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Anti-PEG antibodies: Properties, formation, testing and role in adverse immune reactions to PEGylated nano-biopharmaceuticals

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ABSTRACT

Conjugation of polyethylene glycols (PEGs) to proteins or drug delivery nanosystems is a widely accepted method to increase the therapeutic index of complex nano-biopharmaceuticals. Nevertheless, these drugs and agents are often immunogenic, triggering the rise of anti-drug antibodies (ADAs). Among these ADAs, anti-PEG IgG and IgM were shown to account for efficacy loss due to accelerated blood clearance of the drug (ABC phenomenon) and hypersensitivity reactions (HSRs) entailing severe allergic symptoms with occasionally fatal anaphylaxis. In addition to recapitulating the basic information on PEG and its applications, this review expands on the physicochemical factors influencing its immunogenicity, the prevalence, features, mechanism of formation and detection of anti-PEG IgG and IgM and the mechanisms by which these antibodies (Abs) induce ABC and HSRs. In particular, we highlight the *in vitro*, animal and human data attesting to anti-PEG Ab-induced complement (C) activation as common underlying cause of both adverse effects. A main message is that correct measurement of anti-PEG Abs and individual proneness for C activation might predict the rise of adverse immune reactions to PEGylated drugs and thereby increase their efficacy and safety.

Contents

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1. Introduction

Polyethylene glycols (PEGs) are synthetic polymers with a great variety of industrial and welfare use [1,2]. As one of the few biocompatible synthetic polymers approved by the US FDA as ingredient of food, cosmetic and hygienic products it is all around in our daily life, and its regulatory acceptance for internal use gave rise to numerous medicinal applications, among others as osmotic laxative, a fusogen repairing injured nerves and cell membranes [3], suppressor of oxidative stress [3] and promoter of axonal regeneration [3]. In focus of this review, PEG is gaining increasing use in pharmacotherapy as the polymer of choice for bioconjugation [1,2].

Regarding the latter application, covalent or non-covalent conjugation of PEG to therapeutic proteins, such as antibodies (Abs) and enzymes, or other macromolecular pharmaceuticals, e.g., nucleotides, or drug delivery nanosystems (DDSs), such as liposomes, micelles, dendrimers, polymeric nanoparticles (NPs), solid lipid NPs, increases their hydrodynamic size and water solubility, thus decreasing their self-aggregation and interactions with blood proteins and cells of the mononuclear phagocyte system (MPS). The result is increased *in vitro* and *in vivo* stability entailing longer storage time, decreased renal, proteolytic and phagocytic clearance with consequent increase of circulation time, reduction of adverse effects, an overall increase of applicability and therapeutic index [1,2,4–13]. To date, ≥ 21 PEGylated proteins, peptides, Ab fragments and oligonucleotides have been approved by the FDA for marketing (Table 1). The attached PEGs in these drugs are in the 2–40 kDa range.

Nevertheless, all these medicinal benefits are not without a price. Although the shielding of immunogenic epitopes on proteins initially led to the belief that PEG is poorly immunogenic, recent progress in experimental and clinical research with different PEGylated drugs revealed the rise of anti-PEG IgM and IgG both in animal models and in patients. These anti-drug Abs (ADAs) may neutralize the therapeutic effect of the drug and thereby reduce its clinical efficacy and, in addition, these ADAs may cause adverse immune effects, specifically an acceleration of the blood clearance of the PEGylated drug (ABC phenomenon), resulting in efficacy loss, and hypersensitivity reactions (HSRs) which can lead to anaphylactic shock and death [23–26].

The goal of this review was to recapitulate some basic information of PEGand then to expand on the factors influencing its immunogenicity, the prevalence, features, mechanism of formation and detection of anti-PEG IgG and IgM, and the mechanisms by which these Abs induce ABC and HSRs via complement (C) activation.

2. PEG: structural features

PEGs are highly flexible linear or branched polymers in the 0.4-40 kDa MW range, synthetized with different end-groups. One of the end-groups is used for covalent attachment to free carboxy, amino, or sulphydryl groups on macromolecules, on DDS or on linkers that bind the PEG to DDS, such as phosphatidylethanolamine, via one of a variety of chemically reactive functional groups (acrylate, methacrylate, maleimide, dibenzocyclooctynol, vinyl sulfonate or vinyl or allyl ethers) [3]. The other end-group is most frequently a methyl group (methoxy-PEG), although hydroxy (–OH), amino ($-NH_3^+$), butoxy ($-O-(CH_2)_3-CH_3$) and tert-butoxy ($-O-(CH_3)_3$) terminal endings are also used [27,28]. Fig. 1 shows different presentations of the polymer.

According to X-ray diffraction and other means of molecular modeling of PEG, the chain builds up from repeating S-shaped fragments, or S-modules, consisting of 10 oxyethylene units [13] (Fig. 1). The imbedded cavities along the polymer may accommodate H_2O explaining its strong hydrophilic character providing a water-shield around the anchor nanostructure [13]. Obviously, this shielding effect depends on the length of PEG, the end-groups and the distance of PEG grafts from each other.

It is also known that in addition to Abs, a large number of other proteins and macromolecules in plasma also bind to PEGylated NPs and influence Ab binding [29, 30].

3. The immunogenicity of PEGylated drugs

3.1. Physicochemical features influencing Ab induction

Free PEG, in solution exhibits no or very weak immunogenicity even in the presence of Freund's adjuvant [31,32]. However, when conjugated to a macromolecule or a DDS, it becomes immunogenic, just as small haptens become immunogenic when combined with a larger carrier [31,32]. The Ab response is not limited against different parts of PEG but it is a combination of differential responses against the individual components of the whole PEGylated nanostructure. Accordingly, the immunogenicity of PEGylated drugs depends on numerous features of the polymer and its carrier.

The critical polymer features include the length (MW), branching and the chemical nature of the terminal end-group. In general, Abs induced against backbone epitopes have weaker binding affinities than those induced against the terminal end-groups, whose size and hydrophobicity are critical in determining the binding affinity [28]. In one example for such differential formation of anti-PEG Abs, Sherman et al. [27] reported that Abs elicited by hydroxy PEG-conjugated proteins have similar affinity to both the methoxy PEG and hydroxy PEG, while Abs induced by methoxy PEG-conjugated proteins recognize methoxy PEG more effectively than hydroxy PEG. Among the frequently used PEG end-groups the binding affinities of formed Abs increase in the following order: hydroxy (–OH) < amino ($-NH_3^+$) < methoxy ($-O-CH_3$) < butoxy ($-O-(CH_2)_3-CH_3$) < tert-butoxy ($-O-(CH_3)_3$) [27,28]. Yet another physicochemical factor that may influence immunogenicity is the hydrophilicity of the NP core structure, for example a PEG-shell-possessing polymeric micelle with hydrophobic inner core elicited anti-PEG IgM response, while similarly PEGylated micelles with hydrophilic core did not [33]. Nevertheless, none of the monoclonal or polyclonal anti-PEG Abs are absolutely end-group, linker, or backbone-specific, they display distinct relative selectivities.

Beyond the PEG chain and end-group features, another basic determinant of immunogenicity of PEGylated drugs is the chemical nature of PEG-acceptor core structure, whether it is a protein, a polynucleotide or DDS. This variable may determine the type of immune response in terms of spectrum of Ab subtypes and kinetics of primary and secondary Ab response. Notably, if the PEGylated carrier is a protein, immunogenicity proceeds mostly in a T cell-dependent (TD) pathway [34], and the intrinsic immunogenicity of the protein facilitates the secretion of PEG-specific Abs in a cooperative manner. This phenomenon is exemplified by the immunogenicities of Omontys® and Krystexxa® (Table 1) where the proteins themselves are immunogenic, but their PEGylated formulations induce much stronger anti-PEG immune

Table 1
PEGylated drugs reaching advanced clinical trials or the market.

