Exploring the Relationship between Anti-PEG IgM Behaviors and PEGylated Nanoparticles and Its Significance for Accelerated Blood Clearance

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Abstract

Surface PEGylation on nanoparticles has greatly helped prolong their blood circulation half-lives. However, The injection of PEGylated nanoparticles into mice induced poly(ethylene glycol) (PEG)-specific IgM antibodies (anti-PEG IgMs), significantly changing PEG-liposomes' pharmacokinetics. In this study, we used various PEG-conjugates to conduct a mechanistic study of anti-PEG IgMs' binding behavior. The conventional belief has been that anti-PEG IgMs bind to PEG main chains; however, our findings reveal that anti-PEG IgMs did not bind to PEG main chains, whereas anti-PEG IgMs did bind to PEG-hydrophobic polymer blocks. The insertion of a hydrophilic polymer between each PEG chain and each hydrophobic polymer block suppressed anti-PEG IgMs' binding. We prove here that hydrophobic blocks are essential to anti-PEG IgMs' binding, and also that anti-PEG IgMs do not bind to intact PEGs without hydrophobic moiety. These results support our conclusion that anti-PEG IgMs exhibit specificity to PEG; however, the presence of a hydrophobic block at a proximity position from each PEG chain is essential for the binding. Also in the present study, we elucidate relations between anti-PEG IgMs and PEGylated nanoparticles. In one of our previous studies, anti-PEG IgMs scarcely affected the pharmacokinetics of PEG-*b*-poly(β-benzyl L-aspartate) block copolymer (PEG-PBLA) micelles, whereas anti-PEG IgMs significantly decreased PEG-liposomes' blood circulation half-life. Finally, we found that the ratio of anti-PEG IgM molecules to PEG-liposome particles is critical to these pharmacokinetic changes, and that a 10-fold increase in the number of anti-PEG IgM molecules permitted them to capture the PEG-liposome particles, thus leading to the aforementioned changes.

Keywords

anti-PEG IgM antibody; PEG-liposomes; polymeric micelles; accelerated blood clearance (ABC) phenomenon; hydrophobicity of conjugates

1. Introduction

Poly(ethylene glycol) (PEG) possesses non-toxic and bio-inert properties, and these properties have led to frequent uses in pharmaceutics, cosmetics, and food applications. In pharmaceutics, PEG conjugation to drugs, proteins, and carriers is called PEGylation, and this conjugation technique helps prolong the half-lives of the conjugates in blood because PEGylated drugs can escape from reticuloendothelial system (RES) uptake [1–7]. PEGylation has become a common method for—and has greatly contributed to—the drug targeting field. PEGylation onto liposomes (resulting in PEG-liposomes) prolongs these liposomes' blood half-lives, in contrast to conventional liposomes' blood half-lives. [3–7] Recently, an "anticancer drug"-encapsulated PEG-liposome system was approved for clinical use [8, 9]. Not only liposomes, but also other nanoparticle systems frequently use PEGylation on surface areas [10–15]. In spite of the usefulness and importance of PEGylation, research on PEG-related immune responses has recently reported that the injection of PEG-liposomes into mice can trigger a PEG-related immune response [16–18]. After i.v. injections, PEG-liposomes induced the generation of PEG-specific IgM antibodies (anti-PEG IgMs) [19–21]. Anti-PEG IgMs rapidly eliminate secondary injected PEG-liposomes from the bloodstream. PEG-liposomes that include no drug in their aqueous cavity (empty PEG-liposomes) induce

anti-PEG IgM generation, while cytotoxic anticancer-drug-incorporated PEG-liposomes do not induce anti-PEG IgM generation owing to the loaded cytotoxic drug's suppression of immune responses [22,23]. Therefore, the phenomenon of accelerated blood clearance (ABC) affects repeated injections of PEGylated drug carriers when the carriers incorporate non-cytotoxic drugs or diagnostic agents. This PEG-related immune response (i.e., the ABC phenomenon) can be applied also to other types of PEGylated carrier systems such as polymeric micelles and nanoparticles. Indeed, weak cytotoxic-drug-incorporated carriers, such as siRNA-loaded PEG-liposomes or prostaglandin-E1-loaded PEG-poly(lactic acid) (PEG-PLA) nanoparticles, can induce the ABC phenomenon [24,25]. We have also reported that PEG-liposomes incorporating MRI contrast agents can induce the ABC phenomenon [26]. These obtained results show that immune-response induction against PEG strongly restricts the use of PEGylated drug carriers, owing not only to rapid elimination of PEG nanoparticles from the bloodstream, but also to PEG nanoparticles' serious side effects resulting from undesired biodistribution.

In many studies regarding anti-PEG IgM generation, direct ELISA exhibited the specificity of IgM antibodies against PEG (i.e., against anti-PEG IgMs) [19,20], and our own research also showed that anti-PEG IgMs can exhibit a cross-reactivity to PEG-coated plates [26]. Namely, anti-PEG IgMs induced by PEG-liposomes can bind to both PEG-distearoylphosphatidylethanolamine (PEG-DSPE) (a component of PEG-liposomes) and PEG-PBLA (a component of PEG-PBLA micelles). Also, anti-PEG IgMs immunized by PEG-PBLA micelles can bind to PEG-coated plates. The binding behavior indicates the specificity of anti-PEG IgMs for PEG, and it is generally believed that anti-PEG antibodies bind to PEG main chains (-$(OCH_2CH_2)_n$-), although previous research has yet to obtain clear proof of this binding specificity. In fact, most ELISA experiments used PEG conjugates for coating instead of an intact PEG chain, since the water solubility of the intact PEG chain prevents the chain from being coated on the ELISA plate. In a previous study, we presented new findings regarding the ABC phenomenon as it relates to PEGylated polymeric micelles [27]. The secreted IgM antibodies were known to be PEG-specific antibodies (anti-PEG IgMs); however, in the previous study, we suggested the importance of an interface consisting of a PEG chain and a hydrophobic moiety for anti-PEG IgM binding, and we noted that, in general, induced IgM antibodies do not bind to intact PEG main chains that lack a hydrophobic moiety. Moreover, we uncovered interesting differences between the ABC phenomenon relative to PEG-liposomes and the ABC phenomenon relative to PEGylated polymeric micelles (PEG-PBLA micelles). Even though we injected the same amount of both PEG-liposomes and PEG-PBLA micelles for the second dose, only PEG-liposomes exhibited a rapid blood clearance in the presence of anti-PEG IgMs, and this clearance was induced by the first dose of either PEG-liposomes or PEG-PBLA micelles. The PEG-PBLA micelles did not exhibit rapid clearance. In addition to these two carriers, PEG-hydrophilic block copolymer micelles that were loaded with an MRI contrast agent, PEG-P(Lys-DOTA-Gd) micelles (Gd-micelles), exhibited no induction of anti-PEG IgMs and no rapid blood clearance in the presence of anti-PEG IgMs. Table 1 summarizes our previously obtained findings.

