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Size-dependent induction of accelerated blood clearance phenomenon by repeated injections of polymeric micelles

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ABSTRACT

An accelerated blood clearance (ABC) phenomenon is induced by repeated injections of poly(ethylene glycol)-modified (PEGylated) liposomes. We previously indicated that the phenomenon was induced by polymeric micelles possessing PEG chains like as liposomes, although, the induction mechanism of the ABC phenomenon is not fully elucidated. In the present study, we investigate whether repeat-injection of the polymeric micelles having PEG chains trigger the phenomenon or not. Two polymeric micelles, PM-30 (polymeric micelles with 33.6 nm in diameter) and PM-75 (76.2 nm), were prepared with PEG-poly[Asp(pentyl)] and PEG-poly[Asp(nonyl)], respectively. We firstly examined the ABC-triggering effect of these micelles, and observed that both polymeric micelles, especially PM-75, induced the production of anti-PEG IgM antibody in treated mice. Then, PM-30 or PM-75 was preadministered into mice as a preconditioning. Seven days later, AlexaFluor594-labeled PM-30 or PM-75 was administered to determine the susceptibility of the phenomenon. As a result, rapid clearance of AlexaFluor594-labeled PM-75 from the bloodstream and accumulation in the liver were observed in PM-75 pretreated mice. Although, the ABC phenomenon of AlexaFluor594-labeled PM-30 was less obvious in PM-30 pretreated mice. Our present results indicated that the repeated injections of polymeric micelles caused the ABC phenomenon in a size-dependent manner.

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1. Introduction

Polyethylene glycol (PEG)-modified nanoparticles exhibit long-circulating characteristics in their injection into the bloodstream owing to the avoidance of interactions with plasma proteins (Lasic et al., 1991; Torchilin et al., 1994; van Rooijen and van Nieuwmegen, 1980). Therefore, PEGylation has been widely used for preparations of nanoparticles possessing the long-circulating characteristics in the bloodstream. However, it has been reported that repeated injections of PEGylated liposomes induce rapid clearance from the bloodstream and enhanced accumulation in the liver of the injected liposomes at the second and later injections (Dams et al., 2000; Ishida et al., 2003). This phenomenon is called "accelerated blood clearance (ABC) phenomenon". Previous reports including ours indicated that the ABC phenomenon is related to secretion of IgM antibody against PEG chain from splenic B cells, however, the detail of the immune response is not fully understood (Cheng et al., 1999; Ishida et al., 2005, 2006, 2007).

Polymeric micelles, a kind of nanocarriers for drug delivery system (DDS), are formed from block copolymers, and possess a hydrophobic, cationic, or anionic cores and hydrophilic shells (Gaucher et al., 2005). The hydrophilic shells usually consist of PEG. At present, a number of polymeric micelles encapsulating anticancer drugs are proceeding to clinical studies (Matsumura et al., 2004; Matsumura, 2008; Hamaguchi et al., 2007;). However, little has been known whether the polymeric micelles with the PEG shells trigger and are susceptible to the ABC phenomenon (Koide et al., 2008; Ma et al., 2010) like PEGylated liposomes. We previously reported that the mice preadministered with a large-sized polymeric micelles showed rapid clearance from the bloodstream and enhanced accumulation at liver of PEGylated liposomes at the second dose. In contrast, a small-sized micelle did not induce the ABC phenomenon (Koide et al., 2008). We also reported that gadolinium-encapsulated polymeric micelles lacked both induction and susceptible characteristics of the

Abbreviations: ABC phenomenon, accelerated blood clearance phenomenon; MPEG-DSPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy(polyethylene glycol)-2000]; PM-30, polymeric micelles composed of PEG-poly[Asp(pentyl)]; PM-75, polymeric micelles composed of PEG-poly[Asp(nonyl)].

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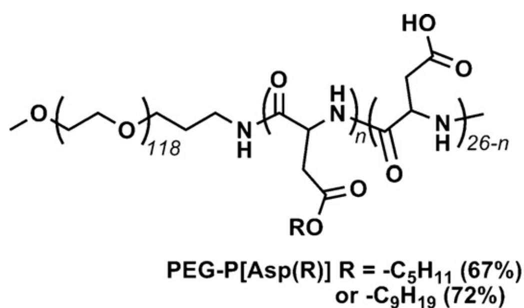

Fig. 1. Structure of block copolymers.

