Ultrasound-Enhanced Delivery of an Aptamer-Doxorubicin Conjugate to Breast Cancer Cells

JU-YAO CHANG[1], TSAI-KAI JU[3], NELSON G. CHEN [ID][2], CHUN-JEN HSIAO[3], AND CHIN-YU WANG[3]

[1]Institute of Biomedical Engineering, National Chiao Tung University, Hsinchu 300, Taiwan
[2]Department of Electrical and Computer Engineering, National Chiao Tung University, Hsinchu 300, Taiwan
[3]Genomics Research Center, Academia Sinica, Taipei 115, Taiwan

Corresponding author: Nelson G. Chen (ngchen@nctu.edu.tw)

ABSTRACT When treating cancer with chemotherapy, serious side effects are caused by the inability of the drug to be solely delivered to the tumor. As a result, a portion of the drug agents is inevitably delivered elsewhere and destroy normal cells. We report the first results on combining ultrasound with an aptamer–doxorubicin conjugate to treat cancer cells. Enhancement of therapeutic effects combined with a reduction in side effects indicates the potential of this approach. Although many studies have noted that ultrasound can enhance drug delivery, ultrasound has not addressed the goal of reducing side effects. To both reduce side effects and enhance the efficiency of killing cancer cells, this study mainly uses a specially targeted aptamer conjugated to the anticancer drug doxorubicin (DOX), which was applied in combination with ultrasound for cancer treatment. We also compared the results between cancer and normal cell lines to explore the targeting effect of the aptamer. Both breast cancer cells (MCF-7) and breast cells (MCF-10A) were used for the experiments. The results show that the aptamer conjugated to an anticancer drug can be used to target cancer cells and ultrasound can enhance drug delivery. This method can significantly reduce the side effects of the anticancer drug and achieve favorable therapeutic effects.

INDEX TERMS Aptamer, cancer, drug delivery, ultrasound.

I. INTRODUCTION

Breast cancer has the highest incidence among cancers in women [1]. Doxorubicin is one of the most commonly used chemotherapeutic drugs in the treatment of breast cancer [2]. Doxorubicin (DOX), also known as adriamycin, is a compound of anthracycline and has been widely used in hematological malignancies, as well as breast, prostate, uterine, ovarian, stomach, and liver cancer [3]. However, DOX is also associated with serious side effects, such as increased lipid oxidation, inhibition of nucleic acid and protein synthesis, abnormal calcium ion regulation, tumor cell resistance, and cardiotoxicity [4]. To effectively treat cancer, high drug concentrations are required at the tumor site. However, only 5% or less [5] of the drugs enter the tumor cells. The remaining dose can result in damage or even death of normal cells.

Ultrasound has been used for medical applications for a long time. Ultrasound drug delivery is one of the major

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research areas. Ultrasound frequencies ranging from 1 to 10 MHz have been used to increase drug molecular delivery into cells [6]–[10]. The ultrasonic intensity at 0.64 W/cm^2 did not cause substantial cell damage [11], whereas ultrasound exposure at 1 and 2.1 W/cm^2 could induce serious cell death [11]. The damaging effect of ultrasound on the cell membrane and cell viability is intensity dependent. Most studies of ultrasound drug delivery were performed with microbubble liposomes. Although this method can increase drug delivery, it is difficult to achieve targeted therapy [6], [12], [13]. If a targeting molecule such as aptamer specific for a specific cancer biomarker can be used and conjugated with a cancer drug, the conjugated molecule can simultaneously increase the therapeutic effect and reduce side effects.

Aptamers are oligonucleic acids (DNA, RNA, and XNA) or peptides that bind to a specific target [14]. Oligonucleic acids or peptides can be combined with a variety of targeted molecules, such as proteins and nucleic acids, through the systematic evolution of ligands by exponential

enrichment (SELEX) screening technology to achieve high affinity and specificity [15], [16]. In recent years, aptamers have become increasingly widely used in biomedicine because not only they have similar functions as antibodies, but they can also recognize more targets [17] than just antigens. Possible targets recognized include ions, peptides, small molecule compounds, proteins, and nucleic acids. Aptamers are stable in the presence of heat, acids, and bases. Aptamers compared to proteins are much easier to synthesize. Therefore, aptamers are often used in medicines such as drug carriers [18].

