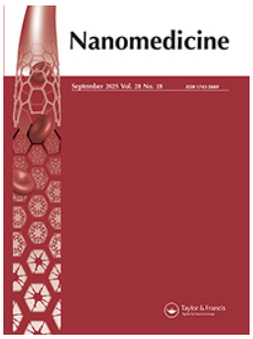

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# Considering the immunogenicity of PEG: strategies for overcoming issues with PEGylated nanomedicines

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PERSPECTIVE



# Considering the immunogenicity of PEG: strategies for overcoming issues with PEGylated nanomedicines

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**ABSTRACT**

In the field of nanomedicine, there is considerable familiarity with both the various applications of poly (ethylene glycol) (PEG) and the related topic of anti-PEG antibodies (anti-PEG Abs). The worldwide spread of mRNA-LNP vaccines has focused much attention on the sometimes problematic relationship between immune responses and the various possible pharmacological uses of PEG. In this paper, which is a perspective review, I summarize the properties of PEG, the properties of anti-PEG Abs, and the various methods for evaluating the relationship between these two factors. I then offer suggestions for addressing the adverse effects that anti-PEG Abs have on the medicinal power of PEGylated nanomedicines. Ultimately, by exploring important developments in the above areas, this review offers an organized, synthesized presentation of information that should prove useful for the development of nanomedicines.

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Poly(ethylene glycol) (PEG); immunogenicity; antigenicity; anti-PEG antibodies; PEG conjugates; antigen–antibody interactions

## 1. Background

In the field of nanomedicine, the development of biopharmaceuticals based on proteins, antibodies, and drug carriers has attracted considerable attention. A particularly recent development centers on lipid nanoparticles containing mRNA (mRNA-LNPs). In fact, lipid nanoparticles are a leading carrier technology consisting of four notable lipids 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, poly(ethylene glycol)-lipid conjugates (PEG lipids), and cationic lipids: the first three have an LNP-stabilizing function while the fourth has an mRNA-stabilizing function [1–3]. The PEG lipids in LNPs have a PEG molecular weight (MW) of 2,000 and are formed from the C14 myristoyl group, whose acyl chains are shorter than those associated with PEG-modified 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) (C18). The use of the short-acyl group has advantages and disadvantages: one major advantage is that, in the short-acyl group, the release of PEG lipids from LNPs induces the efficient uptake of LNPs by immune cells; one major disadvantage, however, is that this release also induces a notable degree of instability in LNPs.

A central concern surrounding the use of mRNA-LNPs in the nanomedical field centers on the immunogenicity of LNPs, especially with respect to PEG. We know that anti-PEG antibodies (anti-PEG Abs) have been found in blood from healthy donors who have never received PEGylated therapeutics [4–8] and in blood from individuals who have received mRNA-LNP vaccinations [9–11]. Growing concerns about the adverse effects of PEGylated therapeutics and the possible role of anti-PEG Abs [12–19] therein underline the need for the nanomedical community to investigate and clarify the immunogenicity issues arising from PEGylated nanomedicines.

## 2. General characteristics of PEG

PEG is a synthetic, linear, nonionic polymer composed of a repeating unit of ethylene glycol (-$CH_2CH_2O$-) with hydroxyl groups at both termini. These structural characteristics are well-known. The hydration of the repeating ethylene glycol units with a minimum of two to three water molecules makes PEG water-soluble [20–22]. Despite its status as a nonionic and nonpolar polymer, PEG is water-soluble and is thus frequently referred to as a hydrophilic polymer. Such a terminology is not entirely accurate: in reality, PEG is a nonpolar polymer that "exhibits" hydrophilicity. The nonpolar characteristic of PEG influences the polymer's interactions with proteins. For example, when hydrated PEG is inserted into the surface of liposome membranes, the PEG inhibits the nonspecific adsorption of serum proteins onto the liposome membranes [23,24]. As we know, this advantage of PEG has generated considerable excitement surrounding PEG conjugation methods (PEGylation) [25–27]. Methoxy-terminus PEG, referred to simply as mPEG, is often used for PEGylation in the nanomedical field. PEG has also served as a substance in PEG-mediated cell fusion [28–30] and as a co-substance in the crystallization of proteins [31–33]. These functions of PEG are possible owing to its "excluded volume" effect, which itself stems from PEG's ability to be in close proximity to proteins without interacting strongly with them. In other words, hydrated PEG can interact with proteins, but only weakly [34–36].

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**Article highlights**

**Differences between PEG and PEG conjugates with respect to anti-PEG Abs**

(1) PEG exhibits antigenicity but no immunogenicity and is therefore thought to be a hapten.
(2) Anti-PEG Abs exhibit specificity for PEG but bind weakly to it.
(3) Many PEG conjugates exhibit immunogenicity and are therefore thought to be immunogens.
(4) Anti-PEG Abs exhibit specificity for PEG conjugates and bind stably to them.

**Why does the immunogenicity of PEG occur?**

(5) Any chemical modification of a single PEG terminal enhances not only the immunogenicity of PEG but also the affinity of anti-PEG Abs for PEG.
(6) Although anti-PEG Abs are nonspecific to protein, lipid, and polymer molecules when they are unattached to PEG, once they are chemically attached to PEG, these molecules – as non-PEG moieties – become notably susceptible to the binding behaviors of anti-PEG Abs.
(7) Results indicate that non-hapten moieties in hapten protein carriers help to bind stably to hapten specific antibodies.

**Strategies to diminish the deleterious effects of anti-PEG Abs on PEGylated therapeutics**

(8) Two strategies (the use of polar group and the use of bulky group at the terminus) may reduce the specificity of anti-PEG Abs for PEG conjugates, and a third strategy (the use of hydrophilic polymer between PEG and non-PEG moiety) may inhibit the stable binding of anti-PEG Abs to PEG conjugates.

## 3. Anti-PEG Abs

In 1977, Abuchowsky et al. were the first to report that, owing to the bio-inertness of PEG, PEG can conjugate with such proteins as bovine serum albumin (BSA) [37]. This technique, which is commonly referred to as PEGylation, today has many uses worldwide. In their pioneering study, the researchers uncovered no evidence pointing to the immunogenicity of PEG or BSA. However, a later study by Richter et al. showed that three types of PEGylated proteins (PEG ovalbumin, PEG bovine superoxide dismutase, and PEG ragweed pollen extract), when administered with Freund's Complete adjuvant, elicited anti-PEG Ab responses in test animals [4]. Since then, we have learnt that anti-PEG Abs can facilitate the rapid clearance of subsequent injected doses of PEGylated therapeutics – a process known as the accelerated blood clearance phenomenon (ABC) [38–43]. However, once again, we should sound a note of caution related to a widespread misunderstanding about PEG and its immunogenicity. Although macrogol, which is simply a generic name for pure PEG, has been used for various clinical purposes, including as a laxative, most uses of PEG harness it not as a biopharmaceutical but as a conjugation material. Therefore, we must consider the difference between the immunogenicity of PEG and the immunogenicity of PEG conjugates, and we should thus use these two terms accordingly and properly [13,14].

In the current paper, I distinguish between two forms of PEG-related immunogenicity: the immunogenicity of PEG itself and the immunogenicity of PEG conjugates. Although PEG is known to be a hapten that does not elicit specific antibodies, it is recognized by specific antibodies. This interesting aspect of PEG explains why it is inaccurate to state that PEG has no immunogenicity. A more accurate assertion would be that PEG's immunogenicity is very low. In contrast to PEG, PEG conjugates – which possess proteins, lipids, or polymers – exhibit more or less immunogenicity. Indeed, this aspect of PEG conjugates has been confirmed in research examining serum anti-PEG Abs. These findings indicate that the immunogenicity of haptenic PEG benefits from conjugation and depends on the nature of its chemically modified materials (proteins, lipids, polymers) [13–16]. For an example, consider poloxamers: these clinically used drug excipients, which are PEG derivatives possessing poly(propylene oxide)s, are known to elicit anti-PEG Abs [44]. Another interesting finding is that these elicited antibodies exhibit cross-reactivity for PEG. In short, the immunogenicity of PEG that I have been discussing above is that of PEGylated materials, not that of PEG itself. This pattern of immunogenicity is, in principle, a mirror image of what happens when hapten – protein conjugates elicit "hapten-derived" antibodies. The difference between the two categories of immunogenicity, though sometimes overlooked, is a critical one.

Another interesting aspect of immunogenicity concerns terminology and definitions: the term 'immunogenicity' has several meanings, or referents. For example, the term can refer to the elicitation of specific antibody responses, which we call humoral immune responses. The term can also refer to cell-mediated immunity. In the current study, my focus is more on humoral immune responses, but even with this narrowed area of inquiry, we should keep in mind that immunogenicity in humoral immune responses has many implications. Thus, it is necessary to clarify whether such immunogenicity translates into (1) an ability to elicit immunoglobulin M (IgM) responses, (2) an ability to perform class-switch recombination, or immunoglobulin-isotype switching, leading to the production of immunoglobulin G (IgG), and (3) an ability to elicit a T-cell response. Although the three phenomena are interconnected, rigorous research on these immune responses must clarify which of the three is the focus. My focus in the present study is on immunogenicity in which PEG conjugates elicit IgM responses to B cells in the early phase of humoral immune responses [45–47].

