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Article

Antigenicity Extension: A Novel Concept Explained by the Immunogenicity of PEG

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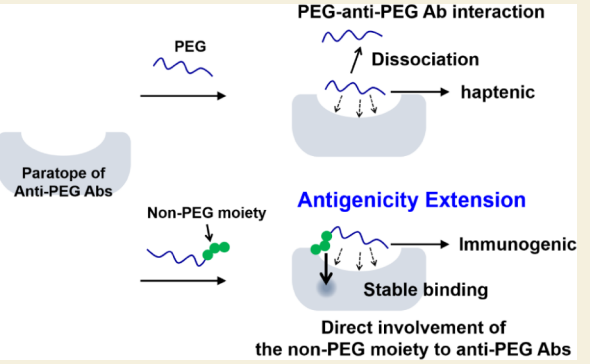
ABSTRACT: Poly(ethylene glycol)-related immune responses have been a great concern regarding mRNA vaccination for the SARS-CoV2 virus, because PEG-lipids are an essential component for the lipid nanoparticles of mRNA vaccines. Meanwhile, no research has elucidated the mechanisms underlying hapten-like PEG-related immunogenicity. For the current study, we uncovered a process by which haptenic PEGs transition into immunogenic PEG-conjugates by means of ELISA and microfluidic diffusional sizing (MDS). We named the process "antigenicity extension." Although PEGs exhibit specific interactions with anti-PEG antibodies, the specific interactions of PEGs with anti-PEG Abs are relatively weak. By contrast, we revealed that exposure of non-PEG moieties to the PEG-specific paratope greatly and directly contributes to PEG's stable bindings through the specific interaction between PEG and anti-PEG antibodies by MDS measurements. This indicates that non-PEG moieties are directly involved in the molecular recognitions between PEG and the PEG-specific paratope to improve the affinity. Occurring antigenicity extension makes PEG-conjugates immunogenic by strengthening the affinity for PEG-specific paratopes. Thus, additional interactions at non-PEG moieties with the PEG-specific paratope are key to the transition of haptenic PEGs into immunogenic PEGs. To this extent, antigenicity extension is a commonly occurring phenomenon in the hapten-to-immunogen transitions occurring in both antigen−antibody interactions and ligand−receptor interactions.



KEYWORDS: *poly(ethylene glycol) (PEG), anti-PEG antibodies, immunogenicity of PEG, hapten-to-immunogen transitions, antigenicity extension*

INTRODUCTION

Poly(ethylene glycol) (PEG) possesses simple repeating units of ethylene oxide ($-CH_2CH_2O-$) and exhibits nonionic and nonpolar characteristics. However, hydrated ethylene oxide can form hydrophilic PEG chains, resulting in PEG that resists the adsorption of serum proteins. Responding to the potential benefits of hydrophilic PEG, researchers have made great strides in conjugating PEG onto proteins and nanoparticles (NPs), using a technique called PEGylation. PEGylation improves the aqueous solubility of proteins and NPs and reduces serum protein adsorption, resulting in reduced immunogenicity of parent proteins. Thus, PEGylation has become the gold-standard method for this type of conjugation.[1,2]

PEG has long been thought of as an inert and non-immunogenic polymer. However, much attention has turned to PEG-related immunological concerns, especially in the wake of the worldwide vaccination effort targeting the SARS-CoV2 virus.[3−8] Anti-PEG antibodies (anti-PEG Abs) have been found in not only patients who were treated with PEGylated proteins but also healthy subjects who were injected with SARS-CoV2 vaccines.[9−14] Ju et al. measured levels of both anti-PEG IgG and anti-PEG IgM in participants who received two doses of BNT162b2 and mRNA-1273. The researchers found that mRNA-1273 was associated with a significant boost in levels of anti-PEG IgG and anti-PEG IgM, whereas BNT162b2 was associated with only modest boosting effects.[13] Another study, this time conducted by Guerrini et al., revealed that even after three vaccine doses, there was no dramatic increase in anti-PEG IgG levels. Nevertheless, the researchers found that (1) some individuals exhibited very high levels of anti-PEG IgG, which could constitute a high-risk factor for anaphylactic reaction, and (2) levels of anti-PEG IgM rose significantly after the first and the third vaccine doses.[11−13]

Even before SARS-CoV2 vaccinations became available, the researchers in pharmaceutical fields was well-aware that repeatedly injected PEGylated proteins, as well as nano-

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particles, induced anti-PEG Abs.[15,16] PEGylated nonhuman enzymes (e.g., uricase and asparaginase) have given rise to severe problems related to anti-PEG Abs, but other PEGylated human proteins have proven to be less immunogenic than the two nonhuman enzymes.[17,18] Anti-PEG Abs have also been found in animals, and anti-PEG Ab-related phenomena have been linked to the accelerated blood clearance (ABC) phenomenon.[19−21] The ABC phenomenon can involve the rapid clearance from blood subsequent or repeated injections of PEGylated drug carriers and proteins and is closely related to the number of induced-anti-PEG Abs and the number of injected PEGylated molecules.[22−24]

Antigens are classified into haptens and immunogens, with haptens capable of binding to specific antibodies but incapable of eliciting antibody responses. In contrast, immunogens induce antibody responses. There is generally a consensus that PEG itself is nonimmunogenic and exhibits haptenic-like characteristics; consequently, PEGs are thought to exhibit both a weak capacity to bind to antibodies (i.e., "low affinity") and an inability to elicit antibody responses.[3−7] In addition, the immunogenicity of PEG in PEGylated proteins is highly reliant on the immunogenicity of parent proteins, as we noted above with respect to both uricase and asparaginase.[25] These facts indicate that a PEG's terminal moieties (i.e., terminal lipids, proteins, or polymers) may play important roles in the PEG's immunogenicity.

Previous studies by Sherman et al. revealed that the α−terminal hydrophobic moieties of PEG chains may play a crucial role in both the immunogenicity and the antigenicity of PEGylated proteins.[26,27] These studies further revealed that *tert*-butoxy-terminated PEG-protein conjugates exhibited immunogenicity higher than those of both methoxy- and hydroxyl-terminated PEG-protein conjugates. In our own past research, we revealed that hydrophobic moieties at the other terminal of mPEG play an important role in anti-PEG IgM induction.[28,29] One of our central findings was that mPEG possessing hydrophobic-block micelles (poly(ethylene glycol)-*b*-poly(β-benzyl L-aspartate) (PEG-PBLA) micelles) effectively elicited anti-PEG IgM, but that no such elicitation occurred with mPEG possessing hydrophilic-block micelles (poly(ethylene glycol)-*b*-poly(L-lysine-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid gadolinium) (PEG-P(Lys-DOTA-Gd) micelles). Both of these micelles used the same terminal PEG, had the same molecular weight, and exhibited nearly the same pharmacokinetics. However, the two micelles differed dramatically from each other in regard to their immunogenicity. Another critical difference between the two micelles lies in the fact that the formation of PEG-PBLA micelles depended on hydrophobic interactions between PBLA chains, whereas the formation of PEG-P(Lys-DOTA-Gd) micelles depended on electrostatic interactions.[29] This fact implies that the hydrophobicity at the terminal of mPEG is deeply associated with PEG's immunogenicity. Although most research has addressed specific peptide−antibody interactions and ligand−receptor interactions, we have decided to focus, in the present study, on roles of not only the PEG moiety but also non-PEG hydrophobic moieties in proximity to PEG chains in the relationship between PEG and anti-PEG antibodies. In fact, the role of non-PEG moieties attached to PEGs in PEG-conjugates on their binding to anti-PEG Abs and PEGs' immunogenicity if not adequality was explored. We suggest that both the location of the non-PEG moieties and their hydrophobicity are key factors in the binding of PEGs and anti-PEG Abs.

In this study, we thus focus on a missing link between haptenic PEGs and immunogenic PEGs. In studying the mechanisms that underlie the transition from haptenic PEGs to immunogenic PEGs, we explore chemical modifications to PEG, the characterization of modified PEGs in solution, binding assays to anti-PEG Abs, and the resulting immunogenicity. The characteristics of haptenic PEG are thought to center on PEG's very low binding affinity to anti-PEG Abs. By using chemically modified PEG, we reveal that chemically modified moieties are directly involved in the binding between binding sites of anti-PEG Abs (paratopes) and PEG, a process that boosts the PEG's binding affinity for anti-PEG Abs. We term this gaining of affinity "antigenicity extension." It occurs when the non-PEG hydrophobic moieties are in the proximity of PEG chains. Antigenicity extension explains not only the transition from haptenic PEG to immunogenic PEG but also the transition from haptenic antigens to immunogenic hapten−protein conjugates. Antigenicity extension is a novel concept and rests on the premise that we should reassess the mechanisms underlying molecular recognition.

