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RESEARCH ARTICLE

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Development of a validated novel bead extraction method for the detection of anti-PEG antibodies in human serum

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ABSTRACT

Aims: Polyethylene glycol (PEG) is used in many applications including drug development. Due to exposure to environmental products, there is a high prevalence of preexisting anti-PEG antibodies in the global human population. The presence of anti-PEG antibodies is a concern for potentially reducing the efficacy of therapeutics after administration and represents a risk of safety events after exposure to PEGylated drug products. We developed and validated a creative and sensitive method for the detection of anti-PEG antibodies in human serum to support clinical programs for PEGylated drugs.
Methods: In this method, biotin-PEG streptavidin beads were used to extract anti-PEG antibodies from human serum for analysis in an anti-PEG ELISA assay. The same serum sample was analyzed in an anti-drug antibody assay.
Results: The anti-PEG antibody assay was validated with a screening cut point of 1.41 normalized signal, confirmatory cut point of 32.2% inhibition, sensitivity of 7.81 ng/mL and sufficient reproducibility, selectivity, and drug tolerance in accordance with the FDA 2019 Immunogenicity guidance.
Conclusion: This method of removal of anti-PEG antibodies enables the use of a single sample to detect anti-drug and anti-PEG antibodies to support drug development programs.

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KEYWORDS
PEG; anti-PEG antibodies; ADA; immunogenicity; BEAD

1. Introduction

Polyethylene glycol (PEG) is a synthetic hydrophilic polymer of ethylene oxide and is used in several applications including pharmaceuticals, cosmetics, and household products. Due to its polymerizing ability, PEG can be a mixture of different molecular weights and can be linear or branched and conjugated to various target molecules. The process of PEGylation can increase the solubility and stability of compounds [1,2] and thus has been attractive in drug development for its usefulness in improving pharmacokinetics and pharmacodynamics of therapeutics. Several pegylated drugs have already been approved by the FDA for various indications including oncology, autoimmune diseases, fungal infections, genetic disorders, and rare diseases [3,4]. In the gene therapy space, the inclusion of pegylated lipids in the formulation of lipid nanoparticles (LNPs) has also become an appealing strategy for drug delivery [5]. The prevalence of anti-PEG antibodies in the population has also been enhanced in the post-COVID era of LNP-based mRNA vaccinations [6,7]. Since PEG is a foreign compound and is used in a wide range of products, it is possible that a large number of the population already has had an immune response to PEG, resulting in anti-PEG antibodies.

The high prevalence of preexisting anti-PEG antibodies in the global human population is a concern for potentially reducing the efficacy of therapeutics after administration, as well as representing a risk of serious infusion reactions and anaphylaxis after exposure to PEGylated drug products [8–11]. Anti-PEG antibodies have been characterized as IgG and IgM antibodies and described as both immunogenic and antigenic. Several studies have demonstrated in preclinical models that anti-PEG IgM antibodies can be associated with accelerated blood clearance of PEGylated proteins after repeat dosing [12]. There have been several studies that have been conducted to better characterize the prevalence of preexisting anti-PEG antibodies in the normal population along with elucidating the isotypes of anti-PEG antibodies to help understand the potential implication of these antibodies in the population on drug treatment. Several of the studies demonstrate varying results of percentage of total anti-PEG antibodies and have explored the effect of sample collection time, gender, age, and race on prevalence of anti-PEG antibodies [13,14]. Given the high incidence of preexisting anti-PEG antibodies in the population along with the increased usage of PEG in drug development, regulatory agencies have expanded the requirements for screening and detection of anti-PEG antibodies in clinical studies associated with PEGylated compounds.

There are several important considerations for developing assays for the detection of anti-PEG antibodies, including the ability to detect both IgG and IgM antibodies, as both could have clinical relevance. The detection of anti-PEG antibodies based on the extent of PEGylation, linear versus branched, and molecular weight of the PEG used in therapeutics has also been

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Article highlights

- This method was developed to extract anti-PEG antibodies from human serum by capture with magnetic beads followed by acid dissociation.
- Removal of anti-PEG antibodies from human serum was demonstrated to be specific for anti-PEG and did not impact the ability to detect anti-drug antibodies.
- The method was validated with an anti-PEG antibody ELISA assay.
- Based on the high prevalence of preexisting anti-PEG antibodies, a cut point strategy was determined by evaluating the upper quantile of the negative population and the lower quantile of the positive population.
- The anti-PEG antibody assay was characterized in method development to be sensitive, selective, and robust.
- This extraction method was shown to be advantageous in removal of anti-PEG antibodies that were interfering with the ability to measure ADA to the non-PEG portion of a PEGylated drug product.
- The method also allows for anti-PEG antibody detection in human serum while also allowing detection of ADA from the same sample, thus allowing efficient use of clinical study samples.

explored in studies that have shown that the molecular weight and extent of PEGylation influences immune response, whereas branching of PEG may not [15]. The assays used to detect anti-PEG antibodies must be sensitive and have the ability to detect low affinity antibodies. Oftentimes, these assays utilize surrogate positive controls from a different species (i.e., mouse or rabbit antibodies) and thus require a dual detection system to include reactivity for human anti-PEG or may require a separate detection reagent for controls versus clinical samples. Additionally, the nature of PEG's repeating structure mimics surfactants and therefore antibodies can cross react with assay reagents and buffers that contain surfactants. Repetitive binding sites also make it difficult to use standard bridging anti-drug antibody (ADA) assays. A number of methods have been published and used to support the detection of anti-PEG antibodies in clinical studies to try to overcome some of these technical challenges including direct assays, conventional bridging assays [16] and affinity capture elution assays [17].

