HHS Public Access

Author manuscript

J Control Release. Author manuscript; available in PMC 2017 December 29.

Published in final edited form as:

J Control Release. 2016 December 28; 244(Pt B): 184–193. doi:10.1016/j.jconrel.2016.06.040.

Anti-PEG antibodies in the clinic: current issues and beyond PEGylation

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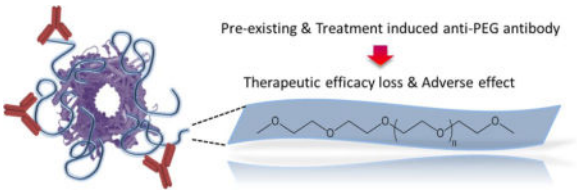
Abstract

The technique of attaching the polymer polyethylene glycol (PEG), or PEGylation, has brought more than ten protein drugs into market. The surface conjugation of PEG on proteins prolongs their blood circulation time and reduces immunogenicity by increasing their hydrodynamic size and masking surface epitopes. Despite this success, an emerging body of literature highlights the presence of antibodies produced by the immune system that specifically recognize and bind to PEG (anti-PEG Abs), including both pre-existing and treatment-induced Abs. More importantly, the existence of anti-PEG Abs has been correlated with loss of therapeutic efficacy and increase in adverse effects in several clinical reports examining different PEGylated therapeutics. To better understand the nature of anti-PEG immunity, we summarize a number of clinical reports and some critical animal studies regarding pre-existing and treatment-induced anti-PEG Abs. Various anti-PEG detection methods used in different studies were provided. Several protein modification technologies beyond PEGylation were also highlighted.

Graphical abstract



Keywords

PEGylation; anti-PEG antibody; protein therapeutics; polymer conjugation; immunogenicity; antibody detection

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1. Introduction

Biopharmaceutical drugs, including peptides, proteins, monoclonal antibodies, drug-antibody conjugates and aptamers, offer significant advantages over small molecule therapeutics due to their specific bioactivity and high potency. More than 200 biotech products were marked during last three decades, with over 900 new products currently in the pipeline.[1] Despite the huge success achieved by biopharmaceutics, these structurally complex biomacromolecules usually face great challenges including instability, inadequate circulation half-life and immunogenicity.[2, 3] A short half-life limits therapeutic efficacy and requires a frequent administration regimen. Immune responses against many biological drugs not only result in accelerated blood clearance during chronic use, but also threaten patients' lives with adverse effects including anaphylaxis and infusion reactions.[4]

The most successful strategy thus far to overcome these shortcomings is the conjugation of polyethylene glycol (PEG) to these biomacromolecules, a process known as PEGylation.[5] PEG is a water-soluble synthetic polymer consisted of ethylene glycol (-CH_2-CH_2-O-) repeating units. It has been recognized as a classic "non-fouling" material that resists non-specific protein adsorption, and is used to coat various surfaces from biomedical devices to drug delivery nanoparticles. In aqueous solution, PEG holds a stable hydration layer through hydrogen bonding to water molecules; this hydration layer in tandem with PEG's flexible chains can resist protein adsorptions to the underlying surfaces.[6–8] Similar to this adsorption-resistance mechanism, covalently conjugating PEG to biomacromolecules significantly increases their hydrodynamic size through the strong hydration effect, helping them avoid rapid renal clearance and prolong circulation half-life. In addition, it is believed that hydrated PEG brushes can shield antigenic epitopes from immune system recognition, helping the underlying biomacromolecule escape from clearance by the reticuloendothelial system and thus mitigate immunogenicity.[9] Since the launch of the first commercial PEGylated protein pegademase bovine used to treat severe combined immunodeficiency disease (SCID) in 1990, at least 10 PEGylated drugs have been approved by the United States Food and Drug Administration (US FDA) and more than 20 are currently in clinical trials. Several review articles have summarized the progress and future prospects of this technology.[5, 10–12]

PEG has been considered as a biologically inert material with no immunogenicity and antigenicity in early studies.[9, 13] For example, the first publication utilizing PEG to alter protein immunogenicity tested PEGylated bovine serum albumin (BSA) in rabbits, in which neither anti-BSA nor anti-PEG Abs were found in the study.[9] However, animal studies in the ensuing decade have found anti-PEG Abs after immunization with PEGylated proteins, although whether it was clinically relevant remained unclear at that time.[14] Until recently, with more PEGylated products entering the clinic, several reports correlated the generation of anti-PEG Abs with loss of therapeutic efficacy and there has been an increase of reported adverse effects after repeated administrations.[15–19] In addition to PEGylated proteins, PEG-modified nanoparticles, e.g. liposomes and micelles, have also been reported to stimulate anti-PEG Ab generation in animal models.[20–27] Ishida et al. did extensive studies on PEG-liposome stimulated immune responses and summarized their findings in a recent review.[28] Although more reports regarding PEG immunogenicity came out during

recent years, and monoclonal anti-PEG Abs became commercially available and have been used to develop analytical tools for PEGylated therapeutics[29–31] or drug delivery[32], studies on the existence of anti-PEG antibody and its clinical relevance are in its infancy.[33] Yang and Lai provided a summary of research relating to anti-PEG immunity.[34] Herein, we will focus on recent clinical reports regarding anti-PEG responses, factors affecting PEG immunogenicity, anti-PEG Ab detection methods, and provide some perspectives on technologies beyond PEGylation.

