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Thermosensitive and Conductive Hybrid Polymer for Real-Time Monitoring of Spheroid Growth and Drug Responses

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssensors.0c02266>.

Chemical structure of components in a DFPS, the fabrication of a DFPS in a cell chamber, illustration of a cancer cell spheroid growth and drug treatment in the DFPS, additional characterizations of the prepared materials (including X-ray photoelectron spectroscopy spectrum and TEM), cytotoxicity of the prepared materials, and impact of gNPs and AgFs on the sensor (PDF)

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Abstract

Three-dimensional (3D) cell culture based on polymer scaffold provides a promising tool to mimic a physiological microenvironment for drug testing; however, the next-generation cell activity monitoring technology for 3D cell culture is still challenging. Conventionally, drug efficacy evaluation and cell growth heavily rely on cell staining assays, using optical devices or flow cytometry. Here, we report a dual-function polymer scaffold (DFPS) composed of thermosensitive, silver flake- and gold nanoparticle-decorated polymers, enabling conductance change upon cell proliferation or death for *in situ* cell activity monitoring and drug screening. The cell activity can be quantitatively monitored via measuring the conductance change induced by polymeric network swelling or shrinkage. This novel dual-function system (1) provides a 3D microenvironment to enable the formation and growth of tumor spheroid *in vitro* and streamlines the harvesting of tumor spheroids through the thermosensitive scaffold and (2) offers a simple and direct quantitative method to monitor 3D cell culture *in situ* for drug responses. As a proof of concept, we demonstrated that a breast cancer stem cell line MDA-MB-436 was able to form cell spheroids in the scaffold, and the conductance change of the sensor exhibited a linear relationship with cell concentration. To examine its potential in drug screening, cancer spheroids in the cell sensor were treated with paclitaxel (PTX) and docetaxel (DTX), and predicted quantitative evaluation of the cytotoxic effect of drugs was established. Our results indicated that this cell sensing system may hold promising potential in expanding into an array device for high-throughput drug screening.

Graphical Abstract

Keywords

cell sensor; drug screening; 3D cell culture; breast cancer; thermosensitive polymer

As the second leading cause of death worldwide, cancer fatally threatens numerous lives. Chemotherapy, surgery, and radiotherapy are still the major, but sometimes ineffective, treatments of cancer.¹ During preclinical drug development, cell viability assays have been used as the primary technique for fast and cost-effective screening of new anticancer drugs in the pharmaceutical industry. Traditionally, two-dimensional (2D) cancer cell culture-based cytotoxicity assays have been the most common approaches. However, cytotoxicity

assays based on 2D cell culture have significant limitations, such as misleading and unpredictable tissue responses.^{2–6} The unacceptable toxicity of drugs is one of the major reasons in clinical trials, and this is especially the case in the most expensive phase III clinical trials.⁷ Therefore, it is of importance to develop an efficient cell culture system to overcome the above problems. Recently, three-dimensional (3D) cell culture systems, which could provide a mimic of the physical microenvironment of tissues or organs for cell growth and differentiation via improvement of cell–cell or cell–extracellular 3D hydrogel breast cancer models for studying the effects of hypoxia on epithelial-to-mesenchymal transition matrix interactions, have been extensively studied and demonstrated as an efficient approach in pharmaceutical compound testing and development, compared to conventional 2D cell culture. Apart from the investigation of drug responses from cancer cells, 3D cell culture systems also allow us to better monitor and understand the development of cancers.^{8–12} There are two major approaches for 3D cell culture: scaffold-based 3D cell culture and scaffold-free 3D cell culture, which reproduce a tissue environment for the development of several 3D cell models such as spheroids and organoids. For scaffold-free 3D cell culture, electrical impedance spectroscopy is one of the major approaches for monitoring cell growth and activity; however, the lack of scaffolds limits its further applications due to an inability to mimic a physical 3D microenvironment.^{13–17} Since the scaffold can offer a suitable and stable 3D space with proper mechanical properties and shape maintenance during cell growth, scaffold-based cell culture has been gaining considerable attention in tissue engineering.^{18–25}

Although various conductive polymer scaffolds including poly(3,4-ethylenedioxythiophene) (PEDOT) have been investigated as 3D cell culture and show the potential in cell activity monitoring,^{26–29} the difficulty in live-cell harvesting and detection limits its further application in drug screening. *N*-Isopropylacrylamide (PNAAM) and its copolymer with high biocompatibility are fluids at low temperatures (near 4 °C), while they can form an elastic hydrogel polymer at higher temperatures (near 33 °C).^{30–33} As such, PNAAM copolymers have been used as 3D cell culture matrices to allow cell growth at proper temperatures and low-temperature cell harvesting. This renders PNAAM one of the most popular polymers for the fabrication of a 3D cell culture scaffold.^{34–38}

