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Ultrasound Triggered Cell Death in vitro with Doxorubicin Loaded Poly Lactic Acid Contrast Agents

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Abstract

Traditional chemotherapy generally results in systemic toxicity, which also limits drug levels at the area of need. Two ultrasound contrast agents (UCA), with diameters between 1–2 μm in diameter and shell thicknesses of 100–200 nm, composed of poly lactic-acid (PLA), one loaded by surface adsorption and the other loaded by drug incorporation in the shell, were compared *in vitro* for potential use in cancer therapy. These poly lactic-acid (PLA) UCA platforms contain a gas core that in an ultrasound (US) field can cause the UCA to oscillate or rupture. Following a systemic injection of drug loaded UCA with external application of US focused at the area of interest, this platform could potentially increase drug toxicity at the area of need, while protecting healthy tissue through microencapsulation of the drug. *In vitro* toxicity in MDA-MB-231 breast cancer cells of the surface-adsorbed and shell-incorporated doxorubicin (Dox) loaded UCA were examined at 5 MHz insonation using a pulse repetition frequency of 100 Hz at varying pressure amplitudes. Both platforms resulted in equivalent cell death compared to free Dox and US when insonated at peak positive pressure amplitudes of 1.26 MPa and above. While no significant changes in cell death were seen for surface adsorbed Dox-UCA with or without insonation, cell death using the platform with Dox incorporated within the shell increased from 16.12 to 25.78% ($p = 0.0272$), approaching double the potency of the platform when insonated at peak positive pressure amplitudes of 1.26 MPa and above. This mechanism is believed to be the result of UCA rupture at higher insonation pressure amplitudes, resulting in more exposed drug and shell surface area as well as increased cellular uptake of Dox containing polymer shell fragments. This study has shown that a polymer UCA with drug housed within the shell may be used for US triggered cell death. US activation can be used to make a carrier significantly more potent once in the area of need.

Introduction

Traditional chemotherapy is plagued by its nondiscriminatory approach, allowing drug to circulate throughout the body, causing systemic effects as well as at the tumor location. This approach leads to the familiar side effects of cancer treatments such as organ toxicity, hair loss, weakness, and nausea, while providing sub optimal drug concentration at the tumor site. Over 80 % of cancers involve solid tumors, roughly half of which lead to metastasis and death [1]. Direct drug delivery to solid tumors is inhibited by their irregular vasculature, high internal pressure, fibrillar collagen in the extracellular matrix and uneven blood flow [2]. Doxorubicin (Dox) can be used as part of the regime to treat liver, breast, ovarian, and lung cancer [3]. Despite these successes, side effects of Dox include nausea, decreases in white blood cell

counts, alopecia, and heart arrhythmias [3]. Studies have also shown a dramatic risk in congestive heart failure when the cumulative dosage of Dox exceeds 550 mg/m² [3]. The success Dox has shown in a variety of solid tumor cancers combined with the desire to minimize the well documented side effects makes it an ideal candidate for targeted delivery.

One particular approach involves the use of ultrasound contrast agents (UCA). UCA consist of an inner core (generally gas) that provides an impedance mismatch capable of reflecting ultrasound (US) waves, providing contrast to US images. These agents must be smaller than 5 μ m in order to pass through the capillary beds and require an outer shell for stability. They have been successfully fabricated from lipids, surfactants, and various polymers and are well reviewed by Goldberg et al. [4]. Within our laboratory we have developed UCA from both poly lactic-acid (PLA) and poly lactic-co-glycolic acid (PLGA). These agents provide over 20 dB of US enhancement *in vitro* and *in vivo* [5], and have an acoustic half life of over an 50 mins [5]. Similar polymer-shelled agents have also been developed on the commercial scale [6].

Current efforts involving US employ both injection of free drug combined with UCA, and UCA loaded with drug, circulated throughout the body and triggered to release drug using external US at a desired location [7,8]. To date the majority of research has focused on lipid-shelled agents. One of the first groups to investigate this was that of Unger who encapsulated paclitaxel contained in an oil layer inside phospholipid-stabilized UCA and insonated with 100 kHz therapeutic US using a pulsed wave with average temporal intensity of 0.8 W/cm² over 30 mins [9].

