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A generic method for the detection of polyethylene glycol specific IgG and IgM antibodies in human serum

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ABSTRACT

Detection of anti-drug antibodies is a critical step in the development of large molecule biopharmaceuticals. In the case of multicomponent/multifunctional molecules, such as fusion proteins and protein conjugates such as covalent polyethylene glycol (PEG)~protein conjugates, it is useful to further characterize anti-drug antibody (ADA) binding to key domains of the drug. The detection of anti-PEG antibodies poses special challenges that if overlooked can result in underreporting antibody responses. Here we describe the development and characterization of a novel ELISA to detect anti-PEG antibodies that provides a more complete interpretation of anti-PEG than other published methods. Being specific to the PEG moiety alone, this method is intended to detect anti-PEG antibodies independent of the protein to which PEG is conjugated.

Based upon early indications that our assay could detect anti-PEG antibodies at a surprisingly high frequency in the general population, our emphasis throughout method development and validation was to ensure that non-specific signals and unintended interactions were not falsely contributing to detection of anti-PEG antibodies. Techniques, including orthogonal methods used to ensure that this ELISA detected antibodies specific to PEG included competition, immunodepletion, immunoprecipitation/western blot and an Octet kinetic binding analysis. The validated ELISA can detect 100 ng/mL of an anti-PEG IgG positive control and 800 ng/mL of an anti-PEG IgM positive control in the presence of 7.5 μg/mL of the PEGylated therapeutic (MW 64 kDa). The intra-assay percent co-efficient of variation (CV) and inter-assay CV of the low positive control samples in the screening method were 4.1 to 7.2% and 16.7 to 17.7%, respectively. Additional assay performance parameters that were validated are also described. When the validated assay was applied to a population of 200 healthy blood donors with no known exposure to biopharmaceutical PEG conjugates it indicated a pre-existing anti-PEG antibody prevalence of 97.5%. We suggest this surprising result is a consequence of exposure to PEG additives in everyday products, such as cosmetics, processed foods and over-the-counter (OTC) pharmaceuticals.

1. Introduction

Polyethylene glycol (PEG) or polyethylene oxide is a polymer of ethylene oxide with a repetitive structure of $(O\text{-}CH_2\text{-}CH_2)_n$. Some commonly encountered forms of PEG are referred to as polysorbate or tween. PEG polymers are synthesized at different molecular weights and in their simplest forms are linear or branched chains that terminate with either a methoxy group or an alcohol depending on the intended use. PEG may also be incorporated into larger chemical structures or coupled to biochemicals. Humans are exposed to PEG through its use in a variety of commercial products such as personal hygiene products, cosmetics, pharmaceutical additives and some processed foods (Fruijtier-Polloth, 2005). Through a process referred to as PEGylation it can be covalently attached to proteins, peptides or nucleotides, making PEG part of the active ingredient in some biotherapeutics (Chen et al., 2016). Biopharmaceuticals may be PEGylated to improve physicochemical properties, increasing aqueous solubility for example. They may be PEGylated to extend in vivo half-life, for example, PEGylation of a small biomolecule can increase in the hydrodynamic radius so that it is too large to be excreted by the kidneys, while PEGylation of a protease/nuclease sensitive biomolecule can make it resistant to degradation (Hamidi et al., 2006; Chen et al., 2016). In general, PEG has been considered non-toxic and is generally recognized as safe by regulatory agencies worldwide.

Abbreviations: PEG, polyethylene glycol; ADA, anti-drug antibody; OTC, over-the-counter; DDM, n-Dodecyl-beta-D-maltoside; NHS, normal human serum; mPEG-SVA, methoxypolyethylene glycol succinimidyl valerate; HRP, horseradish peroxidase; DDT, dithiothreitol; SA, streptavidin; ELISA, enzyme-linked immunosorbent assay; kDa, kilodalton; BSA, bovine serum albumin; PBS, phosphate-buffered saline; MW, molecular weight; PAL, phenylalanine ammonia lyase

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Low titer anti-drug antibodies often have no discernable clinical impact, but the immune system is dynamic and can be altered by changes in antigen; concentration, route of exposure, or co-administered molecules. Thus, a pre-existing low titer antibody response to PEG may adapt when presented with a PEGylated biopharmaceutical. Antibodies that recognize the repeating glycol structure of PEG are thought to be the cause of hypersensitivity reactions and the loss of efficacy of some biotherapeutics. Even though the reported anti-PEG incidence remains low, recent publications have associated anti-PEG antibodies with loss of efficacy in patients treated with PEGylated enzymes such as Pegasparaginase (Armstrong et al., 2007) and Pegloticase (Hershfield et al., 2014). Induction of hyper-sensitivity reactions due to anti-PEG antibodies have also been reported and can result in serious and possibly life-threatening adverse events (Povsic et al., 2016; Wenande and Garvey, 2016). It has been shown that anti-PEG antibodies influence the clearance of PEGylated nanocarriers (Cheng et al., 2000; Laverman et al., 2001; Ishida et al., 2003). Such information has gradually overturned old notions that PEG was non-immunogenic, or that PEGylation concealed a biotherapeutic from the immune system (Abuchowski et al., 1977; Pasut and Veronese, 2012). The ability to detect anti-PEG antibodies has improved over the years, although unfortunately, outdated methods remain prevalent. A consequence of this is that many published studies are based on assays that do not robustly detect antibodies to PEG or suffer from interference caused by the use of Tween® 20 (Schellekens et al., 2013), which makes it difficult to draw firm conclusions about the impact of anti-PEG antibodies. Nonetheless, PEGylated compounds have been approved and can be successfully used to treat many patients.