Although 3D cell culture is a promising tool, cell counting and cell activity monitoring in 3D cell cultures are of great challenge, which limits its application in drug screening. Due to the large size and multiple cell layers of spheroids in the scaffold of cell cultures, it is difficult to use the conventional optical methods of 2D cell cultures to analyze and count the cell spheroids.¹⁵ Traditionally, the cells are harvested and stained using various methods including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a cell counting kit-8 assay, water-soluble tetrazolium salts-1 (WST-1) or fluorescent dyes (Cultrex 3D spheroid fluorometric proliferation/viability assay), followed by using a cell counter or fluorescence microscope. These procedures are harmful to the cells and spheroids and can only show the static status of the live/dead cells but not *in situ* real-time monitoring of cell growth or death. The cells used in these methods are unable to be further investigated for drug treatment.³⁹

As a consequence of the advancement in bioelectronics, electrical sensors have been investigated for label-free measurement of electrical activity and membrane potential or to monitor cell activity.^{40–43} Metallic micro- or nanoparticles possess high conductivity and can be functionalized easily; thus, they are widely utilized in the fabrication of electrical devices and sensors.^{43–46} Previously, we have demonstrated that a gold nanoparticle (gNP)-based polymeric network can be employed to precisely measure the aptamer–protein binding.⁴⁷ Silver flakes (AgFs) have been incorporated into a polymer that was then extensively investigated in biomedical applications.^{48,49} Recently, Matsuhisa et al. reported a stretchable, printable elastic conductor by the dispersion of silver flakes and silver nanoparticles in an elastomer and demonstrated its high sensitivity in pressure sensing.⁵⁰ These studies demonstrated the promising potential of metallic nanoparticle–polymeric composites in biosensing systems.

We hypothesize that cell spheroid formation or cell death can cause a change of the polymer structure and the interaction with metallic nanoparticles due to polymer scaffold deformation, including swelling and shrinkage, followed by the conductance change of the scaffold. The cell proliferation will cause the polymer swelling, while cell death will induce polymer shrinkage. In the present study, we developed a dual-function polymer scaffold (DFPS) composed of thermosensitive polymers, silver flake-decorated polymers, and gNP-decorated polymers to monitor cancer cell proliferation and cell death *in situ* (Figure 1). Our data from spheroid growth-induced volumetric changes of DFPS supported our hypothesis that this system indeed could be used to monitor 3D cell culture. This assay allows for *in situ* quantitative measurement of growth or death of cancer stem cells, particularly spheroids, by monitoring the changes in electrical conductance of the DFPS. Besides, the cells can be easily harvested for further studies in virtue of the thermosensitive property of PINAAm.

RESULTS AND DISCUSSION

As shown in Figure 1a, the dual-function polymer scaffold was designed as a 3D cell culture system that allows for cell growth in a chamber with built-in electrodes. The major component of the DFPS is a copolymer of poly(*N*-isopropylacrylamide) and poly(ethylene glycol) (PNIPAAm–PEG) (Mebiol Gel), and its chemical structure is displayed in Figure S2a in the Supporting Information. Thioglycolic acid was used to modify chitosan to obtain a chitosan derivative (TGA–chitosan) with the thiol group. TGA–chitosan was then utilized to immobilize gNPs or AgFs. Cancer cells, gNP-decorated TGA–chitosan, and AgF-decorated TGA–chitosan were mixed with PNIPAAm–PEG uniformly at 4 °C, after which the polymeric network solidified when the temperature was increased to 33 °C. This resulted in the formation of a dual-function scaffold due to the physical cross-linking polymer structure formed by hydrophobic and entangled chains (Figure 2b in the Supporting Information). When the temperature was lowered down to 4 °C, the polymer structure turned liquid again, which allowed us to harvest the cells from the 3D polymer scaffold in the liquid phase by centrifugation. Due to the thermoreversible property of the DFPS, the cells can be easily seeded and harvested in the polymer scaffold.

