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Original article

A double antigen bridging immunogenicity ELISA for the detection of antibodies to polyethylene glycol polymers

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ABSTRACT

Introduction: Polyethylene glycol (PEG) polymers attached to biotherapeutic molecules enhance in vivo delivery and stability of these large molecular weight drugs. However, these polymers may by themselves be immunogenic and elicit antibodies that can reduce the efficacy of the drug and contribute to potential patient morbidity. A double antigen bridging ELISA immunogenicity assay for the detection of anti-drug antibodies (ADAs) specific to PEG polymers of various sizes has been developed. **Methods:** Hapten-labeled conjugate of 40 kDa PEG polymer was synthesized and used in a double antigen bridging ELISA. The hapten-labeled PEG is incubated with the patient sample, then this mixture is added to a 96-well microplate precoated with 40 kDa PEG, allowing PEG-specific ADA to form a bridge complex with the PEG conjugate and the PEG coated on the microplate. After incubation, the reaction mixture is removed and replaced by horseradish peroxidase (HRP)-labeled anti-hapten antibody. After sufficient incubation, the plate is washed and substrate reagent is added. Enzyme color development, directly proportional to ADA, is stopped after 20 min with 2N sulfuric acid and the absorbance in each well is measured at 450/630 nm. Dose response, drug tolerance, matrix effects, reproducibility, specificity/free drug depletion experiments and screening cut-point determination of 350 naïve normal human sera were performed. **Results:** Using an anti-PEG mouse monoclonal IgM as a positive control, a reproducible dose response curve was demonstrated for the PEG Immunogenicity ELISA. Pre-existing PEG-specific antibodies which were proven to be highly specific to the PEG polymer structure were found in 15 human serum samples in a total population of 350 naïve donors. The assay exhibited no significant matrix effects and was shown to be highly reproducible. **Discussion:** A double antigen bridging immunogenicity assay for the detection of antibodies to PEG in the typical polymer size ranges used in biotherapeutics has been successfully developed in ELISA format. The antibodies detected in positive samples displayed a diverse spectrum of specificities for different PEG polymer lengths and linking functional groups. The discovery of 15 confirmed positive samples among 350 naïve patient samples calls into focus the need for testing PEG-specific immunogenicity of PEGylated biotherapeutics.

1. Introduction

Immunogenicity assays to detect circulating anti-drug antibodies (ADAs) have become an important and necessary tool in the development of biotherapeutics since these high molecular weight drugs can elicit potentially deleterious immune responses that affect drug efficacy and safety. Among many strategies, the potential immunogenicity of such large molecules can be minimized by attaching polyethylene glycol (PEG) polymers. The covalent attachment of PEG to a drug or therapeutic protein can protect the biotherapeutic from the host's immune system, reducing its immunogenicity by masking its immunogenic epitopes, while prolonging the drug's circulatory half-life by increasing the drug's hydrodynamic size, thus reducing renal clearance. PEGylation can also provide water solubility to otherwise hydrophobic small molecule drugs and proteins.

PEG has been generally considered non-immunogenic due to its structure and low charge density, but until recently, this view has largely been anecdotal. Based on size alone, PEG polymers in the molecular size ranges typically used in drug designs (10 kDa to 40 kDa) are potentially immunogenic. Richter and Akerblom (1983, 1984) successfully generated anti-PEG antibodies in animals immunized with PEGylated proteins and in 1984 had already reported the existence of anti-PEG antibodies in humans many years before the first PEGylated drug was approved. They calculated that 0.2% of the healthy population had anti-PEG antibodies, which were mostly IgMs. They had also concluded that for purposes of hyposensitization therapy with PEGylated allergens, these antibodies posed no significant problems.

The occurrence of anti-PEG antibodies in the healthy population is much higher (22–25%) according to later research by Leger et al. (2001), Armstrong et al. (2003), and Garratty (2004). Furthermore, drug-

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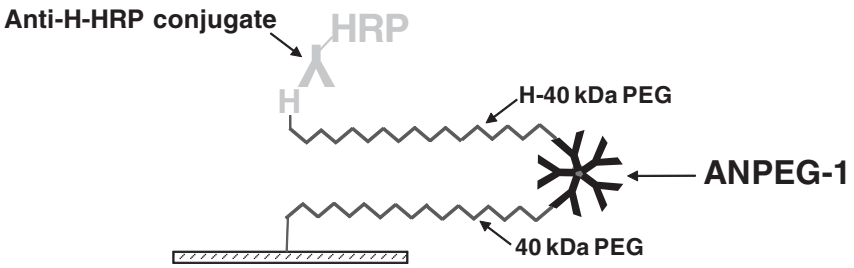

Fig. 1. Schematic of the double antigen bridging assay for the detection of anti-PEG antibodies. H represents a hapten that is conjugated to PEG which is subsequently reacted with a monoclonal anti-hapten antibody attached to HRP.

induced PEG immunity is not as innocuous as originally thought. Ganson et al. (2006) reported that PEG-specific antibodies generated in patients treated with a PEGylated enzyme for the treatment of refractory gout resulted in a higher rate of clearance of the drug compared to non-immune patients. Armstrong et al. (2007) reported similar results of rapid drug clearance in the case of PEG-immune lymphoblastic leukemia patients undergoing treatment with PEGylated asparaginase. The presence of anti-PEG antibodies in both examples potentially limited the efficacy of the administered drug. The fact that several animal IgM and IgG antibodies have now been successfully developed against PEG polymers further attests to the immunogenicity of these structures [Cheng et al. (2000), Cheng et al. (2005)]. Immunogenicity assays to detect ADAs specific to the PEG moieties attached to biotherapeutic molecules are therefore a necessary part of drug safety investigations that require a standard method universally applicable to all PEGylated protein drugs. A double antigen bridging immunogenicity enzyme-linked immunosorbent assay (ELISA) has been successfully developed for such a purpose. The fact that the method can detect antibodies to PEG polymers of various lengths and attached with a variety of functional groups makes it a potentially universal method for detecting PEG-specific anti-drug antibodies generated by any PEGylated biotherapeutic.

