Real-Time Evaluation of Live Cancer Cells by an in Situ Surface Plasmon Resonance and Electrochemical Study

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ABSTRACT: This work presents a new strategy of the combination of surface plasmon resonance (SPR) and electrochemical study for real-time evaluation of live cancer cells treated with daunorubicin (DNR) at the interface of the SPR chip and living cancer cells. The observations demonstrate that the SPR signal changes could be closely related to the morphology and mass changes of adsorbed cancer cells and the variation of the refractive index of the medium solution. The results of light microscopy images and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide studies also illustrate the release or desorption of HepG2 cancer cells, which were due to their apoptosis after treatment with DNR. It is evident that the extracellular concentration of DNR residue can be readily determined through electrochemical measurements. The decreases in the magnitudes of SPR signals were linearly related to cell survival rates, and the combination of SPR with electrochemical study could be utilized to evaluate the potential therapeutic efficiency of bioactive agents to cells. Thus, this label-free, real-time SPR—electrochemical detection technique has great promise in bioanalysis or monitoring of relevant treatment processes in clinical applications.

KEYWORDS: surface plasmon resonance, electrochemistry, real-time evaluation, live cells, cancer

INTRODUCTION

Investigation of cells allows us to better understand cell proliferation, function, and death and thereby to improve the diagnosis and treatment of diseases, especially cancer. Dozens of classic and modern techniques have been applied in evaluation of cells, such as 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric methods, chemical labeling imaging, etc.1–3 The MTT assay and related colorimetric methods are usually used to evaluate the survival rate of cells. However, the colorimetric methods are time-consuming (usually more than 48 h) and cannot be used in a real-time monitoring process.4,5 Fluorescence imaging techniques, which have also been widely used in cell analysis, are strongly reliant on the fluorescent probes.6–8

The surface plasmon resonance (SPR) technique is designed to detect the changes in the refractive index near the surface.7 It is well-known that SPR is a label-free, real-time, sensitive, and fast method for the investigation of the kinetics and affinity constant for molecular interactions,10–13 conformational changes of macromolecules,14,15 etc. Additionally, SPR has been applied in analysis and diagnosis of living cells and clinical samples.16–19 Ona and co-workers20 applied SPR sensors to study the short-term cytotoxicity of anticancer drugs in live cancer cells cultured on a sensor. However, it is not a real-time process because of the subsequent removal of the drugs before SPR measurements.

Electrochemical techniques are widely used in biomedical applications, such as detection of bioactive molecules21,22 and determination of interactions between bioactive agents and biological supramolecules.23–25 Jiang and co-workers26 proposed a highly sensitive electrochemical method for analyzing daunorubicin (DNR) with a low detection limit of 10 nM and a wide linear range of 10–500 nM. Moreover, analysis of cell features27–30 and evaluation of the therapeutic efficiency of drugs31,32 are also possible because of their simple operation process, fast response speed, high sensitivity, and good accuracy. Zhu and co-workers33 developed an electrochemical cytosensing platform for apoptotic cell detection. This platform presented a significant tool for monitoring the early stages of apoptosis. However, the electrochemical methods were hardly used in label-free and real-time analysis of living cells.

Combination of SPR and electrochemical techniques can facilitate the label-free, real-time, and highly sensitive analysis of cells in solution or under physiological conditions.34 SPR can offer the changes in refractive index and other biophysical
properties of cells. Electrochemical methods reflect the redox chemistry of the surroundings of cells. The combined techniques can provide multiparametric information about molecules and cells. The combined technique of electrochemical—surface plasmon resonance (EC—SPR) could have great potential applications in monitoring of the survival rate of cells. Robelek and co-workers introduced a combination of impedance and SPR to monitor living cells simultaneously. Cui and co-workers presented an EC—SPR system to characterize the optical and electrical properties of cells. However, none of these previous studies has attempted to evaluate the treatment efficiency of agents in cells by EC—SPR. Hence, in this study, we aimed to explore the possibility of taking this in situ combination strategy to evaluate live HepG2 cancer cells treated with DNR. The electrochemical technique was applied to monitor in real time the concentration changes of DNR treated with DNR. The electrochemical technique has great potential application in monitoring of relevant clinical treatments.

MATERIALS AND METHODS

Chemicals and Instrumentation. DNR was purchased from Pharmacia Italia, and its stock solution was freshly prepared and stored in the dark at 4 °C. MTT was purchased from Amresco. Other reagents used in this study were of analytical grade.

EC—SPR studies were performed on a model BI-2000 instrument with an EC—SPR analysis module (Biosensing Instrument Inc., Tempe, AZ), and the SPR signal was calibrated in refractive index units (RIU) with the ethanol method. A 670 nm, 1 mW laser was employed as the light source. Their combination is shown in Figure 1.

