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# Cell chip with a thiolated chitosan self-assembled monolayer to detect the effects of anticancer drugs on breast normal and cancer cells

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ABSTRACT

Cell-based chips are an effective *in vitro* analysis tool; however, the sensitivity of the cell chip to biomaterials is high, which is crucial for immobilizing cells on the electrode surface without conductivity. In this study, we report on a cell chip with a thiolated chitosan monolayer that was easy to fabricate, highly adhesive to cells, and enhanced electrochemical signals. Thiolated chitosan containing thiol groups was synthesized and self-assembled on a gold electrode to immobilize cells, and showed superior electrochemical performance to that of poly-L-lysine and collagen. Cyclic voltammetry (CV) was performed to distinguish the redox characteristics of normal (HMEC) and breast cancer cells (MCF-7); then, two anticancer drugs (doxorubicin and cyclophosphamide) were added to the cell cultures to analyze their effects on the redox environment of normal and cancer cells derived from the same origin. As a result, the CV cathode peaks decreased differently with respect to the cell line (normal and cancer) and anticancer drug, which was validated by a conventional MTT viability assay. Hence, the proposed cell chip with a thiolated chitosan modified layer could be used in various fields, including discriminating normal from cancer cells, to evaluating the efficiency of newly developed drugs, and to assessing cytotoxicity of various chemicals.



## 1. Introduction

A variety of *in vitro* tools have been widely used in fields such as toxicity analysis of different chemicals [1] and new pharmaceuticals [2] and detection of pathogen infections [3], and safety assessments of environmental pollutants [4]. In particular, cell chips based on electrical or electrochemical techniques have come into the spotlight as a new *in vitro* tool.

Electrochemical impedance spectroscopy (EIS), electric cell–substrate impedance sensing (ECIS), cyclic voltammetry (CV) [2,5–10] and differential pulse voltammetry (DPV) [11,12] are the most widely used electrochemical techniques in cell-based chip studies. These electrochemical measurement methods are used to estimate the effect of anticancer drugs in various fields. Materials that are most important for increasing the electrochemical signals of cells are needed to measure the electrochemical signals of cells using these methods. The strong attachment of cells on the electrode has been revealed as a key factor that may lead to successful cell growth. Hence, various materials have been utilized to construct a cell-friendly environment [13,14] and extracellular matrix (ECM) proteins [15] or components such as elastin, fibronectin [16], and collagen [17], are most often used. These ECM proteins are effective for attaching cells but they are quite expensive and they must be able to decrease cell chip sensitivity by increasing resistance of the interface between the electrode and cell membrane. We reported previously that a variety of cell chip studies have been conducted using cytophilic peptide binding cysteine. A modified RGD peptide terminated with cysteine residues makes self-assembly on a gold electrode possible [5,18–20]. However, such synthetic peptides have disadvantages in that they are difficult and time consuming to synthesize and are expensive. Hence, in this study, a thiolated chitosan modified cell chip was fabricated *via* a self-assembly technique. Thiolated chitosan is the most proper biomaterial for developing a cell-friendly environment and improving electrochemical sensitivity of a cell-based chip.

Thiolated chitosan, a monomer of glucose, is not only an essential material for cell metabolism but also an effective material for immobilizing and growing cells [21,22]. Based on these characteristics, we synthesized thiolated chitosan, self-assembled it on a gold electrode, and immobilized human cancer cells. Hence, the efficiency of the electrochemical sensitivity between cell chips

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using thiolated chitosan and ECM proteins was compared. After the electrochemical properties of normal human breast cells were compared, two typical anticancer drugs, cyclophosphamide and doxorubicin, were added to the cell chips to estimate the effectiveness of the drugs on normal and cancer cells. Then, we compared results using CV of the cell chips with thiolated chitosan and by the MTT viability assay [23,24].

## 2. Experimental details

### 2.1. Materials

Doxorubicin, cyclophosphamide, poly-L-lysine (PLL), collagen, phosphate buffered saline (PBS), *N*-hydroxysuccinimide (NHS), 3-mercaptopropionic acid, chitosan, and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimidehydrochloride (EDC) were purchased from Sigma-Aldrich (St. Louis, USA). Thiolated chitosan was synthesized or purchased from Carbomer (USA). RPMI, fetal bovine serum (FBS), penicillin/streptomycin, and trypsin were obtained from Welgene (Korea). Deionized (DI) water was obtained from a Millipore water system and was used throughout the experiments. All other chemicals used were obtained commercially as reagent grade.

