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Review Article

Antibodies against polyethylene glycol in human blood: A literature review

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ABSTRACT

Polyethylene glycol (PEG) conjugation, i.e. PEGylation, is a successful strategy to improve the pharmacokinetics and pharmacodynamics of biopharmaceuticals. In the past few decades, PEGylation technology has developed tremendously, and > 15 PEGylated therapeutics have been brought to market, with more in development. However, the widely accepted assumption that PEG would have no antigenicity or immunogenicity is increasingly challenged with popularization of PEGylation technique. Although PEGylation indeed reduces the immunogenicities of the modified molecules, and even appears to completely eliminate their immunogenicities, yet emerging clinical evidence of anti-PEG antibodies (including both pre-existing and PEGylated therapeutics-treatment induced anti-PEG antibodies) have been attracted more and more attention. Anti-PEG antibodies were detected in not only patients treated with PEGylated therapeutics but also PEGylated drugs treatment-naïve individuals with a prevalence from < 1% to 72%. In patients, the existing anti-PEG antibodies may attenuate therapeutic efficacy of PEGylated drugs and increase adverse effects. Although there is no golden standard avenue, several types of methods, including passive hemagglutination, Western Blot, enzyme linked immunosorbent assay, flow cytometry, Meso Scale Discovery technology, Acoustic Membrane Microparticle assay, and surface plasmon resonace technique, were established and used to screen, confirm and quantitatively detect anti-PEG antibodies. Herein, we focused on reviewing the prevalence of anti-PEG antibodies in healthy and PEGylated therapeutics-treated patients, and highlighting the detection methods for pre-screening and quantitative detection of anti-PEG antibodies.

1. Introduction

Poly ethylene glycol (PEG) is an inert, biocompatible synthetic polymer with repeating units of ether oxygen (-CH_2-CH_2-O-) formed by polymerization of ethylene oxide or ethylene glycol under alkaline catalysis (Harris, 1992). Generally speaking, PEG is a mixture of homologues (i.e., ethylene glycol) with different molecular weights (e.g. 2000 Da, 8000 Da, 20 kDa) and shapes. Although the common PEGs are linear in structure, of which drugs can only bind to the end, yet there are other forms of PEGs with different geometries. For instance, branched PEGs have 3–10 PEG chains emanating from a central core group while star PEGs have 10 to 100 PEG chains from a core group (D'souza & Shegokar, 2016). The more PEG chains, the more hydroxyl groups for drug conjugation it provides. From the enthalpic point of view, each ether oxygen subunit is demonstrated to be firmly associated with two or three water molecules in solution. PEGylation refers to covalent conjugation of one or more PEG chains to a target molecule. It also involves formulating of a drug into PEGylated drug-delivery vehicles in the pharmacy (X. W. Zhang, Wang, Ma, & Wu, 2014). In the 1970s, Abuchowski and colleagues conducted two pioneering studies on investigating immunogenicities of PEGylated bovine liver catalase (PEG-catalase) and PEGylated bovine serum albumin (PEG-BSA) using a rabbit model (Abuchowski, McCoy, Palczuk, van, & Davis, 1977; Abuchowski, Van, Palczuk, & Davis, 1977). They demonstrated that PEGylation significantly reduced the immunogenicities of catalase and BSA, and even appeared to completely eliminate their immunogenicities when sufficient PEG was attached. Furthermore, no evidence of immune response to attached PEG, or of tissue or organ

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damage was seen. Since then, PEGylation has developed tremendously and been an important approach to improve the pharmacokinetics and pharmacodynamics of biopharmaceuticals (e.g., proteins, peptides).

PEGylation of drugs can increase the water-solubility and stability, lower the enzymatic degradation, reduce the phagocytosis by reticuloendothelial system (RES), decrease renal clearance, control release and downregulate the immunogenicity or antigenicity (Veronese & Pasut, 2005). Up to now, several PEGylated proteins have been approved for clinical use, including Oncospar (Rau et al., 2018), PEGasys (X. Liu et al., 2018), PEGintron (Lin et al., 2017), Krystexxa (Ganson, Kelly, Scarlett, Sundy, & Hershfield, 2006), etc..

However, drawbacks of PEGylation also emerge with popularization of this technique. There are increasing concerns about the use of therapeutic PEGylated pharmaceuticals due to emerging clinical evidence of anti-drug antibodies (ADA) recognizing PEG, i.e. anti-PEG antibodies (including IgG and IgM), which suggests that PEG is both immunogenic and antigenic. Circulating anti-PEG antibodies have been detected in patients treated with some PEGylated therapeutics, and in clinical trials of PEG-uricase (Hershfield et al., 2014) and PEG-asparaginase (Rau et al., 2018). Even worse, existing anti-PEG antibodies in blood can markedly accelerate blood clearance of the PEGylated drugs, lower clinical efficacy, and increase the risk and severity of infusion reactions. Circulating anti-PEG antibodies have also been found in PEGylated therapeutics treatment-naïve individuals, possibly evoked by chronic exposure to free PEGs which are present in commonly used consumer products (including cosmetics, pharmaceuticals, processed food, etc.) (Garay, El-Gewely, Armstrong, Garratty, & Richette, 2012). High levels of such pre-existing anti-PEG antibodies have recently been linked to serious first-exposure allergic reactions to a PEGylated RNA aptamer, which led to early termination of a clinical trial (Povsic et al., 2016). Despite of the serious consequences of circulating anti-PEG antibodies, influence of anti-PEG antibodies in the therapeutic effect and causing side effect is still in the mystery of the unanswerable questions. Neither concentrations of anti-PEG antibodies in human beings (both healthy populations and PEG-drug-treatment individuals) nor the detection methods of anti-PEG antibodies are well acknowledged.