Humoral immune responses, because they are triggered by the signaling pathways that B-cell receptors (BCRs) create upon encountering an antigen, are fundamentally dependent on the binding affinity of an antigen-specific BCR for a given antigen – and, from the reverse perspective, on the binding affinity of the antigen for the BCR [48]. In the present study, my focus is not on the relationship between antigens and BCRs, the latter of which belong to an immunoglobulin category known as antigen-specific membrane-bound immunoglobulin (mIg). Rather, in the context of PEG, I examine the extent to which the relationship between antigens and antigen-specific antibodies induces an IgM response triggered by B cells. By considering this relationship and its effects, I seek to clarify the differences between haptens and immunogens. I quite deliberately omit from my analysis any T cell-dependent responses because they occur *after* IgM responses. By understanding how certain factors trigger BCRs, we can better understand the relationship between antibodies and antigens both in general and in relation to PEG.

## 4. Difficulty evaluating antigens and their antibodies

In this section, I describe some of the difficulties that researchers face when attempting to evaluate the relationships between antigens and antibodies. A rigorous assessment of these difficulties can shed light on the characteristics of the aforementioned relationships (Figure 1).

Before delving into this task, we should consider five important points. First, BCRs possessing mIg receptors recognize the epitopes of antigens. Continuous linear epitopes that are recognizable to the antigen-binding sites of antibodies have a range of sizes extending from approximately 4 to 12 amino acids [49–51]. Specific peptide sequences in proteins act as epitopes, and most of the selected peptides act as haptens. Second, it is generally the case that, in order to elicit hapten-specific antibodies capable of recognizing specific haptenic-peptide epitope sequences, these peptides must first have been chemically conjugated to carrier proteins [52]. (In this respect, a notable factor to consider is the important role that adjuvants play in the elicitation of antibodies.) Third, in laboratory experiments using indirect ELISA, elicited specific antibodies by peptide carrier proteins exhibit bindings to peptide carrier proteins which were immobilized onto the plates. Fourth, co-crystals of specific antibody fragments and haptenic peptides take shape under conditions involving high substrate concentrations. This co-crystallization indicates that specific antibodies recognize the given peptides. And fifth, when we analyze the binding not of antibodies for immobilized antigens but the other way around by means of sandwich ELISA, although specific antibodies – some of which are polyclonal and thus structurally variable – can recognize a specific haptenic peptide, the peptide's binding affinity for immobilized peptide-specific antibodies is not high, enabling the peptide to dissociate easily from the antibodies. Importantly, however, peptide carrier proteins exhibit significant bindings for the immobilized peptide-specific antibodies. In short, the binding affinity of haptenic peptides for specific antibodies and for specific mIg receptors is low, not high.

How can we understand the often-times highly complicated relationship between antigens and antibodies? One point of confusion has to do with why haptens are nonimmunogenic, whereas hapten carrier proteins are immunogenic. To explore this topic, we should consider the extent to which hapten carrier proteins can stimulate BCRs. We know that the stimulation of BCRs (i.e., "BCR triggering") can initially elicit peptide-specific IgM antibodies. For example, previous researches have examined T cell-independent antigens possessing many epitopes, and it has been proposed that such T cell-independent antigens are capable of cross-linking certain BCRs. In contrast to T cell-independent antigens, T cell-dependent protein antigens, though they possess one or few epitopes, can elicit IgM responses via BCR triggering. A curious fact, however, is that although several explanatory models have been proposed for BCR triggering, the precise mechanism underlying the phenomenon remains unclear [48].

Regarding PEG-related immunogenicity, the chemical modifications involved in the creation of PEG conjugates result in immunogens [13–16]. Because the structure of PEG is simple, one can easily compare haptenic PEG with highly immunogenic PEG conjugates. In addition, although PEG can be chemically modified with lipids, proteins, and polymers, this characteristic of chemical modification is much rarer in PEG than in other low-MW haptens. This aspect of PEG conjugation

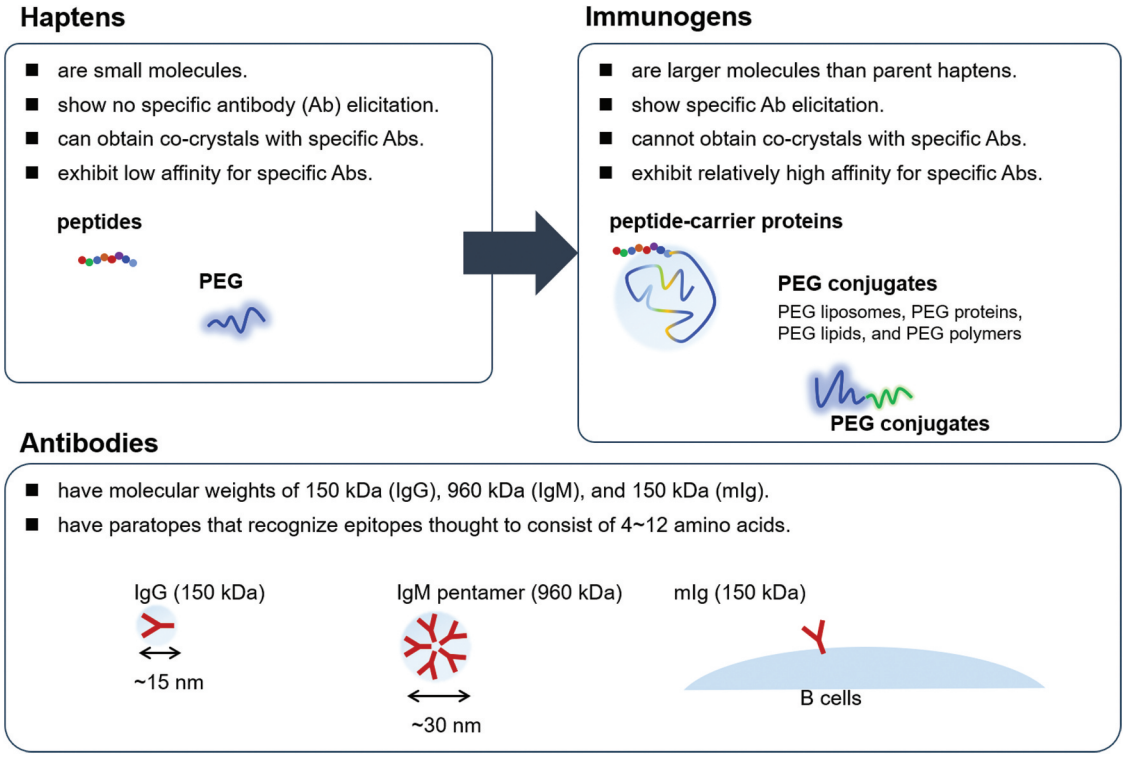


Figure 1. Characteristics of haptens, immunogens, and antibodies.

enables us to examine PEG-related responses with respect to both haptenic PEG and immunogenic PEG conjugates [14]. By clarifying the differences between haptenic PEG and immunogenic PEG conjugates, we can clarify the intrinsic behavior exhibited by immunogens and distinguish this behavior from that of haptens.

## 5. Understanding the relationship between PEG and anti-PEG Abs

PEG lipids are associated with a predominantly IgM response that can be thought of as a T cell-*independent* response [45–47], whereas PEG proteins are associated with an IgM response and an IgG response perhaps best understood to be a T cell-*dependent* response [53–57]. IgM responses are the first antibody responses in both PEG – lipid cases and PEG – protein cases. Although IgM-isotype Abs in both of these cases can switch to either IgG-isotype Abs or other isotype Abs, the IgG response is more efficient in PEG proteins than in PEG lipids. However, it is worth reiterating that the antibodies elicited in each of the two cases are PEG-specific.

PEGylated lipids and PEGylated proteins exhibit specific antibody elicitations at a given dose, while PEG does not exhibit specific antibody elicitations at the same dose. The relationship between PEG and anti-PEG Abs is specific in that anti-PEG Abs are cross-reactive in relation to PEG. However, when PEG is chemically modified, whether through conjugation with lipids, proteins, or polymers, the resulting PEG conjugate exhibits higher immunogenicity than does the parent PEG, and specifically, the binding affinity of anti-PEG Abs is higher for the PEG conjugate than for the parent PEG. As mentioned above, IgM responses are commonly observed phenomena regardless of differences among lipids, proteins, and polymers. Consequently, we should consider why IgM responses can be induced (Figure 2).

When we think of a relationship between specific antibodies and the antigens, we might assume that the antibodies must capture antigens and that the binding is specific and probably sufficiently stable. These assumptions, I suggest, are due in part to previous research findings showing co-crystals (e.g., PEG co-crystals) of antigens and the specific antibody fragments in solution [58–60]. However, certain conditions are necessary for the creation of these co-crystals, and it is difficult to imagine that these conditions can be present in solution right after the mixing of antigens with specific antibodies. Furthermore, and again as mentioned above, highly immunogenic PEG conjugates exhibit immunogenicity, in the form namely of antibody responses, at very low doses, whereas PEG exhibits no antibody response at these low doses or, for that matter, even at high doses. In other words, the ability of antibodies to recognize PEG is a critical, but certainly not the only, factor in immunogenicity.