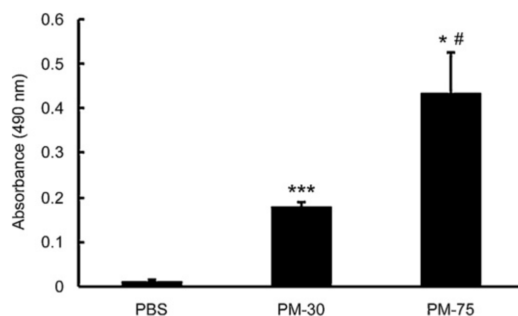

Fig. 2. Anti-PEG IgM production by injection of polymeric micelles. BALB/c mice were intravenously injected with PBS, PM-30 (33.6 nm) or PM-75 (76.2 nm) at the concentration of 2.9 mg/kg. Seven days later, the mice were sacrificed and anti-PEG IgM in the serum was determined. Each value represents the mean ± S.D. of 3 separate experiments. Significant differences: $^*p < 0.05$ and $^{***}p < 0.001$ *vs.* PBS; $^{\#}p < 0.05$ *vs.* PM-30.

ABC phenomenon (Ma et al., 2010). Much more matters remain unknown for the polymeric micelles than for PEGylated liposomes concerning the ABC phenomenon.

In the present study, we investigated the ABC triggering effect of two kinds of polymeric micelles (PM-30, 33.6 nm and PM-75, 76.2 nm in diameter, respectively) in terms of anti-PEG IgM production as well as these micelles' susceptibility to the ABC phenomenon by the use of AlexaFluor594-labeled micelles at the second (test) doses.

2. Materials and methods

2.1. Materials

1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy(polyethylene glycol)-2000] (MPEG-DSPE) was kindly gifted from Nippon Fine Chemical Co., Ltd. (Takasago, Hyogo, Japan). All other reagents were analytical grade.

2.2. Experimental animals

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

Structures of block copolymers used for polymeric micelle preparations are shown in Fig. 1. Around 70% esterified two block copolymers, PEG-poly[Asp(pentyl)] and PEG-poly[Asp(nonyl)], were prepared by esterification of aspartic acid residues of PEG-*b*-poly(aspartic acid) block copolymer (Yamamoto et al., 2007). Briefly, aspartic acid residues of PEG-poly(Asp) were activated with an equivalent of 1,8-diazabicyclo[5,4,0]7-undecene (DBU), followed by a reaction with pentyl iodide or nonyl iodide at 50 °C in dry DMF. The reaction mixture was reprecipitated in diethyl ether at 0 °C. The resulting precipitate was collected, and the polymer was dissolved in DMF, followed by the addition of 6 M HCl (1.0 equiv. of DBU). This mixture solution was dialyzed against water, and the solution was lyophilized. The number of esterification was measured by ^1H NMR in DMSO-d_6 containing 3% trifluoroacetic acid.

For fluorescence-labeling of polymeric micelles, AlexaFluor594-conjugated block copolymers were also prepared. In brief, AlexaFluor594 (0.5 mg) was conjugated to PEG-poly[Asp(pentyl)] (100.0 mg) or PEG-poly[Asp(nonyl)] (101.0 mg) by EDC activation of aspartic acid residues in dry DMSO. The obtained AlexaFluor594 conjugated block copolymers were dialyzed against DMSO, and then dialyzed against H_2O. Lyophilization gave AlexaFluor594 conjugated PEG-poly[Asp(pentyl)] or PEG-poly[Asp(nonyl)].

Polymeric micelles were prepared from these block copolymers by a solvent evaporation method (Yamamoto et al., 2007) as follows: non-labeled PEG-poly[Asp(pentyl)] (21.0 mg) and AlexaFluor594-labeld PEG-poly[Asp(pentyl)] (9.0 mg) were dissolved in chloroform (6.0 mL) and the solutions were evaporated at 40 °C with N_2 flow. The obtained polymer films were further dried under reduced pressure, and saline (3.5 mL) was added to the dried polymer film. Polymeric micelles were formed by sonication equipped with a 5 mm microtip of VCX-750 sonicator (Sonic & Materials Inc., CT, USA). The obtained polymeric micelle solutions were centrifuged and filtered through a 0.22 μm sterile PVDF membrane (Millipore). The size of these polymeric micelles at 2.0 mg/mL concentration was determined by means of dynamic light scattering. Obtained polymeric micelles from PEG-poly[Asp(pentyl)] and PEG-poly[Asp(nonyl)] block copolymers are coded the PM-30 (33.6 nm) and the PM-75 (76.2 nm) based on their size.

