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Note

Particle size-dependent triggering of accelerated blood clearance phenomenon

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ABSTRACT

A repeat-injection of polyethylene glycol-modified liposomes (PEGylated liposomes) causes a rapid clearance of them from the blood circulation in certain cases that is referred to as the accelerated blood clearance (ABC) phenomenon. In the present study, we examined whether polymeric micelles trigger ABC phenomenon or not. As a preconditioning treatment, polymeric micelles (9.7, 31.5, or 50.2 nm in diameter) or PEGylated liposomes (119, 261 or 795 nm) were preadministered into BALB/c mice. Three days after the preadministration [^{3}H]-labeled PEGylated liposomes (127 nm) as a test dose were administered into the mice to determine the biodistribution of PEGylated liposomes. At 24 h after the test dose was given, accelerated clearance of PEGylated liposomes from the bloodstream and significant accumulation in the liver was observed in the mice preadministered with 50.2–795 nm nanoassemblies (PEGylated liposomes or polymeric micelles). In contrast, such phenomenon was not observed with 9.7–31.5 nm polymeric micelles. The enhanced blood clearance and hepatic uptake of the test dose (ABC phenomenon) were related to the size of triggering nanoassemblies. Our study provides important information for developing both drug and gene delivery systems by means of nanocarriers.

1. Introduction

PEGylated liposomes possessing a long-circulating characteristic have been widely used for delivery systems of both drugs and genes. PEG provides a steric barrier to nanocarriers for avoiding interaction with plasma proteins including opsonins and the cells of mononuclear phagocyte system (MPS) (Allen and Hansen, 1991; Sakakibara et al., 1996; Lasic, 1996). However, our recent reports demonstrated that the intravenous injection of PEGylated liposomes might significantly alter a pharmacokinetic behavior of them injected thereafter (Ishida et al., 2006a,c; Wang et al., 2007). A repeat-injection of PEGylated liposomes causes a rapid clearance of them from the blood circulation in certain cases. This phenomenon, referred to as the accelerated blood clearance (ABC) phenomenon, is considered to be related with anti-PEG IgM secretion from splenic B cells (Ishida et al., 2006a,c). Anti-PEG IgM, produced in response to an injected dose of PEGylated liposomes, selectively binds to them injected secondary (Wang et al., 2007).

However, the immune response against polymeric micelles was not known at all. Polymeric micelles are formed from block copolymers typically consisting of hydrophilic and hydrophobic polymer blocks (Kwon and Kataoka, 1995). They are of particular interest as a drug carrier because of their small particle sizes, efficiency in entrapping a satisfactory amount of hydrophobic drugs within the inner core, stability in the circulation, and their ability of sustained release of the drugs. Polymeric micelles were also considered as a less immune response carrier (Yokoyama et al., 1991; Gaucher et al., 2005).

In this study, we examined whether the preadministration of polymeric micelles possessing PEG chains alters the biodistribution of PEGylated liposomes or not. Moreover, we investigated the

Abbreviations: ABC phenomenon, accelerated blood clearance phenomenon; [^{3}H]-CHE, [^{3}H] cholesterylhexadecyl ether; MPEG-DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy(polyethylene glycol)-2000]; MPS, mononuclear phagocyte system; PEG-PBLA, poly(ethylene glycol)-*b*-poly(β-benzyl L-aspartate); PEGylated liposomes, polyethylene glycol-modified liposomes.

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particle size-dependency for triggering the phenomenon by use of PEGylated liposomes and polymeric micelles.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC), cholesterol and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy(polyethylene glycol)-2000](MPEG-DSPE) were kindly gifted from Nippon Fine Chemical Co., Ltd. (Takasago, Hyogo, Japan). [^{3}H]cholesterylhexadecyl ether ([^{3}H]-CHE) was purchased from Amersham Pharmacia (Buckinghamshire, UK). All other reagents were analytical grade.

2.2. Animal

Five-week-old male BALB/c mice were purchased from Japan SLC Inc. (Shizuoka, Japan). The animals were cared for according to the animal facility guidelines of the University of Shizuoka. All animal experiments were approved by the Animal and Ethics Review Committee of the University of Shizuoka.

