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Ultrasound-Enhanced Tumor Targeting of Polymeric Micellar Drug Carriers

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Abstract: Cancer chemotherapy is often complicated by toxic side effects of anticancer drugs. We are developing a new modality of tumor chemotherapy aimed at circumventing side effects of treatment *via* drug targeting to tumors. The technique is based on drug encapsulation in polymeric micelles followed by a controlled drug release at a target site triggered by ultrasonic irradiation. The encouraging *in vitro* results of previous years warranted animal experiments for verification of the *in vivo* feasibility of the proposed technique. We report here on the effect of ultrasound on a biodistribution of a micellar drug carrier (fluorescently labeled Pluronic micelles) in ovarian cancer-bearing nu/nu mice. The degree of carrier accumulation in the cells of various organs was characterized by flow cytometry. Polymeric micelles were formed in either pure Pluronic P-105 solutions (unstabilized micelles) or 1:1 (weight) mixtures of Pluronic P-105 with PEG-diacylphospholipid (stabilized micelles). Intraperitoneal (ip) and intravenous (iv) injections were compared. The data showed that a 30 s ultrasonic irradiation by 1 or 3 MHz ultrasound applied locally to the tumor significantly enhanced accumulation of Pluronic in the tumor cells, which was observed for both ip and iv injections and for unstabilized and stabilized micelles. The data indicated targeting of Pluronic micelles to the tumors; the degree of targeting was enhanced by a local tumor sonication.

Keywords: Ultrasound; polymeric micelles; drug carriers; targeting; ovarian carcinoma; biodistribution

Introduction

The technique of drug targeting to solid tumors that we are currently developing is based on the drug encapsulation in polymeric micelles followed by a controlled and localized release at a target site triggered by focused ultrasound.[1−15] The rationale behind this approach is that drug encapsulation in micelles decreases the systemic concentration of free drug, inhibits intracellular drug uptake, and provides for a passive drug targeting to the tumor interstitium via the enhanced penetration and retention (EPR) effect.[16] The unwanted drug

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interactions with healthy tissues are therefore inhibited. However, as sites of action of most drugs are inside the cells, drug accumulation in the tumor interstitium is not sufficient for the effective tumor treatment. For successful chemotherapy, upon micelle accumulation at the tumor interstitium, efficient intracellular drug uptake by the tumor cells should be ensured. To achieve this, we suggested a localized ultrasonic irradiation of the tumor.[1−5,7−10,12−15] In *in vitro* experiments, ultrasound induced drug release from micelles[12,13] and enhanced the intracellular uptake of both released and encapsulated drug; the latter was presumably caused by the perturbation of cell membranes.[10] Ultrasound was shown by others to enhance the extravasation of a polymeric MRI contrast agent[17] and liposome-encapsulated doxorubicin.[18] For a micellar-encapsulated drug, this effect is expected to increase the selectivity of the drug accumulation in the tumor.

Important advantages of ultrasound are its noninvasive character, its ability to penetrate deep into the interior of the body, and its ability to be focused and carefully controlled.

The promising *in vitro* results warranted continued study for verification of the *in vivo* feasibility of the proposed technique.

The first step in this direction was evaluating the biodistribution of polymeric micellar drug carriers in tumor-bearing mice and the effect of ultrasound on the carrier biodistribution. We report here the results of *in vivo* experiments on the ultrasound-triggered targeting of polymeric micellar drug carriers to the ovarian carcinoma tumors in nu/nu mice; the effect of 1 and 3 MHz ultrasound on a biodistribution of unstabilized and stabilized fluorescently labeled Pluronic P-105 micelles was studied.

Pluronic P-105 is a triblock poly(ethylene oxide)-*co*-poly(propylene oxide)-*co*-poly(ethylene oxide) copolymer with a PEO/PPO/PEO molar ratio of 37/56/37. The micelle size at room temperature is 17.5 nm. The critical micelle concentration (CMC) of Pluronic P-105 at 37 °C is close to 0.1 wt %.

Pluronic micelles have "soft" PPO cores and easily degrade into individual molecules (unimers) upon dilution below the CMC; if drug-loaded, they release drug upon dissolution. Intravenous injections are associated with substantial dilutions of injected solutions in circulating blood. To prevent premature micelle degradation upon intravenous (iv) injections, various approaches to micelle stabilization were suggested.[14] In the study presented here, Pluronic micelle stabilization was achieved by mixing micellar solutions of Pluronic P-105 and PEG-diacylphospholipid, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy-(polyethylene glycol)-2000] (PEG2000-DSPE). PEG2000-DSPE micelles have more hydrophobic cores and therefore a significantly lower CMC than Pluronic P-105. The drawback of pure PEG2000-DSPE micelles is a small core volume resulting in a low drug loading capacity; the micelle size of PEG2000-DSPE at room temperature is 8.9 nm. In a mixed 5% Pluronic P-105/5% PEG2000-DSPE solution, only one dynamic light scattering peak with a low polydispersity was observed, indicating a formation of mixed micelles. The mixed micelle size of 12.9 nm was intermediate between the sizes of pure Pluronic P-105 and PEG2000-DSPE micelles.

Mixed Pluronic P-105/PEG2000-DSPE micelles manifested a stability upon dilutions (and therefore superior drug retaining properties) much higher than that of pure P-105 micelles while retaining a relatively high drug loading capacity of pure Pluronic P-105 micelles (see below).

The biodistribution of fluorescently labeled Pluronic P-105 molecules was measured by flow cytometry. This technique allowed a comparison of the degree of internalization of fluorescent Pluronic molecules by the cells of various organs. A significant advantage of this technique is that it does not require organ homogenization, thus providing for direct measurement of the carrier (or drug) concentration inside the cells, i.e., at the site of action.

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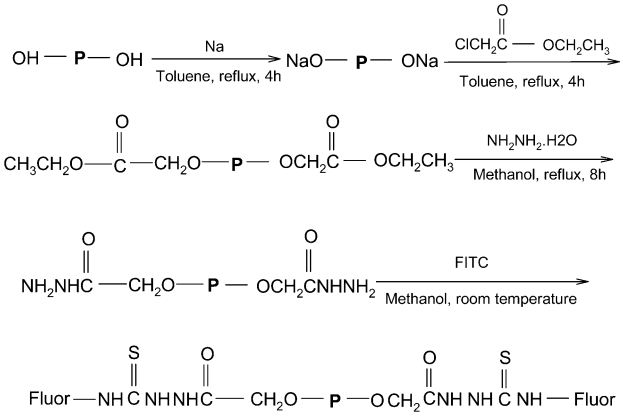
Scheme 1. FPII Synthesis[a]



[a] P is the Pluronic P-105 triblock copolymer.

Experimental Section

Micelle-Forming Polymers. Pluronic P-105 was kindly provided by BASF Corp. Micelles were formed in 5% Pluronic solutions in phosphate-buffered saline (PBS, pH 7.4) via self-assembly of the individual Pluronic molecules (unimers).

PEG-diacylphospholipid, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (PEG2000-DSPE), was bought from Avanti Polar Lipids, Inc. (Alabaster, AL). Mixed micelles were prepared by mixing equal volumes of 5% Pluronic P-105 and 5% PEG2000-DSPE solutions.

Fluorescently Labeled Pluronic Unimers and Micelles. The effect of ultrasound on the biodistribution of Pluronic unimers and micelles in tumor-bearing mice was monitored using fluorescently labeled Pluronic P-105. Two types of fluorescently labeled Pluronic molecules were used. One comprised the ester linkage between the polymer and the label [Fluorescent Pluronic I (FPI)]; the synthesis of this compound developed by Y. Luo (at that time, a postdoctoral fellow in the Department of Medicinal Chemistry, University of Utah) was described in detail previously.[5] Carboxydichlorofluorescein (mixed isomers) was used as a fluorescent label. In cell culture studies, the polymer−label bond in FPI was found to be intact during the 3 h of cell incubation with the polymer, upon which the first signs of a fluorescent label leakage out of the cells (presumably due to the degradation of the polymer−label bond) were observed. In the study presented here, FPI was used for the experiments involving the intraperitoneal injections, the duration of which did not exceed 75 min.

The protocol of the synthesis of a second compound labeled with FITC [Fluorescent Pluronic II (FPII)] was also developed by Y. Luo. The FPII synthesis is presented in Scheme 1. Briefly, end hydroxyl groups of a toluene-dissolved Pluronic P-105 were activated by sequential reactions first with metallic sodium and then with ethyl chloroacetate in toluene. Upon toluene evaporation, the intermediate reaction product reacted with hyrdrazine hydrate in methanol. Finally, fluorescein isothiocyanate (FITC) was added to a reaction mixture that was allowed to react while being stirred for 5 days at room temperature. All reaction conditions are given in Scheme 1. Fluorescently labeled Pluronic P-105 was purified by gel filtration (Sephadex G-25, 20−80 μm, molecular weight cutoff of 5000; eluent: 20% methanol in water). The isothiourea linkage between the polymer and the label in this compound was much more stable in biological fluids than in FPI;[19] in a control experiment, the linkage remained intact upon the overnight incubation with A2780 cells in a culture. FPII was used for the experiments with the intravenous injections that lasted up to 8 h. Fluorescence parameters of FPII are as follows: excitation maximum at 494 nm and emission maximum at 515 nm.