2.4. Stability of polymeric micelles in the presence of serum

Polymeric micelles, PM-30 or PM-75, were incubated in the presence or absence of 50% fetal bovine serum (FBS; Sigma–Aldrich, St. Louis, MO) for 24 h at 37 °C. The particle size of the polymeric micelles was measured by using a Zetasizer Nano ZS (Malvern, Worcs, UK).

2.5. Detection of anti-PEG IgM antibody

Mice were intravenously injected with PM-30 (2.9 mg/kg), PM-75 (2.9 mg/kg), or PBS. Seven days after the injection of each sample, these mice were sacrificed under deep anesthesia for the collection of the blood. Serum was collected after centrifugation (700 × *g*, 15 min, 4 °C) of the blood. To prepare the ELISA plates, 10 μg of mPEG-DSPE in 20 μL ethanol was added onto 96-well plates (Nunc, Roskilde, Denmark). Then, the plates were air dried for 2 h to complete dryness and subsequently blocked with 10% fetal bovine serum (FBS, Sigma–Aldrich, St. Louis, MO) in PBS for 1 h. The 50-time diluted serum samples (100 μL) were added to the plates, incubated for 1 h, and washed 5 times with 1% FBS–PBS. Antibodies bound to mPEG-DSPE were detected with HRP-conjugated goat anti-mouse IgM antibody (Bethyl Laboratories, TX, USA). After incubation with the anti-IgM antibody for 1 h, each well was washed 5 times with 1% FBS–PBS. The coloration was initiated by the addition of *o*-phenylene diamine dihydrochloride (Sigma, St. Louis, MO, USA) that had been diluted with distilled water. After a 15-min incubation, the reaction was stopped by adding 100 μL of 2 M H_2SO_4, and the absorbance was recorded with a Tecan Infinite M200 micro plate reader at a test wavelength of 490 nm.

2.6. Near-infrared fluorescence imaging of AlexaFluor594-labeled polymeric micelles ex vivo

Mice were received an intravenous injection of PM-30 (2.9 mg/kg), PM-75 (2.9 mg/kg) or PBS. Seven days later, AlexaFluor594-labeled PM-30 (2.9 mg/kg) or AlexaFluor594-labeled PM-75 (2.9 mg/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 was centrifugally separated to obtain the serum. Then, the organs (heart, lungs, liver, spleen, and kidneys) were collected. The biodistribution of AlexaFluor594-labeled PM-30 or AlexaFluor594-labeled PM-75 was assessed by near-infrared fluorescence (NIRF) imaging with a Xenogen IVIS Lumina System coupled to Living Image software for data acquisition (Xenogen Corp., Alameda, CA). The fluorescence activity in the serum was measured a with a Tecan Infinite M200 micro plate reader (E_x; 590 nm, E_m; 630 nm). Mice were fed an alfalfa-free feed (Oriental Yeast Co. Ltd., Tokyo, Japan) to reduce the influence of background fluorescence.

2.7. Statistics

Differences in groups were evaluated by analysis of variance (ANOVA) with the Tukey post hoc test.