Because our previous research yielded novel findings about PEG-related immune responses and the ABC phenomenon, we decided to perform a mechanistic study of anti-PEG IgM antibodies for

the current research. Here, we try to prove that the generated IgM antibodies are PEG-specific and bind only when PEG is in the vicinity of a hydrophobic moiety. We compare the binding affinity of anti-PEG IgMs to PEG-liposomes with the binding affinity of anti-PEG IgMs to PEG-PBLA micelles in order to elucidate the differences between the pharmacokinetics of PEG-liposomes and those of PEG-PBLA micelles. In this study, we offer new insights into a role that anti-PEG IgMs can play in the ABC phenomenon; namely, the role where the ratio of anti-PEG IgM antibody molecules to PEGylated nanoparticles can strictly determine the pharmacokinetics of PEGylated nanoparticles in the presence of anti-PEG IgMs.

Table 1. Our previously obtained findings regarding immune responses related to anti-PEG IgMs

	Anti-PEG IgM induction	Rapid clearance
PEG-liposome	**Yes**	**Yes**
PEG-PBLA micelle	**Yes**	**No**
PEG-P(Lys-DOTA-Gd) micelle (Gd-micelle)	**No**	**No**

All the PEG compounds possess a methoxy group at the PEG terminal.

2. Materials and Methods

2.1. PEG-*b*-poly(aspartic acid)-*b*-poly(L-phenylalanine) triblock copolymer synthesis

First, we synthesized PEG-PBLA by undertaking the ring-opening polymerization of β-benzyl L-aspartate N-carboxy anhydride from α-methyl-ω-aminopropoxy poly(ethylene glycol), CH_3O-PEG-NH_2 (molecular weight of PEG = 12,000) according to previous reports [28,29]. PEG-PBLA (0.31 g) was dissolved in dry CH_2Cl_2 (11.0 mL), and 25 equivalent moles of *N*-carboxyanhydride of L-phenylalanine (Phe-NCA) (0.11 g) in dry CH_2Cl_2 (5.0 mL) was added to the solution, and the mixture was stirred for 20 h at 38°C under N_2. The reaction mixture was reprecipitated into diethylether at 0°C, and obtained white precipitate was filtrated, washed with diethylether, and dried (0.37 g). The obtained triblock copolymer (0.33 g) was hydrolyzed with an aqueous solution of NaOH (0.5N, 3.5 mL). We further added H_2O (4.0 mL) to the mixture and stirred at r.t. The solution, which we protonated by adding hydrochloric acid (6N, 0.35 mL), was dialyzed against H_2O with a dialysis membrane (SpectraPor 6, molecular weight cut-off (MWCO) = 1k). After lyophilization (0.25 g), we obtained PEG-*b*-poly(aspartic acid)-*b*-poly(L-phenylalanine), PEG-P(Asp)-P(Phe) triblock copolymers.

2.2. Preparation of PEG-liposomes

First, an empty PEG-liposome was prepared according to the lipid film hydration method [26]. In brief, a mixture of hydrogenated soya phosphatidyl choline (HSPC), cholesterol, and mPEG-DSPE ($M_{w\ PEG}$ = 2k) in a molar ratio of 1.85:1.0:0.15 was dissolved in $CHCl_3$. The organic solvent was evaporated, and the obtained lipid film was hydrated with saline. The PEG-liposomes were extruded with a polycarbonate membrane of 0.4, 0.2, and 0.1 μm pore sizes at 60°C, respectively. We prepared (1) fluorescence probe (CM-DiI) loaded PEG-liposomes via the incorporation of

CM-DiI at 1 mol%. The average particle size of PEG-liposomes used in this study was 130 nm.

2.3. PEG-PBLA micelle preparation

PEG-PBLA micelles were prepared according to the dialysis method [28,29]. PEG-PBLA ($M_{w\ PEG}$ = 12k) was dissolved in DMF and dialyzed against normal saline with a dialysis membrane (MWCO = 1k). The obtained micelle solution was filtered through a Millex 0.22 μm sterile PVDF membrane (Merck Millipore K.K., Tokyo, Japan). The weight-average diameter of the PEG-PBLA micelles was determined by means of dynamic light scattering (DLS-7000, Otsuka Electronic Co. Ltd., Osaka, Japan). The average particle size of PEG-PBLA micelles was 90 nm.

2.4. Immunization by PEG-PBLA micelles

PEG-PBLA micelles were injected at a dose of 0.05 mg/kg (n = 5) into the tail vein of a male C57BL/6 mouse (6 weeks old, Sankyo Lab., Tokyo, Japan). The serum was collected at seven days after the injection, and we confirmed that the obtained sera included anti-PEG IgMs by means of ELISA (see Supplemental Figure S2(A)).

2.5. Direct ELISA

To detect anti-PEG IgMs, we performed a direct ELISA and used a goat anti-mouse IgM (Bethyl Laboratories, TX, USA). To a 96-well plate, we added PEG(2k)-DSPE in ethanol/H_2O (1/1), PEG-PBLA in ethanol/H_2O (1/1), or PEG-P(Asp)-P(Phe) in 5% TFA containing DMSO/H_2O (1/1). All the PEG-conjugates were initially dissolved in organic solvent, and then water was added to the solution. And the polymers are coated on a plate at 4°C for incubation overnight. The PEG-conjugate-coated plates were washed three times with a wash solution (0.05% Tween-20 in 50mM tris-buffered saline, pH = 8.0) and blocked with a blocking buffer solution (1% BSA in 50mM tris-buffered saline, pH = 8.0) for 1 h, and the wells were washed with the wash solution three times. One hundred microliters of diluted sera (100 times diluted in saline) were applied in wells for 1 h, and the wells were washed with the wash solution three times. It should be noted that we synthesized a fluorescence probe conjugated-PEG-PBLA block copolymer and a fluorescence probe conjugated-PEG-P(Asp)-P(Phe) triblock copolymer to confirm actual coated amounts of block copolymers by means of fluorescence spectroscopy. Most ELISA experiments have not mentioned the actual coated amounts of substrates, and in the present study, we observed no drastic difference in the coated amounts among the three polymers (see Supplemental Fig. 1).

To perform a competitive ELISA, we mixed an anti-PEG IgM serum (ABC(+) serum) with PEG-liposomes ($M_{w\ PEG}$ = 2k), PEG-PBLA micelles ($M_{w\ PEG}$ = 12k), PEG-DSPE ($M_{w\ PEG}$ = 2k), or PEG-OH ($M_{w\ PEG}$ = 5k), we incubated the mixture at 37°C for 1 h, and then we added it to the well for 1 h. The concentration of PEGs in the immunized serum was the same as the concentration associated with the in vivo condition. Namely, we calculated the concentration of PEG-liposomes and PEG-PBLA micelles in plasma (dose = 4.0 mg/kg) [30]. The calculated initial concentration of PEG-liposomes or PEG-PBLA micelles was 80 μg/mL in plasma. We conducted an ELISA in the following conditions and steps: 1.0 μL of serum was mixed with 0.08 μg of PEG-nanoparticles or the same mole of PEG-conjugates (80 μg/mL); horseradish peroxidase (HRP)-labeled anti-mouse

IgMs were added to the well and incubated for 1 h before being washed with the washing solution 4 times; 100 μL of 3, 3′, 5, 5′-tetramethylbenzidine (TMB) were added to the well for 15 min; and 100 μL of 0.2 N H_2SO_4 were added to the well so that the reaction would stop. Absorbance at 450 nm was recorded with a microplate reader. To determine the dissociation constant (K_d), we conducted an inhibition ELISA using the same method as in the direct ELISA described above. The serum was mixed with various concentrations of PEG-liposomes or polymeric micelles, and incubated at 37°C for 1h before being added to the well, and we determined the dissociation constant (K_d) of the anti-PEG IgMs.