### 2.2. Cell Culture

Breast cancer and normal breast cells collected from a human breast were purchased from the Korean cell line bank. The cell lines were cultured in RPMI supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were incubated under standard cell culture conditions at 37 in an atmosphere of 5% $CO_2$. The medium was changed every 2 days.

### 2.3. Synthesis of thiolated chitosan by EDC-NHS coupling

We used EDC-NHS coupling mechanism to synthesize thiolated chitosan. One ml of 0.2 M EDC solution and 2 ml of 0.2 M NHS solution were mixed and 4.5 μl of 3-mercaptopropionic acid was added to the solution. Then, this solution was well-mixed, and 10 mg of thiolated chitosan was dissolved in 10 ml of PBS buffer (10 mM, pH 7.4) including 100 μl of hydrogen chloride (HCl). The EDC-NHS solution and chitosan solution were mixed and reacted for 6 h at room temperature.

### 2.4. Fabrication of a substrate with cytophilic materials on a gold electrode

The gold electrode was cleaned with piranha solution at 70 for 3 min before fabricating the cytophilic material layer. Then, the gold electrode was washed with 100% ethanol and DI water. A chamber was attached to the gold surface using polydimethylsiloxane, and the gold electrode was washed in PBS. The thiolated chitosan layer was immobilized by adding 1.0 mg/ml thiolated chitosan solution onto the gold surface at 25 for about 2 h *via* a self-assembly technique. In such a way, PLL was immobilized by adding 0.01% solution, and collagen was immobilized by adding 0.1 mg/ml solution. Substrate topology was investigated by field emission scanning electron microscope (FE-SEM, JSM-6700F, JEOL, Japan) in semi-contact mode at room temperature under air conditions.

### 2.5. Electrochemical detection of the thiolated chitosan modified cell chip by CV

CV was performed using a potentiostat (CHI-660, CH Instruments, USA). The three-electrode system was composed of a cell-based chip as the working electrode, Ag/AgCl (RE-1B Ag/Ag/Cl reference electrode, ALS, Japan) as a reference electrode, and a platinum wire (Platinum Wire Counter Electrode, CH Instruments, USA) as a counter electrode. PBS buffer was used as the electrolyte for the CV measurements. The CV scan rate was 50 mV $s^{-1}$.

### 2.6. MTT assay

About $2 \times 10^4$ cells were seeded in a 96-well plate to validate the results of electrochemical measurements by determining viability of cells treated with anticancer drugs using the MTT (3-(4, 5-fimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, Sigma) assay. After 1 day, the media in the 96-well plate were replaced with new media including the anticancer drugs and incubated for 24 h. A 20 μl aliquot of MTT solution was added. After 2 h incubation at 37 and 5% $CO_2$, media were removed, and 100 μl DMSO was added to each well. Finally, the 96-well plate was shaken on a shaking machine for 5–10 min. Cell viability was measured at an absorbance of 540 nm using a Benchmark microplate reader (Bio-Rad, Mississauga, ON, Canada).

## 3. Results and discussion

### 3.1. Topological analysis of the thiolated chitosan substrate

Thiolated chitosan was synthesized using an EDC-NHS coupling mechanism to fabricate the thiolated chitosan film on a gold surface by attaching a thiol group to a gold electrode. Synthesized thiolated chitosan solution was added to the gold electrode with a chamber and immobilized on the surface. Fig. 1 shows the general outline of the EDC-NHS coupling mechanism (Fig. 1(a)) and a schematic design of the thiolated chitosan modified cell chip (Fig. 1(b)). Field emission scanning electron microscope (FE-SEM) was used to confirm immobilization of the thiolated chitosan self-assembled monolayer on the gold surface and to compare it with that of the bare gold surface. Synthesized thiolated chitosan increased cell attachment, proliferation, and viability. As shown in Fig. 2, the FE-SEM images showed significant differences between thiolated chitosan and the bare gold surface. Fig. 2(a) shows the topology of the bare gold surface and depicts small clusters of gold nanoparticles. The degree of surface roughness was slight, whereas Fig. 2(b) shows the topography of the thiolated chitosan layer, which was different from that of Fig. 2(a). As thiolated chitosan is a polymer, much larger clusters were formed, and the degree of surface roughness was more substantial, which helped increase cell growth and viability because cell adhesion was improved with increased surface roughness. These results were obtained fur to the interactions between the gold and the cell surface and demonstrated the advantage of thiolated chitosan immobilized on a gold surface compared to that of a bare gold surface. By helping to provide rapid electron transfer kinetics, the thiolated chitosan modified substrate provided a more stable and stronger redox signal on CV than that of a bare gold substrate.