MATERIALS AND METHODS

Synthesis of Poly(ethylene glycol)-*b*-poly(β-benzyl L-aspartate) (PEG-PBLA)

Following the literature, we synthesized PEG-PBLA block copolymers with α-methoxy-ω-amino poly(ethylene glycol) (PEG-NH_2) (M_W = 12000, NOF Corporation, Tokyo, Japan)-initiated ring-opening polymerization of β-benzyl L-aspartate *N*-carboxy anhydride (BLA-NCA).[30,31] A macroinitiator PEG-NH_2 (M_W = 12000) was mixed with the monomer BLA-NCA in DMF, and BLA-NCA polymerization was monitored on the IR spectrum. The reaction mixture was poured into cold diethyl ether, and the obtained white solid was filtered and further dried under a vacuum. To determine the average number of BLA units, we used ^{1}H NMR spectroscopy (400 MHz, INOVA NMR spectrometer, Agilent Inc.) in $CDCl_3$ (see Figure S1). We created codes indicating the BLA units of each of the three types of PEG-PBLA that we synthesized: a code of "PEG-PBLA6" indicates PEG-PBLA possessing 6 BLA units, a code of "PEG-PBLA8" indicates PEG-PBLA possessing 8 BLA units, and a code of "PEG-PBLA31" indicates PEG-PBLA possessing 31 BLA units.

General ELISA Procedure

We purchased monoclonal anti-PEG Abs (AGP4, AGP3, 6.3, and 15-2b) from the Institute of Biomedical Sciences at Academia Sinica (Taipei, Taiwan). AGP4 and AGP3 antibodies are mouse IgM antibodies, and 6.3 and 15-2b antibodies are mouse IgG antibodies. AGP4, AGP3, and 6.3 antibodies are main-chain-specific antibodies, and 15-2b antibodies are terminal-methoxy-specific antibodies. We used the following terms for anti-PEG Abs. A PEG main-chain-specific anti-PEG IgM (anti-PEG IgM), a PEG main-chain-specific IgG antibody 6.3 (anti-PEG IgG), and a terminal-methoxy-specific IgM antibody 15-2b (anti-PEG IgG (methoxy)). From Abcam, we purchased biotin-conjugated monoclonal anti-PEG IgG (biotin-conjugated anti-PEG IgG) We used 50 mM tris-buffered saline (TBS), pH = 8.0) as a washing solution for well washing. All procedures for ELISA were examined in duplicate and performed at 24−25 °C unless otherwise noted. Although appropriate washing buffer has been recommended for human serum in ELISA,[12] our experiments were used a mixture of monoclonal anti-PEG Abs and PEG, or PEG-PBLA, and no

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other proteins, and lipids were contained in the mixture. Therefore, we used TBS as a washing buffer in the current study. We used a 1% BSA in 50 mM tris-buffered saline (pH = 8.0) as a blocking buffer solution for 1h at r.t.

Indirect and Inhibition ELISA

To prepare mPEG-immobilized 96-well plates, we relied on a PEG-PBLA31 solution (100 μL of 20 μg/mL in ethanol/H_2O (50% v/v) mixture) at 4 °C. The wells were washed three times with the wash solution and were blocked with the blocking buffer solution for 1 h. After undergoing three washings, the wells were filled with either freshly prepared anti-PEG Ab solutions in Dulbecco's Phosphate Buffered Saline (D-PBS) or 1 h premixed solutions of anti-PEG Ab-PEG samples in D-PBS. After 1 h, we washed the wells three times with the washing solution. Then, we added to the wells a solution of horseradish-peroxidase (HRP)-conjugated anti-mouse IgM antibody (0.01 μg/mL) or a solution of HRP-conjugated protein A/G detection antibody (0.02 μg/mL). After 1 h, we washed the wells three times with the washing solution. To the wells, we added a solution of 3,3′,5,5′-tetramethylbenzidine (TMB) (100 μL). After 15 min, we added 0.36 N H_2SO_4 (100 μL) to the wells in order to stop the reaction. Absorbance (450 nm) was recorded with a microplate reader (Multiskan GO, Thermo Scientific Inc. Ltd.).

Double Detection in Sandwich ELISA

A 100 μL solution of either antimouse IgG antibody (TCI, 25.0 μg/mL in D-PBS) or antimouse IgM antibody (TCI, 25.0 μg/mL in D-PBS) was added to 96-well plates and was kept overnight at 4 °C. We washed the wells with the wash solution, and the wells were blocked with the blocking buffer solution. We washed the wells with the wash solution, and premixed solutions of anti-PEG IgG (2.0 μg/mL, 13.3 nM) or anti-PEG IgM (2.0 μg/mL, 2.0 nM) and various concentrations of PEG samples in D-PBS were added to the washed wells. After 1 h, we washed the wells three times with the wash solution. To detect bound anti-PEG IgG or anti-PEG IgM, we used a solution of HRP-conjugated antimouse IgG antibody or HRP-conjugated antimouse IgM antibody (0.01 μg/mL), and to detect bound PEG samples, we first used a solution of biotinylated mouse anti-PEG IgG (Abcam, PEG-B-47b, 0.01 μg/mL) and then used a solution of HRP-conjugated streptavidin (0.0075 μg/mL). To the wells was added a solution of TMB (100 μL). After 15 min, we added 0.36 N H_2SO_4 (100 μL) to the wells in order to stop the reaction. Absorbance (450 nm) was recorded with a microplate reader.

Sandwich ELISA for Terminal-Methoxy-Specific Anti-PEG IgG

A solution of the anti-PEG IgG (methoxy) (5.0 μg/mL in D-PBS) was added to 96-well plates and kept overnight at 4 °C. Then, we washed the wells with the washing solution. After the wells were blocked with the blocking buffer solution, we added various concentrations of PEG sample solutions in D-PBS to anti-PEG IgG (methoxy)-immobilized plates and kept the wells for 1 h. Then, we washed the wells three times with the washing solution. To detect bound PEG samples, we added biotinylated mouse anti-PEG IgG (0.04 μg/mL) to the wells and then added HRP-conjugated streptavidin (0.025 μg/mL). To confirm whether or not there were nonspecific bindings of PEG samples to 96-well plates, we used noncoated plates and performed exactly the same experiments as we did the procedure following blocking the wells.

It is noteworthy that we have examined sandwich ELISA results with and without immobilized anti-PEG antibodies. Because PEG-PBLAs possess hydrophobic blocks, hydrophobic interactions may immobilize PEG-PBLAs on BSA-blocked plates, even in the absence of immobilized-anti-PEG Abs. This immobilization indicates that we may have observed incorrect binding behaviors of PEG-PBLAs. Therefore, using both dilute concentrations of PEG-PBLAs and sandwich ELISAs, we observed that the PEG-PBLAs exhibited (1) only negligible bindings to BSA-blocked plates (without anti-PEG Abs immobilization) in sandwich ELISA but (2) substantial bindings to anti-PEG Ab-immobilized plates. (see Figure S2).

Biolayer Interferometry (BLI) for Observing Binding Behaviors of PEG-PBLA6 to Anti-PEG IgM Antibody

The octet K2 system (Sartorius AG.) was used for BLI studies. Anti-PEG IgM (5.0 μg/mL in D-PBS) was immobilized onto aminopropyl silane (APS) sensors for 20 min. The anti-PEG IgM-bound sensor was washed with D-PBS and measured for 1 min for baseline. Serial dilution of PEG-PBLA6 solutions were examined for association (10 min) and dissociation (5 min).

Microfluidic Diffusional Sizing (MDS) Platform for Characterizing Anti-PEG Ab-Bound Immune Complexes

We labeled PEG-NH_2 and PEG-PBLA with *N*-hydroxy succinimide ester of AlexaFluor 647 (Alexa647) for MDS experiments (see Supporting Information text)). MDS measurements were performed with a Fluidity One-W Serum instrument (Fluidic Analytics, Cambridge, UK). On an MDS tip, we loaded premixed solutions (5−6 μL) of either PEG-PBLA6 (25 nM) or Alexa647-labeled PEG (100 nM) with anti-PEG Ab. We inserted the MDS chip into the instrument and used the microfluidic system to detect diffused and undiffused molecules. MDS provided us with the K_D and R_h values (hydrodynamic radius) of immune complexes. In observing the temperature-dependent conformational changes of PEG-PBLA6, we made a dilute solution of PEG-PBLA6 on ice and mixed it with anti-PEG Abs at 4 °C. The final concentration of PEG-PBLA6 was set at 25 nM.

In Vivo Immunogenicity Assay of PEG-PBLAs

We injected 100 μL of PEG samples into the tail vein of a C57BL/6 mouse (6 weeks old, male, Sankyo Lab., Tokyo, Japan), and we collected serum on days 7 and 14 after the injection. The sera were 100 times diluted by normal saline and were subjected to ELISAs. We confirmed the anti-PEG IgM Abs of the collected sera by relying specifically on mPEG-PBLA-immobilized indirect ELISAs. All animal experiments were reviewed and approved by the Jikei University's committee responsible for assessing the care and use of laboratory animals. Moreover, the committee found that all the experiments were in accordance with Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. Our animal facility for the mice had a light−dark cycle of 12 h, offered them free access to food and water, and maintained temperatures of 22−24 °C and humidity levels of 40−60%. We performed Dunnett's multiple-comparison test and used α-methoxy ω-hydroxy poly(ethylene glycol) (mPEG-OH) (M_w = 12000) as the control for the comparison. Between-group differences were considered significant with p-values <0.05.