Micelle size was measured by dynamic light scattering (DLS) using Zetasizer 3000HSA (Malvern Instruments, Inc., Southboro, MA).

Measuring the Micelle Stability upon Dilution. The electron spin resonance (ESR) technique was used to measure micelle stability and drug retaining properties upon dilution as described previously.[14] Two ESR probes were used, namely, 16-doxylstearic acid methyl ester (16-DSME) and ruboxyl (Rb) (the latter is a paramagnetic anthracyclin, an analogue of Rubomicin).[20] The ESR probe was introduced into either a 5% Pluronic P-105 solution or a 5% mixed micelle solution to produce an initial probe concentration of 1.25 mM for 16-DSME or 1.0 or 3.0 mM for Rb. The initial solutions were sequentially diluted with either PBS or bovine fetal serum (BFS). The ESR spectra were recorded with the Bruker (Billerica, MA) X-band EMX ESR spectrometer with the following parameters: center field of 3340 G, power of 6.269 mW, modulation frequency of 100 kHz, and modulation amplitudes of 0.1 and 2.0 G. Lorentzian components and double integrals of the spectra were measured by fitting each spectrum to the inhomogeneous line shape model using a computer program described in ref 21. The program provides for a separation of overlapping ESR signals.

Animals. Four-week-old male nu/nu mice were bought from Charles River Labs (Wilmington, MA). Ovarian carcinoma A2780 cells (1×10^6) were inoculated intraperitoneally (ip) or subcutaneously (sc). Upon the ip inoculations, by day 25, the growth of two internal tumors was clearly manifested in control untreated mice (Figure 1A). Subcutaneous tumors were inoculated in the right flank of the mice.

Sonication. Ultrasound was generated by an Omnisound 3000 instrument equipped with a 1 cm^2 piezoceramic crystal and a 1 or 5 cm^2 transducer head. Unfocused continuous wave 1 or 3 MHz ultrasound was applied through the Aquasonic coupling gel to one of the two tumors shown in Figure 1A. A nominal output power density of 1.2 W/cm^2 (1 MHz, intraperitoneal tumors), 1.7 W/cm^2 (1 MHz), or 1.8

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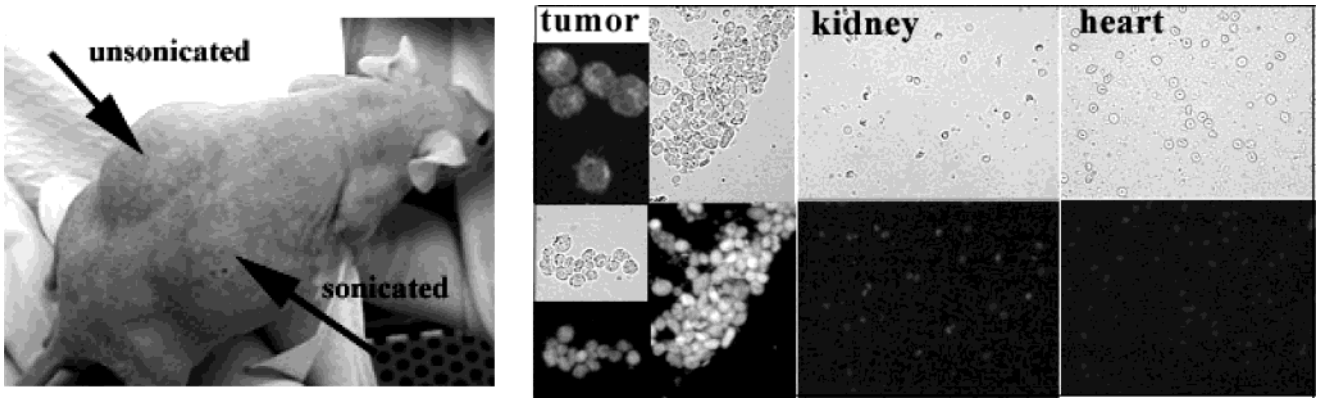

Figure 1. (A) Ovarian carcinoma tumors grown in controlled nu/nu mice. These tumors have been used in biodistribution experiments. After drug injection, one of the two tumors has been sonicated while another was not. (B) Phase contrast and fluorescence micrographs of the tumor, kidney, and heart cells derived from an FPII-injected and sonicated mouse. FPII Pluronic micelles (5%) were injected intravenously; 4 h after the injection, a tumor was sonicated for 30 s with 1 MHz ultrasound at a power density of 3.4 W/cm^2. Ten minutes after the sonication, a mouse was sacrificed, and the tumor and other organs were excised and individual cells derived as described in the Experimental Section. The inset in the phase contrast image of the tumor cells is the confocal image of the cultured tumor cells incubated with 50 μg/mL FPII; Pluronic molecules are confined in the cytoplasmic vesicles and do not penetrate into cell nuclei.

W/cm^2 (3 MHz, subcutaneous tumors) was delivered for 30 s. The temperature increase induced in the tumor by ultrasonic irradiation was measured by a hairlike thermocouple inserted through a syringe needle into various sites of the tumor volume and other sites of a body of an anesthetized mouse. Interestingly, the tumor temperature of 28.1−28.2 °C was lower than the mouse body temperature of 33.3 °C (n = 5). The temperature increase under ultrasound was moderate. When CW 1 MHz ultrasound with a power density of 3.4 W/cm^2 was applied to the tumor for 30 s, the temperature in the middle part of the tumor reproducibly increased by 2.3 °C. The temperature increase was only 0.4 °C at the site of a second (nondirectly sonicated) tumor shown in Figure 1A.

Using a thin hydrophone (model TNU100A with PFS017A Preamplifier, NTR Systems, Inc., Seattle, WA) inserted into the tumor or other sites of an anesthetized mouse, we monitored the ultrasound power distribution over a mouse body. These experiments showed that a power density measured in the tumor was close to the nominal output power density of the transducer. The 1 MHz ultrasound penetrated deep into the interior of a mouse body; the energy loss was very small along the pathway of the ultrasound beam. For 3 MHz, the energy loss on the path of the ultrasound beam was more pronounced but has not been quantitatively measured because of the experimental difficulties.

Micelle Biodistribution Studies. A unimeric (0.1%) or micellar (5%) fluorescently labeled Pluronic solution (100 μL) was injected intraperitoneally or intravenously into ovarian carcinoma-bearing mice.

Two degrees of micelle fluorescence labeling that differ by 25-fold were used in the biodistribution studies. In the first experimental setting, Pluronic micelles were formed either entirely by a labeled polymer or, in the case of mixed micelles, in a 5% FPII/5% PEG2000-DSPE mixture. In the second experimental setting, micelles were formed by the unlabeled 5% P-105 or 5% P-105/diacylphospholipid mixture, and 0.1% of a labeled Pluronic FPII was added to micelles; this concentration of a labeled polymer roughly corresponded to one fluorescence label per micelle. These experiments were performed to rule out possible (though unlikely) complications associated with the intracellular self-quenching of Pluronic fluorescence.

In cell culture studies, in the concentration range of 0.001−1.25%, the fluorescence of A2780 cells was proportional to the external concentration of FPI and FPII; at concentrations of >1.25%, the slope of the standard curve gradually decreased, because of a lower efficiency of the intracellular uptake of Pluronic micelles in comparison to unimers.[11]

Two modes of micelle injection were explored.

(1) Intraperitoneal injections were used with the intraperitoneal tumors (see Figure 1A). One hour after the ip injection, 1 MHz ultrasound was applied for 30 s to one of the two tumors shown in Figure 1A. Mice treated with the same formulations but not subjected to ultrasound were used as controls. One hour after intraperitoneal injections, even nonsonicated tumors already manifested noticeable fluorescence, in contrast to intravenous injections, for which noticeable tumor fluorescence was observed 4−8 h after the injection. This time course difference was presumably related to differences in the pathways of the drug being transported to the tumor tissue, which for the intraperitoneal injections probably did not require the intravasation and extravasation steps.