2.6. Sandwich ELISAs

Rabbit monoclonal anti-PEG IgG antibodies (PEG-B-47, Epitomics Co. Ltd., USA) were coated on plates with a coating buffer (pH = 9.6), the coated plates were stored overnight at 4°C. The plates were washed with PBS containing 0.05% Tween-20 and blocked with a blocking solution (10% BLOCK ACE) for 1 h, and the wells were washed three times. We added various concentrations of either PEG-DSPE or PEG-OH to the well and incubated them for 1h. After we washed the plate three times with PBS containing 0.05% Tween-20, biotinylated rabbit anti-PEG IgG (Epitomics Co. Ltd., times) was added to the plates. HRP-labeled streptavidin (Thermo Scientific Co. Ltd., USA) was added to the well, incubated for 1 h, and washed 3 times. A TMB solution was added to the well for 15 min, and then 100 μL of 0.2 N H_2SO_4 were added to the well so that the reaction would stop. Absorbance at 450 nm was measured with the microplate reader.

2.7. Effect of the second dose of PEG-liposomes on the ABC phenomenon

PEG-PBLA micelles were injected at a dose of 0.2 mg/kg into the tail vein of male C57BL/6 (n = 3) mice. At 6 days after the injection, serum was collected for ELISA. At day 7, CM-DiI-loaded PEG-liposomes were injected into the mice at doses of 5.0, 10, and 20 μmol lipid/kg, and the serum was collected 6h after the second injection. Having measured the fluorescence intensity of CM-DiI (λ_{ex}=550 nm, λ_{em}=570 nm), we determined PEG-liposome concentrations remaining in the serum.

2.8. The Gel Permeation Chromatography (GPC) measurement of PEG-PBLA micelles and PEG-liposomes

GPC measurements of PEG-PBLA micelles and PEG-liposomes were carried out with an HPLC system (LC 2000 series, Jasco, Tokyo, Japan) equipped with a TSK-gel G4000-PW_{XL} column and a TSK-gel guard column PW_{XL} (eluent = H_2O, flow rate = 1.0 mg/mL, detector = refractive index (RI)) at 40°C. We mixed a constant concentration (80 μg/mL) of either PEG-PBLA micelles or PEG-liposomes with monoclonal anti-PEG IgMs (PEG-2-128, Epitomics Co. Ltd., USA) in a concentration ranging from 4.0 ng/mL to 5.0 μg/mL, and the mixture was incubated at 37°C for 1 h. This concentration of nanoparticles is nearly the same as the calculated concentration of nanoparticles in plasma at a dose of 4.0 mg/kg. We performed the same procedure for PEG-liposomes. The mixture was injected into the GPC, and we evaluated the obtained peak area.

3. Results and discussion

3.1. Elucidation of anti-PEG IgMs binding to PEG chains

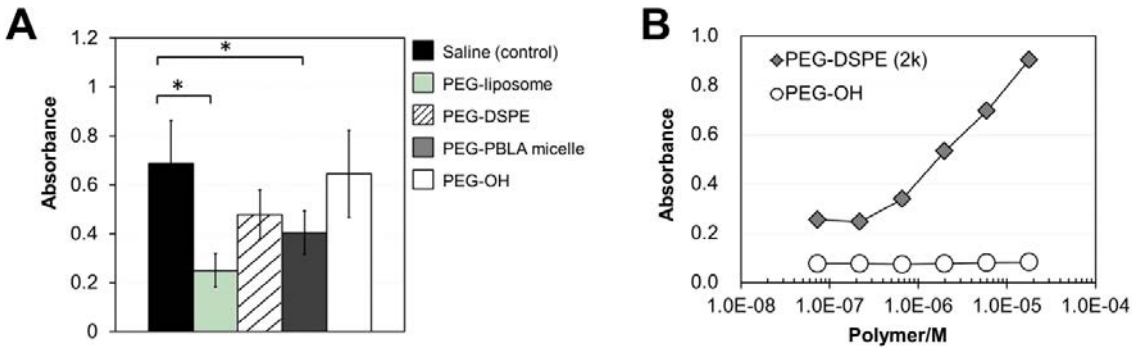

Figure 1. Binding assays of Anti-PEG antibodies to PEG-conjugates. (**A**) An inhibition assay of anti-PEG IgM to PEG-DSPE as determined by a competitive ELISA. An ABC(+) serum was mixed with PEG(2k)-liposomes, PEG(2k)-DSPE, PEG(12k)-PBLA, and PEG-OH(5k), and then, the serum sample was added to the PEG-DPSE-coated plates. All PEGs possessed a methoxy group at the PEG terminal. All samples' PEG concentration was 50 nM in a 100-times diluted ABC(+) serum with normal saline. Dunnett's method was used for multiple comparisons between the control and the other samples. Data are presented as the average ± SD. Symbol * indicates p value < 0.05. (**B**) The binding behavior of PEG-DSPE and PEG-OH relative to anti-PEG mIgG as determined by a Sandwich ELISA.

Previously, we and other groups reported that PEGylated nanoparticle-immunized mouse serum contained PEG-specific IgM (anti-PEG IgMs) and that anti-PEG IgMs could bind to PEG-coated plates by means of ELISA [19,20,26,27,31]. In our previous paper, we suggested the importance of an interface consisting of the PEG chain and a hydrophobic moiety (the PBLA chain for the PEG-PBLA block copolymer, and the DSPE group for the PEG-liposome) for anti-PEG IgM's binding [27].