The reported prevalence of individuals with anti-PEG antibodies has changed dramatically over the last 40 years. Detection of human anti-PEG antibodies based on an early hemagglutination assay yielded an incidence of 0.2% in healthy individuals (Richter and Akerblom, 1984). Many of the later assays described in the literature are standard immunoassays that use either PEG (Liu et al., 2011), PEG-diacylglycerol (Yang et al., 2016), PEGylated proteins (Lubich et al., 2016) or similar molecules as capture reagents and mouse anti-PEG positive control samples (PCs) that are detected via anti-mouse antibody probes while ADA in test samples are supposedly detected by anti-human antibody probes. Obviously, the use of non-equivalent probes for control samples and samples makes it impossible to verify the assay performance relative to clinical test samples. Although a consensus has not been reached regarding the true incidence of anti-PEG in the general population, the reported incidence has climbed over the years, most likely as a result of improved test methods. In recent years the incidence has been reported to be 23% (Lubich et al., 2016), 44% (Chen et al., 2016) and 72% (Yang et al., 2016). The study by Yang et al. examined individuals with no prior history of treatment with PEGylated biotherapeutics and included both contemporary specimens and preserved specimens collected from 1970 through 1999. They detected anti-PEG antibodies in 59% of historical samples and 72% in contemporary samples. During the same period of time assays improved and the reported incidence of antibodies to PEG rose from 0.2% to as much as 72%, and so it appears that most of the reported change in incidence has been the result of improved test methods, perhaps superimposed on a comparatively small in vivo increase resulting from increased environmental and food-based PEG exposure. The reported incidence of antibodies to FDA-approved PEGylated drugs is generally < 5%, but the groups reporting those low incidences do not detect a substantive level of pre-existing antibodies to PEG when such information is provided, so if pre-existing anti-PEG antibodies are common but not detected by assays supporting claims of low induced anti-PEG responses it raises questions about the sensitivity of those assays and the true patient impact, if any, of anti-PEG antibodies.

In this manuscript we present an ELISA that sensitively detects anti-PEG antibodies in human serum. The assay design is intentionally generic so that it may be applied across a variety of PEGylated drugs. Over the years it has become clear that one of the most significant assay improvements (and one included in our assay but not applied universally by others) has been the removal of Tween-based surfactants from assay diluents and wash solutions because such surfactants have been shown to act as competitive inhibitors capable of reducing or abolishing detection of anti-PEG antibodies (Liu et al., 2011; Sherman et al., 2012). We provide additional data to support this and show a clear distinction between PEG-like and non-PEG-like surfactants through comparison of Tween® 20 and alternative non-ionic surfactants, some of which, such as n-Dodecyl β-D-maltoside do not interfere with detection of anti-PEG antibodies. Another factor that may have a substantial impact on anti-PEG assay performance is isotype. A recent report of a cell based FACs assay for mouse antibodies to PEG indicated that a hybridoma expressing IgG was detected with much greater sensitivity than those expressing IgM (Shimizu et al., 2018) but that only serum IgM seemed to corelate with detection of anti-PEG B cells. Likewise, we found that certain assay conditions preferentially skew detection of anti-PEG IgG or IgM, although the reason for this hasn't been elucidated. Where anti-PEG data has been reported without regard to isotype we recommend results be interpreted with caution because some isotypes will not be detected even though an assay was in a bridging format or used an anti-IgGAM detection reagent. Here, we were not able to optimize detection of both IgG and IgM within one assay, therefore we selected conditions that provide adequate (albeit suboptimal) detection of both isotypes.