(2) Intravenous injections were performed through the tail veins. At various times after the iv injections ranging from 2 to 12 h, mice were sonicated for 30 s with 1 or 3 MHz ultrasound. Measuring the level of micelle accumulation in the tumor cells at various times of ultrasound application enabled selection of the optimal regime of ultrasound application, which was found to be between 4 and 8 h after the micelle injections. At shorter times, a large

number of micelles were still circulating in the vasculature while a lower concentration of micelles accumulated in the tumor interstitial space; at longer times, some clearing of micelles from the tumor had already occurred. For a successful treatment, the maximal micelle accumulation in the tumor volume is desirable. Therefore, in the experiments with the intravenous injections, ultrasound was applied 4 or 8 h after the micelle injections. Ten minutes after the ultrasound application, the mice were sacrificed, and the tumor and other organs were excised, dried with filter paper, weighed, and chopped in trypsin. To standardize possible Pluronic leakage from the cells, the volume of trypsin was proportional to the weight (i.e., volume) of the organ. The individual cells of various organs were separated and fixed with 2.5% glutaraldehyde. Phase contrast and fluorescence micrographs of individual cells derived from the tumor, kidney, and heart of an injected and sonicated mouse are given in Figure 1B. The fluorescence of Pluronic P-105 internalized by the cells was measured by flow cytometry (FACSCAN, Becton Dickinson). Fluorescence was excited at 488 nm and measured in FL1 and FL2 channels (FL1 is used for the FITC measurements, while FL2 is used for the propidium iodide and DOX measurements). In some cases, the fluorescence of FPII in the tumor cells measured in the FL1 channel was too high to produce reliable data; in these cases, the data accumulated in the FL2 channel were used. Note that the flow cytometry technique does not provide absolute fluorescence values; the measured value for the same sample may vary from day to day because of the variations in the instrument sensitivity and setting. Therefore, in this work, the data for a particular series of samples were recorded on the same day with the same instrument setting. The experiments were usually carried out in duplicate; a surprisingly low scatter of the results that did not exceed 10% was observed for parallel sets of samples. The biodistribution data are presented in the paper as actual fluorescence histograms and as the ratios of the mean fluorescence of the tumor cells to a mean fluorescence of the cells of other organs of a particular mouse (T/O).

Important Note. Since no correction was introduced for the autofluorescence of the tumor and organ cells, the actual tumor-to-organ Pluronic *uptake* ratios are significantly higher than the T/O Pluronic *fluorescence* ratios presented in the paper. The autofluorescence of tumor cells was significantly lower than that of organ cells (except lung). In many cases, the fluorescence of the organ cells (but not tumor cells!) in experimental mice was equal or close to the autofluorescence of the corresponding control cells. Subtracting a mean autofluorescence of the control cells from that of the corresponding experimental cells would dramatically reduce *the denominator* (but not the numerator!) in the T/O fluorescence ratio, thus substantially increasing the T/O ratio (see an example presented in Figure 10). However, subtraction of two close (and statistical) numbers would introduce a very large error in the tumor-to-organ fluorescence ratios. Therefore, we preferred to present here the T/O values that were calculated using directly measured mean fluorescence values for the tumor and organ cells of a particular mouse. These values should be treated as the lowest limit of the actual T/O uptake ratios; however, the presented T/O fluorescence ratios are useful as they can be precisely calculated and show the correct trends.

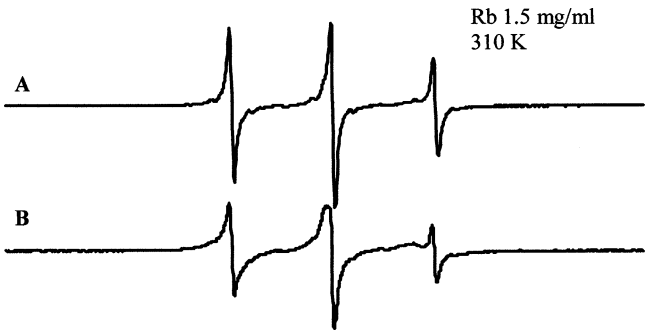

Figure 2. EPR spectra at 37 °C of Rb encapsulated in 5% Pluronic micelles (A) or 5% Pluronic P-105/5% PEG2000-DSPE mixed micelles (B).

Results

Confirming Formation of Mixed Micelles. In a mixed 5% Pluronic P-105/5% PEG2000-DSPE solution, only one DLS peak with a low polydispersity was observed. A mixed micelle size of 12.9 nm was intermediate between the sizes of pure Pluronic P-105 (17.5 nm) and PEG2000-DSPE micelles (8.9 nm). Also, micelle aggregation observed by DLS in pure PEG2000-DSPE solutions was completely prevented in mixed 5% Pluronic P-105/5% PEG2000-DSPE solutions.

Micelle Stability. Figure 2 shows the EPR spectra of a paramagnetic anthracyclin drug Ruboxyl (Rb) encapsulated in the 5% Pluronic P-105 micelles (A) or mixed 5% P-105/5% PEG2000-DSPE micelles (B); the spectra simulations indicated that for mixed micelles, a much higher degree of drug encapsulation in the hydrophobic environment of micelle cores was observed (91.5 ± 1.0% in mixed micelles vs 66.7 ± 3.0% in Pluronic P-105 micelles). Also, a hydrophobicity of the drug environment in the micelle cores was higher for mixed micelles. The hydrophobicity of the ESR probe environment is characterized by the hyperfine splitting constant N_A. A larger N_A corresponds to a lower hydrophobicity. For Rb, $N_A = 15.28$ G in mixed micelles versus 15.46 G in Pluronic P-105 micelles (in PBS, $N_A = 16.01$ G). Interestingly, the hydrophobicity of the Rb environment outside of micelle cores was slightly higher than that of Rb dissolved in PBS: $N_A = 15.86$ G for mixed micelles versus $N_A = 16.01$ in PBS, suggesting that some fraction of the drug was located in the micelle shells; note that the absolute error of the N_A measurements did not exceed 0.001 G.

The higher hydrophobicity of the cores of mixed micelles implied their higher stability. In accordance with this prediction, the electron paramagnetic resonance measurements showed that upon a 50-fold dilution by a fetal bovine serum (FBS), 65% of the encapsulated Rb was retained in

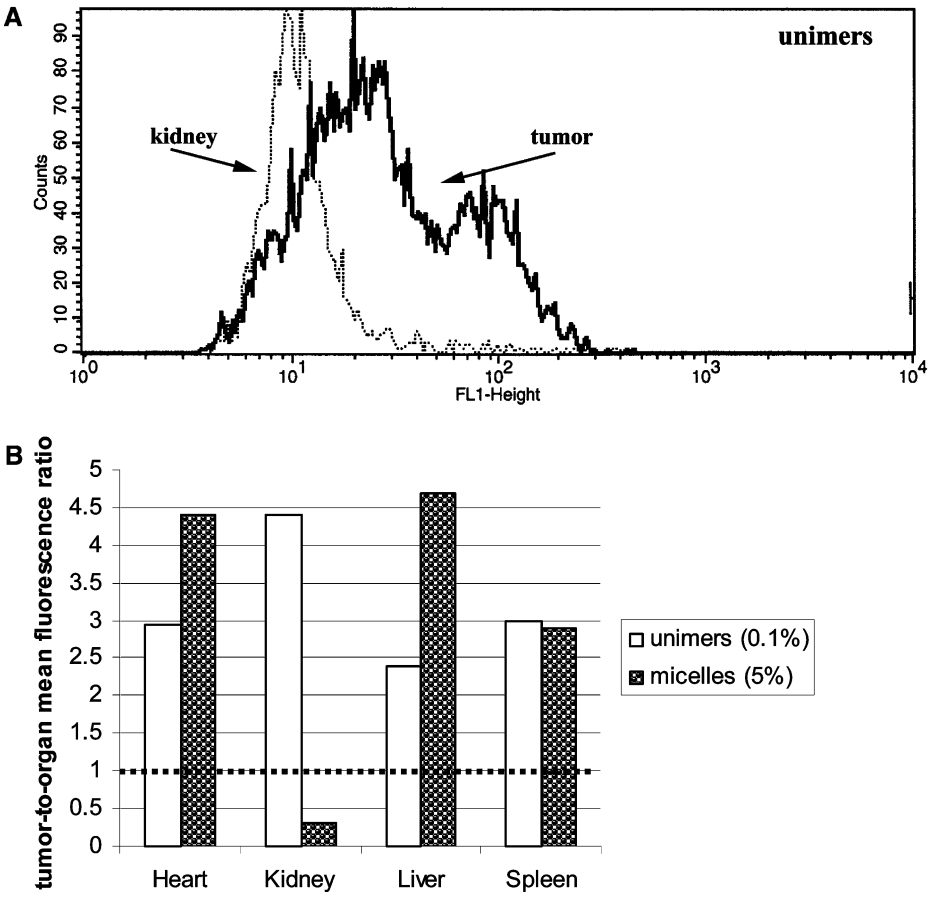

Figure 3. (A) Fluorescence histograms of the tumor cells (thick line) and kidney cells (dotted line) observed upon the intraperitoneal injections of fluorescently labeled Pluronic P-105 unimers (0.1 wt %) into a nu/nu mouse bearing the internal ovarian carcinoma tumor. A mouse was sacrificed 70 min after the injection. (B) Tumor targeting efficiency expressed as a tumor-to-organ mean fluorescence ratio for Pluronic P-105 unimers (white bars) and micelles (gray bars) upon the intraperitoneal injections into internal tumor-bearing mice; mice were sacrificed, and the fluorescence of the organs was measured 75 min after the ip injections. The dotted horizontal line along unity outlines the equality of the tumor and organ cell fluorescence; the bars rising above the dotted line signify a predominant Pluronic fluorescence in the tumor cells.

the mixed micelles while the drug was completely released from the pure Pluronic P-105 micelles (data not shown). These data suggest that upon intravenous injections that are associated with 20−25-fold dilution of the injected system, unstabilized Pluronic P-105 micelles will degrade to unimers while at least 65% of mixed micelles will still be preserved in the circulation.