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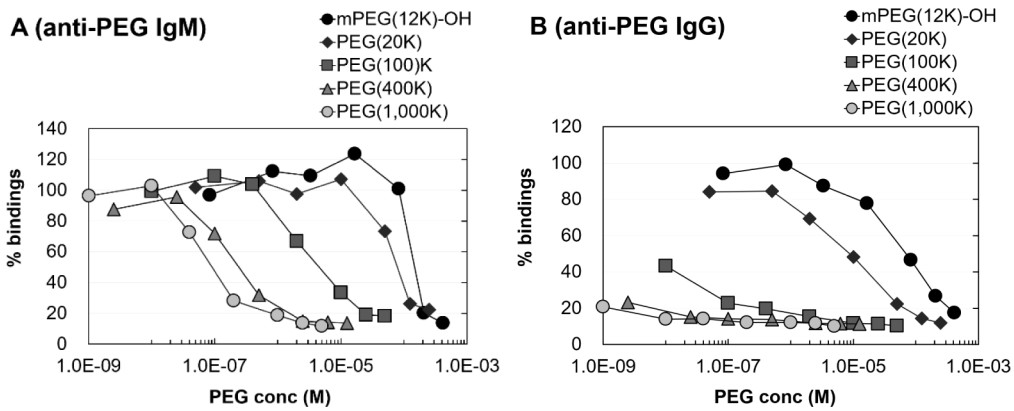

Figure 1. Using inhibition ELISA, we assessed the extent to which mPEG(12k)-OH and PEG with varying molecular weights (20 k, 100 k, 400 k, and 1000 k) inhibited (A) anti-PEG IgM binding to immobilized mPEG(12k) and (B) anti-PEG IgG binding to immobilized mPEG(12k).

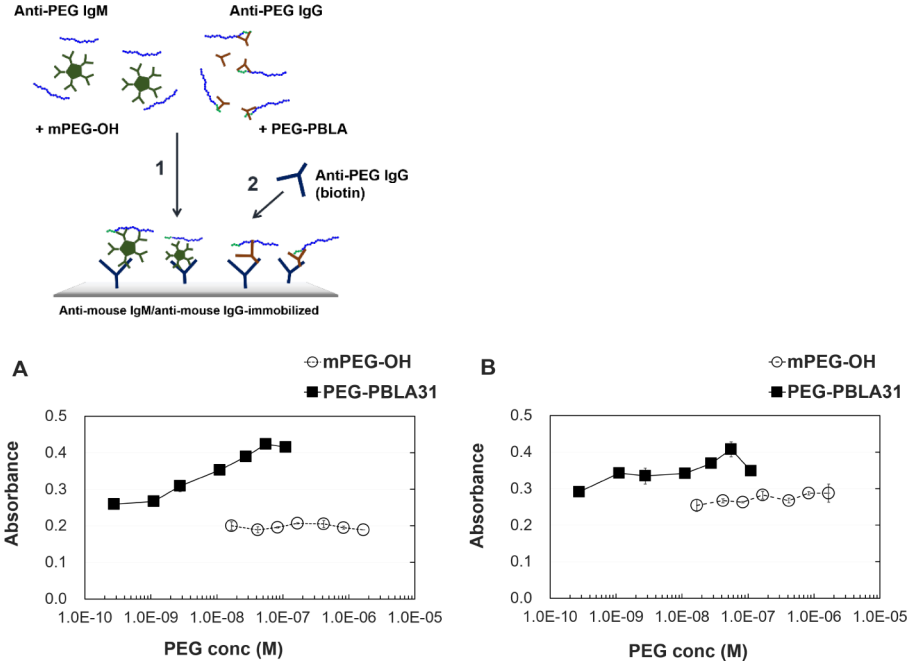

Figure 2. Detection of PEG−anti-PEG Ab immune complexes by means of sandwich ELISA (top). We first separately premixed (A) anti-PEG IgG (13.3 nM) and (B) anti-PEG IgM (2.0 nM) with various concentrations of either mPEG-OH or PEG-PBLA31 for 1 h at 37 °C and added these mixtures to plate-immobilized antimouse IgM Abs or antimouse IgG Abs (**1**). To detect PEG−anti-PEG Ab immune complexes, we used biotin-conjugated anti-PEG IgG (**2**).

RESULTS

Detecting PEGs Bound to Anti-PEG Antibodies

We examined the binding behaviors of anti-PEG Abs (IgM and IgG) using mPEG(12k) immobilized on plates. It should be noted that we examined both different isotypes (IgG and IgM) and different specificities of anti-PEG IgG. Anti-PEG IgMs are mouse IgM antibodies, and anti-PEG IgG and anti-PEG IgG (methoxy) are mouse IgG antibodies. Both the IgM antibodies and anti-PEG IgG are PEG-main-chain-specific antibodies. As for the anti-PEG IgG (methoxy), it is a PEG-related terminal-methoxy-specific antibody. To date, most of the anti-PEG Ab research has used indirect ELISA for detection of anti-PEG Abs, and in the present study, we have similarly used conventional indirect ELISA, this time to test the binding of anti-PEG Abs to immobilized mPEG (see Figure S3). Our indirect ELISA results indicate that the isotypes and specificities of anti-PEG Abs do not affect PEG-specific binding to immobilized mPEG (M_w = 12000). Also in the present study, we used inhibition ELISA to assess how PEG with varying molecular weights might inhibit anti-PEG Abs' binding to mPEG(12k) immobilized on plates (Figure 1). We used main-chain-specific anti-PEG IgM and anti-PEG IgG for the inhibition ELISAs. The PEGs inhibited both anti-PEG Abs' binding to the immobilized mPEG in ways that were clearly dependent on molecular weight. Our indirect ELISA results and our inhibition-ELISA results seem to indicate that anti-PEG Abs bind to PEG; however, these experiments do not prove that the binding was stable. In fact, we required high concentrations of α-methoxy ω-hydroxy poly(ethylene glycol) (mPEG-OH) (M_w = 12000) to prevent anti-PEG Abs from binding to the mPEG immobilized on plates. The results of our inhibition ELISA suggest that mPEG-OH weakly interacts with anti-PEG Abs. More specifically, we estimate that (1) the K_D of mPEG-OH to anti-PEG IgM was on an order of 10^{-4} M and

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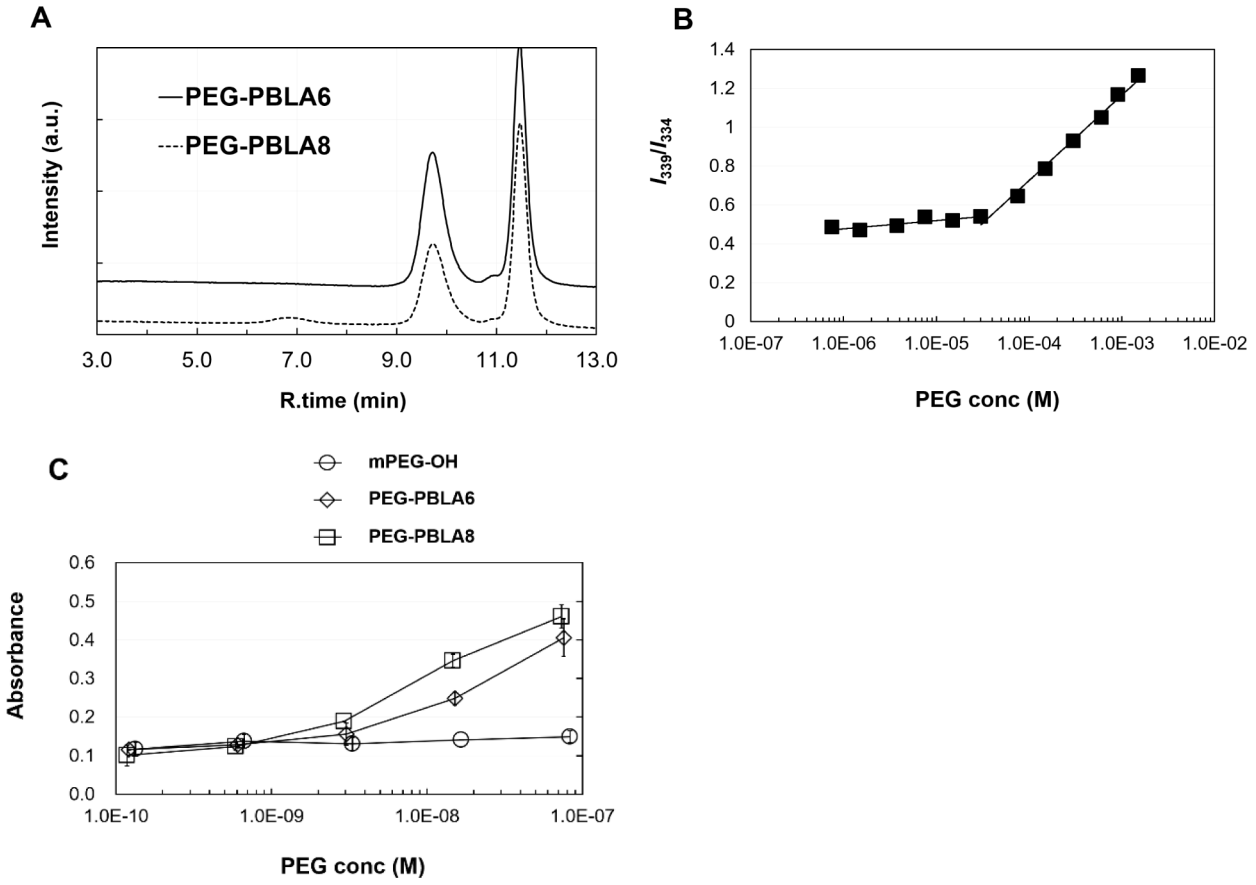