### 3.2. Comparison of the thiolated chitosan substrate with substrates coated with different materials

PLL and collagen are useful biomaterials for cell attachment, proliferation, and spreading through interactions between various cellular receptors and biomaterials. Therefore, thiolated chitosan was synthesized as a new cell-friendly biomaterial to interact with cells. To demonstrate the utility of the thiolated chitosan modified cell chip among various biomaterial modified cell chips, we compared the effect of thiolated chitosan with that of existing biomaterials. The redox behavior of breast cells when using a variety of cytophilic materials was investigated by

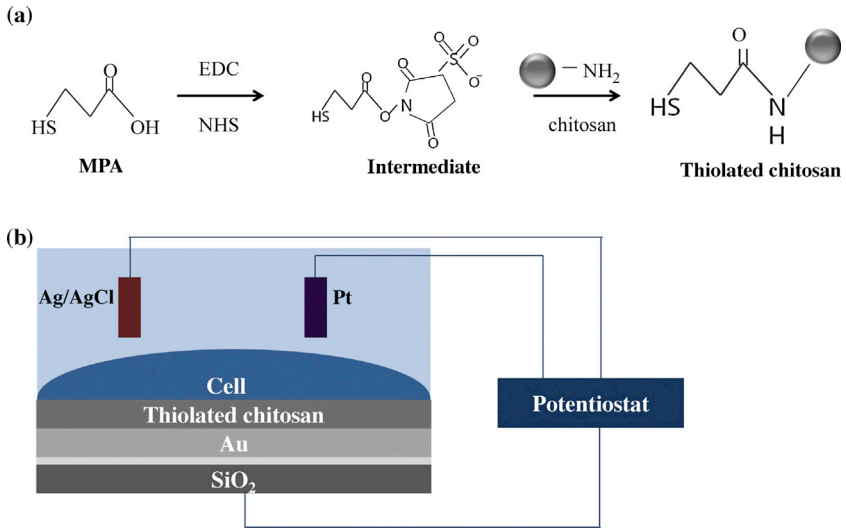


**Fig. 1.** Schematic outline of the experimental setup showing the synthesis process of thiolated chitosan using the EDC-NHS coupling mechanism. (a) Schematic diagram of cell chip with thiolated chitosan. (b) Measurement of redox properties at the cell-substrate interface.

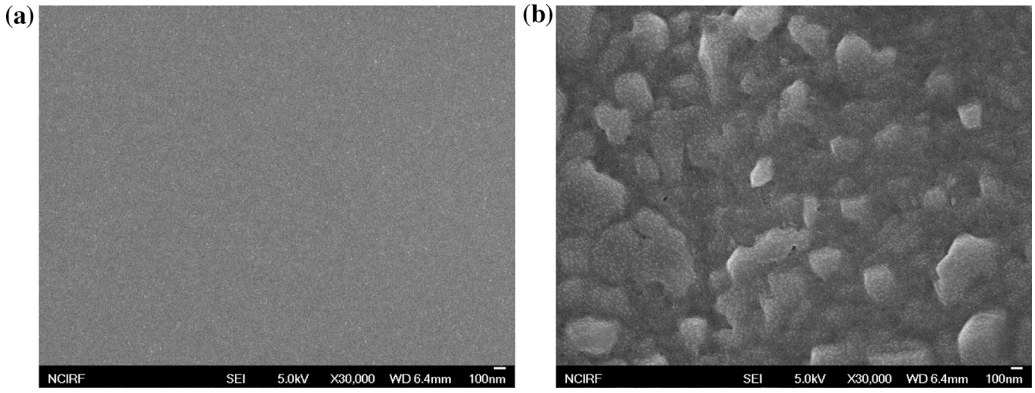


**Fig. 2.** (a) Field emission scanning electron microscope (FE-SEM) images of bare gold. (b) Modified thiolated chitosan on gold.

