Drug delivery in pluronic micelles: effect of high-frequency ultrasound on drug release from micelles and intracellular uptake

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Abstract

The effect of high-frequency ultrasound on doxorubicin (DOX) release from Pluronic micelles and intracellular DOX uptake was studied for promyelocytic leukemia HL-60 cells, ovarian carcinoma drug-sensitive and multidrug-resistant (MDR) cells (A2780 and A2780/ADR, respectively), and breast cancer MCF-7 cells. Cavitation events initiated by high-frequency ultrasound were recorded by radical trapping. The onset of transient cavitation and DOX release from micelles were observed at much higher power densities than at low-frequency ultrasound (20–100 kHz). Even a short (15–30 s) exposure to high-frequency ultrasound significantly enhanced the intracellular DOX uptake from PBS, RPMI 1640, and Pluronic micelles. The mechanisms of the observed effects are discussed.

Keywords: Doxorubicin; High-frequency Ultrasound; Pluronic micelles; HL-60 cells; A2780 cells; A2780/ADR cells; MCF-7 cells; Multidrug resistance; Micellar drug delivery

1 . Introduction

A new modality of drug targeting to tumors that we are currently developing is based on the drug encapsulation in polymeric micelles followed by the localized release at the tumor site triggered by focused ultrasound. The rationale behind this decreases systemic concentration of drug, diminishes intracellular drug uptake by normal cells, and provides for a passive drug targeting to tumors; the micelles should be stable enough to withstand dilution associated with the administration into the circulatory system [1–12]. Due to passive targeting, micellar-encapsulated drugs accumulate at the interstitial space in the tumor; however, the *intracellular* uptake of the micellar-encapsulated drug is much lower than that of a free drug. To enhance the intracellular uptake, we use ultrasonic irradiation. An important advantage of ultrasound is that it is noninvasive, can penetrate deep into the interior of the body, can be focused and carefully controlled. The technology for ultrasonic wave control and delivery is well advanced in the area of biomedical imaging. Ultrasound is also used in hyperthermic cancer therapy. Hyperthermia clinical trials using focused ultrasound (FUS) systems integrated into MR scanners are currently conducted in several sites in the USA and Europe, including Mayo Clinic, Brigham and Women's Hospital, and M.D. Anderson Cancer Center. Ultrasound technology allows a high degree of spatial and temporal control.

Ultrasound of various frequencies may be used in the proposed application. High-frequency ultrasound allows sharper focusing than low-frequency ultrasound but does not penetrate as deep into the interior of the body. Typical penetration depth (the depth at which 50% of the supplied ultrasonic energy is absorbed) for 1-MHz ultrasound in various tissues is 5 cm for fat, 2.7 cm for muscle, 0.9 cm for tendon, and about 0.3 cm for bone; for 3-MHz ultrasound, penetration is about 3-fold lower. In contrast, low- frequency ultrasound (20–100 kHz range) can penetrate to the depth of tens of centimeters in various tissue types [13]. In this respect, high-frequency ultrasound may be advantageous for targeted drug delivery to small superficial tumors while low-frequency ultrasound should be used for treating large and deeply located tumors.

In our previous publications, we have shown that low-frequency ultrasound partially released drug from micelles and enhanced the intracellular uptake of both released and encapsulated drug [1–12]. The polymeric micelles of these studies were formed by a Pluronic P-105 copolymer, which is a tri-block ABA-type copolymer of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO), with 37 ethylene oxide units in each side block and 56 propylene oxide units in the central block. Previously, using a custom ultrasonic exposure chamber with real-time fluorescence detection, we observed an effective drug release from micelles under the action of low- frequency

10.1016/s0168-3659(02)00262-6

ultrasound [6,10].However, at equal ultrasound power density, the degree of drug release dropped with increasing ultrasound frequency in the range of 20–100 kHz [6]. Information on the effect of high-frequency ultrasound on drug release from micelles and intracellular uptake is absent in the literature. Here we report on the effect of 1-MHz ultrasound on drug release from micelles and intracellular drug uptake by ovarian carcinoma, breast cancer, and promyelocytic leukemia cells. Megahertz range frequencies are typically used in physical therapy, ultrasound diagnostics, and hyperthermia; therefore technologies for ultrasonic delivery at these frequencies are readily available.