Despite the technical challenges of developing ADA assays, through our collective knowledge of antibody enrichment, understanding of the limitations with traditional immunoassays, and extensive understanding with statistical methods for determining cut points in populations with preexisting antibodies, we have demonstrated the development and validation of a creative and sensitive method for the detection of anti-PEG antibodies in human serum. This method is especially useful in allowing extraction of anti-PEG antibodies from human serum that could interfere with ADA detection of therapeutics or PEGylated therapeutics. This method proves to be efficient to support clinical study sample analysis for PEGylated drugs by allowing anti-drug and anti-PEG antibody detection in the same sample.

2. Materials and methods

2.1. Serum samples

All normal, lipemic, and hemolyzed serum samples were collected from whole blood. All the individual serum samples were purchased from BioIVT (Hicksville, NY, USA) and stored at −20°C or colder until use. A pooled serum sample (NC, Negative Control) was prepared by combining volumes from selected normal individuals. Individuals were selected as the lowest screening and confirmatory responses in the described assay and the pool was tested during assay qualification to demonstrate minimal reactivity (data not shown).

2.2. PEG reagents

Linear monofunctional methoxyl polyethylene glycol (mPEG) with a 40K molecular weight was purchased from Nanocs Inc (New York, NY, USA). The biotinylated version (mPEG Biotin) or the unlabeled version (mPEG) were used as described in the following methods sections. Multi-PEGylated BSA or mPEG-SVA (PEGylated with 17-fold excess of 20kDa mPEG succinimidyl valerate) was purchased from Life Diagnostics, Inc (West Chester, PA, USA) and used as described. A commercial surrogate positive control (SPC) anti-PEG mouse monoclonal antibody (Life Diagnostics, West Chester, PA, USA) was utilized to prepare varying levels of assay controls for the anti-PEG antibody ELISA.

2.3. Labeling of biotin-PEG streptavidin magnetic beads

Sera Speed Magnetic Streptavidin beads (Cytiva, Marlborough, MA, USA) were brought to room temperature and resuspended by transferring the appropriate volume of beads into a centrifuge tube. The tube with the beads was pelleted on a DynaMag stand for 2–3 minutes and then removed. 1X PBS Wash Buffer was added at a volume equal to the bead volume, and the tube was vortexed 5–10 seconds. The tube was then returned to the magnetic stand for 2–3 minutes to pellet the beads. All liquid was removed and discarded. This wash was repeated five more times. After the last wash, mPEG Biotin diluted in Superblock PBS buffer to 1 mg/mL was added at a volume equal to the bead volume, the tube was vortexed for 5–10 seconds, and then sealed and incubated for a minimum of one hour at room temperature on end over an end rotator set to 37–40. After incubation, the beads were pelleted for 2–3 minutes on the magnetic stand, the liquid was removed and discarded, and then the washing steps with 1X PBS Wash Buffer were performed eight times. After the final wash, the beads were resuspended with a volume of Super Block PBS Buffer equal to the original bead volume, and the beads were stored at 2–8°C until use.

2.4. Bead extraction method

An appropriate volume of labeled Biotin-PEG Streptavidin beads was transferred to a centrifuge tube and placed on a DynaMag stand to thoroughly pellet the beads. The liquid was removed, and an equal volume of 1X PBS was added to the bead pellet. The tube was vortexed to resuspend the beads and placed back on the DynaMag to pellet the beads. This wash step was performed two additional times. The bead pellet was resuspended in casein buffer. Beads were transferred into the appropriate wells of a polypropylene plate and placed on a magnetic plate. While holding the plate magnet against the polypropylene plate, the liquid was

removed from the wells by gently tapping onto the paper towel, with caution not to lose beads.

Samples or controls were diluted in casein buffer or confirmatory buffer at minimum required dilution (MRD) 10 in a separate polypropylene plate. For the confirmatory, samples and controls were incubated after MRD dilution for one hour at room temperature, shaking at approximately 450 RPM. Samples/controls were added to the beads and mixed well by pipetting up and down to ensure sufficient mixing. The plate was incubated at room temperature for one hour with plate shaking at approximately 600 rpm. Then the plate was moved to 2–8°C for overnight incubation while shaking at 600 rpm.

After overnight incubation, the plate was placed on a plate magnet, and the supernatant was removed with a multi-channel pipette and discarded. The plate was washed with an automated plate washer while using a magnet. The plate was washed 3 times with 1X PBS. 0.3 M acetic acid was added to the beads and incubated for 10 minutes on a shaker at approximately 700 rpm. After incubation, the plate was set on a plate magnet, and the supernatant was transferred into a separate polypropylene plate containing 1 M Tris HCl (pH 9.5) in each well to neutralize. The plate containing neutralized bead extract was incubated for 5 minutes while shaking at 450 rpm at room temperature and used in the anti-PEG ELISA method.