The aptamer used in this study is a DNA aptamer that was synthesized against the MAGE-A3 antigen. This aptamer was obtained by SELEX [19], [20]. Since the sequence consists of 52 base pairs (ATCCAGAGTGACGCAGCAAGCAC TCAATATTCCCTGGACACGGTGGCTTAGT), it is abbreviated AP52.

SELEX was extensively used for aptamer screening [20]. The screening process of SELEX, briefly stated consists of synthesized oligonucleotide libraries that are subsequently allowed to bind to the target, followed by the removal of weakly binding ligands. Strongly binding ligands are amplified with the polymerase chain reaction (PCR) to generate a new aptamer library for the next round of screening. After 8 to 20 rounds of screening, the final collection of the active nucleic acid aptamer is produced together with the identification of the aptamer sequence [21].

The major histocompatibility complex (MHC) is a cell surface glycoprotein complex found in most vertebrates. Human MHC glycoproteins are known as human leukocyte antigens (HLAs) [22]. Human MHC proteins can be divided into two major classes. MHC class I proteins are located on all nucleated cells and work by allowing infected cells to present antigens to cytotoxic T cells. MHC class II proteins are only distributed on antigen-presenting cells (APCs), such as macrophages, B cells, and dendritic cells, and its function is to present antigens to helper T cells through endocytosis. Both of the above MHC classes recognize antigens presented by T cells, thereby activating the immune response in vivo [23]. MAGE is known as the melanin antigen gene [24] that is widely used to detect cancer cells. MAGE-A3 has one of the highest expression levels of tumor-specific antigens. MAGE-A3 is presented in both MHC class I and II proteins [25], so MAGE-A3 is considered to be an important biomarker for various cancers. MAGE-A3 is expressed in 56% of head and neck squamous cell carcinomas, 30% of non-small cell lung carcinomas, 16% of colorectal carcinomas, and 12% of breast tumors [26], [27]. Therefore, targeting MAGE-A3 could be an efficient method for either cancer treatment, diagnosis, or both.

II. MATERIALS AND METHODS

The experimental scheme examined three items: (1) the effect of ultrasound on cell viability; (2) aptamer and cell bonding at different times; and (3) confirmation of aptamer conjugates entering into cells by cytotoxicity assay. The overall goal is to understand changes of cancerous and normal cells after ultrasound treatment with and without aptamer-conjugated drugs. First, we treat cancer cells and normal cells with constant parameters without drug exposure. If these conditions do not cause substantial cell mortality, we can subsequently conclude that cell mortality is caused by drugs in later experiments. Then, the aptamer was added to both types of cells for ultrasonic treatment with different incubation times to determine the optimal incubation time for maximal binding. Finally, the aptamer and free DOX (DOX without attachment to aptamer) were added to the cells, and the treated cells were incubated for 48 hours to analyze cell viability with a cytotoxicity test.

A. MATERIALS
Doxorubicin in HCl salt was purchased from Sigma-Aldrich (St. Louis, MO). DOX was dissolved in Milli-Q water at a concentration of 2 mM and stored frozen at -20 °C in the dark. The aptamer AP52 was purchased from Genomics BioSci & Tech (Taipei, Taiwan). It was purified by high-pressure liquid chromatography (HPLC), and the sequence was based on a sequence from Wang et al. [19]. AP52 was dissolved in Milli-Q water at a concentration of 1 mM and stored frozen at -20°C in the dark.

B. CELL CULTURE
Human breast adenocarcinoma cells (MCF-7, ATCC: HTB-22) were derived from a metastatic site from a pleural effusion. These cells were obtained from American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS), and 1X penicillin-streptomycin-glutamine (PSG). Human breast cells (MCF-10A, ATCC: CRL-10317) were derived from a nontumorigenic epithelial cell line. These cells were obtained from American Type Culture Collection (ATCC). Cells were cultured in Mammary Epithelium Basal Medium (MEBM), 5% fetal bovine serum (FBS), PSG, 1 μM hydrocortisone, 10 ng/ml epidermal growth factor (EGF), and 5 μg/ml, human recombinant insulin in zinc solution.