Cell morphologies and images were observed via inverted fluorescence microscopy (IX51, Olympus, Tokyo, Japan). The cytotoxicity of DNR to HepG2 cells was assessed with the MTT assay and measured by using a microplate ELISA reader (Bio-Rad550, Bio-Rad Laboratories, Hercules, CA).

Cell Culture. HepG2 (human hepatocellular carcinoma) cells were purchased from the Shanghai Institute of Cells of the Chinese Academy of Sciences and cultured in DMEM (high-glucose, Gibco) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO), 100 units/mL penicillin (Sigma), and 100 mg/mL streptomycin (Sigma) at 37 °C with 5% CO2 in a 95% humidified atmosphere.

Preparation of Cell Chips. SPR chips (18 mm × 18 mm, 47 nm Au and 2 nm Cr coated on BK7 glass, Biosensing Instrument Inc.) were tightly placed into the wells of a six-well plate and sterilized using 75% ethanol. Then the six-well plate and SPR chip were rinsed with cell culture medium. The dilution of cells with different concentrations (i.e., 2, 5, 8, and 10 × 10^4 cells/mL) was added to wells of the six-well plate and covered the surface of the SPR chip. The six-well plate was put in an incubator at 37 °C with 5% CO2 in a 95% humidified atmosphere for various amounts of time (2, 4, and 6 h). The adhesion profile of HepG2 cells on the SPR chip was investigated by using an inverted microscope. After incubation, the suspension was removed. Then wells of a six-well plate with the SPR chip were washed three times with culture medium to remove excess floating cells. SPR chips with cells were carefully taken out of wells by using a fine point tweezer, and their backs were carefully washed with ethanol on a cotton swab and dried with a rubber suction bulb.

In Situ EC—SPR Experiments. The dynamic changes in the SPR angle were recorded, and electrochemical signals of DNR residue in medium were collected at the same time. Briefly, the prism surface of the SPR equipment was cleaned by using a cotton applicator dampened with ethanol. A small drop of the refractive index matching liquid was placed near the center of the prism. SPR chips with HepG2 cells were gently positioned onto the center of the prism surface without bubbles being trapped at the sensor—prism interface. The EC—SPR analysis module was slightly released over the SPR chip. Then, 700 μL of culture medium with DNR (0, 1, 5, and 10 μM) was injected into the EC—SPR analysis module with a pipet. The Pt auxiliary electrode and Ag/AgCl reference electrode were placed into the holes of the EC—SPR analysis module, as shown in Figure 1.

The dynamic changes in the SPR angle were recorded, and electrochemical signals of the DNR residue in the medium were also collected by using an electrochemical workstation with a cyclic voltammetry (CV) technique over a certain period of time. The experiment was repeated at least three times. The cells were incubated in the buffered medium at room temperature. The initiative potential was at 0 V, while the high and low potentials were separately at 0.8 and −0.8 V, respectively. The scan rate was performed at 0.1 V s⁻¹. The group without incubation with DNR was taken as control, and data for discussion in this paper have been recorded with the control group.

In Vitro Cytotoxicity Studies by the MTT Assay. The MTT assay was taken to verify the results of SPR experiments. Cells were trypsinized and seeded onto 96-well plates (3 × 10^3 cells/well). After incubation for 4 h at room temperature, cells were rinsed in DMEM and incubated with different concentrations of DNR for 2, 4, and 6 h. A MTT solution was added after treatment and the mixture incubated for an additional 4 h. Dimethyl sulfoxide was added to solubilize the formazan crystal, and the optical density (OD) at 490 nm was recorded.

Cell Morphological Assessment. Before the cells had been added to the SPR instrument, the morphologies of cells adsorbed on the SPR chip were assessed by optical microscopy. After the EC—SPR experiment (i.e., treated with DNR for 6 h), morphologies of cells were immediately investigated by optical microscopy. Cells treated with DNR for 6 h were also examined with an inverted fluorescence microscope to study the uptake of DNR by HepG2 cells. The fluorescent images of cells after treatment with DNR were excited at 488 nm. The images were captured with a CCD (QCapture, QImaging Corp.).

Figure 1. Schematic illustrations of combination of SPR and electrochemical techniques in real-time evaluation of live cancer cells after their treatment with DNR.
RESULTS AND DISCUSSION

Optimization for the Adhesion of Cell Chips. Initially, the morphologies of HepG2 cells that adhered on the SPR chip were observed on an optical microscope (Figure 2). To determine the optimal conditions for preparing cell chips, the cell concentration and incubation time were investigated. As shown in Figure 2, the SPR chip was not fully occupied by HepG2 cells at low cell concentrations. However, there were excess cells suspended over the SPR chip at high cell concentrations. The cell concentration was maintained at $\sim 8 \times 10^5$ cells/mL. When the incubation time was 2 h, there were many HepG2 cells suspended and not adhered to the surface of the SPR chip. After being incubated for 4 h, most cells tightly adhered to the SPR chip. Thus, the cellular adhesion time was set to 4 h for the EC–SPR experiments described here. Consequently, the cell chip was prepared with a cell concentration of $8 \times 10^5$ cells/mL and incubated for 4 h before being positioned on the prism surface.