2. Materials and methods

2.1. Materials

Hapten (digoxigenin)—40 kDa PEG polymer conjugate was produced by ANP Technologies, Inc. Anti-PEG mouse monoclonal IgM (ANPEG-1) antibody was generated and characterized by ANP Technologies, Inc using a 40 kDa PEG polymer as immunogen. Calibrators were prepared by serial dilution of this antibody in a naive human serum pool. IM assay diluent (PBS buffer with HAMA blockers) and assay wash buffer (PBS with detergent) were prepared by ANP Technologies, Inc. PEG (40 kDa)-coated 96-well microplates were obtained from ANP Technologies, Inc. Horseradish peroxidase (HRP)-conjugated mouse anti-hapten antibody and assay diluents were prepared by ANP Technologies, Inc. Tetramethylbenzidine/Peroxide Substrate Reagent (TMB) was purchased from Moss Inc. Stop solution (Sulfuric acid, 2.0N) was obtained from VWR, Inc.

2.2. ELISA method optimization

The ELISA method was optimized by testing two possible capture configurations, with 40 kDa PEG directly coated on the plate or its biotin-labeled version coated on a streptavidin-activated plate. The signal dynamic range obtained with the latter was superior and the streptavidin plate coated with the biotinylated PEG was used. The hapten (digoxigenin)-labeled drug which forms the other half of the bridging complex is mixed with the sample, added to the biotin-PEG-coated microwell and then reacted with an HRP-labeled anti-digoxigenin monoclonal antibody in a subsequent step. The concentrations of the two labeled PEG conjugates and the assay incubation times were optimized to attain the best sensitivity and signal dynamic range. The assay method wherein the serum sample was preincubated with hapten-labeled and biotinylated drug then added to a well coated with either streptavidin or the mouse anti-hapten antibody did not yield good sensitivity and dynamic range. The best sensitivity and signal range were obtained when the biotinylated PEG was precoated on a streptavidin-coated plate. In all optimization experiments a Design of Experiments (DOE) approach was employed.

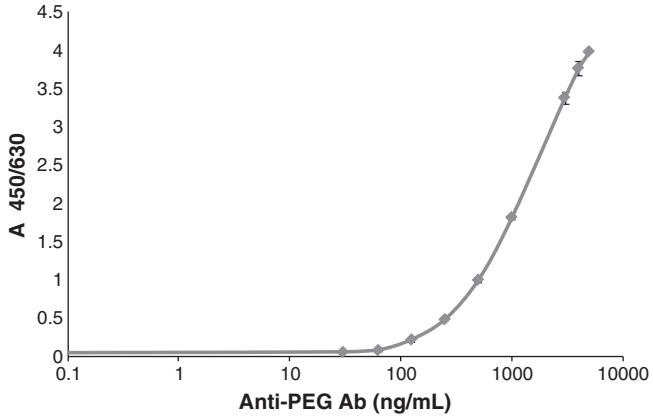

Fig. 2. Dose-response curve of the immunogenicity ELISA for the detection of ADA to PEG. The observed limit of detection was 125 ng/mL.

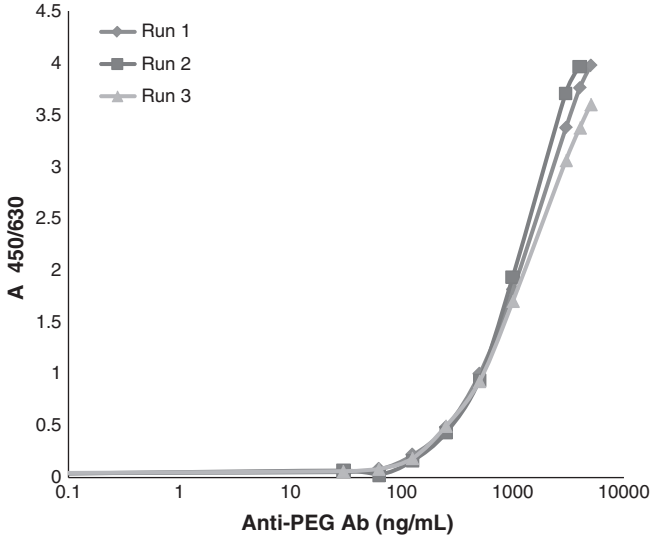

Fig. 3. Reproducibility of dose response curves obtained in three separate assay runs by two different operators.

Table 1
Recovery of QC controls obtained in three separate assays performed by two different operators.

QC Control ng/mL Ab	Run 1 ng/mL(recovery)	Run 2 ng/mL (recovery)	Run 3 ng/mL (recovery)
200	189.9 (94.9%)	195.9 (98.0%)	193.3 (96.7%)
1000	901.2 (90.1%)	934.8 (93.5%)	939.2 (93.9%)

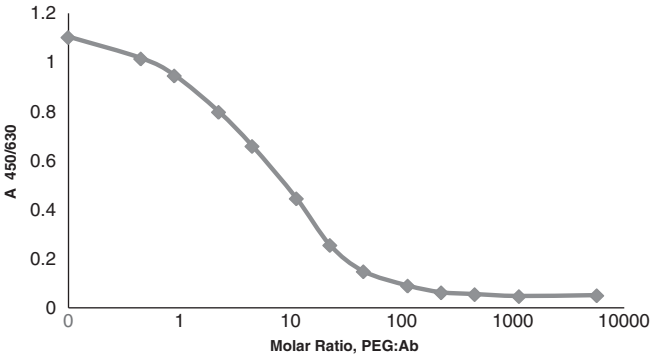

Fig. 4. Drug Tolerance of the immunogenicity ELISA using 500 ng/mL of the ANPEG anti-PEG mouse monoclonal IgM antibody spiked into a negative human serum pool. A positive result can be detected beyond 100X excess free PEG. Complete inhibition occurs at a PEG:Ab ratio of 1000:1.