Figure 3. (A) GPC refractive index (RI) elution profiles for the PEG-PBLAs. GPC was performed in D-PBS at 40 °C. The concentration of PEG-PBLA was 0.25 mg/mL. Detection = RI. A peak at 11.5 min corresponds to the solvent peak. (B) CMC was determined for PEG-PBLA6 by means of the pyrene method. (C) The binding of three types of PEGs to terminal-methoxy-specific anti-PEG IgG was measured by means of sandwich ELISA. PEG-PBLA6, PEG-PBLA8, and mPEG-OH were added to anti-PEG IgG (methoxy) immobilized on plates. PEGs bound to anti-PEG IgG (methoxy) were detected with biotin-conjugated anti-PEG IgG.

(2) the K_D of mPEG-OH to anti-PEG IgG was on an order of 10^{-5} M.

After the above steps were completed, we performed a series of experiments to identify and assess any differences between the stable and weak binding of PEGs to anti-PEG Abs. In this way, we sought to reveal how immune complexes might form between anti-PEG Abs and PEGs. We premixed anti-PEG IgM with mPEG-OH, and we premixed anti-PEG IgM with PEG-PBLA31 ($M_{w,\,PEG}$ = 12000). We followed this same step for anti-PEG IgG. We then added each of these mixtures separately to antimouse IgM immobilized on plates or to antimouse IgG immobilized on plates. We proceeded on the reasonable premise that, if mPEG-OH, PEG−PBLA31, or both are stably bound to anti-PEG IgM or anti-PEG IgG, we should be able to detect—by means of biotin-conjugated anti-PEG IgG Abs—the PEG−anti-PEG Ab immune complexes that bind to the immobilized antimouse IgM or antimouse IgG Abs. As shown in Figure 2, we observed a stark contrast between the mPEG-OH and PEG-PBLA31 results. Specifically, we confirmed that the anti-PEG IgG was able to bind to the immobilized antimouse IgG Abs in both the mPEG-OH case and the PEG-PBLA31 case, and anti-PEG IgM was also able to bind to the immobilized antimouse IgM antibody (see Figure S4). No mPEG-OH bound to anti-PEG Abs in the mixtures. By contrast, we detected PEG in two mixtures: a mixture consisting of anti-PEG IgG and PEG-PBLA31 and a mixture consisting of anti-PEG IgM and PEG-PBLA31. These results indicate that PEG-PBLA31 exhibited the capacity to bind stably to anti-PEG Abs, whereas mPEG-OH exhibited a tendency to dissociate from anti-PEG Abs. In fact, our inhibition ELISA results suggest that mPEG-OH exhibited an inhibitory effect on the capacity of anti-PEG Abs to bind to immobilized PEG. Nevertheless, the overall results indicate that the interaction between mPEG-OH and anti-PEG Abs did not enable PEG to bind stably to anti-PEG Abs in ELISA experiments.

It is important to distinguish between specific interactions (shown in Figure 1) and specific stable bindings (shown in Figure 2). Using conventional sandwich ELISA, we immobilized anti-PEG IgM and anti-PEG IgG onto the plates. We then prepared separate serial dilutions of mPEG-OH and PEG-PBLA31 solutions and added them to the plates. A stark contrast emerged between those of mPEG-OH and PEG-PBLA31. Specifically, PEG-PBLA31 exhibited bindings to anti-PEG IgM, anti-PEG IgG, and anti-PEG IgG (methoxy) but no bindings to normal mouse IgM (see Figure S5). In contrast to the PEG-PBLA31 results, no mPEG-OH bindings were observed. Thus, the question naturally arises as to how PEG-PBLA31 can bind to anti-PEG Abs. To begin with, we know that the PBLA moiety of PEG-PBLA31 exhibited a high degree of hydrophobicity, just as PEG-PBLA31 exhibited stable micelle formulation.[22] These two facts suggest that there are two possible reasons PEG-PBLA31 was able to bind to anti-PEG IgM: first, the PBLA moiety directly supported the

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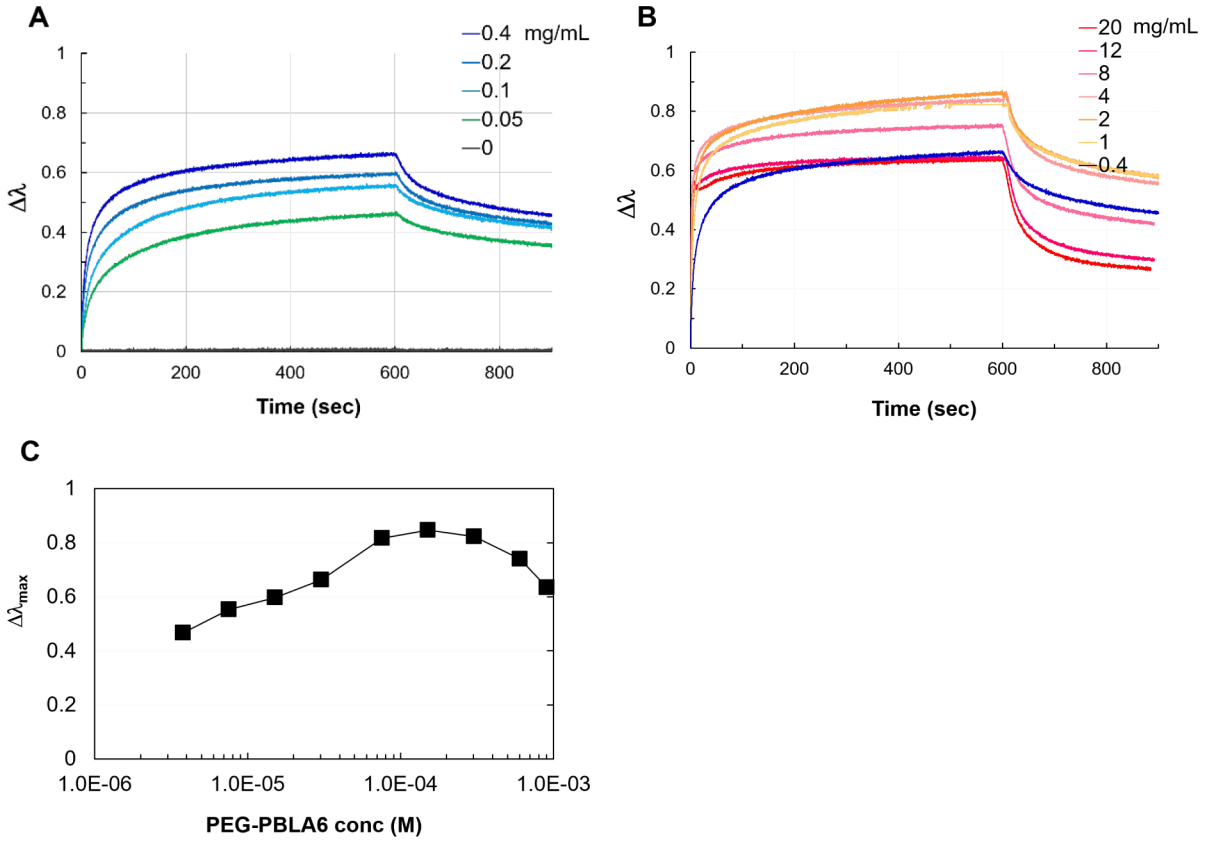

Figure 4. Binding curves for association and dissociation of PEG-PBLA6 (A) below the CMC and (B) above the CMC in a concentration range between 0.05 mg/mL (3.8×10^{-6} M) and 20 mg/mL (1.5×10^{-3} M). Anti-PEG IgM was immobilized on APS sensors. (C) $\Delta\lambda_{max}$ plots of PEG-PBLA6.

binding of PEG to anti-PEG Abs; second, aggregations of the PBLA moiety resulted in crowded PEG chains, which, in turn, led to the presentation of multivalent PEG chains. Although the PEG-PBLA31 concentrations for the experiments were below the critical micelle concentration (CMC = 1.1×10^{-7} M), our results above cannot prove with certainty that either of the two aforementioned possibilities is, indeed, the reason why PEG-PBLA31 was able to bind to the anti-PEG Abs. To resolve this issue, we further examined the binding behaviors.