2. Materials and methods

2.1. Materials

Sera from 200 healthy, 50 obese, and 50 type II diabetic subjects and a normal human serum (NHS) pool were purchased from BioIVT (Hicksville, NY, USA). Immunoglobulin depleted pooled human serum was prepared by passing an aliquot of the NHS pool over protein A MabSelect SuRe antibody purification resin (GE Healthcare Life Sciences) and this served as an antibody-depleted negative control serum. Multi-PEGylated BSA (bovine serum albumin covalently labeled with 20 kDa mPEG succinimidyl valerate (mPEG-SVA) using a molar ratio of approximately 16 PEG:1 BSA) and mono-pegylated BSA unconjugated and conjugated with biotin were purchased from Life Diagnostics, Inc. (West Chester, PA, USA). Human chimeric anti-PEG antibodies, c3.3-IgG and cAGP4-IgM, were purchased from the Institute of Biomedical Sciences, Academia Sinica (Taipei, TW) and used as positive control samples. Mouse anti-human IgG and IgM antibodies conjugated with horseradish peroxidase (HRP) were purchased from SouthernBiotech (Birmingham, AL, USA). Biotin-PEG-OH (MW 10 k) was purchased from Creative PEGWorks (Chapel Hill, NC, USA). Protein A and protein L 96-well filter plates were purchased from GE Healthcare (Buckinghamshire, UK). Surfact-Amps™ Detergent Sampler, paramagnetic Dynabeads™ MyOne™ Streptavidin C1, goat anti-human IgM HRP, and StartingBlock™ TBS were purchased from ThermoFisher Scientific (Grand Island, NY, USA). Modified StartingBlock™ consisted of StartingBlock™ TBS supplemented with 0.25 M NaCl, 0.25 M glutamic acid, and 0.5 M aspartic acid adjusted to pH 7.95 ± 0.05. Goat anti-human IgG HRP and WES™ 12–230 kDa Separation Module Kit were purchased from ProteinSimple (San Jose, CA, USA). Corning® DPBS (Dulbecco's Phosphate-Buffered Saline) without calcium and magnesium was purchased from Corning (Corning, NY, USA). n-Dodecyl-beta-D-maltoside (DDM) was purchased from Cayman Chemical (Ann Harbor, MI, USA) and Millipore Sigma (St. Louis, MO, USA). Therapeutics A and B and the irrelevant therapeutic are proprietary molecules under development. Therapeutic A is comprised of a novel peptide chemically coupled to human IgG4 Fc via a 10 kDa linear PEG. Therapeutic B is a human Fab coupled to 1 branched 40 kDa PEG. The irrelevant therapeutic is a non-PEGylated human IgG4.

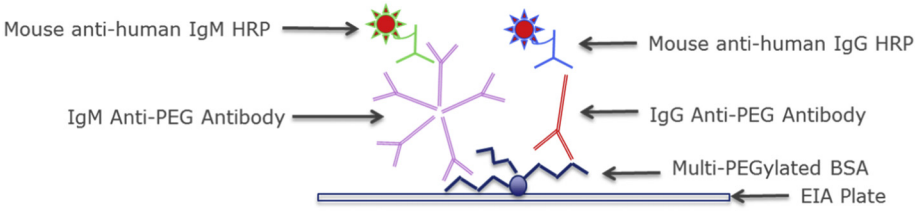

Fig. 1. Conceptual Diagram of the Generic Anti-PEG ELISA: The illustration shows multi-PEGylated BSA coated on the assay plate followed by samples/control samples containing IgG and/or IgM anti-PEG antibodies and HRP labeled mouse anti-human isotype specific antibodies.

2.2. Methods

Generic ELISA for Human Antibodies to PEG: This assay is comprised of two main parts, screening and confirmation. Principal steps are shown in Fig. 1.

2.2.1. Anti-PEG Screening Assay

Costar 96-well high binding plates (Millipore Sigma (St. Louis, MO, USA)) were coated with 50 µL/well of 0.5 µg/mL multi PEG-BSA (in Tris Coating Buffer (10 mM Tris, 150 mM NaCl) for 60 min at 37 °C. Plates were washed three times with 1XPBS + 0.5% DDM then blocked with 250 µL/well of assay diluent for 60 min at 37 °C. Except as specified, assay diluent for the ELISA method was our modified StartingBlock™ supplemented with 0.5% DDM. Samples, positive control samples (c3.3-IgG and cAGP4-IgM in Ig depleted human serum), assay diluent and Ig depleted NHS were diluted 1/10 in assay diluent. Plates were washed as before, and 50uL of samples and control samples were added to the wells in duplicate then incubated for 60 min at 37 °C. Plates were washed as before and 50 µL of assay diluent containing 1/20,000 HRP labeled mouse anti-human IgG plus 1/40,000 HRP labeled mouse anti-human IgM was added to the wells then incubated for 60 min at 37 °C. Plates were washed as before, and 100 µL/well of TMB was added then incubated for 30 min at 37 °C. Color development was halted by adding 100 µL/well of 4 N sulfuric acid. Absorbance at 450 nm - 650 nm was measured using a spectrophotometric plate reader (Molecular Devices).

2.2.2. Anti-PEG Confirmation Assay

The anti-PEG confirmation assay followed the same steps as described for the screening assay, however, in addition to samples and control samples being diluted 1/10 in assay diluent, samples and control samples were also diluted 1/10 in assay diluent containing 100 µg/mL of multi-PEG-BSA as a competitor that binds anti-PEG antibodies, thereby inhibiting assay signal.

2.2.3. Surfactant Comparison

The anti-PEG screening assay was modified in one of two ways in order to test the effect of different surfactants. Modification 1) Plates were washed with wash buffer supplemented with 0.05% Tween® 20 instead of 0.5% DDM. Modification 2) Samples and control samples were prepared in assay diluent that was supplemented with 0.5% of a surfactant other than DDM.