2.3. ELISA procedures

The ELISA method developed for the detection of ADAs to PEG polymers uses a conventional double antigen bridging format. PEG is coated onto a 96-well microplate. ADAs in the patient serum form bridging complexes wherein one ADA binding site binds the hapten-labeled PEG while another binds the coated PEG, thus immobilizing the complex to the plate. A horseradish peroxidase (HRP) conjugated mouse anti-hapten antibody is then allowed to react with the bridging complexes containing the hapten-labeled PEG followed by a color development step with TMB. The intensity of the color developed by the immobilized HRP with TMB is directly proportional to the concentration of ADA in the patient sample. The design of this ELISA is shown in Fig. 1.

Calibrators were prepared by serial dilution of the ANPEG-1 antibody in the normal human serum pool to concentrations of 5000, 4000, 3000, 1000, 500, 250, 125, 62.5, 30, and 0 ng/mL. Quality Control samples were prepared by diluting the same antibody to 1000 and 200 ng/mL in the normal human serum pool.

To perform the assay, the sample and the hapten-labeled PEG conjugate are first mixed together prior to transferring an aliquot of the mixture to the biotin-PEG-coated 96-well microplate. A 7.5 μg/mL solution of the hapten-labeled 40 kDa PEG was prepared in IM assay diluent then 10 μL of this solution was added to 290 μL of calibrators, controls, and samples to make a final hapten-labeled 40 kDa PEG concentration of 0.25 μg/mL in each mixture. These mixtures were transferred at 100 μL per well in duplicate, then incubated for 1.5 h at room temperature. The contents of each well were then aspirated, and 100 μL of anti-H-HRP conjugate diluted in IM assay diluent added. After incubation at room temperature for 0.5 h, the liquid in each well was aspirated and the plate washed three times with assay wash buffer. TMB/hydrogen peroxide color reagent (100 μL) was pipetted into each well, then incubated for 20 min. The 2N sulfuric acid stop solution (100 μL) was then added to each well to stop the enzyme reaction. Absorbance (A) of each well was measured at 450 nm subtracted by the absorbance at 630 nm.

2.4. Assay reproducibility

The assay was run 3 times by 2 different operators to determine the reproducibility of the dose response curves and quality control sample recoveries.

2.5. Drug tolerance testing/free drug depletion assays

To determine the ability of the assay to detect anti-PEG antibody in the presence of increasing level of free PEG polymer, increasing concentrations of 40 kDa PEG were added to the negative human serum pool containing 500 ng/mL of ANPEG-1 anti-PEG mouse monoclonal IgM antibody. Each treated sample was then tested on the immunogenicity ELISA.

2.6. Screening cut-point with normal human serum samples

Naive normal human sera (n = 350) were tested in several batches along with a negative control (NC) human serum pool and the

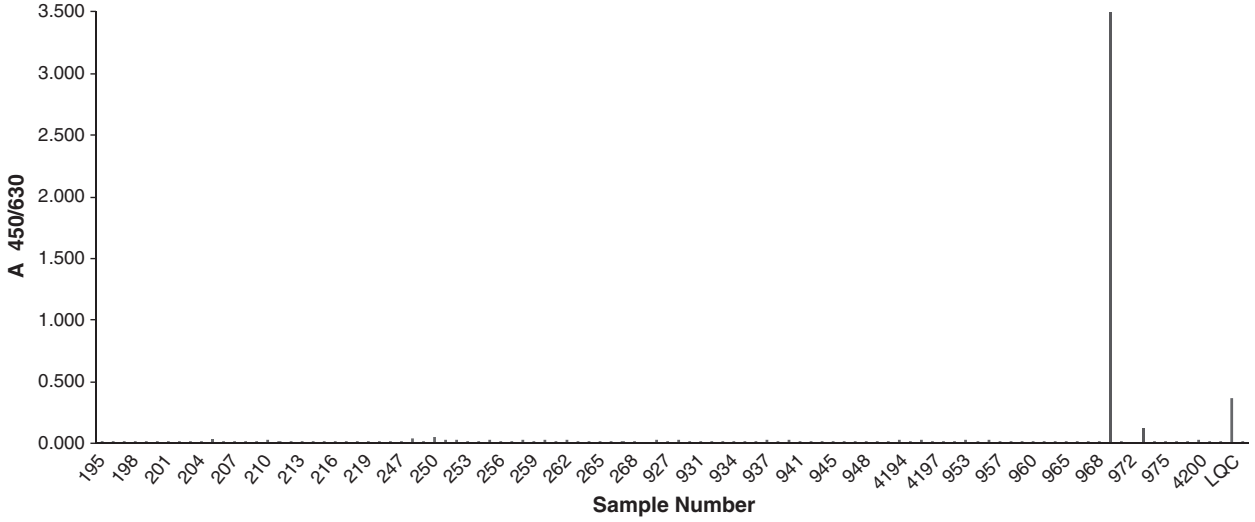

Fig. 5. Results of the PEG ELISA immunogenicity assay screening cut-point determination with the first batch of 102 normal human serum samples. Samples 969 and 973 were found to be positive and were selected for confirmation testing.