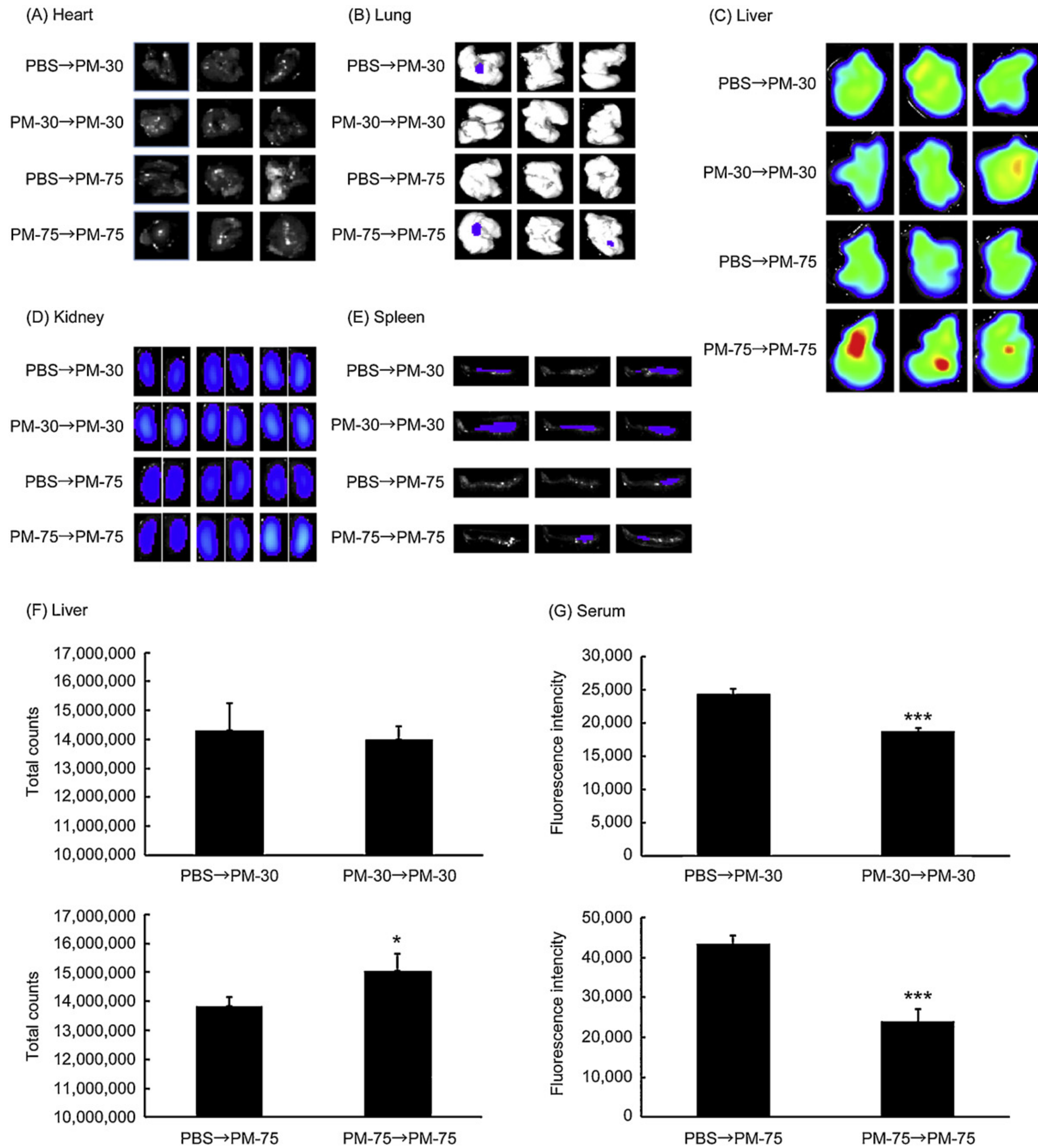

Fig. 3. Biodistribution of test-dose AlexaFluor594-labeled PM-30 or PM-75 after preadministration of PM-30 or PM-75, respectively. BALB/c mice were intravenously injected with PBS, PM-30 (33.6 nm) or PM-75 (76.2 nm) at the concentration of 2.9 mg/kg. Seven days later, AlexaFluor594-labeled PM-30 or PM-75 (2.9 mg/kg) were administered via a tail vein. Twenty-four hours later, the mice were sacrificed, and fluorescence intensity in each organ was determined *ex vivo* by IVIS. Descriptions indicate preconditioning and test-dose: for example, PBS-PM-30eans preconditioning with PBS and test-dose with PM-30. Data represent image and fluorescence activity of heart (A), lung (B), liver (C), kidney (D) and spleen (E). Total photon count of AlexaFluor594 in the liver (F) and fluorescence intensity of it in the serum (G) was determined. Significant differences are shown with asterisks: $^{*}p < 0.05$ and $^{***}p < 0.001$.

Table 1
Stability of polymer micelles.

	Size (nm)	
	PBS	50% serum
PM-30	33.6 ± 2.8	42.4 ± 3.3
PM-75	76.2 ± 1.3	70.4 ± 3.1

Particle size of polymer micelles incubated with PBS or 50% serum for 24 h at 37 °C were measured by using the Zetasizer Nano ZS.