2.2.4. Immunodepletion

Protein A and protein L 96-well filter plates were prepared following the manufacturer's instructions. Samples and control samples were diluted 1/2 in 1XPBS and 60 µL/well was added to the protein A filter plate. Filter plates were incubated for 60 min at room temperature while shaking at 900 rpm and then centrifuged at 1500 RCF for 3 min. The total flow through volume was transferred to wells of a protein L 96-well filter plate and incubated for 60 min at room temperature while shaking at 900 rpm. The plate was centrifuged at 1500 RCF for 3 min. Immunodepleted samples and control samples were then diluted 1/5 in assay diluent and analyzed in the anti-PEG ELISA method without further sample dilution.

2.2.5. Kinetic Binding

As an alternative to ELISA, an Octet RED96 (Forté Bio/Molecular Devices, San Jose, CA, USA) was used to examine the kinetic binding of antibodies to PEG. Wells of a black flat bottom polypropylene plate were loaded with the following samples: baseline buffer (also referred to as dissociation buffer 1, Ig depleted human serum diluted to 40% in 1XPBS + 1%BSA), biotin conjugated mono-PEG BSA, test samples (human serum samples (n = 5) diluted to 40% in 1XPBS + 1%BSA), positive control samples (IgG or IgM anti-PEG antibodies c3.3 or cAGP4, respectively, spiked into Ig depleted human serum and diluted with 1XPBS + 1%BSA to 40% serum and 6 µg/mL c3.3 or 4 µg/mL cAGP4), a negative control (Ig depleted human serum diluted to 40% with 1XPBS + 1%BSA), dissociation buffer 2 (40% Ig depleted human serum in 1XPBS + 1%BSA + 0.5% Tween® 20), or dissociation buffer 3 (40% Ig depleted human serum in 1XPBS + 1%BSA + 0.5% DDM). The Octet RED96 was run in the Dip and Read™ format, such that streptavidin (SA) biosensor tips were sequentially submerged in wells containing: baseline buffer, biotin conjugated mono-PEG BSA, baseline buffer, test samples or control samples, dissociation buffer 1, 2 or 3.

2.2.6. Immunoprecipitation and Western Blot

Immunoprecipitation of anti-PEG antibodies was performed by incubating 200 µL of control samples and individual human sera for 60 min at room temperature with 50 µL of 10 mg/mL streptavidin paramagnetic beads coated with biotinylated PEG. Beads were washed with PBS and then 40uL of WES™ sample buffer containing 5% SDS and 400 mM DDT (Dithiothreitol) was added. Bead-captured samples were denatured at 95 °C for 10 min. A magnet was used to separate eluent from beads. The eluted samples were separated on WES™ (an automated capillary western blot instrument) then probed with anti-IgG/IgM-HRP followed by the addition of chemiluminescent substrate (luminol-S and peroxide).

3. Results

3.1. Surfactant Effects- ELISA

The importance of excluding PEG-based surfactants such as Tween® 20 from lengthy incubations during anti-PEG assays is well known (Liu et al., 2011; Sherman et al., 2012), however the effect of PEG-based surfactants on anti-PEG antibodies during brief contact times (such as the minute or less that is typical of an automatic plate wash step) may be overlooked or thought to be unimportant. To look at the impact of Tween® 20 in wash buffer, we screened control samples and 40 randomly selected drug-naïve sera in our anti-PEG ELISA with wash buffer that contained either 1XPBS + 0.5% DDM or 0.05% Tween® 20 (Fig. 2). The comparison between wash buffers showed a 37% decrease in anti-PEG responses when wash buffer included 0.05% Tween® 20 instead of 0.5% DDM (Fig. 2).

To further explore the impact of different surfactants on the anti-PEG ELISA, we prepared various surfactants at 0.5% in modified StartingBlock™ TBS. Control samples and 19 individual drug-naïve human sera were diluted 1/10 in modified StartingBlock™ TBS containing 100 µg/mL multi-PEG BSA or 0.5% of 11 different surfactants (Fig. 3a and b). Ideally, surfactant should not influence specific binding, whereas addition of a specific competitive antigen should cause

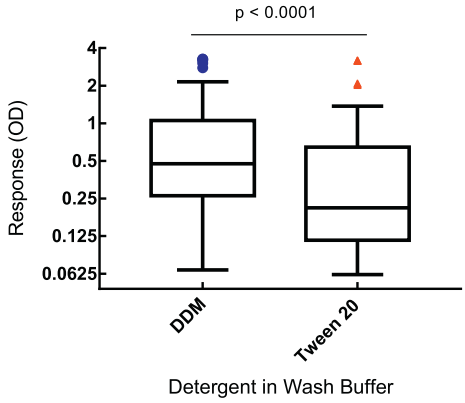

Fig. 2. Impact of Tween® 20 Added to Wash Buffer: Control samples and individual human sera (n = 40) were screened in the generic anti-PEG ELISA using wash buffer containing 0.5% DDM or 0.05% Tween® 20. The data are depicted using Tukey's method for box-and-whisker plots, with samples outside of the whiskers shown as points. A paired t-test indicated Tween® 20 was associated with significantly lower signals (p < .0001).

immunoassay signal to decline. The PEG-like surfactants yielded results that closely matched the responses of samples spiked with excess multi-PEG BSA, while samples spiked with non-PEG-like surfactants yielded higher anti-PEG responses (Fig. 3c).