We utilized X-ray photoelectron spectroscopy (XPS) to confirm the binding interactions between the thiol group and the metal nanoparticle surface. Since the gNP-decorated TGA–

chitosan and TGA–chitosan used were obtained from our previous study, we only presented the XPS spectra of AgF-decorated TGA–chitosan in this study (Figure S1 in the Supporting Information).⁴⁷ The S 2p peak in the spectrum of AgF-decorated TGA–chitosan was deconvoluted into two peaks 163.5 and 162.3 with a branching ratio of 1:2 and a splitting of 1.2 eV, which were assigned to S 2p_{1/2} and S 2p_{3/2} peaks.⁵¹ These peaks were significantly shifted compared to the corresponding peaks in the S 2p peak in TGA–chitosan, suggesting that the AgFs were bound to the thiol groups of TGA–chitosan. The result is consistent with our previous study.⁴⁷ Next, scanning electron microscope (SEM) was used to investigate the morphology and microstructure of the dual-function scaffold (Figure 2a,c). We can clearly observe that the AgFs were dispersed in the scaffold uniformly but the size of AgFs was difficult to measure because they were embedded within the polymer scaffold. Thus, we used transmission electron microscopy (TEM) to determine the size of AgFs. We found that the size of AgFs was about 10 μm (Figure S3 in the Supporting Information). The size of the gNPs was between 2 and 5 nm, as described previously.⁴⁷ We employed energy-dispersive X-ray spectroscopy (EDS) chemical mapping of the element Au to verify the distribution of the gNPs within the polymeric network, as shown in Figure 2c. It is pronounced that gold is evenly dispersed on the surface of the scaffold, indicating that the gNPs were incorporated into the polymeric scaffold as well. Besides, we employed TEM to observe an ultrathin piece of DFPS, as shown in Figure S4 in the Supporting Information. By utilizing a hollow grid, it is notable that the gNPs and AgFs were evenly dispersed within the polymer scaffold, and the size of gNPs and the results in SEM image (Figure 2a) were confirmed as well.

Given that the soft and adhesive properties of the hydrogel in the scaffold, and the metal properties of gNPs and AgFs with stiffness in a force spectroscopy, atomic force microscope (AFM) analysis of the surface of the scaffold was used to further confirm the integration of metal particles and polymeric scaffold. Figure 2d shows the topography of the dual-function scaffold. It is obvious that large AgFs were surrounded by several gNPs. LogDMTModulus images were carried out to analyze the stiffness and adhesion of the surface of the polymer scaffold to confirm that the peaks in the surface topography represent silver flakes and gNPs, at the corresponding positions in the LogDMTModulus (Figure 2e), which are consistent with the position of AgFs and gNPs in the height images. As expected, the softness and high adhesion properties of the dual-function scaffold were observed in these AFM images.

To examine the versatile feature of DFPS, we first investigated the 3D cell culture using DFPS. Triple-negative breast cancer, a subtype of breast cancer negative for estrogen, progesterone, and human epidermal growth factor 2 receptors, accounts for 15–20% of all new breast cancer cases and is highly aggressive.⁵² We selected a triple-negative breast cancer cell line MDA-MB-463 cell to test our DFPS sensing system. The time-lapse microscopic images of cell morphology in DFPS were recorded, as shown in Figure 3a, and the cell morphology using planar cell culture is presented in Figure S5 in the Supporting Information. We can clearly observe the significant difference in morphology between 3D and 2D cell cultures. Obviously, cancer cell spheroid was formed in the 3D polymer network structure. In contrast, the shape of the same cancer cell line in 2D culture was sheet-like flat in the monolayer. There are several stages involved in the morphological changes during cancer cell spheroid formation (Figure 3a). In the first stage, small cell aggregates are spontaneously self-assembled by several single cells (5–6 cells) within the polymeric

network on day 1. Next, the cell proliferation of cell aggregates began on day 2. It is noticeable that the cell aggregate was dramatically enlarged during days 3–6. Finally, the solid spheroids with smooth surfaces were formed as described by others.⁵³

Next, we employed DFPS to monitor the cell growth status by calculating the conductance change of the scaffold every 12 h, and the results are displayed in Figure 3c,d. To obtain the conductance change, the initiated conductance of each DFPS was recorded when cancer cells were seeded in the scaffold at the beginning, and then the conductance at each time point was measured. At the same time, we measured the volumetric change of DFPS. To validate the performance of the DFPS, we performed side-by-side conventional cell counting using a cell counter during 3D cell culture of breast cancer to establish a growth curve of breast cancer stem cells. As shown in Figure 3b, there was an increase in cancer cell number within the scaffold at indicated time points during cell culture. It is obvious that the conductance change of DFPS is proportional to cell concentration. Besides, although we can observe that the volumetric change of DFPS was associated with the change of conductance change and there was no change in the control groups, they are not overlapped over time. This is because the volumetric measurement is inaccurate and there is a huge variety (Figure S6). Besides, the process of volume measurement is tedious, and we need to take the cells out from the incubator and placed them under a microscope, followed by imaging and analysis via software (such as imageJ), while our approach is much simpler. It is obvious that the decreased conductance of the DFPS accompanied by the increased cell numbers is due to the swelling of the polymer network, which confirmed our hypothesis. However, the results exhibit a strong correlation between the conductance change and cell concentration. We then plotted the conductance change as the function of the cell concentration. Importantly, as shown in Figure 3e, it is worthwhile to note that the conductance change exhibited a highly linear ($R^2 = 0.9865$) relationship with the concentration of cancer cells, indicating that a robust cell growth curve can be obtained using this DFPS system.