2. Anti-PEG Abs in the clinic

2.1 The case of PEG-uricase

Uricase, also called urate oxidase, is an enzyme capable of catalyzing uric acid to allantoin. It attracts great interest as a therapeutic by its ability to rapidly clear the urate load in severe gout patients. As humans naturally lack this enzyme, it induces high immunogenicity when used therapeutically and cannot be used for repeated dosing.[35] In 2010, pegloticase, a PEGylated recombinant mammalian uricase under the trade name Krystexxa, was approved by the FDA to treat refractive gout. Each uricase monomer has been conjugated with 10 ± 1 strands of 10-kDa monomethoxy-PEG (mPEG) to reduce its immunogenicity.[36] However, anti-PEG Abs have been detected since the early phase 1 trial: a single subcutaneous (SC) injection of pegloticase induced the generation of both anti-PEG IgM and IgG Abs in 5 out of 13 patients (38%).[37] These antibodies were associated with the quick drug clearance and three anti-PEG positive subjects had injection-site reactions at 8–9 days post-injection. In a following trial of intravenous (IV) administration of the same drug, 9 out of 24 patients (38%) developed anti-PEG Abs after a single injection of 0.5–12 mg PEG-uricase conjugates.[38] These antibodies were mainly of the IgG_2 isotype and caused a rapid drug clearance. However, no allergic reactions were found and all adverse reactions were considered to be mild to moderate. Several subsequent trials also found high incidence rates of anti-drug antibodies (ADAs), but their specificity was not determined.[36, 39]

To further study the influence of observed ADAs on long term therapeutic efficacy, 2 replicate, 6-month, randomized controlled trials were conducted.[19] A total number of 169 patients received pegloticase biweekly or monthly at a dosage of 8 mg through IV injections. Only 65 (38%) patients were classified as responders when the trial completed, where a responder was defined as a patient whose plasma urate level was well-controlled during treatment. Throughout the trial, a high rate of 89% patients developed measurable anti-pegloticase Ab titers. A clinically relevant Ab titer (1:2430) has been determined by an empirical approach. (Fig. 1a, b) Mean anti-pegloticase antibody titers were <1:2430 at all study visits for responders, whereas the mean titers rose to >1:2430 by the week-4 visit for non-responders. Antibody specificity was determined using various PEGylated proteins as competitors in competitive enzyme-linked immunosorbent assay (ELISA), and the anti-pegloticase was found to be predominantly anti-PEG. Although anti-PEG IgM was found to be the major cause of accelerated clearance of PEGylated protein in rodent models,[40] both IgM and IgG were found in human non-responders' (NR's) serum samples. In addition, there is a tendency for patients who generate anti-PEG IgG to become NRs. This suggests that T-cell dependent B cell activation may be crucial in human anti-PEG immunity.

In another independent trial involving thirty patients, more detailed work was done to analyze the relationship between anti-PEG Abs and pegloticase efficacy loss.[17] Interestingly, this trial also included seven organ transplant recipients to evaluate the influence of chronic immunosuppression on anti-PEG susceptibility. The major findings were summarized as follows. 1) Among 30 patients: 1 was classified as NR due to a syncopal reaction before completing the first infusion and was subsequently withdrawn from the study; 12 were transient responders (TR) whose plasma acid concentration lost control during the trial; and 17 were persistent responders (PR); thus in total pegloticase lost efficacy in 43% of patients following multiple administrations. 2) None of the 17 PR mounted significant, sustained anti-PEG response; while in all other 13 patients, anti-PEG response is associated with efficacy loss. 3) All 3 non-naïve patients (those who had received pegloticase in previous clinical trials at least one year prior this study) quickly developed anti-PEG Abs and became TR. 4) Anti-PEG Abs were pre-existing in 5 of 27 (19%) pegloticase naïve patients, all 5 patients became NR or TR in the trial. 5) In 27 pegloticase-naïve patients: 1 in 7 (14%) organ-transplant recipients developed anti-PEG Abs; while 9 of 20 (45%) non-recipients developed anti-PEG Abs. 6) In anti-PEG-positive patients, 8 of 13 (62%) had infusion reactions and 5 were withdrawn from the trial; while in anti-PEG-negative patients 5 of 17 (29%) had reactions and none led to the discontinuation of treatment. These results provided us an opportunity to look into human anti-PEG immunity. Several key conclusions can be drawn from these data: first, anti-PEG Abs are responsible for the therapeutic efficacy loss of pegloticase after multiple injections; second, existence of anti-PEG Abs increased the chance of infusion reactions; third, re-exposure to PEGylated therapeutics may induce B cell memory responses; and finally, immunosuppression may be an effective strategy to manage anti-PEG immunity.

2.2 The case of PEG-asparaginase

Asparaginase is an enzyme expressed by microorganisms that catalyzes the hydrolysis of asparagine to aspartic acid. It is used for the treatment of acute lymphoblastic leukemia (ALL). ALL cells are unable to synthesize the non-essential amino acid asparagine, whereas normal cells are able to make their own asparagine; thus leukemic cells are dependent on the extracellular availability of asparagine.[41] Administration of asparaginase deprives the leukemic cell of circulating asparagine, which leads to cell death. Current commercially available medical asparaginases are from two sources, *Escherichia coli* (*E. coli*) and *Erwinia chrysanthemi.* To avoid asparaginase therapies associated hypersensitivity reactions and improve pharmacokinetics, PEGylated *E. coli* asparaginase was developed under the trade name of Oncaspar. However, rapid clearance of PEG-asparaginase has been observed for a subgroup of pediatric patients treated for ALL.[42] Armstrong *et al.* analyzed blood samples from Oncaspar-treated ALL patients, and concluded that anti-PEG Abs were very closely associated with the drug's rapid clearance.[16] In their analysis, samples from 28 patients who received Oncaspar were included, among which 15 subjects had undetectable asparaginase activity and 13 had effective asparaginase activity. Anti-PEG Abs were screened using both serology and flow cytometry methods. Both anti-PEG IgM and IgG were found, and strong correlations have been demonstrated between plasma asparaginase activity and the occurrence of anti-PEG Abs. (Fig. 1c, d) The positive/negative predictive

values of anti-PEG Abs for blood asparaginase activity were 92%/80% and 100%/68% for flow cytometric and serological detection methods.