Trade names	API	Indication	Producer	Approval	Ref to immunogenicity
Jivi®	60K-PEG recombinant Factor VIII antihemophilic factor	Hemophilia A	Bayer	2018	
Palynziq®	2K-PEG-rhu-Phenylalanine ammonia-lyase, Pegvaliase-pqpz	Phenyl-ketonuria	Biomarin	2018	[14]
Revolixys® kit[a]	40K-PEG-RNA aptamer + reverse agent, a Factor-IXa blocker, Pegnivacogin /Anivamersen	Anti-coagulation	Regado/Tobira	2016	[15]
Adynovate®	20K-PEG-Factor VIII Antihemophilic Factor VIII	Hemophilia A	Baxalta	2015	
Onivyde®	2K-PEG-Liposomal irinotecan hydrochloride trihydrate	Metastatic pancreatic cancer	Ipsen	2015	
Plegridy®	20K-PEG-Interferon beta-1a	Relapsing forms of multiple sclerosis.	Biogen	2014	
Movantic®	<1K-PEG-Naloxegol	Opioid-induced constipation	AstraZeneca	2014	
Omontys®[b]	*40K-PEG-Erythropoietin-mimetic peptide, Peginesatide*	*Anemia associated with chronic kidney disease*	*Affymax/Takeda*	*2012*	[16]
Sylatron™	12K-PEG-Interferon alpha 2b	Melanoma	Merck	2011	
Krystexxa®[c]	*10K-PEG-Uricase, Pegloticase*	*Gout*	*Savient*	*2010*	[17]
Cimzia®	40K-PEG-Certolizumab	Rheumatoid arthritis, Crohn's disease, Axial spondyloarthritis and psoriatic arthritis	Nektar/UCB Pharma	2008	
Mircera®	30K-PEG- erythropoietin (epoetin) beta	Anemia associated with chronic kidney disease	Hoffman-La Roche	2007	[18]
Macugen®	40K-PEG-anti-VEGF aptamer, Pegaptanib	Age-related macular degeneration	Pfizer	2004	[19]
Somavert®	5K-PEG-rhuGH (human growth hormone), Pegvisomant	Acromegaly	Pfizer	2003	
Neulasta®	20K-PEG-Granulocyte colony stimulating factor, Pegfilgrastim	Neutropenia	Amgen	2002	[20]
Pegasys®	40K-PEG-interferon alpha-2	Hepatitis C and B	Hoffmann-La Roche	2001	
PegIntron®	12K-PEG-interferon alfa-2b	Hepatitis C and B	Schering-Plough/Enzon,	2000	
Doxil®/Caelyx®	2K-PEG-Liposomal doxorubicin HCl	Cancer	Alza	1995	[21]
Oncaspar	5K-PEGylated L-asparaginase, Pegaspargase	Acute lymphoblastic leukemia	Enzon	1994	[22]
Adagen®	5K-PEG-adenosine deaminase (bovine), Pegademase	Severe combined immunodeficiency disease (SCID)	Enzon	1990	

[a] *withdrawn from Phase III clinical trials*[a]; *or marketing in the US*[b] *or EU*[c].

response than non-immunogenic protein-bound PEGs do. The immune response is so strong, that these drugs had to be withdrawn from clinical use [17,23,24,35,36].

In contrast to the TD immunization, if the PEG anchor is a DDS, immunogenicity may proceed in a T cell-independent (TI) pathway with a different kinetics and Ab spectrum. The underlying mechanisms of these immunogenicities will be detailed later in Section 5.

3.2. Impact of PEG amount and surface topography on Ab induction

Beyond the above physicochemical features of PEG and its carriers, the extent of PEGylation of different proteins and DDS is another critical determinant of immunogenicity. Proteins can be covered with up to a few molecules of PEG, usually not more than 3, while DDSs obviously have much more sites for PEG conjugation. In the latter case, the variable is not the number of polymers attached to the macromolecule, but the density of PEG on the NP surface, often expressed as mole %.

Information on the relationship between immunogenicity and PEG surface density is mainly available for PEGylated liposomes, where the PEG molecules assume different spatial configuration depending on grafting density. Specifically, a density of <4 mol% PEG2000 on liposomes forms a 3–4 nm thick layer wherein the PEG molecules are separated on the vesicle surface, referred to as "mushroom" configuration. Over about 9–10 mol% PEG2000 forms a 4–10 nm continuous PEG layer, dubbed as "brush" configuration, where the PEG chains may interact, and possibly intertwine with each other.

As for the influence of PEG density on the immunogenicity of PEGylated drugs, the inverse relationship between the extent of PEGylation and immunogenicity of proteins has been known since the rise of PEGylation technology in the 1970s [37]. Nevertheless, the consequences of anti-PEG immune responses to differently PEGylated drugs is not so easy to predict, considering the large number of iterations of chemical and topographical variables in membrane-attached PEG and host immune factors. In one study demonstrating this complexity, Li et al. [38] reported that liposomes with low (3%) and high (9%) PEG density induced similar extent of anti-PEG IgM following intravenous injection, yet the vesicles with high density PEG induced faster

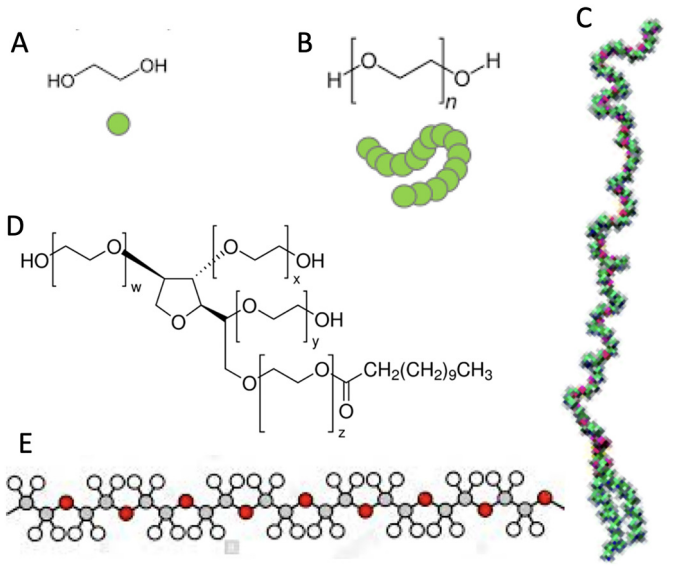

Fig. 1. Chemical details of PEG structure via different presentations. A, ethylene glycol, green; B, linear polyethyleneglycol (PEG), green; C, 2-K-PEG-Phosphatidylethanolamine (PE), red: oxygen, green: carbon atoms, white: H atoms; D, branched PEG, E, schematic illustration of the atomic structure of linear PEG, gray: carbon atoms, white: hydrogen atoms, red: oxygen. The figure was prepared by modifying illustrations in Refs. [8, 13] and in web images accessible at google search "polyethylene glycol/images", among others, at links: https://en.wikipedia.org/wiki/Polyethylene_glycol and https://www.oxiteno.us/what-is-polyethylene-glycol-peg-uses-side-effects/.

clearance of subsequent doses than those with low density. Accordingly, in this example, the high density of PEG enhanced a secondary consequence of immunogenicity, without affecting immunogenicity *per se*.

In addition to PEG surface density, PEG chain length affects the extent of anti-PEG Ab induction. As described above, the minimum MW of an anti-PEG epitope on PEG was reported to be 750 Da with positive relationship between the length of PEG and anti-PEG Ab induction [39]. Namely, $PEG_{30,000}$-BSA and $PEG_{20,000}$-OVA induced higher anti-PEG IgM response compared with PEG_{2000}-BSA and PEG_{5000}-OVA. On the other hand, PEG on nanoparticles, such as liposomes and adenoviruses, are relatively short and their extension does not necessarily increase immunogenicity anymore [39,40].