PEG-PBLAs' Binding Behaviors to Anti-PEG Antibodies

We synthesized two PEG-PBLAs ($M_{W,\,PEG}$ = 12000) while carefully controlling the number of BLA units (see Table S1). The obtained PEG-PBLAs were highly soluble in aqueous media, and as shown in Figure 3A, we confirmed their aggregation behaviors by means of gel-permeation chromatography (GPC). PEG-PBLA possessing 6 BLA units (PEG-PBLA6) exhibited only a single unimodal peak at 9.76 min, and this result indicates that PEG-PBLA6 was totally soluble in a unimer form at 0.25 mg/mL. The addition of 2 BLA units to the PEG-PBLA6 led to a different set of outcomes: PEG-PBLA possessing 8 BLA units (PEG-PBLA8) exhibited two peaks, which were a minor fraction of the aggregation peak at 6.88 min and a major fraction of the unimer peak at 9.76 min at 0.25 mg/mL. We confirmed that PEG-PBLA6 behaves exactly as a unimer does. Also, as shown in Figure 3B, we used the pyrene method to confirm the CMCs. The CMC of PEG-PBLA6 was 3.0×10^{-5} M (0.36 mg/mL). Because, in the ELISA, PEG-PBLA6 was completely soluble in an aqueous solution without aggregation, there was no need to consider the possible contribution of aggregation to the binding capacity of anti-PEG Abs within a certain PEG-PBLA6 concentration range; therefore, we went ahead and subjected PEG-PBLA6 to further experiments.

While relying heavily on indirect ELISA, researchers rarely perform anti-PEG Ab-immobilized sandwich ELISAs for the evaluation of PEG bindings. The common decision to privilege the indirect ELISA over the sandwich ELISA is chiefly due to the fact that PEG itself does not strongly bind to anti-PEG Abs immobilized on plates. However, indirect ELISAs can help detect the binding of anti-PEG Abs to PEG immobilized on plates. Figure 3C presents the results of our anti-PEG IgG (methoxy)-immobilized sandwich ELISA: as can be seen, we obtained a very clear contrast between mPEG-OH and PEG-PBLAs. Anti-PEG IgG (methoxy) is an IgG Ab that recognizes terminal-methoxy groups of mPEG, but in our present study, only PEG-PBLAs exhibited an ability to bind to anti-PEG IgG (methoxy) in a concentration-dependent manner; that is, we detected no stable binding of mPEG-OH to anti-PEG IgG (methoxy). The trends we observed with respect to other anti-PEG Abs were similar to the PEG-related binding trends in the sandwich ELISA (see Figure S6).

Although all three polymers in our study possess an α-terminal methoxy group, only PEG-PBLA6 and PEG-PBLA8 were bound to anti-PEG IgG (methoxy). This particular set of results indicates that anti-PEG IgG (methoxy) initially recognized the terminal-methoxy group of PEGs. However, binding of mPEG-OH to anti-PEG IgG (methoxy) was not stable. Thus, our results indicate that stable binding of PEG to anti-PEG IgG (methoxy) is highly reliant on PBLA moieties.

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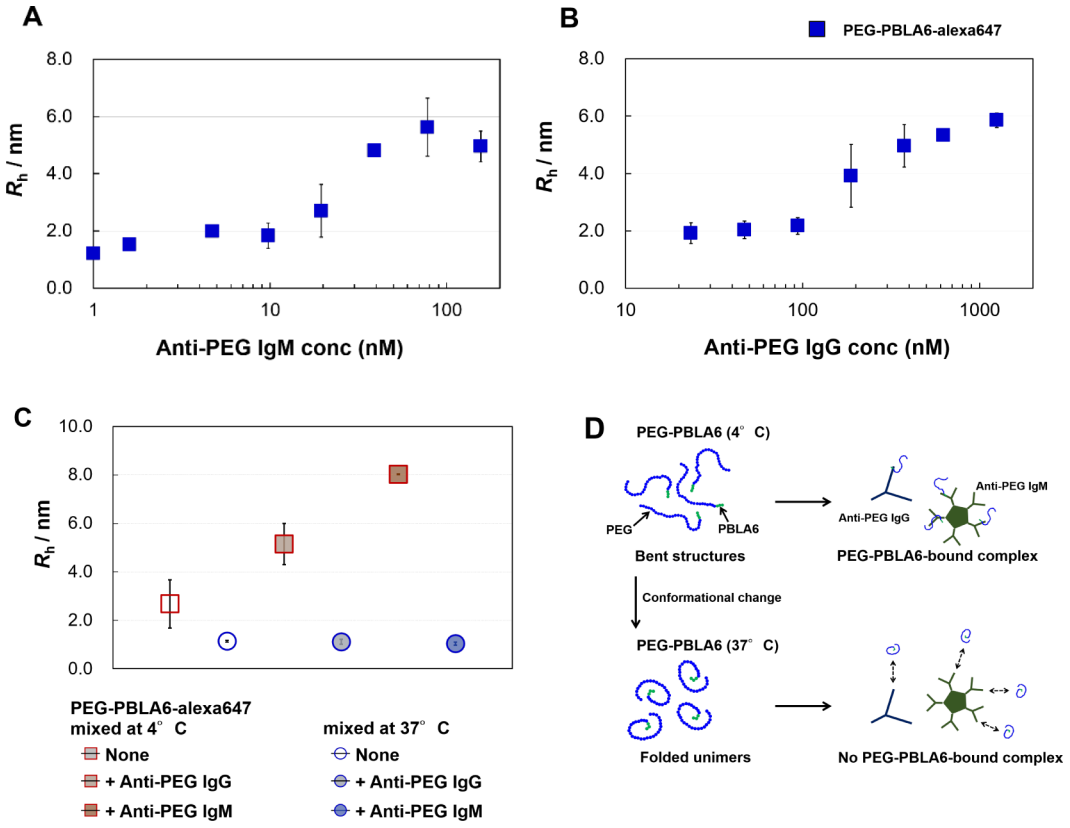

Figure 5. Microfluidic diffusional sizing (MDS) detection of PEG-PBLA6−anti-PEG Ab-immune complexes. (A) Immune complex of anti-PEG IgM with PEG-PBLA6-Alexa647 (25 nM) in solution. (B) Immune complex of anti-PEG IgG with PEG-PBLA6 (25 nM) in solution. (C) Structure-dependent immune complex formation of PEG-PBLA6 with anti-PEG IgM (75 nM) or anti-PEG IgG (625 nM). PEG-PBLA6 exhibited a folded unimer form at 37 °C and a bent structure at 4 °C. We assessed our experiments at 4 and 37 °C for 1 h. The folded unimer of PEG-PBLA6 exhibited no binding to anti-PEG Abs. We repeatedly assessed our experiments (N = 3−4). Structure-dependent binding behaviors of PEG-PBLA6 to anti-PEG Abs are shown in (D).

The results also point to a very interesting structural matter: the PBLA moieties were located on the opposite ends of the terminal-methoxy group. Furthermore, we have confirmed that PEG-PBLA6 was in an aggregate-free form; in other words, PEG-PBLA6, when in a unimer form, exhibited stable binding to anti-PEG IgG (methoxy). Put another way, the non-PEG moiety, the PBLA moiety, directly supported binding of PEG to anti-PEG Abs.

The Hiding of Hydrophobic Moieties in the Micelle Inner Core and Decreased Stable Binding to Anti-PEG IgM

Using biolayer interferometry (BLI), we examined the binding behaviors of PEG-PBLA6 to anti-PEG IgM. In the BLI experiment, we measured the association and dissociation of PEG-PBLA6 after immobilizing anti-PEG IgM to aminopropyl silane (APS) sensors. As shown in Figure 4, we observed concentration-dependent association curves and dissociation curves, yet these results indicate the presence of two notably distinct behaviors: on the one hand, PEG-PBLA6 starts to exhibit association to anti-PEG IgM in a concentration-dependent manner, with very slow dissociation observed below the CMC (Figure 4A); on the other hand, association of PEG-PBLA6 to anti-PEG IgM occurred more quickly above the CMC than below it. Nevertheless, we should bear in mind that rapid dissociation from anti-PEG IgM also occurs. Formation of PEG-PBLA6 micelles exposed mPEG chains to anti-PEG IgM; however, PEG-PBLA6 dissociation from anti-PEG IgM occurred more quickly above the CMC (Figure 4B). Furthermore, $\Delta\lambda_{max}$ of PEG-PBLA6 was highest at 1.5 × 10^{-4} M (2.0 mg/mL) and exhibited a decrease in signal intensities with increases in concentrations (Figure 4C). These behaviors are deeply rooted in PEG-PBLA6's micelle formation, which facilitates association behaviors to anti-PEG IgM, as well as dissociation behaviors from anti-PEG IgM.