We have confirmed that the induction of anti-PEG IgMs exhibited no significant variation in a PEG-PBLA dose range between 0.01 and 0.2 mg/kg (data not shown). And we confirmed that PEG-PBLA micelle-immunized serum (ABC(+) serum) included approximately 5.0 μg of anti-PEG IgM by priming (the anti-PEG IgM concentration = approximately 6.0 nM in plasma; data shown in Supplemental Fig. 2(B)) and used ABC(+) serum for competitive ELISA. Figure 1(A) shows an inhibition assay of anti-PEG IgM binding to a PEG-DSPE-coated plate. We mixed ABC(+) serum with PEG-liposome, PEG-DSPE, PEG-PBLA, and PEG-OH. In this experiment, we tried to mimic a similar condition with the ABC phenomenon in mice. The mixed PEG concentration was constant at 50 nM in serum, and the ratio of the PEG moles to the anti-PEG IgM moles was similar to the condition in which we observed the ABC phenomenon in mice. All the PEG compounds for this experiment possessed a methoxy group at the PEG terminal; therefore, chemical structures of PEG chains are the same. However, the PEG-liposomes, PEG-PBLA micelles, and PEG-DSPE exhibited suppression of anti-PEG IgM binding to the PEG-coated plates, whereas PEG-OH did not exhibit inhibition of anti-PEG IgM binding. Since an intact PEG chain without a hydrophobic conjugate could not be coated on a plate, we also performed a sandwich ELISA in which we coated a

commercially available monoclonal anti-PEG IgG (anti-PEG mIgG) on a plate in order to capture PEG molecules. However, we found that anti-PEG mIgG exhibited no binding to the intact PEG (PEG-OH), whereas anti-PEG mIgG exhibited bindings to PEG-DSPE (Figure 1(B)). Although we used commercially available anti-PEG mIgG for a sandwich ELISA, the aforementioned results of the direct ELISA and the sandwich ELISA indicate that, in general, "PEG-specific" IgM and IgG do not bind to PEG-OH, whereas these antibodies bind to PEG-conjugates.

3.2. Proximity of a hydrophobic block to a PEG chain is essential for anti-PEG IgM binding

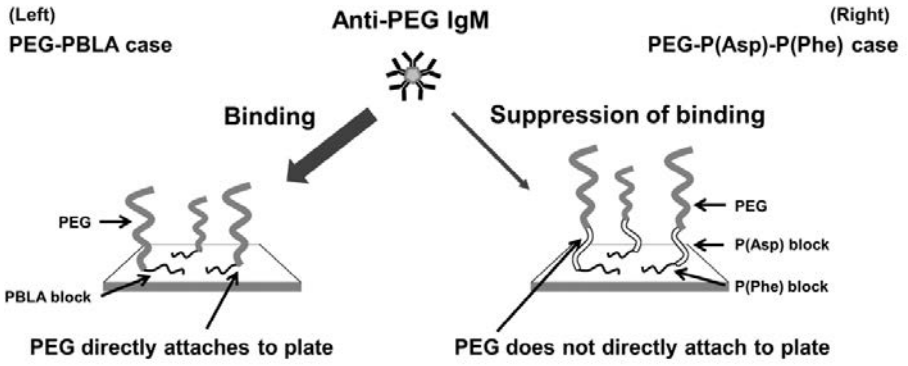

Figure 2. Scheme of anti-PEG IgM binding to a PEG-coated plate: (left) a PEG-PBLA-coated plate; (right) a PEG-P(Asp)-P(Phe)-coated plate.

To elucidate anti-PEG IgM's binding behavior in detail, we synthesized new PEG containing triblock copolymers, PEG-P(Asp)-P(Phe) triblock copolymers. The triblock copolymers have a hydrophilic poly(aspartic acid) block, P(Asp), between the PEG block and the hydrophobic poly(L-phenylalanine) block, P(Phe) (Figure 2). The coating of the PEG-conjugate is achieved by hydrophobic interactions between the hydrophobic block of the PEG-conjugate and the plate for an ELISA. One terminal of the PEG chain in PEG-PBLA is the methoxy group, and the other terminal of the PEG chain directly attaches to the plate, as shown in Figure 2 (left). In contrast, one terminal of the PEG chain in PEG-*b*-P(Asp)-*b*-P(Phe) is the methoxy group, and the other terminal of the PEG chain does not directly attach to the plate, as shown in Figure 2 (right). If anti-PEG IgM binds to a PEG main chain, we observe no difference in anti-PEG IgM binding behavior between the coated polymers. In contrast, if a hydrophobic block is essential for anti-PEG IgM's binding, we observe a significant difference in the binding behaviors of anti-PEG IgM. We have confirmed that anti-PEG IgM exhibits binding to PEG-poly(L-phenylalanine) (PEG-P(Phe)) block copolymers (without a P(Asp) block) (see Supplemental Fig. 3).

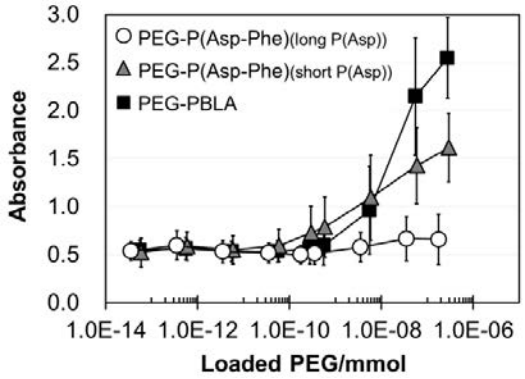

Figure 3. Anti-PEG IgM binding to the three different PEG polymer-coated plates. PEG-PBLA micelle-immunized serum (n = 3) was used for ELISA experiments. The polymer that we used was composed of PEG-PBLA (number of BLA = 30), PEG-P(Asp-Phe) (short P(Asp)) (number of Asp = 24, number of Phe = 17), and PEG-P(Asp-Phe) (long P(Asp)) (number of Asp = 87, number of Phe = 44).

As shown in Figure 3, we observed concentration-dependent anti-PEG IgM binding to PEG-PBLA (black square). In contrast, we found that PEG-P(Asp)-P(Phe) triblock copolymers exhibited suppression of anti-PEG IgM's binding in a hydrophilic-chain-length-dependent manner. This finding indicates that the proximity of the hydrophobic block to the PEG chain is an essential part of anti-PEG IgM's binding. This secondary finding points to two important qualifications: (1) that there be an interface between PEG and hydrophobic blocks for anti-PEG IgM's binding, and (2) that there be no binding of anti-PEG IgM to PEG main chains. In PEG-P(Asp)-P(Phe) triblock copolymers possessing 24 hydrophilic Asp repeating units (short P(Asp)), each PEG chain was 2.4 nm away from the plate, and this distance was calculated according to a mushroom regime of polymers grafted onto the plates [32]. Further elongation of hydrophilic blocks in the PEG-P(Asp)-P(Phe) triblock copolymer (the long P(Asp) possesses 87 hydrophilic Asp repeating units) resulted in a distance (5.1 nm) that was long enough to suppress anti-PEG IgM's binding.