2.3. Preparation of polymeric micelles

Three block copolymers were used for polymeric micelle preparations. Their structures and compositions are summarized in Table 1. Poly (ethylene glycol)-*b*-poly(β-benzyl L-aspartate) (PEG-PBLA) was synthesized by polymerization of β-benzyl L-aspartate *N*-carboxy anhydride from an amino terminal of α-methyl-ω-aminopoly(oxyethylene), as reported previously (Yokoyama et al., 1992). Two partially esterified block copolymers, PEG-P(Asp(pentyl)) and PEG-P(Asp(nonyl)), were prepared by esterification of PEG-*b*-poly(aspartic acid) block copolymer by a reported method (Yamamoto et al., 2007). In brief, aspartic acid residues of PEG-*b*-poly(aspartic acid) block copolymer was activated with 1,8-diazabicyclo[5,4,0]7-undecene, followed by reaction with corresponding alkyl bromides, pentyl bromide and nonyl bromide.

Polymeric micelles were prepared from these three block copolymers by a dialysis method (Yamamoto et al., 2007). Block copolymers were dissolved in DMF at a concentration of 7.5 mg/ml. These polymer solutions were dialyzed against distilled water by the use of a dialysis membrane (Spectra/Por 6, molecular weight cut-off: 1000, Spectrum Japan, Tokyo, Japan). After overnight dialysis, the micelle solutions were concentrated by ultrafiltration (Millipore ultrafiltration membrane PBHK, molecular weight cut-off: 100,000, Nihon Millipore, Tokyo, Japan). By dynamic light scattering, weight-averaged diameters of the obtained polymeric micelles were found to be 50.2, 31.5, and 9.7 nm for PEG-PBLA, PEG-P(Asp(pentyl)), and PEG-P(Asp(nonyl)), respectively.

2.4. Preparation of PEGylated liposomes

PEGylated liposomes composed of DPPC and cholesterol with MPEG-DSPE (10:5:1 as molar ratio) were prepared as described previously (Maeda et al., 2004). In brief, lipids dissolved in chloroform were evaporated to form thin lipid film. Then liposomes were formed by hydration with 10 mM phosphate-buffered 0.3 M sucrose solution (pH 7.4). Then liposomes were sized by five times extrusion through a polycarbonate membrane filter with 100, 400 or 800 nm pores (Nucleopore, Maidstone, UK). For a biodistribution study, a trace amount of [^{3}H]-CHE (74 kBq/mouse) was added to the initial chloroform solution. Particle size of PEGylated liposomes diluted with PBS, pH 7.4, was measured by dynamic light scattering.

2.5. Biodistribution of PEGylated liposomes

Mice were received intravenous injection of polymeric micelles (2.9 mg/kg), PEGylated liposomes (2.0 μmol phospholipids/kg, 2.4 mg total lipids/kg) or phosphate-buffered sucrose. At three days later [^{3}H]-labeled test-dose PEGylated liposomes (5.0 μmol phospholipids/kg) were injected into them via a tail vein. Twenty-four hours after the test-dose administration, the mice were sacrificed for the collection of the blood from the carotid artery. Then the blood treated with heparin was centrifugally separated to obtain the plasma. After the blood was withdrawn, the heart, the lung, the liver, the spleen and the kidney were removed and weighed. The radioactivity in plasma and each organ was determined with a liquid scintillation counter (LSC-3100, Aloka, Tokyo, Japan). Distribution data are presented as % dose per wet tissue. The total amount in plasma was calculated based on the average body weight of the mice, where the average plasma volume was assumed to be 4.27% of the body weight based on the data on total blood volume.

2.6. Statistics

Variance in a group was evaluated by the *F*-test, and differences in biodistribution data, by Student's *t*-test.

