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Label-free electrochemical impedance spectroscopy biosensor for direct detection of cancer cells based on the interaction between carbohydrate and lectin

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ABSTRACT

A novel label-free electrochemical impedance spectroscopy (EIS) biosensor for direct cancer cell detection based on the interaction between carbohydrate and lectin has been developed with good sensitivity and selectivity. In the present work, concanavalin A (Con A), a mannose specific lectin, was immobilized on a gold disk electrode to fabricate the Con A sensor. This sensor was incubated with the cancer cell sample, and the binding of cancer cells with Con A resulted in a change of charge transfer resistance (R_{ct}). EIS measurement was employed to measure the impedance change which reveals the concentration of cancer cells. This method has been successfully applied in human liver cancer cell Bel-7404 for direct and sensitive detection with a detection limit of 234 cells/mL. This method could be extended to carry out multi-component diagnosis applications, thus providing enormous potential for applications of cancer monitoring and therapy.

1. Introduction

Cancer is a leading cause of death worldwide and it is notoriously difficult to diagnose especially in its early stages. At the time of diagnosis, many patients experience related health complications. According to this situation, several approaches, such as tumor markers, imaging and biopsy, were introduced to screen and diagnose cancer in its early stages (Etzioni et al., 2003). Tumor markers, which are substances released by cancer cells either into the blood or urine, or substances created by the body in response to cancer cells, have increased tremendously providing great opportunities for enhancing the efficiency of detection and efficacy of treatment in recent years (Glas et al., 2003; Goonetilleke and Siriwardena, 2007; Schneider, 2006; Zhou et al., 2006). These approaches have enabled us to more precisely detect cancers, guide therapeutic decision making, monitor a patient's response to treatment, and track the progression of cancer (Schrohl et al., 2003). Tumor markers such as alpha-fetoprotein (AFP) for liver cancer (Beneduce et al., 2004) and prostate-specific antigen (PSA) for prostate cancer (Lilja et al., 2008), have provided a clear indication of disease and are used as an arsenal of molecular tests for cancers. However, according to the USA National Cancer Institute (NCI), there are some limitations to the use of tumor markers. Sometimes, noncancerous conditions can cause the levels of certain tumor markers to increase. In addition, not everyone with a particular type of cancer will have a high level of a tumor marker associated with that cancer. Moreover, tumor markers have not been identified for every type of cancer. Based on these points, biomarker-based diagnosis may be more complicated and indirect for cancer screening. Imaging tests such as Computed Tomography (CT; Bastarrika et al., 2005; Gohagan et al., 2005), X-rays (Liu et al., 2007) and magnetic resonance imaging (MRI; Lehman et al., 2007; Saisho and Yamaguchi, 2004) are used to identify the precise location of the tumor and to determine the extent of the disease. While imaging tests are helpful in detecting masses or areas of abnormality, they alone cannot differentiate cancerous cells from noncancerous cells, and also depend on the precision instruments. For the majority of cancers, biopsy works as method of confirmation, such as tissue diagnosis, cytogenetics and immunohistochemistry. Biopsies can indicate the type of cell that is proliferating, its histological grade, genetic abnormalities, and other features of the tumor. Together, this information is useful to evaluate the prognosis of the patient and to choose the best treatment.

As we know, glycosylation of cell surfaces is a critical factor in many biological processes. Cancer is also associated with glycosylation alterations in glycoproteins and glycolipids which play a crucial role in cancer formation and in metastasis (Dube and Bertozzi, 2005; Saldova et al., 2007; Zhao et al., 2007b). At present, the link between glycan structures and disease

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progression, especially cancer, receives particular attention, and glycobiology has also become a new tool for cancer therapeutics and diagnostics (Kreunin et al., 2007; Ueda et al., 2009). Some glycoproteins have been identified as diagnostic markers for many cancers (Reis et al., 2010), and some of the changes in the glycosylation pattern, which have great diagnostic values, are also employed to detect the cancer cells by carbohydrate binding proteins (lectins; Kreunin et al., 2007; Ueda et al., 2009; Zhao et al., 2007a). The carbohydrate-binding specificities of different lectins offer a biological affinity approach (Guo et al., 2012; Szunerits et al., 2010).