Regarding the relationship between anti-PEG Abs and PEG, I would like to offer three tentative proposals. I propose, first of all, that the issue of whether or not anti-PEG Abs bind stably to PEG is not equivalent to the issue of whether or not anti-PEG Abs "recognize" PEG as PEG [61–63]. The specificity of antibodies to PEG refers to the selectivity of both anti-PEG Abs and PEG-specific BCRs for PEG epitopes. Second, I propose that, regarding the immunogenicity of PEG conjugates, the

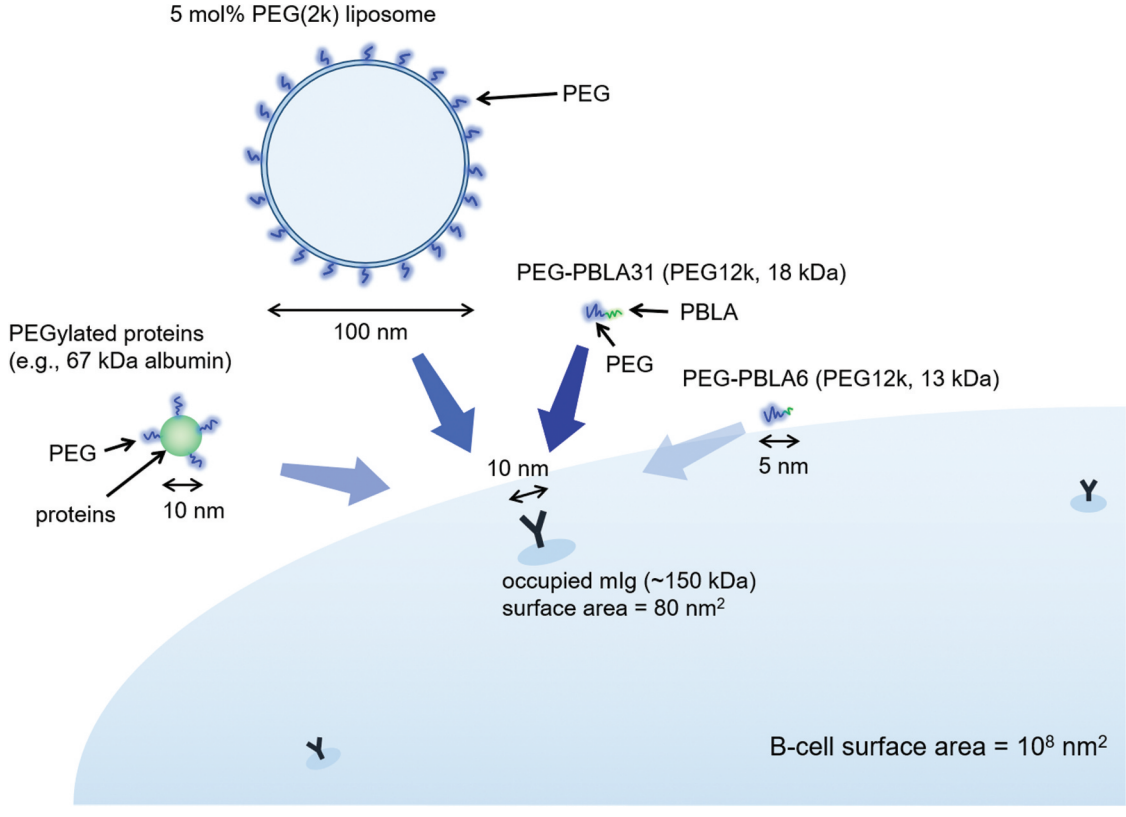


Figure 2. What induces B-cell triggering when PEG-conjugate size varies significantly?

stable binding of PEG-specific BCRs to PEG conjugates (i.e., the slow dissociation of PEG conjugates from PEG-specific BCRs) is highly dependent on two critical factors: (1) the specific interactions between PEG itself and PEG-specific BCRs, and (2) the presence and the types of lipids, proteins, or polymers in the PEG conjugates. In other words, although the anti-PEG Abs exhibit specificity for both PEG and PEG conjugates, the anti-PEG Abs bind more stably to PEG conjugates than to PEG. In general, the essential meanings of the stable binding can be found in two factors: (1) the relatively slow dissociation of antigens from specific BCRs and, as a result, (2) the ability of antigens to induce conformational changes in the BCRs. Therefore, I propose, third, that we should train our attention on the differences between the binding behaviors of anti-PEG Abs in the presence of PEG itself and the binding behaviors of anti-PEG Abs in the presence of PEG conjugates. Once we better understand these differences, we can better understand – and better harness – anti-PEG IgM responses both to PEG *in vivo* and to PEG conjugates *in vivo*.

## 6. Additional factors contributing to the stable binding of anti-PEG abs to PEG

Research has shown that the immunogenicity of PEG conjugates is greater, to varying degrees, than that of the parent PEG. This finding suggests that we should consider how anti-PEG Abs interact with lipids, proteins, and polymers, all of which are molecular non-PEG moieties and none of which induce specificity in the anti-PEG Abs. However, can we reasonably argue that, because anti-PEG Abs exhibit no specificity for these lipids, proteins, and polymers, no interaction occurs between the anti-PEG Abs and the moieties? To answer this question, we must consider the binding affinity of anti-PEG Abs not for PEG itself but for PEG conjugated possessing the aforementioned moieties.

If a binding assay (e.g., ELISA, SPR) demonstrates that there is no specificity between the aforementioned non-PEG moieties (i.e., proteins, lipids, polymers) and anti-PEG Abs, we would probably expect to observe no specific bindings between them. However, binding assays are unable to establish whether or not interactions occur between anti-PEG Abs and the moieties when the latter are part of a PEG conjugate. Specificity indicates that two molecules can interact with each other when they are in close proximity to each other. Thus, if the specificity of anti-PEG Abs for PEG can close the distance between anti-PEG Abs and non-PEG moieties, this new structural arrangement may induce unexpected or atypical intermolecular interactions between the anti-PEG Abs and the moieties. However, the task of proving the existence of these interactions is likely very difficult.

## 7. Stable binding between PEG conjugates and anti-PEG Abs

In general, the conjugation of low-affinity haptens to proteins such as BSA and keyhole limpet hemocyanin (KLH), which is a high molecular weight protein, can elicit specific antibodies. Research on this topic has been conducted in relation to PEG. For example, Li et al. examined the immunogenicity of PEG-carrying protein conjugates and found that PEG-possessing KLH conjugates exhibited the highest immunogenicity [64]. There are several methods for detecting the presence of elicited specific antibodies, and one particularly convenient method is indirect ELISA, as mentioned above. Research harnessing this method has shown that elicited antibodies bind more easily to immobilized haptens than to non-immobilized ones, and, in general, this is thought to be because the immobilization not only decreases the movements of haptens but also accentuates the multivalency of haptens on the immobilized surface. These two reasons, though they certainly pertain to interactions between elicited antibodies and haptens, cannot directly result in stable bindings.

Consider, for example, anti-PEG Ab-immobilized assays: although anti-PEG Abs specifically recognize PEG, stable bindings of PEG to immobilized anti-PEG Abs are difficult to detect [61]. As I discussed above, even when anti-PEG Abs exhibit specificity for PEG, PEG can quickly dissociate from the anti-PEG Abs, thus demonstrating their weak affinity for PEG. Nevertheless, research has shown that PEG possessing chemically modified ends can bind, with varying degrees of affinity, to immobilized anti-PEG Abs. This binding behavior is strikingly present in cases where PEG has been chemically modified with proteins, lipids, or polymers, with the binding behavior being highly dependent on the characteristics of these moieties (e.g., molecular weight, hydrophobicity, and positive charge) [62]. Specifically, the terminal ends of these PEG conjugates can be modified by functional groups whose complexity ranges from the simple (e.g., methoxy and amino groups) to the more complex (e.g., lipids, polymers, and proteins). These terminal ends affect the interaction between anti-PEG Abs and PEG. This indicates, for example, that anti-PEG Abs bind more stably to mPEG-OH than to PEG itself. Regarding PEG lipids and PEG-hydrophobic block copolymers, attention must obviously be paid to their hydrophobicity-related self-assembly behaviors. Bindings of PEG lipids or PEG-hydrophobic block copolymers to immobilized anti-PEG Abs have been observed at concentrations below the critical micelle concentration (CMC) [63]. In addition, PEG proteins, which do not engage in self-assembly, easily bind to immobilized anti-PEG Abs.