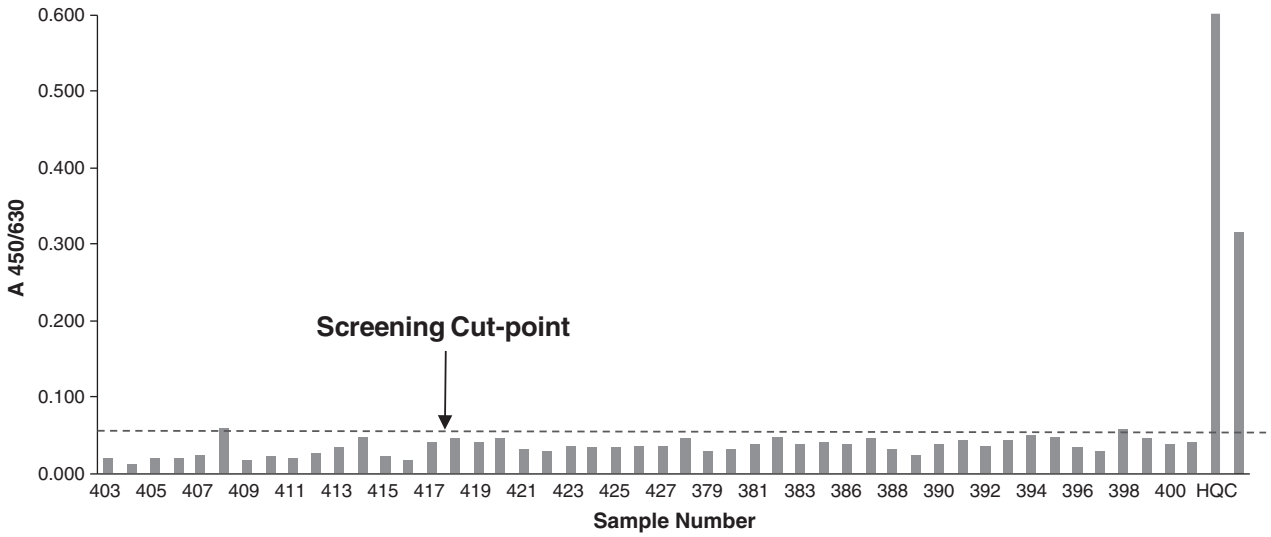

Fig. 6. Results of the PEG ELISA immunogenicity assay screening cut-point determination with the second batch of 48 normal human serum samples. The last 2 samples are the high and low QC control samples. No samples from this batch were selected for further testing.

positive controls (PCs) using the immunogenicity ELISA. A floating screening cut-point was determined for each batch screened with the immunogenicity ELISA. The cut-points were calculated using the following equation:

$$\text{Screening Cut-point} = \text{Mean of absorbance of all samples} + 1.645 \text{ Standard Deviation of absorbance of all samples.}$$

Any samples that showed an average absorbance higher than the low positive QC control were excluded from this calculation as outliers.

2.7. Determination of antibody specificity in positive human serum samples

Positive samples uncovered in the screening cut-point determination with 350 naïve human serum samples were further analyzed by spiking 10 μg/mL of amine-functionalized 20 kDa PEG (designated 20kPEG-NH2), aldehyde-functionalized 20 kDa PEG (20kPEG-CHO), amine-functionalized 40 kDa PEG (40kPEG-NH2), aldehyde-functionalized 40 kDa PEG (40kPEG-CHO) and 40 kDa polyvinyl pyrrolidone (PVP), another polymer that is used in medical applications and pharmaceutical formulations. These spiked samples were then tested in the ELISA.

2.8. Determination of sample matrix effects

Four randomly selected negative human serum samples were spiked with 200 and 500 ng/mL of ANPEG-1 anti-PEG mouse monoclonal IgM. All samples were then tested in duplicate to determine if the immunogenicity ELISA was affected by sample matrix variations.

