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Effect of Local Dual Frequency Sonication on Drug Distribution from Nanomicelles

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***Abstract*—**The nanosized polymeric micelles release the drug due to acoustic cavitation, which is enhanced in dual frequency ultrasonic fields. In this study, adult female Balb/C mice were transplanted with spontaneous breast adenocarcinoma tumors and were injected with a dose of 1.3 mg/kg doxorubicin in one of three forms: free doxorubicin, micellar doxorubicin without sonication and micellar doxorubicin with sonication. To increase cavitation yield, the tumor region was sonicated with low level dual frequency of 3 MHz and 28 kHz. The animals were sacrificed 24 h after injection, and their tumor, heart, spleen, liver, kidneys and plasma were separated and homogenized. The drug content in their tumor, heart, spleen, liver, kidneys and plasma was determined using tissue fluorimetry. The results show that in the group that received micellar doxorubicin with sonication, the drug concentration in the tumor tissue was nine and three times higher than in the free doxorubicin group and the micellar doxorubicin without sonication group, respectively. In the micellar doxorubicin with sonication group, the drug concentration in other tissues was lower than other groups ($p<0.05$). We conclude that dual frequency sonication improves drug release from micelles and increases the drug uptake by tumors due to sonoporation.

***Keywords*—**Nanomicelles, Dual frequency ultrasound, Drug delivery

I. INTRODUCTION

THE main mechanisms of biological action for ultrasound include the generation of thermal energy, sonoporation, the enhancement of local microjets due to inertial cavitation which are further enhanced by multifrequency sonication and the enhanced permeability of blood capillaries. All of these mechanisms could potentially be used to enhance drug uptake locally. Doxorubicin is of great importance in the treatment of leukemia and solid, but its clinical use is hampered by its myelotoxicity and its cumulative cardiotoxicity [1, 5]. By targeting this drug to the desired site of action and increasing its uptake using sonication, besides increasing therapeutic efficiency, its side effects minimize. The experimental results from studies in sonochemistry indicate that activity of cavitation generated by multi-frequency ultrasound irradiation is higher than by single frequency irradiation [6-8]. Therefore, we used dual frequency ultrasound irradiation in continuous mode.

Various nanosized carriers have been reported which are used for passive targeting [5, 9-14]. Polymeric micelles [15] have received considerable attention due to their self assembling characteristics in aqueous solution. These properties offer a unique biodistribution of drugs and target solid tumors [10] and it has reported that doxorubicin-conjugated block copolymeric micelles were effective in treatment of solid tumors [11, 16]. Generally, it was reported that anticancer agent incorporated in polymeric micelles had enhanced blood circulation time and suppressive effect on the solid tumor growth [11, 12, 16-18]. Many polymeric micelles are convenient to use since they have been reported to be suitable for escaping the reticuloendothelial system and renal extraction because of their small size which is approximately from 20 to 100 nm [16]. Various methods for the quantification of doxorubicin in biological fluids have been reviewed. However, fluorescence detection is probably the most appropriate detection method for doxorubicin, considering its simplicity of use, selectivity and sensibility [1].

In this study, polymeric micelles using Pluronic P105 was prepared and loading doxorubicin into theses micelles and checking drug release upon sonication as in vitro were applied. The drug distribution pattern in animal tissues and their tumor were studied into three groups as following: injection of free Doxorubicin, injection of micellar Doxorubicin and injection of micellar Doxorubicin with combined dual frequency ultrasound irradiation and their anti-tumor effect was tested in vivo. Ultrasound was used to enhance intracellular drug uptake from micelles. Using spectrophotometric methods, drug content in several tissues were quantified.

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II. MATERIALS AND METHODS

Female inbred Balb/C mice were purchased from the breeding colony at the Pasteur Institute of Iran (Tehran, Iran). The tumor model was a syngenic of murine spontaneous breast adenocarcinoma, which was chopped into fresh pieces of about 2-3 mm diameter and then transplanted into the flank region of the mice. Tumors reached a diameter of about 7-9 mm, at which point they were ready to be used in experiments. All animal experiments and protocols were evaluated and approved by the Animal and Ethics Review Committee of the Tarbiat Modares University (Tehran, Iran).