I authored a 2025 study examining the binding behaviors of the block copolymer PEG(12k)-*b*-poly(b-benzyl L-aspartate) (PEG-PBLA) relative to anti-PEG Abs [61]. We studied how the chemical features of the antigen influenced the affinity of the Abs for the antigen. To examine a single copolymer chain (i.e., a unimer) of PEG-PBLA, I selected BLA consisting of only a small number of units (an average of six BLA repeating units) (PEG-PBLA6). We found that PEG-PBLA6 exhibited not only temperature-dependent conformational changes but also conformation-dependent binding behaviors to anti-PEG Abs. PEG-PBLA6 had a *unimer micelle* structure ($R_h = 1.1 \pm 0.1$ nm) at 37°C. By contrast, at 4°C, PEG-PBLA6—because it was hydrated – had an *extended bent* structure ($R_h = 2.7 \pm 0.0$ nm) (Figure 3). The results we obtained were expected. While the unimer micelle structure, whose PEG-chain movements are thought to be slow, exhibited no formation of bound complexes vis-à-vis anti-PEG Abs, the extended bent structure did exhibit such formations, an outcome suggesting not only that

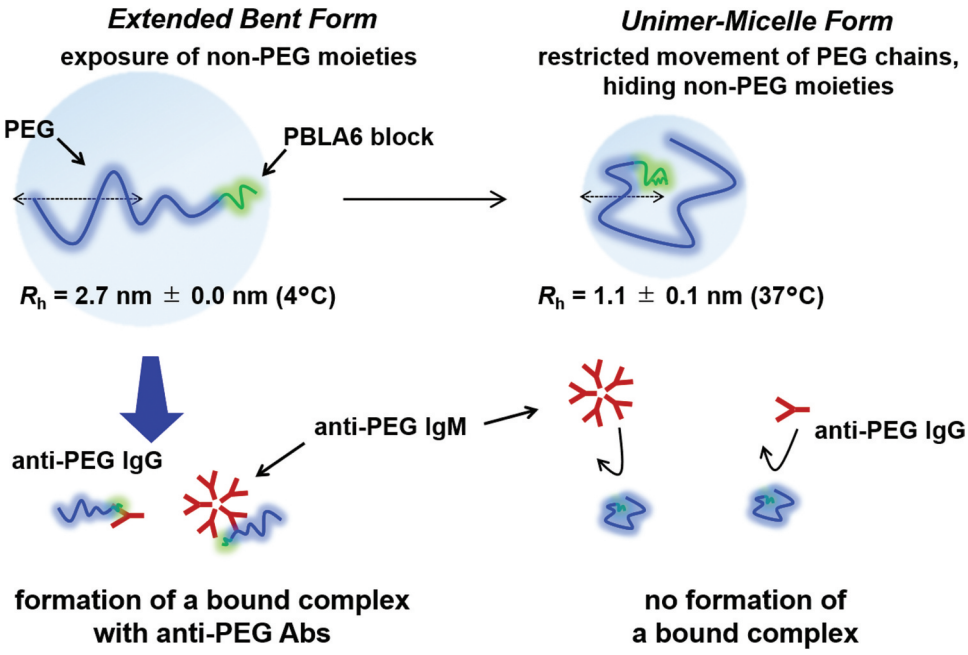


Figure 3. Changes in the conformation of single PEG-PBLA6 unimers are temperature-dependent, and anti-PEG abs preferentially bind to the extended bent form of PEG-PBLA6 whereas no bindings are observed for the unimer-micelle form of PEG-PBLA6.

anti-PEG Abs (IgM and IgG) were stably binding to PEG-PBLA6 but also that the exposed PBLA6 blocks were directly involved in the binding process. Further, we confirmed that non-aggregated PEG-PBLA6 unimers elicited anti-PEG IgM antibodies, whereas mPEG-OH did not. In other words, the immunogenicity of PEG conjugates stems from the ability of PEG conjugates to bind stably to PEG-specific BCRs. And the stability of this binding was attributable mainly to the non-PEG moieties in the PEG-PBLA6. Our observation of haptenic PEG becoming an immunogenic PEG conjugate is a phenomenon we have termed 'antigenicity extension' [61].

The existence of antigenicity extension has important implications for the research field addressing the immunogenicity of hapten – protein conjugates. One important implication is that the field should focus not only on haptens themselves but more broadly on the roles that non-hapten moieties play in the interactions between antigens and antibodies. My own view is that non-hapten moieties are most likely to have direct involvement to these interactions. Two main findings uncovered from our previous research support this assertion: first, haptenic PEG possessing highly hydrophobic PBLA blocks became a highly immunogenic antigen, and second, the immunogenicity of PEG-PBLA was dependent on the chain length of the PBLA [61–63]. More specifically, these studies found that, when hydrophobic PBLA blocks interacted with each other, the cohesive force arising from the blocks helped to form stable self-assembling structures (i.e., polymeric micelles), and when the cohesive force of the blocks was directed to anti-PEG Abs, they would stably bind to the PEG-PBLA6.

A critical factor in the binding of antibodies to antigens is the avidity effect; that is, the total strength with which specific antibodies bind directly to chemically modified epitopes consisting of proteins, lipids, or polymers. In a previous research [65], I and my colleagues observed that mPEG hydrophilic block copolymer micelles, when formed from electrostatic interactions between carboxylic acid and tertiary amines, exhibited no immunogenicity and were associated with very low antibody – antigen binding affinity; by contrast, mPEG hydrophobic block copolymer micelles (PEG-PBLA31 micelles) exhibited substantial immunogenicity and were associated with very high antibody – antigen binding affinity. The two aforementioned polymers have been synthesized using the same mPEG-$NH_2$, and the two aforementioned micelles exhibited nearly identical blood half-lives in mice [66]. Although researchers currently regard the avidity effect as relatively comparable to multivalent effects [67], the drastic contrast between the two types of micelles regarding binding affinity and immunogenicity indicate that the functions of multivalent effects differ, to a notable degree, from the main functions of the avidity effect.

In an ELISA involving PEG-immobilized plates, researchers used BSA to block the binding of non-PEG specific substrates to PEG-immobilized plates and found that the blocking process suppressed the nonspecific adsorption of other substrates on the plates. However, when, in the ELISA, the specific interactions between PEG and anti-PEG Abs enabled the anti-PEG Abs to get sufficiently close to the PEG, the anti-PEG Abs were able to interact directly to the various molecules (e.g., BSA molecules, lipid molecules) around the immobilized PEG. Furthermore, this indicates that the non-paratope region of the anti-PEG Abs is also directly involved in the specific interactions between PEG and anti-PEG Ab. This sequence of events indicates that, in contrast to common perception, non-epitope moieties play important roles in the above relationships between anti-PEG Abs and PEG conjugates.

Why do chemical modifications of a hapten upon its binding to a protein enable a specific antibody or a specific receptor on an antibody to bind to the hapten – protein conjugate? And why does the immobilization of a hapten on plates facilitate the ability of specific antibodies to bind to the hapten? My suspicion, based on the available evidence, is that these binding behaviors are most likely due to the avidity effect, which itself is a function of antigenicity extension. This knowledge should prove highly useful in efforts to design biopharmaceutics for applications in the field of nanomedicine.

## 8. Concerns about existing anti-PEG Abs

Because most of us acquired anti-PEG Abs after receiving an mRNA-LNP vaccine, researchers in the field of nanomedicine should identify and study the possible undesirable consequences stemming from anti-PEG Abs. These consequences fall into two major categories: injection-related adverse effects and therapeutic inefficacy [68–76].

Hypersensitivity reactions (HSRs), also referred to as infusion-related reactions, are an important type of injection-related adverse effect. They have previously been linked to IgE and non-IgE reactions and can lead to a number of symptoms, including shortness of breath, fever, excessive sweating, general aches and pains, and, interestingly, a sense of panic immediately upon drug administration. Many of these symptoms can be managed, but some may lead to severe pseudo-anaphylaxis. It should be noted that some pseudo-anaphylaxis responses have been associated with complement activation in people whose bodies had already developed anti-PEG Abs. However, such responses have been traced not only to PEGylated biopharmaceuticals but indeed to many other drugs, such as therapeutic antibodies and diagnostic agents [77–79]. Furthermore (and quite obviously), not all instances of pseudo-anaphylaxis stem from the mere existence of anti-PEG Abs: elicitation of specific antibodies is a very common phenomenon produced by a wide array of therapeutic molecules (proteins, antibodies, and others), not just PEG conjugates.

The second type of adverse effect that I mentioned above in relation to anti-PEG Abs is therapeutic inefficacy. The presence of specific biopharmaceutical-elicited antibodies in the human body can reduce the intended benefits of biopharmaceuticals [13,80]. Again, and as is widely known, this issue pertains to a vast array of biopharmaceuticals, not just to PEGylated ones. Given that the elicitation of antibodies specific to a foreign antigen is a common and appropriate immune response to the presence of the antigen in the human body, we should strive to maximize our understanding of all the possible situations in which this elicitation adversely affects treatments. In the following section, I discuss this topic in greater detail.

## 9. The importance of considering the number of anti-PEG Abs

The therapeutic inefficacy of PEGylated biopharmaceuticals stemming from the presence of anti-PEG Abs is a matter of considerable concern in various clinical and extra-clinical settings. We know that treatments involving PEGylated biopharmaceuticals can repeatedly elicit both anti-PEG Ab responses and the formation of immune complexes that lead to an increased risk of HSRs [70]. The binding of anti-PEG Abs to PEGylated biopharmaceutics can compromise the long blood circulation of the drug that is critical for therapeutic efficacy. In essence, the drug becomes useless or, at best, limited in its usefulness. Regarding nanomedicines, many have larger molecular weights and longer blood-circulation traits than conventional drugs. The latter trait is particularly important, as the therapeutic efficacy of nanomedicines is highly dependent on their blood-circulation characteristics. Therefore, ABC (insofar as it is an antibody-dependent loss of PEGylated nanomedicines from the circulatory system) is a particularly concerning threat to the therapeutic efficacy of these nanomedicines.

