Polyethylene Glycol-Diacyllipid Micelles Demonstrate Increased Acculumation in Subcutaneous Tumors in Mice

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Purpose. The purpose of this work is to study the potential of micelles prepared from amphiphilic polyethelene glycol/phosphatidylethanolamine (PEG-PE) conjugates as a particulate drug delivery system capable of accumulation in tumors via the enhanced permeability and retention (EPR) effect.

Methods. Micelles were prepared from PEGs of different molecular lengths conjugated with PE. The micelles were characterized by fluorescence-based critical micellization concentration (CMC) measurements, dynamic light scattering, and HPLC. Blood clearance and tumor accumulation of ^{111}In-labeled micelles were studied in mice with subcutaneously established Lewis lung carcinoma (LLC) and EL4 T lymphoma (EL4) tumors.

Results. Various versions of PEG-PE conjugates with PEG blocks ranging from 750 to 5000 Da formed very stable low CMC micelles at all concentrations down to 10^{-5} M. The size of the micelles varied between 7 and 35 nm depending on the length of the PEG block. Micelles remained intact after prolonged incubation with the blood serum. Upon intravenous administration into mice, the micelles demonstrated circulation longevity, and they efficiently and selectively accumulated in both subcutaneous Lewis lung carcinoma and EL4 T lymphoma tumors.

Conclusions. PEG-PE conjugates form very stable, long-circulating micelles. These micelles efficiently accumulate in tumors *in vivo* and may potentially be used as a tumor-specific delivery system for poorly soluble anticancer drugs.

KEY WORDS: Polyethelene glycol; phospholipid; CMC; polymeric micelles; tumor drug delivery.

INTRODUCTION

Micelles prepared from certain amphiphilic polymers possess a number of properties that distinguish them from other particulate drug carriers (1). The micelles are lacking the interior water compartment, however, they do have a hydrophobic interior (micelle core), which may be effectively loaded with various poorly soluble drugs. Some polymeric micelles have a very low critical micellization concentration (CMC), which makes them extremely stable and prevents their dissociation upon *in vivo* applications. The average size of micelles is usually smaller than that of liposomes and microparticles permitting their more efficient extravasation, especially in body areas with leaky vasculature (1).

Usually, microparticulate drug carriers are removed from the circulation via the opsonization-mediated phagocytosis by organs and tissues of the reticuloendothelial system (RES) (2). The use of certain polymers as micelle-forming materials may, however, result in micellar drug carriers with extended circulation times. The micelle corona formed by water-exposed hydrophilic polymer blocks provides the protective effect to micelles and determines their longevity *in vivo* by repelling opsonizing blood components (3). This allows micelles to stay in the blood (tissues) relatively long avoiding the interaction with opsonins and recognition by RES. The ability of hydrophilic polymers to inhibit RES uptake has been demonstrated with numerous examples of microparticular carriers having their surface modified with certain "protective" water-soluble polymers (4–7).

Because of their characteristic size (normally, between 5 and 50 nm), the micelles may represent an ideal delivery system for pharmaceuticals utilizing enhanced permeability and retention (EPR) effect also known as a "passive" targeting or accumulation via an impaired filtration mechanism. The EPR effect is based on the spontaneous penetration of long-circulating molecular aggregates and small particles into the interstitium through the leaky vasculature in certain pathological sites in the body, such as tumors, infarcts, and inflammations (8,9). The transport efficacy of microparticulates, such as liposomes and/or micelles, into the tumor interstitium and their accumulation there are, to a great extent, determined by their ability to penetrate tumor vascular endothelium (10,11). Diffusion and accumulation parameters were shown to be strongly dependent on the cutoff size of the tumor blood vessel wall, and this cutoff size may vary for different tumors (11–13). The ability of certain types of polymeric micelles to accumulate in tumors has been demonstrated in a number of models *in vivo* (14,15).

Micelles prepared from conjugates of polyethyleneglycol (PEG) and diacyllipids (16) are of particular interest. Phospholipid residues attached to PEG moieties represent short, however, extremely hydrophobic blocks due to the presence of two long-chain fatty acyl groups, and effectively form a hydrophobic core of the micelle (16). PEG chains, on the other hand, are known to be highly water soluble, highly hydrated, and able to serve as efficient steric protectors for various microparticulates (such as micelles, liposomes, nanoparticles and nanocapsules) in biological media (17).