3. Results and discussion

To examine the stability of the polymeric micelles, we measured the size change of both PM-30 and PM-75 in the presence of 50% serum for 24 h at 37 °C (Table 1) before *in vivo* studies. The notable particle size changes of both PM-30 and PM-75 were not observed in the presence of serum. It has been indicated that induction of the ABC phenomenon is related to the secretion of anti-PEG IgM from splenic B cells (Ishida et al., 2006; Koide et al., 2010). Therefore, we firstly demonstrated whether the PM-30 or PM-75 induced anti-PEG IgM production in mice. Mice were administrated with PBS (control), PM-30, or PM-75 at a concentration of 2.9 mg/kg. Seven days after the injection, the amount of anti-PEG IgM was measured by ELISA. As shown in Fig. 2, anti-PEG IgM was notably secreted by the administration of the PM-30 and PM-75. In addition, the amount of the anti-PEG IgM by the injection of PM-75 was higher than that of PM-30. The result indicated that these polymeric micelles having PEG chains triggered the induction of ABC phenomenon. In addition, size was revealed to be an important factor for the ABC phenomenon-triggering action. These results are consistent with our previous study that the size of polymeric micelles is important factor for inducing ABC phenomenon (Koide et al., 2008).

Since, both polymeric micelles induced anti-PEG IgM secretion, the ABC phenomenon was expected to be induced in pretreated mice with both micelles. Therefore, we next measured biodistribution of the second dose (test-dose) by the use of AlexaFluor594-labeled PM-30 or PM-75 in the mice pretreated with PBS (control), the PM-30, or the PM-75 (Fig. 3). In the PM-30 pretreated mice, the biodistribution of the AlexaFluor594-labeled PM-30 had no significant difference from the PBS-pretreated mice. On the other hand, fluorescence intensity in the serum was slightly but significantly decreased compared with the PBS treated mice. This difference is considered to result from differences in sensitivity of the liver and serum measurements; The ABC phenomenon can be more sensitively detected in serum than in liver due to a considerably high accumulation amount in liver of the control (PBS-pretreated). On the other hand, the PM-75-treated mice significantly increased the accumulation of the AlexaFluor594-labeled PM-75 in the liver compared with the PBS-treated mice. In addition, the mice pretreated with the PM-75 showed a significant decrease of test-dose AlexaFluor594-labeled PM-75 in the serum compared with the PBS pretreated mice. Biodistribution of AlexaFluor594-labeled PM-30 or PM-75 in other organs such as heart, lung, spleen and kidney was not significantly difference between PBS- or polymeric micelle-treated mice. Another important finding of this study was long-circulating properties of both the micelles. Fluorescence intensity values of serum shown in Fig. 3(G) indicated 8.5% dose for PBS-PM-30 and 18.2% for PBS-PM-75 at 24 h after intravenous injection. Both these dose % values represent characteristics of typical long-circulating carriers. Therefore, the weak induction of ABC phenomenon by PM-30 is not due to the rapid clearance from the bloodstream.

These results reveal that repeated injections of the polymeric micelles may induce the ABC phenomenon as PEGylated liposomes do, and that the ABC phenomenon of the polymeric micelles is substantially dependent on their size. In fact, we previously demonstrated that the mice pretreated with relatively large polymeric micelles (50.2 nm) showed rapid clearance of the test-dose PEGylated liposomes (100 nm), and induced the ABC phenomenon. However, the mice pretreated with relatively small polymeric micelles (31.5 nm) did not induce the phenomenon (Koide et al., 2008). Moreover, to consider the present and previous results, it is suggested that the chemical structure of the hydrophobic inner core polymer block is not important for the ABC induction, since small polymeric micelles, which did not induce ABC phenomenon in the previous study, consisted with the same kinds of the hydrophobic moieties to the polymeric micelles used in the present study.

Therefore, our results strongly suggest that the induction of and susceptibility to the ABC phenomenon is determined with the particle sizes.

4. Conclusions

This study is first report that repeated injections (both pretreatment and test doses) of polymeric micelles induce the ABC phenomenon induction like as PEGylated liposomes. In addition, this study reveals that triggering of and susceptibility to the ABC phenomenon should be micelle size-dependent, since the ABC phenomenon was more greatly induced for the larger polymeric micelles. Since PEGylated nanocarriers such as polymeric micelles and liposomes are now used as carriers for drugs and nucleic acid medicines, this study should provide important information for the development of the DDS medicines using PEGylated carriers.

References

Cheng, T.L., Wu, P.Y., Wu, M.F., Chern, J.W., Roffler, S.R., 1999. Accelerated clearance of polyethylene glycol-modified proteins by anti-polyethylene glycol IgM. Bioconjug. Chem. 10, 520–528.

Dams, E.T., Laverman, P., Oyen, W.J., Storm, G., Scherphof, G.L., van Der Meer, J.W., Corstens, F.H., Boerman, O.C., 2000. Accelerated blood clearance and altered biodistribution of repeated injections of sterically stabilized liposomes. J. Pharmacol. Exp. Ther. 292, 1071–1079.