Doxorubicin was obtained from Pharmacia (Italy). Pluronic P-105 was provided by the BASF Corp. (Mount Olive, NJ, USA). N-N-Diethylacrylamide (NNDEA) was obtained from Polysciences (Warrington, USA).N,N'-Bis(acryloyl)cystamine (BAC) was obtained from Fluka (Sigma-Aldrich, UK), and benzoyl peroxide (BP) was obtained from Merck (Merck KGaA, Darmstadt, Germany).

Polymeric micelles were prepared using Pluronic P-105, which is a triblock copolymer consisting of blocks of poly (propylene oxide) (PPO) and poly (ethylene oxide) (PEO) in the form PEO37-PPO56-PEO37. A solution of NaCl and 10 Wt.% Pluronic P-105 in distilled water was added to a round-bottom balloon, which was stirred for 20 minutes while immersed in a water bath under a nitrogen purge at a temperature of 65°C . A mixture of BAC, BP and NNDEA (26:1:55 weight ratio) was added to the above solution, and it was allowed to stir at this temperature under a nitrogen purge for 3.5 h. After turning the nitrogen off, the mixture was allowed to polymerize for 19.5 h. The size distribution of the micelles was measured by dynamic light scattering (DLS) (Malvern Instruments Ltd, Malvern, UK). To load doxorubicin into injectable micelles, it was necessary to find the optimal drug loading into micelles through a standard curve, which was measured using a UV spectrophotometer (Shimadzu, Model RF-1500, Japan) at 350 nm. Several known concentrations were prepared, and their fluorescence emissions were read. It is notable that at higher concentrations, the fluorescent emission decreases due to self-quenching of the doxorubicin. Fluorescence intensity (counts) versus doxorubicin concentration (mg/ml) yielded a straight line of positive slope for increasing concentrations. The results show that there is a significant correlation between the fluorescence intensity and the doxorubicin concentration (R^2 = 0.96, $p<0.01$). A linear regression analysis was applied between the fluorescence intensity and the doxorubicin concentration.

Two ultrasonic system, a 28 kHz source (Ultrasound Lab, TMU, Tehran, Iran; with 27.7 kHz center frequency, 421 Hz bandwidth and 60 mm diameter) and a 3 MHz therapeutic unit (SM3670, Shrewsbury Medical Ltd, Shropshire, UK with 3.3 MHz center frequency and 30 mm diameter) which was used in a cubic water tank in an orthogonal geometry. Acoustic calibration for the power and intensity of the device was carried out in degassed water in the tank. Different single frequency sonication conditions including 28 kHz (I_{SATA}=0.04 W/cm^2) and 3 MHz (I_{SATA}=2 W/cm^2) was done, continuously (Hasanzadeh et. al. 2010). In each frequency conditions, all of the acoustical subharmonic signal amplitudes; 14 kHz; at the water tank were searched and recorded with hydrophone duration 3 min irradiation. Each signal from the experiments comprised of 16384 data points collected with a sampling rate of 10 MHz that is a combination of primary frequency and the pressure wave coming from oscillating wall of the cavities. To extract frequency contents, signals were analyzed in MATLAB software version 7.0.1 (Math Software Co., Mathworks, USA) using FFT function with hamming window. Five measurements were performed for various sonication conditions.

To examine drug release in vitro, a custom exposure chamber was constructed according to the spectrophotometric characteristics of doxorubicin. A digital with a multi-band filter (Alexa Fluor® 350/488/594, USA) was used to record images (BMP images with dimensions of 640×480 pixels), and a background was recorded without sonication. The frequency and intensity were selected using a subharmonic analysis method to enhance the acoustic cavitation at 28 kHz and 3 MHz (Hasanzadeh et al. 2010).

Each sonication protocol was run 5 times, and the RMSE (root mean square error) values of the images were used to compare between images. The average of the RMSEs of the image components was used to compare the ability of different sonication conditions to release the drug from the micelles.

Doxorubicin was stirred into the micellar solution to load it into the micelles; to separate the free drug from the encapsulated drug, the solution was filled into a dialysis bag and dialyzed against water for 2 h. According to the standard curve of the drug in micelles, the optimum drug loading into micelles was determined. To investigate the stability of the micellar drug, the fluorescence amplitude of the micellar drug at 350 nm was measured over time, and the amplitude of this emission from each day was normalized to day zero (the time of drug encapsulation) to find the stability of the complex. The complex was stored at 4° C, and its stability was measured for one month.