Herein, we present a novel lectin-based label free electrochemistry method for direct detection of liver cancer cell. This strategy can detect the cancer cells directly by using lectin probing the glycoproteins or glycopeptides on the surface of the cancer cells, which would give much more accurate reflection of the cancer situation in vivo. It also integrated the advantages of label free from electrochemical impedance spectroscopy (EIS) measurement and the high sensitivity from carbohydrate lectin interaction. Most of the electrochemical-based sensors developed require labeling of the probes or targets. However, the labeling process would make the experiments relatively complex and might affect the bioaffinity between the probes and their targets. EIS, which is based on the change of the charge transfer resistance (R_{ct}) using a redox couple, has received much attention due to its high sensitivity and label-free characteristics. As indicated in Scheme 1, the electrodes were pretreated after which the Con A was immobilized on the electrodes to fabricate the Con A sensor. This sensor was incubated with the cancer cell sample, and the binding of cancer cells with Con A resulted in a change of charge transfer resistance (R_{ct}). EIS measurement was employed to measure the impedance change which reveals the concentration of cancer cells. Bel-7404 cell, a human hepatocellular carcinoma with membrane-associated glycoprotein (gp 43; Fuhrer et al., 1991) which presents high-mannose oligosaccharide chains (Almeida et al., 1996), was used as an example to develop the proposed direct cell detection system. In the absence of the Bel-7404, the cell cannot be recognized and probed by its corresponding lectin, Con A, and gain lower impedance change. Upon adding cells, the impedance change increases with the recognition and probing. The cancer cells in samples estimated from the interaction between the carbohydrate and their corresponding lectin correspond to the measurement of an increase in impedance. The normal human liver cell lines L02, which without membrane-associated glycoprotein of high-mannose structure, was used as a control for this carbohydrate-lectin based detection.

2. Materials and methods

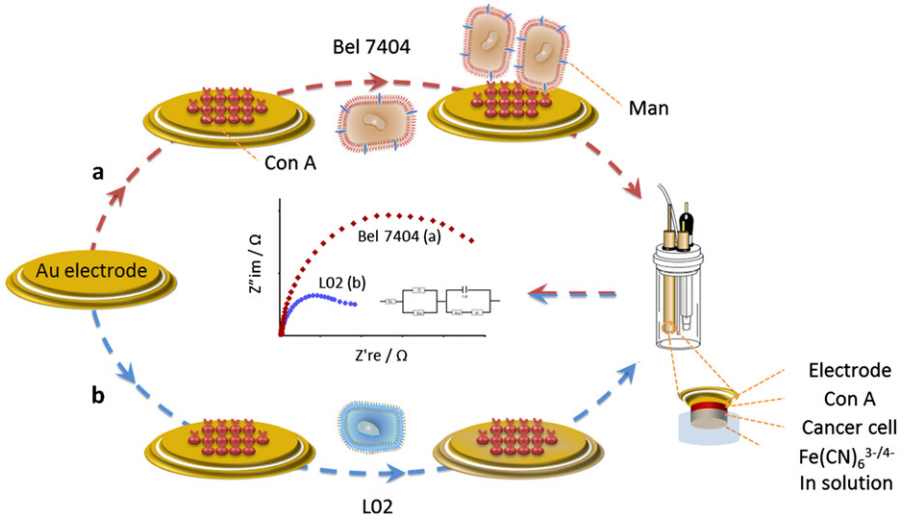
2.1. Chemicals and materials

Gold disk electrodes and alumina powders (0.3 and 0.05 μm) were purchased from CH Instruments (Chenhua Instrument, Shanghai, China). $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ were from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Con A and D-mannose were from Sigma-Aldrich (St. Louis, MO); Bel-7404 and L02 cells were from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All chemical reagents were of analytical grade, used as supplied without further purification unless indicated. All other mentioned chemicals and solvents were purchased from Sigma (St. Louis, MO) and used without further purification unless stated otherwise. Unless otherwise noted, all solutions were prepared with ultrapure water (Milli-Q water, 18.2 MΩ cm) from a Millipore Milli-Q system (Bedford, MA, USA).