The relationship between anti-PEG Abs and ABC is nothing more than the stoichiometric ratio of an anti-PEG Ab to a PEGylated nanomedicine [63]; that is to say, the number of anti-PEG Abs that is required to alter the pharmacokinetic properties of the aforementioned nanomedicine. For example, if one IgG antibody having an MW of 150 kDa has a high affinity for – and is bound to-one molecule of a therapeutic protein having an MW of 50 kDa, the pharmacokinetic properties of the protein will decline significantly (Figure 4(A)). In such a situation, therapeutic efficacy is possible only when an excess amount of the therapeutic proteins is administered in relation to the given amount of IgG in the blood.

As we know, a major category of nanomedicines is PEG liposomes [81–83], which noticeably exhibit ABC in the presence of anti-PEG IgM. This trait is due chiefly to the ratio of elicited anti-PEG IgM molecules to injected PEG-liposome particles [63,81,84]. To better understand this ratio, consider a comparison between PEG liposomes and PEG-block copolymers, which are another major category of nanomedicine. In theory, the number of PEG lipids (in, for example, PEG-DSPE) necessary for a PEG liposome with a diameter measuring 100 nm would be 10 to 100 times greater than for the number of PEG-block copolymers necessary for a PEG-block copolymer micelle with a diameter measuring 100 nm. This fact indicates that the number of PEG-block copolymer micelle particles is 10 to 100 times higher than the number of PEG-liposome particles at the same PEG dose [63]. Therefore, in the presence of identical numbers of elicited anti-PEG IgM, PEG-block copolymer micelles exhibit less ABC, if any, than PEG liposomes exhibit. In short, regarding the number of nanomedicines' molecules to be injected (i.e., administrations), PEG liposomes are not advantageous.

An issue just as important as the injected-carrier number is the molecular weight and shape of injected carriers. IgG and IgM differ from each other regarding the number of antibodies that are required for altering the pharmacokinetic properties of nanomedicines, and this difference depends entirely on what types of nanomedicines are being administered. PEG liposomes consist of a short mPEG chain (MW = 2k) and lipid membranes. This shape greatly helps anti-PEG Abs capture PEG liposomes. The shortness of the mPEG chains (3–4 nm) on liposomes can help close the physical distance between the PEG liposomes' lipid membranes [85,86] and anti-PEG Abs (the

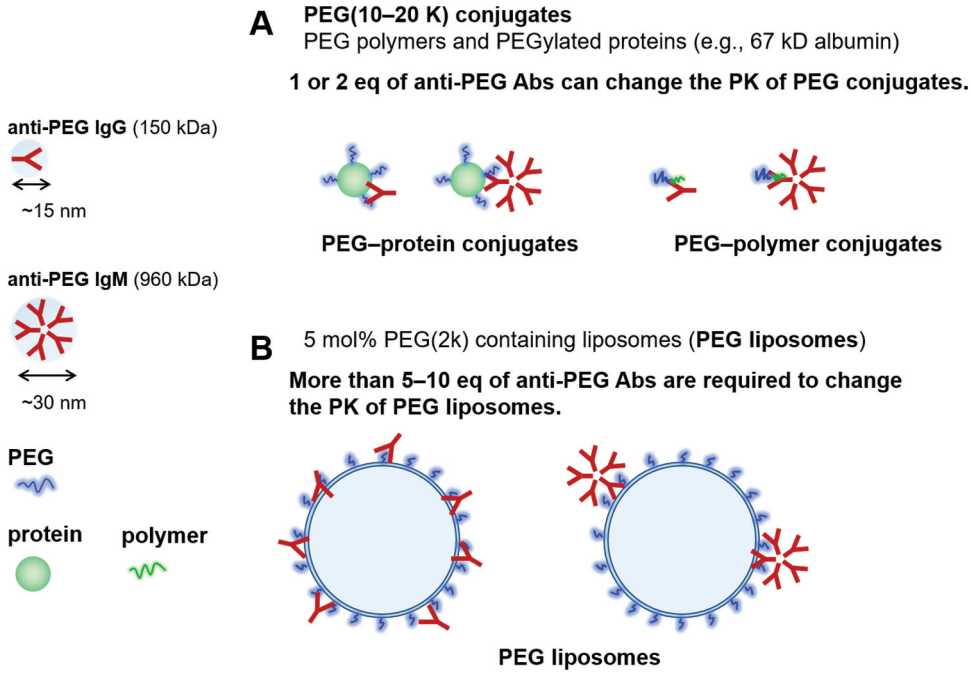


Figure 4. Equivalent ratios of anti-PEG abs to PEG conjugates determine the fate of PEG conjugates *in vivo*.

diameter of the latter being around 15–30 nm). For the above reasons, I strongly suspect that anti-PEG Abs can easily and stably bind to mPEG(2k) that has been inserted into liposome membranes.

If we compare PEG liposomes with PEG-block copolymer micelles, we can better understand the two aforementioned characteristics of the liposomes: (1) the number of PEG-lipids that are required for each PEG liposome particle and (2) the ability of PEG liposomes to facilitate the binding efficacy of anti-PEG Abs. In a previous study that I conducted with my colleagues, PEG-liposome injections and PEG-PBLA31 micelle injections in mice elicited anti-PEG IgM, with the elicitation confirmed by ELISA 1-week post-injection [63]. Once the anti-PEG IgM had been elicited, we separated the mice into two groups, one group receiving injected PEG liposomes and the other group receiving nearly equal PEG moles of injected PEG-PBLA31 micelles. Only the PEG liposomes – not the PEG-PBLA31 micelles – exhibited ABC. However, in each of the two groups, the anti-PEG IgM was able to bind to the injected substances (i.e., the PEG liposomes and the PEG-PBLA31 micelles) and, at 6 h post-injection, was completely used up. Interestingly, although the PEG-PBLA31 micelles consumed all the anti-PEG IgM, the pharmacokinetic behavior of the micelles exhibited no change during the 6 h period. This finding indicates that the number of PEG-PBLA31 micelles was sufficiently higher than the number of elicited anti-PEG IgM antibodies. Furthermore, we found that anti-PEG Abs were able to bind more stably to PEG-PBLA31 polymers that were dissociated from PEG-PBLA31 micelles than to PEG-PBLA31 micelles. The favorable binding behavior of anti-PEG IgM relative to the dissociated PEG-PBLA31 polymers indicates that they are much more immunogenic than is PEG-DSPE. In fact, in our study, even very low doses of PEG-PBLA31 polymers elicited a significant anti-PEG IgM response [63].

The ratio of anti-PEG Abs to the administered units of a given nanomedicine is important for obvious reasons, and for equally obvious reasons, the effective ratio varies greatly depending on the class of antibodies present and the type of nanomedicine being administered. This variance, in particular, is observable in large-particulate nanomedicines (e.g., PEG liposomes, PEGylated micelles, PEGylated metal nanoparticles) and in protein-based nanomedicines, all of which are highly half-life dependent and all of which are susceptible to reduced therapeutic effects when the number of anti-PEG Abs is relatively high.

For example, it is reasonable to assume that anti-PEG IgM (MW = 960 kDa) is better than anti-PEG IgG (MW = 150 kDa) at decreasing the blood half-life of PEG liposomes having a diameter of 100 nm (Figure 4(B)). This difference indicates that ABC, in order for it to occur, requires more anti-PEG IgG than anti-PEG IgM. However, if both anti-PEG IgM and anti-PEG IgG are equally capable of binding to PEGylated proteins having diameters of 5 to 10 nm, one anti-PEG IgM molecule and one anti-PEG IgG molecule should be equally capable of decreasing the blood half-life of the PEGylated proteins. The assertion that anti-PEG IgG is less capable than anti-PEG IgM at promoting ABC rests on two factors: anti-PEG IgM has more binding sites than anti-PEG IgG; and the molecular weight of anti-PEG IgM is greater than the molecular weight of anti-PEG IgG. All things equal, the more binding sites an antibody has, and the bigger the antibody is, the more likely the antibody will change the pharmacokinetics of nanomedicines. Two types of PEG conjugates are important in the field of nanomedicine: PEGylated lipids (i.e., PEG-DSPE and PEGylated polymers) and PEGylated proteins. Because various PEG liposomes and PEGylated micelles act as T cell-independent (TI) antigens, an IgM response to these antigens should be substantial insofar as the response requires no assistance from T cells [40,45,46,61–63]. By contrast, PEGylated proteins, acting as T cell-dependent (TD) antigens [53–56], can induce IgM.

However, TD antigens are more effective than TI antigens at promoting isotype-class switching (i.e., a B cell's ability to shift production from IgM class of antibody to another). With respect to PEGylated proteins, their TD status induces (1) the clonal expansion of PEG-related B cells and (2) affinity maturation by means of somatic hypermutation. Understandably, therefore, concern arises regarding the possibility that PEGylated proteins, because of their TD status, will elicit high amounts of high-affinity anti-PEG IgG, thus threatening the viability of nanomedicines that are dependent on PEGylation. In general, the effect of TI antigens on B cells is often considered a transient one, whereas the effect of TD antigens on B cells is considered a long-term or even permanent one; thus, regarding nanomedicines, especially those reliant on PEGylated proteins, the latter effect is a more serious issue than the former effect to result in desirable therapeutic outcomes.