3.3. Impact of linker on immunogenicity of PEG

As mentioned in section 2, PEGylation is performed by various conjugation chemistries and linkers to optimize the solubility, immunogenicity and biological function of the drugs [41]. The linker between PEG and proteins/carriers might affect the immunogenicity of PEG. In an earlier study by Poppenborg et al. [42] showed that both amide and succinate bonds between PEG and asparaginase induced anti-PEG Abs at similar extent following injection of PEG-asparaginase. Recently, in addition to anti-PEG Abs, anti-succinate linker Ab was observed in patients who suffered from HSRs to PEG-asparaginase [43].

3.4. Host immune and genetic factors influencing PEG immunogenicity

Among the host factors influencing Ab induction by PEGylated drugs, the route and schedule of drug administration, presence of preformed Abs and immune status of the patient are obviously critical. In addition, there is apparently a genetic factor as well. Chang et al. [44] have identified seven single-nucleotide polymorphisms (SNPs), most prominently "rs12590237" localized in the variable segment of immunoglobulin heavy chain gene to be significantly associated with high prevalence and concentrations of natural (pre-existing) anti-PEG IgM in the general population in China.

4. Features of anti-PEG Ab binding to PEG

As mentioned, anti-PEG Abs have specificities to either the backbone or the terminal end-groups of PEG, both having different binding characteristics. The binding to the backbone has relatively low affinity, and the number of bound Abs is limited by the length of PEG. Considering the length and MW of an oxyethylene group (0.34 nm, 44 Da, respectively), and the epitope size that the variable region of Fab covers (2–3 nm) it can be estimated that approximately 3–6 oxyethylene groups can bind one IgG molecule [13,28]. Thus, the most frequently used 2-K-PEG can bind up to 8–15 IgG molecules, at least in theory [13,45]. The actual number, however, depends on more preconditions, such as Ab-binding by "satellite" PEGs, which are small, 1/3 S-module PEG segments (i.e., 3 oxyethylene units) whose link to the core chain cannot be identified (Fig. 2).

5. The adverse consequences of Ab binding to PEGylated drugs

As mentioned, the clinical significance of PEG immunogenicity lies in the possibility that binding of anti-PEG Abs decreases the therapeutic efficacy and safety of PEGylated drugs. The therapeutic efficacy can be reduced either by blocking the drug's therapeutic effect in some way, an effect referred to as neutralization. The other way may be the enhancement of the drug's clearance, the ABC phenomenon, decreasing the drugs bioavailability. Finally, hypersensitivity reactions (HSRs) represent a health risk, with potentially severe, life threatening or deadly (pseudo)allergic reactions. The next subsections briefly recapitulate these phenomena highlighting the role of anti-PEG Abs therein.

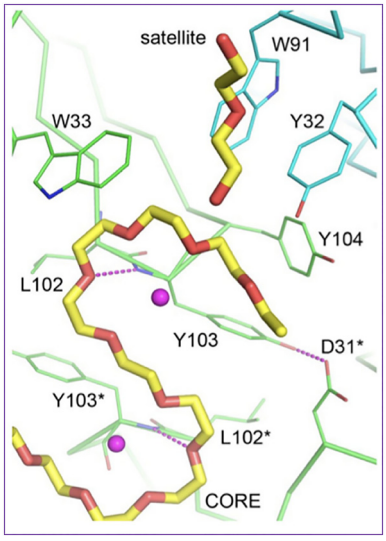

Fig. 2. Molecular details of Ab binding to PEG. The stick model based on X-ray diffraction data shows the intercalation of the aromatic rings of amino acids in the variable region on the Fab arms of 2 IgG molecules (green and magenta thin sticks) into the groves of an S-shaped core PEG fragment consisting of 10 oxyethylene units, and to a satellite PEG fragment (thick stics with yellow carbons and red oxygen). Bound water molecules within the groves are shown as red spheres. Reproduced from Ref [13] with permission.

5.1. The ABC phenomenon and involvement of anti-PEGAbs

The essence of the phenomenon is that in case of repeated *i.v.* injections of animals or man with a complex, nanoparticulate drug, such as PEGylated proteins or NPs, the second and subsequent doses display progressively decreasing circulation time, and hence, reduced efficacy. Since its first report in 2000 [46], the ABC phenomenon has been intensely studied in our group (TS-TI) and many of its details have been clarified [25].

We established, among others, its presence and variable extent in mice, rats [47], guinea pigs [48], minipigs [49] and beagle dogs [50], its dependency on the drug type (liposomes, ovalbumin, micelles) and chemical composition (phospholipids with different acyl chains and charges, cholesterol and different PEGs) and physicochemical properties (surface charge and liposome size) of PEGylated NPs [51]. We have studied and established the dependence of ABC on lipid dose (inverse correlation with dose) [52–54] and administration schedule (administrations within 3 days to 4 weeks) [55,56]. Notably, high dose of PEGylated liposomes administered as a first dose does not preconditions for ABC upon repeated administrations but rather elicits immunological tolerance, or anergy of B cells in mice [54,57], and the effective period for inducing ABC is 4–7 days after the first dose, beyond which the phenomenon gradually vanishes over weeks [46,55,56]

Further studies identified the molecular mechanism of ABC in the following major steps: proliferation and differentiation of specific B cells in the marginal zone of the spleen in T-cell independent manner, resulting in anti-PEG IgM formation, complement (C) activation, opsonization by C3 fragments and clearance from systemic circulation by cells of the MPS (Kupffer cells) [34,50,52,53,58–63] (Fig. 3). Of note, the above time window for observing ABC is consistent with the biological half-life of IgM (3 weeks) [64] and the causal role of anti-PEG IgM in ABC via C activation and opsonization of PEGylated liposomes [26].

Among the unexpected, sometimes counter-intuitive findings regarding the ABC phenomenon, the anti-PEG IgM response was found to be not specific to PEGylated liposomes, since bare, non-PEGylated liposomes also induced the ABC phenomenon of a second dose PEGylated

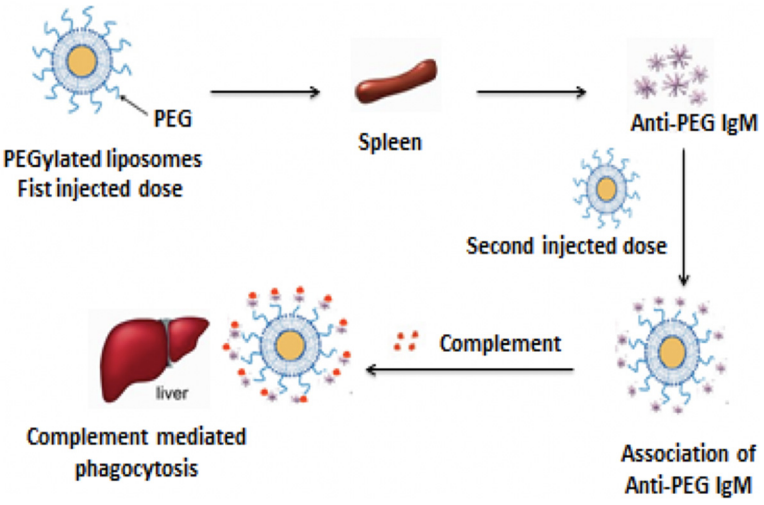

Fig. 3. Mechanism of accelerated blood clearance (ABC) phenomenon. Reproduced from Ref. [65] with permission.

liposomes in mice [51]. Furthermore, liposomes containing anticancer drugs (doxorubicin, oxaliplatin, mitoxantrone, topotecan) have been found not to affect, decrease or increase the ABC response, depending on dose and mechanism of cytotoxicity [57,58,66]. For example, therapeutic doses of Doxil suppress the anti-PEG IgM production as a result of cytotoxicity on B cells, as there is no report that Doxil would be immunogenic or cause efficacy loss because of the ABC phenomenon [21,67,68]. On the other hand, Suzuki et al. [49] found that *i.v.* injection of Doxil at low, subtherapeutic doses, triggers the ABC phenomenon in various animal species. The importance of cytotoxicity in preventing ABC is examplified by the finding that PEGylated liposomal Topotecan, a cell cycle specific drug, exerts its inhibitory action on ABC only in the S phase of the cell cycle, when B cells are responsive to cytotoxicity [55,66].