In terms of polymer UCA for drug delivery Kooiman et al. have successfully encapsulated Sudan Black as a model drug within an oil/gas core and successfully released it using a 10 cycle sine wave generated from a 1 MHz transducer with peak-negative-pressures of 0.51 MPa [10]. This same group has shown that paclitaxel can successfully be encapsulated within the core of this agent and released *in vivo* using 1 MHz pulsed US at a PRF of 50 Hz and mechanical index of 0.7 within MC-38 mouse colon adenocarcinomas, substantially halting tumor growth for 4 days compared to controls [11].

Despite these advances little work has examined the efficacy of a polymer shelled UCA delivery vehicle *in vitro*. *In vitro* experimentation provides opportunities for cost effective optimization of parameters such as agent type, acoustics, treatment cycle and dosage that would otherwise be costly and wasteful of animals when using *in vivo* trials. *In vitro* trials are expected to translate well into future *in vivo* work by providing a measure of the agent's ability to become more potent once activated at a particular site within the body. Additionally, efforts were made to keep parameters (cell type, insonation parameters, CA dosage, and treatment schedule) within a clinically viable range. However, it is acknowledged that full *in vivo* environmental conditions cannot be completely simulated *in vitro*.

This paper examines two drug loaded polymer UCA, one in which Dox is surface-coated on a PLA agent (S-Dox-UCA) and one in which Dox is incorporated in the shell of a PLA agent (I-Dox-UCA). The agents were used *in vitro* against MDA-MB-231 cells, a breast cancer cell line, to show the agent's potential for *in vivo* use as well as to investigate the mechanisms of US-triggered cell death. These findings bring a new perspective to the field in that to our knowledge they are the first reported efficacy study using chemotherapeutic loaded, polymer shelled contrast agents *in vitro*.

Materials and Methods

Materials

Poly-lactic-acid (PLA) (100 DL Low IV, MW = 83 KDa) was purchased from Lakeshore Biomaterials (Birmingham, AL). Poly (vinyl alcohol) (PVA), 88% mole hydrolyzed, with a MW of 25 KDa, camphor and Dox were all purchased from Sigma-Aldrich (St.Louis, MO). Ammonium carbonate was purchased from J.T. Baker (Phillipsburg, NJ). MDA-MB-231 breast cancer cells (passage 5) and RPMI cell culture media were originally purchased from ATTC (Manassas, VA). Fetal bovine serum (FBS) and Opticell® culture cassettes were purchased from Fisher Scientific (Waltham, MA). All other chemicals were reagent grade from Fisher Scientific (Waltham, MA). A live/dead reduced biohazard viability/cytotoxicity assay (Kit #1 L-7013) was purchased from Molecular Probes (Eugene, OR). All other chemicals were reagent grade from Fisher Scientific (Waltham, MA).

Sample Preparation

UCA were fabricated using a (W/O)/W double emulsion method developed in our laboratory, with some modifications [12,13]. Modifications include addition of drug (discussed later), and pulsed sonication in an ice bath rather than continuous insonation at room temperature. For UCA fabrication, aliquots of 0.5 g of PLA and 0.05 g of camphor were dissolved in 10 ml of methylene chloride while stirring. After the PLA had dissolved, 1 ml of ammonium carbonate (4% w/v) was added and the mixture sonicated in an ice bath at 20 kHz using 110 Watts of applied power for 30 seconds at 3 seconds on, 1 second off to a sonicator probe (Misonix Inc. CL4 tapped horn probe with 0.5" tip, Farmingdale, NY). The resulting (W/O) emulsion was then added to 50 ml of 4°C, 5 % PVA and homogenized for 5 minutes at 9000 rpm (Brinkmann Instruments, Westbury, NY). After homogenization, UCA was collected using centrifugation and washed with hexane, after which the capsules were flash frozen and lyophilized for 48 hours. As the agent undergoes freeze drying, ammonium carbonate and camphor sublime out of the capsule, leaving a void in their place. This void later fills with the gas (in this case air) when exposed to atmospheric pressure.

Two methods of loading Dox on or within these UCA have been developed in our laboratory. Using one method, 15 mg of Dox (the point at which maximal drug payload is reached) is added during the primary (W/O) emulsion, resulting in Dox incorporation within the shell of the agent (I-Dox-UCA). The second technique relies on bathing prefabricated UCA in a deionized water solution of Dox at 4°C for 24 hours, resulting in Dox being adsorbed to the surface (S-Dox-UCA). This adsorption process is due to the electrostatic attraction between the UCA and drug and results in Dox coated UCA [14]. These agents maintain roughly 75% of their original US enhancement, show smooth surface morphology, have diameters of 1-2 µm, shell thicknesses of 100-200 nm, and show an encapsulation efficiency of 22-30% (partially unpublished results) [14].