Electrochemical characterisations, including cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) measurements, were carried out on a CHI 660D electrochemical workstation (Chenhua Instrument Company, Shanghai, China) with a conventional three-electrode cell. The three-electrode electrochemical cell consisted of a bare gold electrode (2 mm in diameter) as working, platinum wire as auxiliary, and a saturated calomel electrode (SCE) as reference electrode (saturated with 3.0 M KCl). All electrochemical measurements were carried out in the laboratory at ambient room temperature (RT, 22–25 °C).

2.2. Pretreatment of electrode

Here the electrodes used for impedance spectroscopy measurements were designed to incorporate a standard three electrode system comprising a saturated calomel electrode as reference electrode, a gold working electrode, and a platinum counter electrode. Briefly, the gold electrode (2 mm in diameter, CH Instruments Inc.) was first polished sequentially with 0.3 and 0.05 μm alumina slurry for 5 min, followed by ultrasonic cleaning in ethanol and Milli-Q water for 5 min. Then, the electrode was washed thoroughly with Milli-Q water and dried in a nitrogen stream to obtain a clean gold surface. The cleanness of the



Scheme 1. Schematic illustration of label-free EIS strategy employed cancer cell direct detection based on the interaction between carbohydrates and lectin.

electrode was checked by performing CV measurement in a fresh 0.5 M H_2SO_4 solution with the following parameters: potential range from −0.3 to 1.5 V vs. Hg/Hg_2Cl_2 and scan rate of 0.1 V/s. A typical single sharp reduction peak located at ~0.88 V and multiple overlapping oxidation peaks in the range of 1.1–1.5 V could be clearly observed. After that, the electrodes were washed thoroughly with Milli-Q water and dried in a nitrogen stream to obtain a clean gold surface. Then the electrodes were ready for Con A attachment.

2.3. Electrochemical Con A sensor fabrication

The immobilization of Con A on gold electrode followed the optimized conditions of Jantra et al. Briefly, Con A was first dissolved in Tris–HCl (10 mM Tris–HCl with 1 mM Mn^{2+} and 1 mM Ca^{2+}, pH 7.0) at the final concentration of 100 nM, and then activated at 4 °C for 6 h. The pretreated electrodes were first incubated with 10 μL activated Con A at RT for 2 h. After that the gold electrodes were washed thoroughly with Milli-Q water and dried in a nitrogen stream. The cleanness of the electrode was checked again by performing CV measurement with the following parameters: potential range from −0.2 to 0.6 V and scan rate of 0.1 V/s, and EIS measurement with the following parameters: initial potential was 0.175 V with a 5 mV amplitude. The electrodes were then ready for the electrochemical detection experiments.

2.4. Bel-7404 and L02 cell culture

The Bel-7404 cell culture conditions followed the reported protocols (Zhang et al., 2002). Briefly, Bel-7404 was maintained in RPMI 1640 media (Gibco, Rockville, MD, USA) supplemented with 10% calf serum, 100 kU/L of penicillin and 100 mg/L of streptomycin, at 37 °C in a humidified 5% CO_2-in-air atmosphere. The L02 cell culture conditions followed the reported protocols (Wang et al., 2008). Briefly, L02 cells were cultured at 37 °C, in a humidified 5% CO_2-in-air atmosphere in RPMI 1640 medium containing 10% fetal calf serum, 1% penicillin (100 IU/mL), and 1% streptomycin (100 μg/mL).