In a recent report regarding the safety of PEG-asparaginase, 79 out of 615 Oncaspar-treated patients developed clinical allergy against PEG-asparaginase and discontinued therapy for that reason, although whether it was related to anti-PEG Ab was not determined.[43] One common protocol to manage allergic reactions for patients who received unmodified *E. coli* asparaginase is switching to *Erwinia* asparaginase, which is antigenically distinct from *E. coli*-derived asparaginase.[44, 45] Following a similar strategy, Jazz Pharmaceuticals has developed a PEGylated *Erwinia* asparaginase named pegcristantaspase, aiming to replace PEGylated *E. coli* asparaginase in patients who developed hypersensitivity to Oncaspar. A phase 2 study (NCT02257684) involving four patients has been conducted to evaluate the effectiveness, safety and dosage of pegcrisantaspase in patients with ALL / Lymphoblastic Lymphoma (LBL) following hypersensitivity to Oncaspar. Unfortunately, this trial was terminated early before completion. Although no study results have been released about this trial yet, one possibility for the trial failure is that anti-PEG Abs generated during Oncaspar therapy may cross-react with pegcristantaspase.

2.3 The case of pegnivacogin and pre-existing anti-PEG Abs

In addition to treatment-induced antibodies, a recent clinical study regarding pegnivacogin has indicated the potential clinical significance of pre-existing anti-PEG Abs in patients before their first exposure to PEG containing therapeutics.[46] Pegnivacogin is a RNA aptamer, which was conjugated to a 40-kDa branched mPEG. When administering pegnivacogin, three acute coronary syndrome patients experienced serious allergic reactions (one life-threatening) during their first exposure to the drug, which led to early termination of the trial. Subsequent analysis using two independently validated ELISA designs has correlated the severe allergic reactions to pre-existing anti-PEG Abs in the patients' blood, predominantly IgG.

Although clinical significance of pre-existing anti-PEG Abs needs to be studied further, current evidence already suggested that pre-existing anti-PEG Abs may increase the chance of treatment failure and adverse effects of some PEGylated therapeutics. In a clinical trial discussed above, pegloticase lost efficacy rapidly in all 5 treatment-naïve patients with pre-existing anti-PEG Abs, while only 27% (4/15) patients (non-organ transplant recipients) without pre-existing anti-PEG became TR during study.[17] Moreover, the pegloticase-naïve patient who experienced a syncopal reaction during initial drug infusion was found to have the highest pre-existing anti-PEG Ab level. Besides PEGylated therapeutics, free PEG in drug formulations may also trigger allergy in the presence of pre-existing anti-PEG Abs. A case study in 2005 reported that a severe hypersensitivity reaction occurred when a patient with no known allergies received an intraarticular injection of a corticoid preparation containing 4-kDa PEG as an excipient. Systematic testing revealed that the response was mediated by an IgE Ab that was specific to PEG.[47]

Anti-PEG immunity was also reported to have memory B cell response, thus re-exposure to the same or different PEG-containing therapeutics may also result in reduced efficacy or increased frequency of adverse reactions.[17, 18] In a phase 1 trial for treating

phenylketonuria, a PEGylated phenylalanine ammonia lyase induced anti-PEG Abs in all 25 participants, 2 of whom had hypersensitivity reactions when administered medroxyprogesterone acetate, a drug that contains PEG as an excipient.[18] By contrast, some other studies showed pre-existing anti-PEG Abs did not have any adverse impact on the tested PEGylated therapeutic.[48, 49] Concluding from all these results, effects of pre-existing anti-PEG Abs depend on Ab level, individual susceptibility, as well as the exposed PEGylated therapeutics. To reduce the chance of adverse reactions in treatment-naïve patients, it is strongly recommended to conduct an anti-PEG Ab screening prior to administering PEG-containing therapeutics.

Pre-existing anti-PEG Abs in the general population was first reported by Richter and Akerblom in 1984, as 0.2% in healthy blood donors and 3% in untreated allergy patients, mostly anti-PEG IgM.[48] Two decades later, Armstrong et al. reported a significantly higher incidence rate of ~27% in healthy blood donors, with an isotype pattern distribution of 19% IgG only, 5% IgM only, and 3% subjects possessed both.[50] Recently, Yang and Lai reported an even higher anti-PEG Ab incident rate of 42% (13/31) in healthy adult individuals, with 26%, 3% and 13% of the total subjects exhibiting IgG only, IgM only and both IgM and IgG antibodies.[34] There seems to be an increasing trend of anti-PEG Ab incidence rate over the last thirty years, although the sample size was not big enough to give a definitive answer. Anti-PEG Abs have also been found in pre-treatment sera or untreated controls in clinical trials.[16–18, 46, 49, 51] The mechanism of anti-PEG Ab generation in individuals who have never received PEGylated therapeutics has not been clearly studied yet. However it is likely to be related with the increased daily exposure to PEG-containing household, hygiene and cosmetic products. Yang and Lai proposed a mechanism that exposure of human body insults or wounds to PEG products may provide a chance to induce anti-PEG Abs.[34] When PEG-containing products contact with local inflammatory sites, environmental microbes may stimulate immune cells and trigger anti-PEG immunity.

3 Factors affecting PEG immunogenicity

3.1 Carrier protein and modification density

As most PEGylated therapeutics is based on PEG-protein conjugates, it is of particular interest to study the influence of various parameters on PEG-protein immunogenicity. Over thirty years ago, Richter and Akerblom did a pilot study on the generation of anti-PEG Abs in animal models, [14] after the introduction of PEGylation as a way to alter protein immunogenicity. In their study, PEG has been conjugated to three immunogens, including ovalbumin (OVA), bovine superoxide dismutase (SOD) and ragweed pollen extract (Rag). These conjugates were administered in mice and rabbits subcutaneously with or without the presence of Freund’s adjuvant (FA). Both degree of conjugation and PEG molecular weight were adjusted to explore potential factors affecting immunogenicity. Free PEG only exhibited no or very weak immunogenicity in the presence of FA, whereas PEG-protein conjugates induced significant anti-PEG Ab generation in the immunized animals. The haptenic characteristic of PEG was further revealed by its dependence on carrier protein immunogenicity. This result explains why PEGylated nonhuman enzymes, e.g., uricase and asparaginase, have been reported to present the most severe issues resulting from anti-PEG