3.3. Binding affinity of anti-PEG IgM to PEG carriers

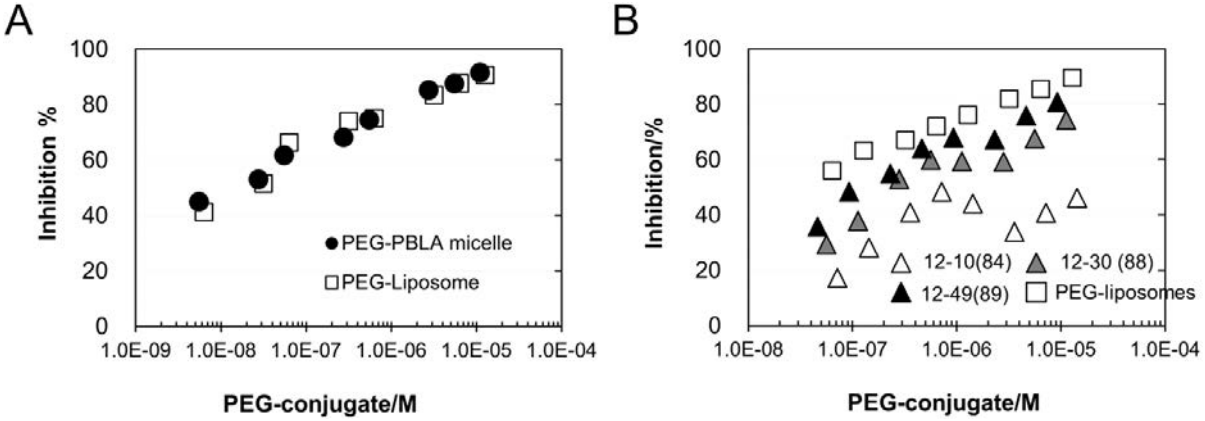

Figure 4. Determination of the K_d of anti-PEG IgMs by means of inhibition assays. (**A**) Inhibition of anti-PEG IgM binding to PEG-PBLA-coated plates by the addition of the PEG-PBLA micelle

(black circle) and the PEG-liposome (white square). (**B**) The PEG-liposome (white square), the 12-49(89) micelle (black triangle), the 12-30(88) micelle (gray triangle), and the 12-10(84) micelle (white triangle) were used for inhibition assays.

By conducting an inhibition assay on anti-PEG IgM, we determined the dissociation constant (K_d) of anti-PEG IgM relative to each PEG-DSPE of the PEG-liposome and to each PEG-PBLA block copolymer of the PEG-PBLA micelle. Figure 4(A) depicts anti-PEG IgM binding behaviors inhibited by various concentrations of either the PEG-liposome or the PEG-PBLA micelle as a function of PEG concentration. As shown in Figure 4(A), the binding behaviors of anti-PEG IgM to these PEG-conjugates exhibited no difference, and this finding indicates that the binding affinity of anti-PEG IgM to each PEG-DSPE of the PEG-liposome was the same as the aforementioned binding affinity to each PEG-PBLA block copolymer of the PEG-PBLA micelle. This outcome was a little unexpected because, in ABC-induced mice, we had previously observed significant differences between blood-clearance behaviors involving PEG-liposomes and those involving PEG-PBLA micelles [27]. A binding-affinity difference attributable to anti-PEG IgMs would initially appear to be a possible reason for the very different blood-clearance behaviors. However, no such binding-affinity difference was detected. We also found that anti-PEG IgM exhibited two K_ds (Table 2). Anti-PEG IgM exhibited high affinities to PEG-conjugates below the PEG-conjugate concentration at 20 μg/mL. In contrast, anti-PEG IgM exhibited low affinities to PEG-conjugates above the PEG-conjugate concentration at 20 μg/mL (see Supplemental Fig. 4A). This concentration was nearly the same as critical micelle (aggregation) concentrations (cmc) of PEG-conjugates in both the liposome case and the polymeric micelle case. This concentration-dependent K_d may be related to the formation of self-assembled nanoparticles. We performed further experiments to examine the binding behavior of anti-PEG IgM relative to varying hydrophobic chain lengths of PEG-P(Asp-Bzl) micelles.

Table 2. K_d values of anti-PEG IgM

Code	K_d/nM*	K_d/nM**
	Conc. > 20 μg/mL	Conc. < 20 μg/mL
PEG-PBLA micelle	97	9.2
PEG-liposome	139	16.0

We mixed serum with either PEG-liposomes or PEG-PBLA micelles in a concentration ranging from 0.1 to 200 μg/mL.
* K_d values obtained a concentration greater than 20 μg/mL. ** K_d values obtained a concentration less than 20 μg/mL.

Figure 4(B) shows that anti-PEG IgM binding was suppressed by different hydrophobic chain lengths of PEG-P(Asp-Bzl) block copolymer micelles. We synthesized PEG-P(Asp-Bzl) block copolymer micelles according to our previous report [28,29]. In contrast to the PEG-PBLA block copolymer, the PEG-P(Asp-Bzl) block copolymers possess both a hydrophilic aspartic acid group (11–16%) and a hydrophobic benzyl-esterified Asp group (84–89%) in the P(Asp-Bzl) block. The prepared PEG-P(Asp-Bzl) micelles exhibited monodispersed micelle formation without large aggregates, and large aggregates possibly affect anti-PEG IgM's binding behaviors. All the micelles

exhibited critical micelle concentrations in the range between 1.6 and 15.6 μg/mL. For example, the code 12-49(89) indicates a 12,000-molecular-weight PEG involving 49 Asp units with 89% benzyl-esterification (the average number of benzyl groups is 43.6). As shown in Figure 4(B), the affinity of anti-PEG IgM to PEG-P(Asp-Bzl) micelles was highly dependent on the length of the given hydrophobic block (while all the micelles possessed a 12,000-molecular-weight PEG). As was the case with the PEG-liposome, the 12-49(89) micelle effectively inhibited anti-PEG IgM binding to PEG-coated plates. In contrast, the 12-10(84) micelle, which possessed the shortest hydrophobic block, exhibited much weaker inhibition than the 12-49(89) micelle. Table 3 summarizes the obtained K_d values. Anti-PEG IgM exhibited high affinities to PEG-P(Asp-Bzl) block copolymers that were below concentration at 20 μg/mL in all the cases (see Supplemental Fig. 4B). The 12-49(89) micelle and PEG-liposome exhibited a 10-fold higher affinity for anti-PEG IgM than the 12-10(84) micelle. We found that the affinity of anti-PEG IgM relative to PEG-conjugates depends on the hydrophobic chain length of the PEG-P(Asp-Bzl) block copolymer.

Table 3. K_d values of the PEG-P(Asp-Bzl) micelles and the PEG-liposome

Code	Asp	Bzl	K_d/nM *	K_d/nM **
	/N	/N	Conc. > 20 μg/mL	Conc. < 20 μg/mL
12-49(89)	49	43.6	671	42
12-30(88)	30	26.4	1048	78
12-10(84)	10	8.4	1969	179
PEG-liposome	–	–	254	20

* K_d values obtained a concentration greater than 20 μg/mL. ** K_d values obtained a concentration less than 20 μg/mL.