2.5. Electrochemical detection of Bel-7404

After culture, the cells were collected by spin at 2000 rpm for 10 min from the culture medium and washed by phosphate buffer saline buffer (PBS, 100 mM, pH 7.3); after that, the cell numbers were calculated and the cell concentration was adjusted to 1,000,000 cells/mL. Bel-7404 cells were then diluted in PBS to 4 different cell concentrations, which were 100,000 cells/mL, 50,000 cells/mL, 10,000 cells/mL and 1000 cells/mL. L02 cells, the normal liver cells used as a control, were also prepared in PBS at 1,000,000 cells/mL, 100,000 cells/mL and 10,000 cells/mL.

For the Bel-7404 cell detection, a 10 μL droplet of the prepared Bel-7404 cells was placed on the Con A attached electrodes and left at RT for 1 h. After that, the gold electrodes were washed thoroughly with Milli-Q water and dried in a nitrogen stream and then tested using CV and EIS measurement. CV measurement was performed at a scan rate of 0.1 V/s between −0.2 V and 0.6 V. For EIS measurement, the initial potential was 0.175 V with a 5 mV amplitude. For the control experiments, the gold electrodes were incubated with L02 cells (1,000,000 cells/mL) at RT for 1 h, then washed thoroughly with Milli-Q water, dried in a nitrogen stream and then tested using CV and EIS measurement. Unless otherwise noted, each electrochemical measurement was repeated using at least three electrodes under the same conditions and all electrochemical measurements were carried out at laboratory ambient room temperature (RT, ~25 °C).

3. Results and discussion

3.1. Fabrication and characterization of Con A sensor

CV and EIS were used to monitor the process of the Con A sensor fabrication. Con A was first immobilized on the electrode as probe. The immobilization of Con A on gold electrode was referred to the procedure reported in the literature with some modifications. The formation of the Con A layer was confirmed by CVs of 2.5 mM $K_3Fe(CN)_6$ and 2.5 mM $K_4Fe(CN)_6$ at 0.1 V/s with a potential range from −0.2 to 0.6 V, and EIS at an initial potential of 0.175 V with a 5 mV amplitude. As shown in Fig. 1a, the CV behavior of $Fe(CN)_6^{3-/4-}$ in buffers was sensitive to the concentration of the Con A immobilized. Charge (Q) on CV measurements decreased gradually along with the increase of the concentration of Con A. Even the peak of the curve became inconspicuous while we added the solution with a high concentration. It indicated that the exchange of charge between solution and the surface of electrode had been hindered by the immobilization of lectin. The CV signal decreased with the Con A concentration increase from 1 nM to 1 μM; for bare gold electrode, the peak-to-peak separation ΔE_p was 149 mV. The immobilization of Con A on the surface of gold electrode led to a significant increase in the peak-to-peak separation ΔE_p to 343 mV (100 nM Con A). When the Con A concentration was higher than 1 μM, the CV response could hardly be observed; therefore, we chose 100 nM as the optimized immobilization concentration to probe the cancer cells. Fig. 1b shows the EIS Nyquist plots; from Fig. 1b, the change in semicircle diameter was the result from the change in the interfacial resistance R_{ct} to electron transfer from ferricyanide in solution to their modified electrode. At a bare electrode, the electron transfer process was very fast and the