Abs, whereas others are relatively safer.[49, 51, 52] Another key finding from this study is that the degree of modification is a crucial factor determining PEG-protein conjugate immunogenicity, as more PEG chains on a protein elicited weaker antibody responses, possibly due to better masking of immunogenic epitopes. Finally, it has been determined that anti-PEG Ab binds to 6–7 ethylene glycol repeating units. With these conclusions, it is easy to understand why anti-PEG Ab was not found in the earlier work evaluating immunogenicity of PEGylated bovine serum albumin (BSA).[9] First, BSA shares a large degree of similarity with rabbit serum albumin (RSA),[53] which makes it less immunogenic as a model protein. Second, a high degree of modification can be easily attained with BSA. Serum albumin is a class of protein with extraordinary ligand binding capacity,[53] but this is not the case for most therapeutic proteins. Third, immunological assays of the time, e.g., immunodiffusion and complement fixation tests, were not capable of detecting low-titer antibodies. In summary, Richter and Akerblom's work provides valuable information to direct the development of safer PEGylated protein therapeutics: the choice of low immunogenic protein and high degree of PEG modification should be considered whenever it is possible.

3.2 PEG material hydrophobicity

Besides carrier protein properties and conjugation parameters, the molecular structure of PEG itself is another possible factor affecting overall immunogenicity and antigenicity. In a study conducted by Sherman *et al*, the results suggested that hydrophobicity of the PEG end-group has a notable effect on the conjugate immunogenicity and antigenicity.[54] The immunogenicity of three different PEG analogs is in the order of t-butoxy-PEG (tBu-PEG) > mPEG > hydroxyl-PEG (OH-PEG). Abs elicited by OH-PEG have similar affinity to both mPEG and OH-PEG, while antibodies inducted by mPEG recognize mPEG more effectively than OH-PEG, suggesting the existence of "methoxy-specific" and "backbone-specific" anti-PEG Abs. Recently, the same group did a more detailed competitive assay to study the binding affinities of different anti-PEG Abs to a series of PEG or oligo(ethylene glycol) analogs.[55] The results demonstrated that: 1) PEG molecular weight and end-group hydrophobicities have greater influence on anti-mPEG Ab binding affinity than their resemblance to the methoxy terminus of the haptenic polymer; 2) anti-PEG Abs inducted by OH-PEG protein conjugates are mainly against the PEG backbone but not the hydroxyl end-group; 3) rabbit anti-PEG Abs bind to as few as three ethylene glycol repeating units; 4) neither "methoxy-specific" nor "backbone-specific" anti-PEG Abs was absolutely specific, but showed distinct relative selectivities. All these finds suggest that PEG immunogenicity and antigenicity is related to its hydrophobicity. Although OH-PEG might be better than mPEG for PEGylated therapeutics, "backbone-specific" anti-PEG Ab still remains.

4 Anti-PEG Ab detections

Based on the clinical findings reviewed above, it would be beneficial to screen and monitor both pre-existing and induced anti-PEG Abs during the treatment of PEGylated therapeutics. Clinically relevant anti-PEG Ab titers need to be defined to control the risk of adverse effects for patients exposed to PEGylated products. Thus a highly reliable quantitative anti-PEG Ab detection methodology is essential for both academic study and clinical

applications.[56] Over the last four decades, a variety of techniques have been used to detect anti-PEG Abs and determine Ab specificities. Those methods ranged from some of the most traditional immunoassays to state-of-the-art sensor technologies. However, the results obtained with different methods are usually incomparable, and some of them are only qualitative rather than quantitative. A brief description of each anti-PEG Ab detection method is listed below.

4.1 Passive hemagglutination

Passive hemagglutination was first used in Richter and Akerblom's work to identify anti-PEG Abs[14, 48] and subsequently in Armstrong's work to screen anti-PEG Ab in healthy blood donors[50]. Passive agglutination is done by modifying erythrocyte surfaces with antigen; the erythrocytes then agglutinate in the presence of antiserum specific for the adsorbed antigen. When detecting anti-PEG sera, mPEG was used for sensitization of homologous erythrocytes. The sensitized red blood cells were mixed with serial dilutions of tested serum. The titers were defined as the highest serum dilution giving complete hemagglutination. This serological method is qualitative and cannot indicate antibody isotypes.

4.2 Flow Cytometry

Armstrong et al. developed an anti-PEG Ab detection method using flow cytometry.[50] The tested serum and plasma samples were incubated with 10 um diameter TentaGel-OH particles, which are composed primarily of PEG. The particles were subsequently washed with phosphate-buffered saline (PBS) and stained for bound IgG and IgM with fluorescein-labeled anti-human IgG and phycoerythrin-labeled anti-human IgM. Then the particles were washed again by PBS, re-suspended, and the mean fluorescence intensity of 10,000 single TentaGel-OH particles was determined by flow cytometry. Compared with the serological method, this method possessed advantages in providing quantitative results and ability to differentiate antibody isotypes.

4.3 Western Blot

Sroda et al. used the protein immunoblot technique to confirm the existence of anti-PEG Abs generated in rabbit sera after repeated injections of PEG-phosphatidyl ethanolamine liposomes.[57] In their procedure, PEGylated liposomes containing patent blue violet were incubated with rabbit serum. Liposomes with adsorbed Abs were then separated by gel filtration through a microcolumn. Finally, the liposome fraction was subjected to SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose filter and identified by reaction with anti-rabbit IgG. A competition assay has to be conducted to confirm PEG specificity if the same PEGylated liposomes were used for immunization. This method is qualitative and capable to distinguish antibody isotypes.