5.2. Hypersensitivity reactions and involvement of anti-PEG Abs

The HSRs caused by PEGylated drugs are essentially the same as caused by a great variety of *i.v.* medications without PEGylation, including liposomal and micellar drugs, radiocontrast agents, biologicals, enzyme therapies, iron compounds, even small molecules [69–72]. The mild to severe allergy symptoms arise shortly after the first treatment, although reactions starting later, or after repeated treatments are also observed. In most cases the problem spontaneously resolves, but occasionally, the reaction can escalate into fatal anaphylaxis.

One mechanism of HSR, arising as a consequence of C activation, has been intensely studied and reviewed over the past 20 years [73–76]. The C activation triggering the HSR (C activation-related pseudoallergy, CARPA) can proceed via all three known pathways, i.e., the classical, alternative and lectin. Free PEG (PEG-600), used to treat spinal cord injury, can activate C in the absence of anti-PEG Abs via by the alternative and lectin pathways [77], but the alternative pathway can be involved in any way, even if the trigger mechanism proceeds via the classical pathway, as an amplification mechanism. In fact, recent evidence highlight the causal role of anti-PEG Abs-triggered classical pathway initiation of CARPA, at least for the case of PEGylated liposomes. Notably, we have shown in pigs [78] that 2-K-methoxy-PEGylated liposomes (called Doxebo), corresponding to liposome-encapsulated doxorubicin (Doxil) without the drug, induced massive anti-PEG IgM formation within 3 days, reaching peak between days 6–9 and titer declining slowly over >6 weeks (Fig. 4A). Testing the reactogenicity of the same liposomes by *i.v.* injection during the period of seroconversion led to fatal anaphylaxis within 2–3 min only in immunized animals, while the reaction was minor in naïve control pigs (Fig. 4B) [78].

The causal role of anti-PEG Ab-induced classical pathway C activation in pseudo-anaphylaxis in this study was suggested by the simultaneous elevation of the C terminal complex (sC5b-9), a C activation byproduct, in the blood of pigs and rise of pulmonary arterial pressure, an immediate cause of circulatory collapse and death (Fig. 5A). The fact that Doxil can bind anti-PEG Abs and activate C through this mechanism was recently proven *in vitro* and *in vivo*, showing sC5b-9 induced membrane damage with subsequent release of free doxorubicin from PEGylated liposomal doxorubicin (Doxisome) following the addition of monoclonal anti-PEG Abs [79]. Fig. 5B is a reproduction of the striking electron microscopic image of membrane damage caused by the binding of sC5b-9 to liposomes following the addition of monoclonal anti-PEG Abs in the presence of serum C.

5.3. Experimental and clinical examples of anti-PEG Ab triggered adverse effects feeding an immune stimulatory vicious cycle

The pig experiment described above, wherein anti-PEG IgM-triggered C activation led to major HSR [78], provides a model for those clinical observations wherein a PEGylated drug displayed severe HSRs along with loss of efficacy and direct or indirect evidence of C activation. Among the PEGylated drugs listed in Table 1, the followings were reported to cause more or less severe HSRs: *Doxil®/Caelyx®* [21], *Oncaspar®* [22,80], *Neulasta®* [20], *Macugen®* [19], *Mircera®* [18], *Palynziq®* [14], *Omontys®* [16], *Krystexxa®* [17] and *Revolixys®* [15,81–83], leading to market withdrawal of the last 3 medicines. Among these reactogenic drugs significant formation of anti-PEG Abs and/or treatment failure and/or C activation were described for *Oncaspar®* [17,23,24,35,36] and *Revolixys®* [15, 81-83].

Considering the known causal relationships among C activation, ABC, HSRs, opsonization and immunogenicity, we proposed the possible rise of an immune stimulatory vicious cycle among these effects (Fig. 6), a concept [26,84] consistent with some of the above listed clinical observations.

6. Mechanism of formation of natural and induced anti-PEG Abs [16,17]

As mentioned in the section on physicochemical features influencing Ab induction (3.1.), the immunogenicity of PEGylated drugs may be TD or TI, depending on the chemical nature of PEG-anchor molecule or DDS. PEGylated proteins induce immune responses mainly via the classical TD manner, while PEGylated NPs, like PEGylated liposomes, may induce Ab production via TI immunogenicity. However, neither of these

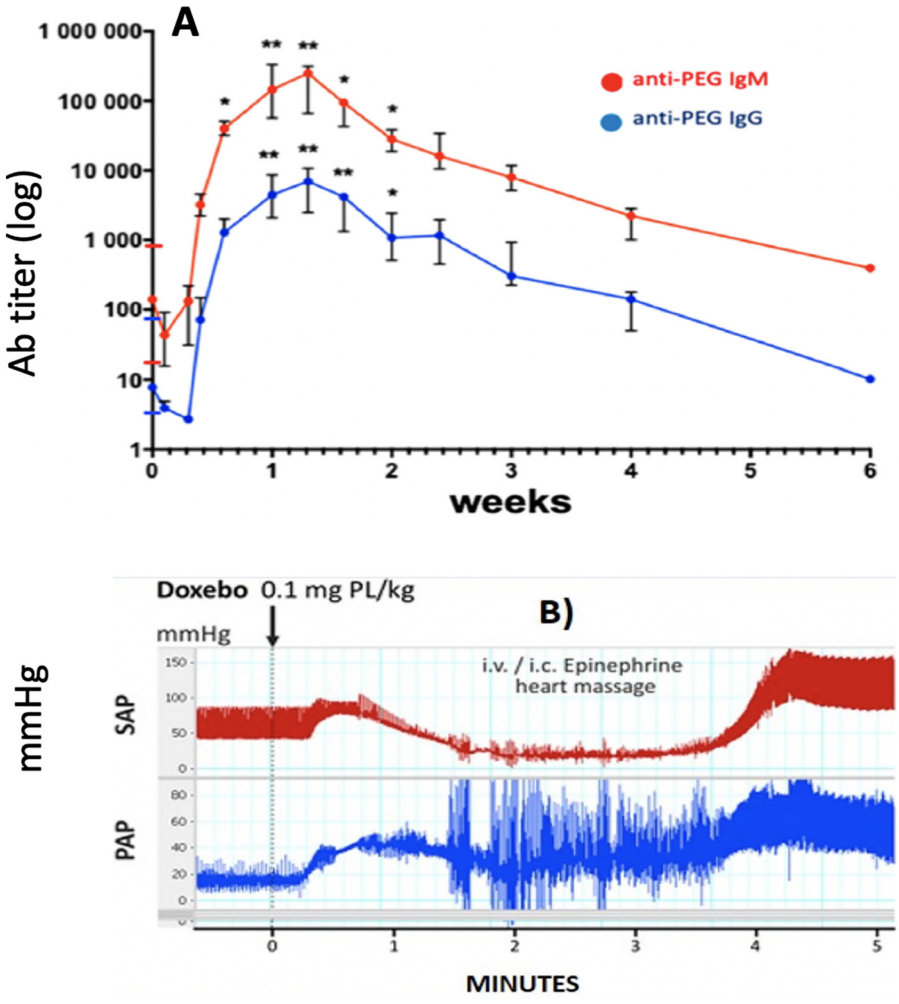

Fig. 4. Correlation between immunogenicity and PEGylated liposome-induced pseudo-anaphylaxis in pigs. A) Immunogenicity of Doxebo: Time course of anti-PEG IgM and IgG levels in blood, expressed as log titer. B. Changes of systemic and pulmonary arterial blood pressures (SAP, PAP) in pigs immunized with Doxebo 3 weeks earlier. The lethal anaphylactic reaction is reversed by epinephrine with heart massage. Figures reproduced from Ref. [78].

mechanisms is identical to the pure (non-conjugated) protein or liposomal vaccine-induced primary and secondary immune responses [7].