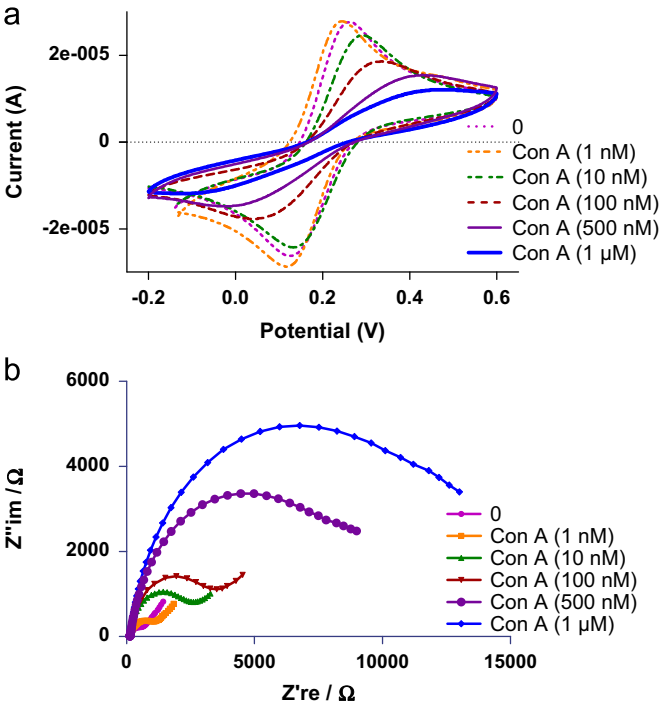


Fig. 1. Fabrication and characterization of Con A modified gold electrode. a) Cyclic voltammograms of Con A modified electrodes based on different Con A concentrations. b) Nyquist plots of impedance spectra of Con A modified gold electrode with different Con A concentrations. Z″ is imaginary and Z′ is real part of impedance. The CV spectra were recorded from −0.2 V to 0.6 V at the rate of 0.1 V/s. The impedance spectra were recorded from 100 kHz to 1 Hz, and the amplitude was 5 mV vs Hg/Hg_2Cl_2 using 10 mM PBS buffer (pH 8.0), containing 100 mM NaCl, 2.5 mM $K_3Fe(CN)_6$ and 2.5 mM $K_4Fe(CN)_6$.

electrochemical response was a nearly straight line (Fig. 1b). After Con A was immobilized on the gold electrode surface, it effectively blocked the electron transfer process and thus led to an enhanced electron-transfer resistance, which also demonstrated that Con A had been successfully immobilized on the electrode. The R_{ct} markedly increased with an increase of the concentration of the immobilized Con A. This result extracted from EIS measurements (Fig. 1b) is in good agreement with the results obtained from CV (Fig. 1a).

3.2. Mannose binding assay

To demonstrate the specific binding of the Con A conjugate to cell surface mannose, a mannose binding experiment was first performed to demonstrate the specific binding of the Con A conjugated to cell surface mannose moieties. CV and EIS were also employed for the binding assay investigation. As shown in Fig. 2a, when the Con A sensor combined with 100 nM mannose, the peak-to-peak separation further increased (ΔE_p=421 mV) compared with that in the bare gold electrode (ΔE_p=149 mV) and that in the absence of Con A (ΔE_p=343 mV). This indicated that the immobilized Con A could respond to mannose. As shown in Fig. 2b, after mannose was incubated with the immobilized Con A onto the surface of gold electrode, the R_{ct} markedly increased. This was most likely attributed to the fact that the interaction of the Con A with mannose occurs, leading to an increase of R_{ct}. Different from the change of R_{ct} value, electronic capacitor (C_d) value of model equivalent circuit had its own trend of transformation. The C_d value went down while we dropped the solution of Con A and mannose on electrode surface step by step, which showed that Con A and mannose had been bound with each other successfully.