The goal of the current study was to work below the level of hyperthermia, therefore it was necessary to measure the temperature rise due to sonication in vivo. Three female Balb/C mice were selected and placed into the sonication condition after being anesthetized. The temperatures of the tumors and their surrounding environment were monitored every second during sonication with a portable digital thermometer containing a dual thermocouple input. The tissue temperature was monitored invasively using K-type wire thermocouples (TP-01, Lutron Electronic Enterprise Co., Taiwan). Each of these thermometers had two wire thermocouples placed in the animal's body from 1 cm below the skin in the center of the tumor (close to the ultrasonic probes) to about 4 cm below the skin in 1 cm steps. All of the thermocouples provided inputs to the control unit, which

contained a microprocessor connected to a computer via an RS-232 port. The temperatures were recorded every second (as .doc files) using thermometer software (Multilogger Thermometer CHY502A, Taiwan), which was connected to a PC, and the temperature rise under sonication conditions was recorded every 30 s for 20 min. The water temperature was controlled using a digital thermometer and an electrical heater and was fixed at 32 °C. The results indicate that a 2.5 min sonication causes a temperature rise below the level of hyperthermia (T≤42°C) (Fig. 1).

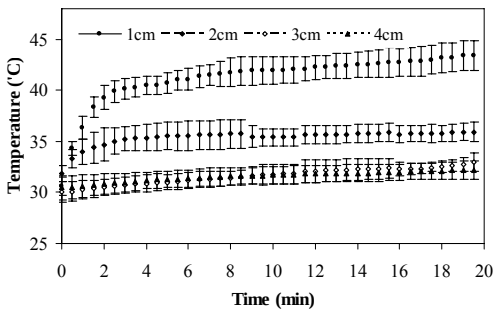

Fig. 1 The mean± SD of temperature changes (°C) at different depths in mice versus duration of sonication under dual frequency sonication conditions

To study the drug distribution in animals, 9 female Balb/C mice with tumor diameters of about 7-9 mm were randomly divided into 3 groups as follows: IV injection of free doxorubicin, IV injection of micellar doxorubicin and IV injection of micellar doxorubicin with sonication. After drug injection via the tail vein at a dose of 1.3 mg/kg (the amount of drug injected was 26 µg for each animal in different groups), animals were anesthetized and placed in a special cage in the vicinity of the probes in the ultrasonic field; in only the sonication group, the tumor region was exposed for 2.5 min to the selected sonication protocol, which was determined by the subharmonic amplitude analysis. After 24 h, the animals were sacrificed, blood was collected using a heparinated syringe from the heart, and tissues (heart, liver, spleen, kidney and tumor) were dissected and lyophilized. The blood was centrifuged (1500 rpm for 10 min), and the plasma was separated and kept for later analysis. Tissue extracts were obtained according to available protocols (Shiah et al. 1999). The doxorubicin concentration in the tissues and plasma was determined by a fluorescence assay using a UV spectrophotometer at 350 nm. Standard curves for each tissue were established by adding known amounts of doxorubicin to tissue extracts of control mice to obtain various concentrations; these curves were used later to obtain the drug concentration in different tissues from the experimental groups.

Statistical analysis was performed using Microsoft Excel 2003 and SPSS 16 (SPSS/PC Inc., Chicago, IL). The drug content in tissues is presented as the mean±SD. To analyze differences between groups, a one way ANOVA was used with a level of significance of 0.05 ($P<0.05$).

III. RESULTS

The results of measurements as subharmonic amplitudes of primary frequencies; 14 kHz; in single frequency irradiation and simultaneous dual frequency irradiation are shown in Figure 2. This increase is a synergistic effect with respect to single irradiation and shows an enhanced cavitation activity in combined fields.