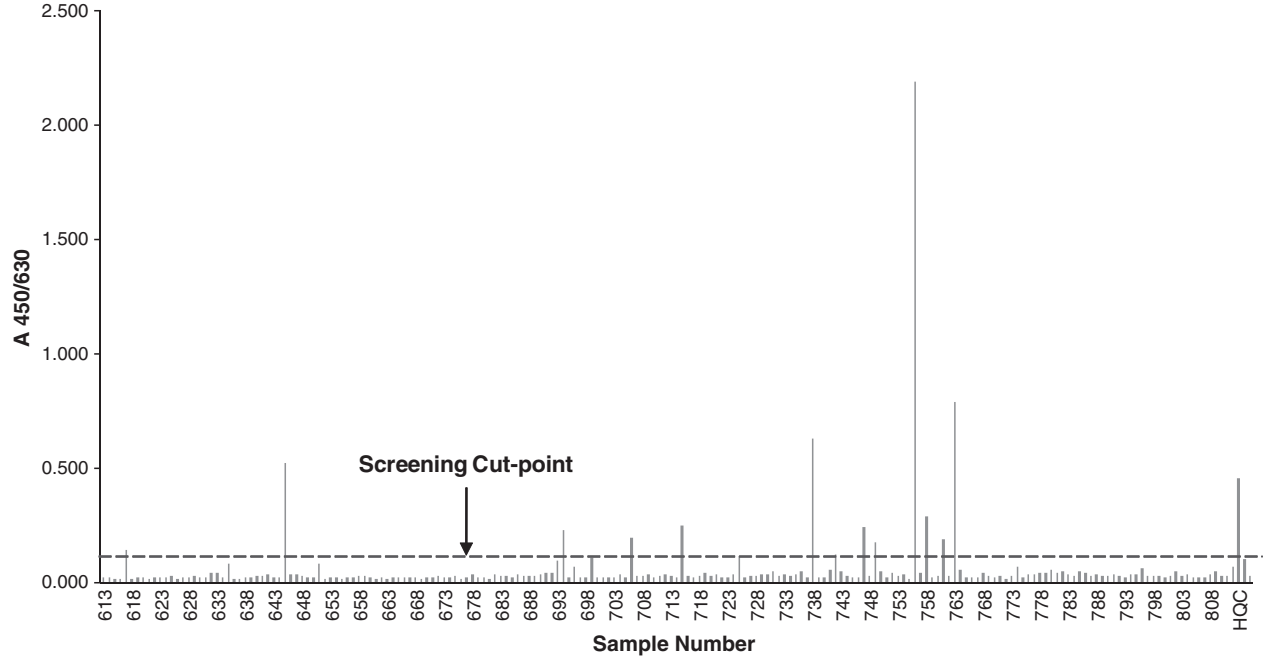

Fig. 7. Results of the PEG ELISA immunogenicity assay screening cut-point determination with the third batch of 200 naive human serum samples. The last 2 samples are the high and low QC control samples. Fourteen samples found positive were selected for further testing (samples 617, 645, 694, 706, 715, 725, 738, 742, 747, 749, 756, 758, 761 and 763).

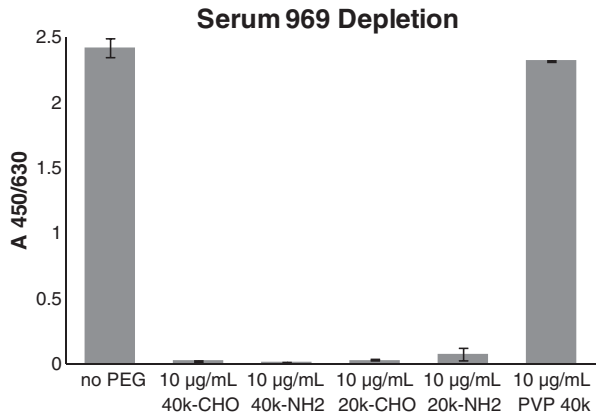

Fig. 8. Depletion of sample 969 with 40 and 20 kDa PEG compounds with aldehyde (CHO) and amine (NH2) functions and 40 kDa PVP. The antibody response recognized all PEG species but not PVP.

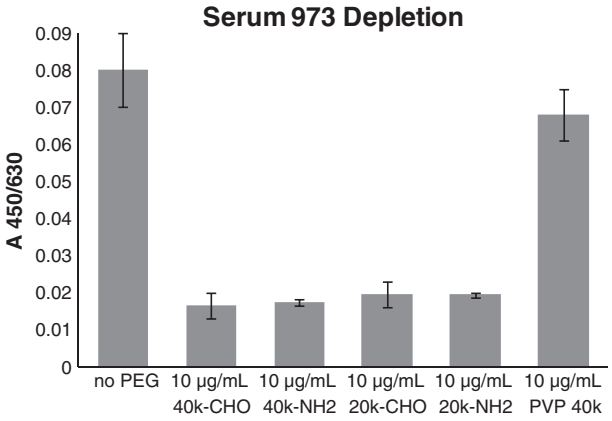

Fig. 9. Depletion of sample 973 with 40 and 20 kDa PEG compounds with aldehyde (CHO) and amine (NH2) functions and 40 kDa PVP. The antibody response recognized all PEG species but not PVP.

3. Results

3.1. Dose response curves and assay reproducibility

A dose response curve was successfully generated with the optimized ELISA procedure (Fig. 2). The dose response curve was reproduced three times by two operators (Fig. 3). The values obtained for the QC samples for each run are summarized in Table 1. The observed limit of detection is 125 ng/mL of anti-PEG antibody. From Fig. 3, the assay is highly reproducible from run to run and from operator to operator. The recoveries of QC controls ranged from 90.1% to 98.0% in three separate assays.

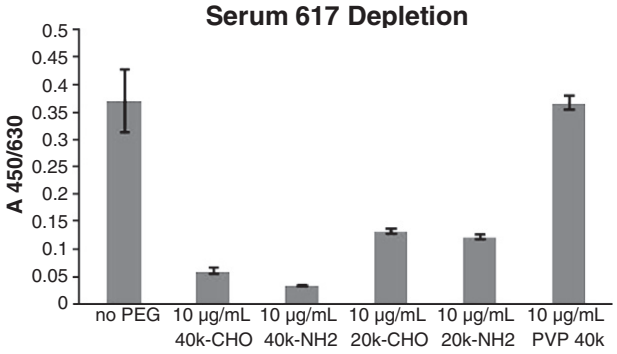

Fig. 10. Depletion of sample 617 with 40 and 20 kDa PEG compounds with aldehyde (CHO) and amine (NH2) functions and 40 kDa PVP. The antibody response recognized 40 kDa PEG better than 20 kDa PEG. PVP was not recognized.

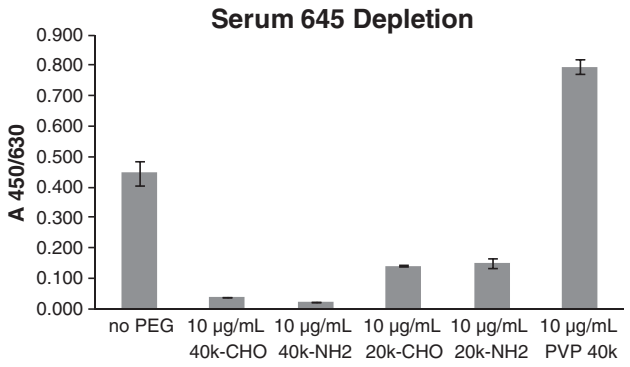

Fig. 11. Depletion of sample 645 with 40 and 20 kDa PEG compounds with aldehyde (CHO) and amine (NH2) functions and 40 kDa PVP. The antibody response recognized 40 kDa PEG better than 20 kDa PEG. PVP was not recognized.