4.4 ELISA

Among all methods, ELISA is the most popular technique for anti-PEG Ab detection and isotype classification due to its high sensitivity. Most of the clinical reports reviewed above adopted ELISA as their method to screen anti-PEG Abs. Different formats of ELISA,

including direct,[17, 27, 40] sandwich[22], competitive,[17, 54, 55] and double antigen bridging[58], have been employed to detect and study anti-PEG Ab selectivity. Taking the simplest direct ELISA as an example, free PEG or PEGylated materials (protein, lipid, biotin, etc) are usually used as antigens to coat plate surface. Non-specific adsorptions from detection samples are blocked by high concentration irrelevant protein solutions, e.g. BSA or dry milk powder. Then serial dilutions of serum or plasma samples are added to the plates. After washing, secondary antibodies such as anti-IgG or anti-IgM conjugated with an enzyme reporter are added to detect and identify the anti-PEG Ab subclass. Extra care should be taken in the selection of antigen coatings to avoid antibody cross-reactions. PEG-like detergents (e.g. Tween-20) should also be avoided in the washing buffer as they could cross-react with anti-PEG Abs and decrease the detection sensitivity.[54] To achieve the quantitative detection, standard curves can be generated using commercially available anti-PEG Abs. ELISA provides a reliable quantitative detection, and is powerful in differentiating antibody isotype and selectivity. However, a small protocol difference (antigen coating, incubation time, agents, etc.) usually significantly affects final readings, which makes it difficult to directly compare results from different experiments.

4.5 Acoustic Membrane MicroParticle

Dong et al. reported the use of BioScale's Acoustic Membrane MicroParticle (AMMP®) technology via the ViBE® Workstation to measure anti-PEG Abs in human serum samples.[59] Test samples were diluted and incubated with paramagnetic beads coated with linear chain mPEG to capture anti-PEG Abs. The complex was then captured on an acoustic membrane coated with Protein A. The change in mass on the membrane resulted in a signal proportional to the mass of anti-PEG Abs. As a quantitative method, the limit of detection is as low as 1 μg/mL of anti-PEG IgG.

4.6 Surface plasmon resonance sensor (SPR)

Our group developed a sensitive and quantitative method for anti-PEG Ab detection using optical SPR sensors.[60] SPR is a powerful technique to monitor label-free biomolecular interactions and biomolecule/surface interactions in liquids with detection limits as low as nanogram levels.[61] We grafted PEG-based polymers onto the SPR sensor chips as antigens to detect anti-PEG Abs in samples. As PEGs are directly immobilized, no other antibody could specifically bind to the surface other than anti-PEG Abs. In addition, the non-fouling PEG coatings can simultaneously resist non-specific protein adsorptions from diluted blood serum, thus diminishing the background noise and enabling direct detection in complex media. The diluted serum samples were directly flown through the PEG-modified SPR sensor, and specific binding between anti-PEG Abs and PEG polymers was directly monitored by wavelength shift. (Fig. 2) Recently, we have optimized this technique to achieve ng-level detection sensitivities (unpublished results). We showed that SPR detection is at least ten times more sensitive than direct ELISA, and is capable in distinguishing antibody isotypes with quantitative concentrations.

5 Protein modification beyond PEGylation

Clinical findings of anti-PEG Abs have casted a shadow over the future of PEGylation technology. A safer and more effective approach beyond PEGylation is highly desirable. Since the early 1980s, a series of synthetic polymers, including poly(N-vinylpyrrolidone),[62] polyoxazoline,[63] poly(N-acryloyl morpholine),[64] poly(vinyl alcohol),[65] polyglycerol,[66] and polyzwitterions,[67, 68] have been proposed to conjugate to proteins and function in a similar way as PEG does (Polymer structures shown in Fig. 3). In addition, naturally degradable polymers, such as polysaccharides[69] and polypeptides[70–72] were also developed as PEGylation alternatives. Polymer-protein conjugation chemistries have been reviewed in details by Maynard[73] and Chilkoti[74]. Herein we will focus more on immunological studies of these materials.

5.1 Synthetic polymers

Although many synthetic polymers have been studied as protein conjugating materials, only several have been evaluated regarding immunological properties. Immunological studies of poly(N-vinylpyrrolidone) (PVP) can be dated back to 1956, when Maurer reported the discovery of anti-PVP Abs from medical volunteers who accepted intramuscular injections of high molecular-weight PVP polymers.[75, 76] Following Maurer's work, Andersson confirmed the specificity of anti-PVP Ab elicited in mice using competitive serological methods.[77] It was shown that anti-PVP Ab binding can be inhibited by the monomer N-Vinyl-2-pyrrolidone. Caliceti et al. conducted a research to compare the immunological properties of protein-polymer conjugates prepared from different neutral soluble polymers.[78] Uricase from *Candida utilis* was modified with 6-kDa PVP, poly(N-acryloyl morpholine) (PAM, 6 kDa), branched mPEG (b-mPEG, 10 kDa), and linear mPEG (l-mPEG, 5 kDa) at similar degrees of modification. All conjugates were injected into mice intraperitoneally with Freund's adjuvant to induce Ab responses. As anticipated, all conjugates induced a lower production of anti-native uricase Abs compared with the native protein. Interestingly, the ranking order of immunogenicity was native uricase > uricase-PVP ≥ uricase-PAM > uricase-l-mPEG > uricase-b-mPEG. All four conjugates induced anti-polymer immune responses, while Anti-PVP and anti-PAM Abs were generated earlier and with higher titers compared with anti-PEG Abs. These results suggested that with higher immunogenicity, PVP and PAM might not be the appropriate substituents for future protein conjugations.

Zwitterionic materials, bearing simultaneously a pair of oppositely charged ions in the same moiety while maintaining overall neutral charge, have been identified recently as a class of extremely hydrophilic materials.[79, 80] Taking poly(carboxybetaine) (PCB) as an example, its strong electrostatically induced hydration confers an ability with superior resistance to nonspecific protein adsorption.[81] Zwitterionic polymer coatings also endow stealth properties to nanoparticles, enabling significantly prolonged in vivo circulation half-lives.[60, 82] In a previous study, PCB has been conjugated to the model enzyme chymotrypsin to test its protein stabilizing effect.[68] It has been shown that PCB polymers exhibited a more robust ability than PEG to protect conjugated proteins against environmental stressors. More importantly, PEG conjugation decreased the binding affinity of the enzyme to a peptide

substrate, while conjugation to PCB of similar R_h increased the binding affinity. It is hypothesized that super-hydrophilic PCB zwitterions draw water molecules away from hydrophobic regions of the protein, thus facilitating the interaction between catalytic site in the enzyme and the substrate. These encouraging results motivated subsequent in vivo studies involving PCB-protein conjugates to evaluate protection against immunological responses, this time using uricase from *Candida sp.* as the model protein.[83] The antibody detection assays utilized rat proteins conjugated with PCB or PEG polymers to selectively detect polymer-specific responses and were validated using commercial antibodies in competition assays. The data demonstrated that PCB-uricase conjugates performed better than their PEG counterparts: anti-PCB IgM (Fig. 4a) and IgG (Fig. 4b) titers were not detectable above negative control, while the corresponding anti-PEG antibodies were clearly identified and increased in titer with experimental time (Fig. 4c). All these results make PCB a competitive candidate in substituting PEG for future design of polymer-protein conjugates.