3.4.The anti-PEG IgM consumption by the PEG-liposome

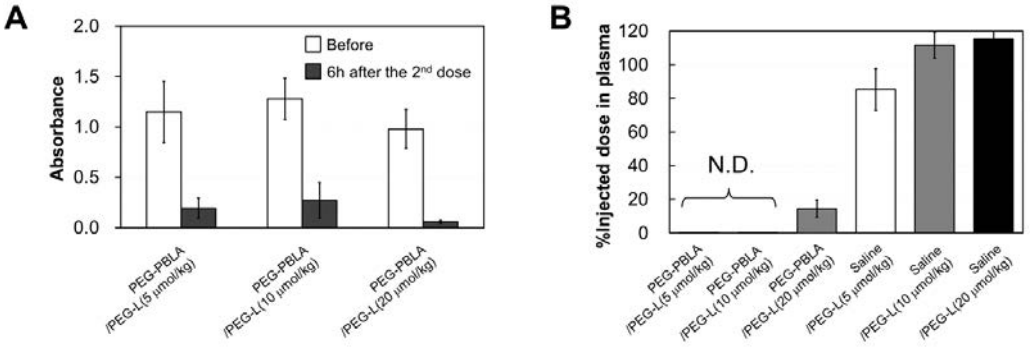

Figure 5. (**A**) Concentration changes in anti-PEG IgM before and after the 2nd dose of the PEG-liposome in PEG-PBLA-immunized mice. Here, 'PEG-PBLA' refers to the PEG-PBLA micelle, and 'PEG-L' refers to the PEG-liposome. (**B**) Plasma concentrations of PEG-liposomes in either PEG-PBLA micelle-immunized or saline-injected mice at 6 h after the 2nd PEG-liposome dose. 'N.D.' = not detected.

Figure 5 shows the second-dose effects on the PEG-liposome's behavior in ABC(+) mice. We

primed mice with the PEG-PBLA micelle at 0.05 mg/kg in the first dose. Then, we injected the PEG-liposome (the second dose) in doses of 5.0, 10.0, and 20.0 μmol lipid/kg at 7 days after the first dose. Figure 5(A) shows the anti-PEG IgM amounts in serum before the PEG-liposome injection (white bar) and 6 h after the PEG-liposome injection (black bar). Differences between white bars and black bars indicate anti-PEG IgM consumption due to binding to the PEG-liposome at the second dose. Anti-PEG IgM persisted at 6 h after the PEG-liposome injection at both 5.0 and 10.0 μmol lipid/kg, while complete anti-PEG IgM consumption was observable at 6 h after the PEG-liposome injection at 20.0 μmol/kg. Figure 5(B) shows PEG-liposomes' plasma concentration at 6h after the PEG-liposome injection. The first injection was of either PEG-PBLA micelles or saline. When we observed the remaining anti-PEG IgM after the PEG-liposome injection (black bar) in Figure 5(A), we found no PEG-liposomes in plasma, as illustrated in Figure 5(B). Indeed, when we observed no remaining anti-PEG IgM, we found the PEG-liposome in plasma. This indicates complete anti-PEG IgM consumption by the second dose of the PEG-liposome at 20 μmol lipid/kg, and the amount of anti-PEG IgM was not enough to eliminate the 20.0 μmol lipid/kg dose of the PEG-liposome.

3.5. Effects of anti-PEG IgM on PEG nanoparticles' concentration in blood

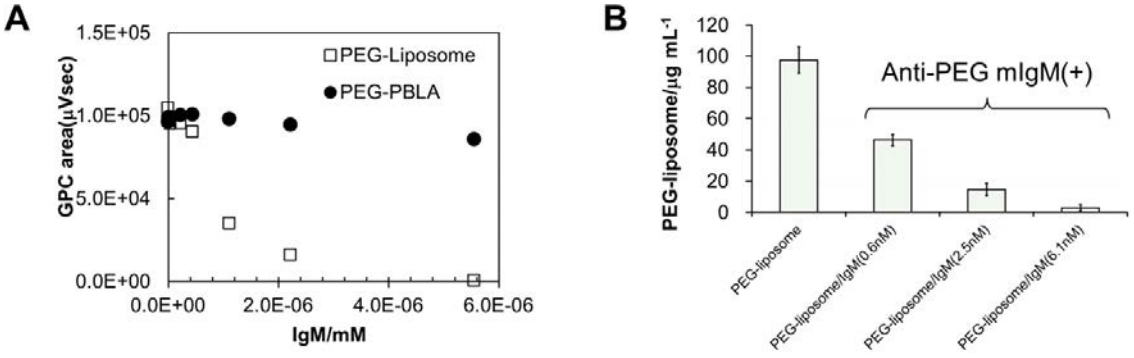

Figure 6. Anti-IgM binding significantly changes PEG-liposome behaviors. (**A**) The GPC assay of PEG-PBLA micelles and PEG-liposomes mixed with anti-PEG mIgM as a function of various concentrations: the concentration of the PEG-PBLA micelle and the PEG-liposome was constant at 80 μg/mL. (**B**) The effect that the addition of anti-PEG IgM to the PEG-liposome had on the plasma concentration at 6 h after the injection: the PEG-liposome was injected at a dose of 5.0 μmol lipid/kg (4.0 mg/kg), and was mixed with anti-PEG mIgM at 0.6, 2.5, and 6.1 nM, respectively. The mixed anti-PEG mIgM concentrations are shown in parentheses.

We examined the anti-PEG IgM binding effect on PEG-nanoparticles' behaviors. Figure 6(A) depicts effects of an addition of commercially available monoclonal anti-PEG IgM (anti-PEG mIgM) on PEG-nanoparticles' GPC refractive index (RI) peak area. We uncovered these effects by using GPC measurement, which detects the eluted RI peak area of PEG-nanoparticles' fraction. We have established this measurement method in which the eluted RI peak area strongly correlates with *in vivo* pharmacokinetics of polymeric micelles [33]. We injected either PEG-PBLA micelles or

PEG-liposomes at a constant concentration (80 μg/mL) in the presence of various concentrations of anti-PEG mIgM. This concentration at 80 μg/mL corresponds to a concentration of the PEG-liposome or the PEG-PBLA micelle in plasma at a dose of 4.0 mg/kg (we injected this dose for the second dose). The maximum plasma concentration of anti-PEG IgM was approximately 6.0 nM. Therefore, we varied from 0.0 to 6.1 nM the anti-PEG IgM concentration. As shown in Figure 6(A), we observed no drastic change in the eluted PEG-PBLA micelle's peak area (black circle), and this result indicates that anti-PEG mIgM did not change the PEG-PBLA micelle's peak area in the anti-PEG mIgM's concentration range between 0.0 and 6.1 nM (the maximum concentration of anti-PEG IgM was approximately 6.0 nM in plasma). By contrast, we observed drastic decreases in the eluted PEG-liposome's peak area. Both the PEG-PBLA micelles' concentration and the PEG-liposomes' concentration were constant at 80 μg/mL; however, only the PEG-liposome exhibited a significant change in the eluted GPC peak area. This indicates that a significant amount of the anti-PEG mIgM-PEG-liposome complex was formed in the mixture, and the anti-PEG mIgM-PEG-liposome complex could not elute owing to the complex formation, while the PEG-PBLA micelle did not exhibit a change in the eluted amounts. We performed further experiments to clarify the effect of the mixed ratio on the pharmacokinetics of the PEG-liposome. We intravenously injected PEG-liposomes mixed with different concentrations of anti-PEG mIgM into naïve mice and collected serum at 6h after the injection. Figure 6(B) depicts the PEG-liposome concentration at 6 h after the injection. When we mixed PEG-liposomes with between 0.6 and 6.1 nM of anti-PEG IgM, we observed a decrease in the PEG-liposomes' concentration in plasma, in contrast to PEG-liposomes without anti-PEG mIgM. These results indicate that anti-PEG IgMs' direct binding to PEG-liposomes significantly affects pharmacokinetics at a 4.0 mg/kg dose of the PEG-liposome with an anti-PEG mIgM concentration range between 0.0 and 6.1 nM.