2 . Materials and methods

2 .1. *Drug*

Doxorubicin (DOX) was obtained from the University of Utah Hospital (SLC, UT) in a 1:5 mixture with lactose; pure DOX was bought from Sigma (St.Louis, MO). Stock solutions of DOX were kept frozen.

2 .2. *Cells*

Promyelocytic leukemia HL-60 cells grown in suspensions were kindly provided by Dr.B.K. Murrey (Department of Microbiology, Brigham Young University, Provo, UH). They were cultured in RPMI-1640 medium (Sigma) supplemented with 20% fetal calf serum, 2 mM L-glutamine, 0.2% sodium bicarbonate, and 50 mg/ml gentamicin at 37 8C in humidified air containing 5% CO_2

Drug-sensitive A2780and multidrug-resistant (MDR) ovarian carcinoma A2780/ADR cells growing in adherent monolayers were kindly provided by Dr. T.C. Hamilton (Fox Chase Cancer Center, PA). Cells were cultured in a complete RPMI 1640 medium, which in the case of A2780/ADR cells included 800 ng/ml doxorubicin (DOX) for maintaining resistance.

2 .3. *Micelles*

A micelle-forming block copolymer, Pluronic P-105 was kindly supplied by the BASF Corporation (Mount Olive, NJ) and used as a 10% solution in either phosphate-buffered saline (PBS) (Sigma) (for micellar release measurements) or a complete RPMI 1640 medium (for the intracellular uptake measurements). DOX in desired concentration was introduced into the micellar Pluronic solution from a stock solution; it spontaneously partitioned into the core of Pluronic micelles [3]. Pluronic solutions were sterilized by filtration through a 0.2-mm filter.

.4. *Measuring drug release from micelles*

The measurements of the degree of drug release are based on the decrease of DOX fluorescence intensity when DOX is transferred from the hydrophobic environment of micelle cores to the aqueous environment. A custom ultrasonic exposure chamber with real-time fluorescence detection was described previously [6]. Briefly, an argon-ion laser beam of 488 nm was directed to a drug-containing cuvette to excite fluorescence. The emissions were collected using a fiber optic collector and filtered to remove the excitation wavelength. The emissions were quantified using a photodetector, digitized with a 12-bit A/D converter, and stored in a Macintosh computer for further analysis.

Digitized fluorescence intensity data were analyzed to calculate the percent of the drug release from micelles as described previously [6]. Briefly, fluorescence intensity of a 10-mg/ml DOX solution in PBS (I_{PBS}) was measured first; the PBS solution was then carefully sucked out of the cuvette and replaced with a 10-mg/ml DOX solution in 10% Pluronic micelles. Fluorescence of this solution (I_{mic}) was measured, and a difference Imic - I_{PBS} was assumed to correspond to a 100% drug release from micelles. Then ultrasound was switched on, and DOX fluorescence under sonication (I_{us}) was recorded; if sonication induced partial drug release from micelles into the aqueous environment, I_{us} was lower than I_{mic}; the 'ultrasound on'–'ultrasound off' cycles were repeated several times to check reproducibility. The length of each ultrasound exposure cycle was 1–2 min. The scatter of the data obtained in various ultrasound cycles did not exceed 20%. The degree of drug release (DDR) was calculated as follows:

$$DDR = \frac{I_{mic} - I_{US}}{I_{mic} - I_{PBS}}$$

2 .5. *Sonication*

For sonicating HL-60 cells, a 3-ml cell suspension in DOX-containing medium was placed in the test tube inserted in the water thermostat maintained at 37 8C; ultrasonic transducer was installed next to the test tube at a distance of 2 mm, as shown in Scheme 1a. Before sonication, cells were equilibrated at 37ºC for 5 min. A2780, A2780/ADR, and MCF-7 cell were grown in six-well plates; transducer was placed under a particular well of the plate as shown in Scheme 1b; acoustic contact between the transducer and the plate was provided by the Aquasonic 100 ultrasound transmission gel (Parker Laboratories, Orange, NJ) placed on the transducer surface.