Earlier, we reported some preliminary data on polymeric micelles formed by PEG-PE conjugates (18). In particular, we demonstrated that PEG-PE conjugates with various PEG lengths provide extremely stable and long-circulating micelles that can be loaded with a variety of poorly soluble drugs including anticancer drugs such as dequalinium (19), and are capable of delivering their load into low cutoff size tumor in mice with a higher efficiency than even PEG-modified liposomes (15). As shown in (15), these micelles firmly retain a micelle-incorporated hydrophobic protein in the circulation and during their accumulation in tumor sites.

Here, we present the detailed results on the formation of micelles from different versions of PEG-PE conjugates and additional characterization of PEG-PE micelles with respect to their size and stability. Blood clearance characteristics of these micelles and their ability to effectively accumulate in subcutaneous LLC and EL4 tumors in mice are also reported.

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MATERIALS AND METHODS

Materials

All lipids and diacyllipid-PEGs were from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. All other chemicals and components for buffer solutions were analytical grade preparations. Distilled and deionized water was used in all experiments. ^{111}In with specific radioactivity of 395 Ci/mg of In equivalent was purchased from Perkin Elmer Life Sciences, Inc. (Boston, MA) and was used within 7 days.

Methods

Synthesis of Diethylene Triamine Pentaacetic Acid-Phosphatidylethanolamine Conjugate (DTPA-PE)

To prepare the amphiphilic DTPA-PE used for micelle labeling with ^{111}In, a solution of 0.1 mmol of egg PE in 4 ml of chloroform supplemented with 30 μl of triethylamine was added drop-wise to a stirred solution of 1 mmol of DTPA anhydride in 20 ml of DMSO. The mixture was incubated for 3 h at room temperature under argon. After incubation, the solution was dialyzed against 6 l of water at 4°C for 48 h with several changes. Purified DTPA-PE was freeze-dried and stored frozen at –80°C.

Preparation and Characterization of Micelles

Micelle Preparation. To prepare micelles, first, one of the compounds listed in Table I was dissolved in chloroform. Then, the diacyllipid-PEG film was formed by organic solvent evaporation, and micelles were formed by an extensive vortexing of this film for 5–15 min in HBS (10 mM HEPES, 150 mM NaCl, pH 7.4). The final concentration of PEG-PE conjugate was 5 mM. PEG_{2000}-DOPE micelles were prepared at room temperature. Micelles from PEG modified with saturated PE were formed at 50°C. In some cases, 0.5% mol of the fluorescent probe, dipalmitoyl phosphatidylethanolamine (lisamine rhodamine B) (Rh-PE), was added to the micelle composition. For labeling the micelles with ^{111}In, DTPA-PE was also added in trace amount.

Radiolabeling of Micelles. For animal experiments, DTPA-PE-containing micelles in HBS were mixed with 50–100 μCi of $^{111}InCl_3$ in 0.1 M Na-citrate, pH 3.7. The mixture was incubated for 1 h at room temperature and dialyzed against at least 3000-fold excess of HBS overnight at 4°C to remove unbound ^{111}In. Purified ^{111}In-labeled micelles were used fresh for animal experiments.

Micelle Size Measurement. The micelle size (hydrodynamic diameter) was measured by dynamic light scattering (DLS) using a N4 Plus Submicron Particle System (Coulter Corporation, Miami, FL) at PEG-PE concentration of 2–10 mM.

Critical Micelle Concentration Determination. CMC was estimated by pyrene method adapted from a protocol published earlier (20). Pyrene was dried from a 10 mg/ml solution in chloroform to obtain 1 mg of dry crystals. To these crystals, 1 ml of 10^{-10}–10^{-4} M micellar solution of PE-PEG in HBS was added. The mixtures were incubated for 17 h at 25°C with shaking in the darkness under argon. Free pyrene was removed by filtration through polycarbonate membranes with 0.2 μm pore size (Poretics Products, Livermore, CA). The fluorescence of filtered samples was measured at the excitation wavelength of 339 nm and emission wavelength of 390 nm ($F_{339/390}$) using F2000 fluorescence spectrometer (Hitachi, Japan). CMC values were determined from the pyrene fluorescence in solution as a function of PEG-PE concentration, and have corresponded to PEG-PE concentrations, at which a sharp increase in the solution fluorescence has occurred.