All cells were incubated at 37 °C in a humidified atmosphere with a 5% CO_2 incubator. Cells were sub-cultured after filling their growth plates. Cells were harvested in a 15 ml centrifuge tube using 0.25% trypsin-EDTA (1X) and centrifuged at 400 relative centrifugal force (rcf) for five minutes. Then, the cells were mixed and shaken with trypan blue. The level of cell staining, indicative of cell viability, was measured by a cell counter. Finally, cells were transferred to a disk for ultrasound exposure.

C. APTAMER DOXORUBICIN CONJUGATION
An aptamer-doxorubicin conjugate was produced as follows. The first step was DNA denaturation, and the appropriate amount of AP52 was placed in a rapid temperature gradient nucleic acid replicator (Bio-Rad C1000) and heated to 95 °C for 5 minutes; then, the sample was removed and placed on ice
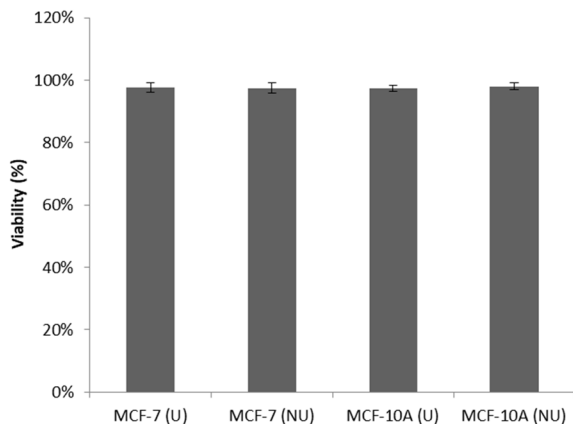
FIGURE 1. Effect of ultrasound on cell viability. Cancer cells (MCF-7) and normal cells (MCF-10A) were treated with ultrasound without any drugs. Labels denote cells given ultrasonic treatment (U) and not given ultrasonic treatment (NU). The cell viability was assessed with trypan blue. Data are expressed as the mean ± standard error calculated from four independent experiments (n = 4).

for 30 minutes. The second step was mixing DOX and AP52. DOX and AP52 were prepared in cell culture medium at a molar ratio of 1:1.5, resulting in a final DOX concentration of 5 μM. Finally, the aptamer and DOX conjugate were synthesized. DOX and AP52 were placed in a rotary mixer and mixed in the dark for one hour to complete the synthesis process for the DOX and AP52 conjugate.

D. ULTRASOUND EXPOSURE

The ultrasonic exposure was delivered using the setup described below. It includes an ultrasonic applicator (Sonicator® 740, Mettler Electronics, Anaheim, CA, USA), acrylic constant temperature circulating water tank, 6 cm cell culture plate rotating rod, and degassing device. Before the experiment, the water tank was filled and degassed at 37°C. Experiments were initiated after dissolved gas levels were reduced to 1 ppm (mg/l). An ultrasonic frequency of 1 MHz, intensity of 0.8 W/cm^2, and a 100% duty cycle of 60 s was used in all experiments. The temperature of the samples was kept at 37°C throughout the sonication process.

E. CELL VIABILITY ASSAY

A cell viability assay was used to test the viability of cells after ultrasonic treatment. Before the treatment, the dead cells in the culture medium were removed with PBS (phosphate buffered saline), and the culture medium was replaced. Afterward, the cells were harvested by trypsinization, and the suspension was centrifuged at 400 rcf for five minutes. The cells were stained in an equal volume of trypan blue and counted with an automated cell counter (Invitrogen) to assess cell viability.