A reasonable conclusion to be drawn from these results is that first anti-PEG IgM interacted with exposed-dense mPEG chains on PEG-PBLA6 micelles, and sensorgrams of PEG-PBLA6 exhibited fast association to anti-PEG IgM. Second, because hydrophobic PBLA6 moieties were embedded in the micelles at higher PEG-PBLA6 concentration than the CMC, the exposed mPEG chains did not exhibit fully stable bindings to anti-PEG IgM. Therefore, sensorgrams of PEG-PBLA6 exhibited fast dissociation from anti-PEG IgM at high PEG-PBLA6 concentrations.

It is possible that micelle dissociation occurred with respect to PEG-PBLA6. As we described above, the dissociated form of PEG-PBLA6 exhibited slow dissociation from anti-PEG IgM at concentrations below the CMC. Therefore, we conclude that some PEG-PBLA6 remained bound to the anti-PEG IgM.

The above-mentioned patterns were further confirmed when we observed the behaviors of PEG-PBLAs possessing different BLA units (data not shown). In examining mPEG-OH binding behaviors to anti-PEG IgM, we observed that the binding seems to have required a relatively high concentration of mPEG-OH. We also observed that sensorgrams of high concentrations of mPEG-OH exhibited fast association with

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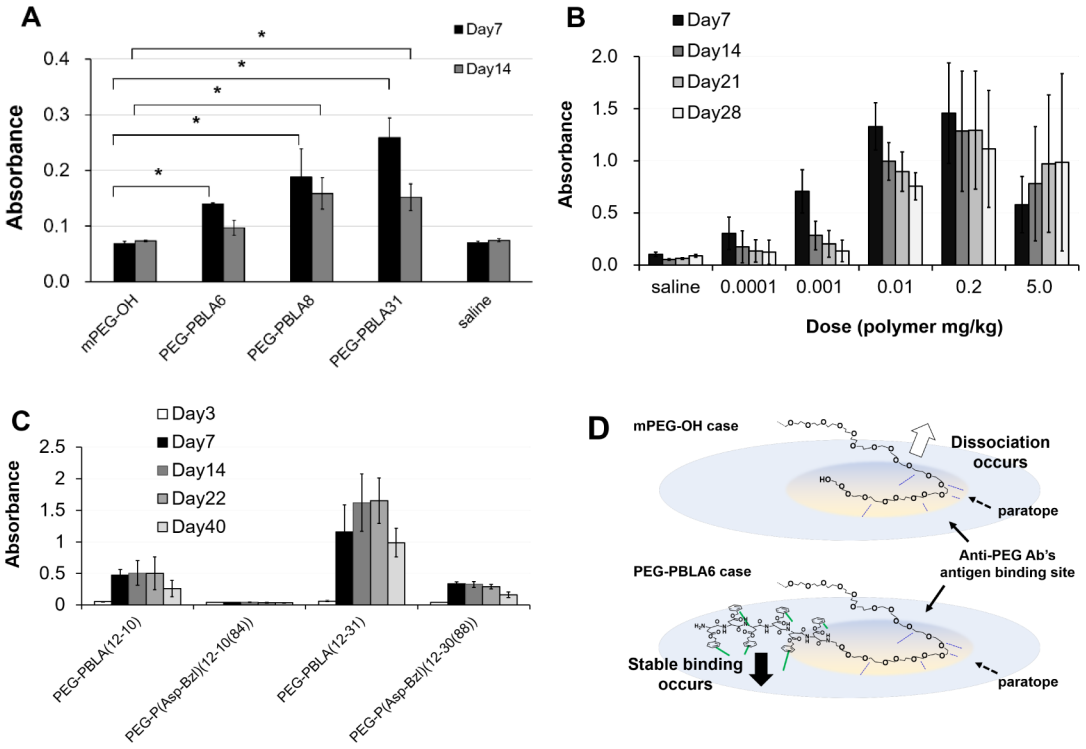

Figure 6. (A) Hydrophobic PBLA block-dependent anti-PEG IgM titers. Elicitation of anti-PEG IgM was achieved at a dose of 10 mg polymer/kg ($N = 3$). Highly immunogenic PEG-PBLA31 was injected at a dose of 0.01 mg polymer/kg as a positive control ($N = 4$). Saline (100 μL) was used as a negative control ($N = 4$). (B) PEG-PBLA31 dose-dependent anti-PEG IgM responses. (C) Anti-PEG IgM responses induced with four different kinds of conjugate blocks of PEG at a dose of 0.2 mg polymer/kg ($N = 3$). Data represent the mean value ± SD. (D) Schematic image of antigenicity extension. The stable binding of PEG to anti-PEG Abs is promoted by PBLA6 through PEG-specific interactions.

anti-PEG IgM and fast dissociation from anti-PEG IgM (see Figure S7).

PEG-PBLA–Anti-PEG Ab Immune Complexes in Solution

Both the PEG-immobilized ELISA and the anti-PEG Ab-immobilized ELISA involve substrate immobilization. We explored the binding between PEG-PBLA6 and anti-PEG Abs in a dilute solution. To this end, we used a microfluidic diffusional sizing (MDS) system, which detects hydrodynamic sizes (R_h) of a fluorescence-labeled molecule with or without the addition of a binding partner molecule. For the titration of anti-PEG Abs, we used a constant PEG concentration of Alexa647-conjugated PEG-PBLA6 (PEG-PBLA6-Alexa647) (25 nM). Figure 5A exhibits the titration curves of anti-PEG IgM in relation to those of PEG-PBLA6-Alexa647. We clearly observed immune complexes for anti-PEG IgM with PEG-PBLA6-Alexa647, but no immune complexes were detectable for anti-PEG IgM with mPEG-Alexa647 (Figure S8). Moreover, our results indicate that, for PEG-PBLA6-Alexa647, the K_D value of anti-PEG IgM was 1.0 ± 2.0 nM. Regarding anti-PEG IgG with PEG-PBLA6-Alexa647, we detected immune complexes, and the binding affinity (K_D = 0.3 ± 0.1 μM) was lower than the aforementioned binding affinity (Figure 5B). According to our inhibition-ELISA results, the inhibitory effects of mPEG-OH were much more pronounced on anti-PEG IgG ($K_D = 10^{-5}$ M) than on anti-PEG IgM ($K_D = 10^{-4}$ M). The MDS results seem to contradict the results corresponding to the aforementioned inhibitory effects. However, a point worth considering is that the affinities of these two antibodies for mPEG-OH are relatively low ($K_D = 10^{-4}$ to 10^{-5} M), whereas the antibodies' affinities for PEG-PBLA6 are relatively high ($K_D = 10^{-7}$ to 10^{-9} M). As we mentioned above, specific stable binding depends not only on PEG but also on PBLA. Because we found that high affinities were strongly linked to PBLA6, we concluded that the anti-PEG IgM made effective use of PBLA moieties for stable bindings involving interactions between anti-PEG IgM and PEGs.

We further found that PEG-PBLA6 exhibited temperature-dependent conformation changes and binding behaviors of PEG-PBLA6 to anti-PEG Abs were PEG-PBLA6's conformation-dependent. (Figure 5C). Freshly prepared PEG-PBLA6-Alexa647 (25 nM) was kept at either 4 or 37 °C for 1 h, and we performed MDS measurements of the solutions. The PEG-PBLA6 (4 °C) had an R_h of 2.7 ± 0.0 nm, whereas PEG-PBLA6 (37 °C) had an R_h of 1.1 ± 0.1 nm. Because PEG-PBLA6 was well-soluble in D-PBS, and a very low concentration of PEG–PBLA6 was used in the MDS experiments, it is very likely that a single PEG-PBLA6 molecule was involved in the formation of these structures. PEG-PBLA6 was hydrated at 25 nM. Therefore, PEG-PBLA6 exhibited a bent structure possessing an R_h of 2.7 ± 0.0 nm. When we heated the PEG-PBLA6 solution to 37 °C, the PBLA moiety had shown dehydration, and the dehydration of PBLA6 induced conformational changes to PEG-PBLA6. The formation of these structures involved single PEG-PBLA6 molecules, and we confirmed that the R_h of PEG-PBLA6 gradually became small R_h at 25 °C, and it took more than 4 h to exhibit the R_h of 1.1 ± 0.1 nm (see Figure S9A). Conformations of PEG-PBLA6 are thought to be highly concentration- and temperature-dependent. It should be noted that mPEG-Alexa647 (25 nM) had an R_h of 3.7 nm at 4 °C, and the R_h of mPEG was larger than that of PEG-PBLA6.

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Additionally, mPEG also had an R_h of 3.1 nm at 37 °C (see Figure S9B). Therefore, we concluded that the hydrophobic PBLA6 moiety was embedded by a PEG chain immediately after the temperature was increased from 25 to 37 °C, and at this point in time, PEG-PBLA6 formed folded unimers (a form of unimer micelle) (Figure 5D). Therefore, we conducted our MDS and ELISA experiments before the occurrence of the folded unimer formation.