Therefore, we highlight the existing global evidences on the current situation of anti-PEG antibodies in human blood and advances in detection methods of anti-PEG antibodies which are useful for the diagnosis and management of PEG-drug-treated individuals.

2. Survey methodology

Four online databases, including Google Scholar, PubMed, Scopus and Web of Science, were used to collect relevant studies. Articles were screened by the following key combinations in English: (1) anti-PEG antibody, (2) detection of anti-PEG antibody, (3) concentrations/levels of anti-PEG antibody, (4) PEGylation, PEGylated therapeutics or PEG conjugates. Articles no related with anti-PEG antibody were exclude regardless of the impact factor of the journal, authors or published date.

3. Anti-PEG antibodies in human blood

3.1. Anti-PEG antibodies in clinic

Since the approval of Gris-PEG® in 1975, the number of therapeutic PEGylated pharmaceuticals has steadily increased. So far, nineteen PEGylation of therapeutics have been approved by FDA (Table 1), including Omontys® and Omontys Preservative Free® (Takeda Pharms USA) which have been voluntarily discontinued due to 19 serious hypersensitivity reactions, involving three deaths (DeFrancesco, 2013), with more in research & development. Widely promoted and used in clinic, these drugs are increasingly reported to induce severe anaphylaxis and infusion reactions associated with PEG. Searching studies of interventions associated with the keyword “Polyethylene glycol” or “PEG” in *ClinicalTrials.gov*, eighty-four active studies of a PEG-containing intervention were found, in addition with 355 open trials (including not yet recruiting, recruiting, or enrolling by invitation) (Searching date: Sep. 8, 2019).

PEGylation technology is now considered to be one of the most advanced approaches in modern pharmaceutical industry. As PEGylated drugs are widely promoted and used in clinic, there are increasing reports on severe anaphylaxis and infusion reactions associated with PEG. Furthermore, anti-PEG antibodies are becoming an ever-growing problem. The potential immunogenicity and antigenicity of PEG which can induce PEG-specific antibodies were first reported by Richter and colleagues in 1983 (Ary Wolfgang Richter & Åkerblom, 1983). However, they identified that, in allergic patients, the anti-PEG antibodies were predominantly IgM, not IgG. Since then, numerous studies have revealed that typical PEGylated therapeutics, such as PEG-uricase and PEG-asparaginase, induce both anti-PEG IgM and IgG in patients who first receive injection of PEGylated drugs (Ganson et al., 2006; Lipsky et al., 2014) or have been injected into these products several times (Y. Liu et al., 2019; Rau et al., 2018). Y. Liu et al. (2019) conducted a clinical trial in which PEG-asparaginase and native L-asparaginase were respectively used to treat acute lymphoblastic leukemia patients. Unexpectedly, they discovered that, of all the specific anti-PEG-asparaginase antibodies (including both anti-PEG antibody and anti-L-asparaginase antibody), anti-PEG antibody but not anti-L-asparaginase antibody was the predominant component (96%). Similar results were also reported on inducing anti-PEG antibodies (including IgG and IgM) in 5 of 13 patients treated for chronic refractory gout with PEG-uricase, though the titer of anti-PEG antibodies was low (Hershfield et al., 2014). All of these suggest that PEG has the potential of immunogenicity and antigenicity, and anti-PEG antibodies are very common in patients treated with PEGylated therapeutics.

3.2. Anti-PEG antibodies in healthy populations

About two or three decades ago, the majority of experts on PEG-related research generally accepted the assumption that PEG was not antigenic or immunogenic (Garratty, 2004). To the best of our knowledge, A. W. Richter and Akerblom (1984) pioneered in analyzing anti-PEG antibody in healthy blood donors by employing the method of passive hemagglutination. Maybe due to less exposure to PEG and limitation of detection approaches at that time, anti-PEG antibody was only observed in approximately 0.2% healthy donors, which was considered to be of no clinical significance and probably not interfere with the clinical use of PEGylation therapeutics. Entering the 21st century, however, things change a lot. Armstrong et al. (2007) discovered that asparaginase activity was undetectable in 15 patients treated with PEG-asparaginase and anti-PEG antibody was detected in 9 by serology method (passive hemagglutination) and in 12 by flow cytometry approach. It was supposed that the existing anti-PEG antibody might be elicited by PEG-asparaginase and entail rapid clearance of PEG-asparaginase. Nevertheless, anti-PEG antibody was also detected in 6 of 16 native asparaginase treatment patients who had never been exposed to PEG-asparaginase. Compared with the study of Richter et.al, a significantly higher occurrence (~25%) of anti-PEG antibodies in healthy blood donors by the approach of serology was reported in this study (Armstrong et al., 2003; Garratty, 2004). It should be pointed out that only 5% showed strong reactions (3+ or 4+). Simultaneously, anti-PEG antibodies were detectable by flow cytometry which showed that the incidence rates of only IgG were 18%, only IgM were 14%, and IgG + IgM were 13%, respectively. These findings suggest that anti-PEG antibodies are pre-existing (naturally occurring) in some group of population. To further investigate the effect of anti-PEG antibody on PEGylated drugs and optimizing dosage regimen containing PEG or PEG derivatives, it is crucial to screen and monitor anti-PEG antibody levels in target populations.

A few years ago, a comprehensive study on anti-PEG antibodies in 1504 (756 male and 748 female) healthy Han Chinese individuals

Table 1
Approved therapeutic PEGylated pharmaceuticals.