F. DRUG UPTAKE

Before the ultrasound exposure, the dead cells in the culture medium were removed with PBS. Culture medium or the aptamer drug were then separately added to the cells and incubated for different time durations. Then, the cells were
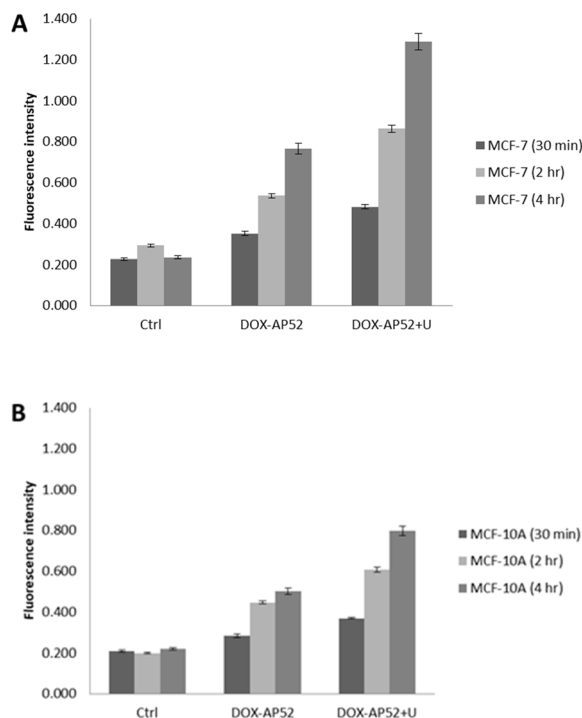
FIGURE 2. Intracellular doxorubicin fluorescence intensity at different incubation times. MCF-7 (A) and MCF-10A (B) cells were incubated with the aptamer drugs for different times (30 min, 2 hours, and 4 hours) and then underwent ultrasonic treatment. Labels indicate the following: Cells that did not undergo ultrasonic treatment or receive drugs (Ctrl), cells that underwent ultrasonic treatment and received AP52 + DOX (DOX-AP52 + U), cells that did not undergo ultrasonic treatment but did receive DOX + AP52 (DOX-AP52). The fluorescence intensity of intracellular DOX was measured by a fluorescence analyzer. Data are expressed as the mean ± standard error calculated from eight independent experiments (n = 8).

treated with ultrasound. After the treatment, the cells were harvested as described above. Then, the cells were mixed with culture medium and placed in a 96-well plate for fluorescence analysis. The fluorescence from DOX was detected with an excitation wavelength of 485 nm and an emission wavelength of 590 nm. Intracellular drug uptake was judged by the results of a fluorescence analyzer (Fluoroskan & Luminoskan, Thermo Scientific).

G. CYTOTOXICITY ASSESSMENT

After ultrasonic treatment, the cells were placed in a 96-well plate and incubated for 48 hours. Test compounds (Alamar-Blue cell viability assay reagent) and vehicle controls were added so that the final volume was 100 μl in each well. The cells were cultured at 37 °C in an incubator for 4 hours and then measured by a fluorescence analyzer. Cell viability was calculated as the ratio of the experimental value of the sample to that of the control cells that received no treatment.