2.5. Anti-PEG antibody ELISA

96-well Nunc Immuno Maxisorp plates were coated by addition of 100 µL/well mPEG-SVA in carbonate coating buffer, human IgG in 1X PBS (Jackson ImmunoResearch, West Grove, PA, USA), human IgM in 1X PBS (Jackson ImmunoResearch, West Grove, PA, USA) or PBS buffer only. Plates were sealed, tapped to ensure equal distribution of the liquid in each well, and then incubated at room temperature overnight without shaking. On Day 2 of the assay, plates were washed three times by addition of wash buffer followed by inversion of the plates to remove the liquid and tapping dry on absorbent paper. Casein blocking buffer was then added to all wells, and the plates were covered and incubated at room temperature with shaking for 1–3 hours. The blocked plates were washed three times with 1X PBS and tapped dry. 100 µL/well of the neutralized bead extract from the bead extraction plate described in the previous section was then transferred to the coated plates in duplicate wells. Plates were sealed and incubated on an orbital shaker at ~450rpm at room temperature for 60–80 minutes. After incubation, plates were washed three times with 1X PBS and tapped dry. 100 µL/well of the working detection antibodies (Goat anti-Mouse IgG-HRP, Fitzgerald Industries, Acton, MA, USA), and goat anti-Human IgG-HRP (MilliporeSigma, Burlington, MA, USA) were added to the plates. Plates were sealed and incubated on an orbital shaker at ~450rpm at room temperature for 60–80 minutes. After incubation, plates were washed three times with 1X PBS and tapped dry. 100 µL/well of the room temperature TMB was added to the plates. Plates were incubated on an orbital shaker at ~450rpm at room temperature, and the OD was monitored. When OD 650 reached ~1.0 for the HPC, the reaction was stopped by the addition of 50 µL/well TMB Stop Solution. Plates were read at 450 nm on a BioTek Synergy 2 plate reader.

2.6. Anti-drug antibody core assay

The core ADA assay refers to a bridging assay that was used to detect ADA to a proprietary PEGylated peptide drug product. The assay used a surrogate positive control (SPC) proprietary to the drug product. Positive controls were prepared by spiking the SPC into human serum at varying concentrations (HPC, MPC, LPC). Details of this method are not required for understanding the novel bead extraction anti-PEG antibody method described herein. This core ADA assay required a high drug tolerance (~1 mg/mL) for this drug program.

3. Results

3.1. Effect of anti-PEG removal from serum samples

The bead extraction method is depicted in Figure 1. To determine the effect of anti-PEG antibody extraction, samples were untreated or treated with 0.1, 1, 5, and 10 mg/mL of biotinylated mPEG beads to remove preexisting anti-PEG from the sample. After treatment, anti-PEG antibodies were acid eluted and tested for binding in the anti-PEG ELISA assay. This experiment was performed with 8 individual serum samples with varying levels of anti-PEG antibodies (data not shown). All samples with high anti-PEG antibodies showed a reduction in anti-PEG binding after anti-PEG removal treatment, thus demonstrating that the extraction treatment removed anti-PEG antibody from the samples. Figure 2(a) shows a representative sample from this experiment. In comparison to no treatment, samples exposed to bead capture at all concentrations showed a significant reduction in anti-PEG antibody binding. The amount of capture reagent did not have a significant impact on the signal post removal with 0.1 mg/mL appearing to be a saturating concentration for anti-PEG removal.

The effect of anti-PEG removal was further evaluated in the core ADA assay and in the anti-PEG antibody assay. For this evaluation, control samples were prepared with ADA SPC spiked at high (aDrug HPC), medium (aDrug MPC), and low concentration (aDrug LPC) in human serum. Three negative serum (NC) samples, and high (aPEG HPC) and low (aPEG LPC) anti-PEG antibody serum sample that was previously identified, were also evaluated in this experiment. All samples were treated with 0.1 and 10 mg/mL capture beads, then acid eluted and assayed in the anti-PEG ELISA assay (Figure 2(b)). Only samples with anti-PEG antibody showed activity in the anti-PEG ELISA thus confirming specificity for anti-PEG in the removal step. Signal was slightly reduced for the anti-PEG control samples (aPEG HPC, aPEG LPC) upon extraction, but the reduction was not significant. The SPC spiked samples (aDrug H/M/LPC) and the NC samples remained unaffected pre- and post-extraction.

The same samples prepared previously were also evaluated in the core ADA assay to determine the effect of anti-PEG removal on the core ADA assay. Controls were analyzed from a supernatant that was subjected to an ACE (affinity capture elution) method with an acid dissociation step (300 mm and 600 mm acetic acid) prior to analyzing in the core ADA assay (Figure 3(a)). The same controls were analyzed from