3.2. Surfactant Effects- Kinetic Binding Assay

In an orthogonal analysis of antibody binding to PEG, we tested control samples and five donor sera using an Octet instrument. The individual sera were chosen based on anti-PEG ELISA results that indicated two sera were "high positive" (BRH1275698 and BRH1275701), two were "low positive" (BRH1275598 and BRH1275654), and one was "negative" (BRH1275625). In the absence of surfactant, antibodies showed gradual dissociation from Octet sensors decorated with biotin conjugated PEG~BSA, whereas addition of the PEG-based surfactant Tween® 20 resulted in rapid dissociation from sensors leading most anti-PEG signals to return to background levels within seconds after exposure to Tween® 20 (Fig. 4a). As stated by the manufacturer, the IgG anti-PEG c3.3 was selected to be resistant to competition by Tween® 20. This is in agreement with our results, which indicate that Tween® 20 effected dissociation of c3.3 less than other antibodies. Nonetheless, dissociation of c3.3 was also greatly accelerated by Tween® 20 and approached baseline within minutes. Addition of the non-PEG-based surfactant DDM resulted in persistent binding similar to dissociation in the absence of surfactant (Fig. 4b). From these experiments we conclude that an orthogonal Octet method detected positive and negative anti-PEG signal in agreement with the generic ELISA method and that addition PEG-based surfactants result in rapid and dramatic loss of anti-PEG binding that might generate false negative results.

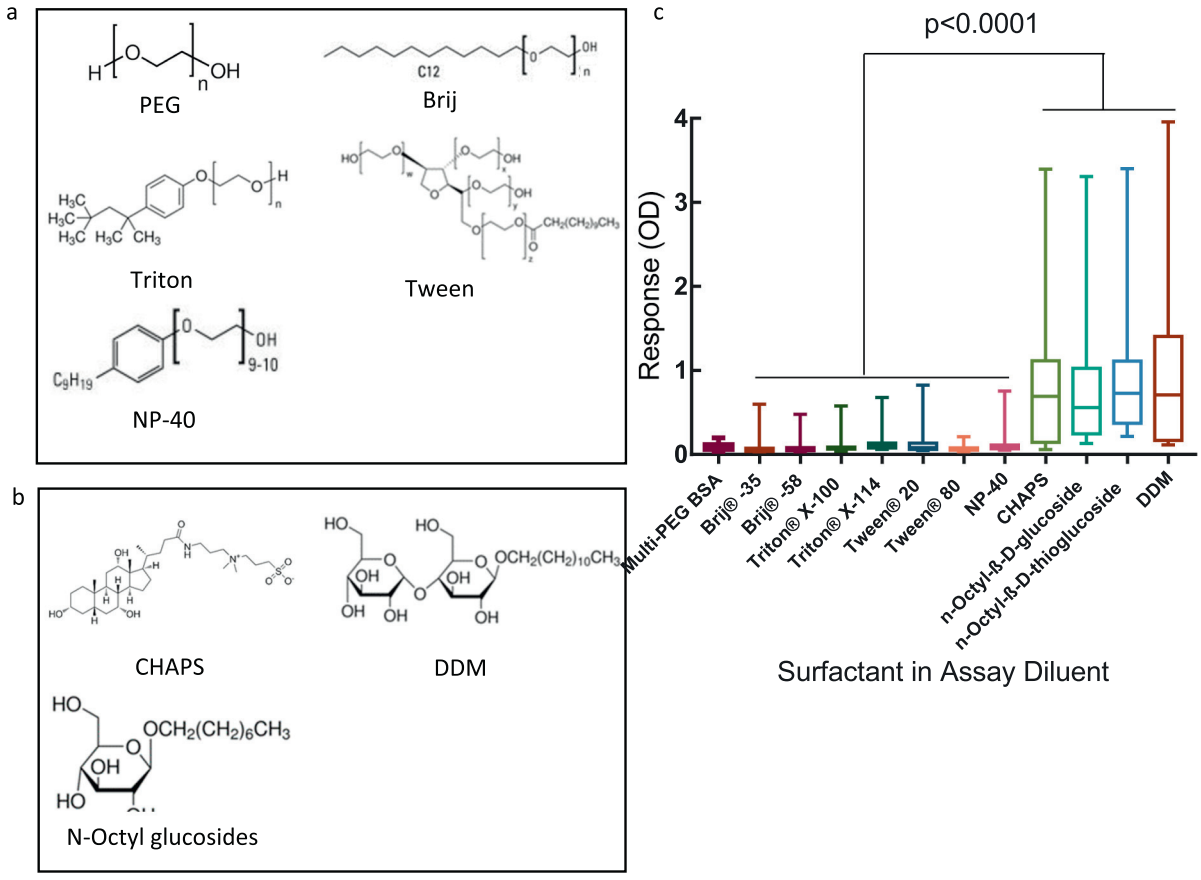

Fig. 3. Impact of Surfactants Added to Assay Diluent: Individual human sera (n = 19) were diluted 1/10 in modified StartingBlock™ TBS containing 100 µg/mL multi-PEG BSA or 0.5% of 11 different surfactants including (a) PEG-based surfactants or (b) non-PEG-based surfactants. Samples were then screened in the generic anti-PEG ELISA without further dilution. (c) The data are depicted using box-and-whisker plots. A paired t-test indicated that results obtained from samples diluted in any of 7 PEG-based surfactants were significantly different from results obtained from samples diluted in any of 4 non-PEG-based surfactants.