H. STATISTICAL ANALYSIS

The experimental data in this study are expressed as the mean ± standard error in each independent experiment. To further confirm the effect of the aptamer on cells in this study, we analyzed the samples with t-tests. Significance was
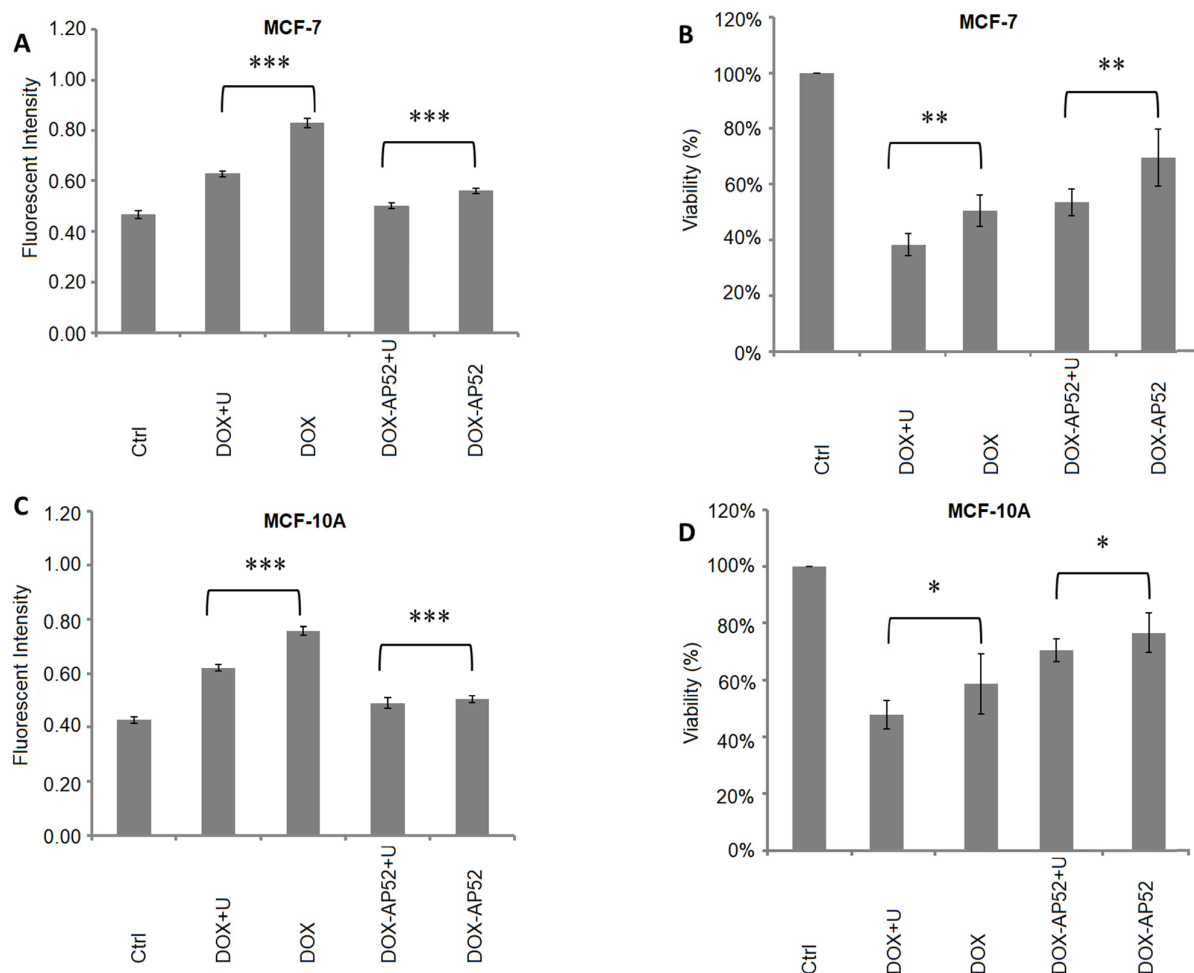
FIGURE 3. Relationship between the fluorescence intensity of intracellular DOX and cell viability. Graphs denote MCF-7 fluorescence intensity (A), MCF-7 viability (B), MCF-10A fluorescence intensity (C), and MCF-10A viability (D) respectively. Column labels indicate the following: Cells that did not undergo ultrasonic treatment and did not receive any drugs (Ctrl), cells that did undergo ultrasonic treatment and received free DOX (DOX + U), cells that did not undergo ultrasonic treatment and received free DOX (DOX), cells that did undergo ultrasonic treatment and received DOX + AP52 (DOX-AP52 + U), and cells that did not undergo ultrasonic treatment and received DOX + AP52 (DOX-AP52). Data are expressed as the mean ± standard error calculated from eight independent experiments. Statistical analysis was performed using t-tests, and the differences were considered significant when p < 0.05 (NS, not significant, * p < 0.05, ** p < 0.01 and *** p < 0.001).

defined as p < 0.05 (NS, nonsignificance, *p < 0.05, **p < 0.01 and ***p < 0.001).

III. RESULTS AND DISCUSSION

A. EFFECT OF ULTRASOUND ON CELL VIABILITY

Ultrasonic parameters were: intensity of 0.8 W/cm^2, frequency of 1 MHz, and time duration of 60 s. The results (Fig. 1) show that the cell viability of the cancer cells and normal cells after ultrasonic treatment was over 97%, indicating that the ultrasound intensity at this level did not cause serious cell death. Therefore, the direct cause of cell death in subsequent experiments is not by ultrasound.