Fig. 7 illustrates the kinetics of typical protein-induced immunogenicity, characterized by a start of specific IgM production around day 5 and peak at about day 10. The rise and peak of IgG following isotype switching during the primary response is delayed by about 5 days, and the extent of IgG secretion slightly exceeds that of IgM. In contrast, during a secondary response, the IgM production is less and is followed by subtype switch with a rise and peak of IgG at around 11 and 15 days, respectively. During the secondary response the IgM production lessens while the IgG response starts and peaks earlier at a substantially increased quantity.

The TD immunogenicity to PEGylated proteins differs from the above scheme. For example, in rabbits, PEGylated human IFN-α, human serum albumin or porcine uricase, used as immunogens together with complete (1st injection) and incomplete (1, 2, 4, 8, 10 weeks later) Freund's adjuvant led to the production in 2 weeks predominantly IgG Abs [27,28]. In our previous study, anti-PEG IgM was produced from day 5 and reached a peak at day 7 following single injection of PEGylated OVA (ovalbumin, the main protein found in egg white, a TD antigen) in mice without secretion of IgG [34]. In humans, anti-PEG IgG was detected in 37% of patients after a single *i.v.* injection of pegloticase [85]. As mentioned, protein anchors of PEGs may enhance the immunogenicity of PEG by presentation of its peptides by PEG-specific B cells to helper T cells [86,87]. Therefore, anti-PEG Ab induction by TD antigens vary with carrier proteins, the presence of adjuvants and species.

TI immunogenicity, which has been proposed to be the mechanism of PEGylated liposome-induced Ab induction, is typical of repetitive structures. Studies from our (TS-TI) laboratory point to this mechanism underlying the immunogenicity of PEGylated DDS [52,60,64,78]. The process is localized to B cells in the marginal zone of the spleen [52,60,64] and is characterized by only minor IgG response running parallel with massive IgM production, with lack of isotype switching and absence of secondary response.

An example for this type of immune response, obtained in pigs, was shown in Fig. 4A. Fig. 7B and C show the murine prototype of this immunogenicity, wherein maximal rise of anti-PEG IgM was observed within 5 days after *i.v.* injection of PEGylated liposomes in mice followed by a decline to near baseline level in 2–4 weeks (Fig. 7B). This was paralleled with a smaller rise of anti-PEG IgG (Fig. 7C) without secondary response despite repetitive weakly immunization over 6 weeks (Fig. 7C).

As for the molecular mechanism of TI immunogenicity, the phenomenon has been attributed to a subset of B cells specialized for rapid and massive IgM production in response to foreign antigens. These IgM memory B cells, also called natural memory or natural effector memory B cells, derive from a particular developmental pathway characterized by somatic hypermutation. Thus, their Ig heavy chain repertoire differs

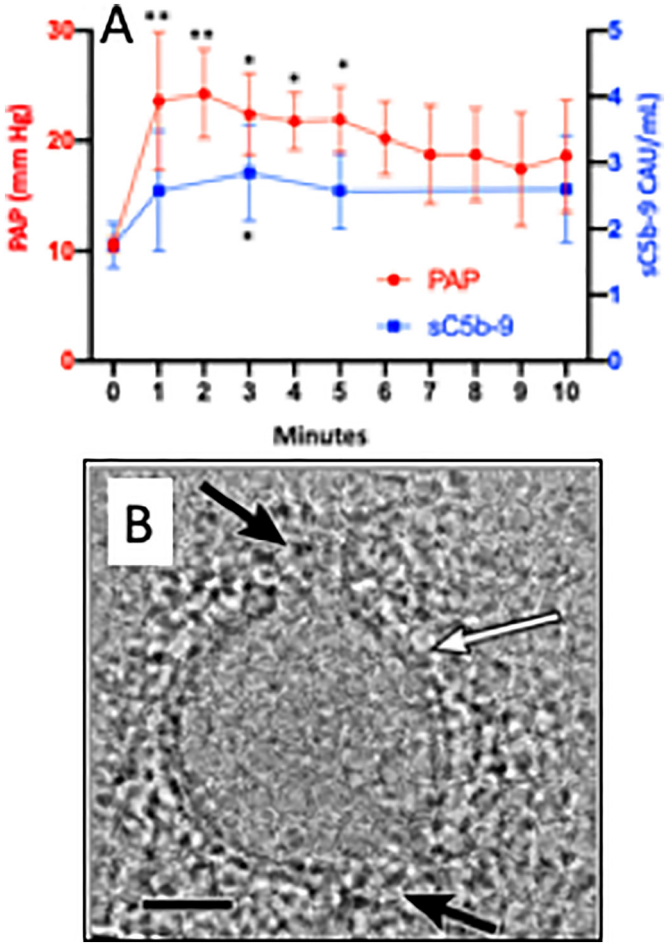

Fig. 5. Evidence for anti-PEG Ab-mediated C activation in pigs *in vivo* (A) and in human serum, *in vitro* (B). Panel A shows the changes of pulmonary arterial pressure (PAP) and sC5b-9 following i.v. injection of Doxebo in animals immunized with Doxebo a week before. The paralleling courses of rapid rise and slower decline of these variables on the minute time scale reflect close correlation between liposome-induced cardiopulmonary distress and C activation. Reproduced from Ref. [78] with permission. In B, human serum was incubated with PEGylated doxorubicin-containing liposomes (Doxisomes) together with a monoclonal anti-PEG IgG. The cryo-EM image shows the damage of liposomal bilayer (black arrows) following incubation of vesicles with human serum in the presence of anti-PEG IgG. White arrow: intact bilayer. Scale bar represents 25 nm, reproduced from Ref. [79] with permission.

from that of T-dependent B cells in the germinal center [44]. In man, they make about 25% of B cells in the peripheral blood [44], while in rodents, they have been localized at the marginal zone of spleen follicles [57,63,88,89]. Marginal zone B cells react with polymeric antigen through crosslinking of B cell receptor, entailing rapid, but low affinity Ab production without help of T cells. Thus, PEGylated liposomes can massively produce anti-PEG IgM within 5 days. However, the affinity of anti-PEG Ab may be different between TD and TI antigens. We have recently demonstrated [34] that anti-PEG IgM induced by PEGylated OVA cleared both PEGylated OVA and PEGylated liposomes from blood, but that induced by PEGylated liposomes cleared only PEGylated liposomes. This suggests that the anti-PEG IgM secreted from marginal zone B cells without help of T cells in response to PEGylated liposomes have low avidity, resulting in weak clearance of PEGylated OVA.

Fig. 8 shows a scheme of possible steps whereby PEGylated liposomes induce TI immunogenicity in the marginal zone of spleen follicles. The PEG on liposomes bind to receptors (BCR) on these IgM memory B cells that express specificity against PEG. The size of liposomes and configuration of PEG on their surface fits in-between 2 or 3 specific B cell receptors and crosslink them to trigger an intracellular signal cascade leading to the proliferation of PEG-specific B cells and

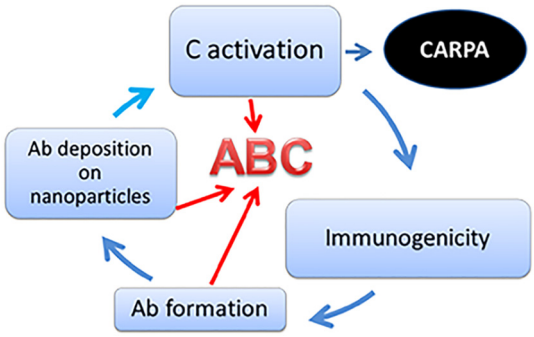

Fig. 6. Complement-activation fed immune stimulatory vicious cycle. The cycle starts with C activation by PEGylated drugs entailing anaphylatoxin (C3a, C5a) release that causes HSRs, i.e. CARPA. The other consequence of C activation, opsonization of PEGylated drugs by C3b and its derivatives (C3dg and C3d), explains their ABC. C activation, a known "bridge" between innate and specific immunity, enhances the rise of anti-PEG Abs which bind to the drugs further enhancing C activation and ABC to close the cycle. Reproduced from Ref. [84] with permission.

differentiation to plasma cells with production and release of specific anti-PEG IgM into the blood [90].