Real-Time Monitoring of Live HepG2 Cells via EC–SPR. The EC–SPR instrument is schematically shown in Figure 1, and the sensitivity of the instrument was $8.23 \times 10^{-7}$ RIU (see the Supporting Information). SPR signals were collected continuously while cyclic voltammetry (CV) was applied to determine the relevant DNR residue in the medium. HepG2 cells treated with DNR were monitored with in situ EC–SPR at room temperature in culture medium. The SPR

Figure 2. Optical images of cells adhered to the SPR chip with different cell concentrations and incubation times.

Figure 3. (A) Dynamic plots of magnitudes of SPR signals of HepG2 cells on the SPR chip after incubation with DNR (5 $\mu$M) and a control. (B) Partial enlarged view of SPR signals of HepG2 cells on the SPR chip after incubation with DNR (5 $\mu$M). (C) Three-dimensional plots of SPR signals of HepG2 cells on the SPR chip after incubation with 1, 5, and 10 $\mu$M DNR. The DNR was injected at 0 h, i.e., the start of the EC–SPR experiment.
signals of HepG2 cells with or without DNR treatment were dependent on time and concentration (Figure 3). Magnitudes of SPR signals of DNR treatment groups declined, while the magnitude of the SPR signal of the control group increased as shown in Figure 3A. The slow increase in the magnitude of the SPR signal for the control group was related to the growth of cells and attachment of them to the surface of the SPR chip. The SPR signals of DNR treatment groups with 5 μM shifted approximately −107.913, −238.170, and −350.563 mdeg for 2, 4, and 6 h, respectively. The SPR signals of HepG2 cells treated with DNR (1 and 10 μM) are illustrated in Figure S3 of the Supporting Information. It is evident that SPR could be readily used to evaluate live HepG2 cancer cells treated with DNR and thus monitor the relevant treatment efficiency of DNR in HepG2 cells.

Meanwhile, it is observed that SPR signals were also sensitive to changes in mass and refractive index near the interface of the SPR chip (<200 nm), as shown in Figure 3. The initial fluctuations of the SPR signal were due to the addition of DNR to culture medium. The refractive index of DNR is predicted to be 1.692, which is larger than the refractive indices of 1.369 and 1.337 for HepG2 cells and DMEM culture medium, respectively.38,39 Because the relevant HepG2 cells treated with DNR underwent the stage of apoptosis, it is obvious to note that the refractive index near the SPR chip remarkably decreased because of the detachment of apoptotic cells from the SPR chip.40 The sensitivity of the SPR instrument is high; however, a relatively small decline was detected in the magnitude of the SPR signal of HepG2 cells treated with DNR. Previous works have also observed a subtle shift in SPR signals of cells treated with therapeutic agents.36,41 As shown in Figure 1, the EC–SPR module was a closed system, so the apoptosis or dead cells as well as cell fragments remained in the medium. Therefore, the relevant decline of the magnitude of the SPR signal was not as large as that of the classic flow SPR system.

The SPR signal fluctuated during the potential applications (Figure 3A,B). Scan voltages based SPR signals are presented in Figure 4B and Figure S5, but there was no significant influence on the trend of SPR signals after the application of electrochemical techniques.35 When the potential became negative, the magnitude of the SPR signal decreased. Meanwhile, a positive potential introduced an increased magnitude of the SPR signal. Compared to the CV curve, the cyclic SPR signal was not closed because of the decreased magnitude of the SPR signals. These results are in agreement with previous reports in the literature.36 When potentials were applied to the SPR chip, the electron densities and electroactive species adsorbed on the SPR chip surface varied. The positive potential led to the increase in surface electron intensities and the thickness of the oxidation layer (gold and electroactive species); hence, the magnitudes of the SPR signals increased.36,62

Because cells did not entirely capture the surface of the SPR chip, as shown in Figure 2, the DNR molecule can readily diffuse to the electrode surface through the cell layer. Compared with that of DNR alone, the relevant peaks at approximately −0.6 V of DNR residues outside the cells were found to shift slightly in the positive direction after incubation with HepG2 cells, which did not appear on the cyclic voltammogram of HepG2 cells in medium without DNR (Figure S2). The results of the CV study of DNR residues outside HepG2 cells are shown in Figure 4A and Figure S2. Only one reduction peak, 1c, was recorded immediately after the addition of DNR to culture medium. It is noted that two oxidation peaks, namely, 1a and 2a, appeared after the second scan, and the current of peak 1a was increased, which could be attributed to the reduced products of DNR adsorbed on the SPR chip.