5.2 Nanogel encapsulation

Learning from immunogenicity studies of PEGylated proteins, we concluded that two issues are responsible for their immune response: limited polymer coverage of protein surfaces and the immunogenicity/antigenicity of PEG itself. As suggested by Richter and Akerblom, the degree of modification is a crucial factor determining PEG-protein conjugate immunogenicity.[14] To completely inhibit immune responses, a high degree of modification is needed to better mask the protein epitopes. However, due to the limited number of surface functional groups, or as a tradeoff to preserve protein activity, complete concealment of all danger signals from the protein surface is usually infeasible for therapeutic proteins. To overcome this key challenge of incomplete surface camouflage, we proposed that surface conjugation of a crosslinked polymer hydrogel layer would be an effective tactic to reduce immunogenicity.[60] As systematic studies have shown that PEG immunogenicity strongly correlates its hydrophobic characteristics, we have chosen zwitterionic PCB to coat protein surfaces as a less-immunogenic PEG alternative. The conjugation process was done by protein acylation and subsequent *in situ* radical polymerization. After being individually encapsulated in the super-hydrophilic zwitterionic gel (Fig. 5a), the model therapeutic protein uricase from *Candida sp.* showed exceptional stability and long *in vivo* circulation half-life. Repeated administrations did not induce accelerated clearance of the PCB nanocapsules, whereas PEGylated control group was cleared from blood nine times faster for the third dosage than the first dosage (Fig. 5b). Two techniques, including ELISA and SPR, have been applied to detect anti-polymer Ab generation. No detectable antibody response was observed against either the protein or the PCB polymer for nanocapsules, by contrast both anti-uricase and anti-PEG Abs were found in sera from PEG-uricase-treated group. Following uricase, a similar result was also observed from PCB-coated butyrylcholinesterase, demonstrating the effectiveness of PCB coatings on prolonging protein circulation and reducing immunogenicity.[84] Besides PCB encapsulation, nanogels made from another zwitterionic polymer poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC) was also reported to bestow proteins with long half-lives and reduced immunogenicity, although the generation of anti-polymer Abs was not studied.[85] Covering the whole protein surface with a super-hydrophilic polymer seems a promising strategy for enzymatic therapeutics, as the coated hydrogel layer allows

small substrates to diffuse into and reach the catalytic site. For protein therapeutics functioning by binding to large molecules, e.g. antibodies and cytokines, nanogel encapsulation is not appropriate as the gel coating would significantly hinder protein bindings.

5.3 Polypeptide fusion

While synthetic polymers have long been used for protein-polymer conjugation, some recent studies substituted PEG with hydrophilic polypeptide chains. Since the conjugation of polypeptide to a protein can be done genetically by recombinant technology, the specific characteristics of the peptide, such as sequence and length, can be precisely tuned during the production process. In addition, the naturally attached polypeptide has the similar chemical components as the protein itself, making it degradable in the native metabolic process.

XTEN technology has been reported to extend the therapeutic peptide exenatide's circulation half-life by genetically fusing with a non-structured hydrophilic polypeptide.[70] The polypeptide is composed of alanine, glutamic acid, glycine, proline, serine and threonine. By adjusting the XTEN sequence length, blood circulation half-lives of the fused peptide can be tuned from minutes to more than thirty hours in rats, due to the decreased removal via renal filtration. Immunogenicity tests showed that only weak or no IgG response specific to XTEN was detected when rabbits were immunized by XTEN conjugated green fluorescent protein with adjuvant, due to the exclusion of hydrophobic amino acids and the non-structured characteristic. However, since only one peptide chain is attached to a protein, XTEN conjugation did not prevent anti-protein Ab generation. Several XTEN-modified peptides and small proteins have been tested in pre-clinical or clinical studies, including glucagon, [86] glucagon-like peptide 2,[87] human growth hormone (hGH),[88] and annexin A5.[89]

Another peptide fusion technology, called PASylation, has been established using a similar design principle as XTEN. In the case of PASylation, only proline, alanine and serine were selected to build the polypeptide.[72] A random sequence of the three small, uncharged hydrophilic amino acids maximizes the peptide chain flexibility and hydrodynamic volume. The PAS polypeptide has been fused to interferon, hGH, F_{ab} fragments and leptin using the *E. coli* expression system, and all fused proteins showed significantly enhanced circulation half-lives.[72, 90, 91] Immunogenicity of PAS-conjugated proteins has been evaluated by testing ADAs in plasma from mice treated daily with PAS-hGH. Although anti-hGH Ab was induced by PAS-hGH, no anti-PAS Ab was found in the sera.

Based on the design principles of zwitterionic or mixed charged moieties, the EKylation technology has been developed and demonstrated.[71] Bioinformatics studies of over 1,000 proteins have revealed the predominance of positively charged lysine (K) and negatively charged glutamic acid (E) residues on protein surfaces at balanced ratios.[92] It is believed that the distribution of equal amounts of oppositely charged E and K residues provides a zwitterionic layer to stabilize protein and resist non-specific adsorptions. Both random and alternating EK sequences have been shown to confer nonfouling zwitterionic characteristics to surfaces and nanoparticles.[93–95] Thus, it was hypothesized that fusion of a simple alternating EK peptide would provide protection to the protein in a similar way as

zwitterionic PCB polymer. As a proof of concept, polyEK peptides of different lengths were fused to the model enzyme β-lactamase via *E. coli* expression.[71] As expected, the fused enzyme showed enhanced stability against thermal stress over its native version.