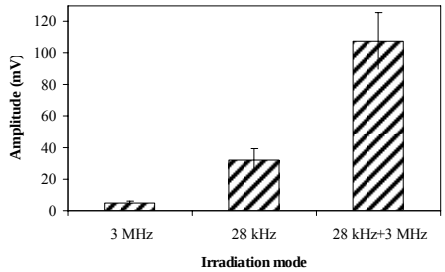

Fig. 2 Subharmonic signal amplitudes (mV) of 28 kHz and 3 MHz sources presented as Mean±SD in single frequency and in combination of frequencies

The result of DLS (Dynamic Light Scattering) showed that average size of synthesized micelles were 14 nm and were lower than 21 nm with unimodal and narrow size distributions as we expected.

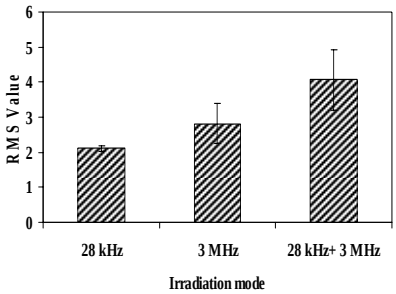

Fig. 3 RMSE values of images (Mean±SD) with single and combined dual frequency sonication

Our previous results showed that the 28 kHz subharmonic amplitude for 28 kHz (0.04 W/cm2) and 3 MHz (2 W/cm2) combined dual frequency in continuous mode was about 5 time higher than that obtained from the algebraic sum of 28 kHz and 3 MHz irradiation. RMSE values of images with combined dual frequency sonication (28 kHz and 3 MHz) in vitro are as Figure 3. Drug release from micelles is due to cavitation activity which has shown that is enhanced in dual frequency field; this object is observable in RMSE values too.

The stability of capsulated drug was evaluated for one month. The fluorescence amplitude of micellar drug at 350 nm was evaluated. The amplitude of this emission at each day was normalized to day zero. Results showed that capsulated drug is stable until 4 days and about 90% of the maximum possible stability was maintained during ten days.

To study drug distribution pattern in animal tissues, free Doxorubicin, micellar Doxorubicin and micellar Doxorubicin with sonication at optimum condition were injected to female

Balb/C. the result of Doxorubicin concentration in tissues and plasma by a fluorescence assay are shown in Table 1. In this Table, Standard curves for each tissue were established by adding known amounts of Doxorubicin to tissue extracts of control mice to obtain different concentrations. The results are shown as linear regression functions. Fluorescence intensity (count) versus concentration of Doxorubicin (µg/ml) for each tissue yielded a straight line of positive slope for concentrations. The results show that there is a significant correlation between the fluorescence intensity and concentration of Doxorubicin ($R>0.96$, $p<0.01$) for each tissue.

TABLE I
TABLE 2 DRUG CONTENT (µG) PRESENTED AS MEAN (SD) IN DIFFERENT TISSUES OF DIFFERENT GROUPS STUDIED 24H AFTER INJECTION

Tissue	Linear regression function	Correlation of coefficient	P-value
Spleen	Y = 0.2X - 2.6	*0.98*	<0.01
Heart	Y = 0.1X - 1.6	*0.98*	<0.01
Liver	Y = 0.07X - 3.6	*0.96*	<0.01
Kidney	Y = 0.2X - 4.8	*0.98*	<0.01
Tumor	Y = 0.2X - 4.5	*0.99*	<0.01
Plasma	Y = 0.1X - 1.0	*0.99*	<0.01

These curves were later used to obtain drug concentration in different tissues in groups studied. Table 2 shows the results of tissue spectroflourimetric measurements in different groups; free Doxorubicin, micellar Doxorubicin and micellar Doxorubicin with 28 kHz (0.04 W/cm^2) and 3 MHz (2 W/cm^2) combined dual frequency in continuous mode sonication. Drug content in group received micellar drug in tumor tissue is significantly higher (3.34 times) than group receiving Doxorubicin in its free form ($P<0.05$). In other tissues, drug content in micellar form is lower (1.35 times) than the group received free Doxorubicin ($P<0.05$). It is obviously shown that sonication causes a significant increase (2.60 times) in tumor uptake from micellar drug and a decrease in drug uptake in other tissues (from 1.5 times for spleen to 9.6 times for heart). It is expected that the micellar drug accumulation and uptake in tumor tissue be higher (8.69 times) than free drug which is due to tumor nature and EPR (Enhanced Penetration and Retention) effect. Besides, sonication causes an increased drug uptake because of sonoporation which is observed in group which sonicated at optimum sonication condition.