HPLC. HPLC of the micelles was performed using D-7000 based HPLC system equipped with diode array and fluorescence detectors (Hitachi, Japan). Separation was done using a size-exclusion Shodex® column (Shoko Co., LTD, Japan) with HBS as a mobile phase at room temperature and at the flow rate of 1.5 ml/min. Peaks of different components were detected by optical density at corresponding wavelengths and/or by following the fluorescence intensity at the excitation wavelength of 550 nm, and emission wavelength of 590 nm ($F_{550/590}$).

Micelle Stability in Blood Serum. To test the stability of micelles in the blood serum, Rh-PE-labeled micelles were incubated with the fetal bovine serum (FBS) at the diacyllipid-PEG concentration of 5 mM at room temperature for 48 h. The samples obtained were diluted in HBS by 1000-fold and analyzed for the presence of micelles and their size by HPLC as described above.

Table I. CMC Values and Particle Sizes of Micelles Formed from PEG-PE with Various Lipid and Polymer Constituents

PEG-diacyllipid*	CMC	Particle size
PEG_{750}-DSPE	1.0×10^{-5} M	7–15 nm
PEG_{2000}-DSPE	1.1×10^{-5} M	7–20 nm
PEG_{5000}-DSPE	6.2×10^{-6} M	10–40 nm
PEG_{2000}-DOPE	8.7×10^{-6} M	7–20 nm
PEG_{5000}-DOPE	7.3×10^{-6} M	10–35 nm
DPPC	9.5×10^{-6} M	N/A

* DSPE, distearoyl-PE; DOPE, dioleoyl-PE; the number associated indicates the molecular weight of PEG; DPPC, dipalmitoylphosphatidylcholine.

Cell Cultures

Murine tumor cell lines, LLC and EL4, were purchased from the American Type Culture Collection (Manasas, VA) and grown at 37°C, 5% CO_2. LLC cells were maintained in DMEM cell culture medium supplemented with 10% of heat inactivated FBS, penicillin/streptomycin, pyruvate, L-glutamine and non-essential amino acids. For mice inoculation, LLC cells were grown until confluency, washed with Hank's solution and detached using a 0.25% trypsin, 0.1% EDTA solution. Detached cells were washed 2 times with Hank's buffer at 700× g, re-suspended in the same buffer and immediately used for the inoculation. EL4 cells were grown in RPMI 1640 medium supplemented with the same additives as the medium for LLC cells. For mice inoculation, EL4 cells were centrifuged at 700× g and the supernatant was replaced with the Hank's buffer immediately before use. All products for cell culturing were purchased from Mediatech, Inc. (Herndon, VA).

Blood Clearance and Tumor Accumulation Experiments

The experiments were performed in female C57BL/6J mice (Charles River Laboratories, Wilmington, MA) following a protocol approved by Northeastern University Institutional Animal Care and Use Committee in accordance with "Principles of Laboratory Animal Care" (NIH publication #85-23, revised in 1985). The animals were allowed free access to food and water. To grow tumors, mice were inoculated subcutaneously with 20,000 of LLC cells or 5,000 EL4 cells into left rear flanks. When tumor diameters reached ca. 0.5–1 cm (1–2 weeks post inoculation), the mice were injected with 100 μl of 0.5 mM ^{111}In-labeled micellar formulations via the tail vein. At the required time points within 0.5–17 h post injection interval, mice were sacrificed by cervical dislocation. Blood, tumors and samples of muscle tissue were collected and analyzed for the presence of the micelle-associated ^{111}In radioactivity. Two pharmacokinetic parameters were calculated from these data. First, micelle circulation half-lives assuming first order elimination process have been determined from the % of injected dose in blood vs. time plots. Second, areas under the curve % of the dose per gram of tumor or muscle vs. time (AUC) have been estimated. Both pharmacokinetic parameters have been calculated using a computer program, Origin (Microcal Software, Inc, Northampton, MA). There were 4–5 animals in a group for each time point.

