirmed by EIS and QCM measurements. The analytical signals of the sensor in EIS and QCM experiments were compared for three control human cancer cells, including breast (MCF-7), colon carcinoma (HCT116), prostate (PC3), and the test hepatocellular (HepG2) cells. When the control cells were incubated with the TLS11a aptamer-modified electrode at the same concentration of 1×10^5 cells/mL, only small R_{et} changes to the control cells were observed, indicating the excellent selectivity of the sensor for HepG2 cells (Figure 3A). Furthermore, interaction of HepG2 cells with

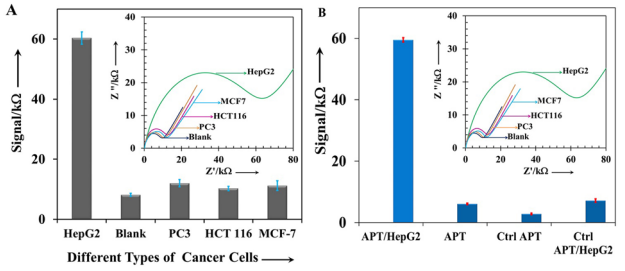

Figure 3. (A) Comparison between HepG2 cells with other control cancer cells including PC3, HCT 116, and MCF-7 cells using EIS in 5×10^{-4} M $[Fe(CN)_6]^{3-/4-}$ (1:1) solution at a constant dc potential of +0.23 V (the same concentration, 1×10^5 cells/mL). (B) The comparison between interaction of HepG2 cells (1×10^5 cells/mL) with TLS11a aptamer (APT) and another negative control sequence [sgc8 (Ctrl APT)].

another control aptamer [sgc8 as anti-leukemia (CCRF-CEM) aptamer] was investigated (Figure 3B) and small R_{et} changes were also observed for negative control sequence. QCM results were also confirmed the same as EIS results (Figure 4). Very

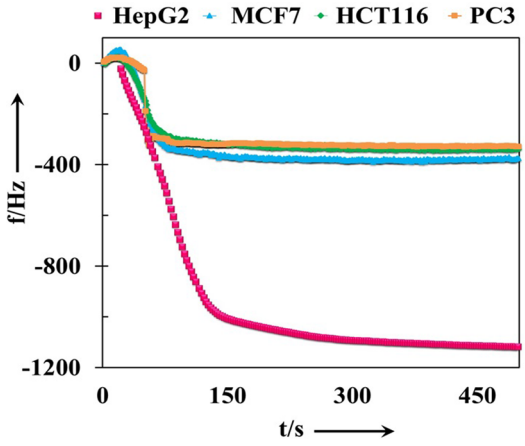

Figure 4. Comparison between HepG2 cells with other control cancer cells including PC3, HCT 116, and MCF-7 cells using QCM techniques (the same concentration, 1×10^4 cells/mL).

small frequency shifts were observed when TLS11a aptasensor was treated with control cells instead HepG2 cells. The results indicate that TLS11a aptamer has high binding affinity to the biomarkers on the HepG2 cell surface compared with other cells, and this aptasensor can discriminate HepG2 cells and other ones very well.

Another unique feature of the sensor is its reproducibility. A relative standard deviation (RSD) of 2.8% was obtained for a

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series of three sensors used for the detection of 5.0×10^5 cells/mL. Also, in order to evaluate the storage stability of the developed aptasensor, aptamer-modified electrodes were stored in PBS 1×, pH 7.4 at 4 °C. After 7 days, a decrease of 3.7% was observed for the detection of the same cell counts, implying remarkable electrode stability.

TLS11a aptamer did not show significant cytotoxicity under optimal conditions as tested by the MTT assay.[24] The MTT assay is a colorimetric method for determination of the number of viable cells through cell proliferation. The HepG2 cells were plated in 96-well plates and grown overnight at 37 °C. A plate reader was used to measure the absorbance at 570 nm to evaluate the cell viability. These cells indicated no significant cytotoxicity at the different concentrations over range of 1−10 μM. The cell viability of 96 ± 2 was obtained at working concentration of 4 μM of TLS11a aptamer.

CONCLUSION

A sensitive diagnosis of liver cancer cells remains one of the challenges in the field of biosensors and biomedical engineering. In this study, one of the most sensitive protocols so far reported for the detection of these cells using aptamer-based electrochemical biosensors has been introduced. The TLS11a aptamer has high affinity to human liver cancer cells when applied as the recognition layer in an electrochemical sensor. The specific and high-affinity interaction between the liver cancer cells and TLS11a enabled the biosensor to identify HepG2 at concentrations as low as 2 cells/mL. The presented aptasensor is a simple, selective, and label-free diagnostic tool for the detection of the HepG2 cells. A linear relationship between R_{et} and the logarithmic value of cell concentration was found over a range of 1.0×10^2 to 1.0×10^6 cells/mL. In comparison with previously reported protocols for cancer diagnosis using aptamers, this sensor offers superior overall performance.

ASSOCIATED CONTENT

Supporting Information

Whole-cell aptasensing using a quartz crystal microbalance, quantitation of aptamer surface density, and some characterization experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: m.mehrgardi@chem.ui.ac.ir.

*E-mail: ashutosh.tiwari@liu.se. Phone: +46 1328-2395. Fax: +46 1313-7568.

Notes

The authors declare no competing financial interest.

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