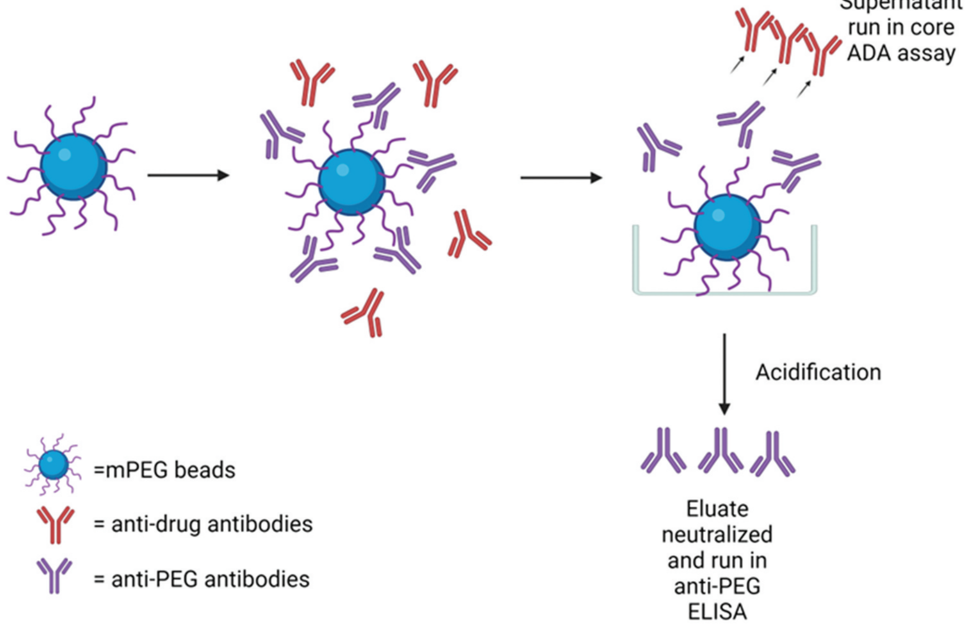

Figure 1. The BEAD extraction method. Magnetic streptavidin beads were labeled with mPEG Biotin and incubated with serum samples in a polypropylene plate overnight. After overnight incubation, the plate was placed on a plate magnet and the supernatant containing non-peg antibodies removed to be run in the core ADA assay. Acetic acid was added to the beads to elute the anti-peg antibodies and neutralized into a separate polypropylene and used in the anti-peg ELISA method.

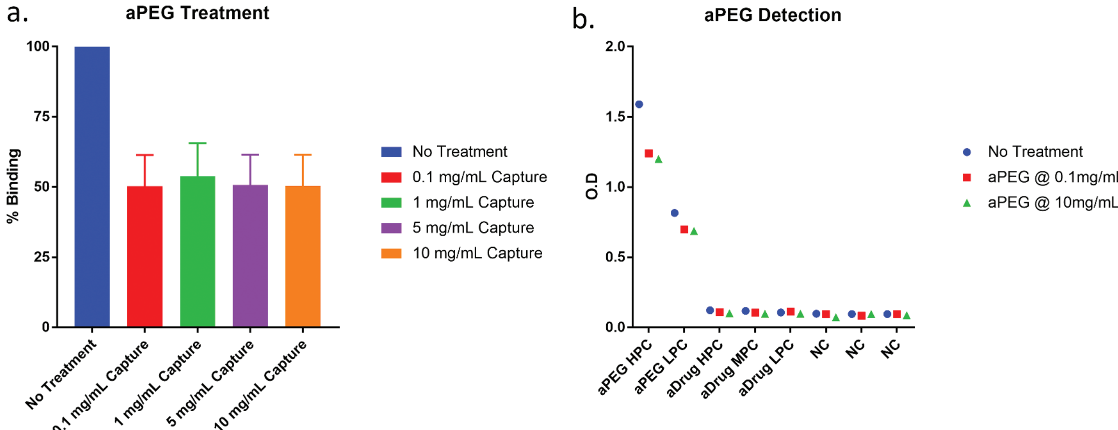

Figure 2. Removal of anti-peg antibodies from samples. (a) Samples were untreated (blue bar) or treated with 0.1 mg/mL (red bar), 1 mg/mL (green bar), 5 mg/mL (purple bar), and 10 mg/mL (orange bar) capture to remove anti-peg antibodies from sample. The samples were analyzed for anti-peg binding activity. (b) Human serum samples containing high (aPEG HPC) and low (aPEG LPC) levels of anti-peg antibodies, samples prepared with positive control spiked at high (aDrug HPC), medium (aDrug MPC) and low concentration (aDrug LPC) in human serum and negative serum pool samples (NC) were untreated (blue circles) or treated with 0.1 mg/mL (red squares) or 10 mg/mL (green triangles) capture beads, then acid eluted and assayed in the anti-peg ELISA assay.

supernatant directly into the core ADA assay after anti-PEG removal with 0.1 and 10 mg/mL bead capture for comparison of both assay formats (Figure 3(b)). When analyzed in the ACE method, the anti-PEG positive samples (aPEG HPC, aPEG LPC) showed no signal above background in the core ADA assay. The data shows that both formats of sample pretreatment do not dramatically lower or affect the detection of anti-drug antibodies in the core ADA method. In addition, results indicate that removal of anti-Peg antibodies does not impact the ability to detect ADA in the sample.