CV. As shown in Fig. 3, redox peaks were achieved for the various cell-friendly biomaterials, which helped to increase cell immobilization and improve cell viability. We confirmed the characteristic cathode peak currents of each biomaterial in Fig. 3(a) and then seeded breast cancer cells on gold substrates including PLL, collagen, thiolated chitosan, and a bare gold substrate. The effect of the cytophilic materials on the electrochemical signals, which was one of the ways to detect states of cells, was studied by CV in Fig. 3(b). As shown in Fig. 3(c), the thiolated chitosan modified cell chip showed significantly the highest cathode peak current ($I_{pc}$) when it was compared with collagen and PLL. To prove the difference of modified electrodes performance, $p$ value ($p < 0.05$) was calculated through significance test ($T$-test). An asterisk (*) means that a $p$ value was occurred as less than 0.05 between each modified electrode. CV was measured using PBS buffer as electrolyte at scan rate was 50 mV $s^{-1}$ and using Ag/AgCl and Pt as

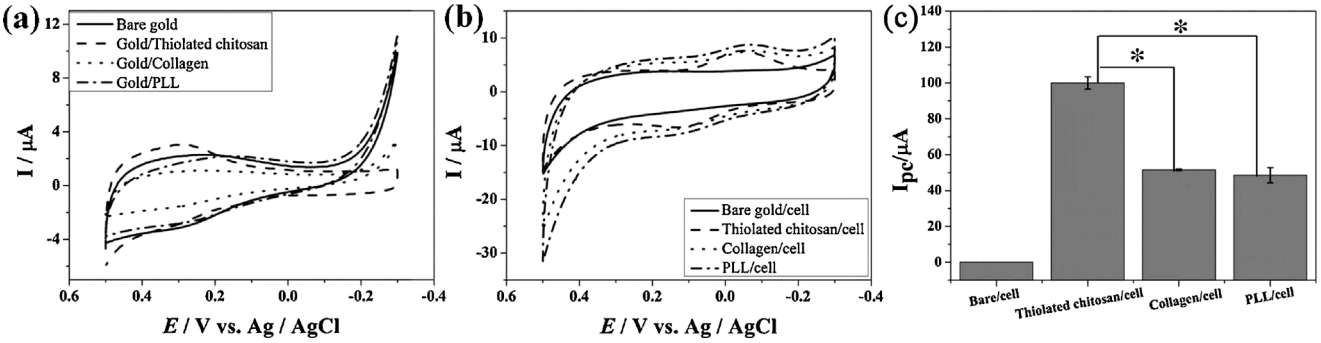


**Fig. 3.** Comparison of substrates coated with different materials: (a) Cyclic voltammograms to compare electrochemical signals using modified gold electrode without cells (— Bare gold, – – Gold/Thiolated chitosan, • • • Gold/Collagen, –• Gold/PLL). (b) Cyclic voltammograms for comparing electrochemical signals using modified gold electrode with MCF-7 (— Bare gold/cell, – – Gold/Thiolated chitosan/cell, • • • Gold/Collagen/cell, –• Gold/PLL/cell). (c) comparison of reduction peak current ($I_{pc}$) from MCF-7 on modified gold electrode.

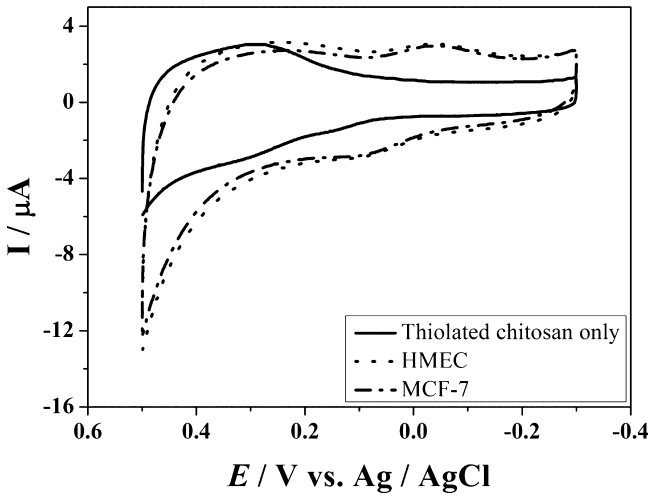


**Fig. 4.** Cyclic voltammograms with normal and cancer cell lines on thiolated chitosan substrate: electrochemical signals among bare electrode, electrode with breast normal cells, and breast cancer cell (— Thiolated chitosan, • • • HMEC, –• MCF-7).

reference and counter electrode respectively. Data obtained from mean ± SD of triplicate experiments maintaining identical condition. Pre-existing cytophilic materials were not self-assembled and just coated on substrate by physical absorption. However, the thiolated chitosan can make monolayer easily with self-assembly technique on gold surface. A monolayer film of extracellular matrix (ECM) is very important in electrochemical detection because the ECM was working as insulator with increase of thickness. And also, a thiolated chitosan modified cell chip provides stable condition while measuring electrochemical signal of cells. In contrary to physical absorption which can detach ECMs from substrate while doing wash step, the immobilization strength of thiolated chitosan by chemical bond was stronger than that of ECMs such as PLL and collagen by physical absorption. Therefore, the thiolated chitosan modified cell chip showed superiority to the collagen and PLL modified cell chip, and the bare gold substrate. These results demonstrated the advantage of thiolated chitosan as a cell-friendly material used to increase cell adhesion and proliferation and showed great potential for safety assessments of new medicine and for the cytotoxicity evaluation of environmental pollutants.