## 10. Various perspectives on issues related to anti-PEG Abs

In the section above, I discussed two major issues related to the presence of anti-PEG Abs in the human body: injection-related adverse effects and therapeutic inefficacy. In this section, I discuss various perspectives from the literature regarding how we might avoid or at least diminish the adverse effects stemming from these issues.

### *10.1. Methods for inducing immune responses to PEG-related anergy or tolerance*

The literature has suggested several methods for reducing antibody elicitation. One such method, tested on mice, involves decorating Siglec (sialic acid-binding Ig-like lectin) ligands to inhibit the propagation of immune-activating signals (i.e., to promote the propagation of immune-suppressive signals) [87–89]. Two major B cell inhibitory co-receptors belonging to the Siglec family are CD22 (i.e., Siglec-2) and Siglec-G, both of which recognize sialic acid-terminus glycans. Both CD22 and Siglec-G are expressed on B cells and contribute to tolerance induction. One way of inducing tolerance is to induce non-PEG responsive states, such as an anergic state specific to PEG; and another way is to induce a deletion state specific to PEG. In these ways, it is thought that the adverse effects of anti-PEG Abs on therapeutic outcomes can be reduced. Temporal anergic states for PEG may also be able to reduce these adverse effects.

Duong et al. prepared a high-MW poly(acrylamide) (MW = 1,000 k) complexes consisting of about 400 sialylated glycans and nitrophenol (NP) haptens. The researchers examined the binding of various sialylated glycans to B cells and reported that select sialic acids (bNeuGC or NeuGC) suppressed the elicitation of anti-NP Abs [90]. Several factors could be responsible for this suppression because both small molecular NP haptens and large sialylated glycans conjugate to polyacrylamide main chains. One major reason for the aforementioned suppression is the strong inhibitory signal of B cells: as the authors intended, this inhibitory signal suppresses the elicitation of anti-NP Abs. An important point to consider is the possibility that sialylated glycans may hide the process whereby NP haptens are exposed to B cells. However, if sialylated glycans are present on liposome surfaces, the inhibitory effect of these glycans on B cells would be clear, because an array of different ligands can be easily inserted into the outer membranes of liposomes. Macauley et al. prepared PEG liposomes that expressed both antigen ligands and CD22 ligands at the terminus of PEG chains [91]. In the study, the prepared PEG liposomes – which the authors referred to as "tolerogenic liposomes" – induced antigen-specific apoptosis. Ohmae et al. reported that ligands for Siglec-G and CD22 were able to attach to the outer ends of poly(sarcosine)-*block*-poly (L-lactic acid) (PLA) micelles [92]. Then, by suppressing the elicitation of anti-sarcosine Abs, these micelles managed to reduce their exposure to the ABC. Similarly, Mima et al. focused on gangliosides serving as CD22 ligands and reported that liposomes containing both PEG and gangliosides suppressed anti-PEG IgM in a manner that was dependent on the amount of gangliosides inserted into the membrane of the liposomes [93].

All of these very interesting approaches to Siglec-related responses considerably reduce the elicitation of specific antibodies and, thus, reduce the elicitation's various adverse effects, including, most importantly, the adverse effects on therapeutic efficacy [55]. In these approaches, a critical aim is to reduce the absolute amount of the specific antibodies. However, we would be remiss if we neglected the other roles of Siglec ligands. Researchers have prepared synthetic polymers possessing Siglec ligands that bind to Siglec receptors on immune cells. This binding activity has an immunosuppressive effect on the cells [88,94]. Indeed, sialic acids on some human pathogenics are recognized by Siglecs, and therefore, Siglec ligands are important for promoting infection or mediating immune responses [88]. In such scenario, we should remind that risk of not only infection diseases but also autoimmune diseases will be increased.

McSweeney et al. reported that, prior to the administration of PEG liposomes, the administration of pre-treatment high-MW PEG (MW = 40 k) suppressed the elicitation of anti-PEG IgM [95]. This suppression may have been due to the possibility that the presence of low-affinity PEG on PEG-specific BCRs complicates the binding of subsequently administered PEG liposomes to the BCRs. However, PEG-specific BCRs may exhibit low affinity for high-MW PEG. Hence, the suppressive effect of the high-MW PEG on the anti-PEG Abs is likely to be temporary owing to a pair of factors: the amount of time during which the high-MW PEG is on the PEG-specific BCRs and the long half-life of the PEG liposomes. It should be noted that in the aforementioned study, the pre-treatment high-MW PEG only partially suppressed the anti-PEG IgM response.

### *10.2. Pre-treatments for clearance of anti-PEG Abs*

Next, I will discuss possible methods for reducing the adverse effects of existing anti-PEG Abs on therapeutic efficacy. One such method is to induce temporal clearance of anti-PEG Abs from blood by forming anti-PEG Ab immune complexes. The method is highly dependent on the binding affinity of PEG or of PEG conjugates for anti-PEG Abs.

Although PEG conjugates exhibiting a high affinity for anti-PEG Abs might be a useful pre-treatment component in therapies involving PEGylated drug-delivery systems, there are potential drawbacks as well as benefits to such an approach. For example, on the one hand, just a small number of PEG liposomes can capture a large number of anti-PEG Abs, yet, on the other hand, the PEG liposomes can trigger the formation of immune complexes, which in turn might have deleterious complement-related side effects [70,74,75]. Immune complexes of antigen-bound antibodies are well-known to activate the classical complement pathway capable of inducing an inflammatory response to pathogens [52]. Indeed, Chen et al. reported that (1) immune complexes of PEG liposomes with anti-PEG Abs recruited complement factors and that (2) the complement system's terminal components (C5b to C9), when activated, formed a membrane attack complex, which created pores in liposome membranes [74]. In the study, the membrane attack complex triggered the release of a cytotoxic drug – doxorubicin – from the inner cavity of PEG liposomes. This example is just one of the many involving complement-system activation. Compounding this unwanted outcome would be the further possibility that the PEG-liposome pre-treatment would induce the generation of even more anti-PEG Abs. A similar set of problems is likely to arise with high-affinity PEG conjugates such as PEG-block copolymers and PEG proteins.

The above difficulties naturally suggest that low-affinity PEG would be a preferable pre-treatment component. McSweeney et al. investigated this suggestion and reported that in the presence of anti-PEG Abs, the ABC of PEG liposomes diminished in a manner dependent on the MW of pre-treated PEG: reductions were observed when 10 k, 20 k, and 40 k of PEG were administered 30 minutes prior to the administration of PEG liposomes [96]. Similarly, Talkington et al. reported that the ABC of PEG-uricase diminished in the presence of anti-PEG Abs when 40 k of PEG was administered prior to the PEG-uricase administration [97]. Very high doses of PEG are required to fully capture anti-PEG Abs. Talkington et al. used (1) a dose of 2,200 mg/kg for PEG having an MW of 10 k and (2) a dose of 550 mg/kg for PEG having an MW of 40 k. In neither case did the authors observe adverse effects. Even though the capturing effect would be more efficient with high-MW PEG than with the PEG used for current PEGylation, adverse effects would be more likely to occur with this high-MW PEG than with, say, PEG having an MW of 40 k because the higher the MW of PEG is, the more pronounced the hydrophobic and viscous properties of PEG are.

In sum, although PEG conjugates exhibiting high affinity for anti-PEG Abs can rapidly eliminate them from blood, the PEG conjugates can simultaneously induce the generation of these anti-PEG Abs, and the formation of an immune complex that ironically increases the risk of adverse effects. To get around these unwanted outcomes, we could turn to low-affinity PEG, but it would have to be administered in extremely high doses. Hence, advocates of PEG for drug-delivery systems face a dilemma-one that, as of this writing, remains unresolved.

### 10.3. Possibility of PEG conjugates that do not exhibit stable binding to anti-PEG Abs

Next, I consider the issue of whether or not there is a mechanism that prevents anti-PEG Abs from recognizing and binding stably to PEG conjugates. Thus, a PEG conjugate exhibiting rapid dissociation from anti-PEG Abs would be in a good position to avoid interacting with them. In other words, because PEG is haptenic and because the current array of therapeutic PEG conjugates is immunogenic, the research community should strive to design PEG conjugates that are non-immunogenic or haptenic: such conjugates would be in a good position either to avoid interactions with or quickly dissociate from anti-PEG Abs. Therefore, to synthetically reduce the interactions between PEG conjugates and anti-PEG Abs, I propose that researchers find ways both to reduce the specificity of anti-PEG Abs for PEG and to inhibit stable bindings of anti-PEG Abs for PEG conjugates.