B. APTAMER AND CELL BONDING AT DIFFERENT TIMES

Then, the aptamer was added to the cancer cells and normal cells, and the cells were incubated with the aptamer for different durations (30 minutes, 2 hours, and 4 hours) followed by ultrasonic treatment. The intensity of DOX fluorescence

in the cells was determined by fluorescence analysis. Results indicate that longer incubation time ends in greater binding between cell and drug, regardless of whether the cells are normal or cancerous (Fig. 2A).

1) OPTIMAL BINDING TIME

The intensity of DOX fluorescence in the cells changed significantly after 2 hours of incubation, and the DOX fluorescence value in MCF-10A cells was close to saturation after 2 hours (Fig. 2B). Therefore, in all subsequent experiments, the aptamer and cell binding process was carried out for 2 hours with the experimental parameters described above.

2) ULTRASOUND ENHANCES APTAMER DRUG UPTAKE

Regardless of whether the cells were cancer cells or normal cells, the intensity of DOX fluorescence in the cells after ultrasonic treatment was higher than in untreated

cells (Fig. 2), which shows that ultrasound can increase the cellular uptake of the drugs. To further confirm that the aptamer-drug conjugates are absorbed by the cells, we also measured cell viability with cytotoxicity assays described in the following section.

3) TARGETING APTAMER DRUGS TO CANCER CELLS

Regardless of whether ultrasound was applied, the fluorescence from cancer cells due to the aptamer-DOX complex was higher than that from normal cells. The results showed that the aptamer (AP52) we used in the experiment did target breast cancer cells. Therefore, the active targeting and aggregation levels into the cancer cells are high.

C. CONFIRMATION OF CELLULAR UPTAKE OF THE APTAMER CONJUGATES BY CYTOTOXICITY ASSAY

1) COMPARISON OF DRUG UPTAKE AND CELL VIABILITY

In the next step, the cancer cells and normal cells were separately incubated with the aptamer-drug conjugates and free DOX (DOX not attached to aptamers) for 2 hours and then subjected to ultrasonic treatment. The intensity of DOX fluorescence in the cells was determined by fluorescence analysis. Finally, the cytotoxicity test was performed after the treated cells and drug were incubated for 48 hours, and the cell survival rate was recorded. The fluorescence intensities of the cells, both with and without ultrasound treatment after being incubated with the different drugs, were compared with each other with t-tests. The results (Fig. 3A, C) show that the P values are all less than 0.01, indicating that ultrasound had a significant effect on drug delivery. In addition, as seen in Fig. 3, fluorescence intensity of DOX within the cells and cell survival rate are negatively correlated, thereby demonstrating that the drug did enter the cells and caused cell death to achieve therapeutic effects.

2) COMPARISON OF MCF-7 (DOX + U) AND MCF-7 (DOX-AP52 + U)

DOX primarily works by damaging DNA and thereby killing cells; however, it cannot differentiate between tumor cells and fast-growing normal cells, thus causing drug side effects. In Fig. 3A, the signal from MCF-7(DOX + U) differs from that of MCF-7(DOX-AP52 + U) depending on whether the aptamers (AP52) were added. The results show that the intracellular fluorescence concentration of MCF-7(DOX-AP52 + U) is not higher than that of MCF-7(DOX + U). Since the expression level of MAGE-A3 is only approximately 12%, the selective effect is not very pronounced. In addition, free DOX penetrates the cell membrane easier than the aptamer-DOX conjugates. Therefore, the fluorescence in MCF-7(DOX-AP52 + U) cells was slightly lower than that in MCF-7(DOX + U) cells.

3) IMPROVEMENT OF DRUG DELIVERY

The survival rate of cancer cells and normal cells was analyzed, and the ratios of the cancer cell survival rate to the

TABLE 1. Cell viability after the cytotoxicity test (n = 8). Data are expressed as mean ± standard errors.