Biodistribution of Pluronic Unimers after Intraperitoneal Injections. After the intraperitoneal injections of Pluronic unimers, the distribution of the fluorescence of the tumor cells was bimodal, with the presence of highly fluorescent cells in all studied tumor sections (Figure 3A, thick line). No cells with an equally high fluorescence were found in other organs; actually, the fluorescence of other organ cells was very similar to the autofluorescence of the corresponding cells. As an example, fluorescence histograms of the tumor (thick) and kidney cells are compared in Figure 3A. The mean tumor-to-organ (T/O) cell fluorescence ratio is shown in Figure 3B (white bars); the dotted horizontal line along the unity outlines the equal fluorescence of the tumor and organ cells. However, since the autofluorescence of tumor cells is significantly lower than that of the organ cells, the T/O ratio of 1 still corresponds to a predominant Pluronic accumulation in the tumor cells.

On the basis of panels A and B of Figure 3, a significant tumor targeting was observed for Pluronic unimers.

Biodistribution of Pluronic Micelles after Intraperitoneal Injections. In contrast to the unimers, for Pluronic P-105 micelles, the highest fluorescence was observed in the kidney cells; the fluorescence of the tumor cells was lower than that of the kidney cells but higher than that of the liver, spleen, heart, and lung (in Figure 3B, compare the biodistribution of unimers and micelles; see also Figure 4).

Effect of Ultrasound on the Biodistribution of Pluronic Micelles after Intraperitoneal Injections. A 30 s tumor irradiation with 1 MHz ultrasound dramatically increased the level of Pluronic uptake by the cells of a sonicated tumor (Figure 4A) and substantially decreased the level of uptake by the kidney cells [compare panels B and C of Figure 4 for the kidney cell histograms in the unsonicated and sonicated mice (thin lines)]. In the sonicated mouse, the highest level

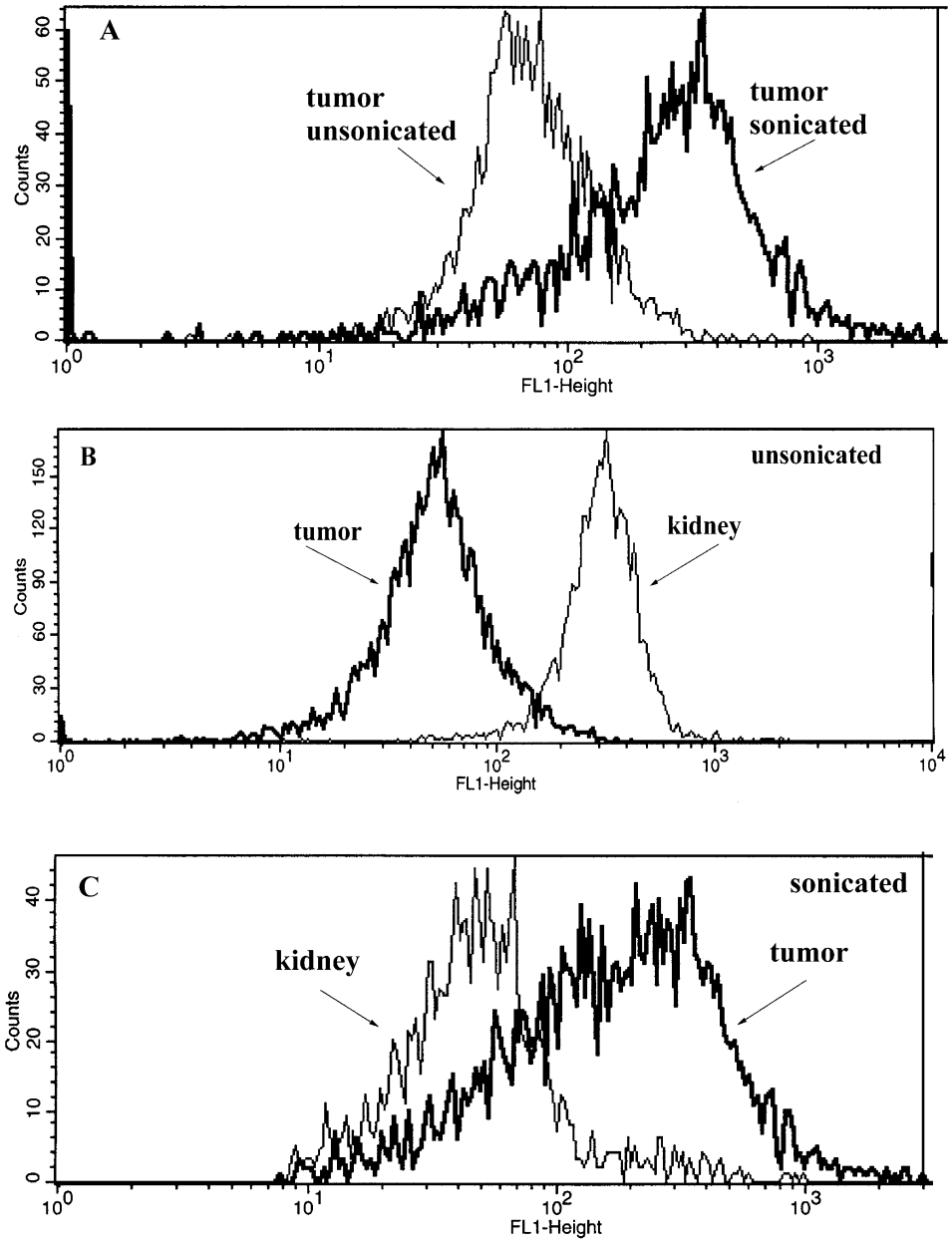

Figure 4. Effect of ultrasound on the biodistribution of fluorescently labeled Pluronic P-105 micelles in nu/nu mice bearing internal ovarian carcinoma tumors. Fluorescence histograms for (A) tumor cells of the unsonicated (thin line) and sonicated (thick line) tumor of the same mouse, (B) tumor cells (thick line) and kidney cells (thin line) from an unsonicated mouse, and (C) tumor cells (thick line) and kidney cells (thin line) from a sonicated mouse. Fluorescently labeled FPI 5% Pluronic P-105 micelles were injected intraperitoneally. One hour after the injection, the tumor was sonicated with 1 MHz ultrasound for 30 s at a power density of 1.2 W/cm^2. Ten minutes after the sonication, a mouse was sacrificed, and tumor and other organs were excised and treated as described in the Experimental Section to produce individual cells; the fluorescence distribution was measured by flow cytometry. Sonication significantly enhanced the Pluronic uptake by the tumor cells and inhibited that by the kidney cells.

of Pluronic accumulation was observed in the tumor cells, indicating an efficient ultrasound-enhanced tumor targeting (Figure 5).

It is noteworthy that the level of Pluronic uptake by the cells of a nonsonicated tumor of a sonicated mouse (see Figure 1A) was higher than that by the tumor cells of a mouse that had not been sonicated (compare fluorescence histograms for the unsonicated tumor cells in panels A and B of Figure 4). Also, the level of Pluronic uptake by the organs of the peritoneal cavity was somewhat higher in a sonicated mouse than in a nonsonicated mouse.

Biodistribution of Unstabilized Pluronic P-105 Micelles after Intravenous Injections. When 100 μL of a 5% Pluronic P-105 micellar solution was injected intravenously, a substantial (~20−25-fold) dilution of the initial solution proceeded, resulting in Pluronic P-105 molecules circulating

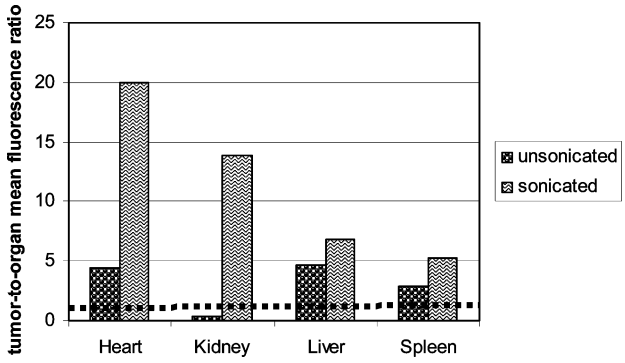

Figure 5. Effect of ultrasound on the tumor-to-organ mean fluorescence ratio for Pluronic P-105 micelles injected intraperitoneally. Sonication conditions were those given in the legend of Figure 4.