RESULTS AND DISCUSSION

Micelle Properties

All studied PEG-PE conjugates (Table I) form micelles upon an intensive vortexing in an aqueous buffer. Conjugates with unsaturated PE blocks form micelles at room temperature. Elevated temperature facilitates formation of micelles from saturated compounds. The size of the micelles is presented in Table I. As the measurements indicate, all compounds form particles with the size of 7–35 nm that is characteristic for micelles. Micelles formed from compounds of a higher molecular weight have a slightly larger size indicating that the micelle size may be tailored for a particular application by varying the length of hydrophilic block. Since the observed size of particles in various micellar systems does not change during more than one month long storage at room temperature, the micelles may be considered stable.

Pharmaceutical micelles should be stable enough to provide a sufficient time for drug delivery and accumulation in the target zone. Stability of micelles both *in vitro* and *in vivo* as well as their clearance from the body depend on their CMC values (1). We used the pyrene incorporation method to evaluate the CMC values of our formulations (20). The results are shown in Table I. CMCs of all compounds are in a 10^{-5} M range, which is at least 100-fold lower than those conventional detergents. CMC values in a 10^{-5} M range indicate that even upon the dilution in the blood pool (during the supposed therapeutic application) PE-PEG micelles will preserve their integrity.

Despite the fact that pyrene method is considered to be one of the most sensitive and precise (20,21), its sensitivity may not be sufficient enough for the measurement of actual CMC values of compounds with double acyl chains, since it has been reported that in the cases of very low CMC values, even the most sensitive conventional methods for CMC determination are not applicable and produce the upper estimation rather than actual values (22). For example, an apparent CMC value produced by the pyrene method for DPPC (9.5×10^{-6} M, Table I) is much higher than that obtained using the radioactive DPPC [5×10^{-10} M] (22). Thus, the numbers shown in Table I should be considered the upper limits of CMC rather than actual values.

We have shown that size-exclusion HPLC may be used to evaluate the size of micelles at concentrations, which are much lower than about 2 mM required by DLS. The latter concentration is far above the one expected upon *in vivo* administration. Figure 1 shows HPLC profiles of PEG_{5000}-DSPE and PEG_{2000}-DSPE micelles compared to other samples of known size. Profiles in the main frame of Fig. 1 were obtained using micelles at the same concentration that was used for DLS. The micelles have sizes between those of liposomes and IgG. HPLC resolves the size difference between PEG_{5000}-DSPE and PEG_{2000}-DSPE. Dilution of the micelles to the concentration of 2.5×10^{-6} M does not change the retention times of the micelles (Fig. 1, inset). The results obtained show, in particular, that the micelles do not dissociate at a concentration even below the apparent CMC values obtained by the pyrene method. Because of extreme stability of the micelles, it is rather difficult to determine the actual, not apparent, CMC values for PEG_{5000}-DSPE and PEG_{2000}-DSPE. From the practical point of view, it is important that both types of micelles remain intact at concentrations much lower than required for drug delivery purposes.

For a potential *in vivo* application, it is important that the delivery system is not affected by the blood plasma components. To investigate the stability of micelles in the blood, we have incubated the PEG_{5000}-DSPE and PEG_{2000}-DSPE micelles in the presence of blood serum for 48 h at room temperature and analyzed the integrity of the particles by HPLC (Fig. 2). There were some minor changes in the shape of peaks indicating that the micelles were somehow affected. The bulk amount of the particles, however, retained the size characteristic for the original micelles (compare with the inset