7. Pre-existing (natural) anti-PEG Abs in healthy humans

In addition to post-immunization, anti-PEG Abs are also observed in healthy subjects without any prior exposure to PEGylated drugs or agents. These are referred to as pre-existing, or natural anti-PEG Abs whose prevalence in the normal human population seems to increase over time. In particular, the prevalence of Ab^+ individuals in the healthy population was estimated to be 0.2% in 1984 [32], 25% in 2012 [91] and about 40% in 2016 [92]. This increase may be due to an improvement of the limit of detection of Abs over time and to greater exposure to PEG. Regarding the latter option, Yang et al. [56] pointed to the fact that the skin is always exposed to external stimuli, which causes inflammatory responses and immune cell recruitment. Under such conditions, daily use of PEG applied to skin might activate recruited immune cells, resulting in the induction of pre-existing anti-PEG Abs. Keeping with the idea of transcutaneous immunization, we recently found in hairless rat that daily skin exposure to a PEG-containing cosmetic, but not shampoo and sunscreen, induced anti-PEG Abs whose level corresponded to that induced by PEGylated liposomes (unpublished data). Accordingly, daily exposure to cosmetics, toothpaste, shampoos, sunscreens, that contain some forms of PEG, might contribute to the increase of the prevalence of pre-existing anti-PEG Abs, although a role of oral immunization with PEG-containing soft drinks and internally taken medicines cannot be excluded, either. This multifactorial background of anti-PEG Ab formation also explains the substantial individual variation of anti-PEG Ab titer in blood [23,24,81,82,91,93], with very high values observed in a small fraction (up to 10%) of humans [93].

8. Strategies to suppress PEG immunogenicity

Based on all above possible problems arising from the immunogenicity of PEG, it is clear that reducing or eliminating this effect will be necessary for successful clinical applications of some PEGylated nanodrugs. Accordingly, there is substantial research effort to find ways to achieve this goal [94,95]. The approaches showing more or less success include structural modification of the PEG moiety, for example by replacing methoxy PEG with other functional groups, such as amino (-NH_2), carboxyl (-COOH) and hydroxyl (-OH), or using alternative polymers, such as polyvinyl alcohol, polyvinyl-pyrrolidone or polyacrylamide instead of PEG. Yet, for liposomes, another approach is the insertion of gangliosides into the bilayer beside PEG, which suppresses the immune response. However, perhaps the easiest approach

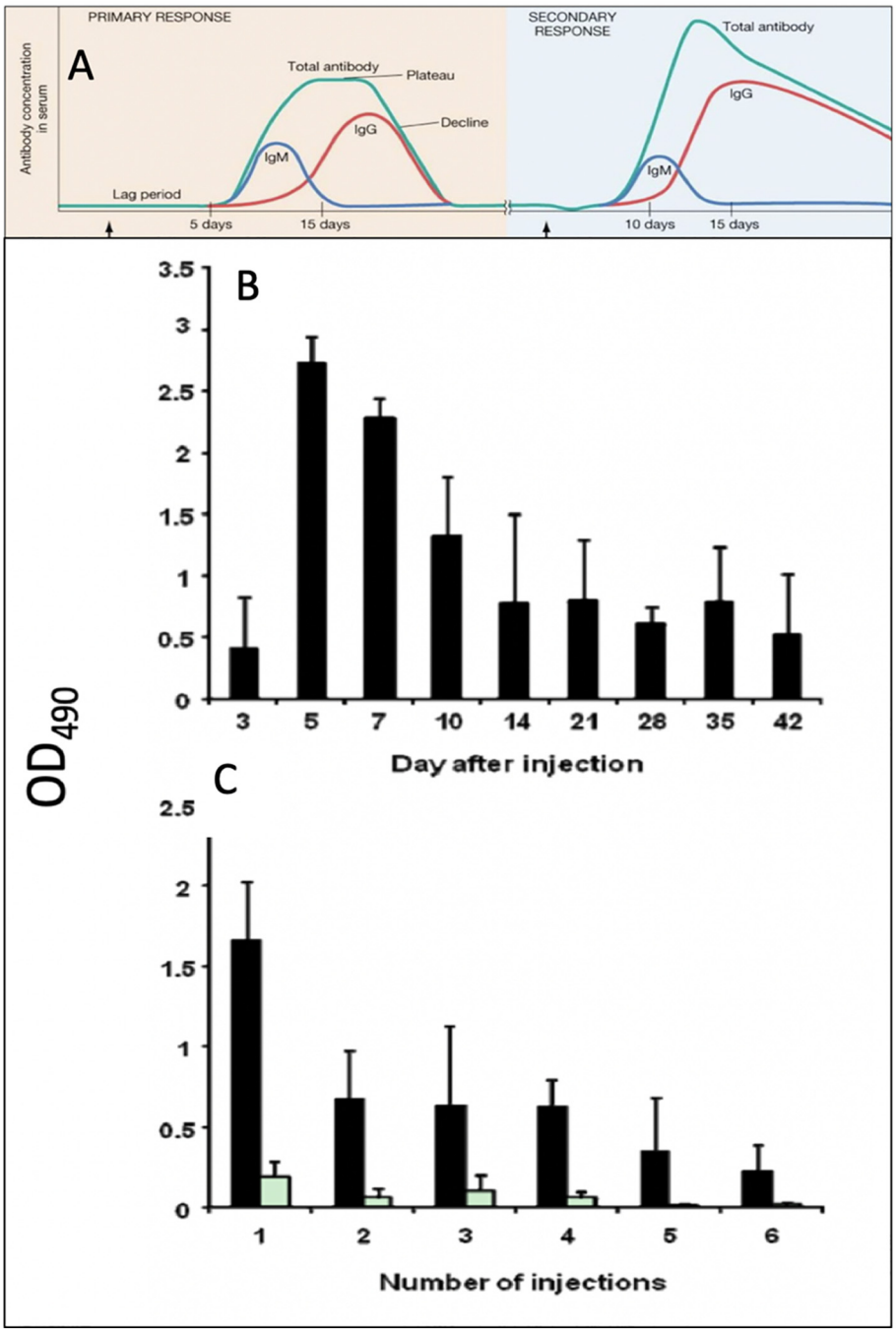

Fig. 7. Features of typical protein-induced T cell-dependent (A) and PEGylated liposome-induced T-cell independent (TI) immuogenicities in mice (B and C). A is a textbook illustration of typical Ab response to protein antigens. B shows anti-PEG IgM production up to 6 weeks following a single i.v. injection of PEGylated liposomes while C presents the anti-PEG IgM production following sequential injections with PEGylated liposomes six times in 7-day intervals in mice,. In C, the closed column represents anti-PEG IgM, and the open column represents anti-PEG IgG. Reproduced from Ref. [60] with permission.

is pretreatment with (a large dose of) drug-free DDS, with or without prolongation of the time interval between injections, since TI immunogenicity may be short-lived [25,26,65,78].

9. Anti-PEG Ab assays

9.1. Assay types and their features

As summarized in Table 2, the earliest method, passive hemagglutination, is rapid and simple, but not sufficiently sensitive and quantitative. To achieve increased sensitivity, Western blot, acoustic membrane microparticle technology (AMMP), enzyme immunosorbent assay (ELISA) and flow cytometry can be used, which amplify the signal by using enzyme reactions or fluorescence. However, these techniques are usually not quantitative in absolute terms, and the detection limits depend on experimental conditions. The surface plasmon resonance (SPR) technology is ultrasensitive, quantitative and fast, but the method is not common due to the special and expensive instrument and reagents needed.