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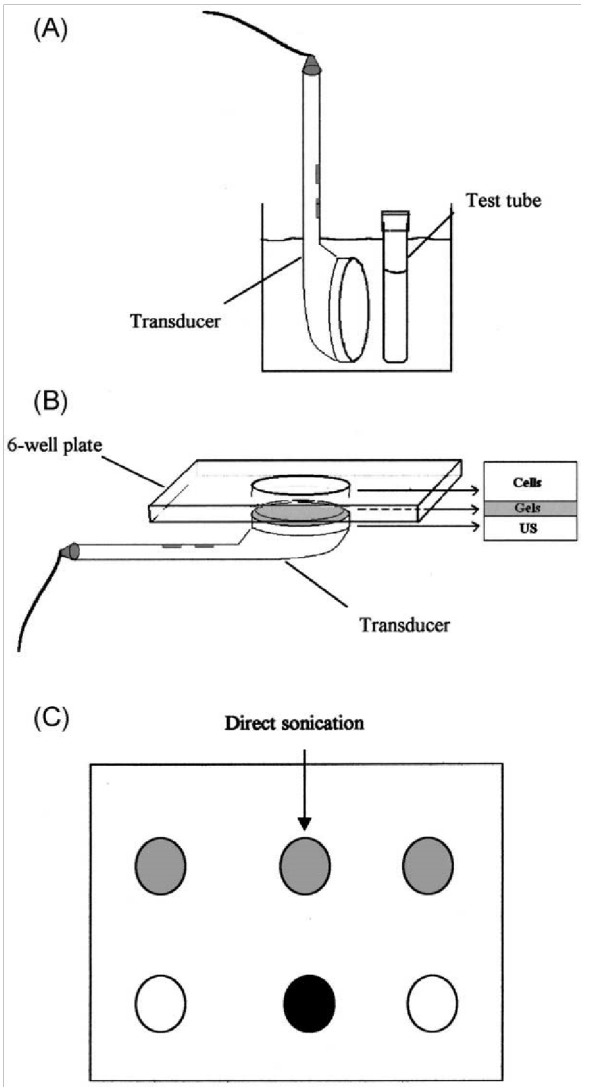

Scheme 1. Experimental settings for 1-MHz sonication of (A) HL-60 and (B,C) A2780, A2780/ADR, and MCF-7 cells; in (C), directly sonicated well is black, three wells with the tested cell suspensions are shaded.

Our experiments showed that there was acoustic contact between the directly sonicated well of the six-well plate and the wells adjacent to the directly sonicated one. These adjacent wells received about 10% of the acoustic energy supplied to the directly sonicated well. While sonication caused temperature increase in the directly sonicated well, no measurable temperature increase was observed in the wells adjacent to it. These adjacent wells

(Scheme 1c) were used for sonicating A2780, A2780/ADR, and MCF-7 cells grown in adherent monolayers; regular cell growth medium was replaced by DOX-containing medium heated to 37 8C before the start of the sonication; the plate was then placed into the incubator and cells were equilibrated for 5 min. Sonication lasted for 15–30 s; as mentioned above, no temperature increase was observed in the cell-containing wells during sonication. Control samples were kept without sonication in the same drug-containing medium at the same temperature and for the same time (in some instances, to produce measurable drug uptake, control samples were incubated for longer time than sonicated samples; this is specified in the figure captions).

To generate 1-MHz or 3-MHz ultrasound, a PTI transducer (Omnisound 3000C Accelerated Care Plus, Sparks, NV) was used. Sonication at 67 kHz was performed in the sonication bath (Sonicor Instruments, Copaique, NY).

The acoustic intensity in the tested samples at these frequencies was measured using a hydrophone (model TNU100A with PFS017A Preamplifier, NTR Systems, Seattle, WA). The voltage was recorded using Tektronix TDS3012 two-channel color digital phosphor oscilloscope (Tektronix, Beaverton, OR). Ten thousand points were recorded in 0.2 s for each measurement. The signal was averaged using mean absolute value (MAV) method; temporal average power density values are presented in the paper.

2 .6. *Measuring the intracellular uptake of DOX*

The initial concentration of cells ranged from $3*10^6$to $5*10^6$ cells/ml as counted using a hemacytometer. After exposure to DOX (10–50 mg/ ml in various experiments) and ultrasound (15–30 s), cells were counted again to measure the degree of sonolysis, upon which they were centrifuged, washed by PBS, fixed with a 3% formalin or 2.5% glutaraldehyde and analyzed by flow cytometry. Fluorescence histograms were recorded with a FAC Scan flow cytometer (Becton-Dickinson) and analyzed using Cell Quest software supplied by the manufacturer. Minimum of 10 000 events was analyzed to generate each histogram. The experiments on the effect of ultrasound on the intracellular DOX uptake with and without micelles were always conducted in parallel, at the same day and using the same batch of the cells.