#### 10.3.1. A strategy to reduce the specificity of anti-PEG Abs for PEG

There are important similarities between the effort to reduce the specificity with which anti-PEG Abs recognize PEG and the above-mentioned Siglec cases. Antigen – antibody interactions must be considered in terms of the chemical structures not only of antibodies but also of antigens, in this case PEG. Typically, a hydrophobic methoxy group is attached to one PEG terminus. The methoxy group is similar to PEG chains in that they both are nonpolar and contribute to the PEG-oriented specificity of anti-PEG Abs. Indeed, previous research reported that the use of butoxy groups at PEG termini increased both the antigenicity and the immunogenicity of PEG [98,99]. From this finding, we can speculate that, by significantly altering the characteristics of the most exposed PEG terminus, we could reduce the ability of anti-PEG Abs both to draw near a PEG main chain and thus to recognize the PEG. Because anti-PEG Abs are polyclonal, some of them may recognize mPEG while others may recognize PEG. Researchers studying the key interactions between nonpolar PEG and anti-PEG Abs have observed a certain subset of Van der Waals forces involving hydrogen bonding [58–60]. This interaction is also closely related to aromatic rings, which themselves are generally considered to be hydrophobic. Therefore, I propose that hydrophobicity is a key characteristic of the interactions between nonpolar PEG and anti-PEG Abs. We should recall that the binding sites of anti-PEG Abs have both nonpolar and polar moieties, which are thought to interact with PEG. These interactions between the moieties (whether nonpolar or polar) and PEG greatly facilitate the ability of anti-PEG Abs to bind to PEG. Therefore, the most effective way to inhibit the initial contact between anti-PEG Abs and PEG is to rely on polar groups such as purely anionic (negatively charged) terminus polar groups or anionic – cationic (negatively and positively charged) zwitterionic groups. It would obviously be counterproductive to rely on cationic (positively charged) groups (e.g., certain amine groups). One last point worth making in this matter is that, from a structural perspective, anti-PEG Abs are less likely to recognize PEG whose termini have bulky structures than PEG whose termini have methoxy structures.

### *10.3.2. A strategy to inhibit stable binding of anti-PEG Abs for PEG conjugates*

Having discussed strategies for reducing the PEG specificity of anti-PEG Abs, let us turn our attention to strategies for inhibiting the ability of anti-PEG Abs to bind stably to PEG conjugates. Non-PEG moieties, when present in PEG conjugates, strengthen the binding affinity of anti-PEG Abs for PEG and, as a result, strengthen PEG immunogenicity. The non-PEG moieties directly involve in the *specific* interactions between PEG and anti-PEG Abs and interact with anti-PEG Abs. Because *non-specific* interactions between biomolecules and other molecules in aqueous media are often hydrophobic and cationic, the chemical modification of such hydrophobic molecules, cationic molecules, or large-MW proteins opposite the methoxy terminus of PEG can increase the ability of anti-PEG Abs to bind stably to PEG conjugates. Thus, if we can minimize the strengthening effects of the non-PEG moieties for anti-PEG Abs' binding to PEG conjugates, *ceteris paribus* the PEG in the given PEG conjugates will remain intact.

In the previous research that my colleagues and I conducted, our approach to inhibiting the stable binding of anti-PEG Abs to PEG conjugates was quite simple [63,100]. We focused on two main areas: (1) how the characteristics of PEG contributed to the specificity (Figure 5(A)), and (2) how chemically modified non-PEG moieties might stabilize the specific interactions between PEG and anti-PEG Abs (Figure 5(B)). Drawn partly from the findings of the above research, my view today is that pharmacologists studying the role of PEG in drug-delivery systems should consider using hydrophilic molecules to create a physical distance between PEG and chemically modified non-PEG moieties. For example, if hydrophilic or anionic molecules are placed between PEG and hydrophobic or cationic molecules, this arrangement will suppress the involvement of non-PEG moieties in the specific interactions between PEG and anti-PEG Abs (Figure 5(C)) [62]. My suggestions here rest not on PEG motility but on factors that inhibit the direct involvement of non-PEG moieties in the specific interactions that occur between PEG and anti-PEG Abs in the very small region near the Ab paratopes. Conversely, PEG termini having very large molecules or very large surfaces may facilitate the interaction with the anti-PEG Abs; therefore, the use of hydrophilic or anionic molecules may not suppress the involvement of non-PEG moieties.

For the above reasons, I propose that PEG possessing liposomes, highly hydrophobic polymers, or very large proteins should be highly immunogenic regarding initial IgM responses to the PEG. The successful separation of PEG from both the large lipid membrane surfaces of liposomes and the surfaces of large macromolecular proteins would, I argue, reduce the adverse effects that the binding of anti-PEG Abs to PEG conjugates can have on the therapeutic value of PEG-related drug-delivery systems. The various research findings that I have presented in this article have led me to conclude that polar hydrophilic groups are an effective way to create physical distance between PEG and liposomes, non-PEG hydrophobic polymers, and very large proteins.

## 11. Possible and actual alternatives to PEG

Let us turn our attention to possible and actual polymer alternatives to PEG (alternative polymers) (Figure 6). Among the many suggested alternatives are nonionic poly(glycerol)s, poly(oxazoline)s (POxs), poly(vinylpyrrolidone)s (PVPs), and such poly(acrylamide) derivatives as poly(N-(2-hydroxypropyl) methacrylamide) (HPMA) [19,101–106]. Some research has pointed to zwitterionic polymer derivatives including poly(carboxy betaine)s, poly(sulfobetaine methacrylate)s, and poly(2-methacryloyloxyethyl phosphorylcholine) [13,107–114]. Additional

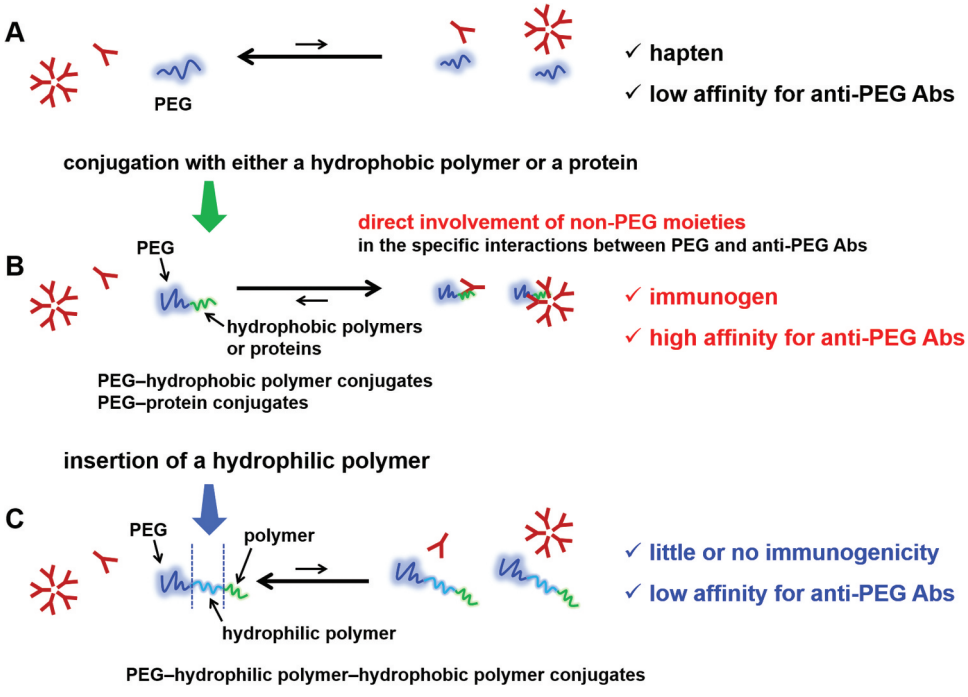


Figure 5. Insertion of a hydrophilic chain between PEG and hydrophobic blocks helps to suppress anti-PEG ab binding.

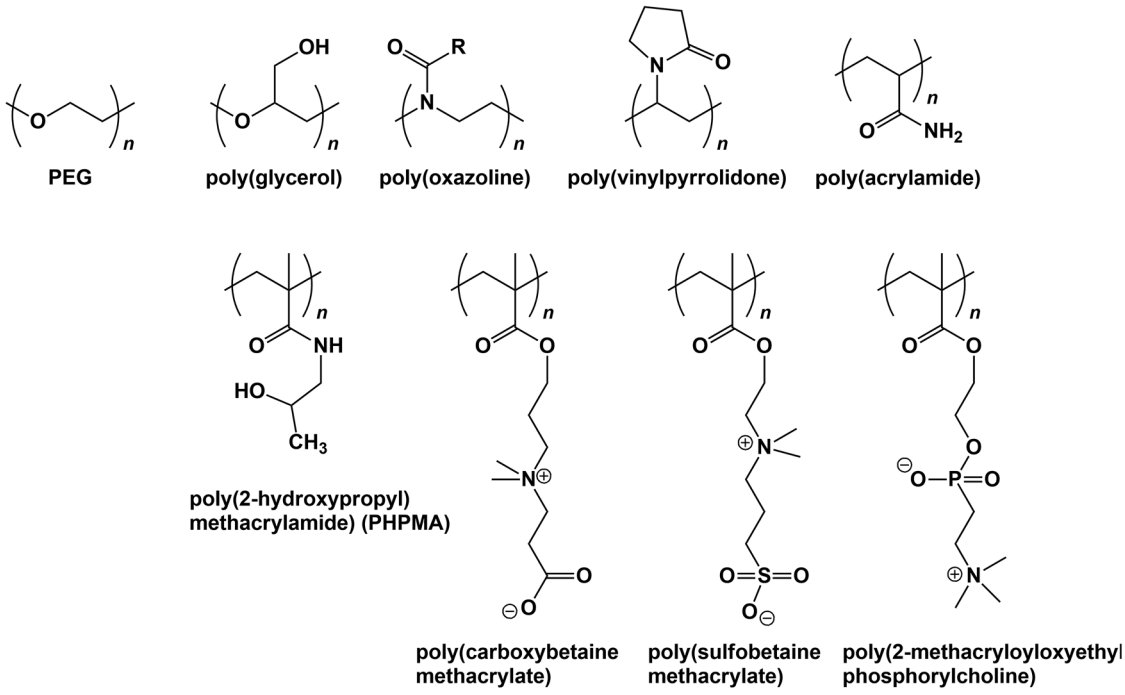


Figure 6. Possible and actual polymer alternatives to PEG.

research has focused on yet other classes of alternatives: specific polypeptide sequences such as nonrepetitive randomized sequences of six amino acids (alanine, glutamate, glycine, proline, serine, and threonine), proline/alanine-rich sequences, and elastin-like polypeptides consisting of randomized repeating sequences of such amino acids as glycine, valine, and proline [115,116]. In attempting to understand the benefits and drawbacks to these alternatives, we should consider PEG characteristics.