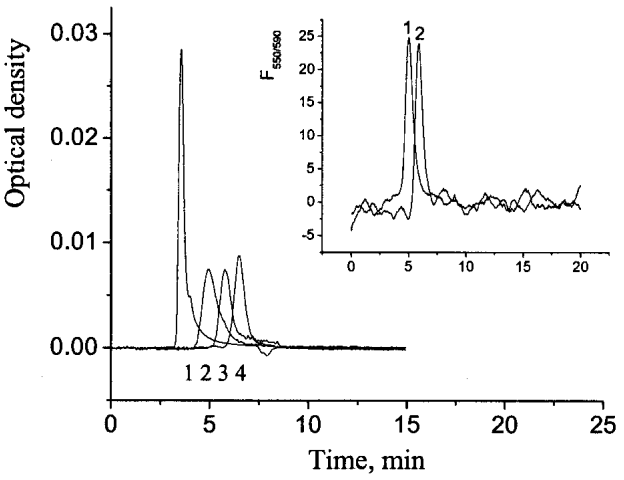

Fig. 1. HPLC profiles of PEG-PE micelles and reference compounds. Main frame: 1. 240 nm liposomes (A_{250}); 2. PEG_{5000}-DSPE micelles at 5 mM (A_{220}); 3. PEG_{2000}-DSPE micelles at 5 mM (A_{220}); 4. IgG (A_{280}). Inset shows HPLC profiles of: 1. PEG_{5000}-DSPE; and 2. PEG_{2000}-DSPE micelles after dilution to 2.5×10^{-6} M.

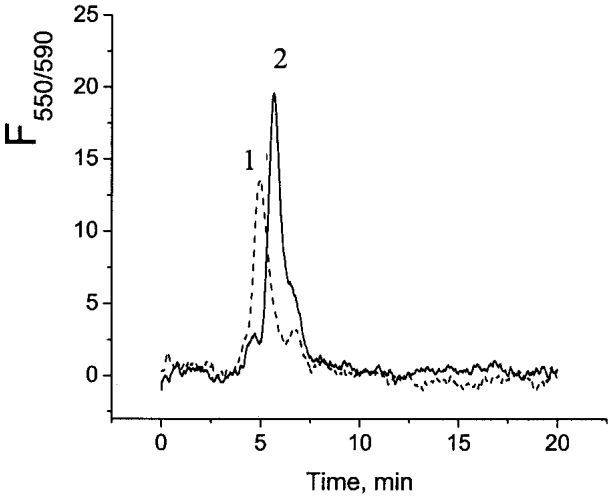

Fig. 2. HPLC profile of the micelles after incubation in FBS: 1. PEG_{5000}-DSPE; 2. PEG_{2000}-DSPE.

in Fig. 1). The *in vivo* situation, however, may be somewhat different because of micelle possible interaction with lipoproteins and/or cells.

Micelle Clearance *in Vivo*

The results of the blood clearance study of some PEG-PE micelles are shown in Fig. 3. The micelle formulations have circulation half-lives from 1.2 to 2.1 h depending on the molecular size of PEG block. PEG_{5000}-DSPE, PEG_{2000}-DSPE and PEG_{750}-DSPE have the circulation half-life of 2.1 h, 2.0 h and 1.2 h, respectively. Evidently, the increase in the size of PEG block increases the micelle circulation time in the blood, probably, by providing a better steric protection against opsonin penetration to the hydrophobic micelle core. The use of unsaturated DOPE instead of saturated DSPE as a lipid component of PEG-PE conjugates somewhat decreases the circulation half-life of micelles (1.3 h for PEG_{2000}-DOPE vs. 2.0 h for PEG-DSPE). The reasons for this difference are not yet clear. All micelle formulations have a longer half-life compared to the majority of non-surface-modified

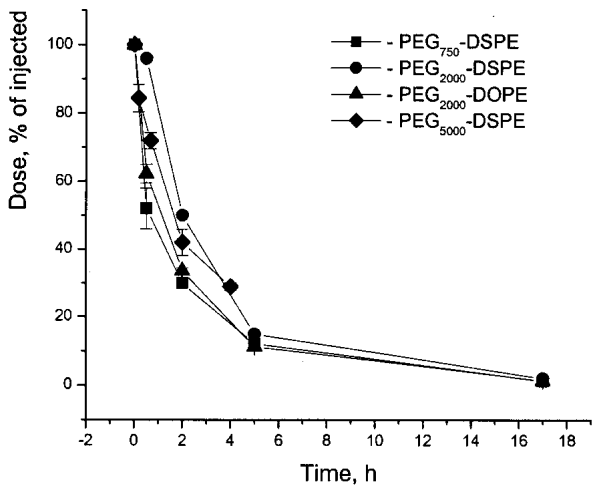

Fig. 3. Blood clearance of ^{111}In-labeled PEG-PE micelles in mice.

particulates (though somewhat shorter than that for PEG-coated liposomes). The shorter micelle half-life compared to PEG-liposomes may be explained by their faster extravasation from the vasculature due to their considerably smaller size when compared with liposomes (15). In addition, accelerated micelle dissociation *in vivo* can in part be responsible for their shorter half-life because of renal clearance of unimers, which results in shifting the micelles/unimers equilibrium towards dissociation.