Lastly, the effect of anti-PEG removal was evaluated on the drug tolerance of the core ADA assay. This was assessed with positive control samples spiked with SPC at varying concentrations, at low (LPC), medium (MPC), and high (HPC) and incubated with increasing concentrations of drug (0 to 2.5 mg/mL) (Figure 4(a)). These samples were evaluated in the ACE format where the supernatant was subjected to an acid dissociation step (Figure 3(a)) or subjected to anti-PEG bead removal (Figure 4(b)) and analyzed in the core ADA assay. Removal of anti-PEG antibodies showed higher levels of drug tolerance at all levels of positive controls tested. The background of the ACE format was slightly higher which could have an impact on assay sensitivity, as compared to the bead extraction method. The drug tolerance was determined to be 313 µg/mL at the HPC, 78 µg/mL at the MPC, and 39 µg/mL at the LPC (100 ng/mL) in the ACE core ADA assay format. Drug tolerance was significantly increased after removal of anti-PEG antibodies with bead

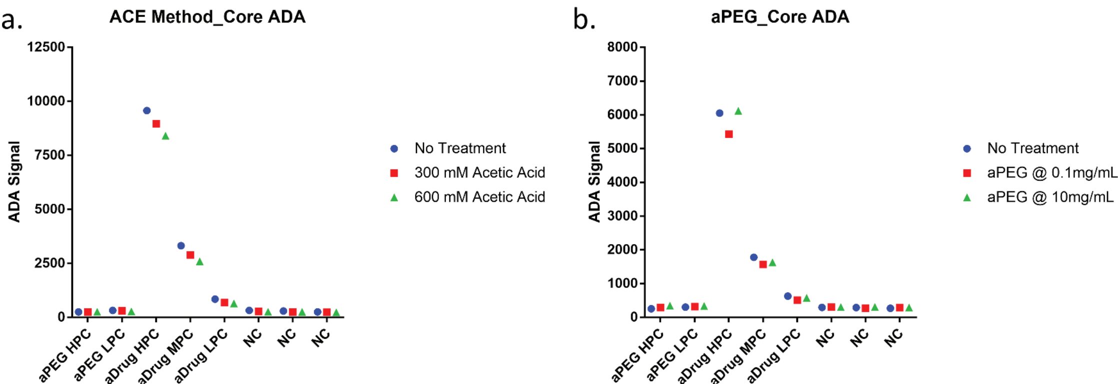

Figure 3. Comparison of anti-drug antibody levels with sample pretreatment. (a) Human serum samples containing high (aPEG HPC) and low (aPEG LPC) levels of anti-peg antibodies, samples prepared with positive control spiked at high (aDrug HPC), medium (aDrug MPC) and low concentration (aDrug LPC) in human serum and negative serum pool samples (NC) were untreated (blue circles) or subjected to an ACE (affinity capture elution) method with an acid dissociation step with 300 mm (red squares) or 600 mm (green triangles) acetic acid prior to analyzing in the core ADA assay. (b) The same samples were subjected to no treatment (blue circles) or treated with 0.1 mg/mL (red squares) or 10 mg/mL (green triangle) anti-peg bead capture and analyzed in the core ADA assay.

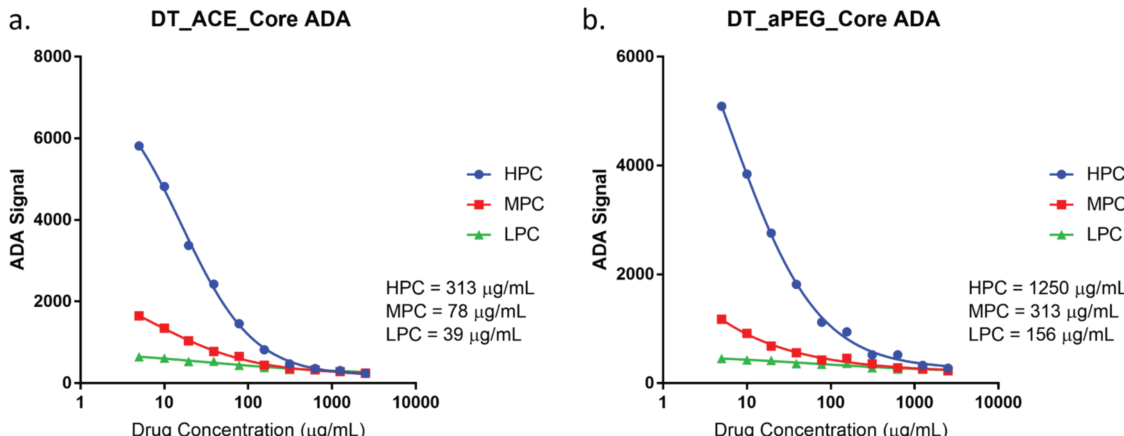

Figure 4. Drug tolerance in core ADA assay after sample pretreatment. (a) This was assessed with positive control samples that were prepared by spiked positive control in human serum at varying concentrations at high (HPC; blue circles), mid (MPC; red squares), and low (LPC; green triangles) and incubated with increasing concentrations of drug (0 to 2.5 mg/mL). Samples were assayed in the conventional ACE ADA core method. (b) The same positive control samples were treated with the anti-peg depletion method and analyzed for drug tolerance in the core ADA method.

capture and was determined to be 1.25 mg/mL at the HPC, 313 µg/mL at the MPC, and 156 µg/mL at the LPC after anti-PEG removal, thus, demonstrating superior drug tolerance achieved post removal of anti-PEG antibodies with bead extraction.