When incubated with cells, DNR molecules should be internalized and consumed by HepG2 cells, and the concentration of DNR in the medium decreased, which readily led to a decrease of the current of DNR. The current of peak 1c was found to decrease after the first scan and further decrease to 50% at 1 h for the group treated with 10 μM DNR, as shown in Figure 4A. After a relatively long incubation time, the cells were killed with DNR and detached from the surface of the SPR chip. DNR molecules, which were internalized into cells, were released into the culture medium. At the same time, the surface of the SPR chip was free after the detachment of apoptotic cells, which increase the electrochemically effective area. Thus, the relevant peak current changes with the change in DNR concentration as well as incubation time, which can be further utilized to evaluate the DNR residues in the related interface outside HepG2 cells.

**Quantitative Analysis of the in Vitro Cytotoxicity by SPR.** The MTT assay of *in vitro* cytotoxicity of DNR to HepG2 cells was taken to confirm the EC–SPR results, as shown in...
Figure 5A and Figure S6. The survival rate of HepG2 cells was >92% over 2 h and decreased to 80% over 4 h after 1 μM DNR treatment. The relative time- and concentration-dependent changes in SPR signals are shown in Figure 5A and Figure S6, respectively. It is evident that the SPR signals were significantly related to the survival rates of HepG2 cells in cultures with different DNR concentrations. As shown in Figure 5B, the decreases in the magnitudes of SPR signals were linearly fitted to cell survival rates, which were expressed via the equation survival rate (%) = 0.0721Δangle (mdeg) + 97.2278, and the adjusted R² value was 0.9791.

Our observations illustrate that the change in the SPR signals pertained to the apoptosis of cells and detachment of them from the interface of the SPR chip. The dynamic SPR signals can be attributed to the living cells, which still adsorbed on the surface of the SPR chip. Herein, the decrease in the magnitudes of the SPR signals in 100 mdeg means that cells in 7.21% had been apoptotic and detached from the SPR chip. These results demonstrate that SPR can be effectively applied in the real-time quantitative evaluation of HepG2 cells.

Moreover, cell morphologies were checked by optical microscopy (Figure 6). Before the EC–SPR experiments, the untreated cells were spindle- or polygon-shaped and tightly adhered to the SPR chip. The SPR chips were almost fully covered with cells. However, in comparison with the control group (as shown in Figure S7), the DNR-treated cells were turned to rundle and detached from the SPR chip after EC–SPR studies. It is obvious that the surface of the SPR chip was still occupied with cells for the control group, while the surface was partly exposed in the treatment group. As the increment of DNR dosage, the coverage of the SPR chip with cells was found to decrease considerably. Cells treated with DNR were also examined by inverted fluorescence microscopy to study DNR uptake and showed obvious fluorescence.

These results also corroborated the SPR studies. The cells after treatment were apoptotic and detached from the SPR chip; therefore, the magnitudes of the SPR signals decreased after 4 h. Cells treated with higher concentrations of DNR were less viable and more detached than those with lower concentrations. Therefore, the magnitudes of SPR signals of...
groups with higher DNR concentrations declined more rapidly than those with relatively lower concentrations.

**CONCLUSIONS**

In this study, we have taken the strategy of the EC–SPR study to evaluate live cancer cells treated with DNR on the interface of SPR chips. Signal changes recorded form the SPR resulted from the morphology and mass changes of adsorbed HepG2 cells and refractive index variation of the medium solution. The DNR residue concentration outside the cells in culture medium can be determined by an electrochemical method. The results of light microscopy images and MTT tests also demonstrated the release or desorption of relevant cells, which was caused by the apoptosis of cancer cells after treatment with DNR. The decreases in the magnitudes of SPR signals were linearly related to the release or desorption of relevant cells, which was caused by the morphology and mass changes of adsorbed HepG2 cells and refractive index variation of the medium solution. The DNR residue concentration outside the cells in culture medium can be determined by an electrochemical method. The results of light microscopy images and MTT tests also demonstrated the release or desorption of relevant cells, which was caused by the apoptosis of cancer cells after treatment with DNR. The decreases in the magnitudes of SPR signals were linearly related to

**REFERENCES**


**ASSOCIATED CONTENT**

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b08066.

Calculated values for the refractive index of ethanol solutions, SPR signals of DNR-treated HepG2 cells with and without electrochemical samplings, scan voltage-based SPR signals, and an in vitro cytotoxicity study using the MTT assay (PDF)

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***Notes***

The authors declare no competing financial interest.

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**ABBREVIATIONS**

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylessimiazolium bromide; SPR, surface plasmon resonance; EC, electrochemistry; CV, cyclic voltammetry; DNR, daunorubicin