	Ctrl	DOX+U	DOX	DOX-AP52+U	DOX-AP52
MCF-7 Viability	100%	38±4%	51±6%	54±5%	70±10%
MCF-10A Viability	100%	48±5%	59±11%	71±4%	77±7%

normal cell survival rate were obtained for each case. Results are as follows: DOX + U (80 ± 0.1%), DOX (86 ± 0.19%), DOX-AP52 + U (75±0.08%), and DOX-AP52 (90±0.15%). The use of aptamers as drug carriers coupled with the use of ultrasound was the best treatment in our study (Table 1).

IV. CONCLUSION

In summary, our data suggest that aptamer drugs combined with ultrasound treatment can enhance the survival ratio of normal cells to cancer cells. Therefore, the resulting side effects can be expected to be minimized. Since the cancer cell line we used only had 12% MAGE-A3 expression, the targeting effect should be much better if the MAGE-A3 expression level is much higher. This improvement could be attributed to the specific targeting of aptamers, which resulted in the drugs having an enhanced targeting effect against cancer cells with MAGE-A3 biomarkers. Our results suggest that the use of the aptamer-DOX conjugate in combination with ultrasound treatment can be a promising new strategy to improve the efficiency and reduce the side effects of conventional chemotherapy.

V. FUTURE PROSPECTS

We plan to select cancer cells with higher expression of MAGE-A3, perform more ultrasonic experiments under different conditions to find the optimal conditions for ultrasound irradiation, and finally apply this combination of aptamer and ultrasound to animal experiments to determine the extent to which this combination can enhance drug delivery and reduce side effects.

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TSAI-KAI JU received the B.S. and M.S. degrees in zoology from National Taiwan University (NTU), in 1995 and 1997, respectively, and the Ph.D. degree from the Institute of Molecular and Cellular Biology, NTU, Taipei, Taiwan, in 2004. He received Postdoctoral training from the Department and Graduate Institute of Pharmacology, NTU, from 2005 to 2008 and then he was a Technical Consultant from 2008 to 2013 with the Instrumentation Center, NTU, National Science Council (now Ministry of Science and Technology), Taiwan. Since 2014, he has been with the Genomics Research Center, Academia Sinica, Taiwan. His current research interests include investigating the next generation sequencing techniques and the cytoskeletal proteome.

NELSON G. CHEN was born in Knoxville, TN, USA, in 1977. He received the B.S. degree in engineering science from the University of Tennessee, in 1999, and the M.S. and Ph.D. degrees in biomedical engineering from the University of Michigan, in 2001 and 2009, respectively. From 2008 to 2013, he was a Research Scientist with Sci-Tec, Inc. Since then, he has been an Assistant Professor with National Chiao Tung University, Hsinchu, Taiwan, where he heads the Applied Ultrasound Laboratory with the Department of Electrical and Computer Engineering. His research interests include the interaction of ultrasound with various suspended particles and associated bioeffects and their measurement, the study of chemical fingerprints and their properties, and applications.

CHUN-JEN HSIAO received the B.S. and M.S. degrees in Medical Technology from National Cheng Kung University, Tainan, Taiwan, in 2002 and 2004 respectively, and the Ph.D. degree from the Institute of Immunology and Microbiology of National Yang-Ming University, Taipei, Taiwan, in 2015. Since 2015, he has been a Postdoctoral Fellow with the Genomics Research Center, Academia Sinica, Taipei, Taiwan. His research interests include developing tools for clinical diagnosis and analysis. His research interests include the rapid diagnosis of disease and identification of microorganisms, biomarker discovery, proteomics, microarrays, and mass spectrometry.
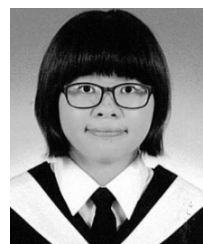
JU-YAO CHANG was a master's student with National Chiao Tung University, Institute of Biomedical Engineering. She is currently with the Genomics Research Center, Academia Sinica. Her research interest includes the application of ultrasound toward drug delivery.

CHIN-YU WANG received the Ph.D. degree in applied chemistry from National Chiao Tung University (NCTU), Hsinchu, Taiwan, in 2002. Her current research interest includes targeted drug delivery using aptamers against shared tumor-specific peptide antigens.

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