3.2. Preliminary cut point assessment

Preliminary anti-PEG screening and confirmatory cut points were determined prior to the assay validation (Figure 5). In the preliminary determination, the screening cut point was determined

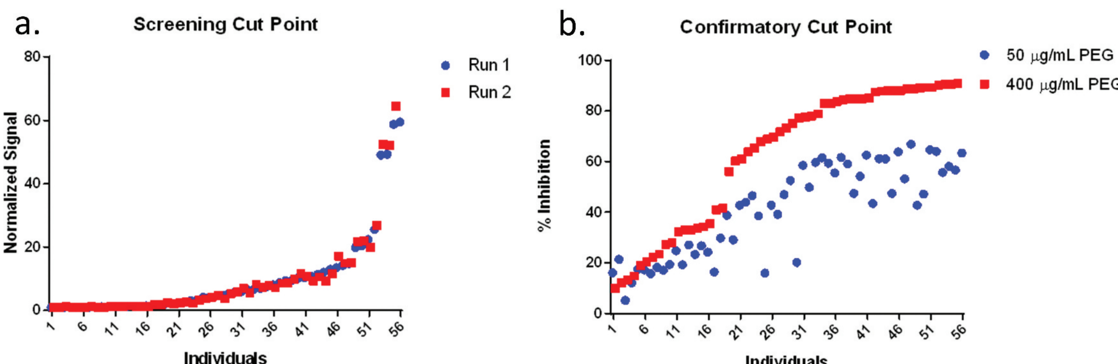

Figure 5. Preliminary anti-peg screening and confirmatory cut point determination. (a) The screening cut point was determined by testing 56 individual serum samples in run 1 (blue circles) and run 2 (red squares) performed by two analysts. The response of each sample was normalized to the mean of the NC. (b) The confirmatory cut point was evaluated with the same 56 individual serum samples with excess PEG at 50 µg/mL (blue circles) or 400 µg/mL (red squares) and analyzed in the anti-peg assay.

by testing 56 individual normal human serum samples that were assayed in two independent runs. Three replicates of the NC were on each plate and were used to normalize the individual sample response to the mean of the NC pool. The resulting preliminary cut point factor was determined to be 1.4. Based on this data, most normal human sera were shown to have some level of response in the screening assay indicating preexisting anti-PEG antibodies (Figure 5). A preliminary confirmatory cut point was evaluated with an excess of 50 µg/mL and 400 µg/mL of PEG in the confirmatory buffer. The preliminary confirmatory cut point was determined to be 42% inhibition at the 400 µg/mL level and 30% inhibition at the 50 µg/mL level. Data from the cut point runs were plotted comparing screening normalized signal to confirmatory percent inhibition (Figure 5). The assay was later validated in accordance with the 2019 FDA Immunogenicity guidance [18] with the screening cut point of 1.41 normalized signal and a confirmatory cut point of 32.2% inhibition (data not shown).

3.3. Establishing assay controls for the anti-PEG assay

To monitor assay performance, anti-PEG controls were prepared and characterized during method development. Positive controls were prepared with the anti-PEG mouse monoclonal antibody at 500 ng/mL (HPC-m), 100 ng/mL (MPC-m), and 20 ng/mL (LPC-m). A second set of positive controls were assessed by using a commercial serum sample with high anti-PEG antibodies that was identified during the preliminary cut point assessment. The sample was diluted 1:5 in NC serum pool (HPC-h) and at a low level generated at a 1:20 dilution (LPC-h). These controls were not assigned a concentration but were spiked into the NC serum pool to give a signal that represented a high and low response endogenous anti-PEG response in the assay. These positive controls were detected with a cocktail composed of anti-mouse IgG and anti-human IgA/G/M antibodies. Control conditions were included on each development plate with uncoated wells, wells coated with IgG, or wells coated with IgM were also used to characterize detection performance. Mean responses and %CV from 4 independent runs are depicted for all controls in preliminary precision experiments and shown in Table 1.

The NC used during early development was derived from a pool of 3 individuals which had low signal in the screening assay. For validation, a larger NC pool needed to be prepared by screening additional serum samples. A total of 117 individuals from mixed race, age, and gender were screened in the assay (Table 2). The data was normalized against the development NC pool and compared to the preliminary cut point of 1.4. Individuals highlighted in red (Table 2) were selected for preparation of the larger NC pool to be used to preliminarily characterize the assay and for use in validation.

3.4. Characterization of the anti-PEG assay

Sensitivity was first assessed by performing serial dilutions of the commercial SPC, anti-PEG antibody, spiked above the HPC-m in a neat matrix, followed by dilution to the MRD and extraction of the anti-PEG antibodies. Using the preliminary cut point of 1.4, the calculated sensitivity was determined at 7.81 ng/mL (Supplemental Figure S1). Drug tolerance was evaluated in method development in the presence of PEG starting at 10,000 µg/mL, then diluted to 7.81 µg/mL in NC pool. Drug tolerance was based on a 2.5-fold increase over background (Table 3). The assay appeared to be tolerant to 500 µg/mL of PEG at the LPC concentration of 20 ng/mL. Lastly, preliminary selectivity was assessed by spiking a high (500 ng/mL) and low (20 ng/mL) concentration of the mouse SPC into 4 individual normal human serum samples followed by dilution to the MRD. The serum samples were also run unspiked at the MRD in the assay (Table 4). Samples were normalized to the mean of the NC. All unspiked samples (NC-SEL-1, NC-SEL-2, NC-SEL-3, NC-SEL-4) screened negative or below the preliminary screening cut point of 1.4. The low and high spiked selectivity samples (HPC-SEL-n

Table 1. Characterization of assay controls.