One of the greatest pharmaceutical traits of PEG is that it does not interact strongly with serum proteins. This fact suggests, conversely, that PEG interacts weakly with serum proteins. Ethylene glycol (EG) possesses hydrophobic characteristics. Despite this hydrophobicity, in principle EG units are always hydrated. According to our recent studies, the hydrophobic hydration of EG units seems to be closely associated with the binding specificity of anti-PEG Abs for PEG because the less hydrophilic the PEG is, the more susceptible the PEG becomes to the binding activity of anti-PEG Abs (data not shown). This characteristic is closely associated with PEG's excluded-volume effect: PEG hinders certain chemical reactions in a system by expanding its spaces that are inaccessible to other molecules [117,118]. These two facts explain why PEG has been a popular choice for cell – cell fusion and for protein crystallization. Thus, because of the complex three-dimensional structure of their receptors, very few types of B cells can selectively bind to a protein. And thus, I propose that linear, nonpolar PEG might interact with a wide range of proteins and, more specifically, with a wide range of B-cell receptors. Such interactions have been observed in the high titer of anti-PEG IgM responses to PEGylated drugs [84].

As PEG does, all alternative polymers must exhibit water solubility and avoid strong interactions with serum proteins. If these alternative polymers possess bulky repeating units, it is reasonable to suspect that the initial antibody titer corresponding to the alternative polymers will be lower than the antibody titer corresponding to actual PEG because, in contrast to the PEG case, no wide range of B-cell receptors will recognize the alternative polymers. This assertion is borne out by previous research showing that concentrations specifically of anti-PEG IgM in mouse plasma were high relative to total IgM concentrations [84]. In addition, it is reasonable to consider the possibility that, owing to their haptenic natures, alternative polymers exhibit no or very low immunogenicity, as is the case with PEG itself. Thus, we should consider the immunogenicity of alternative polymer conjugates. Take, for example, liposomes consisting of both a hydrophilic polymer (it could be PEG or an alternative polymer) and a lipid membrane: this combination creates a good foundation for examining not only the pharmacokinetics characterizing the alternative polymer liposome but also the ability of these alternative polymers to elicit anti-polymer Abs [102–104,114]. Indeed, Kierstead et al. examined the ABC traits of such hydrophilic polymers as PEG, POx, and PVP. The researchers noticed that PEG and POx liposomes exhibiting long blood circulation also exhibited the highest ABC of all the hydrophilic polymers [102]. Another study examined poly(carboxybetaine), which holds promise as a good alternative to PEG; however, the researchers found that poly(carboxybetaine) liposomes exhibited ABC [114]. Any alternative polymer liposome that exhibits a sufficient half-life and that avoids eliciting anti-polymer Abs would seem to have considerable potential as a component of a drug-delivery system. An important point needs to be mentioned, though: despite the correlation between anti-polymer Ab elicitation and ABC, ABC is not always observed in the presence of anti-polymer Abs. As I described above, ABC is quite simply determined by the ratio of anti-polymer Abs to carrier molecules. We should also bear in mind that once

a polymer – protein conjugate triggers an IgG response involving clonal expansion following the generation of anti-polymer IgM Abs, the suppressive effect of alternative polymers on IgM B-cell responses will have a less effect on later T cell-related responses.

## 12. Future perspectives

As of 2025, the research community studying the role of PEG in drug-delivery systems has made the following important findings. Anti-PEG Abs exist, and both the titer tests of anti-PEG IgG and the titer tests of anti-PEG IgM have detected increased Ab levels after mRNA-LNP vaccination [119]. Because anti-PEG Abs have been found in serum before vaccination and have been shown to be predominantly in females, researchers have suggested that cosmetics and other products regularly consumed more by females than by males help trigger the generation of anti-PEG Abs [120]. One thing that is not yet clear is whether or not anti-PEG Abs are directly involved in infusion-related reactions and other adverse effects stemming from the intravenous administration of medicines. In particular, little is known about how these infusion-related reactions might be associated with complement factors immediately after the administration of drugs. Also, few studies have focused substantively on why anti-PEG IgG has been found in the human body both prior to and following the administration of vaccines. Of major interest in this regard is the fact that anti-PEG Abs have been found in healthy donors who never previously received PEGylated therapeutics [4–8]. We know that, just as with cosmetics and other regularly consumed products, mRNA-LNPs use PEG lipids, and we also know that B-cell responses to these PEG derivatives are, in general, TI responses. Of course, TI antigens are known to induce isotype-class switching from the IgM isotype to IgG. Therefore, we cannot rule out the possibility that B-cell gene rearrangement occurs not only in response to PEG lipids but also without the help of T cells and results in IgG-secreting B cells. However, because clonal expansion and affinity maturation are, at least in theory, thought to occur in protein-related TD responses, there seems to be little likelihood that very high anti-PEG IgG titer levels would be detectable without the involvement of T cells. The specificity of PEG rests largely on the distinguishing traits of PEG: it is nonpolar and water-soluble and is free of structural bulkiness. These traits allow PEG to elicit antibody responses from a greater number of B cells than is possible with conventional antigens. Consequently, B cells, without the help of T cells, may produce many IgG antibodies specific to PEG.

Another possible reason for the detection of anti-PEG IgG in people who received mRNA-LNPs concerns the cross reactivity of preexisting non-PEG antigen-elicited antibodies in relation to PEG. The nature of PEG may explain this cross reactivity. Many existing proteins and other potential antigens can induce diverse antibodies, of which a certain number can, in all likelihood, recognize the epitopes belonging to proteins whose chemical characteristics are similar to those of PEG. One idea is that, because of this cross-reactivity, PEG conjugates stimulate existing IgG-producing B-cell receptors. Although this idea is unlikely if we assume that, in the context of PEG, specific Abs recognize only specific antigens, if specific B-cell receptors recognize only a few nonpolar ethylene glycol units or terminal methoxy groups of PEG as epitopes, these B-cell receptors probably or at least possibly will exhibit a broad spectrum of specificities and thus will exhibit a wide degree of affinity. In fact, Ishida et al. reported that anti-PEG IgM recognized not only PEG liposomes but also non-PEGylated liposomes and that the binding affinity of anti-PEG IgM was higher for PEG liposomes than for non-PEGylated liposomes [40]. In contrast to the short-term, "temporal" nature of IgM responses, IgG responses are long-term. Therefore, anti-PEG IgG-related responses may pose a serious threat to PEGylated drug-delivery systems, particularly if the antibody titer is high. Future research should continue to address these potentially deleterious effects [9–11,121,122].

## 13. Conclusion

In this perspective review, I have summarized the literature addressing the various roles of anti-PEG Abs in PEG-related drug-delivery systems. A fundamental finding in the literature is that many of us have anti-PEG Abs circulating in our bodies, often as a result of previous exposure to mRNA-LNP vaccines. Another fundamental finding is that these anti-PEG Abs can reduce the therapeutic efficacy of various PEGylated medical treatments and can trigger adverse side effects. Although the proposed solutions to these reductions in therapeutic efficacy must overcome formidable challenges, we should, I argue, consider the issue from several distinct perspectives.

In conclusion, we can glean a great deal of important knowledge by understanding the differences between two distinct relationships: (1) the relationship between *PEG* and anti-PEG Abs and (2) the relationship between *PEG conjugates* and anti-PEG Abs. Researchers have overlooked a common phenomenon that is inextricably linked to the differences between *haptenic PEG* and *immunogenic PEG conjugates*: in PEG conjugates (but obviously not in PEG), anti-PEG Abs are likely to bind directly to non-PEG moieties. One of the main points in this perspective review is that the conjugation of non-PEG moieties to PEG chemically transforms the moieties from their previously non-specific state to a specific one. Thus, in PEG conjugates, the non-PEG moieties become susceptible – and even vulnerable – to highly selective interactions with other molecules, including anti-PEG Abs. To this extent, non-PEG moieties play important roles as antigens in PEGylated drug-delivery systems. In short, the presence of anti-PEG Abs in our bodies poses a threat to PEGylated therapeutics, and even alternative polymers face challenges. Despite current obstacles, studies such as the present one will help researchers both refine old nanomedicines and design new ones in ways that improve drug-delivery systems reliant on PEG.

## Author contributions

This work was authored solely by Kouichi Shiraishi.

## Disclosure statement

The author has no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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