9.2. Anti-PEG ELISA: formats and limitations

Overall, ELISA is the most widely applied technique to detect anti-PEG Abs due to its high sensitivity and capability to quantify Ab levels

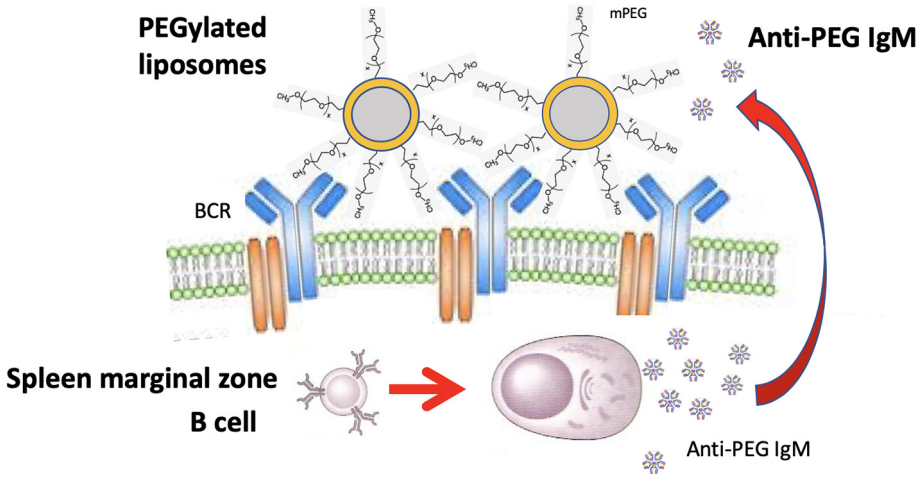

Fig. 8. Molecular mechanism of TI immunogenicity by PEGylated liposomes in splenic marginal-zone IgM memory B cells. Scheme based on Refs. [57, 63, 88–90].

at least in relative terms. In direct anti-PEG ELISAs a color reaction is generated by enzyme-conjugated host IgG or IgM specificAbs recognizing PEG-specific Abs in serum or plasma bound to a PEG coated surface. In bridge-format ELISAs [102], PEG specific surface bound anti-PEG Abs are detected via the conjugated antigen, rather than anti-host IgG or IgM. Thus, the anti-PEG Abs are wedged inbetween two layers of PEGylated antigen due to these Abs' bi- or multivalency: the 1st one is coated on the surface for capture and the 2nd, most often biotinylated antigen, is preincubated with the sample for ultimate detection by a streptavidin-enzyme conjugate. The advantage of bridge ELISAs over direct ones is obvious when immunogenicity of chimeric, humanized, or fully human monoclonal Ab-based drugs are measured whereupon a cross reactivity of conjugated anti-human IgG or IgM with the plate-bound antibody drug cannot be excluded [98]. However, in absence of such danger in case of anti-PEG Abs, the bridge assay, which involves more steps, may have no advantage over the direct ELISA [102]. The use of competitors is also popular in direct systems wherein the concentration of competitors is measured at a constant serum dilution, usually that inhibits 50% of the maximal binding [27].

A main limitation of anti-PEG ELISAs lies in their questionable validity to *in vivo* conditions in light of the likely difference of the Abs' avidity to different PEGylated formulations *in vivo* and in the test system, *in vitro*. In particular, the ELISA cannot truly reflect the binding conditions in blood, since the binding of Abs to flat plate-attached PEG antigens is unlikely the same as the binding in plasma to protein- or DDS-attached PEG.

9.3. Anti-PEG ELISAs: considerations for standardization

As mentioned above, there is no “gold standard” or “authentic calibration standard” that could quantify the exact avidity, and, hence, amount of anti-PEG Abs that bind *in vivo* to PEGylated drugs. There are several reasons for this shortcoming. One is that PEGs on proteins, DDS and plate-bound anchor structures are likely to have different primary-, secondary- and tertiary structures implying, in analogy to proteins, the length, branching and intra- and inter-molecular bonds that stabilize the polymer in space. Yet other variables that cannot be reproduced in a standard include the binding of additional proteins to the PEGylated NPs that have an impact on Ab binding and the variations of individual immune responses to PEGylated drugs. The latter circumstance is manifested in different combinations of anti-PEG Ig classes and IgG subclasses in individual samples, each having different binding characteristics. For all these reasons there is much work ahead to develop a quantitative anti-PEG ELISA for universal use.

At present, the most widely used approach to estimate anti-PEG Ab levels in a way that enables inter-experimental comparisons is titration, which involves the measurement of the dilution where the Ab binding signal reaches the baseline or a preset lower limit value. However, titration assays also depend on test specifics, such as the coating and the detergent used in the ELISAs. To illustrate with an example, in the most commonly used anti-PEG ELISAs, DSPE- or BSA-conjugated PEGs are attached to the surface of ELISA plates by either drying (using alcohol as solvent) or letting the adherence spontaneously proceed due to secondary intermolecular forces (e.g. using plates with different hydrophobicity). Plates with apolar surface coating, like the Polysorp (Nunc) plates, preferentially bind the hydrophobic DSPE part of PEG-DSPE antigens via Wan der Waals forces, providing better access of Abs to the PEG part relative to plates with hydrophilic coatings, such as Maxisorp plates which tend to attract the hydrophilic PEG portion. For the same reason, the length of PEG is critical for Ab access to the different epitopes on PEG. It seems that 2 kDa PEG is sufficiently long to provide reproducible results when an apolar binding surface is used.

Yet another critical assay variable is the detergent used in the blocking and washing steps to reduce unspecific absorption of proteins to the plate and the antigen during ELISA [103]. The issue was raised by questioning the use Tween-20 (polysorbate), a widely applied non-ionic detergent, for anti-PEG Ab detection since it contains polyoxyethylene (in addition to sorbitan monolaurate) which may compete with PEG for Ab binding [92]. The authors suggested the use of zwitterionic 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) instead, which is a common surfactant in electrophoresis and isoelectric focusing. However, we have used Tween-20 in an ELISA measuring anti-PEG IgM in pig blood [78] and obtained no evidence for Tween 20's interference with anti-PEG Ab detection. As shown in Fig. 9, measuring of anti-PEG IgM with ELISA using 0.1% Tween-20 gave a low (0.6) (Fig. 9A) and high (1.4) (Fig. 9B) signal (A_{450} was 0.6 and 1.4, in naïve and a PEGylated liposome (Doxebo)-immunized pigs, respectively) at the lowest dilution for these samples, which is consistent with effective immunization of the animal against PEG. Importantly, the high signal at low dilution in Fig. 9B decreased upon increasing the dilution, and it showed dose dependence, 0.1% Tween-20 giving higher signal than 0.05 or 0.01%. Such dilution and dose dependence of anti-PEG Ab measurement could not be seen if Tween-20 interfered with the assay. Moreover, the experiment in Fig. 9 also shows the unique utility of Tween-20, since its omission from the medium eliminated the capability to dilute out the A_{450} signal. Notably, the experiment in Fig. 9 showed greatest sensitivity with 0.05% Triton X-100, which may be a useful information for future studies.

Table 2
To analyze the immunogenicity of PEG, a great variety of techniques are available whose main features has been tabulated in order of increasing sensitivity (Table 2).