2 .7. *Cavitation*

Transient cavitation was monitored by trapping free radicals formed upon sonolysis of water molecules using 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) radical trap; this trap forms relatively stable adducts with hydroxyl radicals [14]. DMPO was dissolved in PBS at a concentration of 0.1 M. The sonication was performed in darkness; upon completion of the sonication, solutions were immediately frozen and kept in liquid nitrogen until analyzed by electron paramagnetic resonance (EPR) spectroscopy.

3 . Results and discussion

3 .1. *Drug release from micelles*

The onset of DOX release from micelles at 1-MHz ultrasound was observed at much higher power densities than at a frequency range of 20–100 kHz (Fig. 1). For instance, a 10% DOX release from Pluronic P-105 micelles required a power density of 0.058 W/cm^2 at 20-kHz ultrasound, 2.8 W/cm^2 at 67-kHz ultrasound, and 7.2 W/cm^2 at 1.0-MHz ultrasound. At all frequencies, the dependence of the degree of drug release on ultrasound power density projected into the coordinate origin; the absence of a threshold suggested that a process other than transient cavitation was responsible for the drug release [12]. To characterize transient cavitation, we trapped hy-

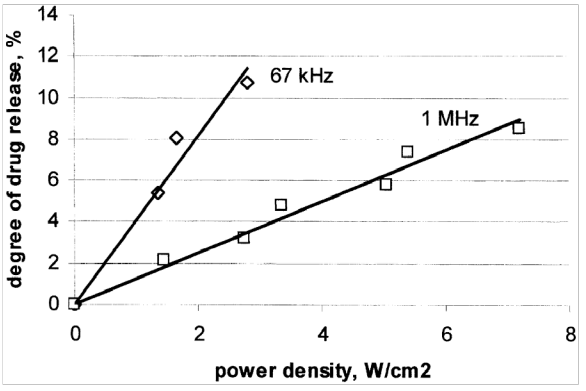

Fig. 1. The effect of ultrasound frequency and power density on the drug release from 10% Pluronic P-105 micelles.

droxyl radicals produced upon the collapse of cavitation bubbles using DMPO radical trap [14].

3.2. Cavitation

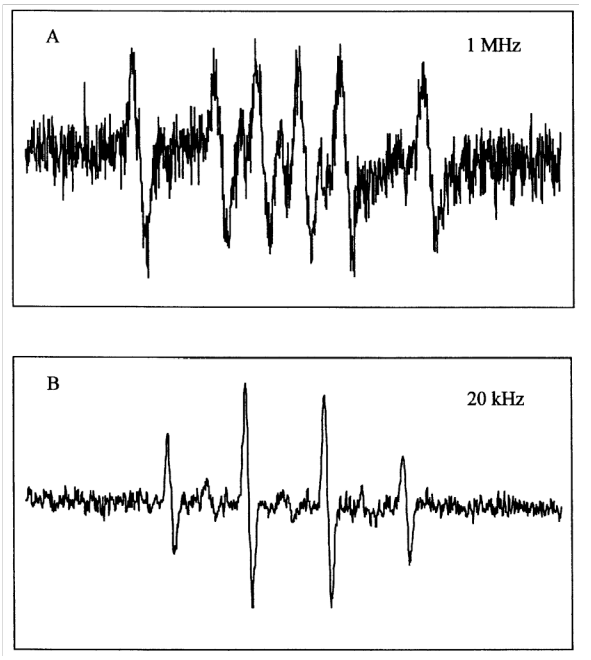

Fig. 2. EPR spectra of the radicals trapped by the DMPO trap upon sonicating DMPO solutions in PBS placed in polystyrene test tubes (diameter 12-mm): (a) sonication frequency 1 MHz, power density 7.2 W/cm , duration 5 min; and b) sonication frequency 20 kHz , instrument amplitude setting 5%, power density 0.18 W/cm , duration 2 min.

The cavitation threshold was found to dramatically increase with increasing ultrasound frequency. For example, upon a 2-min sonication, at 20 kHz, radicals were observed at a power density as low as 0.08 W/cm , while at 67 kHz, a minimum power density of 1.0 W/cm^2 was needed to observe traces of radicals, and at 1 MHz, only traces of radicals were observed at a power density of 3.6 W/cm^2 upon a 5-min sonication. The radicals formed at 1 MHz differed from DMPO/hydroxyl adducts observed at 20 kHz (Fig. 2) suggesting that at 1 MHz, secondary radicals formed upon the ultrasound-induced degradation of primary DMPO/OH adducts were recorded. Similar to our observations at lower ultrasound frequencies [12], noticeable drug release from micelles proceeded at power densities at which no radical formation was recorded supporting the hypothesis that drug release from micelles was not related to transient cavitation.