### 3.3. Electrochemical characterization of breast cancer and normal breast cells

Breast cancer and normal cells originated from the same place making our comparison significant. Before electrochemically characterizing the breast cancer and normal cells, we seeded the cells on a gold substrate coated with thiolated chitosan and grew the cells for 3 days. Then, cyclic voltammetry was conducted using PBS (0.01 mM, pH 7.4), a potential range of −0.3–0.5 V, and a scan rate of 50 mV $s^{-1}$. The cyclic voltammograms of the breast cancer and normal breast cells showed a quasi-reversible process in the anode and cathode peak currents. Fig. 4 shows a slight difference between peak currents of the breast cancer and normal breast cells, and the

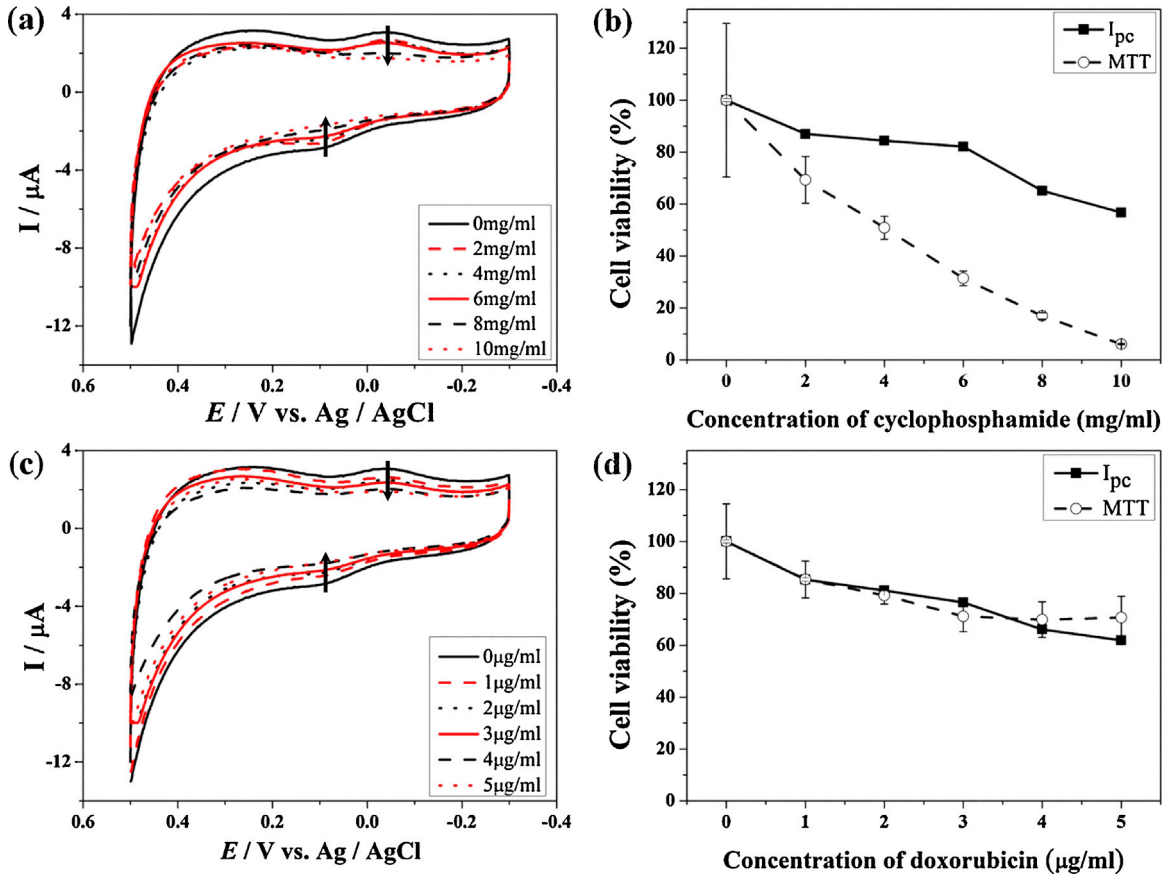


**Fig. 5.** Effects of anticancer drugs with varying concentrations on HMEC: (a) Cyclic voltammogram of normal cells treated with cyclophosphamide (— 0 mg/ml, – – 2 mg/ml, • • • 4 mg/ml, — 6 mg/ml, – – 8 mg/ml, • • • 10 mg/ml), (b) linear plot of $I_{pc}$ value and MTT results. Cyclophosphamide concentrations: 0, 2, 4, 6, 8, and 10 mg/ml, (c) cyclic voltammogram of normal cells treated with doxorubicin (— 0 μg/ml, – – 1 μg/ml, • • • 2 μg/ml, — 3 μg/ml, – – 4 μg/ml, • • • 5 μg/ml), (d) linear plot of $I_{pc}$ value and MTT results. Doxorubicin concentrations: 0, 1, 2, 3, 4, and 5 μg/ml. The scan rate was 50 mV $s^{-1}$ and cell number was $2 \times 10^5$ cells/ml. Data are mean ± standard deviation of triplicate experiments under identical condition.