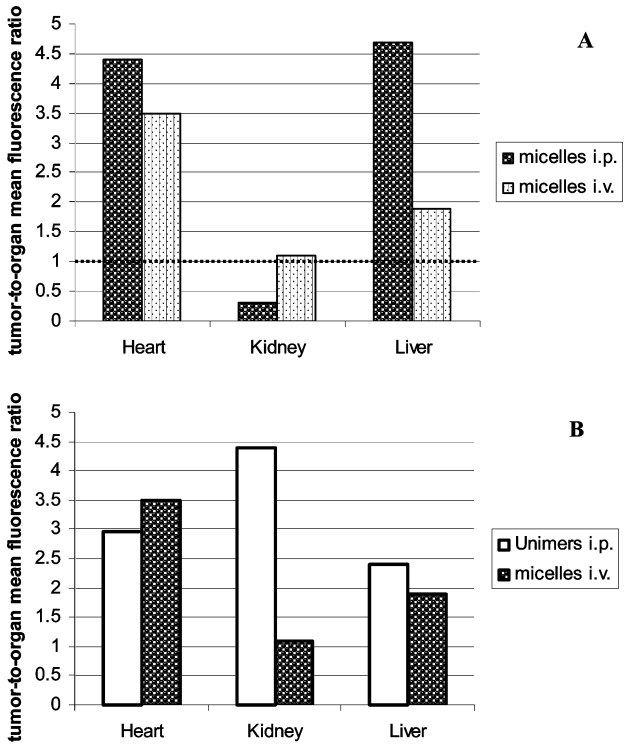

Figure 6. Comparison of the tumor-to-organ mean fluorescence ratio for (A) unstabilized 5% Pluronic P-105 micelles injected intraperitoneally and intravenously and (B) 0.1% Pluronic unimers injected intraperitoneally vs 5% Pluronic micelles injected intravenously. Intraperitoneal injections were used for the internal tumor-bearing mice (see Figure 1), while the intravenous injections were used for the subcutaneous tumor-bearing mice. For the intraperitoneal injections, a biodistribution was measured 70 min after the injection; for the intravenous injections, a biodistribution was measured 8 h after the injection.

in the vasculature predominantly as unimers (see the ESR results given above).

A biodistribution of unstabilized Pluronic micelles after the intravenous and intraperitoneal injections is compared in Figure 6A. After the intravenous injections, relatively more micelles accumulated in the liver cells while fewer accumulated in the kidney cells. Except for the kidney cells, tumor-to-organ fluorescence ratios were close to the pattern characteristic of Pluronic unimers (Figure 6B), confirming micelle degradation in the circulation. As reported above for the Pluronic unimers, the fluorescence histogram of the tumor cells was bimodal [Figure 7A (dotted line)], indicating a nonuniform Pluronic distribution in the tumor volume.

The most important difference between the intraperitoneal and intravenous injections of Pluronic micelles was related to a lower level of Pluronic accumulation in the kidney cells for the intravenous injections, the tumor-to-kidney fluorescence ratio being noticeably higher (Figure 6A); this presumably resulted from the micelle degradation in the circulation, which reduced the extent of kidney cell trapping. However, the level of Pluronic accumulation in the kidney cell was still higher than that observed upon the injection of Pluronic unimers (Figure 6B), supposedly due to a 50-fold higher concentration of Pluronic unimers produced upon the degradation of micelles, which required a longer elimination time.

As shown in Figure 6B, a considerable passive tumor targeting was observed for Pluronic unimers formed upon the degradation of micelles. The corresponding fluorescence histograms for the tumor, spleen, and heart cells are presented in Figure 7 (dotted lines).

The data given above confirm the degradation of unstabilized Pluronic micelles upon the intravenous injections.

Biodistribution of Mixed Micelles after Intravenous Injections. As mentioned above, the ESR data indicated that ~65% of mixed micelles were preserved upon a 50-fold dilution of a 5% mixed micellar solution by FBS. This suggested that upon the intravenous injections, mixed micelles would circulate in the vasculature predominantly in the intact micellar form.

After the iv injections of mixed micelles, without ultrasound, a multimodal distribution of cell fluorescence was observed, with a significant fraction of the cells manifesting a relatively low fluorescence [Figure 8 (thin line)]. This indicates a low efficiency of the intracellular uptake of intact micelles. We want to outline the fact that *a low level of intracellular uptake does not indicate the lack of micelle accumulation in the tumor interstitium; quite the opposite, a strongly enhancing effect of ultrasound on the tumor cell accumulation of micelles (see below) suggests that micelles effectively accumulated in the tumor volume presumably via the EPR effect.*

Effect of Ultrasound on the Biodistribution of Unstabilized and Stabilized Micelles upon Intravenous Injections. Similar to the results observed with the intraperitoneal injections, for both unstabilized and stabilized Pluronic micelles injected intravenously, a 30 s local sonication of the tumor significantly enhanced the intracellular Pluronic uptake by the tumor cells.

For the reasons explained in the Discussion, with the majority of the intravenous injections, tumors were irradiated with 3 MHz ultrasound (rather than 1 MHz ultrasound which was used with the intraperitoneal injections).

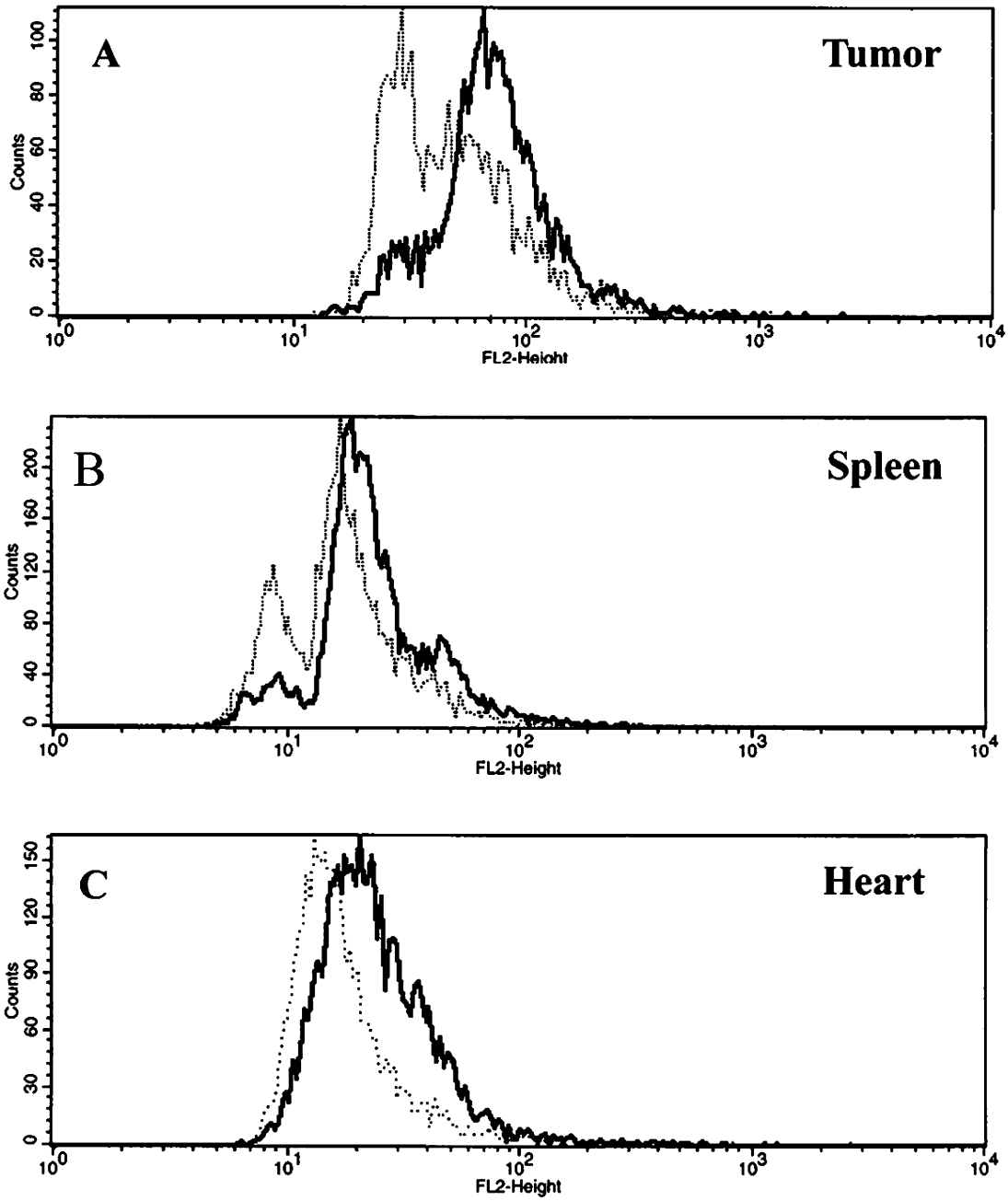

Figure 7. Fluorescence histograms of the unsonicated (dotted line) and sonicated (thick line): (A) tumor cells, (B) spleen cells, and (C) heart cells. The unstabilized 5% FPII Pluronic micelles were injected intravenously into two subcutaneous tumor-bearing mice; 8 h after the micelle injection, the tumor of one mouse was sonicated for 30 s with 3 MHz ultrasound at a power density of 1.8 W/cm^2. Both unsonicated and sonicated mice were sacrificed 10 min after the ultrasonic treatment of a mouse.