3.2. Drug tolerance testing/free drug depletion assays

The ability of the assay to detect anti-PEG antibodies in the presence of excess PEG in a serum sample is exemplified in Fig. 4. Partial depletion of assay signal with a serum sample containing 500 ng/mL of anti-PEG IgM antibody is observed at a PEG:Antibody molar ratio of 10:1, but complete inhibition of binding is not observed until a molar ratio of 1000:1.

3.3. Screening cut-point determination with normal human serum samples

Naïve human sera (n = 350) were tested in three separate batches of 52, 48, and 200 samples to determine screening cut-points. The results are shown in Figs. 5, 6, and 7. The screening cut-point was empirically derived for each batch from the standard deviation of the absorbance measurements generated by the population tested (i.e., the calculated floating cut-point = mean + 1.645 * SD).

3.4. Determination of antibody specificity in positive human serum samples

All naive human serum samples found to be positive by the ELISA were further tested in a depletion assay wherein 10 µg of amine- and aldehyde-functionalized 20 kDa and 40 kDa PEG polymers along with 10 µg 40 kDa PVP were individually spiked into each sample and tested in the ELISA to characterize the specificity of the antibodies detected by the ELISA. The results for each sample are presented in

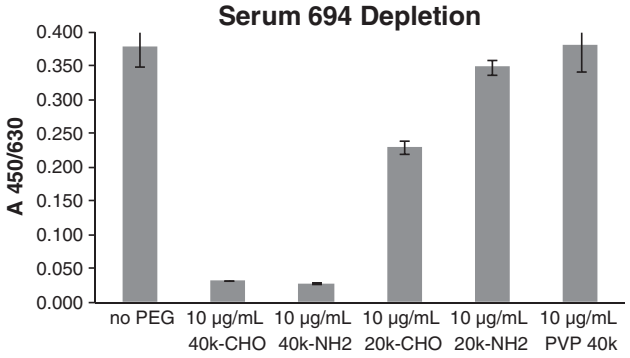

Fig. 12. Depletion of sample 694 with 40 and 20 kDa PEG compounds with aldehyde (CHO) and amine (NH2) functions and 40 kDa PVP. The antibody response recognized 40 kDa PEG better than 20 kDa PEG. PVP was not recognized.

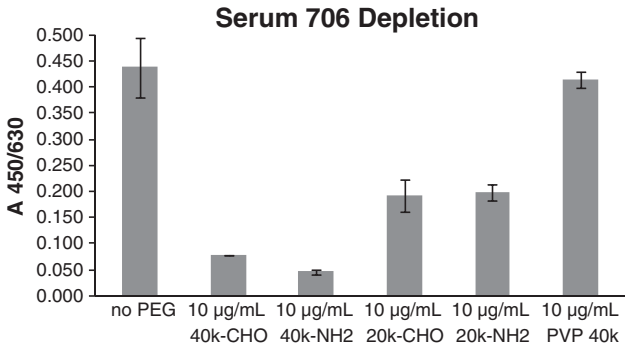

Fig. 13. Depletion of sample 706 with 40 and 20 kDa PEG compounds with aldehyde (CHO) and amine (NH2) functions and 40 kDa PVP. The antibody response recognized 40 kDa PEG better than 20 kDa PEG. PVP was not recognized.

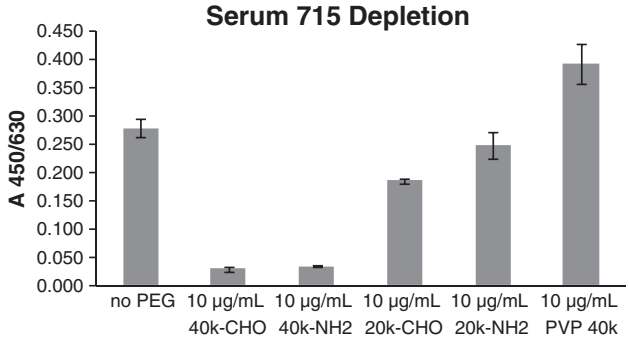

Fig. 14. Depletion of sample 715 with 40 and 20 kDa PEG compounds with aldehyde (CHO) and amine (NH2) functions and 40 kDa PVP. The antibody response recognized 40 kDa PEG better than 20 kDa PEG. PVP was not recognized.

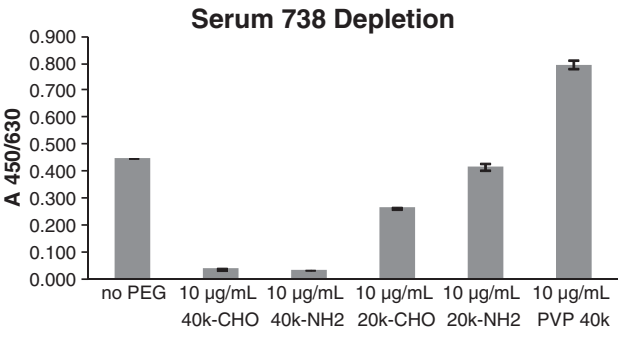

Fig. 16. Depletion of sample 738 with 40 and 20 kDa PEG compounds with aldehyde (CHO) and amine (NH2) functions and 40 kDa PVP. The antibody response recognized 40 kDa PEG better than 20 kDa PEG. PVP was not recognized.

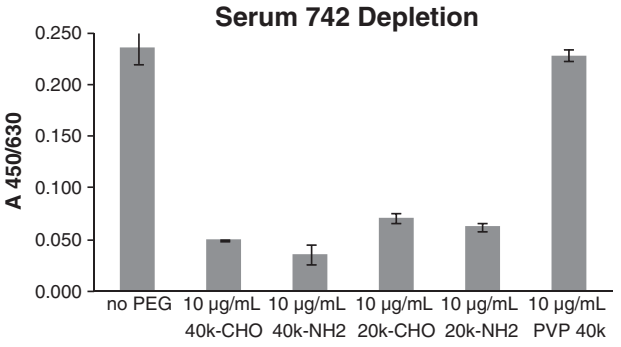

Fig. 17. Depletion of sample 742 with 40 and 20 kDa PEG compounds with aldehyde (CHO) and amine (NH2) functions and 40 kDa PVP. The antibody response recognized all PEG species but not PVP.