Sterilization of UCA

Plasma sterilization was performed with oxygen using a Harrick PDC-32G Plasma Sterilizer (Ithaca, NY). Seventy mg of agent was sterilized for 3 mins on the machine's high power setting (25 mA, 18W applied to the RF coil). These settings were optimized to provide fully sterile, acoustically sensitive agents. Sterilization at longer time intervals proved to dramatically decrease *in vitro* enhancement of the agent, while shorter time periods or lower power levels resulted in unsterilized samples. We have noticed that this process fully sterilizes PLA UCA without sacrificing the agent's acoustic properties, while also modifying the surface for possible drug or ligand attachment (unpublished results).

Ultrasound Triggering of Drug Loaded Agent

MDA-MB-231 cells were cultured to 70% confluency within Opticell® culture systems. Prior to treatment, media was removed and replaced with 10 ml of fresh media containing the desired Dox-loaded agent. I-Dox-UCA and S-Dox-UCA experimental groups both contained 2 mg of drug loaded agent, for a total Dox concentration of roughly 10 μ M assuming complete release of drug into the cell media (0.055 mg Dox). Each arm of the study included controls of US alone, US plus 10 μ M of free Dox, US with 2 mg of UCA containing no drug, uninsonated drug loaded UCA, uninsonated UCA containing no drug, no treatment, and 10 μ M free Dox with 2 mg UCA and US.

Opticell® cassettes were held in a 20 gallon tank of 18.6 M Ω -cm, 37°C deionized water using a clamp arm. A Panametrics (Waltham, MA) 5 MHz transducer with a 12.7 mm diameter, -6 dB bandwidth of 91%, and focal length of 50.8 mm was focused on one quadrant of the Opticell® surface. Previous studies have shown similar agents to resonate in a range of 3-5 MHz, making this frequency ideal for UCA destruction [5]. A pulser/receiver (5072 PR Panametrics, Waltham, MA) was used to generate pressure amplitudes with pulse repetition frequency equal to 100 Hz. Pressure amplitude levels were varied using the pulser/receiver's energy level setting and later determined using a 0.5 mm polyvinylidene fluoride (PVDF) needle hydrophone (Precision Acoustics, Dorset, UK). All given pressure amplitudes correspond to the peak positive values of the asymmetrical pulses at the focal plane. For the setup's varying energy levels, peak positive pressure amplitudes were measured as 0.00, 0.69, 0.92, 1.26, and 1.65 MPa, with corresponding peak negative pressure amplitudes of 0.00, 0.45, 0.57, 0.74, and 0.94 MPa.

Each quadrant of the Opticell® was insonated for 5 mins for a total of 20 minutes of insonation. After insonation, cells were stored in an incubator until determination of cell death was performed. Orientation of cells after treatment (face up vs. face down) did not result in any differences of cell death for either the insonated group or uninsonated controls. Future work will focused on media replacement post insonation to remove drug/UCA not uptaken by cells, for a more realistic simulation of *in vivo* conditions. However, this proves difficult due to the possibility of temporary, but substantial cell detachment in the presence of inertial and transiently cavitating UCA, as shown by Ohl et al. who used a single finite wave of 2.5 μ s and -4MPa amplitude to induce UCA rupture and cell detachment [15].

Determination of Cell Death

Cell death was calculated as the percentage of dead or cytotoxic cells to the total number of cells. For viability after treatment a Live/Dead Reduced Biohazard Viability/Cytotoxicity Kit #1 (Molecular Probes, Eugene, OR) was used. Cells were incubated for 15 mins at room temperature with a live stain (SYTO 10 green fluorescent nucleic acid stain) and a cytotoxic stain (DEAD Red (ethidium homodimer-2) nucleic acid stain). After staining, cells were fixed with 4 % glutaraldehyde (Sigma, St. Louis, MO) for 1 hour at room temperature. Fluorescent images were then obtained using an Olympus IX71 fluorescence microscope. Live cells (SYTO stain) were read using a 510-540 nm wavelength filter, while dead (DEAD stain) cells were read using a 570-630 nm filter. Images were processed using SPOT image software to adjust intensity levels. Images of stained cells in 10 adjacent fields were then counted blindly for each marker after the images and had been overlaid.