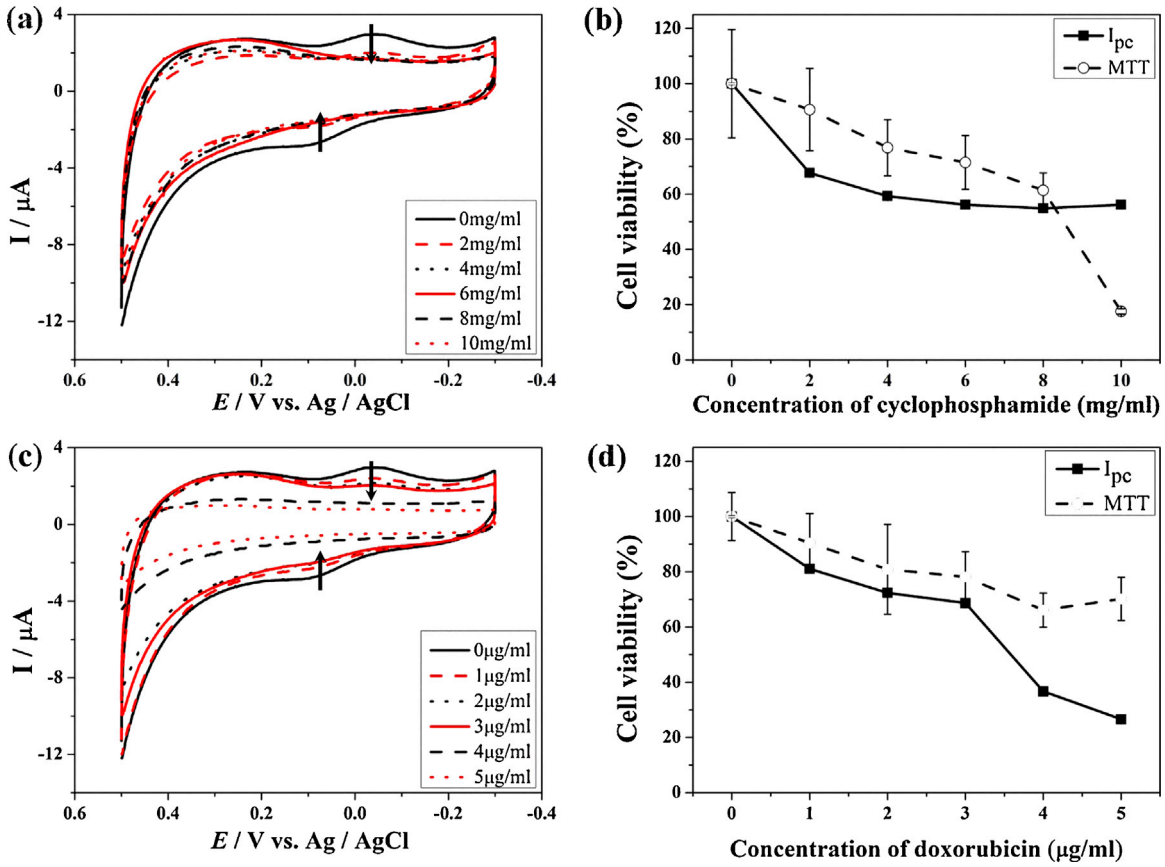


**Fig. 6.** Effects of anticancer drugs with varying concentrations on MCF-7: (a) Cyclic voltammogram of MCF-7 treated with cyclophosphamide (— 0 mg/ml, – – 2 mg/ml, • • • 4 mg/ml, — 6 mg/ml, – – 8 mg/ml, • • • 10 mg/ml), (b) linear plot of $I_{pc}$ value and MTT results. Cyclophosphamide concentrations: 0, 2, 4, 6, 8, and 10 mg/ml, (c) cyclic voltammograms of breast cancer cells treated with doxorubicin doxorubicin (— 0 μg/ml, – – 1 μg/ml, • • • 2 μg/ml, — 3 μg/ml, – – 4 μg/ml, • • • 5 μg/ml), (d) linear plot of $I_{pc}$ value and MTT results. Doxorubicin concentrations: 0, 1, 2, 3, 4, and 5 μg/ml. A scan rate was 50 mV $s^{-1}$ and cell number was $2 \times 10^5$ cells/ml. Data obtained from mean ± standard deviation of triplicate experiments under identical conditions.

Table 1 also shows a −0.046 V difference in $I_{pc}$ in breast cancer and −0.042 V in normal breast cells. These results seemed to be similar but the cancer and normal cells had a slight difference in oxidation potential, and the $I_{pc}$ was also slightly different. However, it was difficult to distinguish the difference in peak currents between the cancer and normal cells ($p > 0.05$) because of both cell lines from same organ. Therefore, we found that cells with the same origin had similar cathode peak currents and that it was difficult to distinguish cells by CV.

### 3.4. Electrochemical characterization of breast cancer and normal cells treated with anticancer drugs by CV

Breast cancer and normal cells were seeded at $2 \times 10^5$ cells/ml and grown for 2 days. Then, they were treated with doxorubicin and cyclophosphamide for 1 day. As the concentrations of the anticancer drug increased, electrochemical signals indicating cell viability decreased. Fig. 5(a) shows the cyclic voltammograms of normal breast cells treated with cyclophosphamide on the chip, and Fig. 5(b) shows the $I_{pc}$ measured by CV and the MTT result. The MTT assay was performed