Drug Name (Active Ingredients)	Approval Date	Indications
Adagen® (PEG-adenosine deaminase)	1990.3.21	SCID
Asparlas® (Calaspargase Pegol-MKNL)	2018.12.20	Acute lymphoblastic leukemia
Cimzia® (Certolizumab Pegol)	2008.4.22	Crohn's disease; Rheumatoid arthritis, Psoriatic arthritis; Ankylosing spondylitis; Non-radiographic axial spondyloarthritis; Plaque psoriasis
Copegus® (Ribavirin)	2002.3.12	CHC
Fulphila® (Pegfilgrastim-JMDB)	2018.6.4	Patients with cancer receiving myelosuppressive chemotherapy
Gris-PEG® (Griseofulvin Ulreamicrosize)	1975.4.16	Ringworm infections
Krystexxa® (PEGloticase)	2010.9.14	Chronic gout in adult patients refractory to conventional therapy
Macugen® (PEGaptanib Sodium)	2004.9.17	Neovascular (wet) age-related macular degeneration
Neulasta® (PEGfilgastim)	2002.1.31	Neutropenia; HSARS
Omontys® (PEGinesatide acetate)	2012.3.27	Anemia associated with chronic renal failure
Omontys Preservative Free® (PEGinesatide Acetate)	2012.3.28	Anemia associated with chronic renal failure
Oncaspar® (PEGaspargase)	1994.2.1	Acute lymphoblastic leukemia
Palynziq® (PEGvaliase-PQPZ)	2018.5.24	Phenylketonuria
Pegasys® (PEGinterferon Alfa-2A)	2002.10.16	CHC; CHB
Pegintron® (PEGinterferon)	2001.1.19	CHC
Plegridy® (PEGinterferon Beta-1A)	2014.8.15	Multiple sclerosis
Revcovi® (Elapegademase-LVLR)	2018.10.15	ADA-SCID
Somavert® (PEGvisomant)	2003.3.25	Acromegaly
Udenyca® (PEGfilgrastim-CBQV)	2018.11.2	lower the chance of infection in people with bone marrow problems caused by chemo

SCID, severe combined immunodeficiency disease; CHC, chronic hepatitis C; CHB, chronic hepatitis B; ADA-SCID, adenosine deaminase severe combined immune deficiency; HSARS, hematopoietic Subsyndrome of acute radiation syndrome.

residing in Taiwan was conducted (Chen et al., 2016). Chimeric anti-PEG antibodies (c3.3-IgG and cAGP4-IgM) were constructed through genetic engineering technology to serve as positive controls in subsequent direct and competitive ELISA assays. Samples with absorbance values at least 3 times as great as the mean background absorbance in the direct ELISA and absorbance reduced by 35% when PEG-liposomes are added in the competitive ELISA are considered as positive samples. By comparing with c3.3-IgG or cAGP4-IgM standard curves respectively, relative concentrations of anti-PEG IgG or anti-PEG IgM in samples assessed can be gained. After measured by both direct and competitive ELISAs, a total of 666 (44.3%) participants had positive anti-PEG antibodies. More detailedly, 386 (25.7%) of the 1504 healthy donors had detectable anti-PEG IgG, 407 (27.1%) had anti-PEG IgM, and 126 (8.4%) had both anti-PEG IgG and IgM. Of particular note is that gender significant influences the incidence rate of anti-PEG antibodies. The incidence of both anti-PEG IgG (28.3% vs 23.0%, $p = .018$) and IgM (32.0% vs 22.2%, $p < .0001$) were markedly higher in female than in male. Whereas, neither anti-PEG IgG concentrations (4.93 μg/mL vs. 6.77 μg/mL, $p = .262$) nor anti-PEG IgM concentrations (1.98 μg/mL vs. 1.49 μg/mL, $p = .170$) showed significant differences between females and male. Interestingly, the incidence of anti-PEG IgG negatively associated with age (~60% incidence for 20-year-old donors and ~20% for donors > 50 years), however no age-related discrepancies were observed for anti-PEG IgM. Though the mechanism of the significantly higher prevalence of anti-PEG IgG in females than males is currently unclear, yet it could be partially explained by the assumption that females are more exposed to cosmetic products containing PEG or PEG derivatives and females are prone to have more autoimmune responses compared to males. And they also speculated that a higher prevalence of anti-PEG IgG in younger individuals might reflect the fact that immune responsiveness diminishes gradually with age. However, more studies are required to verify these data and to test the hypotheses.

More recently, Yang et al. (2016) established rigorously validated competitive ELISA avenues with engineered chimeric anti-PEG monoclonal antibodies (c6.3-IgG and cAGP.3-IgM) as standards, and quantified the concentrations of anti-PEG IgG subclasses (IgG1, IgG2, IgG 3and IgG4) and anti-PEG IgM. Surprisingly, detectable anti-PEG antibodies were found in as many as 72% of the 377 samples (~18% IgG only, ~25% IgM only, and ~30% both IgG and IgM), which was significantly different from the previously reported incidence of pre-existing anti-PEG antibodies varying widely according to different studies, ranging from 0.2%–44% (Hershfield et al., 2014; Lubich et al., 2016; A. W. Richter & Akerblom, 1984; Tillmann et al., 2010). Although the overall prevalence of anti-PEG antibodies was quite high, yet just low levels of anti-PEG antibodies were detected in majority of these positive specimens, with only 8% of the samples possessing anti-PEG antibodies (~7% IgG and ~1% IgM) in excess of 500 ng/mL. In addition, they reported an interesting phenomenon that IgG2 (57% of the positive participants), instead of IgG1 (26%), was the predominant anti-PEG IgG subclass. Because of the great disparities in prevalence of anti-PEG antibodies when compared with previous studies, they measured anti-PEG antibodies in healthy human sera samples banked from the 1970s ($n = 30$), 1980s (n = 30) and 1990s ($n = 19$) to determine whether these differences reflect a true increase in the prevalence of anti-PEG antibodies among the population over time or are likely attributed to differences in sensitivity of various detection assays. Anti-PEG antibodies were detected in ~56% of the samples (20% IgG only, 19% IgM only, and 16% both IgG and IgM), indicating that the differences are likely attributed in part to the detection methods and anti-PEG antibodies might be longstanding.