Anti-PEG IgM significantly changes PEG-liposomes' pharmacokinetics (rapid blood clearance in the presence of anti-PEG IgM), and this induction of a PEG-related immune response, obviously, restricts the use of PEGylated nanoparticles. Anti-PEG IgM was believed to bind to the PEG main chain; however, we suggested, in our previous study, that PEG and hydrophobic blocks are essential for anti-PEG binding [27]. However, we did not evaluate the chemical structure of PEG-conjugates for anti-PEG IgM binding. In fact, PEG-DSPE for PEG-liposomes possesses hydrophilic anionic phosphoric acid (-PO_4^--NH_4^+-) in between PEG and the hydrophobic diacyl group; however, PEG-DSPE exhibits anti-PEG IgM binding. Therefore, in the current study, we studied the chemical structure of PEG-conjugates for anti-PEG antibody binding.

In our experiment in Figure 3, anti-PEG IgM did not exhibit strong bindings to PEG-P(Asp)-P(Phe) triblock copolymers, while anti-PEG IgM exhibited a binding to PEG-PBLA. This experiment clearly indicates two important roles of anti-PEG IgM. First, anti-PEG IgM does not strongly bind to the PEG main chain, although anti-PEG IgM possesses PEG-specificity. One possibility is that anti-PEG IgM possesses very weak interaction with PEG main chains or the terminal methoxy group, and the main force for bound-complex formations is hydrophobic conjugate dependent. Second, anti-PEG IgM can exhibit cross-reactively PEG-specific bindings when a hydrophobic block is in a position near the PEG chain.

Inhibition of anti-PEG IgM binding by PEG-P(Asp-Bzl) block copolymers revealed anti-PEG

IgM's affinity. All PEG-P(Asp-Bzl) block polymers possess a PEG chain (MW = 12,000); however, we found that the affinity of anti-PEG IgM was dependent on the hydrophobic chain length of PEG-conjugates. Anti-PEG IgM exhibited a weak affinity to the low hydrophobic content of PEG-P(Asp-Bzl) block copolymers. The importance of hydrophobicity relative to immune responses has been recognized in protein antigen cases [34,35], and our experiments also indicate the importance of hydrophobic blocks in immune responses. In contrast to protein antigens, each PEG-containing block copolymer was constituted simply of one hydrophilic PEG chain and one hydrophobic block.

We determined the K_d of anti-PEG IgM relative to both the PEG-DSPE of the PEG-liposome and the PEG-PBLA block copolymer of the PEG-PBLA micelle, and the results indicate that anti-PEG IgM exhibited a similar affinity to two kinds of PEG-conjugates. This was an unexpected result. We thought that the differences between anti-PEG IgM's binding to each PEG-DSPE of PEG-liposomes and anti-PEG IgM's binding to each PEG-PBLA block copolymer of PEG-PBLA micelles were a possible reason for rapid blood clearance of PEG-liposomes; however, results regarding K_ds could not explain why only PEG-liposomes—not PEG-PBLA micelles—exhibited rapid blood clearance in the presence of anti-PEG IgM.

We previously reported that anti-PEG IgM, when induced with PEG-nanoparticles, significantly decreased PEG-liposome concentrations (dose = 4.0 mg/kg) in blood, whereas anti-PEG IgM did not decrease PEG-PBLA micelle concentrations (dose = 4.0 mg/kg) in ABC (+) mice [27]. Although this dose included nearly the same number of PEG chains in both PEG-liposomes and PEG-PBLA micelles (as shown in Table 4, the number of PEG molecules (N) in PEG-liposomes was nearly the same as the number in PEG-PBLA micelles). Anti-PEG IgM strongly affected only PEG-liposomes' pharmacokinetics [27]. Furthermore, we observed consumption of anti-PEG IgM at 6h after the second dose of both PEG-liposomes and PEG-PBLA micelles.

It is worth revisiting the above-mentioned two experimental findings from our previous study: the first finding was that the K_d of anti-PEG IgM for the PEG-liposome's PEG-DSPE was nearly the same as the corresponding K_d value for the PEG-PBLA micelle's PEG-PBLA block copolymer; and the second finding was that only the PEG-liposome exhibited rapid blood clearance in the presence of anti-PEG IgM. These two previous findings led us, in the present study, to consider the number of particles at the injected dose. Thus, for the present study, we observed complete anti-PEG IgM consumption after PEG-liposome injection at 20.0 μmol lipid/kg (Figure 5(A)). We calculated the number of PEG nanoparticles to evaluate how anti-PEG IgM affects PEG-liposomes' pharmacokinetics. Approximately 1.4×10^4 PEG-DSPE molecules are needed for a 130 nm single PEG-liposome particle, and the injected number of PEG-liposome particles in each mouse was 2.1×10^{11} particles at 4.0 mg/kg dose [36,37]. In contrast, the aggregation number of the PEG-PBLA micelle was 1,700, indicating that the injection dose's number of PEG-PBLA micelle particles was nearly 10-fold greater than the same dose's number of PEG-liposome particles (as shown in Table 4, the number of PEG-liposome particles was almost one order of magnitude different from the number of anti-PEG IgM molecules). The amount of induced anti-PEG IgM was approximately 5 μg/mL in serum (see Supplemental Fig. 2); more specifically, the number of generated anti-PEG IgM molecules was 3.3×10^{12} molecules in serum. The number of anti-PEG IgM molecules

secreted in blood was 10-fold greater than the number of injected PEG-liposome particles. Both the GPC experiment and the *in vivo* experiment, shown in Figure 6, revealed that the number of anti-PEG mIgM molecules (6.1 nM), being 10 times greater than the number of PEG-liposome particles (80 μg/mL = 0.4 nM), dramatically changed the PEG-liposome's behavior in GPC and *in vivo*.

Table 4. Calculation of the number of injected PEGylated nanoparticles

	Dose mg/kg	Diameter /nm	PEG molecules N^*/mouse	PEG-nanoparticles N^{**}/mouse
PEG-liposome	4.0	130	3.1×10^{15}	2.1×10^{11}
PEG-PBLA micelle	4.0	90	2.7×10^{15}	1.6×10^{12}

	Concentration in plasma	Number of anti-PEG IgM molecules
Anti-PEG IgM***	6.0 nM	3.3×10^{12}

* Number of PEG-DSPE molecules for PEG-liposomes, and number of PEG-PBLA molecules for PEG-PBLA micelles

** Number of PEG-liposome particles and number of PEG-PBLA micelle particles

*** Amount of anti-PEG IgM was 5.0 μg in plasma, and this indicates 3.3×10^{12} IgM molecules (6.0 nM) in plasma

These possible calculations explain why PEG-PBLA micelles did not exhibit rapid blood clearance (i.e., the ABC phenomenon), whereas PEG-liposomes exhibited the phenomenon. The immunization induced ca. 10^{12} anti-PEG IgM molecules, a number 10 times greater than the number of injected PEG-liposome particles. Around ten anti-PEG IgM molecules bound to each PEG-liposome, and the formed anti-PEG IgM-PEG-liposome complex exhibited the ABC phenomenon. In contrast, the number of anti-PEG IgM molecules was not enough to completely eliminate PEG-PBLA micelles from blood. Therefore, we observed the complete consumption of anti-PEG IgM after the second dose of PEG-PBLA micelles, whereas a change in PEG-PBLA micelles' blood concentration was negligible [27].