to certify the results of CV using the electrochemical detection method. As a result, normal cells were affected by the anticancer drugs as the anticancer drugs were toxic to all cells. Number of breast normal cells was decreased with increasing concentration of cyclophosphamide. To analyze the difference of results between by CV and MTT, *T*-test was performed. From Fig. 5(b), MTT assay result was lower than $I_{pc}$ values of cell chip. MTT assay is one of the cell viability test method and its result is occurred by MTT reduction activity of mitochondrial dehydrogenase. And also, the dehydrogenase has detoxification ability for cyclophosphamide [25]. In this reason, a cyclophosphamide worked as substrates of competitive inhibition in MTT experiment for investigating the cytotoxic effect of cyclophosphamide on cells. Therefore, the result of MTT assay was lower than actual cell viability degree. On the other hand, cyclic voltammograms of normal breast cells treated with doxorubicin on the chip was showed in Fig. 5(c) and $I_{pc}$ values and MTT results showed similar tendency in doxorubicin treatment test (Fig. 5(d), $p > 0.05$, *T*-test).

**Table 1**
Cathode peak currents ($I_{pc}$) of Au-thiolated chitosan, Au-thiolated chitosan-HMEC, and Au-thiolated chitosan-MCF-7 were formed in the table, respectively.

| Materials | Oxidation | Reduction |
|---|---|---|
| Au-chitosan | 0.293 V | 0.286 V |
| Au-chitosan-HMEC | 0.088 V | −0.04 V |
| Au-chitosan-MCF-7 | 0.074 V | −0.046 V |

The results of cyclophosphamide-treated breast cancer cells are shown in Fig. 6(a) and (b). In normal cell culture condition, a contact inhibition was occurred and proliferation was stopped when cells were contacted with neighboring cells. However, the growth of cancerous cells was not affected by contact inhibition and piled up after they filled full area they had.

Fig. 6 showed the effect of anticancer drugs on breast cancer cells. Fig. 6(a) showed cyclic voltammograms of MCF-7 treated