3 .3. *Effect of high-frequency ultrasound on the intracellular drug uptake*

Sonication at 1 MHz substantially increased the intracellular uptake of DOX from PBS or RPMI 1640. An example of the flow cytometry histograms for the unsonicated and sonicated A2780 cells in suspensions is given in Fig. 3. It should be noted that sonication in PBS caused substantial cell lysis; at a power density of 15.2 W/cm^2 , 25% cells were lysed in PBS, whereas no cell lysis was observed in the presence of Pluronic micelles confirming earlier data[12]. As shown in Ref. [12], cell lysis is caused by transient cavitation; sonoprotection property of Pluronic micelles presumably results from quenching transient cavitation [12]. It should be noted that in our experiments, cell lysis was caused exclusively by ultrasound treatment rather than by the cytotoxic action of the internalized drug; at very short incubation/sonication times used in this study, drug did not affect cell viability even at much higher intracellular concentrations. However, as shown in Ref. [12], presence of DOX in the non-micellar systems enhanced cell lysis due to the amplified cavitation.

Note that DOX uptake by A2780 cells sonicated for only 15 s was significantly higher than that by unsonicated cells incubated in suspension with the same concentration of DOX for 30 min (Fig. 3). The same was true for the MDR cells (Fig. 4). High-frequency sonication enhanced the intracellular drug uptake not only from the

conventional medium (PBS or RPMI 1640) but also from (or with) Pluronic micelles. Flow cytometry histograms of the MDR A2780/ADR cells unsonicated or sonicated in the presence of Pluronic micelles are shown in Fig. 4; fluorescence histograms of A2780 cells sonicated in the presence or absence of 10% Pluronic micelles are compared in Fig. 5. Note that without ultrasound, DOX uptake by the drug-sensitive cells from Pluronic micelles was about 2-fold lower than that from PBS [11]; in contrast, under sonication, only marginal differences in the average drug uptake in PBS or Pluronic micelles were observed (Fig. 5). These data imply that the proposed technique can provide for decreasing systemic concentration of free drug without compromising the intracellular drug uptake at the tumor site.

The enhancement of drug uptake from (or with) Pluronic micelles under the action of 1-MHz ultrasound was observed for all cell lines studied. An example for the breast cancer MCF-7 cells is shown in Fig. 6, and an example for the HL-60 cells is shown in Fig. 7.

Two possible mechanisms of the ultrasound-enhanced drug uptake were proposed earlier [8,9]; one mechanism is related to the drug release from micelles while the other is associated with the enhanced uptake of the micellar-encapsulated drug. As suggested by the effect of the ultrasound pulse duration on the drug uptake, at low-frequency ultrasound, both mechanisms presumably worked in concert [8,9]. At high-frequency ultrasound, our equipment did not provide for varying pulse duration; substantial drug release from micelles and noticeable increase

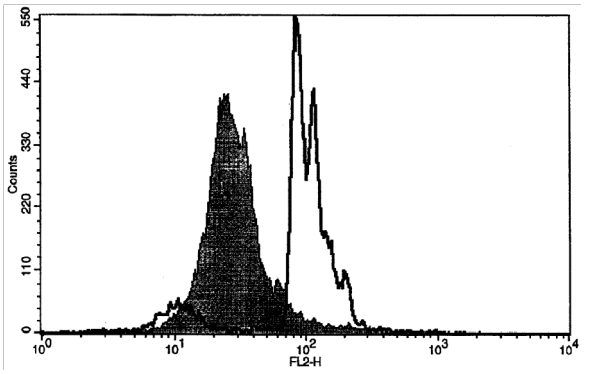

Fig. 3. Flow cytometry histograms illustrating the effect of ultrasound on the DOX uptake from PBS by the drug-sensitive A2780 cells; initial DOX concentration in the incubation medium 20 mg/ml. Unsonicated cells (shaded histogram) were trypsinized, washed by PBS and incubated with DOX for 30 min. Sonicated cells were equilibrated with DOX at 37 8C for 5 min prior to ultrasonic treatment; they were exposed to 1-MHz ultrasound for 15 s, power density 18.1 W/cm . Upon switching ultrasound on,[2] some fraction of the cells was immediately detached from the substrate; fluorescence histogram presented in figure is for the detached cells.