Statistical Analysis

Statistically significant differences for multiple groups were determined using a one way ANOVA with a Newman-Keuls post test. Statistical significance between individual groups was determined using a Student's t-test. All testing was done using Prism 3.0 (GraphPad, San

Diego, CA). Statistical significance was determined using $\alpha = 0.05$. Error bars were displayed as standard error about the mean.

Results and Discussion

Effects of US on Efficacy of Surface-bound Dox

The S-Dox-UCA was first examined as an US-sensitive drug carrier. Dox adsorbed microbubbles were insonated at 5 MHz, 1.65 MPa in the presence of MDA-MB-231 cells as described in the methods section and cell death measured after 48 hours. These results are shown in Figure 1.

As expected, little cell death was seen in untreated cells (2.1 +/- 1.5%), and by introducing US alone (5.6 +/- 6.0 %), UCA alone (10.7 +/- 16.8 %) or US with unloaded UCA (7.2 +/- 4.3%). When Dox with and without US was introduced into the system cell death became significantly different from controls ($p < 0.01$). However, no significant difference was found between treatment with free Dox accompanied by US (23.8 +/- 9.9 %), S-Dox-UCA without US (25.5 +/- 11.7 %), and S-Dox-UCA with US (29.23 +/- 12.2). The most dramatic, and statistically significant ($p = 0.0272$ relative to S-Dox-UCA with US) increase was achieved when cells were treated with free Dox, blank UCA and US (38.3 +/- 12.2 %).

The S-Dox-UCA results suggest that although Dox is initially adsorbed to the polymer shell, this does not prevent drug action, possibly due to release of Dox by polymer degradation at the surface. However, if free Dox and UCA are administered, the interaction of US with the UCA translates into an increase in susceptibility of the cells to the free Dox. Increased cellular membrane permeability in the presence of insonated UCA has been observed by other groups at slightly lower frequencies (1MHz) and varying acoustic pressure amplitudes (.15-1.39 MPa peak-negative-pressure), and is attributed to interactions between the inertial or transiently cavitating bubble and the cell membrane [16-19]. While a strategy of free drug, free UCA and ultrasound may lead to optimal cell death and has been frequently reported in the literature [20-22] this approach does not address the main concern of reducing systemic toxicity in the healthy (uninsonated) areas unless the dose can be drastically reduced by this procedure.

Effects of US on Efficacy of Incorporated Dox

The above investigation was repeated using UCA with incorporated Dox (I-Dox-UCA). The results are shown in Figure 2. No significant difference was measured among the control samples and introduction of Dox in all forms induced the expected statically significant increase in cell death ($p < 0.01$).

However, with I-Dox-UCA, there was a statistically significant difference between the insonated drug loaded agent (25.78%) relative to the uninsonated agent (16.12 %) ($p=0.0272$). The experimental group of insonated drug loaded agent showed equivalent efficacy as the 10 μ M free Dox combined with US. This is important since, unlike the free Dox, the incorporated Dox is not available to cause systemic toxicity. While the free Dox, US and blank UCA still showed significantly more cell death ($p=0.0124$), again this strategy does not limit systemic toxicity.

Comparison of Loading Methods

Figure 3 shows a comparison of the two loading methodologies in the presence and absence of insonation (Data reproduced from figures 1-2).

Both platforms showed statistically equivalent efficacy post insonation, however the I-Dox-UCA showed roughly 10% less cell death compared the S-Dox-UCA samples when not

insonated ($p=0.0336$). These results indicate the incorporated platform is a better choice for targeted cancer therapy as the two platforms show relatively the same efficacy once insonated, but the I-Dox-UCA platform significantly reduces toxicity prior to activation ($p=0.0272$ compared to the uninsonated control). By using a platform with lowered systemic toxicity, overall treatment dosage may be increased, both minimizing side effects and increasing drug efficacy.

Effects of Insonation Pressure Amplitude on the Dox UCA

Insonation pressure amplitudes were varied for both S-Dox-UCA and I-Dox-UCA. Cell death for each of these groups at 48 hours is shown in figure 4.