Micelle Biodistribution and Tumor Accumulation

It has been repeatedly demonstrated that long-circulating PEG-grafted liposomes display an increased accumulation in implanted tumors (23) via the EPR effect. However, it has also been found that in some cases the use of long-circulating liposomes does not enhance tumor accumulation. In (24), it was shown that coating 100 nm liposomes with PEG did not result in an increased accumulation of liposome-encapsulated drug in a subcutaneously established murine LLC tumor. This phenomenon may be explained by the low vascular permeability (small cutoff size) of the LLC as well as some other tumors. In such cases, drug carriers smaller in size than liposomes may provide more efficient tumor drug delivery utilizing EPR mechanism. In our earlier preliminary research, we showed that the micelles formed from PEG_{5000}-DSPE deliver the micelle-incorporated soybean trypsin inhibitor in subcutaneously established LLC tumor in mice with higher efficiency than PEG-liposomes (15).

Here, we investigate the tumor accumulation and the tumor-to-muscle accumulation ratio using an additional tumor model and micelles of some other compositions. The data showing that the micelles formed from PEG_{750}-DSPE and PEG_{2000}-DSPE efficiently accumulate in LLC tumor are presented in fig. 4. The advantage of PE-PEG versions with shorter PEGs is that they have higher hydrophobic core/polymer corona ratio thus being capable of incorporating poorly soluble drugs with higher efficiency.

Both micellar formulations studied accumulate in LLC tumors more efficiently compared to muscle tissue (Fig. 4). Despite their shorter half-live in the circulation (Fig. 3) PEG_{750}-DSPE micelles have a higher targeting index (AUC_{tumor}/AUC_{muscle}) compared to PEG_{2000}-DSPE: 3.8 and 2.8 respectively. However, the PEG_{2000}-DSPE version stays in the tumor for a longer time (Fig. 4A). No indication of micelle elimination from the tumor was detected after as long as 17 h post injection. The results obtained show, in particular, that such parameters characterizing tumor targeting as tumor accumulation and tumor residence time could be controlled by choosing PEG blocks of an appropriate size.

The accumulation of micelles in EL4 tumor is shown in Fig. 5. Both types of micelles demonstrated selective accumulation in this tumor as well. In general, the accumulation of the micelles in EL4 tumor follows the pattern found for the LLC tumor. In the case of EL4, however, the micelles prepared from PEG_{750}-DSPE exhibit slightly better accumulation compared to PEG_{2000}-DSPE. This fact may be explained by a smaller cutoff size of EL4 vasculature compared to that of the LLC tumor.

Some other factors, in addition to the leaky vasculature, can be involved in control over the tumor accumulation of