For the unstabilized Pluronic P-105 micelles injected intravenously and circulating as unimers, a local ultrasonic irradiation of the tumor with 3 MHz ultrasound resulted in an increase in the mean fluorescence intensity of the cell population (Figure 7A); the increase was caused mainly by the increase in the fraction of highly fluorescent cells in the cell population (rather than the increase in the fluorescence intensity of individual cells). The redistribution of the cells in the cell population between the weakly and strongly fluorescent fractions could result from the ultrasound-enhanced diffusion of polymer molecules in the tumor tissue.

Only slight enhancement of the fluorescence of other organs was observed under 3 MHz ultrasound, as illustrated in panels B and C of Figure 7 for the spleen and heart cells; therefore, the tumor-to-heart mean fluorescence ratio, which is an important therapeutic index, was increased by sonication.

For the mixed micelles, a local ultrasonic irradiation of the tumor also resulted in a significant enhancement of the Pluronic P-105 uptake by the tumor cells, as shown in Figure 8A (thick line) for the mixed micelles that comprised 0.1% FPII and in Figure 9 for the mixed micelles formed

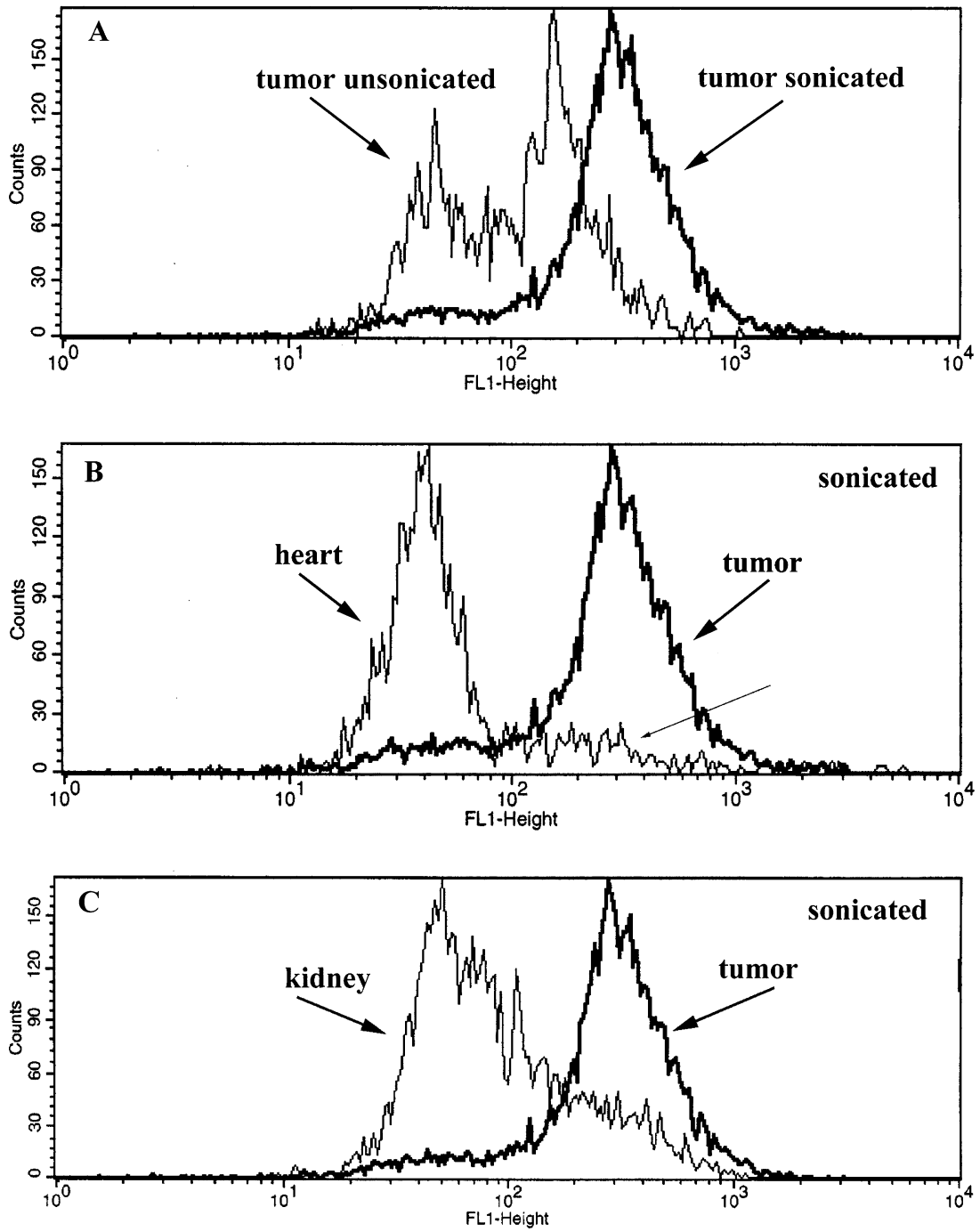

Figure 8. Fluorescence histogram of (A) tumor cells in the unsonicated and sonicated mice, (B) heart and tumor cells in the sonicated mouse, and (C) kidney and tumor cells in the sonicated mouse. Mixed micelles (5%) comprising 0.1% FPII were injected intravenously into two subcutaneous tumor-bearing mice; 4 h after the injections, a tumor of one mouse was sonicated for a total of 60 s with 1 MHz ultrasound at a power density of 3.4 W/cm^2 (two sonications of 30 s each were applied, with a 30 s interval between the sonications for cooling the ultrasound probe). Both mice were sacrificed 10 min after the sonication.

by a 2.5% FPII/2.5% PEG-DSPE mixture. For sonicated mixed micelles, fluorescence histograms of the tumor cells were shifted to higher fluorescence values, indicating a substantial increase in the level of the Pluronic intracellular uptake [rather than the increase in the *number* of the highly fluorescent cells observed for the unstabilized micelles (Figure 7A)]; again, no substantial enhancement of the Pluronic uptake by the liver, spleen, and kidney cells was observed, indicating an effective micelle tumor targeting (Figure 10). The tumor-to-kidney and tumor-to-heart fluorescence ratios were very favorable (see Figures 8 and 10). In addition, a substantially more uniform distribution of the fluorescence of the tumor cells resulted from tumor sonication, suggesting an enhanced micelle diffusion over the tumor volume.

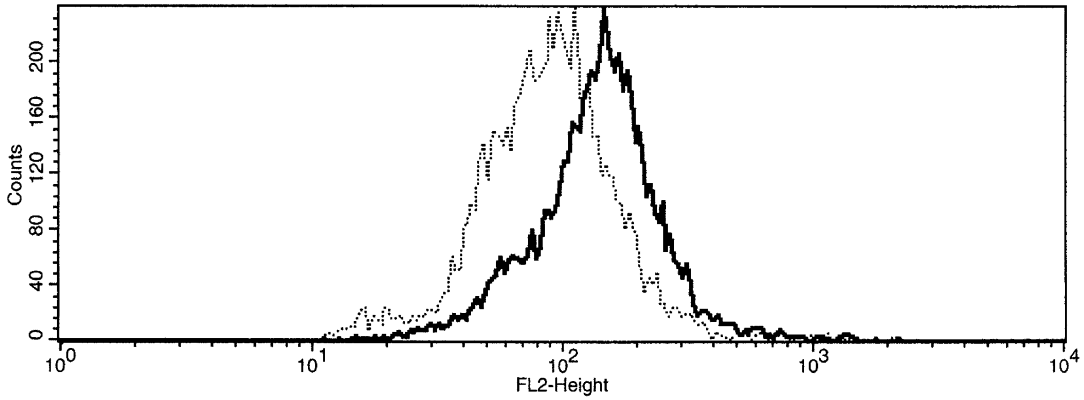

Figure 9. Fluorescence histograms of the tumor cells for the unsonicated (dotted line) and sonicated (thick line) mice. FPII mixed Pluronic micelles (2.5%) were injected intravenously into two subcutaneous tumor-bearing mice; 8 h after the micelle injection, the tumor of one mouse was sonicated for 30 s at an ultrasound frequency of 3 MHz and a power density of 1.8 W/cm^2. Both unsonicated and sonicated mice were sacrificed 10 min after the ultrasonic treatment.