6 Conclusions

In conclusion, in contrast to the common assumption that PEG is biologically inert, both pre-existing and therapeutically induced anti-PEG Abs have been found in the general population as well as patients receiving PEGylated therapeutics. The existence of anti-PEG immunity has brought complications to PEGylated protein therapeutics, especially since several clinical studies have correlated efficacy loss and increased adverse events of certain PEG-containing therapeutic products with anti-PEG Abs. However, our understanding of the mechanisms of anti-PEG immunity and characteristics of anti-PEG Abs are still very limited. To manage these challenges, blood anti-PEG Ab levels of patients should be closely monitored before and during PEGylated drug treatment. Finally, safer and less immunogenic delivery approaches beyond PEGylation are highly desirable as alternatives for therapeutics.

Acknowledgments

The authors are grateful to the financial support from the Defense Threat Reduction Agency (HDTRA1-13-1-0044) and National Institutes of Health (1R21EB020781-01).

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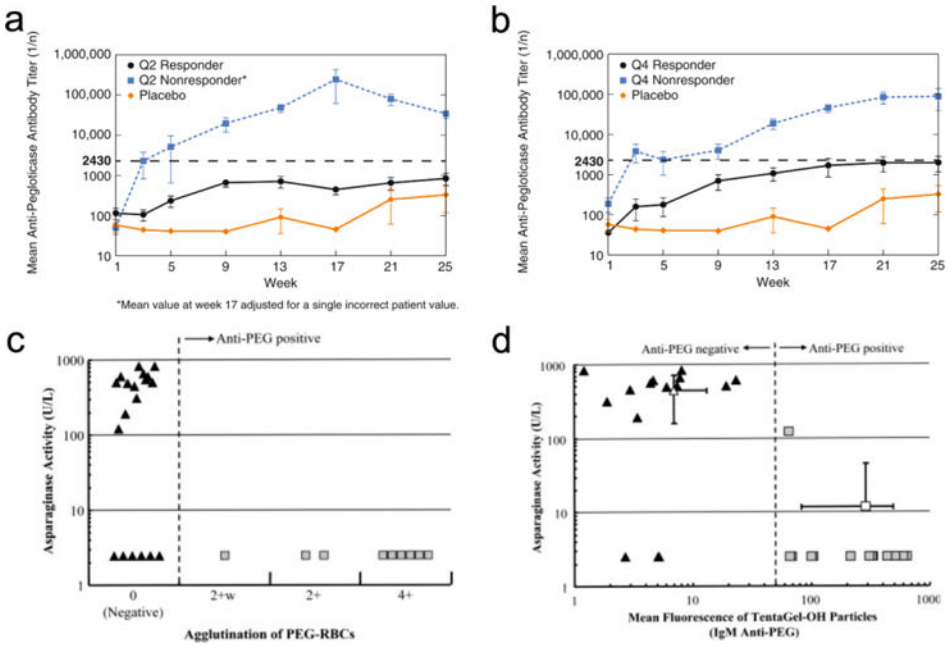

Figure 1.

Mean anti-pegloticase antibody titers over time among serum uric acid responders and NR receiving biweekly (a) and monthly (b) pegloticase. (d). Reprinted with permission from ref[19]. Anti-PEG antibody vs. asparaginase activity for patients treated with PEG-asparaginase tested by serological identification (c) and flow cytometry (d). Reprinted with permission from ref[16].

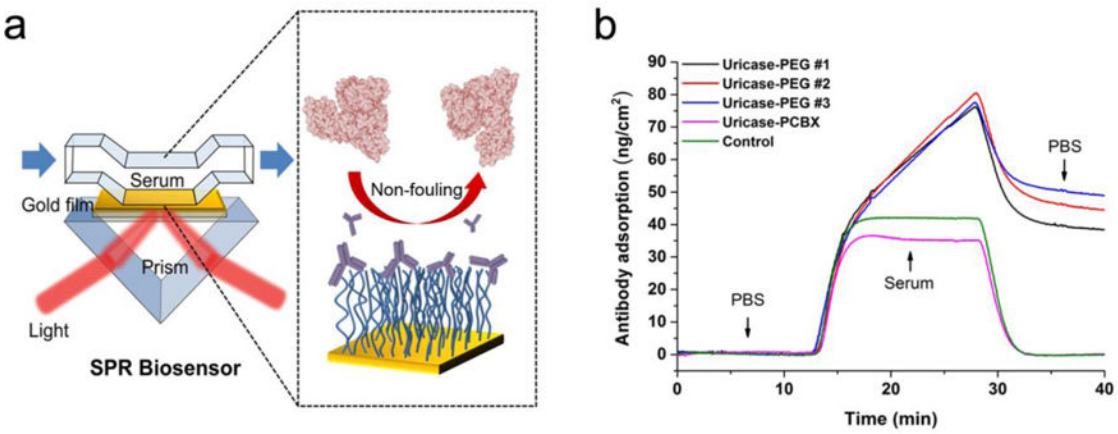

Figure 2.

Detection of anti-PEG Abs by an SPR sensor. (a) Scheme showing the detection setup. The gold chip surface was modified with PEG-based polymer brushes. The polymer brush serves dual functions, providing a nonfouling background while serving as the investigated antigen. (b) Sensor signal-time curves when detecting. Reprinted with permission from ref[60].