As a synthetic polymer for cell culture, the components should be safe for the cells. Since the biocompatibility and biodegradation of PNIPAAm–PEG have been extensively investigated in previous studies,^{54,55} we employed the MTT assay to evaluate the cytotoxicity of the gNP-decorated TGA–chitosan and AgF-decorated TGA–chitosan, as shown in Figure S7 in the Supporting Information. We found that the gNP-decorated TGA–chitosan had no toxicity when below 75 $\mu\text{g/mL}$. However, the toxicity of gNP-decorated TGA–chitosan was increased when the concentration was higher than 75 $\mu\text{g/mL}$. AgF-decorated TGA–chitosan exhibited similar results.

Based on the data from Figure S7, the gNP-decorated TGA–chitosan had no toxicity when below 75 $\mu\text{g/mL}$, yet the toxicity of gNP-decorated TGA–chitosan increased when the concentration was higher than 75 $\mu\text{g/mL}$. Therefore, in the following experiments, we started with 50 $\mu\text{g/mL}$ of gNP-decorated TGA–chitosan in the development of DFPS. For the two metallic nanoparticle-based DFPS (combined DFPS), the sensor was prepared using 50 $\mu\text{g/mL}$ of gNP-decorated TGA–chitosan and 50 $\mu\text{g/mL}$ of AgF-decorated TGA–chitosan. In the gNP-only DFPS, only 50 $\mu\text{g/mL}$ of gNP-decorated TGA–chitosan was included, while in the AgF-only DFPS, only 50 $\mu\text{g/mL}$ of AgF-decorated TGA–chitosan was used. We found

that AgFs may have a larger impact on the conductance change of DFPS in Figure S8a. Hence, we fixed the AgF-decorated TGA–chitosan at 50 $\mu\text{g/mL}$, and various concentrations of gNP-decorated TGA–chitosan were then used to develop DFPSs. As shown in Figure S8b, DFPS made by gNP-decorated TGA–chitosan at a concentration of 25 $\mu\text{g/mL}$ had the best performance. Then, we constructed DFPS with various concentrations of AgFs in a range of 0.00125–0.1 mg/mL and a fixed concentration of gNP-decorated TGA–chitosan (25 $\mu\text{g/mL}$) to optimize the concentration of AgFs. As shown in Figure S9, the electrical conductance change was elevated with the increase of the AgF concentrations between 0.00125 and 0.01 mg/mL. However, the conductance changes were decreased beyond this range, which may be due to the increased cytotoxicity of the AgFs at higher concentrations.

To explore the potential capability of DFPS for drug screening, we selected three different anticancer drugs to treat the MDA-MB-436 cell in the DFPS 3D cell culture system. In docetaxel (DTX), paclitaxel (PTX), and doxorubicin hydrochloride (DOX) treatments, we found that cancer cell spheroids in DFPS exhibit less sensitivity in the drugs compared to the cells in 2D cell culture (Figure 4a–c), especially for DOX. Interestingly, we observed that the cells continued to grow for 12 h after PTX treatment, indicating that the cells were not sensitive to PTX, compared to those of DTX treatment. The fact that the MD-MB-436 cells are relatively less sensitive to PTX compared to those of DTX is in agreement with the previous studies reported by others.⁵⁶ As shown in Figure 4d–f, the conductance change is proportional to the cell survival curve, as shown in Figure 4a–c; however, the signal of change was much more significant than the MTT assay. To validate these results, we used a cell proliferation inhibitor mitomycin C (2 $\mu\text{g/mL}$) to treat the MDA-MB-436 cells. As shown in Figure 4f, there were no significant changes in electrical conductance signals after mitomycin C treatment, indicating that the decreasing signal in fact may reflect the cell proliferation, instead of the result of ECM remodeling. We also examined the volumetric changes of DFPS treated by DTX, PTX, and DOX. It is found that the volumetric changes of DFPS were correlated with the conductance change. Figure 4g demonstrates that the DFPS cell sensor has a linear relationship between conductance change and cell survival, enabling us to predict the drug effect on the cells in response to drugs through monitoring conductance changes. We established a linear equation between the electrical conductance change and cell survival of MDA-MB-436 cells. Then, we can predict the cytotoxicity of the same cells through measurement of conductance change. These results demonstrated that the DFPS can be potentially used as a promising tool for drug screening.