To understand the ABC phenomenon in the drug-targeting field, we should undertake the rather unique task of examining injected dose in terms of not only weight and number of molecules, but also number of injected particles because all the nanoparticles for drug targeting possess their own aggregation (association) number. Normally, we would consider an injected drug dose and a carrier dose (an approximately 10 wt% drug is usually loaded into carriers), but would not consider the injected number of particles. In this study, we revealed that the number of particles can significantly affect the targeting ability of PEG-nanoparticles after induction of the PEG-related immune response (i.e., of anti-PEG IgM).

We raised two possible ways to evade anti-PEG IgM-related rapid clearance in the wake of the PEG-related immune response for a better understanding of the anti-PEG IgM-related rapid-clearance phenomenon. The number of generated anti-PEG IgM molecules is limited (ca. 10^{12} anti-PEG IgM molecules in mouse serum). Therefore, a large number of PEGylated nanoparticles (ca. more than 10^{12} PEGylated nanoparticles) can exhibit negligible clearance. The number of PEGylated particles is dependent on their own aggregation number. Therefore, small particles are

better than large particles, which usually need a large number of molecules for aggregation. Second, highly dense PEGylated nanoparticles can evade anti-PEG IgM capturing (this assertion is reasonable, as we observed less affinity of anti-PEG IgM to PEG-conjugates at high concentrations). Therefore, we should carefully choose drug carriers to avoid failure of drug targeting in the wake of the immune response.

4. Conclusion

We have presented here new insights into the immune response—the ABC phenomenon—induced by PEGylated nanoparticles. Our mechanistic study of anti-PEG IgM confirmed the role that the relationship between anti-PEG IgM and PEG nanoparticles (e.g., PEG-liposomes and polymeric micelles) plays in the ABC phenomenon. Not only induced anti-PEG IgM in our experiments, but also commercially available anti-PEG antibodies (IgG and IgM) exhibited specificity to PEG chains, but these antibodies exhibited no binding to PEG main chains (-$(OCH_2CH_2)_n$-). Anti-PEG IgM exhibited hydrophobic block length-dependent binding. All our data strengthen our hypothesis that the proximity of hydrophobic blocks to PEG chains was essential to the binding. In addition, we introduced a novel consideration regarding a relationship between the number of anti-PEG IgM molecules and the number of injected PEGylated nanoparticles. To change the PEG-liposome's pharmacokinetics, we need ten-fold the number of anti-PEG IgM molecules as PEG-liposomes. In PEG-PBLA micelle cases, the number of anti-PEG IgM molecules is not large enough to trigger the pharmacokinetic change owing to the large number of PEG-PBLA micelle particles. In the induced PEG-related immune response, namely in the presence of anti-PEG IgM, the polymeric micelle carrier system, which exhibits a lower aggregation number than the PEG-liposome and a greater number of particles than the PEG-liposome, is advantageous in some drug-targeting applications.

ACKNOWLEDGEMENT

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Figure captions

Figure 1. Binding assays of Anti-PEG antibodies to PEG-conjugates. (**A**) An inhibition assay of anti-PEG IgM to PEG-DSPE as determined by a competitive ELISA. An ABC(+) serum was mixed with PEG(2k)-liposomes, PEG(2k)-DSPE, PEG(12k)-PBLA, and PEG-OH(5k), and then, the serum sample was added to the PEG-DPSE-coated plates. All PEGs possessed a methoxy group at the PEG terminal. All samples' PEG concentration was 50 nM in a 100-times diluted ABC(+) serum with normal saline. Dunnett's method was used for multiple comparisons between the control and the other samples. Data are presented as the average ± SD. Symbol * indicates p value < 0.05. (**B**) The binding behavior of PEG-DSPE and PEG-OH relative to anti-PEG mIgG as determined by a Sandwich ELISA.

Figure 2. Scheme of anti-PEG IgM binding to a PEG-coated plate: (left) a PEG-PBLA-coated plate; (right) a PEG-P(Asp)-P(Phe)-coated plate.

Figure 3. Anti-PEG IgM binding to the three different PEG polymer-coated plates. PEG-PBLA micelle-immunized serum (n=3) was used for ELISA experiments. The polymer that we used was composed of PEG-PBLA (number of BLA=30), PEG-P(Asp-Phe) (short P(Asp)) (number of Asp=24, number of Phe=17), and PEG-P(Asp-Phe) (long P(Asp)) (number of Asp=87, number of Phe=44).

Figure 4. Determination of the K_d of anti-PEG IgMs by means of inhibition assays. (**A**) Inhibition of anti-PEG IgM binding to PEG-PBLA-coated plates by the addition of the PEG-PBLA micelle (black circle) and the PEG-liposome (white square). (**B**) The PEG-liposome (white square), the 12-49(89) micelle (black triangle), the 12-30(88) micelle (gray triangle), and the 12-10(84) micelle (white triangle) were used for inhibition assays.

Figure 5. (**A**) Concentration changes in anti-PEG IgM before and after the 2nd dose of the PEG-liposome in PEG-PBLA-immunized mice. Here, 'PEG-PBLA' refers to the PEG-PBLA micelle, and 'PEG-L' refers to the PEG-liposome. (**B**) Plasma concentrations of PEG-liposomes in either PEG-PBLA micelle-immunized or saline-injected mice at 6h after the 2nd PEG-liposome dose. 'N.D.'=not detected.

Figure 6. Anti-IgM binding significantly changes PEG-liposome behaviors. (**A**) The GPC assay of PEG-PBLA micelles and PEG-liposomes mixed with anti-PEG mIgM as a function of various concentrations: the concentration of the PEG-PBLA micelle and the PEG-liposome was constant at 80 μg/mL. (**B**) The effect that the addition of anti-PEG IgM to the PEG-liposome had on the plasma concentration at 6 h after the injection: the PEG-liposome was injected at a dose of 5.0 μmol lipid/kg (4.0 mg/kg), and was mixed with anti-PEG mIgM at 0.6, 2.5, and 6.1 nM, respectively. The mixed anti-PEG mIgM concentrations are shown in parentheses.

Figure 7. Effect of the PEG-liposome's immunization on different strains: C57BL6 WT mice (white), MyD88 knockout mice (black), and MyD88 hetero mice (gray) were used for the experiment. The figure depicts the concentration of the injected carrier at 6h after the second dose.

All the mice were injected with either saline or the PEG-liposome 7 days before the second injection.

Graphical Abstract