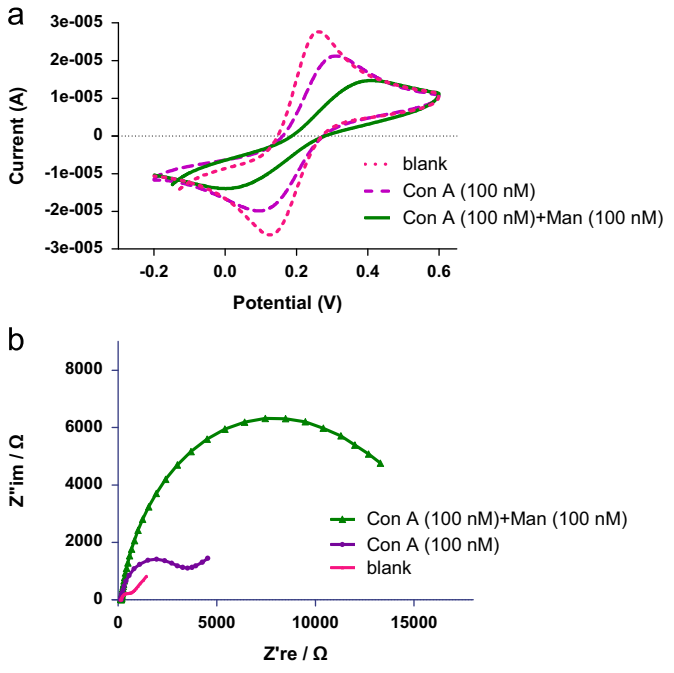


Fig. 2. Cyclic voltammograms (a) and Nyquist plots of impedance spectra (b) of bare gold electrode (pink), 100 nM Con A modified gold electrode (purple , dashed line) and Con A modified gold electrode interacted with 100 nM mannose (Green, solid line). The CV spectra were recorded from −0.2 V to 0.6 V at the rate of 0.1 V/s. The impedance spectra were recorded from 100 kHz to 1 Hz, and the amplitude was 5 mV vs Hg/Hg_2Cl_2 using 10 mM PBS buffer (pH 8.0), containing 100 mM NaCl, 2.5 mM $K_3Fe(CN)_6$ and 2.5 mM $K_4Fe(CN)_6$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Bel-7404 detection sensitivity

The structures of glycans, which decorate all eukaryotic cell surfaces, change with the onset of cancer. In order to develop the label free cancer cells direct detection system, a type of liver cancer cell, Bel-7404, with mannan located outside the cell membrane, provided the binding site for Con A which could probe the cell by anchoring the mannan. From Fig. 3, the change of the electron transfer resistance can be seen markedly after the Con A sensor was incubated with Bel-7404 cells for 1 h and subsequently washed with PBS, while no significant resistance was observed after the Con A sensor was incubated with L02, the normal liver cells, which were used as control under same conditions.

Different concentrations of Bel-7404 cells were employed to react with the Con A sensor for the establishment of calibration curve by plotting the relative change of the R_{ct} against the cell concentration (Fig. 4). The detection limit for Bel-7404 cancer cell was 234 cells/mL based on the usual 3σ definition as 3 times the standard deviation of the blank sample. The factors that contributed significantly to the sensitive Bel-7404 cancer cell detection were the high sensitivity recognition and binding between Con A and mannan and the EIS-based cell direct detection.

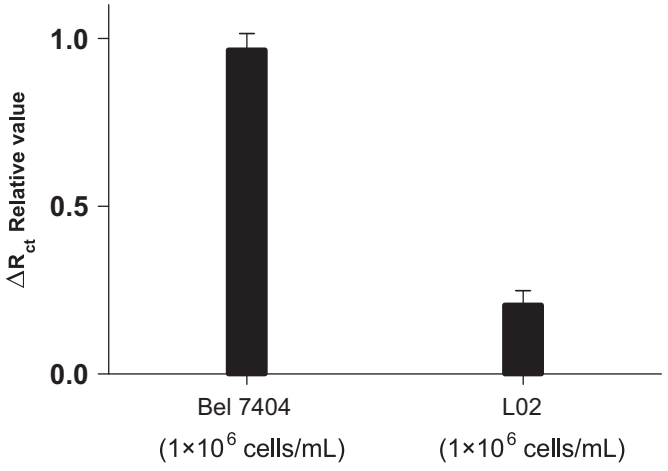


Fig. 3. Change of charge transfer resistance (R_{ct}) comparison with human hepatocellular carcinoma cell Bel-7404 and human normal liver cell L02 presented separately.