Fig. 8–23. With 15 of the 16 samples tested, assay signal was significantly reduced upon the addition of any of 4 variants of PEG polymers, while PVP showed no effect on the formation of bridging complexes with PEG.

Sample 725 (Fig. 15) was a borderline positive that proved to have no PEG-specific antibodies upon depletion testing. When all confirmed positives were removed from the screening cutpoint calculations, new cutoffs yielded 17 out of 335 samples as false positives for a total false positive rate of 5.1%.

Of the 15 PEG-antibody positive samples, 4 samples (969, 973, 742, and 747 in Figs. 8, 9, 17, 18) equally recognized both 20 and 40 kDa PEG with either an aldehyde or amine functional group. The remaining 11 samples displayed antibody activity significantly more specific to 40 kDa PEG than 20 kDa PEG. Seven samples (617, 706, 747, 756, 758, 761, and 763 in Figs. 10, 13, 18, 20–23) appeared to possess greater specificity for the amine-functionalized 40 kDa PEG than for the aldehyde-functionalized counterpart.

3.5. Sample matrix effects

Four naïve normal human serum samples were randomly chosen and spiked with 200 and 500 ng/mL of ANPEG-1 positive control antibody then tested in the ELISA. The results are summarized in Table 2. Each sample showed analytical recoveries within the acceptable 80–120% range at each level of anti-PEG IgM tested.

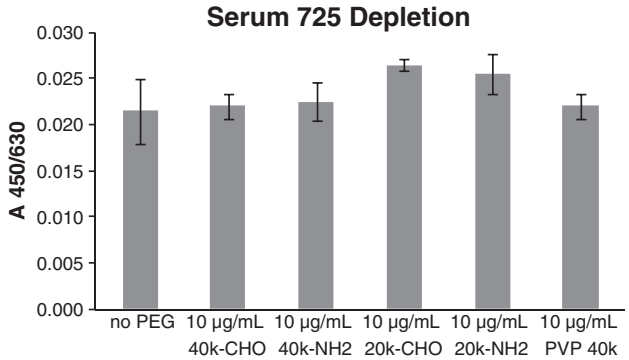

Fig. 15. Depletion of sample 725 with 40 and 20 kDa PEG compounds with aldehyde (CHO) and amine (NH2) functions and 40 kDa PVP. This sample was a borderline positive which proved to be a true negative.

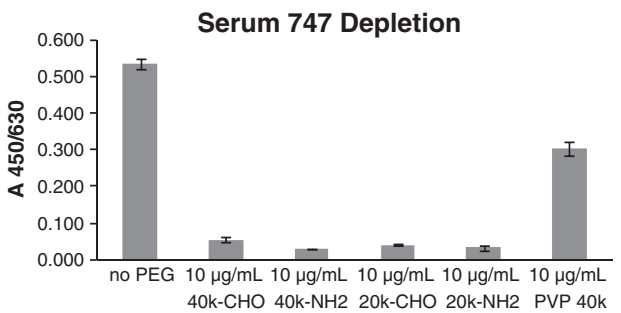

Fig. 18. Depletion of sample 747 with 40 and 20 kDa PEG compounds with aldehyde (CHO) and amine (NH2) functions and 40 kDa PVP. The antibody response recognized all PEG species but not PVP.

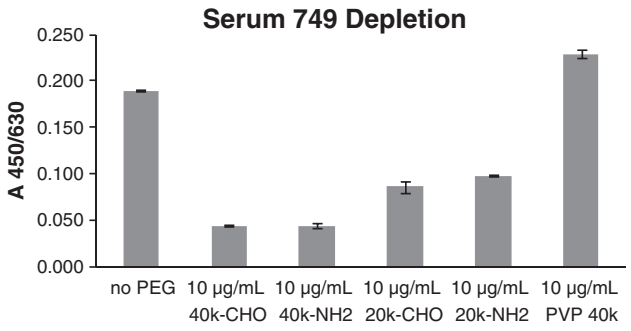

Fig. 19. Depletion of sample 749 with 40 and 20 kDa PEG compounds with aldehyde (CHO) and amine (NH2) functions and 40 kDa PVP. The antibody response recognized 40 kDa PEG better than 20 kDa PEG. PVP was not recognized.

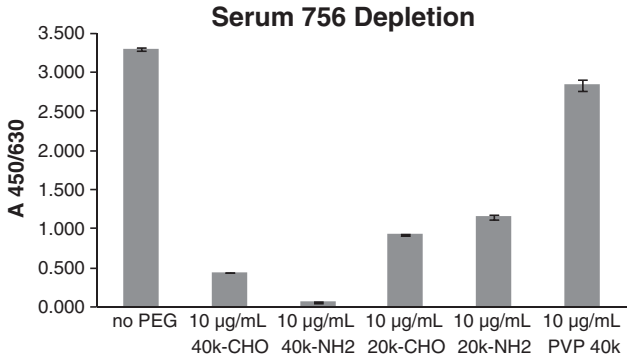

Fig. 20. Depletion of sample 756 with 40 and 20 kDa PEG compounds with aldehyde (CHO) and amine (NH2) functions and 40 kDa PVP. The antibody response recognized 40 kDa PEG better than 20 kDa PEG. PVP was not recognized.