The xCELLigence Real-Time Cell Analysis products made by ACEA can real-time monitor cell behavior and cell viability via impedance measurement on a plate equipped with an electrode,⁵⁷ and the cell sensor developed by Chawla et al. combined impedance sensor and microfluidic cell culture to determine growth rates of cells through impedance measurements.⁵⁸ However, the xCELLigence Real-Time Cell Analysis products can only measure the cells that are attached to the electrode on the bottom of the plate. For the sensor reported by Chawla, only a 2D monolayer of cells can be formed between the PDMS and the glass slide due to the structure of the microfluidic chip. It is apparent that both ACEA and Chawla's technologies are not applicable in 3D cell culture. As discussed in this manuscript, 3D cell culture provides a promising tool to mimic a physiological microenvironment for drug testing.

In this study, we integrated metallic nanoparticles into a biocompatible polymer scaffold to develop a novel 3D cell culture system that allows for cell growth, cell growth monitoring, and drug screening via cell–polymer interaction-induced conductance change of polymer scaffold. This sensing system has two functions: (1) the cancer cells can grow within the polymer scaffold to form 3D cell spheroids, which can simulate cancer cell growth in the body. Although the polymer scaffold-based 3D cell culture has been widely studied, it is not easy to distribute the cells uniformly into the polymer scaffold.⁵⁹ To overcome this problem, we introduced thermosensitive polymer as one of the major components in our polymer scaffold. (2) The cell activity can be monitored by tracking the conductance change of the scaffold. Stretchable elastic conductor-dispersed silver flakes and silver nanoparticles have been reported in pressure sensing.⁵⁰ We assumed that the cell proliferation in a 3D scaffold can cause the polymer network swelling, whereas the drug-induced cell death can lead to the polymer network shrinkage. The results in Figures 3c and 4d demonstrated the cell activity-induced volumetric changes of the polymer scaffold, which supported our hypothesis. All of these changes result in the electrical conductance change of the DFPS by increasing or reducing the distance between the metallic nanoparticles within the polymeric network. Taking advantage of the conductance change of the DFPS, we can establish the relationship between the conductance change of the DFPS and the cellular activity. The results of the functionality of DFPS confirmed our hypothesis. We noticed that the conductance change of DFPS is proportional to cell concentration for both cancer cell proliferation and cell death and both exhibit an excellent linear relationship. Accordingly, in the function of cell growth monitoring, we can quantify the cell number in DFPS to monitor cell growth *in situ* without the need of staining the cells and damaging the cells, which is superior to the current cell counting technologies.

For drug screening studies, since the conductance change has a linear relationship with the cell survival across different time points, the quantitative estimation of the toxic effect of drugs on cell activity can be established. Therefore, we can predict the drug effect through the measurement of conductance change of the DFPS. In this study, we successfully evaluated the toxicity of PTX to the cancer cells using our sensing system. These results demonstrated that the DFPS can provide a reliable and accurate assessment of drug toxicity, which open a new horizon for drug screening without the need for staining and harvesting the cells.

In the traditional drug screen studies, cells in 2D culture are quite different from 3D cell spheroids in morphology, physiology, and metabolism. The cells in the 2D plate were exposed to the drug directly, but the 3D dense multicellular spheroid structure is formed in a natural 3D extracellular matrix, which could prevent the rapid dispersion of drugs within the spheroid, reducing the DTX-, PTX-, and DOX-induced cell deaths.⁶⁰ Therefore, the 2D cell culture system may result in different responses to drug treatment compared to the real situation in the body and may lead to false-positive results.⁶¹ Hence, the 2D culture system could contribute to the failure in clinical trials, whereas the DFPS may provide more accurate and reliable results of drug response in drug screening.