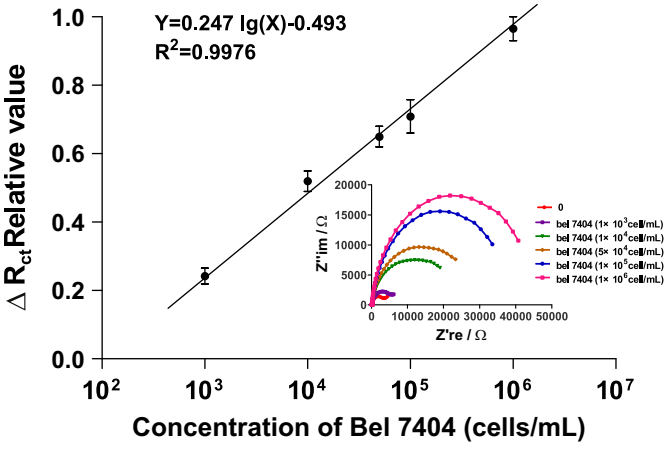


Fig. 4. Human hepatocellular carcinoma cell Bel-7404 EIS detection calibration curve plotting the relative change of R_{ct} against the cell concentration. The inset shows the Nyquist plots of impedance spectra of 100 nM Con A modified gold electrode interacted with different concentrations of Bel-7404 cell measured.

Table 1
Recoveries and reproducibility (indicated by coefficients of variation, CV) of the spiked samples detection by the proposed label free Con A sensor ($n=5$).

Spiked cells/mL	Detected cells/mL	Recoveries (%)	Spiked cells/mL	Detected cells/mL	Recoveries (%)
80,000	79965	99.96	30000	30769	102.56
80,000	80248	100.31	30000	29069	96.90
80,000	82226	102.78	30000	32657	108.86
80,000	77648	97.06	30000	24379	81.26
80,000	80437	100.55	30000	28833	96.11
	AVE (%)	100.13		AVE (%)	97.14
	CV (%)	2.04		CV (%)	10.55

3.4. Bel-7404 sample testing

Ten spiked samples at two different concentrations of 30,000 and 80,000 cells/mL were tested by the Con A sensor, and the results can be seen in Table 1. The recoveries were determined by testing those cell samples at different concentrations. As shown in Table 1, this method revealed good average recovery rates from 97.14% to 100.13% and coefficients of variance (CV) from 2.04% to 10.55%. These values are in accordance with the required accuracy in trace analysis.

4. Conclusions

Cancer formation and metastasis is always associated with glycosylation alterations in glycoproteins and glycolipids. According to this property, we have described a label-free electrochemical impedance spectroscopy biosensor for cancer cell direct detection based on the interaction between carbohydrates and proteins. This method has several significant advantages toward the improvement of sensitivity and selectivity detection of cancer cells. First, our sensor requires no label on the probe or target. EIS measurement was employed to identify the cancer cells present or absent. Second, this assay is more straightforward; compared with common tumor markers based diagnosis, this method directly targets the cancer cell, which is more direct and accurate. Third, this method exploits the advantages of high specificity and sensitivity of carbohydrate and protein interactions. In summary, this proposed straightforward method combines label free detection by EIS method with the high sensitivity and selectivity of carbohydrate and protein interactions with the limit of detection (LOD) of 234 cells/mL and the recovery range from 97.14% to 100.13%. Comparing with the earlier reports based on cell biosensors using different electrochemical methods (Andreescu and Sadik, 2005; Maltez-da Costa et al., 2012; Osibote et al. 2011) this method is sensitive enough to guarantee the application of the method for cancer cells direct detection. Moreover, this method could be extended to carry out multi-component diagnosis applications, thus providing enormous potential for applications of cancer monitoring and therapy.

Acknowledgments

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