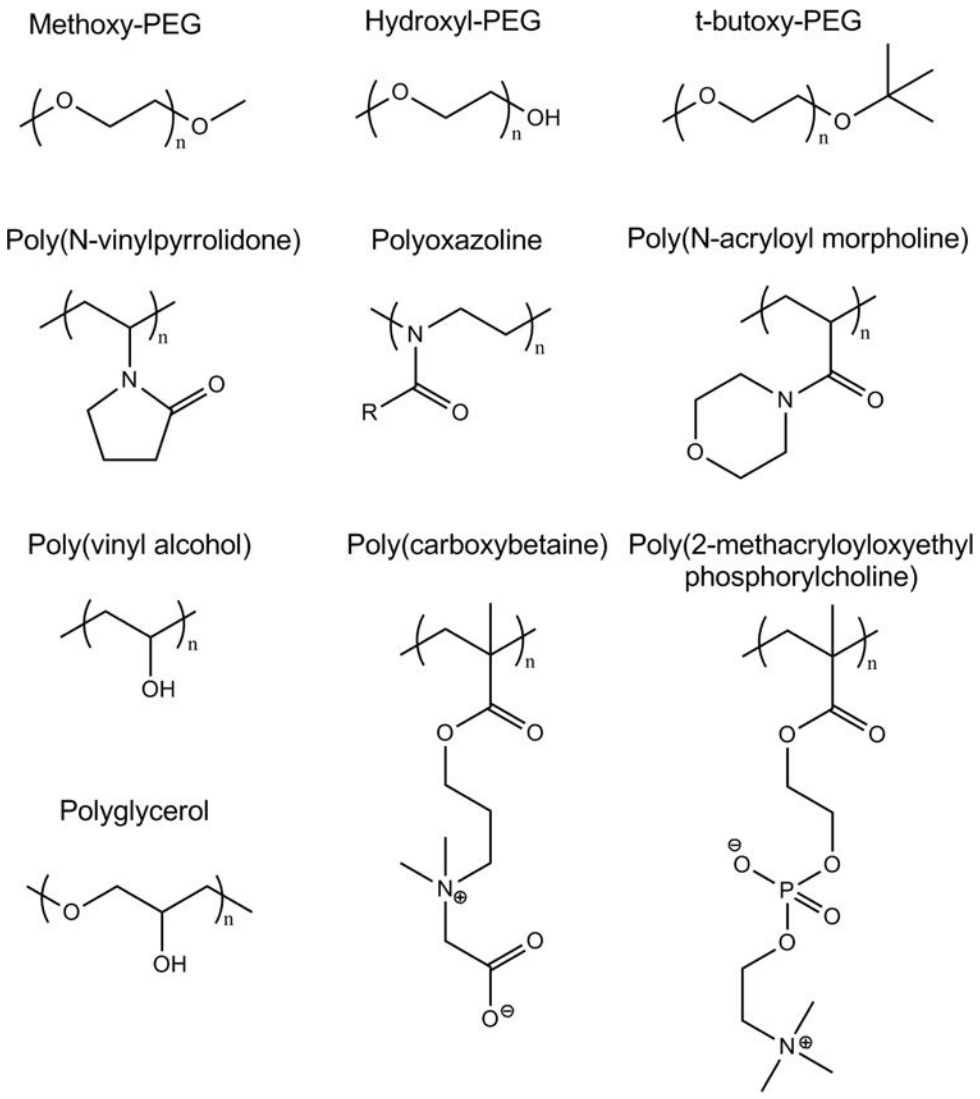

Figure 3.
Chemical structures of various synthetic polymers used for protein-polymer conjugation.

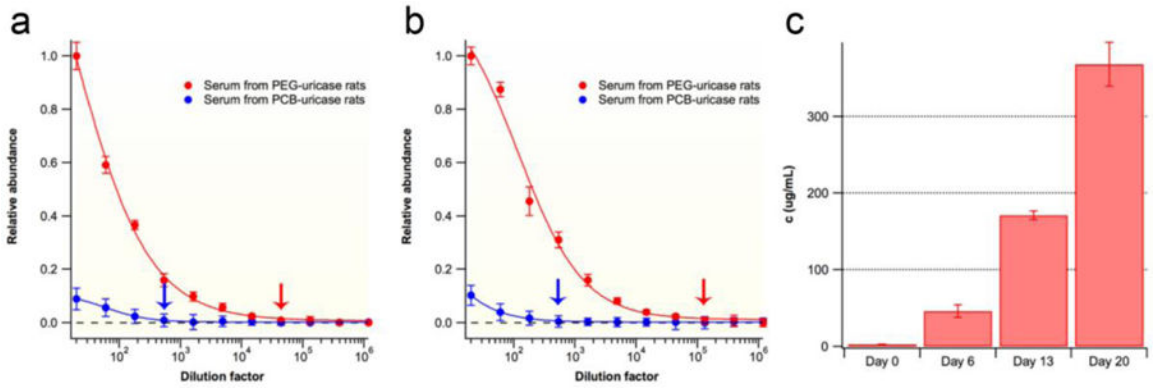

Figure 4.

Antibody titer against either polymer detecting for a) IgM and b) IgG after three repeated administrations of polymer-conjugated proteins. As rat anti-PEG IgM is commercially available, the IgM titer over the course of the study is quantified in c). Reprinted with permission from ref[83].

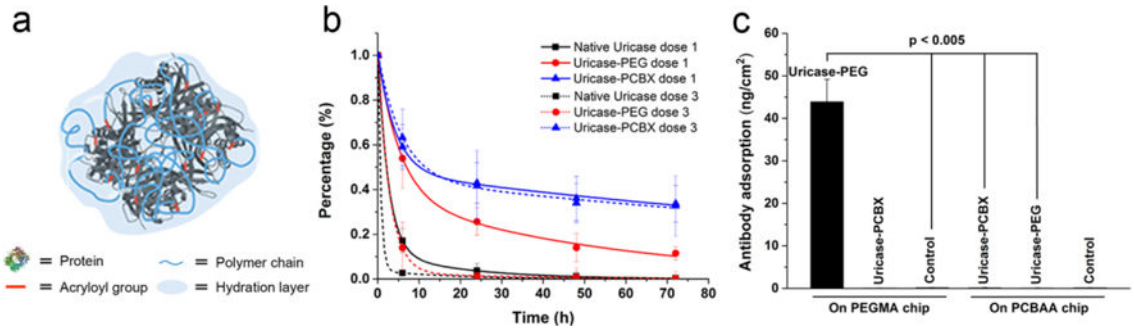

Figure 5.

a) Scheme shows the protein nanocapsule structure. b) Circulation profiles of the modified uricase after the first and third administrations. c) Anti-polymer Ab detected by SPR sensor. Reprinted with permission from ref[60].