Interestingly, we found that the AgFs contributed more to the electrical conductance change than GNPs. However, it is still challenging to understand the exact mechanism

of this phenomenon using the theoretical models from current literatures. The volumetric change of the scaffold induces the change in nanoparticle density; meanwhile, several other factors might be involved, including the connectivity and mobility of the flakes due to the deformation of the polymeric structure.⁶² Future work will be needed to further understand the electrical mechanism. We also expect to combine this cell sensor system with array systems to develop a high-throughput drug screening device to improve the efficiency in new drug discovery.⁶³

CONCLUSIONS

Collectively, we have established a rapid and noninvasive cell sensing system for *in situ* monitoring of cancer cells and spheroid growth and drug screening. In this novel system, the breast cancer cells can grow into spheroids with the physiological extracellular matrix environment and can be tested for drug responses simultaneously. Importantly, this system allows us to predict cell responses to anticancer drugs without the need for cell staining. Finally, this system can be easily adapted to an array format, which allows for real-time and high-throughput screening of multiple drugs or multiple cell lines for the purpose of drug discovery and cancer research.

EXPERIMENTAL SECTION

Materials.

Mebiol Gel PNIPAAm-PEG 3D thermoreversible hydrogel was obtained from Cosmo Bio (Tokyo, Japan). Chitosan, gold(III) chloride hydrate (HAuCl₄), thioglycolic acid (TGA), potassium phosphate (dibasic), sodium acetate, acetic acid, doxorubicin hydrochloride (DOX), mitomycin C, and silver flakes were purchased from Sigma-Aldrich (St. Louis, MO). Phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), MEM nonessential amino acid solution (MEM NEAA), fetal bovine serum (FBS), TrypLE Express enzyme, and penicillin-streptomycin (Pen-Strep) were obtained from ThermoFisher Scientific (Carlsbad, CA). *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) was obtained from Carbosynth (San Diego). *N*-Succinimidyl acrylate (NHS-AA) and *N*-hydroxysuccinimide (NHS) were purchased from TCI America (Portland, OR). Ethyl alcohol was obtained from Pharmco-AAPER (Shelbyville, KY). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Alfa Aesar (Haverhill, MA). Paclitaxel (PTX) and docetaxel (DTX) were obtained from Avachem Scientific (San Antonio, TX). All distilled water used in this study was generated using a Thermo Scientific Barnstead GenPure Water Purification System (Waltham, MA).

Synthesis of Thioglycolic Acid-Modified Chitosan (TGA-Chitosan).

The method is similar to that reported previously.⁴⁷ Briefly, chitosan was dissolved in acetic acid solution, while TGA was activated by EDC. TGA/EDC mixture was then added to the chitosan mixture solution, followed by the addition of NHS. The mixture was allowed to stir for approximately 5 h. The saturated alkaline ethyl alcohol solution of anhydrous sodium acetate was used to precipitate the raw TGA-chitosan. The raw TGA-chitosan was dissolved in degassed distilled water after washing with ethyl alcohol/distilled water

solution, after which the mixture was centrifuged, and the supernatant was removed. The raw product was then dissolved in degassed distilled water, followed by dialysis for 4 days at 4 °C.⁶⁴ The purified TGA–chitosan was stored in the dark under vacuum at 4 °C.

Synthesis of gNP-Decorated TGA–Chitosan.

The method of synthesis of gNP-decorated TGA-chitosan was according to previous reports with modifications. It was based on modifications of previous reports.^{65,66} Briefly, TGA–chitosan was dissolved in degassed distilled water. H_{Au}Cl₄ was then added into the TGA–chitosan solution to give a TGA–chitosan/H_{Au}Cl₄ molar ratio of 1:10. Ice-chilled NaBH₄ solution (NaBH₄/H_{Au}Cl₄ molar ratio of 10:1) was added to the vigorously stirred TGA–chitosan/H_{Au}Cl₄ solution at room temperature and allowed to react for 24 h under argon. To achieve the gNP-decorated TGA–chitosan, the mixture was centrifuged. The supernatant was then removed, and ultrasonic redispersion–centrifugation was applied to the precipitate by washing twice with degassed distilled water. Finally, the gNP-decorated TGA–chitosan was suspended in PBS.

Synthesis of Silver Flake-Decorated TGA–Chitosan (AgF-Decorated TGA–Chitosan).

TGA–chitosan was first dissolved in degassed distilled water. Silver flakes were then added into the vigorously stirred TGA–chitosan solution at room temperature and allowed to react for 24 h under argon. To achieve the AgF-decorated TGA–chitosan, the mixture was centrifuged, the supernatant was removed, and ultrasonic redispersion–centrifugation was repeated twice to the pellet. Finally, the AgF-decorated TGA–chitosan was suspended in PBS.

Development of a Dual-Function Polymer Scaffold.