Through a genome-wide association study, Chang et al. (2017) provided a new perspective for the induction of anti-PEG antibodies. They demonstrated that single-nucleotide polymorphisms (especially rs12590237) of the immunoglobulin heavy chain locus were significantly associated with anti-PEG IgM response, which may provide novel genetic biomarkers for predicting the immunogenicity and antigenicity of PEG and efficacy of PEGylated therapeutics.

All above, although more and more evidences imply that pre-existing anti-PEG antibodies may attenuate the clinical efficacy and safety of PEGylated therapeutics, the prevalence and concentrations of pre-existing anti-PEG antibodies are not well known so far in not only healthy individuals and treatment-naïve patients but PEGylated drug-treated patients. Based on the available evidences, it is necessary and beneficial to monitor ant-PEG antibodies before, during and post the treatment of PEGylated therapeutics. Hence more rigorous methodological studies are required to survey the incidence and concentrations of pre-existing or PEGylated therapeutics-induced anti-PEG antibodies (IgG, IgM, or both).

4. Detection of anti-PEG antibodies

Recently, McSweeney, Versfeld, Carpenter, and Lai (2018) conducted a survey on physician awareness of immune responses to PEGylated drugs. The average time of the surveyed physicians spent in clinical practice was 14 years, and a total of 83% (66/80) of respondents prescribed PEGylated medicines. Although 91% of the participants were aware of anti-drug antibodies, yet only 22% were aware of the anti-PEG antibody responses. And even worse, limited attention (~35%) was paid to the inclusion of PEG in prescribed PEG-conjugated formulations. The anti-PEG antibodies in human blood significantly shorten plasma half-life of the PEGylated pharmaceuticals, lower the clinical efficacy, and increased the risk and severity of infusion reactions. It should be particularly concerned that it has documented evidences of a link between adverse clinical outcomes and anti-PEG antibodies. Omontys® (PEGinesatide), a PEGylated synthetic peptide to mimic the pharmacological activity of erythropoietin for the treatment of anemia, was withdrawn in the USA due to serious hypersensitivity reaction and caused death of a small proportion of patients (3 cases) administered this drug for the first time (Eckardt, 2013). As aforementioned, anti-PEG antibodies are detrimental to the patients in need of treatment with PEGylated therapeutics and need attract more attentions (Anchordoquy & Simberg, 2017). Although there currently lacks a golden standardized assay protocol to quantitatively measure the levels of anti-PEG antibodies, there have been several types of technologies for the screening and confirmation of anti-PEG antibodies, and the focus of this section is to review some of them.

4.1. Passive hemagglutination

To our knowledge, the earliest study on detecting anti-PEG antibodies was published in 1983 (Ary Wolfgang Richter & Åkerblom). Rabbits were immunized with monomethoxy PEG modified proteins, and the eliciting anti-PEG antibodies were detected by agglutination of PEG-sensitized blood group O red blood cells (PEG-RBCs). Titers were defined as reciprocals of the highest serum dilution giving complete agglutination. They subsequently conducted the first investigation of analyzing anti-PEG antibodies in human blood sample also by the method of passive hemagglutination, and indicated that the prevalence of naturally occurring (pre-existing) anti-PEG antibody were 0.2% and 3.3%, respectively, in healthy blood donor and treatment-naïve allergic patients (A. W. Richter & Akerblom, 1984). After > 2 decades of development, Armstrong et al. (2007) investigated circulating anti-PEG antibodies in individuals with acute lymphoblastic leukemia using this technology. PEG-RBC polymer was prepared by modifying blood group O RBCs with methoxy-PEG-succinimidyl propionate. Test sera were then mixed with PEG-RBCs, and samples that were positive for anti-PEG antibodies could agglutinate the PEG-coated RBCs. Agglutination reflecting anti-PEG antibody concentrations (semi-quantitative analysis) was scored according to the 0–4+ scale. Of the 15 test specimens, nine were positive by the agglutination method while 12 were positive by flow cytometry technique. In recent years, the method of passive hemagglutination has been seldom used in the measurement of anti-PEG antibodies as consequence of lack of accuracy, sensitiveness and quantitativeness.

4.2. Western Blot

To our knowledge, up to now, there is only one report on detecting anti-PEG antibodies by Western Blot (Sroda et al., 2005). Small PEGylated liposome suspension intravenously injected to rabbits once a week for consecutive 6 weeks, and sera was obtained before each injection. With 3-h incubation of the rabbit serum and PEG-liposomes stained by patent blue violet (PBV) at the ratio of 1:1, PBV stained PEG-liposomes can adsorb proteins in the sera to form PEG-liposome-protein complex. This PEG-liposome-protein conjugate was separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. If alkaline phosphatase (AP) goat anti-rabbit IgG can specifically bind to the proteins adsorbed PBV stained PEG-liposomes, it is explicit that the protein obtained is anti-PEG IgG. Although anti-PEG antibody can be successfully recognized, Western Blot is a just method for qualitative or semi-quantitative analysis. Furthermore, there is little information about sensitivity and reproducibility of this method for detecting anti-PEG antibodies.

4.3. Enzyme linked immunosorbent assay

Enzyme linked immunosorbent assay (ELISA), including indirect ELISA, sandwich ELISA and competitive ELISA, is widely applied in the detection and quantification of circulating anti-PEG antibodies (mainly IgG and IgM) due to the easy-to-use procedures. Various types of ELISAs are employed with modification to the basic ELISA steps. The key step of ELISA is the direct or indirect detection of anti-PEG antibodies by adhering or immobilizing the antigen (PEG or PEG derivates) onto a solid-phase support (e.g., 96-well plate).