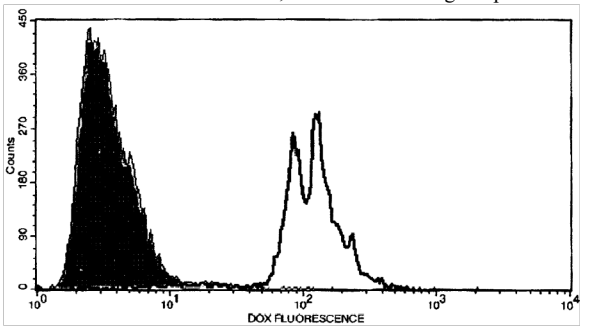

Fig. 4. Fluorescence histograms of the MDR A2780/ADR cells incubated or sonicated in the presence of 10% Pluronic micelles: shaded, unsonicated control (attached cells incubated with DOX for 5.5 min); open, cells exposed to 1-MHz ultrasound for 30 s upon 5-min equilibration with DOX at 37 8C; power density 15.2 W/cm ; initial DOX concentration in the incubation medium 20[2] mg/ml.

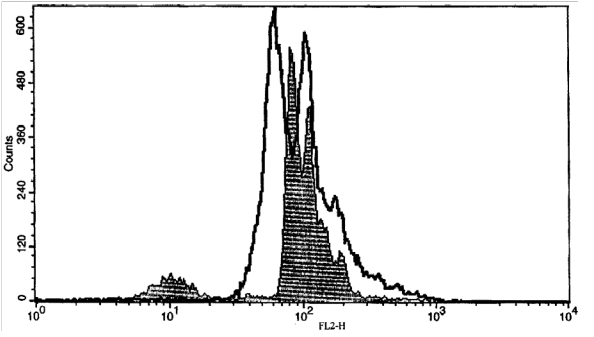

Fig. 5. Flow cytometry histograms of drug-sensitive A2780 cells sonicated with DOX in the presence (open) or absence (shaded) of 10% Pluronic micelles. Sonication by 1-MHz ultrasound for 30 s; power density 15.2 W/cm ; initial DOX concentration in the[2] incubation medium 20 mg/ml. Before sonication, the samples were equilibrated at 37 8C for 5 min.

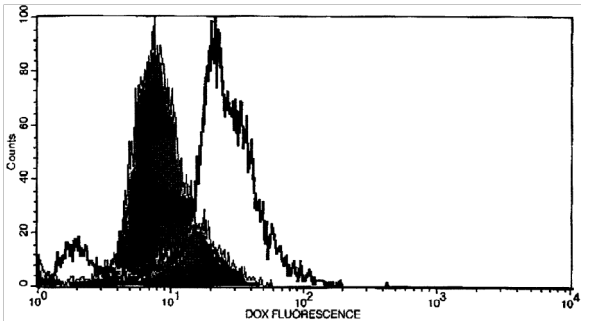

Fig. 6. Fluorescence histograms of the breast cancer MCF-7 cells incubated (shaded) or sonicated in the presence of 10% Pluronic micelles: shaded, unsonicated control (attached cells incubated with DOX for 1 h); cells were sonicated for 30 s upon 1 h incubation with DOX at 37 8C; DOX concentration 20 mg/ml, power density 15.2 W/cm .[2]

of the intracellular drug uptake were observed in the same ultrasound power density range; therefore the data of this work did not allow unambiguous discriminating between the two above mentioned mechanisms of the ultrasound-enhanced drug uptake at high ultrasound frequencies.
Whatever the exact mechanism of the enhancement of drug uptake, the data presented above suggest that high frequency ultrasound can effectively deliver drugs encapsulated in polymeric micelles to cancerous cells. This is an important finding since high-frequency ultrasound is widely used in clinical practice for imaging purposes (though at much lower power densities than used here). The ideal scenario for the clinical application of the above technique would be combining imaging and therapeutic ultrasound ransducer arrays in one instrument that will be used first for tumor imaging followed by the automatic focusing of the therapeutic ultrasound beam. High frequency ultrasound will release drugs from micelles at the tumor site and enhance the intracellular drug uptake. MRI imaging may be used to monitor thermal buildup during ultrasonic irradiation [15].