Very interestingly, but as we expected, the folded unimer PEG-PBLA6 exhibited no binding to either anti-PEG IgM or anti-PEG IgG. In stark contrast to these outcomes, PEG-PBLA6 that was kept at 4 °C exhibited significant binding to both anti-PEG IgM and anti-PEG IgG. Namely, when the hydrophobic PBLA is exposed to anti-PEG Abs, the PBLA directly helps PEG bind to anti-PEG Abs.

Haptenic PEG exhibits specificity to an antibody but lacks enough affinity for the antibody to maintain stable binding. In general, IgG Abs exhibit a higher affinity for specific antigens than IgM Abs. The results of our inhibition ELISA in the current study indicate that anti-PEG IgG exhibited higher affinity than anti-PEG IgM for mPEG-OH (Figure 1). However, the above-mentioned MDS experiments indicate that the affinity of anti-PEG IgM for PEG-PBLA6 was higher than that of anti-PEG IgG for PEG-PBLA6. These differences in affinity would have escaped our attention if we focused only on interactions between PEGs and anti-PEG antibodies. Obviously, in our results, the affinity of both anti-PEG IgM and anti-PEG IgG for PEG-PBLA6 was higher than the affinity of these antibodies for mPEG-OH. The above observations support the assertion that PBLA plays an important role in PEG's bindings to anti-PEG Abs when PEGs approach the paratopes of anti-PEG Abs. Thus, according to our results, the PBLA moiety that was used for the binding of anti-PEG Abs to PEG brought about additional interactions between the epitopes of PEG and the paratopes of anti-PEG Abs. These additional interactions strengthened the affinities between PEG and anti-PEG Abs to a more pronounced level. It is this transition that we have termed "antigenicity extension." Although anti-PEG Abs are PEG-specific antibodies, our term indicates that non-PEG moieties attached to PEG, when the PEG is close to anti-PEG Abs, compensate for the specific but weak interactions between PEG and the anti-PEG Abs.

In Vivo Immunogenicity of PEG-PBLA6

Having uncovered findings that led us to formulate our novel concept termed antigenicity extension", we decided to examine PEG-PBLA6's ability to bind to anti-PEG Abs in relation to in vivo immunogenicity. We injected mPEG-OH, PEG-PBLA6, or PEG-PBLA8 into mice at a dose of 10 mg polymer/kg, and we injected highly immunogenic PEG-PBLA31, which served as a positive control, into mice at a dose of 0.01 mg polymer/kg. We collected sera on days 7 and 14 after the first injection, and to detect anti-PEG IgM in the collected sera, we performed ELISAs (Figure 6A). As we expected, mPEG-OH did not elicit any anti-PEG IgM, whereas PEG-PBLA6, PEG-PBLA8, and PEG-PBLA31 significantly elicited anti-PEG IgM on day 7. Although the level of anti-PEG IgM titers in the PEG−PBLA6 group was not as high as the corresponding level in the PEG-PBLA31 group, we observed significant differences between the anti-PEG IgM titers in the PEG-PBLA6 group and those in the mPEG-OH group (p = 0.027) on day 7. Our results indicate that PEG-PBLA6 became immunogenic without aggregation of PEG-PBLA6. On day 14, we observed that the levels of anti-PEG IgM titers in both the PEG-PBLA8 group and the PEG-PBLA31 group were significantly different from the corresponding level in the mPEG-OH group (p < 0.001) for both comparisons vs mPEG-OH. Significance was not obtained in the case of anti-PEG IgM titers in the PEG-PBLA6 group (p = 0.339 vs mPEG-OH). We further examined the dose response of PEG-PBLA31 and the immunogenic potential of PEG-PBLA(N) (Figure S10). Our central finding was that PEG-PBLA31 elicited anti-PEG IgM at a dose of 0.0001 mg polymer/kg (Figure 6B). The concentration for the dose (20 ng/mL) was far lower than the CMC of PEG-PBLA31 (CMC = 2.0 μg/mL, 10^{-7} M). The dose indicates that PEG-PBLA31 exhibited unimers or unstable aggregate structures in vivo. We then examined the in vivo immunogenicity of moderately benzylated (84−88%) mPEG-*b*-poly(aspartic acid)s (PEG-P(Asp)s), which we obtained by benzylation of PEG-P(Asp)s following hydrolyzation of PEG-PBLA(N) (see Table S2).[32] We found that the levels of anti-PEG IgM titers in the prepared benzylated PEG-P (Asp) groups were far lower than the corresponding levels in the parent PEG-PBLA groups (Figure 6C). This difference indicates that residues of hydrophilic aspartic acid suppressed PEG-PBLA's immunogenicity. The above-mentioned experiments indicate that PBLA moieties can help PEGs to stably bind to PEG-specific receptors in vivo by antigenicity extension and that PEG-PBLAs exhibited immunogenicity.

DISCUSSION

PEG has been known to serve as a hapten.[3−5] However, the molecular mechanisms underlying PEG immunogenicity have not been elucidated. In the current study, we have focused on the transition of haptenic PEGs into immunogenic PEGs by considering how the non-PEG moieties' chemical characteristics of PEG-block copolymers affect the ability of the PEGs to bind to anti-PEG Abs. Using both PEG-main-chain-specific anti-PEG Abs and terminal-methoxy-specific anti-PEG Abs, we concluded that PEGs do not have enough affinity to maintain stable PEG−anti-PEG Ab immune complexes in a dilute solution, and this is why PEGs do not exhibit immunogenicity. This state of affairs perfectly reflects the haptenic characteristics of PEG. McSweeney et al. reported that a high dose of PEG (40 k) pretreatment suppressed anti-PEG IgM responses following a PEG-liposome injection.[33,34] The study's finding indicates that high-molecular-weight (MW) PEG (40 k) will specifically yet weakly interact with PEG-specific B-cell receptors without elicitation of anti-PEG IgM in the spleen and will stay there a long time owing to slow diffusion.

We also confirmed that high-MW PEG (e.g., M_w = 100000) inhibited bindings of anti-PEG Abs more effectively than did low-MW PEG (e.g., mPEG-OH) (Figure 1). However, PEG-PBLA6 exhibited significant signs of stable binding to anti-PEG IgM, whereas PEG (100 K) exhibited no signs of stable binding to anti-PEG IgM (Figure S11). This set of results indicates that PEGs have very little chance of inducing anti-PEG Abs.

On the other hand, crystallographic studies have shown that there are close packing structures between the PEG and the Fab fragments of anti-PEG IgG.[35−37] The researchers found only 500 to 600 Å of the buried surface area of paratopes in the PEG−Fab cocrystals.[35,36] From the perspective of protein−protein interactions, such a small buried surface area of paratopes clearly indicates the presence of high K_D values (low

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affinity).[38] These small buried areas of paratopes also indicate that the PEGs in question possess haptenic characteristics. In addition, relatively high PEG concentrations are necessary to obtain PEG−Fab cocrystals. In such situations, PEGs are thought to exhibit the intrinsic characteristic of hydrophobicity.[39] In the present study, we confirmed that high phosphate salt concentrations (D-PBS) facilitate interactions between PEGs and anti-PEG Abs (see Figure S12). This finding further indicates that PEG hydrophobicity plays an important role in the interactions between PEG and anti-PEG Abs for the crystal formation. Likewise, high PEG concentrations may also help to induce structural changes of the Fab, a process that has been conceptualized as the conformational selection model.[40−43] The interactions between PEGs and anti-PEG Abs are weak enough to dissociate at low PEG concentrations. In fact, high PEG concentrations serve as a macromolecular crowding agent: the function induces depletion forces, which in turn can enhance protein−protein interactions.[44−46] Therefore, we believe that high PEG concentrations further facilitate the interactions between PEG and Fabs and that conformational changes in anti-PEG Abs gradually improve both the suitability and the stability of PEG−Fab cocrystals. The above considerations can explain why we observed, on one hand, stable PEG bindings to the Fabs in the cocrystals but, on the other hand, a high degree of dissociability characterizing the relationship between PEG and anti-PEG Abs in solution.

In the present study, PEG-PBLA6 exhibited bindings to not only the PEG-main-chain-specific Abs but also the terminal-methoxy-specific anti-PEG IgG. Very interestingly, the terminal PBLA moiety helped PEG bind to anti-PEG IgG (methoxy) (Figure 3C). Figure 6D explains the mechanism. The first interaction between mPEG and anti-PEG IgG (methoxy) starts with the terminal-methoxy group and anti-PEG IgG (methoxy): the two molecules are thought to further interact with each other through the PEG main chain, but anti-PEG IgG (methoxy) exhibits a low affinity for both the terminal methoxy group and the PEG main chain; therefore, the interactions between mPEG-OH and anti-PEG IgG (methoxy) easily dissociate. In contrast to mPEG-OH, PEG-PBLA6 exhibited binding to anti-PEG IgG (methoxy). It should be noted that mPEG-Alexa647 (25 nM) exhibited an R_h of 3.7 nm at 4 °C (see Figure S9B), and the R_h of PEG-PBLA6-Alexa647 (2.7 nm) was smaller than that of mPEG-Alexa647. This size difference indicates that PEG-PBLA6, from a structural perspective, is slightly bent and, from a locational perspective, is in the binding site (i.e., the paratope) of anti-PEG IgG (methoxy). Therefore, we concluded that the PBLA6 is directly involved in the low-affinity interaction between the mPEG and anti-PEG IgG (methoxy) (Figure 6D).