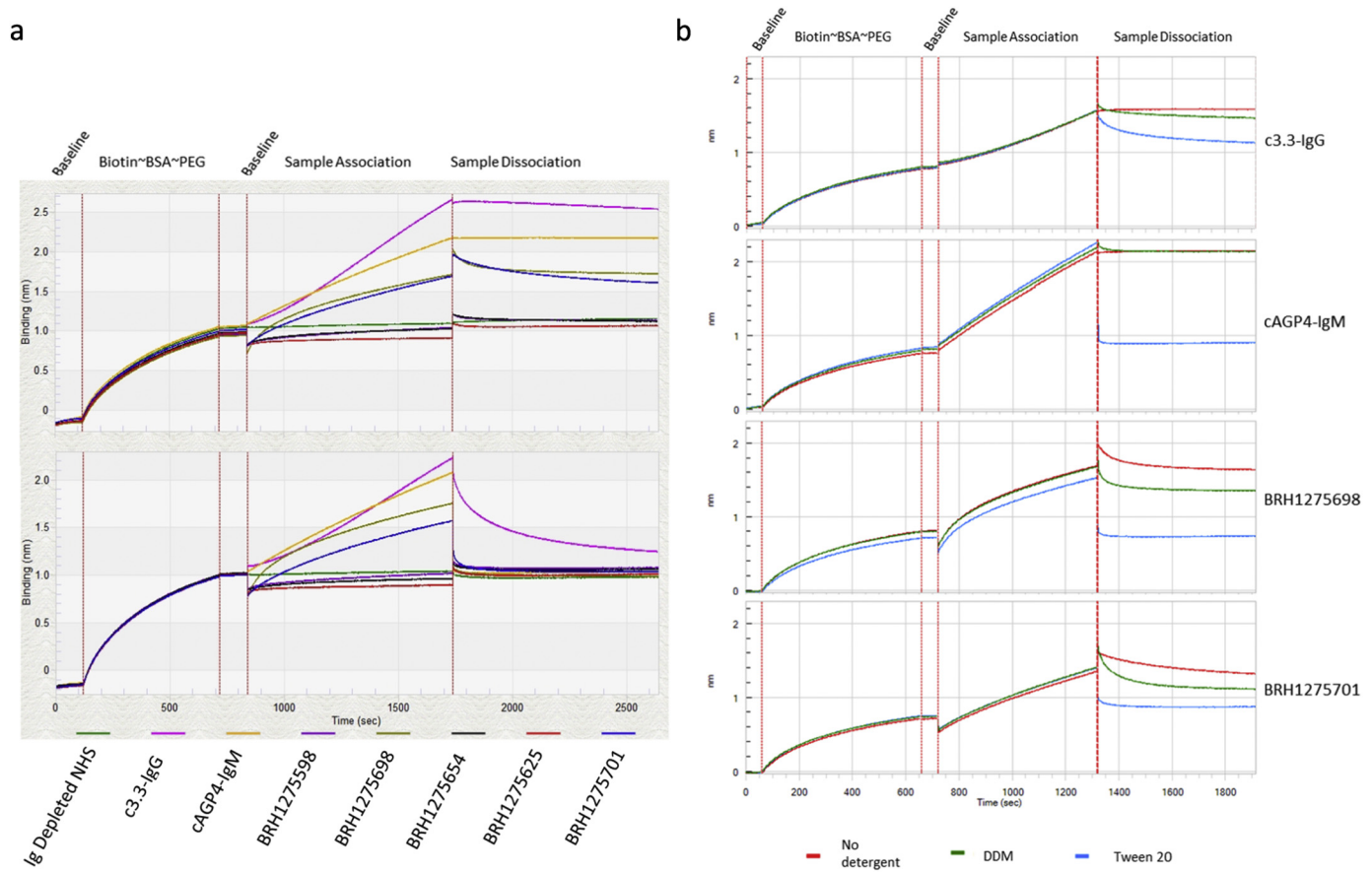

Fig. 4. Effect of Surfactants on the Kinetic Binding of Anti-PEG Antibodies. (a) Individual human serum samples ($n = 5$) and control samples were examined for their ability to bind mono-PEG BSA labeled sensors followed by dissociation from sensors in the absence (top) or presence (bottom) of Tween® 20. (b) A subset of samples was examined for their ability to bind mono-PEG BSA labeled sensors and dissociate from sensors in the absence or presence of DDM or Tween® 20.