IV. CONCLUSION

In present study, in group which received Doxorubicin in its free form, spleen had the highest drug content and tumor had lower drug content than it while in group which received micellar drug, tumor had the highest drug concentration. This problem could be explained by tumor tissue characteristics. The physiological and interstitial properties of tumors such as slower lymphatic drainage than normal tissue which causes

TABLE II
THE STANDARD LINEAR REGRESSION FUNCTIONS FOR EACH TISSUE AS FLUORESCENCE INTENSITY (X: COUNT) VERSUS CONCENTRATION OF DOXORUBICIN (Y: µG/ML)

Group	Spleen	Liver	Kidney	Heart	Tumor	Plasma
Doxorubicin	2.50 (0.09)	1.13 (0.09)	1.90 (0.21)	2.49 (0.09)	1.50 (0.41)	0.82 (0.12)
Micellar Doxorubicin	2.28 (0.26)	0.94 (0.19)	1.48 (0.57)	2.31 (0.01)	5.00 (0.71)	0.65 (0.21)
Micellar Doxorubicin +Sonication	1.50 (0.37)	0.25 (0.16)	0.74 (0.13)	0.24 (0.13)	**13.00 (0.29)**	0.36 (0.03)

extravasation of macromolecules to their interstitial spaces. Also, lack of vascular permeability factors in tumor cells which make the tumor vasculature abnormally leaky to macromolecules and impossibility of tumor tissue to eliminate macromolecules may explain this accumulation of the polymer conjugates in tumors. The notable result was group which received micellar drug in combination with sonication. We used a dual frequency sonication condition which reduces cavitation area [8] and as a result, we can minimize cavitation region in vivo and control sonoporation region which causes enhanced drug uptake locally. It was observed that tumor drug uptake in this group is much more than group which only received micellar drug.

It was concluded that sonication in the time that blood drug content had not reduced significantly can increase drug uptake in tumor and it shows an enhanced drug uptake relative to physiological and interstitial properties of tumors. In other words, local sonication with a dual frequency system enables us to locally induce cavitation which causes drug release from micellar carriers and increases tumor vasculature permeability and so, increases drug uptake. Finally, the data suggest that acoustic cavitation plays an important role in triggering drug release from micelles. We hypothesize that cavitation events due to combined dual frequency irradiation disrupt micelles and release drug into aqueous environment. Based on the results presented above, acoustically activated micellar drug delivery may develop into an effective therapeutic technology for targeted delivery drugs to solid tumors.

REFERENCES

[1] A. Kümmerle, T. Krueger, M. Dusmet, C. Vallet, Y. Pan., H.B. Ris and L.A. Decosterd, “A validated assay for measuring doxorubicin in biological fluids and tissues in an isolated lung perfusion model: Matrix effect and heparin interference strongly influence doxorubicin measurements,” J. Pharm. Biomed. Anal., vol. 33, 2003, pp. 475-494.

[2] P. E. Colombo, M. Boustta, S. Poujol, F. Pinguet, P. Rouanet, F. Bressolle, M. Vert,”Biodistribution of doxorubicin-alkylated poly(l-lysine citramide imide) conjugates in an experimental model of peritoneal carcinomatosis after intraperitoneal administration,” Eur. J. Pharm. Sci. vol. 31, 2007, pp. 43-52.

[3] V. Alakhov, E. Klinski, S. Li, G. Pietrzynski, A. Venne, E. Batrakova, T. Bronitch and A. Kabanov,”Block copolymer-based formulation of doxorubicin. From cell screen to clinical trials,”Colloids Surf. B. Biointerfaces, vol. 16, 1999, pp.113-134.

[4] A. M. M. Osman, M. M. Nemnem, A. A. Abou-Bakr, O. A. Nassier and M. T. Khayyal,”Effect of methimazole treatment on doxorubicin-

induced cardiotoxicity in mice,"Food Chem. Toxicol., vol. 47, 2009, pp. 2425-2430.