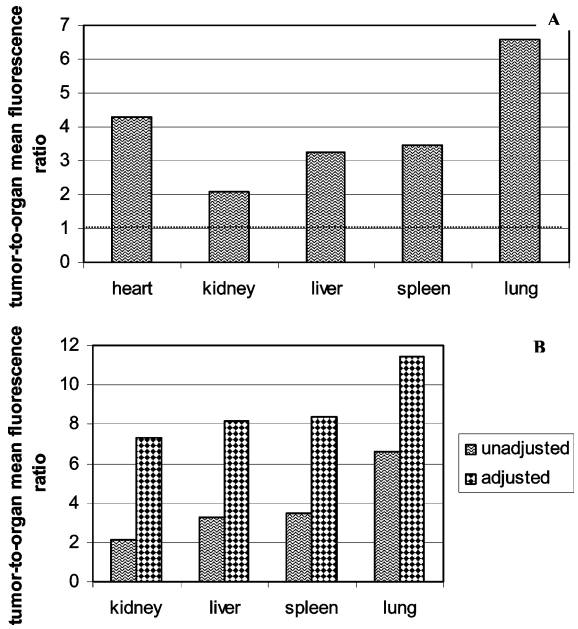

Figure 10. (A) Tumor-to-organ mean fluorescence ratio for the sonicated mouse of Figure 8. (B) Tumor-to-organ fluorescence ratio corrected to the autofluorescence of the cells. For the heart cells, the mean fluorescence of the experimental cells did not differ from that of the control. The corrected mean fluorescence ratios reflected the actual Pluronic tumor-to-organ uptake ratios.

Discussion

Tumor Targeting of Pluronic Unimers and Micelles without Ultrasound. For Pluronic unimers, a predominant fluorescence of the tumor cells compared to those of other organs, i.e., a significant tumor targeting, was observed; the mean fluorescence of the tumor cells was noticeably higher than that of the liver, spleen, kidney, and heart cells. In contrast, Pluronic micelles injected intraperitoneally were effectively trapped by the kidney cells (Figure 3), which could cause renal toxicity reported for a different type of Pluronic micelles.[29]

This raised concerns of the renal toxicity that was reported earlier for a different type of micelles, poloxamer 188, with a molecular mass of 8400 Da (which is equivalent to that of Pluronic P-68).[29]

(21) Smirnov, A. I.; Belford, R. L. Rapid Quantitation from Inhomogeneously Broadened EPR Spectra by a Fast Convolution Algorithm. *J. Magn. Reson.* **1995**, *113*, 65−73.

(22) Batrakova, E. V.; Li, S.; Vinogradov, S. V.; Alakhov, V. Y.; Miller, D. W.; Kabanov, A. V. Mechanism of Pluronic Effect on P-Glycoprotein Efflux System in Blood-Brain Barrier: Contributions of Energy Depletion and Membrane Fluidization. *Pharmacology* **2001**, *299*, 483−493.

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(24) Batrakova, E.; Lee, S.; Li, S.; Venne, A.; Alakhov, V.; Kabanov, A. Fundamental relationships between the composition of Pluronic block copolymers and their hypersensitization effect in MDR cancer cells. *Pharm. Res.* **1999**, *16*, 1373−1379.

(25) Batrakova, E.; Li, S.; Miller, D.; Kabanov, A. Pluronic P85 increases permeability of a broad spectrum of drugs in polarized BBMEC and Caco-2 cell monolayers. *Pharm. Res.* **1999**, *16*, 1366−1372.

(26) Batrakova, E.; Alakhov, V.; Kabanov, A. Selective energy depletion and sensitization of multiple drug resistant cancer cells by Pluronic block copolymer. *Polym. Prepr. (Am. Chem. Soc., Div. Polym. Chem.)* **2000**, *41*, 1739−1740.

(27) Kabanov, A. V.; Batrakova, E. V.; Miller, D. W. Pluronic block copolymers as modulators of drug efflux transporter activity in the blood-brain barrier. *Adv. Drug Delivery Rev.* **2003**, *55*, 151−164.

(28) Venne, A.; Li, S.; Mandeville, R.; Kabanov, A.; Alakhov, V. Hypersensitizing effect of Pluronic L61 on cytotoxic activity, transport and subcellular distribution of doxorubicin in multiple drug-resistant cells. *Cancer Res.* **1996**, *56*, 3626−3629.

(29) Schaer, G. L.; Spaccavento, L. J.; Browne, K. F.; Krueger, K. A.; Krichbaum, D.; Phelan, J. M.; Fletcher, W. O.; Grines, C. L.; Edwards, S.; Jolly, M. K.; Gibbons, R. J. Beneficial effects of RheothRx injections in patients receiving thrombolitic therapy for acute myocardial infarction. Results of a randomized, double-blind placebo-controlled trial. *Circulation* **1996**, *94*, 298−307.

After the intraperitoneal injections of Pluronic micelles, the mean fluorescence of the tumor cells was significantly higher than that of the cells of a reticulo-endothelial system or those of heart and lung. For the organ cells, the fluorescence level was close to the autofluorescence of the control; this suggested that those micelles that were not trapped by the kidney cells were effectively targeted to the tumors (Figure 3).

Renal Toxicity Issues. The purpose of the study reported in ref 29 was to evaluate the safety and efficacy of adjunctive therapy with poloxamer 188 in patients receiving thrombolytic therapy for acute myocardial infarction. The patients received a very high initial intravenous dose of poloxamer 188, a 48 h infusion of 300 mg/kg/h over 1 h followed by 30 mg/kg/h over 47 h; the poloxamer was packaged as a 15% (!) (w/v) solution in saline. Despite the large injected dose, poloxamer 188 was well tolerated without adverse hemodynamic effects or significant organ toxicity. Adjunctive therapy with poloxamer 188 resulted in substantial benefits in this randomized trial, including significantly smaller infarcts and greater myocardial salvage. No alteration in blood pressure or heart rate and no significant increase in the frequency of bleeding complications were observed. However, although poloxamer 188 was not associated with any statistically significant evidence of organ toxicity, reversible elevations in the level of serum creatinine occurred in 5% of treated patients, suggesting renal dysfunction. Further studies showed that an increased incidence of renal dysfunction was observed in patients with a baseline creatinine level of >1.5 mg/dL or an age of more than 75 years and in those receiving larger doses of poloxamer 188.[30] This necessitated modification of the study protocol to exclude patients with a creatinine level of >1.5 mg/dL or an age of more than 75 years and to restrict the poloxamer 188 treatment regimens to those with smaller doses. It was suggested later that the renal toxicity observed in 5% of the patients was caused not by Pluronic itself but by the impurities in the formulation.[31]

In our study, no adverse effects or chronic toxicity was observed in 2 month experiments involving injections of stabilized Pluronic P-105 micelles (four bolus injections with intervals of 3 days); both intraperitoneal injections into internal tumor-inoculated mice and intravenous injections into healthy mice were explored. No weight loss was observed in either case; moreover, there was a slight but statistically significant increase in the survival rate of the micelle-treated mice in comparison to control, most probably due to the cytostatic action of Pluronic micelles[4] (Table 1). These data suggested that the micelles were finally diluted, degraded, and eliminated from the kidney cells.

Table 1. Survival Rates of Mice Inoculated on Day 0 with ip Injections of A2780 Ovarian Carcinoma Cells[a]

group	no. of animals in the group	survival rate (days)
PBS	17	40.6 ± 8.2
PBS and US	5	39.6 ± 6.5
P-105 empty micelles	13	51.9 ± 21.6
P-105 empty micelles and US	13	47.2 ± 9.0

[a] The mice were treated by ip injections of PBS or stabilized micelles on days 1, 4, 7, and 11. Some groups of animals were sonicated for 30 s with 1 MHz ultrasound at a power density of 1.2 W/cm^2; ultrasound was applied to the abdominal region of a mouse 1 h after the injection.