3.3. Specific Detection of Immunoglobulin

To determine specificity, we tested 170 drug-naïve individual sera in a competitive binding version of the anti-PEG ELISA. Control samples and individual sera were diluted 1/10 in assay diluent alone, or assay diluent containing 100 µg/mL of multi-PEG BSA, PEGylated therapeutic A, or PEGylated therapeutic B (Fig. 5a). Compared to the response in assay diluent, the PEGylated proteins led to mean percent signal decreases of 80%, 73% and 75%, respectively. To further verify that the responses were specific to PEG, control samples and 24 of the 170 individual sera were randomly selected, diluted 1/10 in assay diluent alone or assay diluent containing 100 µg/mL of either a non-PEGylated therapeutic or BSA (Fig. 5b). Comparable responses were generated by sera and control samples prepared in assay diluent, non-PEGylated therapeutic, and BSA.

To confirm that anti-PEG ELISA responses are due to immunoglobulin we immunodepleted 264 drug-naïve individual sera and screened them in the anti-PEG ELISA. Replicates of control samples and individual sera were either untreated or passed through consecutive 96-well protein A and L filter plates and then screened in the anti-PEG ELISA (Fig. 6). Compared to untreated sera, immunodepletion caused mean anti-PEG signals to decrease 89.8%, providing further confirmation that the anti-PEG signals are due to pre-existing antibodies.

3.4. Antibodies and Isotypes

The anti-PEG ELISA typically used a mixture of HRP labeled anti-human IgG + IgM probes, however, probing replicate samples with the individual anti-IgG or anti-IgM probes allowed for characterization of anti-PEG Ig isotype. Assays run this way indicated that various healthy donor sera contained differing amounts of IgG and/or IgM anti-PEG activity (Fig. 7). An orthogonal method (PAGE/Western blot analysis) was also used to examine anti-PEG isotypes. In this case, control samples and seven human sera were incubated with biotinylated PEG coated paramagnetic streptavidin beads, then bead-bound substances were eluted, reduced, denatured, and separated by molecular size via polyacrylamide gel electrophoresis. PAGE bands were blotted then probed for IgM (Fig. 8a) or IgG (Fig. 8b).

Based on anti-PEG antibody control samples, the sensitivity of the PAGE/Western blots was 0.750 µg/mL for IgG anti-PEG and 0.125 µg/mL for IgM anti-PEG while the sensitivity of the anti-PEG ELISA was 0.100 µg/mL for IgG anti-PEG and 0.800 µg/mL for IgM anti-PEG. In those instances, in which the anti-PEG ELISA signal for total immunoglobulin was ≥1.522, the western blots also generated positive anti-PEG signals. The PAGE/Western analysis appeared to be less sensitive than the ELISA because total anti-PEG ELISA signals < 1.522 did not generate bands in the PAGE/Western. However, as signal could be detected by the PAGE/Western, there was agreement as to the characterization of IgG and/or IgM anti-PEG isotypes in each serum (Table 1).

3.5. Characterization of the Performance of a Generic Anti-PEG ELISA

3.5.1. Screening and Confirmation Assay

During validation our anti-PEG antibody method 3 analysts generated 6 runs of 200 individual human sera (50 obese, 50 type II diabetic, and 100 normal) in the screening and specificity methods (Fig. 9a). Normalization to the immunodepleted pooled serum consistency