In the literature, the *increase* of the drug uptake under sonication is usually attributed to the formation of micropores in the cell membranes (sonoporation) [16–20]. In a study by Tachibana, scanning electron microscopy of HL-60 cells exposed for 30 s to 255-kHz ultrasound at 0.4 W/cm^2 revealed the formation of multiple surface pores and ‘dimple-like craters’ [16]. While increased membrane permeability due to the sonoporation may be important for the uptake of large molecules (e.g., DNA and proteins), micelles,or nano- and microparticles, the situation is different for small molecules like DOX. Our experiments showed that a real thermodynamic equilibrium was established between the external and internalized drug [9]. This type of the membrane equilibrium does not depend on the properties of membranes separating two compartments. Moreover, the concentration of drug inside the sonicated cells is usually more than order of magnitude higher than that in the external medium [9]. Therefore the poration should result in *leaking* of the free (non intercalated) drug from the cytoplasm into the external medium to equalize drug concentrations in both liquid compartments. This process is opposite in sign to the internalization of more drug observed under sonication and therefore cannot account for it.

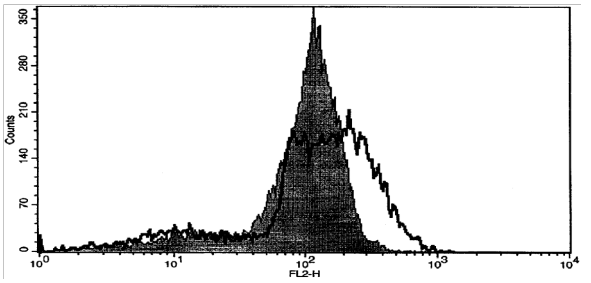

Fig. 7. Flow cytometry histograms of HL-60 cells unsonicated or sonicated in the presence of 10% Pluronic micelles: shaded, unsonicated control; cells were sonicated for 30 s in the test tubes by 1-MHz ultrasound, power density 7.2 W/cm ; initial DOX[2] concentration 5 mg/ml.

The Langmuir-type drug uptake isotherms observed in our experiments [9] are characteristic of the systems with a restricted number of sorption centers. The enhanced uptake of a free drug under sonication may be related to the generation of new sorption centers due to the perturbation of the cell structures; alternatively, shifting the equilibrium between the external and internalized drug may be related to the generation of excited drug molecules, which would increase the enthalpy of drug internalization. This hypothesis is supported by the experimental observation of single-bubble and multiple-bubble sonoluminescence [21,22], which suggested the generation of molecularly excited states in the liquids undergoing sonication; very short (picosecond range) flashes of light were observed at each ultrasound cycle; the spectrum of sonoluminescence was more indicative of the sonicated liquid than of the gas dissolved in it [22], which implied that the molecularly excited solvent molecules were generated. It appears feasible to assume that the excessive energy may be transferred from the solvent molecules to the dissolved drug thus producing 'hot' drug molecules.

This would increase the equilibrium constant of the drug internalization process due to the increased process enthalpy. According to the above mechanism, the generation of excited molecules proceeds under the action of cavitation bubbles that oscillate (but not necessarily collapse) in the ultrasonic field; therefore cavitation (but not necessarily *transient* cavitation) may play an important role in enhancing the intracellular uptake of the free drug. Also, as shown above, ultrasound- induced drug release from micelles proceeds without energy threshold indicating that this process is not related to *transient* cavitation. The latter may be more directly involved into the intracellular uptake of the drug-loaded micelles, which is facilitated by the microporation of plasma membranes. Transient cavitation is also the main cause of cell sonolysis. Due to different mechanisms involved into the drug release from micelles and cell sonolysis, a 'window' of power densities was observed, inside which noticeable drug release from micelles was not accompanied by an extensive cell sonolysis [12]. This window should be used in clinical practice. Based on the above considerations, the mechanism of the ultrasonic enhancement of the intracellular drug uptake from/with polymeric micelles appears different from that of sonophoresis, where *transient* cavitation plays a predominant role [23–27]. Summarizing, high frequency ultrasound may be instrumental in developing a new technique of drug targeting to tumors, based on drug encapsulation in polymeric micelles followed by tumor sonication by focused high-frequency ultrasound beam. This technique is not invasive and provides for a high degree of temporal and spatial control.

A cknowledgements
The authors acknowledge funding from the National Institutes of Health (CA 76562) and thank Dr. David Draper of BYU for providing the transducer for 1 MHz and 3 MHz ultrasound.

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