Method	Test principle	Detection limit*	Advantages	Limitations	Ref.
Passive hemagglutination	Erythrocyte surface is modified with methoxyPEG (or other PEG derivative) and mixed with serial dilutions of test serum. In the presence of anti-PEG Abs, the erythrocytes agglutinate which can be followed easily. Titer is defined as the highest serum dilution giving complete hemagglutination.	No information	Relatively fast and inexpensive assay. PEGylated erythrocytes may be similar to membrane attached PEG derivatives, therefore they model the immunogenicity of larger PEGylated NPs (e.g., liposomes).	It cannot discriminate Ab isotypes. Its sensitivity is relatively low.	[31,91]
Western blot analysis	Dye-conjugated PEGylated antigen (e.g. PEGylated liposome) is incubated with samples (serum). Ab-antigen complex is enriched by gel filtration. Finally, the dye containing fraction is subjected to SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membrane, and anti-PEG Abs are identified and visualized by enzyme reaction after recognizing them by conjugated anti-IgG/IgM Abs.	1 μg/mL anti-PEG or even less depending the efficiency of Ab enrichment.	It models the physiological PEG–anti-PEG Ab interaction inasmuch as the same soluble antigen could be used for detection than for Ab generation (e.g. PEGylated liposomes). As a consequence, functionally active and important Abs are detected. It can discriminate anti-PEG Ab isotypes.	It consists of several steps, which might decrease the accuracy of the measurement. For Ab-antigen enrichment step, antigen must be stained, which might not be obvious. There is no standard to quantitate the data.	[96]
Acoustic membrane microparticle (AMMP®) technology	Samples are diluted and incubated with paramagnetic beads coated with methoxyPEG to capture anti-PEG Abs. The complex is captured on an acoustic membrane coated with Protein A. The change in mass on the membrane results in a signal proportional to the mass of anti-PEG Abs.	1 μg/mL anti-PEG IgG	This method provides absolute results.	Protein A binds mainly IgG, thus the method's quantitation of anti-PEG IgM is unreliable.	[97]
Microtiter plate-based enzyme immunosorbent assays (ELISAs)	The common feature of these tests is that PEG-containing antigen is attached somehow to a 96-well plate, which captures PEG specificmAbs from the test samples. Thereafter Abs are visualized by enzyme reaction after they are recognized by conjugated antigens (bridging type) or antibodies (or other detetector molecules). Further details are discussed below (in 9.2 and 9.3)	100 ng/mL level of anti-PEG Abs	It has relatively high sensitivity It provides reliable and quantitative anti-PEG Ab detection. It is the most widely applied technology for anti-PEG Ab quantitatioin It can discriminate among Ab isotypes. It does not require expensive and special equipment. A substantial knowledge about PEG-Ab interaction is originates from this method.	Concentration needs to be calculated by serial dilution of a standard. However, it is not possible to produce an Ab standard whose binding affinity (avidity)_ to the plated antigens is the same as the Abs in the sample. Therefore, the assays provide relative values instead of concentration in absolute terms. Hence, there is wide variation in the literature regarding anti-PEG Ab units and value ranges.	[98–100]
Flow cytometry	PEG is grafted on a polymer matrix, such as TentaGel-OH polystyrene microparticles. After washing steps, particles are stained for bound IgG and/or IgM with fluorescent dye labeled anti-IgG and/or IgM. The mean fluorescence intensity of particles is determined by flow cytometry.	It is not known exactly. Detection limit of a FACS based bead array is 10–5000 pg/mL.	It can discriminate anti-PEG Ab isotypes. It has great potential because of the high sensitivity of measurement.	PEG is presented by a polymer matrix instead of its treatment-relevant support (i.e., NPs), therefore the binding conditions are different; the obtained values do not necessarily reflect in vivo binding. There is no fluorescence standard.	[7]
Surface plasmon resonance (SPR)	A PEG-functionalized polymer is immobilized onto SPR sensor chips for capturing anti-PEG Abs from the samples flown through the sensor units. Bound anti-PEG Abs are quantified by wavelength shift proportional with the amount of Ab bound to the sensor chip.	1 ng/mL anti-PEG Abs	It is a highly sensitive measurement with low background, enabling quantification of anti-PEG Abs in absolute concentration. It can discriminate anti-PEG Ab isotypes.	SPR is a powerful label-free technique to monitor biomolecule-surface interactions. However, it requires a special, expensive instrument, and like other methods using flat or matrix support for PEG, the measurement does not necessarily mimic the in vivo interactions (avidity) of Abs with protein or NP-conjugated PEG	[101]

Measurements are ranked in descending order according to their estimated detection limit. However it must be emphasize that real detection limit is dependent from test details, particular in the case of Western Blot and ELISAs.

Regarding the question, why Tween-20 escapes from PEG-specific Ab binding, the most likely explanation lies in the substantial structural differences between this surfactant and 2K-PEG-DSPE, the antigen used to coat the ELISA plate in our assay [78]. Tween-20 consists of 5 ethylene oxide units at 4 different sites on the moleculewhile 2K-PEG-DSPE contains 46 ethylene oxide units in a raw (Fig. 1), implying entirely different epitope exposure for Ab binding.

The message from the above example is that one needs to be cautious to make predictions and extrapolations regarding technical details and results in one particular anti-PEG ELISA to another, and that each ELISA test benefits from thorough validation before making generalized conclusions. At least until a gold standard anti-PEG assay will become available.

10. Conclusions

The immunogenicity of PEGylated drugs is critical to the therapeutic success and safety of an expanding number of nano-biopharmaceuticals. In order to reduce the risk of the ABC phenomenon, which might decrease the therapeutic effect upon repetitive treatments, as well as the risk of CARPA, which may cause severe HSRs with potential death, it seems useful to test patients for natural anti-PEG Ab titers

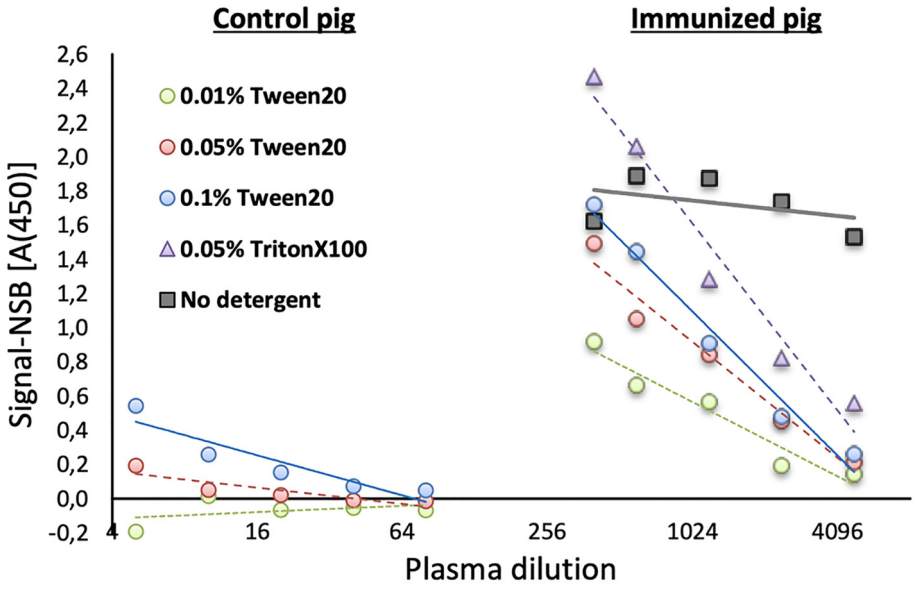

Fig. 9. Plasma dilution-dependent readings [A(450)] of porcine anti-PEG IgM specific direct ELISAs using 0.01, 0.05, 0.1% Tween-20, or 0.05% Triton X-100 surfactants or no detergent in the washing and incubation steps. Experimental details are in Ref. [78] "NSB" means non-specific binding, obtained in wells that were not coated with antigen.

and proneness for C activation in their blood samples prior to and/or after treatment with a PEGylated drugs [26]. Likewise, testing of the immunogenicity of PEGylated drugs in animal models that are sensitive not only to ABC phenomenon but also to the anaphylactic activity of the drugs, such as the pig "CARPA" assay [78,104] could flag potentially strong immunogenicity in man. Needless to emphasize, scientific research into these problems will hopefully unveil more details of these adverse immune effects and thus suggest more preventive measures in the future.

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