4. Discussion

Using ANPEG-1, a mouse monoclonal IgM raised against 40 kDa PEG, a working immunogenicity ELISA for the detection of anti-PEG antibodies in serum has been successfully demonstrated. The ELISA procedure was characterized to be highly reproducible with no detectable matrix effects. The drug tolerance study demonstrated the specificity of the assay with this positive control IgM. Assay signal depletion was observed at the expected PEG:Ab ratio of 10:1 for IgM. However, complete depletion did not occur until a ratio of 1000:1 was reached, showing a high drug tolerance for the immunogenicity test procedure.

Although it has been argued that PEG used as a stabilizing adduct in protein drugs is not immunogenic, the ELISA successfully detected 15 individual naïve human serum samples with apparently pre-existing antibodies specific to PEG in a total population of 350 naïve donors. Using the ELISA, the specificity of the antibody response in all samples was confirmed to be highly PEG-specific in the follow-on confirmatory depletion assays. Moreover, the antibodies in these samples show diversity in specificity. In all cases, both 20 kDa and 40 kDa PEG polymers inhibited the formation of bridging complexes in the assay. However, in 11 of 15 samples, the antibody activity appeared to have greater specificity for the larger 40 kDa PEG. In addition, in 7 of these samples, the antibody activity appears to have preference for a PEG polymer containing an amine function.

An additional indication of the PEG-specific antibody response in all 15 samples is the inability of PVP, a similarly sized water-soluble polymer used in pharmaceutical preparations, to deplete the assay signal at the same levels as PEG polymers. It can be theorized that the addition of a relatively large polymer such as PEG or PVP in high concentrations can disrupt the formation of bridge complexes and reduce assay signal merely because of the high osmolarity of solutes created in the sample. However, the fact that PEG alone causes assay depletion while PVP does not at the same concentrations disproves this theory and points strongly to the presence of highly specific anti-PEG antibodies in these individuals.

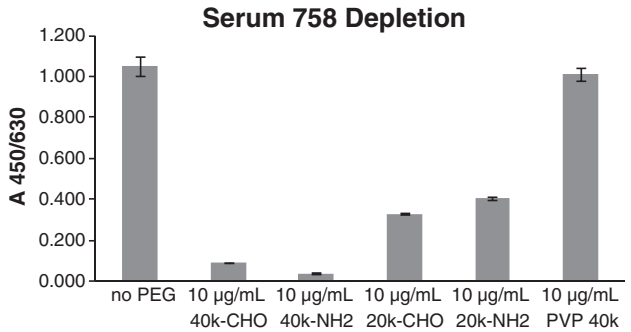

Fig. 21. Depletion of sample 758 with 40 and 20 kDa PEG compounds with aldehyde (CHO) and amine (NH2) functions and 40 kDa PVP. The antibody response recognized 40 kDa PEG better than 20 kDa PEG. PVP was not recognized.

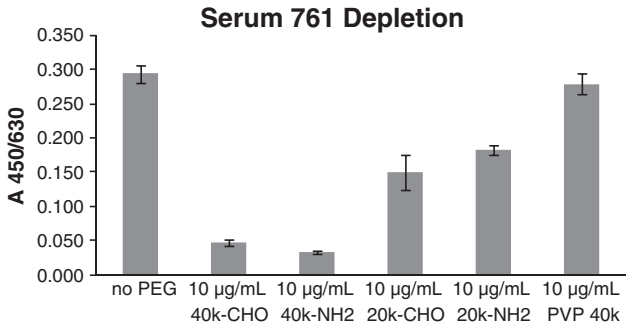

Fig. 22. Depletion of sample 761 with 40 and 20 kDa PEG compounds with aldehyde (CHO) and amine (NH2) functions and 40 kDa PVP. The antibody response recognized 40 kDa PEG better than 20 kDa PEG. PVP was not recognized.

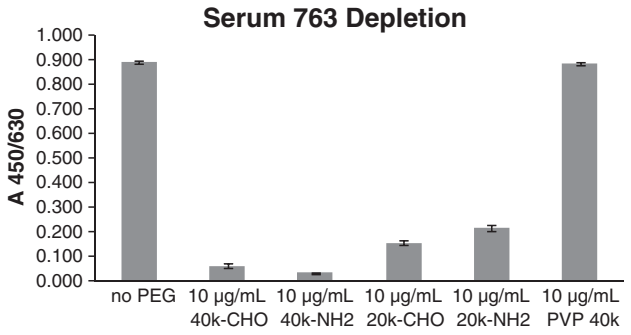

Fig. 23. Depletion of sample 763 with 40 and 20 kDa PEG compounds with aldehyde (CHO) and amine (NH2) functions and 40 kDa PVP. The antibody response recognized 40 kDa PEG better than 20 kDa PEG. PVP was not recognized.

Table 2
Sample matrix effects: recovery of ANPEG-1 positive control antibody in 4 random naïve human sera.

Sample number	200 ng/mL spike ng/mL(recovery)	500 ng/mL spike ng/mL (recovery)
1	162.4 (81.2%)	420.1 (84.0%)
2	172.2 (86.1%)	552.4 (110.5%)
3	170.5 (85.3%)	499.7 (99.9%)
4	189.1 (94.6%)	486.3 (97.3%)

The discovery of 15 positive individuals in a population of 350 naïve human samples underlines the need to study the effect such pre-existing immunity will have on the efficacy of PEGylated drugs. These results also call into focus the requirement to study the PEG-specific immunogenicity of PEGylated biotherapeutics and the potentially deleterious effect on drug efficacy and safety from immunity developed upon exposure in the patient. Since it has shown the ability to detect a diverse population of anti-PEG antibodies in human serum samples, the NIDS® double antigen bridging immunogenicity ELISA for the detection of anti-PEG antibodies is a potential universal tool for such immunogenicity studies involving any PEGylated drug.

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