Gaucher, G., Dufresne, M.H., Sant, V.P., Kang, N., Maysinger, D., Leroux, J.C., 2005. Block copolymer micelles: preparation, characterization and application in drug delivery. J. Control. Release 109, 169–188.

Hamaguchi, T., Kato, K., Yasui, H., Morizane, C., Ikeda, M., Ueno, H., Muro, K., Yamada, Y., Okusaka, T., Shirao, K., Shimada, Y., Nakahama, H., Matsumura, Y., 2007. A phase I and pharmacokinetic study of NK105, a paclitaxel-incorporating micellar nanoparticle formulation. Br. J. Cancer 97, 170–176.

Ishida, T., Maeda, R., Ichihara, M., Irimura, K., Kiwada, H., 2003. Accelerated clearance of PEGylated liposomes in rats after repeated injections. J. Control. Release 88, 35–42.

Ishida, T., Harada, M., Wang, X.Y., Ichihara, M., Irimura, K., Kiwada, H., 2005. Accelerated blood clearance of PEGylated liposomes following preceding liposome injection: effects of lipid dose and PEG surface-density and chain length of the first-dose liposomes. J. Control. Release 105, 305–317.

Ishida, T., Ichihara, M., Wang, X., Yamamoto, K., Kimura, J., Majima, E., Kiwada, H., 2006. Injection of PEGylated liposomes in rats elicits PEG-specific IgM, which is responsible for rapid elimination of a second dose of PEGylated liposomes. J. Control. Release 112, 15–25.

Ishida, T., Wang, X., Shimizu, T., Nawata, K., Kiwada, H., 2007. PEGylated liposomes elicit an anti-PEG IgM response in a T cell-independent manner. J. Control. Release 122, 349–355.

Koide, H., Asai, T., Hatanaka, K., Urakami, T., Ishii, T., Kenjo, E., Nishihara, M., Yokoyama, M., Ishida, T., Kiwada, H., Oku, N., 2008. Particle size-dependent triggering of accelerated blood clearance phenomenon. Int. J. Pharm. 362, 197–200.

Koide, H., Asai, T., Hatanaka, K., Akai, S., Ishii, T., Kenjo, E., Ishida, T., Kiwada, H., Tsukada, H., Oku, N., 2010. T cell-independent B cell response is responsible for ABC phenomenon induced by repeated injection of PEGylated liposomes. Int. J. Pharm. 392, 218–223.

Lasic, D.D., Martin, F.J., Gabizon, A., Huang, S.K., Papahadjopoulos, D., 1991. Sterically stabilized liposomes: a hypothesis on the molecular origin of the extended circulation times. Biochim. Biophys. Acta 1070, 187–192.

Ma, H., Shiraishi, K., Minowa, T., Kawano, K., Yokoyama, M., Hattori, Y., Maitani, Y., 2010. Accelerated blood clearance was not induced for a gadolinium-containing PEG-poly(L-lysine)-based polymeric micelle in mice. Pharm. Res. 27, 296–302.

Matsumura, Y., Hamaguchi, T., Ura, T., Muro, K., Yamada, Y., Shimada, Y., Shirao, K., Okusaka, T., Ueno, H., Ikeda, M., Watanabe, N., 2004. Phase I clinical trial and pharmacokinetic evaluation of NK911, a micelle-encapsulated doxorubicin. Br. J. Cancer 91, 1775–1781.

Matsumura, Y., 2008. Poly (amino acid) micelle nanocarriers in preclinical and clinical studies. Adv. Drug Deliv. Rev. 60, 899–914.

Torchilin, V.P., Omelyanenko, V.G., Papisov, M.I., Bogdanov Jr., A.A., Trubetskoy, V.S., Herron, J.N., Gentry, C.A., 1994. Poly(ethylene glycol) on the liposome surface: on the mechanism of polymer-coated liposome longevity. Biochim. Biophys. Acta 1195, 11–20.

van Rooijen, N., van Nieuwmegen, R., 1980. Liposomes in immunology: multilamellar phosphatidylcholine liposomes as a simple, biodegradable and harmless adjuvant without any immunogenic activity of its own. Immunol. Commun. 9, 243–256.

Yamamoto, T., Yokoyama, M., Opanasopit, P., Hayama, A., Kawano, K., Maitani, Y., 2007. What are determining factors for stable drug incorporation into polymeric micelle carriers? Consideration on physical and chemical characters of the micelle inner core. J. Control. Release 123, 11–18.