This tentative conclusion is supported by previous studies on PEG−Fab cocrystals: researchers have confirmed that van der Waals contacts between aromatic side chains and H-bonding on ethylene glycol enable PEG to bind to Fabs.[33,34] In these studies, cocrystals exhibited evidence that aromatic-ring residues were near PEG chains. In addition to the specific but relatively weak interaction between PEG (for example, less than 12000 MW) and anti-PEG Abs (K_D = 10^{-3} to 10^{-4} M), the hydrophobic interaction between non-PEG hydrophobic moieties of PEG-conjugates and the hydrophobic amino acids of anti-PEG Abs plays the crucial role of stabilizing the binding process (K_D = 10^{-7} to 10^{-9} M). Consequently, PEG-PBLA6 exhibits a high binding affinity to anti-PEG Abs, resulting in a situation where PEG-PBLA6 exhibits immunogenicity. This process, which also occurs for main-chain-specific anti-PEG Abs, explains why we observed that affinity for PEG-PBLA6 was higher in anti-PEG IgM than in anti-PEG IgG. Although affinity for PEG was higher in anti-PEG IgG than in anti-PEG IgM, anti-PEG IgM proved to be better than anti-PEG IgG at compensating for the specific but weak interactions between PEG and anti-PEG Abs by using the non-PEG moiety effectively for the binding.

As we noted above, we have created a term for antigens' transition from a haptenic state to an immunogenic state: "antigenicity extension." We believe that there has been a missing link between haptens and immunogenic hapten−protein conjugates. In general, haptens bind to—but do not elicit—specific antibodies. However, researchers have considered the possibility that hapten−protein conjugates elicit specific antibodies owing to two factors: (1) the cross-linking of B-cell receptors and (2) the stimulation of such immune cells as antigen-presenting cells, macrophages, and helper T cells. Furthermore, researchers have highly relied on cocrystals of hapten-specific antibodies to understand antigen−antibody interactions in many studies, whereas haptens exhibit non-immunogenic characteristics. Despite the importance of different characteristics of haptens and hapten−protein conjugates, the role of the protein part of hapten−protein conjugates on their binding to hapten-specific antibodies has not been sufficiently elucidated, rather excluded because of nonspecificity to the specific antibodies.

In the present study, in contrast, we have demonstrated that a soluble form of PEG-PBLA6 elicited an anti-PEG IgM response to mice and that the soluble form of PEG-PBLA6 was small enough to interact with single B-cell receptors. Although we confirmed that PEG-PBLA6 exhibited a PBLA-embedded folded unimer form possessing small R_h values in a very diluted condition at 37 °C, we should consider the possibility of exposure of non-PEG moieties (hydrophobic PBLA moieties in the current study) to PEG-specific B-cell receptors in vivo. More specifically, the exposure of non-PEG moieties directly involves interactions of PEG with PEG-specific B-cell receptors, and this exposure, in turn, greatly contributes to the stable binding of PEG-PBLA6 to PEG-specific B-cell receptors. This process awakens anti-PEG Ab responses without B-cell receptor cross-linking to mice. We propose that antigenicity extension in the B-cell receptors is essential to awakening B cells to an important function: the function of eliciting a PEG-specific antibody response to PEG-conjugates, regardless of whether antigens are T-cell-dependent or T-cell-independent. Because of the antigenicity extension, hapten−protein conjugates can stably bind to hapten-specific B-cell receptors and can thus promote B-cell stimulation, which in turn promotes antibody elicitation. Namely, once lipids, proteins, and polymers have been conjugated to a PEG, antigenicity extension more or less occurs. Then, PEG-conjugates become new antigens and, thus, bind—with greater efficiency and greater affinity—to PEG-specific receptors. Protein or lipid conjugation to PEGs determines the T-cell-dependent or T-cell-independent responses of PEGs to B cells.

This outcome quite possibly occurs for all of the PEG-materials. For example, researchers found anti-PEG Abs in mice that had been treated with cosmetics containing both polyoxyethylene and polyoxypropylene dimethyl ether or both PEG-11-methyl-ether-dimethicone (PEG-conjugated poly-(dimethyl silane)) and PEG-40-hydrogenated-castor oil.[47]

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We think this was due to the antigen extension of those PEG-analogues to the PEG-specific B-cell receptors. The concept of antigenicity extension also explains why ELISAs easily detected the stable binding of anti-PEG Abs to immobilized PEG but rarely detected the stable binding of PEG to immobilized-anti-PEG Abs. In indirect ELISA, the stable binding of anti-PEG Abs is thought due to effects that the BSA surfaces of PEG's immobilized plates had on anti-PEG Abs' bindings.[22,23] Although, in the present study, we are proposing the concept of antigenicity extension specifically for antigen−antibody interactions, we have strong and reasonable suspicion that our novel concept applies equally well to other molecular-recognition phenomena, such as ligand−receptor interactions.

If one antibody is specific to a hapten but the antibody does not have high affinity for hapten−protein conjugates, then antigenicity extension is unlikely, and the antibody will exhibit little neutralizing effect. Thus, for example, we observed that the inhibition behaviors exhibited by PEG-P(Asp) toward anti-PEG IgM were similar to the corresponding inhibition behaviors exhibited by mPEG-OH (see Figure S13). This similarity indicates the hydrophilic P(Asp) moiety did not significantly bolster the binding affinity of PEGs for anti-PEG IgM. Therefore, we found that a hydrophilic polymer-inserted PEG-conjugates (PEG-hydrophilic polymer-hydrophobic polymer conjugates and PEG-hydrophilic polymer−protein conjugates) significantly suppress anti-PEG Abs' bindings.[22,48] Both specific interactions and antigenicity extension are keys to the effective regulation of antibody elicitation. Because when the various chemical structures conjugating to a hapten induce the antigenicity extension of the hapten, a variety of binding circumstances can arise, and thus, a variety of cell responses are at least a possibility.

There are two reasons for our discovery of the antigenicity extension. First, and importantly, to evaluate antigenicity and immunogenicity, we knew that we could vary the terminal chemical structures and molecular weights of PEG while keeping the specificity unchanged. As compelling as this reason is, the second reason is even more compelling: PEG, being a highly water-soluble macromolecule, can dissolve not only proteins but also lipids and hydrophobic polymers in an aqueous solution. Because highly hydrophobic lipids and hydrophobic polymers possess high-association forces in an aqueous solution, haptens, which are low-molecular-weight compounds, conjugating lipids, or hydrophobic polymers become insoluble. Therefore, neither hapten−lipid conjugates nor hapten−hydrophobic polymer conjugates are possible subjects of investigation. In addition, because a hapten conjugate with a low-molecular-weight compound exhibits water solubility, the hapten conjugate, insofar as it possesses a low association force, exhibits low affinity and low immunogenicity. In order for haptens to be immunogens, haptens need to conjugate with high-molecular-weight carrier proteins; however, roles of the protein in hapten-protein conjugates-specific antibodies are difficult to evaluate. However, because PEG can conjugate with highly hydrophobic lipids and hydrophobic polymers, we proved non-PEG moieties directly interact with anti-PEG Abs in the current study.

CONCLUSION

In summary, we have presented a novel concept, antigenicity extension, which is key for molecular recognition. This concept refers to a matter of great importance: the direct involvement of nonantigen moiety caused by the antigen−antibody-specific interaction plays a crucial role for the stable bindings of antigen−antibody-specific interactions that, to a significant extent, help govern immunogenicity. We propose that protein conjugation to haptens enables haptens to benefit from antigenicity extension. We are very excited to share with both the research community and the medical community the concept of antigenicity extension, as we strongly believe that it will shed considerable light on the critical mechanisms underlying the development of therapeutic antibodies.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsbiomedchemau.4c00042.

Block copolymers' characteristics information and ^{1}H NMR spectrum of PEG-PBLA6; additional information on indirect and sandwich ELISA, association and dissociation behaviors of mPEG-OH to anti-PEG Abs by BLI, time-dependent conformational change of mPEG-OH, and in vivo immunogenicity study (PDF)

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Author Contributions

K.S. performed most of the experimental work and data analysis. M.Y. conceived the study's conceptual goals and supervised our pursuit theme. K.S. and M.Y. participated in the writing and revision of the original manuscript. K.S. acquired funding for the study. All authors participated in the revision of the manuscript and approved the final version. CRediT: **Kouichi Shiraishi** conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, writing - original draft.

Notes

The authors declare no competing financial interest.

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