Indirect ELISA, one of the most typical kinds of ELISAs, involves two binding processes of primary antibody and enzyme-labeled secondary antibody. PEG or PEG conjugates are first coated on the 96-well plate to bind anti-PEG antibodies in specimens, followed by the incubation with the labeled secondary antibody (Fig. 1A). The existence and levels of anti-PEG antibodies can be reflected according to the OD values (X. Y. Wang, Ishida, & Kiwada, 2007). Indirect ELISA is a feasible and widely used way to measure the amount of anti-PEG antibodies and to compare the immunogenicities of PEG with various molecular weights and structures as well as different types of PEG conjugates (Rau et al., 2018; Shimizu et al., 2018; P. Zhang et al., 2018). However, the disadvantages of indirect ELISA are also obvious that it may lead nonspecific binding because of cross-reaction induced by the secondary antibodies.

Another effective ELISA type for detecting anti-PEG antibodies is competitive immunodetection (Fig. 1B). The central event is a competitive binding process in which the pre-coated antigen (PEG or PEG conjugates) in the 96-well plate competes with serum or plasma add-in antigen (competitor antigen) for binding the target antibody (Gan & Patel, 2013; Moreno et al., 2019). The procedures of competitive ELISA are different in some respects when compared with indirect ELISA, sandwich ELISA and direct ELISA. The simplified procedures are as follow. As same as indirect ELISA, competitive ELISA also begins with that PEG antigen (e.g., mPEG2000-DSPE) is coated on the surface of the 96-well plates. Two specific antibodies, competitor antibody (an enzyme-conjugated anti-PEG antibody) and anti-PEG antibody in the specimens are employed. Cumulative competition occurs between the two antibodies for binding the same PEG antigen. If anti-PEG antibody presents in the test samples, the specific binding of antigen and antibody occurs, leaving behind very small amounts of PEG antigen available for binding with the enzyme-labeled competitor antibody. When the unbound enzyme-labeled competitor antibody was washed off, a substrate was added in to elicit a chromogenic or fluorescent signal. The signal changes negatively associate with the levels of anti-PEG antibody in the test specimens (Fix et al., 2018). This method is efficient to analyze specificity of anti-PEG antibodies against separate parts of PEG conjugates (Fix et al., 2018; Henry et al., 2016; Y. Z. Qi et al., 2017; Sherman, Williams, Sobczyk, Michaels, & Saifer, 2012; Shiraishi et al., 2016), and is with high sensitivity to recognize anti-PEG antibodies even when the samples are with very low-abundance of these antibodies(Yang et al., 2016).

Sandwich ELISA has also been used to measure anti-PEG antibodies. Shiraishi et al. (2013) employed this method to measure whether a conventional belief was true that specific anti-PEG IgM antibodies could recognize the PEG main chain. Commercial rabbit monoclonal anti-PEG antibodies are coated on the surface of the 96-well plates, and then mPEG2000-DSPE, PEG-OH, or PBS are added to the wells, respectively. After washing the plates, biotinylated rabbit anti-PEG IgG was added

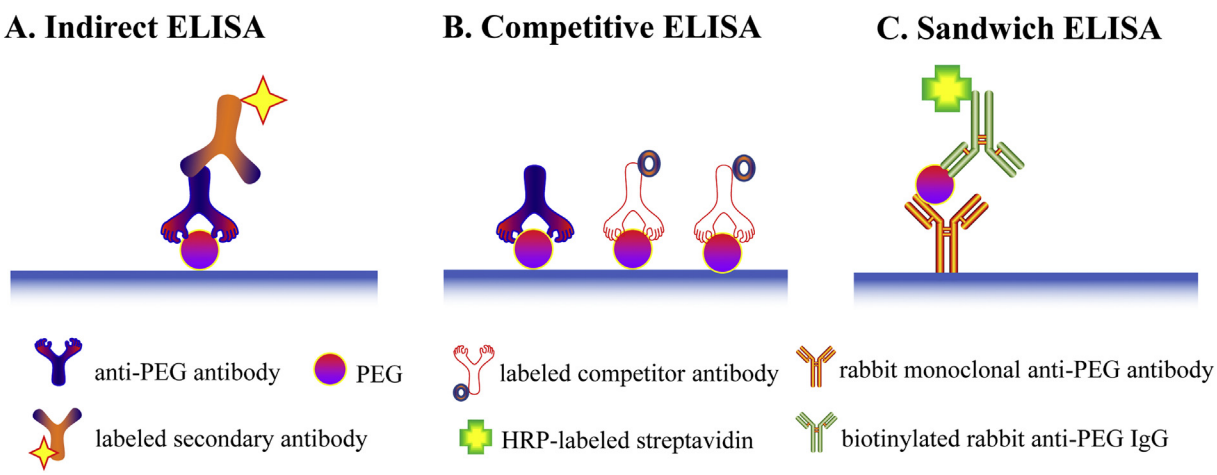

Fig. 1. Enzyme-linked immunosorbent assay (ELISA) to detect anti-PEG antibody. (A) Indirect ELISA. PEG is pre-coated on the 96-well plate to bind anti-PEG antibodies in given samples, followed by the incubation with the labeled secondary antibody. (B) Competitive ELISA. PEG antigen is coated on the surface of the 96-well plates. Two specific antibodies, competitor antibody (an enzyme-conjugated anti-PEG antibody) and anti-PEG antibody in the specimens are employed to competitively bind the pre-coated PEG antigen. (C) Sandwich ELISA. A commercial rabbit monoclonal anti-PEG antibody is pre-coated on the 96-well plates, and then PEG antigen is added. Afterwards, biotinylated rabbit anti-PEG IgG was added, followed by the addition of HRP-labeled streptavidin. The last step of the 3 types of ELISA is the forming antigen-antibody complex reacts with a chromogen, which produces color changes to quantitatively or qualitatively detect anti-PEG antibody.

into the plates, followed by the addition of HRP-labeled streptavidin (Fig. 1C). By the method, they demonstrated that these anti-PEG antibodies exhibited no binding to PEG main chains. In contrast to conventional ELISA, sandwich ELISA is more applied to make clear cross-reactivity of anti-PEG antibodies and different PEG conjugated materials (Bludau et al., 2017; Hao et al., 2017; Lin et al., 2017).