Control	Conc/Dil	Mean	CV (%)
HPC-m	500 ng/mL	43.95	5.2
MPC-m	100 ng/mL	10.76	2.2
LPC-m	20 ng/mL	2.99	3.1
HPC-h	1:5	59.35	2.9
LPC-h	1:20	3.57	16.4
NC ($n = 12$)	0 ng/mL	0.06	4.6
IgG	2 mg/mL	1.40	15.3
IgM	2 mg/mL	0.31	24.4
Uncoated	0 ng/mL	0.04	0.6

HPC-m: high positive control mouse; MPC-m: mid positive control mouse; LPC-m: low positive control mouse; HPC-h: high positive control human; LPC-h: low positive control human; NC: negative control; IgG: immunoglobulin G; IgM: immunoglobulin M.

Table 2. Screening individual serum samples.

Sample ID	Gender	Mean	CV (%)
S1	Female	0.82402	4.2
S2	Female	0.84987	1.5
S3	Male	0.88578	0.9
S4	Female	0.90602	1.1
S5	Male	0.92481	3.4
S6	Female	0.93459	5.6
S7	Female	0.95639	0.9
S8	Female	0.96237	0.2
S9	Female	0.97218	1.2
S10	Female	1.0422	0.7
S11	Male	1.0455	0.3
S12	Female	1.068	3.5
S13	Female	1.0711	1.5
S14	Female	1.1091	4
S15	Male	1.1254	16.8
S16	Female	1.16	0.6
S17	Male	1.1693	3.7
S18	Male	1.1857	7.4
S19	Male	1.1869	15
S20	Male	1.2029	0.9
S21	Male	1.2061	0.7
S22	Female	1.2345	5.7
S23	Female	1.252	2.3
S24	Female	1.2733	1.6
S25	Male	1.2947	5.1
S26	Female	1.3014	1.2
S27	Female	1.3029	7.8
S28	Female	1.3797	0.9
S29	Female	1.4808	5.4
S30	Male	1.503	0.6

S: sample. Samples S1-S18 were selected for preparation of the larger NC pool to be used to preliminarily characterize the assay and for use in validation.

Table 3. Drug tolerance assessment.

PEG concentration (μg/mL)	HPC (500 ng/mL)	MPC (100 ng/mL)	LPC (20 ng/mL)
10,000	0.1445	0.06	0.064
4000	0.157	0.0765	0.083
2000	0.358	0.1325	0.113
1000	0.7635	0.2345	0.149
500	1.335	0.397	0.175
250	1.9385	0.5405	0.177
125	2.266	0.6505	0.219
62.5	2.548	0.7355	0.224
31.3	2.772	0.7835	0.220
15.6	2.447	0.7885	0.216
7.81	2.8215	0.8115	0.215
0	2.6695	0.787	0.186

HPC: high positive control; MPC: mid positive control; LPC: low positive control.

Table 4. Selectivity assessment.

Sample	Concentration (ng/mL)	Normalized Value (O.D.)
HPC-SEL-1	500	46.90
LPC-SEL-1	20	2.69
NC-SEL-1	0	0.99
HPC-SEL-2	500	48.14
LPC-SEL-2	20	3.00
NC-SEL-2	0	1.13
HPC-SEL-3	500	47.51
LPC-SEL-3	20	2.82
NC-SEL-3	0	1.03
HPC-SEL-4	500	50.37
LPC-SEL-4	20	3.36
NC-SEL-4	0	1.09

O.D.: optical density; HPC-SEL: high positive control spike; LPC-SEL: low positive control spike; NC-SEL: unspiked.

and LPC-SEL-n) screened positive (>1.4 cut point) in all four samples that were tested.

After the assay was characterized in method development by assessing preliminary assay cut points, sensitivity, control performance, and drug tolerance, the assay was moved into validation. The assay was validated in accordance with the 2019 FDA immunogenicity guidance [18] (validation data not shown).

4. Discussion

PEG is commonly added to proteins, peptides, and nanoparticles as a means to extend the half-life of therapeutic molecules in circulation and improve therapeutic efficacy. The prevalence of PEG in consumer products, in the environment, and within drug products can be a source leading to a high prevalence of pre-existing anti-PEG antibodies in patient populations. The existence of the preliminary anti-PEG antibodies can theoretically lead to increased clearance of dosed PEGylated therapeutics, resulting in potentially decreased efficacy but also increased risk of hypersensitivity reactions. When monitoring the immunogenicity of the PEGylated biotherapeutic, it is important to be able to track both the reaction to the PEG portion of the molecule, as well as the immune response to the "core" drug itself. In addition, given the prevalence of anti-PEG antibodies, it can be very difficult to measure the drug-specific immunogenicity response. This novel method was developed for two main reasons. First, for this specific drug program the high level of anti-PEG antibodies in human serum was interfering with the ability to measure ADA to the PEGylated drug product which also required a high drug tolerance. Traditional drug tolerant assay formats such as ACE were evaluated and did not provide the level of drug tolerance in the core ADA assay needed to support the clinical study. Second, the method enables use of a single pre-extraction to detect antibodies to the therapeutic drug (core ADA) as well as to PEG in a robust and sensitive anti-PEG ELISA assay.