with cyclophosphamide. As MCF-7 is cancer cells, cells piled up in case they filled full area in cell chip. As a result, the cell chip can measure a signal of cells which was immobilized on electrode only. Thus the measured $I_{pc}$ value of cells was lower than expected cell's $I_{pc}$ value in Fig. 6(b). On the contrary, cell viability was higher in MTT assay than in electrochemical detection because the MTT assay was just related with number of mitochondrial dehydrogenase and its activity. The difference of cell viability between MTT assay and electrochemical method was decreased while increase of cyclophosphamide concentration with competitive inhibition and finally viability of MTT assay was lower than $I_{pc}$ value at over 8 mg/ml condition ($p > 0.05$, *T*-test). Fig. 6(d) is $I_{pc}$ value of Fig. 6(c) and it showed cytotoxicity of doxorubicin on cancerous cell. The electrochemical method was more sensitive than MTT assay in entire treatment conditions of doxorubicin. A doxorubicin showed anti-cancer effects on Fig. 6(d) (20% of cell viability) compared with Fig. 5(d) (normal cell, 60% of cell viability). Comparing these Figures, we found that the cyclic voltammograms of normal breast cells changed and that the $I_{pc}$ decreased according to increasing concentrations of cyclophosphamide. In particular, there is the difference between the $I_{pc}$ and cell viability as assessed by the MTT assay, and the $I_{pc}$ was higher than cell viability measured by the MTT assay at high concentrations in both types of cells. Fig. 5(c) describes the results of cell chips treated with doxorubicin on normal breast cells, and Fig. 5(d) shows the $I_{pc}$ and the results of the MTT assay on normal breast cells. In these results, we confirmed that the viability of normal breast cells decreased as doxorubicin concentrations increased but $I_{pc}$ value was lower than the cell viability measured by the MTT assay at a high concentration. The cyclic voltammograms of cell chips with breast cancer cells treated with doxorubicin are shown in Fig. 6(c) and the measurements of $I_{pc}$ of breast cancer cells and the results of the MTT assay are shown in Fig. 6(d) ($p > 0.05$, *T*-test). Two kinds of anticancer drugs we used showed anticancer effects on breast normal and cancer cells.

CV was a useful tool to measure cell viability. However, we performed the MTT *assay to validate the effects of the anticancer drugs on breast cancer and normal* breast cell lines based on CV. As shown in Fig. 5(b) and Fig. 6(b), viability of breast cancer and normal cells treated with cyclophosphamide decreased according to increasing concentrations of cyclophosphamide. Similarly, the effect of doxorubicin on breast cancer and normal cells was shown in Fig. 5(d) and Fig. 6(d). We were able to verify the effect of anticancer drugs on normal and cancer cells by CV. However, we found that the electrochemical signals and MTT assay showed partially different results for the same drugs and same cell lines. The MTT results were similar to values of $I_{pc}$ but cell viability as measured by the MTT assay decreased in contrast with actual cell viability because the inhibition of dehydrogenase activity by the drug was greater in the MTT assay. The MTT signals seemed to be reduced unlike actual cell viability in cases in which cell dehydrogenase activity was inhibited. Therefore, as shown in Fig. 5(b), $I_{pc}$ values were higher than cell viability as assessed by the MTT assay. Fig. 5(d) shows similar results between CV and MTT but the slight differences were due to the two types of drugs used. Breast cancer cells were more affected by loss of contact inhibition than by the effects of dehydrogenase. Hence, cell movement and proliferation were inhibited by contact among normal cells but cancer cells proliferate infinitely without inhibition based on contact among cells.

Unlike in other related researches, by using electrochemical measurement system compared with other measurement method, sensitivity of a cell chip was higher in Fig. 6(b) and (d) which was obtained through testing effects of anticancer drugs. Therefore, our concept was highly suitable for cell-based chip system.

## 4. Conclusions

We synthesized thiolated chitosan and easily generated a chitosan self-assembled monolayer that was effective for improving cell chip function. Thiolated chitosan was superior to any of the well-known cell-friendly materials. We measured higher cellular redox signal intensities compared with those of PLL, collagen, and a bare substrate. Normal and breast cancer cells showed similar tendencies with respect to the reduction in oxidation potential because both cell types were derived from the same origin. These tendencies can be used to effectively confirm the origin of various kinds of cells. Two anticancer drugs, cyclophosphamide and doxorubicin, were added to thiolated chitosan modified cell chips. A 4 μg/ml concentration of doxorubicin was critical and decreased cell activity significantly, whereas activation of cells decreased greatly with 2 mg/ml cyclophosphamide. Cell viability as assessed by the MTT assay decreased suddenly in the 4 μg/ml doxorubicin concentration and the viability of cyclophosphamide-treated cells was sharply reduced at 10 mg/ml. These results were almost analogous to the results from the thiolated chitosan modified cell chips. Rather, the thiolated chitosan modified cell chips showed higher sensitivity than that of the MTT assay. These results indicate that a cell chip based on thiolated chitosan was highly useful to assess effectiveness of anticancer drugs and to analyze their cytotoxicity in normal cells at the same time. Therefore, we expect that our cell chip based on thiolated chitosan will be applied for cell-mediated cytotoxicity assays, disease diagnosis, and anticancer drug assessments.

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