Cold cell culture medium was added to Mebiol Gel PNIPAAm–PEG powder and stored at 4 °C. The gNP-decorated TGA–chitosan, AgF-decorated TGA–chitosan, and cell suspension were added into the Mebiol Gel PNIPAAm–PEG solution in flask flat with occasional gentle shaking to mix. A 24-well plate was warmed up with the addition of a culture medium to 37 °C, followed by pouring 250 μ L of prepared solution into a well of the 24-well plate. Finally, the plate was placed into the cell incubator at 37 °C to form the dual-function polymer scaffold.

X-ray Photoelectron Spectroscopy (XPS).

AgF-decorated TGA–chitosan was dissolved in degassed distilled water and then was spotted on a silicon wafer and dried under vacuum. The spectrum was recorded on a PHI 5700 XPS instrument equipped with a monochromatic Al K α X-ray source (RBD Instruments, Bend, OR).

Scanning Electron Microscopy (SEM) Study of a DFPS.

The dual-function scaffold was immersed in ethanol sequentially and dried. The dried samples were then coated with 5 nm of iridium. The SEM images of the dual-function scaffold were conducted by using a Nova NanoSEM 230 (FEI, Hillsboro, OR).

Transmission Electron Microscopy (TEM) Study of a DFPS.

AgFs or ultrathin DFPS pieces in distilled water/alcohol (v/v = 1:1) were dropped onto a hollow grid (Ted Pella, Redding, CA), and the extra solution was removed by filter paper and air-dried. The image was obtained by using a JEM-2010F transmission electron microscope (JEOL, Tokyo, Japan).

Atomic Force Microscopy (AFM) Study of a DFPS.

A MultiMode AFM was performed (Bruker, Billerica, MA) to examine the surface properties of the dual-function scaffold through indentation of surface components. The topography and LogDMTModulus (log of DMT mode, a relative stiffness of the surface) on the same area in one AFM scan were recorded by using a PeakForce QNM (Quantitative Nanoscale Mechanical) mode simultaneously. These measurements were carried out by an AFM probe with a soft silicon nitride cantilever and silicon nitride tips (Bruker, spring constant of 0.01 N/m; model: MLCT). AFM image processing was analyzed by using NanoScope Analysis version 1.50 software (Bruker, Billerica, MA).

2D Cell Culture.

The breast cancer cell line MDA-MB-436 was cultured in DMEM (high glucose) medium containing 10% fetal bovine serum (FBS), 0.1 mM MEM nonessential amino acids (NEAAs), 2 mM L-glutamine, 1% Pen–Strep. The cells were cultured in a 25 cm² cell culture flask at 37 °C in an incubator with 5% CO₂ under a humidified atmosphere, and the medium was changed daily. The cells were passaged by harvesting with TrypLE reagents. The harvested cells were stained using trypan blue and counted using a Countess II FL Automated Cell Counter (ThermoFisher Scientific).

Cell Growth Monitoring in DFPS.

To transfer the cell culture from 2D to DFPS, 0.1 mL of the MDA-MB-436 cells (2000 cells/mL) was mixed with 1 mL of copolymer solution in a centrifuge tube in an ice bath. Fifty microliters of copolymer–cell suspension was poured into each well of the plate incubated at 37 °C for 10 min to form a 3D polymer matrix. Finally, 100 μ L of the culture medium was added into each well, and fresh medium was replenished daily. At the time of imaging and cell counting, an ice-chilled medium was added to the 3D culture to dissolve the polymer matrix. Spheroids were collected by centrifuging at 200 $\times$ g for 3 min, incubated with TrypLE reagents at 37 °C for 7 min and dissociated into a single-cell suspension. The time-lapse microscopic images were recorded to observe the growth of breast cancer spheroids. As shown in Figure 1a, each cell culture chamber was equipped with two gold electrodes and warmed up to 37 °C. The conductance of the DFPS was measured using a Model 2450 SourceMeter every 12 h. The conductance change was calculated.

To investigate the volumetric change of the scaffold, we used the same method as described in the Development of a Dual-Function Polymer Scaffold section. Instead of pouring 250 μ L of prepared solution into a well of the 24-well plate, we loaded the scaffold solutions with or without cells into capillary tubes (Fisher brand, inner diameter: 1.1 mm) at 4 °C; then, the capillary tubes were placed in the culture medium, followed by incubation at 37

°C. The lengths of scaffolds in capillary tubes at various time points during incubation were measured to determine the volumetric change of the scaffold using the following equation

$$\text{volumetric change} = \frac{\pi R^2 L_m - \pi R^2 L_0}{\pi R^2 L_0} \times 100\%$$

where R is the inner diameter of a capillary tube, L_m is the length of scaffolds in capillary tubes at various time points during the incubation, and L_0 is the initiated length of scaffolds in capillary tubes when the scaffolds are formed at 37 °C. The volumetric change of the scaffold before and after drug treatment was also conducted using a similar method.