Several considerations need to be made when developing assays for anti-PEG antibody detection. Given that both IgG and IgM anti-PEG antibodies can play a role in affecting the safety and efficacy of PEGylated therapeutics, it is important to understand if anti-PEG assays that are being used to support clinical studies can detect both isotypes. Our method herein is capable of measuring both IgG and IgM anti-PEG antibodies (data not shown). Given the variety in PEG moieties being used in drug development (varying molecular weights, linear, branched) it is also important to understand if the anti-PEG antibody assay being used can detect the anti-PEG antibodies to the PEG of interest. With our novel extraction method, we have also confirmed that our method can be used for 2kDa, 5kDa, 20kDa, and 40kDa PEG molecules, making it a platform assay for the detection of antibodies to varying sizes of PEG.

Our unique method offers some interesting advantages to other methods available for ADA detection for PEGylated drugs. Routinely, two independent assays are developed for the detection of antibodies to the drug and a second method for the detection of antibodies to PEG. This is typically utilized with independent samples or aliquots of samples as it undergoes multiple freeze/thaw cycles and tiered analysis (screening, confirmation, and titration). Our method allows for efficient removal of anti-PEG antibodies from a human serum sample that can be analyzed in two different assays from a single sample aliquot, which can also minimize the volume of sample that is collected from patients. It allows for sample use on other assays that can provide information on the drug's safety and efficacy with use in other bioanalytical assays.

There are other published assays that have been developed and demonstrated to allow an advantage of a single assay validation as opposed to two assays for anti-drug and anti-PEG simultaneous detection. For example, the ACE-AGL method utilizes protein AGL to recapture ADA following acid elution (17). Our method can be adapted for use in cases where acid elution is insufficient for extremely high levels of drug tolerance or for mitigation of interference with anti-PEG antibodies in serum samples. Another challenge in anti-PEG antibody assay development is that the detection system oftentimes is different for controls and samples when surrogate positive controls are used from different species (e.g., mouse or rabbit). In these cases, it requires a dual detection system that utilizes plate real estate as control wells in sample production, thus minimizing the sample throughput of the assay. We have demonstrated in our validation, the use of both surrogate positive control detection and a human serum positive sample used as a control that demonstrated acceptable precision and reproducibility in the assay. This data allows for potentially dropping the commercial positive control and only using the human positive control in sample analysis. This will allow the use of a single detection system and monitoring of assay run performance with a relevant human anti-PEG antibody control.

One of the most challenging aspects of developing and validating anti-PEG antibody assays, or ADA assays that demonstrate high prevalence of preexisting antibodies in the human population, is the ability to determine accurate cut points. In cases where preexisting antibodies make up more than 30% of the baseline samples, standard outlier removal steps might not be feasible. Especially after removal of large data sets from cut point determination that can leave a very small data set to perform statistical analysis [19]. There are published methods for calculating cut points for high preexisting antibody assays that have been described in various publications and caution excessive removal of outlier for cut point determination [20,21]. In this method validation, we sought an interesting approach to determine the screening cut point (SCP). In order to understand true positivity, identifying individuals that have preexisting responses is critical so that the cut point is not set artificially high and therefore would not accurately identify true positive responses in patient samples. As shown in Figure 4, our cut point data set fell along a spectrum of responses, with no clear delineation of preexisting positives versus negative samples. Given the spectrum of positive signal in the screening cut point data, the assay cut points were calculated in two ways by evaluating the populations. An upper 95% quantile of the presumptive negative samples and a lower 95% quantile of the positive samples. For both populations, an ANOVA was fit to analyze the variables isolated in the experimental balanced design and determined the primary sources of variability. The lower 95% quantile of the positive population was ultimately used to set the screening cut point, because the positive population was a larger dataset (and thus more robust) and because the cut point factor yielded a more conservative result of 1.4 normalized signal. As with the screening cut point determination, the confirmatory cut point was evaluated after splitting the individuals into the "negative" and "positive" populations and determined with an upper 99% quantile was calculated for all negative population results, and a lower 99% quantile was calculated for positive population results. The cut point determination strategy is an alternative approach to standard ADA assays given the high prevalence of preexisting antibodies in the population. In both instances, the lower cut point was implemented to ensure positives were not under-reported. Cut point determination for anti-PEG assays needs to be set with caution and evaluated in a way that minimizes missing positive ADA samples.

5. Conclusion

A novel, creative, sensitive, and selective method was developed and validated for the detection of anti-PEG antibodies in human serum using a bead extraction method. This method allows for extraction and detection of anti-PEG antibodies from human serum in an anti-PEG antibody ELISA assay. The method also allows for anti-PEG antibody removal for sufficient detection of anti-drug antibodies from the same human serum sample.

Disclosures

The views and conclusions presented in this paper are those of the authors and do not necessarily reflect the representative affiliation or company's position on the subject. The authors are employed by their affiliated companies and have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

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