Mechanism of Intracellular Uptake. It deserves mentioning that despite a 50-fold difference in the initial concentration of the ip injected Pluronic P-105 unimeric (0.1%) and micellar (5%) solutions, a difference in the resulting intracellular fluorescence did not exceed 2−3-fold; in the cell culture experiments with A2780 cells, the fluorescence of the cells incubated with a 5% fluorescent Pluronic solution was 15-fold higher than that of the cells incubated with a 0.1% solution, ruling out a substantial self-quenching as a possible cause of the *in vivo* effect. Comparing *in vitro* and *in vivo* intracellular uptake data implied that other factors besides a reduced level of internalization, for instance, reduced extravasation, could be involved in the low efficiency of the micelle intracellular uptake by various organs. It appears that in the unimeric and micellar solutions, *the intracellular uptake of Pluronic molecules proceeds mainly because of the unimers*.

The biodistribution data obtained for the intravenous injections of unstabilized Pluronic micelles confirmed their degradation into unimers upon the intravenous injections; this implied that the intravenously injected unstabilized Pluronic micelles could modulate the biological processes as was earlier observed for Pluronic unimers by Kabanov's group; in particular, the sensitization of multidrug resistant (MDR) cells characteristic of Pluronic unimers could be expected.[23−28]

Effect of Ultrasound on Micelle Tumor Targeting. Independent of the injection mode, 1 and 3 MHz ultrasound substantially increased the level of Pluronic micelle uptake by the tumor cells (Figures 4, 5, and 7−10). Unfocused 1 MHz ultrasound also decreased the level of micelle uptake by the kidney cells (Figures 4 and 5). In the sonicated mice, the highest level of Pluronic accumulation was observed in the tumor cells, indicating efficient ultrasound-enhanced tumor targeting.

An ultrasound-induced decrease in the level of Pluronic trapping by the kidney cells (Figure 5) confirmed that micelles were destroyed *in vivo* by the ultrasonic irradiation, which allowed Pluronic elimination through the kidney route. As explained below, unfocused 1 MHz ultrasound penetrated deep in the interior of a mouse body, which could cause the degradation of the micelle trapped in the kidney cells.

The cause of this effect was that at the site opposite from the transducer, 1 MHz ultrasound waves were reflected from

(30) Yusuf, S. Evaluation of RheothRx, a novel hemorheologic agent, in acute myocardial infarction. *Circulation* **1995**, *92* (Suppl. I), I-24 (Abstract).

(31) Toth, K.; Bogar, L.; Juricskay, I.; Keltai, M.; Yusuf, S.; Haywood, L. J.; Meiselman, H. J. The effect of RheothRx injection on the hemorheological parameters in patients with acute myocardial infarction. *Clin. Hemorheol. Microcirc.* **1997**, *17*, 117−125.

a skin−air interface back into the interior of a mouse body; in addition, the interior interfaces and bones located in the path of the ultrasound wave acted as reflection centers, which resulted in ultrasonic energy being spread beyond the tumor volume. Direct measurements by a hydrophone of the ultrasound energy in various sites of a mouse body during the localized irradiation of one of the tumors by 1 MHz ultrasound showed that some fraction of the ultrasonic energy was delivered to other organs.

Spreading of unfocused 1 MHz ultrasound beyond the tumor volume could be responsible for a higher level of Pluronic uptake by the cells of a nonsonicated tumor of a sonicated mouse (left tumor in Figure 1A) in comparison to the uptake by the tumor cells of a mouse that was not sonicated at all (compare histograms of nonsonicated tumors in panels A and B of Figure 4). This effect is also responsible for a somewhat higher absolute level of Pluronic uptake by the organs of a peritoneal cavity of a sonicated mouse in comparison to the unsonicated mouse.

Spreading of ultrasound beyond the tumor volume is therefore undesirable. This problem could be solved by (i) focusing the ultrasound beam in the tumor, which would result in a much lower ultrasound power density reaching the skin−air interface, and (ii) increasing the ultrasound frequency, which would decrease the depth of the penetration of the ultrasonic beam. The second approach was used with the intravenous injections of Pluronic micelles. The depth of penetration of 3 MHz ultrasound is 3-fold shallower than that of 1 MHz ultrasound; in fat and muscle tissues, 1 MHz ultrasound loses half of its energy at depths of 5 and 2.7 cm, respectively, while for 3 MHz the depths are 1.7 and 0.9 cm, respectively. Thus, unfocused 1 MHz ultrasound, though applied locally to the tumor, penetrated all through the mouse body and was reflected from the interfaces, while 3 MHz ultrasound was much more localized in a tumor volume. For 3 MHz ultrasound applied locally to the tumor, the enhancement of the Pluronic uptake by the tumor cells proceeded without a noticeable enhancement of the uptake by other organs; this resulted in the tumor-to-organ Pluronic accumulation ratio being substantially increased by ultrasound.

The enhancing effect of ultrasound on the Pluronic intracellular uptake by tumor cells could be presumably related to four main factors.

(i) The first is the enhanced micelle extravasation that increased the micelle concentration in the tumor interstitial space. However, a weak effect of ultrasound applied shortly after the intravenous injection ruled out a significant enhancement of the micelle extravasation.

(ii) The second is enhanced diffusion of the unimers and micelles. This factor is definitely operative, as manifested by a substantially more uniform Pluronic fluorescence distribution in the tumor cells.

(iii) The third is ultrasound-induced micelle degradation into unimers in the tumor interstitium. As discussed above, the internalization of unimers is much more efficient than that of the micelles and may be responsible for the increased

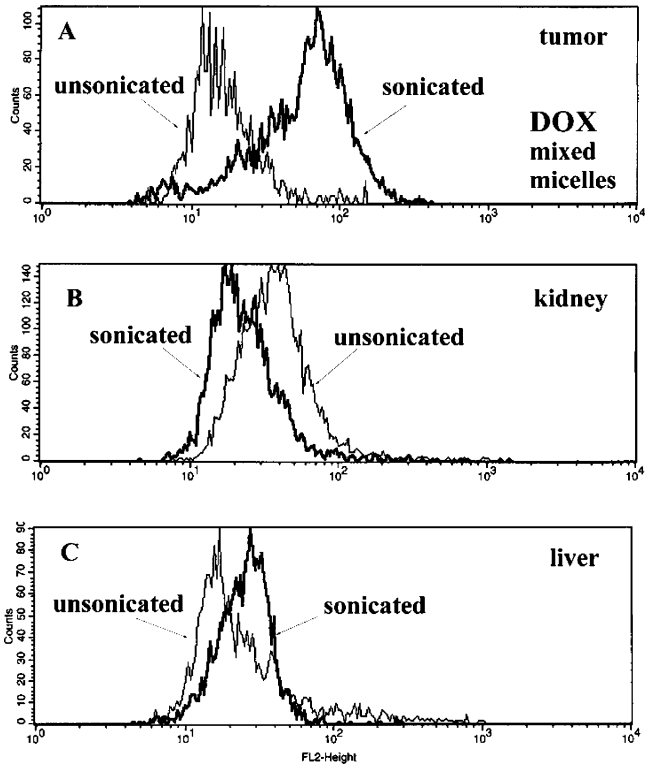

Figure 11. Fluorescence histograms of (A) tumor, (B) kidney, (C) liver cells in nonsonicated (thin lines) and sonicated (thick lines) mice injected iv with DOX (6 mg/kg) encapsulated in mixed micelles. Mice were sonicated 8 h after the drug injection with 1 MHz CW ultrasound at 1.7 W/cm^2; nonsonicated and sonicated mice were sacrificed 10 min later.

level of Pluronic intracellular uptake; a decreased level of micelle kidney trapping suggested that micelle degradation actually proceeded *in vivo* under ultrasonic irradiation. For drug-loaded micelles, a localized micelle degradation in the tumor volume is expected to result in drug release from micelles, which in turn would lead to localized drug uptake by the tumor cells, i.e., drug tumor targeting. The *in vivo* experiments with mixed micelle-encapsulated doxorubicin (DOX) confirmed this prediction, as illustrated in Figure 11. Comparing Figure 11 with Figure 4 showed that DOX uptake with and without ultrasound followed the pattern observed for the drug carrier; in nonsonicated mice, the level of DOX uptake by the tumor cells was lower than that by kidney cells; sonication dramatically increased the intracellular level of uptake of both carrier and drug by the tumor cells while reducing the level of uptake by the kidney cells. Some enhancement of the DOX uptake by the organ cells under the action of 1 MHz ultrasound (exemplified in Figure 11 for the liver cells) was similar to the effect observed for the carrier and explained above.

(iv) The last is the ultrasound-induced perturbation of cell membranes that could enhance the uptake of Pluronic unimers and micelles. The relative importance of this factor remains to be explored.

Conclusions

The *in vivo* data presented above imply that drug delivery in polymeric micelles combined with a local ultrasonic irradiation of the tumor may be developed into a powerful new tool of drug targeting and treatment of cancerous tumors.

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