Varying insonation pressure amplitude showed no statistically significant differences in cell death for all S-Dox-UCA. These findings are consistent with our previous findings and reinforce the hypothesis that all available drug is exposed with or without UCA destruction, resulting in limited additional cell death with sonication.

A statistically significant increase in cell death was seen for I-Dox-UCA for all pressure amplitudes larger than 0.92 MPa ($p=0.0346$). Cell populations insonated at a pressure amplitude of 1.26 MPa showed an 8.11 % increases in total cell death (a 48% increase in drug efficacy) compared to insonation at 0.92 MPa. Several groups have examined the cavitation thresholds of similar polymeric UCA with reports of thresholds as low as 0.96 to over 1.54 MPa depending on UCA shell thickness and insonation frequency [23,24]. These findings provide evidence that the incorporated agent is becoming significantly more toxic after sonication due to rupture of the agent.

Cell Death over Time with Shell Incorporated Dox-UCA

The rate of cell death using I-Dox-UCA was investigated by observing cell death over time. Cell populations were combined with the platform and insonated at 1.65 MPa for 20 mins. These results are shown in figure 5.

No statistically significant differences were seen between the insonated and uninsonated groups over the first eight hours, although cell death was 3.6 and 5.3% higher at 4 and 8 hours respectively. Between 8 and 24 hours the rate of cell death for insonated cells increased, resulting in significantly higher cell death at all time points after 8 hours. This increase in cell death rate after 8 hours in insonated populations is believed to be a result of apoptosis, not physical stresses associated with UCA cavitation, which would present immediate signs of cell death. This is consistent with the low level of cell death seen with insonated, unloaded UCA (7.15%) in figures 1-2. Images showing differences of cell death at the 48 hour time point between non-treated controls and insonated I-Dox-UCA are shown below in figure 6.

Conclusions

Need for a targeted chemotherapy delivery vehicle and benefits of ultrasound assisted drug delivery have both been well documented. We have presented two distinct forms of drug loaded PLA UCA for US-triggered drug delivery. A platform with drug incorporated within the shell has been shown superior, showing equivalent potency in MDA-MB cancer cells to free Dox combined with US. Without activation from US, the agent approaches half the platform's potency. These differences have been linked to UCA rupture and are believed to be due to both an increase in exposed drug and uptake of Dox-UCA shell fragments. This platform shows promise for an *in vivo* platform in which a systemic dose of drug loaded UCA can be used with external US to provide localized cell death at an area of need, while minimizing cytotoxicity of healthy tissue.

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Figure 1.

Percent cell death of MDA-MB-231 cells 48 hours after insonation at 5 MHz ($P=1.65$ MPa) in the presence of 2 mg of S-Dox-UCA ($10\mu\text{M}$ total Dox). No significant differences were seen between the insonated S-Dox-UCA and uninsonated S-Dox-UCA indicating minimal cell death triggered by US.

Figure 2.

Percent cell death of MDA-MB-231 cells 48 hours after insonation (5 MHz, $P=1.65$ MPa) of 2 mg ($10\mu\text{M}$ total Dox) of UCA with Dox incorporated within the shell. A 9.66% increase in cell death was seen between the insonated drug loaded agent relative to the uninsonated agent ($p=0.0272$).

Figure 3.

Cell death comparison of insonated and uninsonated groups for S-Dox-UCA and I-Dox-UCA. Significantly less cell death ($p=0.0336$) was seen between the uninsonated UCA with incorporated drug relative to the surface coated agent, while no difference was seen in efficacy between the insonated (activated) groups.

Figure 4. Effects of insonation pressure amplitude with both S-Dox-UCA and I-Dox-UCA after 48 hours. Significant increases in cell death were seen for shell incorporated agents for pressure amplitudes of 1.26 MPa and above.

Figure 5.

Cell death over 48 hours of cells insonated with the I-Dox-UCA platform. The insonated and uninsonated groups show similar cell death over the first 8 hours, but become significantly different at 24 hours and for all time points afterwards.

Figure 6. Differences in cell death in populations with a) non-treated cells and b) insonated I-Dox-UCA at a pressure amplitude of 1.65 MPa (Green = live cells, Red = dead cells, size bar = 100 µm, time = 48 hours). The decrease in cell population size with drug loaded agent is due to Dox halting cell reproduction, eventually leading to cell apoptosis.