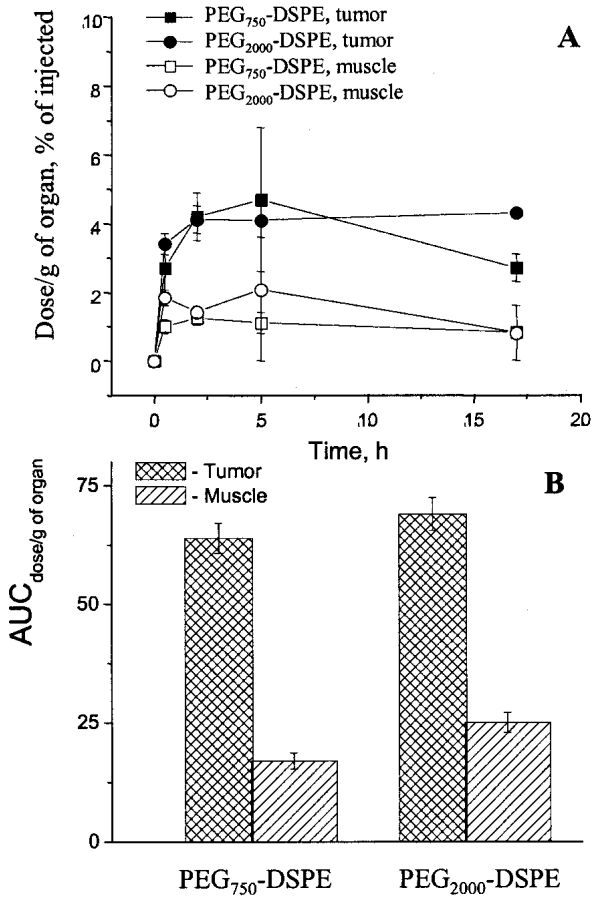

Fig. 4. Selective accumulation of PE-PEG micelles in Lewis lung carcinoma tumor in mice: A—pharmacokinetics; B—AUC. Mean ± SE; number of data set was 4–5 per time point.

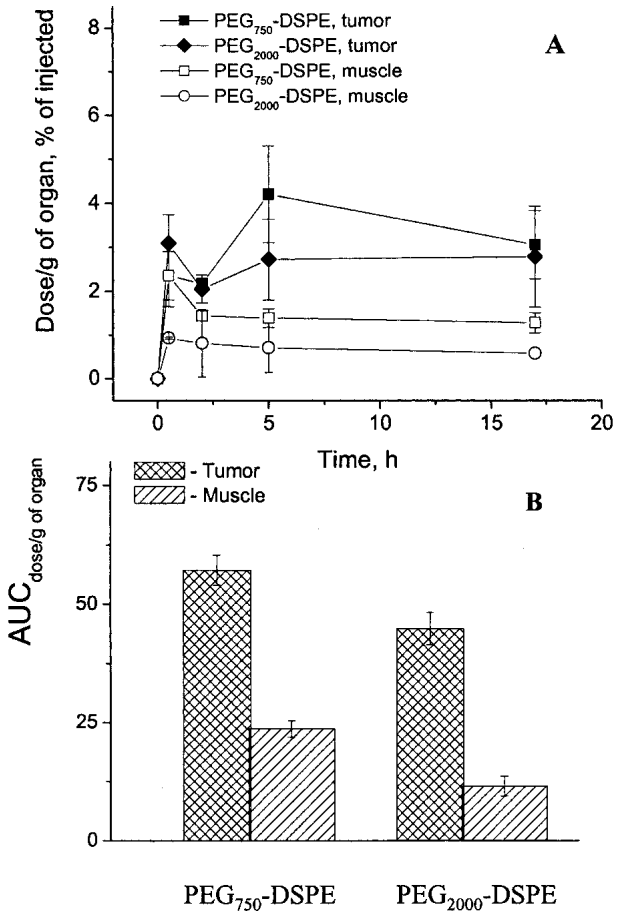

Fig. 5. Selective accumulation of PE-PEG micelles in EL4 T lymphoma tumor in mice: A—pharmacokinetics; B—AUC. Mean ± SE; number of data set was 4–5 per time point.

particulate formulations, such as blood vessel density, blood flow rate, and interstitial pressure in tumors. However, these factors do not seem to play the crucial role in our case, since the net tumor accumulation was rather consistent despite variable tumor sizes in individual mice (i.e. variable blood vessel density and interstitial pressure) in two different tumor types, one of which (LLC) is characterized with high vascularity and lowcut off size (15). The fact that it is namely a small micelle size allowing for their better tumor accumulation is additionally supported by a very limited accumulation of larger size long-circulating liposomes in small cutoff size tumors, such as LLC (24).

In conclusion, we have demonstrated that long-circulating PEG-PE-based polymeric micelles spontaneously accumulate in tumors via the EPR mechanism. Such micelles could be used for the delivery of poorly soluble anticancer drugs into tumors. This is confirmed by earlier findings that different poorly soluble substances can be effectively incorporated into PEG-PE-based micelles without affecting their stability (16,19) together with our recent data on anticancer drug-loaded PEG-PE micelles (25). The efficacy of micelle accumulation depends on the tumor type (cutoff size of tumor vasculature) and can be controlled by varying the molecular size of PEG blocks in PEG-PE conjugates. The therapeutic effect of micelle-incorporated anticancer drugs is the subject of our current research.

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