[5] A. Fundar, R. Cavalli, A. Bargoni, D. Vighetto, G. P. Zara and M. R. Gasco," Non-stealth and stealth solid lipid nanoparticles (SLN) carrying doxorubicin: pharmacokinetics and tissue distribution after i.v. administration to rats,"Pharmacol. Res, vol. 42, 2003, pp. 337-343.

[6] A. H. Barati, M. Mokhtari-Dizaji, H. Mozdarani, S. Z. Bathaie and Z. M. Hassan,"Effect of exposure parameters on cavitation induced by low-level dual-frequency ultrasound," Ultrason. Sonochem, vol. 14, 2007, pp.783-789.

[7] R. Feng, Y. Zhao, C. Zhu and T. J. Mason,"Enhancement of ultrasonic cavitation yield by multi-frequency sonication," Ultrason. Sonochem, vol. 9, 2002, pp. 231-236.

[8] H. Hasanzadeh, M. Mokhtari-Dizaji, S. Z. Bathaie, Z. M. Hassan, V. Nilchiani and H. Goudarzi,"Enhancement and control of acoustic cavitation yield by low level dual frequency sonication: A subharmonic analysis," Ultrason. Sonochem, 2010 to be published.

[9] G. J. R. Charrois and T. M. Allen,"Drug release rate influences the pharmacokinetics, biodistribution, therapeutic activity, and toxicity of pegylated liposomal doxorubicin formulations in murine breast cancer," Biochim. Biophys. Acta, vol. 1663, 2004, pp.167-177.

[10] M. Yokoyama, M. Miyauchi, N. Yamada, T. Okano, Y. Sakurai, K. Kataoka and S. Inoue,"Characterization and anticancer activity of the micelle-forming polymeric anticancer drug adriamycin-conjugated poly (ethylene glycol)-poly (aspartic acid) block copolymer,"Cancer Res, vol. 50, 1990, pp.1693-1700.

[11] M. Yokoyama, M. Miyauchi, N. Yamada, T. Okano, Y. Sakurai, K. Kataoka and Inoue S,"Polymer micelles as novel drug carrier: Adriamycin-conjugated poly (ethylene glycol)-poly (aspartic acid) block copolymer,"J. Control. Release, vol. 11, 1990, pp. 269-278.

[12] Y. I. Jeong, J. W. Nah, H. C. Lee, S. H. Kim and C. S. Cho,"Adriamycin release from flower-type polymeric micelle based on star-block copolymer composed of poly(gamma-benzyl L-glutamate) as the hydrophobic part and poly(ethylene oxide) as the hydrophilic part," Int. J. Pharm, vol. 188, 1991, pp. 49-58.

[13] H. L. Wong, A. M. Rauth, R. and Bendayan, X. Y. Wu,"In vivo evaluation of a new polymer-lipid hybrid nanoparticle (PLN) formulation of doxorubicin in a murine solid tumor model."Eur. J. Pharm. Biopharm, vol. 65, 2007, pp. 300-308.

[14] R. K. Subedi, K. W. Kang and H. K. Choi,"Preparation and characterization of solid lipid nanoparticles loaded with doxorubicin,"Eur. J. Pharm. Sci, vol. 37, 2009, pp. 508-513.

[15] K. Kazunori, S. K. Glenn, Y. Masayuki, O. Teruo and S. Yasuhisa,"Block copolymer micelles as vehicles for drug delivery,"J. Control. Release, vol. 24, 1993, pp.119-132.

[16] M. Yokoyama, T. Okano, Y. Sakurai, H. Ekimoto, C. Shibazaki and K. Kataoka,"Toxicity and antitumor activity against solid tumors of micelle-forming polymeric anticancer drug and its extremely long circulation in blood,"Cancer Res, vol. 51, 1991, pp. 3229-3236.

[17] G. S. Kwon, M. Naito, M. Yokoyama, T. Okano, Y. Sakurai and K. Kataoka,"Physical entrapment of adriamycin in AB block copolymer micelles," Pharm. Res, vol. 12, 1995, pp. 192-195.

[18] Y. I. Jeong, H. S. Na, K. O. Cho, H. C. Lee, J. W. Nah and C. S," Antitumor activity of adriamycin-incorporated polymeric micelles of poly([gamma]-benzyl l-glutamate)/poly(ethylene oxide)," Int. J. Pharm, vol. 365, 2009, pp. 150-156.