Despite numerous studies on detecting anti-PEG antibodies by ELISA as far as it goes, however, ELISA technique is not a perfect method to measure the levels of anti-PEG antibodies in human specimens. Because there lack commercially available human anti-PEG antibodies (IgG or IgM) on the market as standard to make a standard curve for quantifying the existing antibodies, only the relative amounts of anti-PEG antibodies can be estimated which makes comparisons of different studies difficult. Although the engineered chimeric monoclonal anti-PEG antibody (IgM or IgG) generated by combining mouse Fab with human Fc is a better alternation, yet it is costly and inconvenient to yield the chimeric antibodies in a large scale (Chen et al., 2016; Yang et al., 2016). Interference factors, such as Tween-20 (Sherman et al., 2012) and blocking buffer may also effect the real results.

4.4. Flow cytometry

About a decade ago, Armstrong and his colleagues (2007) established a flow cytometry method to detect anti-PEG antibodies in sera specimens from pediatric patients with acute lymphoblastic leukemia. Briefly, TentaGel-OH particles with 10-μm diameter were incubated with serum samples to bind anti-PEG antibodies existing in serums. After washing 3 times, centrifuged pellets were mixed with fluorescein-labeled goat-anti-human IgG (i.e., goat FITC-anti-IgG) and R-phycoerythrin-labeled antihuman IgM (μ-chain specific goat RPE-anti-IgM) in the dark. Then, fluorescence intensity was determined by flow cytometry. Samples with a mean fluorescence intensity of > 100 for IgG and/or > 50 for IgM were identified as positive for anti-PEG antibodies. They used the flow cytometry-based assay only to qualitatively screen anti-PEG antibodies (i.e., positive or negative), and no accurate amount of anti-PEG antibodies were obtained. Then Lubich et al. (2016) upgraded this method in a study enrolling healthy individuals and hemophilia patients. The later version of flow cytometry method to detect anti-PEG antibodies is a multi-tiered approach including sensitive screening assays, competition-based confirmatory assays and titration assays. However only titers were offered to reflect a relative degree of anti-PEG antibody concentrations. In addition, specificity of this method was not validated owing to low titers of anti-PEG antibodies in serum samples. This method is not worth recommended because of lacking accurate anti-PEG antibody concentrations.

4.5. Meso Scale Discovery technology

Meso Scale Discovery (MSD) technology is a type of electrochemiluminescence immunoassay in which high binding carbon electrodes on the bottom of the assay plates allow for easy attachment of biological reagents, leading to light emission at 620 nm read by an MSD instrument. Based on this technology, Myler et al. (2015) established two methods (bridge and direct assays) to detect anti-PEG antibodies in human serum induced by PEGylated therapeutics (PEG-IFN-α and PEG-IFN-λ). Briefly, streptavidin was coated on the MSD plates in the first step in both methods. For the method of bridge assay (Fig. 2A), samples were preincubated with ruthenylated PEG-IFN and biotinylated PEG-IFN, allowing any anti-PEG antibody present in the samples to bind both the labeled PEGylated therapeutics to form a bridge complex. Then the PEG antigen-antibody bridge complex was captured on the streptavidin-coated MSD plates through the biotinylated portion of the bridge complex. Finally, light emission at 620 nm excited by the ruthenylated portion of the complex was proportional to the levels of anti-PEG antibodies present in the specimens. For the direct assay (Fig. 2B), biotinylated PEG-IFN was firstly incubated with the streptavidin-coated MSD plates. Subsequently, samples were added to the MSD plates to make any anti-PEG antibodies present in the samples bind with the immobilized PEG-IFN. A ruthenium labeled goat anti-human Ig was used to detect anti-PEG antibody which had bound to the MSD plates. In this study, sensitivity of bridge assay was reported to be 15.7 ng/mL while that of direct assay was 25 μg/mL. The bridge assay was able to detect anti-PEG IgM and IgG reactive to common PEG derivatives at a sufficient sensitivity. Therefore, this MSD based bridge assay is a reliable and efficient way to detect the levels of anti-PEG antibodies in human specimens. Moreover, MSD method can also be conducted based on a surrogate PEGylated protein. MSD technology produces a great propellant effect on detecting anti-PEG antibodies in human specimens because of needless of human anti-PEG antibody or chimeric antibody as standard and high sensitivity. But the high costs significantly impede promotion of this method.

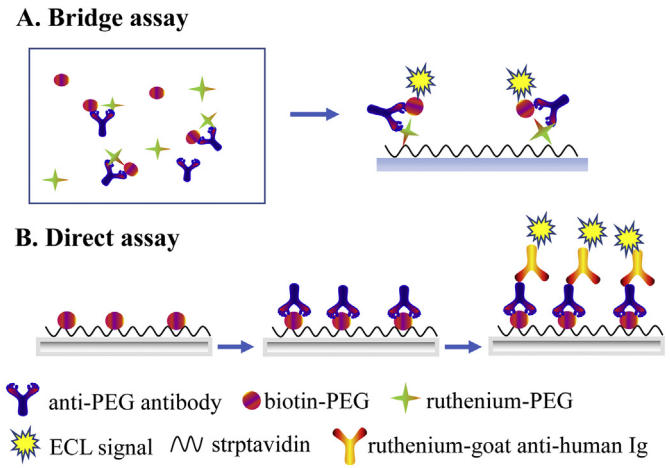

Fig. 2. Meso Scale Discovery (MSD) technology to detect anti-PEG antibody. (A) Bridge assay. Firstly, mix samples with ruthenium-PEG and biotin-PEG, allowing any anti-PEG antibody present in the samples to bind with both the labeled PEGs to form a bridge complex. Then the PEG antigen-antibody bridge complex is captured on the streptavidin-coated MSD plates through the biotin portion of the bridge complex. Finally, anti-PEG antibody is detected by light emission at 620 nm excited by the ruthenium part of the complex. (B) Direct assay. Biotin-PEG is firstly incubated with the MSD streptavidin plate. Subsequently, human sera is added to the MSD plate to make any anti-PEG antibodies present in the serum bind with the immobilized PEG. A ruthenylated goat anti-human Ig is used to detect anti-PEG antibody which had bound to the MSD plate.