3. Results and discussion

At first, we used PEGylated liposomes with 119, 261 or 795 nm diameter as a preconditioning dose. Fig. 1 shows the biodistribution of test-dose PEGylated liposomes (127 nm). The amount of the PEGylated liposomes in the plasma was significantly decreased and that in the liver was significantly increased in the mice preadministered with the PEGylated liposomes. ABC phenomenon was caused by all liposomes tested. Fig. 2 shows the biodistribution of test-dose PEGylated liposomes in the mice preadministered with polymeric micelles (9.7, 31.5 or 50.2 nm) at 3 days before. The mice prereceived

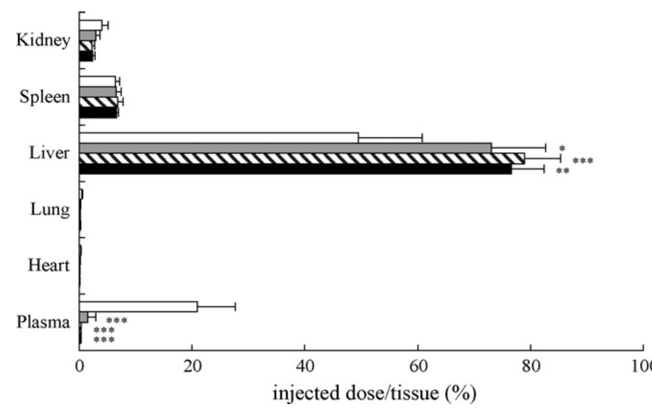
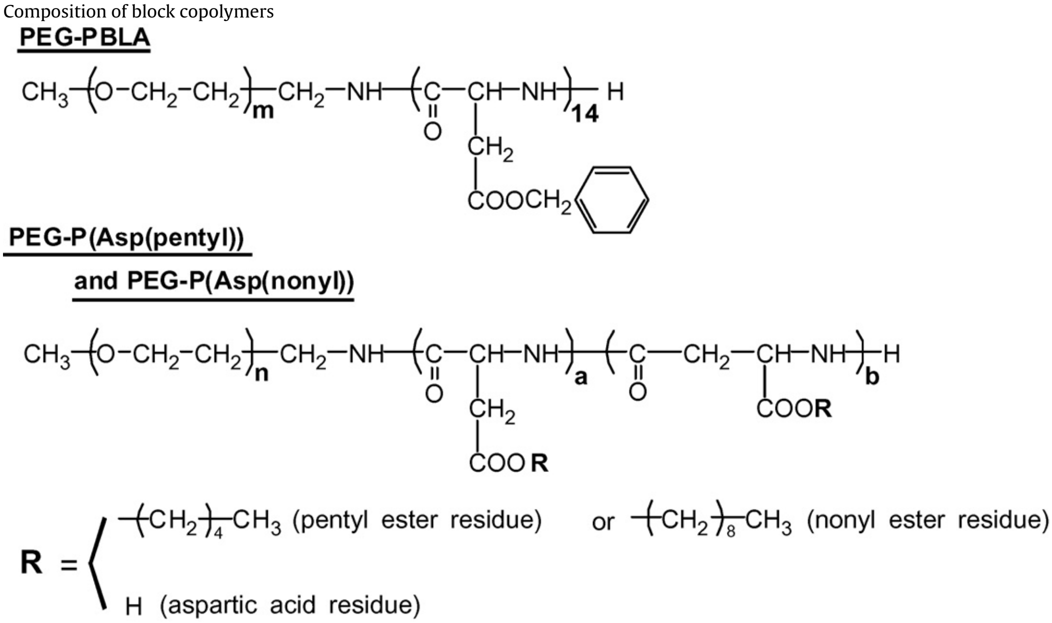


Fig. 1. Biodistribution of test-dose PEGylated liposomes after preadministration of various sized ones. BALB/c mice were intravenously injected with PEGylated liposomes (2.0 μmol phospholipids/kg) with 119, 261 or 795 nm size. Three days later [^{3}H]-labeled test-dose PEGylated liposomes (5.0 μmol phospholipids/kg) were administered via a tail vein. Twenty-four hours later, the mice were sacrificed and the radioactivity in the plasma and each organ was determined ($n = 5$). Data are presented as a percentage of the injected dose per tissue and S.D. Data represent phosphate-buffered sucrose (open bar), 119 nm (gray bar), 261 nm (hatched bar), and 795 nm (closed bar) PEGylated liposomes, respectively. Significant differences against phosphate-buffered sucrose group are shown with asterisks: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Table 1
Composition of block copolymers