Cytotoxicity Study of the Component of Scaffold.

The 3T3 cells were cultured in DMEM containing 10% FBS, 0.1 mM MEM NEAA, and 1% Pen–Strep for 2 days at 37 °C under 5% CO₂, followed by seeding a 96-well plate at a density of 1×10^4 cells/well. After the cells were seeded for 48 h, the culture medium was carefully removed and replaced by 100 μ L of various concentrations of gNP-decorated TGA–chitosans or silver flask-decorated TGA–chitosan medium solutions in each well and incubated for 24, 48, and 72 h at 37 °C under 5% CO₂. Next, 20 μ L of MTT (5 mg/mL) was added into each well and incubated for 3.5 h at 37 °C. The media were carefully removed, and 150 μ L of MTT solvent was added into each well in dark and shacked for 15 min. The plate was read using a UV spectrophotometer (Epoch, Biotek, Winooski, VT).

MTT Assay.

The 3T3 cells were cultured in 100 μ L of the cell culture medium containing DMEM, 10% FBS, and 1% Pen–Strep for 2 days at 37 °C under a 5% CO₂, and then the cells were seeded in a 96-well plate at a density of 1×10^4 cells/well. After the cells were seeded for 48 h, the culture medium was carefully removed and replaced with 100 μ L of the cell culture medium solution containing various concentrations of gNP-decorated TGA–chitosans or silver flake-decorated TGA–chitosans in each well and incubated for 24, 48, and 72 h at 37 °C under a 5% CO₂. In the control group, 100 μ L of the cell culture medium solution was used instead of gNP-decorated TGA–chitosan or silver flake-decorated TGA–chitosan. Next, 20 μ L of MTT (5 mg/mL) was added into each well and incubated for 3.5 h at 37 °C. The medium was carefully removed, and 150 μ L of MTT solvent containing 4 mM HCl and 0.1% NP40 in isopropanol was added into each well and mixed thoroughly using the pipette in dark. The plate was wrapped in aluminum foil and shacked for 15 min. The plate was read using a UV spectrophotometer at 570 nm (Epoch, Biotek, Winooski, VT).

Drug Screening Studies.

To investigate the drug screening capability of DFPS, the MDA-MB-436 cells were cultured and the initiated conductance was recorded. After 3 days of 3D cell culture, we used various concentrations of PTX, 5 nM of DTX, DOX, or PBS, or 20 μ g/mL of mitomycin C in cell culture medium to treat the cancer spheroid in DFPS and measured the conductance change at various time points. In comparison, we also side-by-side performed the same treatment on

3D DFPS cell culture and traditional 2D cell culture and utilized the MTT assay to evaluate the cytotoxicity on the cancer spheroids and cancer cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 2.

Characterizations of DFPS: (a) SEM image of a dual-function scaffold; (b) the schematic illustration of SEM image; (c) energy-dispersive X-ray spectroscopy (EDS) mapping of the Au on the surface of a dual-function scaffold; (d) the topography of AFM image of a dual-function scaffold; (e) the corresponding LogDMTModulus mode; and (f) the schematic illustration of AFM image.

Figure 3.

Functionality of DFPS: (a) microscope images of cancer cell growth into a spheroid in DFPS during days 1, 2, 4, and 6. (b) MDA-MB-231 cell growth curve; (c) the plotted curve of electrical conductance change at different time points during cell culture; (d) the relationship between electrical conductance change and cell concentration; (e) the linear relationship between cell concentration in DFPS and electrical conductance change; and error bars in (d) and (e) represent the standard deviation from three measurements using different devices with the same DFPS.

Figure 4. Drug screening study using DFPS: (a–c) the cell growth curves of MDA-MB-436 cells in response to PTX at various concentrations ranging from 1 to 10 nM, DTX at 0.1–1 μM , and DOX at 0.05–18 μM in cell culture medium using 2D or 3D cell culture; (d–f) the conductance change curve of MDA-MB-436 cells in response to PTX at various concentrations ranging from 1 to 10 nM, DTX at various concentrations ranging from 0.1 to 1 μM , and DOX at various concentrations ranging from 0.05 to 18 μM , or 20 $\mu\text{g}/\text{mL}$ mitomycin C, or phosphate-buffered saline (PBS) in cell culture medium in DFPS, as well as the DFPS without cells; (g) linear relationship between the electrical conductance change

and cell survival of MDA-MB-436 cells; and (h) merged images of MDA-MB-436 cells in response to 0.8 μ M DTX using 2D cell culture.

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