4.6. Acoustic Membrane Microparticle technology

Acoustic Membrane Microparticle (AMMP®) technology for detecting anti-PEG antibodies, first introduced by Dong et al. (2015), basically determines anti-PEG antibody concentrations by measuring the changes in the oscillating frequency of a piezoelectric membrane in the following way (Fig. 3). Firstly, biotinylated PEG (biotin-PEG) was bound to streptavidin-coated paramagnetic beads, and then human sera potentially containing anti-PEG antibodies was mixed with the beads to form bead complex which combined biotin-PEG and anti-PEG antibodies. An engineered chimeric monoclonal anti-PEG IgG was used as standard. Using the ViBE platform from BioScale (Billerica, USA), when samples flowed through the sensor cartridge, the beads with PEG antigen-antibody complex were captured by protein A bound to the piezoelectric membrane surface while the beads without bound anti-PEG antibody fell away from the membrane coated with protein A. The changes in mass on the piezoelectric membrane surface resulting from the binding of the complex to the membrane sensor generated signals proportional to the mass of bound anti-PEGs. In this study, the AMMP® assay for the detection of anti-PEG antibody concentrations in human serum samples provided a promisingly high sensitivity (< 1000 ng/mL) with a great detection range up to 40 μg/mL (0.625–40 μg/mL) and a desirable reproducibility within 7% CV. Meanwhile, they conducted an evaluation of MSD technique with the same chimeric anti-PEG antibody which only achieved sensitivities in the 50–500 μg/mL range which was substantively different from the aforementioned Myler's study (Myler et al., 2015). Besides high sensitivity, it was also claimed that AMMP® technology overcome flaws of ELISA such as reducing working time from a full day to several hours (only 2 h needed to complete incubation), fulfilling walk-away instead of manual manipulations during step-wise incubation, and wider dynamic range. Unfortunately, when compared with this study, a fundamentally different reproducibility which was relatively poor with CV > 20% was obtained in another study conducted by Chilewski, Shields, Mora, and Myler (2018) to validate this method. In addition, this ViBE platform is just available to no more than three 96-well microtiter plates per operation, which limits efficiency of this system in some degree. Therefore, more bioanalysis experiments are needed to verify this novel approach.

4.7. Surface plasmon resonace technique

Surface plasmon resonace (SPR) assay is an ultra-sensitive and label-free method to detect molecular interactions in real time. The sensor chip consists of a matrix layer covalently attached to a quite thin (~50 nm thick) gold film coated on a glass slide. Ligands can be covalently bound to the matrix layer, and subsequent binding to the immobilized ligands can then be quantitatively measured by the SPR signals. The SPR signal was reported in response units (rsp.u.) and corresponded to the mass of captured ligand (Borresen et al., 2018). Zhang et al. (2015) set up an SPR-based method to detect anti-PEG antibodies in human sera specimens by modifying the surface of gold chips with PEG methyl ether methacrylate (PEGMA) polymer (Fig. 4). When samples flow through the surface of the PEGMA modified sensor chip, only anti-PEG antibodies can bind to the PEGMA modified polymer surface and the generating SPR signals showed typical specific binding kinetics with binding mass increasing linearly with time. They reported that the capacity of this SPR-based technique to detect anti-PEG antibodies reached 40–50 ng/cm^2 in 15 min under experimental conditions with no detectable nonspecific binding. Afterwards, the same research team successfully detected clinical blood samples by using the SPR assay (P. Zhang et al., 2017). With application of a labeled secondary antibody amplifying the signals, they differentiated and quantitatively determined the concentrations of different anti-PEG antibody isotypes (IgG and IgM) in human blood specimens. The sensitivity of a custom-built SPR sensor used in this work reached 0.3 ng/

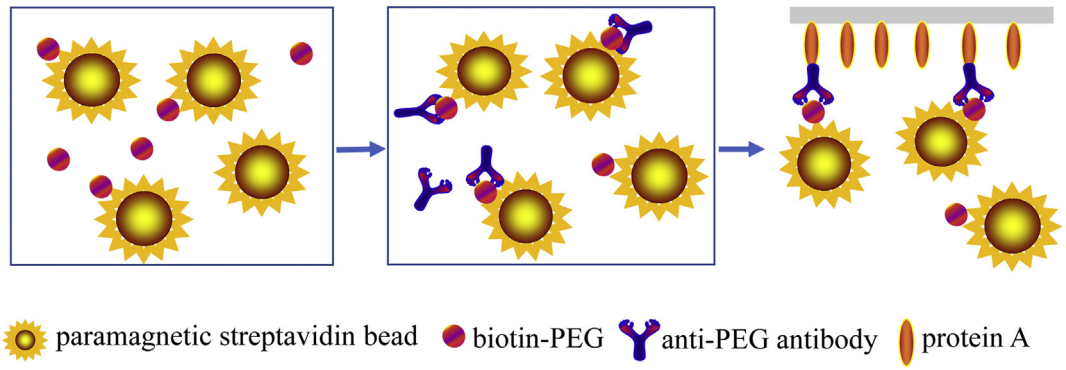

Fig. 3. Acoustic Membrane Microparticle (AMMP) technology for detecting anti-PEG antibody. Firstly, biotin-PEG is bound to streptavidin-coated paramagnetic beads, and then human sera is mixed with the beads to form PEG antigen-antibody complex. When samples flow through the sensor cartridge of the ViBE platform, the beads with PEG antigen-antibody complex are captured by protein A immobilized on the piezoelectric membrane surface while the beads without anti-PEG antibody fall away from the membrane.