Copolymer	Molecular weight (M.W.)	M.W. of PEG block	Number of Asp units ($a+b$)	Esterification degree (%)[a]	Diameter (nm)[b]
PEG-PBLA	15,000	12,000	14	100	50.2
PEG-P(Asp(pentyl))	9,000	5,000	22	75	31.5
PEG-P(Asp(nonyl))	10,000	5,000	22	72	9.7

[a] Esterification degree (%) = (number of ester residues)/(number of ester residues) + (number of aspartic acid residues) × 100. This degree was determined by ^{1}H NMR measurements.
[b] Weight-weightened average diameter determined by dynamic light scattering.

50.2 nm polymeric micelles showed a significant decrease of test-dose PEGylated liposomes in the plasma and a significant increase in hepatic uptake. However, the preadministration of both 9.7 and 31.5 nm polymeric micelles did not change plasma concentration and hepatic uptake of test-dose PEGylated liposomes. It appears that ABC phenomenon was not caused by preadministration with smaller-sized polymeric micelles (31.5 nm or less), while it was triggered by preadministration with larger-sized polymeric micelles (50.2 nm or more). These results indicate that ABC phenomenon was triggered by preconditioning with not only PEGylated liposomes but also PEG-containing polymeric micelles. Furthermore, the size of nanoassemblies presenting PEG moiety on their surface is one of important factors to induce the ABC phenomenon. In case of large particles, they would be recognized easily by immune cells and activate immune systems, presumably in spleen (Ishida et al., 2006b). By contrast, small particles might avoid the recognition by immune cells. In the point of the molecular weight of PEG moiety, we previously reported that elongation of PEG chain length did not show any difference for inducing ABC phenomenon (Ishida et al., 2005). Consequently, the larger particles may produce anti-PEG IgM (Wang et al., 2007) that triggers enhanced blood clearance and hepatic uptake of test-dose PEGylated liposomes, although further investigation should be required to prove this assumption.

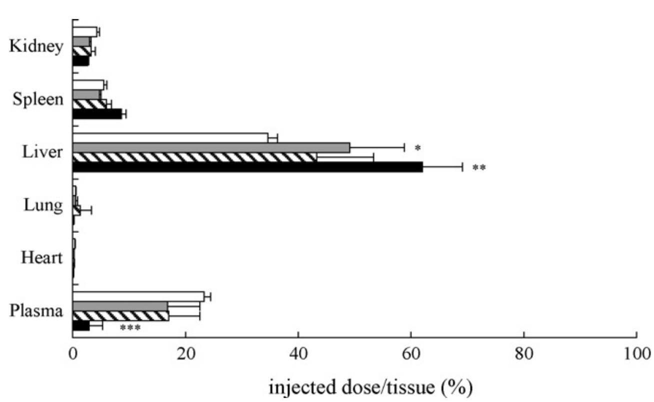


Fig. 2. Biodistribution of test-dose PEGylated liposomes after preadministration of various size polymeric micelles. BALB/c mice were intravenously injected with polymeric micelles (2.9 mg/kg) with 9.7, 31.5 or 50.2 nm size. Three days later [^{3}H]-labeled PEGylated test-dose liposomes (5.0 μmol phospholipids/kg) were administered via a tail vein. Twenty-four hours later, the mice were sacrificed and the radioactivity in the plasma and each organ was determined ($n=5$). Data are presented as a percentage of the injected dose per tissue and S.D. Data represent phosphate-buffered sucrose (open bar), 9.7 nm (gray bar), 31.5 nm (hatched bar), and 50.2 nm (closed bar) polymeric micelles, respectively. Significant differences against phosphate-buffered sucrose group are shown with asterisks: $^*p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

4. Conclusions

This study is the first report to demonstrate that the preconditioning with polymeric micelles sized at around 50 nm, which are most widely used to deliver anti-cancer drug, causes the ABC phenomenon. Furthermore, it is clarified that the size of nanoassemblies is one of important factors for ABC phenomenon. Since nanocarriers are now progressing in the field of DDS, this study points out the important information about unexpected immune reactions against nanocarriers.

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