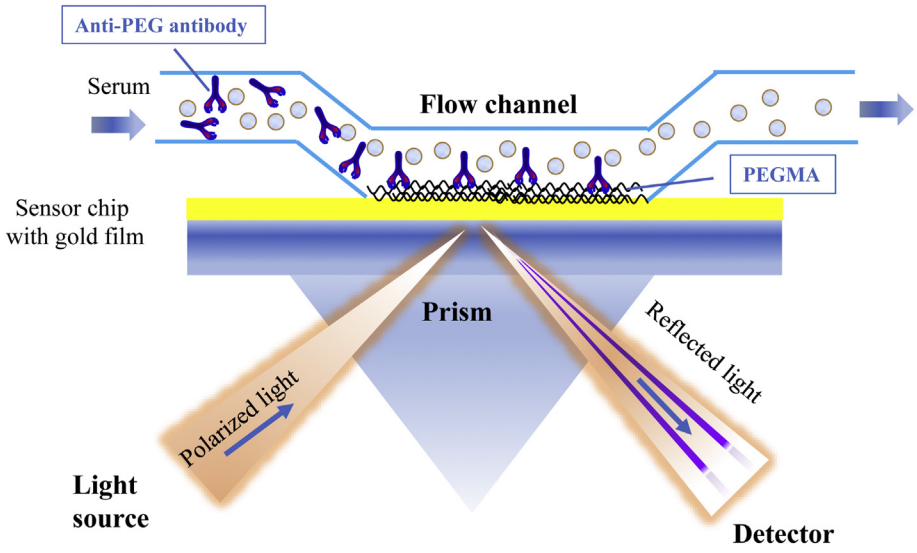

Fig. 4. Surface plasmon resonace (SPR) technique for anti-PEG antibody detection. The gold film of the SPR sensor chip is modified by PEG methyl ether methacrylate (PEGMA) polymer. The PEGMA polymers perform dual functions, serving as a nonfouling background and PEG antigen. When human serum flow through sensor chip, only anti-PEG antibodies can bind to the PEGMA modified polymer surface. The generating SPR signals show typical specific binding kinetics with binding mass increasing linearly with time. This SPR diagram is based on the study of P. Zhang et al. (2015). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cm^2. And the detection limits of this SPR assay were 10 ng/mL and 50 ng/mL for anti-PEG IgM and IgG, respectively, whereas direct ELISA presented the detection limits 100 ng/mL for anti-PEG IgM and 1 μg/mL for anti-PEG IgG.

Admittedly, high costs of the instruments cannot be neglected, but SPR assay is indeed a rapid, convenient, sensitive and reliable technique to pre-screen and quantitatively determine anti-PEG antibodies (IgG and IgM) in human blood samples.

5. Prospects/conclusion

PEGylation of therapeutics (including RNA (Moreno et al., 2019), peptides (P. Zhang et al., 2018), proteins (Paik & Deeks, 2019), liposomes (F. Wang et al., 2019), micelles (Shiraishi & Yokoyama, 2019) and other molecules (Mima et al., 2017)) help to overcome degradation by enzymes, increase water-solubility and stability, lower immunogenicity and antigenicity of the target molecule, and extend circulating half-life (X. W. Zhang et al., 2014). However, anti-PEG antibodies have been observed in both PEGylated therapeutics treated patients and healthy population with prevalence increasing from 0.2% (A. W. Richter & Akerblom, 1984) to 72% (Yang et al., 2016). Numerous studies have also elucidated that anti PEGylated therapeutic antibodies are mainly against the PEG part (i.e., anti-PEG antibodies) not drug (RNA, peptide, protein) or carrier (liposome, micelle) part (Y. Liu et al., 2019; Moreno et al., 2019; F. Qi et al., 2019). Anti-PEG antibodies binding to PEGylated therapeutics is assumed to form antibody-drug complex, accelerating blood clearance of target drugs and weakening efficacy of PEGylated therapeutics. Therefore, blood levels of anti-PEG antibodies of patients should be closely monitored before/during/post treatment of PEGylated drugs.

To investigate the effect of anti-PEG antibodies on PEGylated drugs, several detection approaches (including passive hemagglutination, Western Blot, ELISA, flow cytometry, MSD, AMMP, SPR, etc.) are established and applied to screen and quantitatively measure the levels of anti-PEG antibodies in the human blood samples. However, lack of humanized anti-PEG antibodies as standard, low content of anti-PEG antibodies in the blood samples, and interference from blocking buffer and other components in the blood sample severely hampered progress in this field. Generation mechanisms of anti-PEG antibodies and the effect of anti-PEG antibodies on the PEGylated therapeutics remain a mystery.

With the development of detection techniques, passive hemagglutination and Western Blot may not be suitable for detecting anti-PEG antibodies in human blood specimens because of the qualitative or semi-quantitative results. Despite the lack of human and humanized anti-PEG antibodies, it is extremely exciting that chimeric antibodies successfully constructed from DNA (Chen et al., 2016; Yang et al., 2016) are used as standard in ELISA and electrochemiluminescence detections. Compared to traditional ELISA, the novel techniques, including MSD, AMMP and SPR, demand much smaller amount of samples and can simultaneously deal with different kinds of components of samples with a wider detection range and a much faster detection speed It can be expected that the novel techniques will exceed traditional ELISA in the field of anti-PEG antibody detection after lowering the cost of assay plates and popularization of relevant instruments. It is imperative to establish a rigorous, convenient, rapid, economical, effective and steady avenue to detect and monitor the existence, types and levels of anti-PEG antibodies in human blood.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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