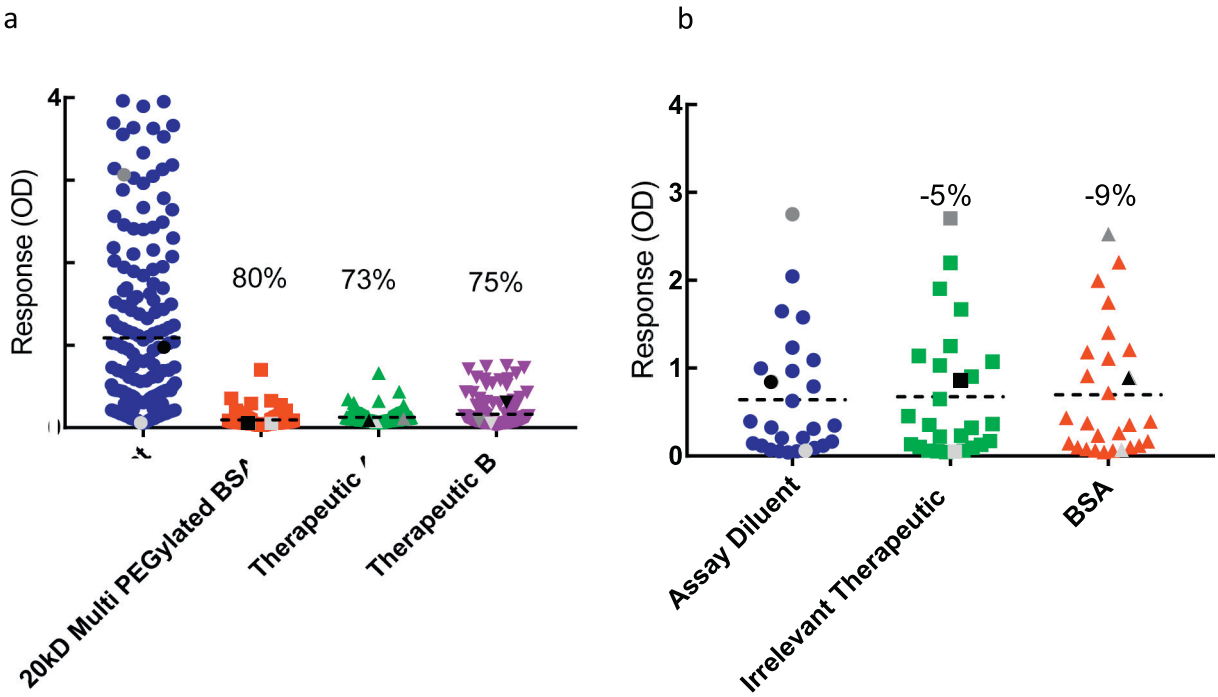

Fig. 5. Confirming Anti-PEG Antibodies in the Generic Anti-PEG ELISA: (a) Individual human sera (n = 170) and control samples (cAGP4 1000 ng/mL, black; c3.3 1600 ng/mL, dark gray; pooled Ig depleted NHS, light gray) were diluted in assay diluent with or without the addition of multi-PEG BSA, or PEGylated therapeutic A, or PEGylated therapeutic B. (b) A subset of individual human sera (n = 24) and control samples were diluted in assay diluent with or without the addition of a non-PEGylated therapeutic or BSA. Data points indicate the mean of 2 replicates. Dotted lines indicate the mean response for each condition. Percent values indicate mean inhibition of ELISA signal for each condition relative to samples prepared in assay diluent alone.

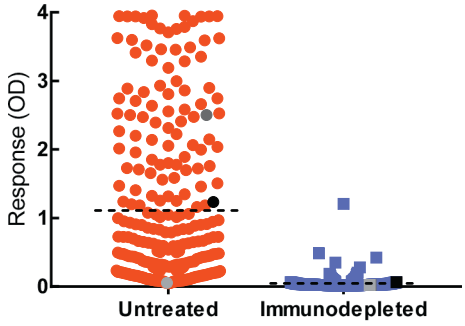

Fig. 6. Confirmation Generic Anti-PEG ELISA Signals are Due to Antibodies: Individual human sera (n = 264) and control samples (cAGP4 2000 ng/mL, black; c3.3 1200 ng/mL, dark gray; pooled Ig depleted NHS, light gray) were screened for anti-PEG antibodies before and after being immunodepleted by passage through 96-well protein A and L filter plates. Data points represent the mean of duplicates and dotted lines indicate the overall mean response before and after immunodepletion.

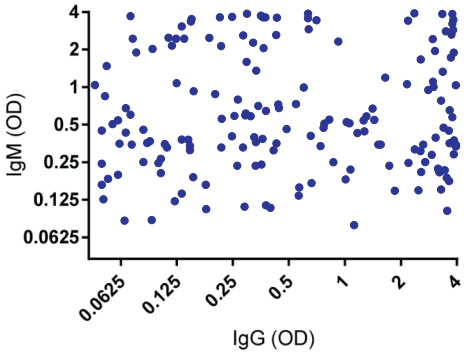

Fig. 7. Anti-PEG IgG and IgM Activity: Individual human sera (n = 170) were screened for anti-PEG antibodies in a modification of the ELISA that detected anti-PEG antibodies using anti-human IgG HRP and anti-human IgM HRP probes separately instead of combined. Data points represent the mean of duplicates with CVs < 20%. Results for 1000 ng/mL cAGP4, 400 ng/mL c3.3, and Ig depleted pooled NHS control samples (not shown in Fig. 7) were 0.032, 2.674, and 0.021 OD, respectively verses IgG and 2.528, 0.152, and 0.150 OD, respectively verses IgM.

control reduced inter-day and inter-analyst variability. Due to the limited number of negative sera, an immunodepleted pooled serum was used to calculate a confirmation cutpoint, following the FDA guidance recommendation for an 80% one-sided lower confidence interval for the 99th percentile of the negative control population to assure at least a 1% FPR with an 80% confidence level (FDA IM assay guidance, 2019). The resulting confirmation cut point was 47.0% which led to the conclusion that 97.5% (195 of 200) of human serum samples contained pre-existing antibodies to PEG (Fig. 9a).

The screening cut point calculation was based on an approach recommended for populations in which there is a high incidence of pre-existing positive results (Schneider, et al., year) which used the NVs of sera diluted with multi-PEGylated BSA and a false positive rate (FPR) of at least 5% with 90% confidence The screening cut point was 3.53 for the non-diabetic population and 2.54 for diabetics.

3.5.2. Variability

The variability of the ELISA screening method was determined by calculating the percent coefficient of variation (CV) for the positive control samples. The low positive consistency control samples generated intra-assay percent CV values ranging from 4.1 to 7.2% and inter-assay percent CV values ranging from 16.7 to 17.7%. The high positive consistency control samples generated intra-assay percent CV values ranging from 4.7 to 7.1% and inter-assay percent CV values ranging from 16.6 to 18.9%. Similarly, for the specificity assay the high positive control samples generated inter-assay CV values between 5.9 and 9.2%.

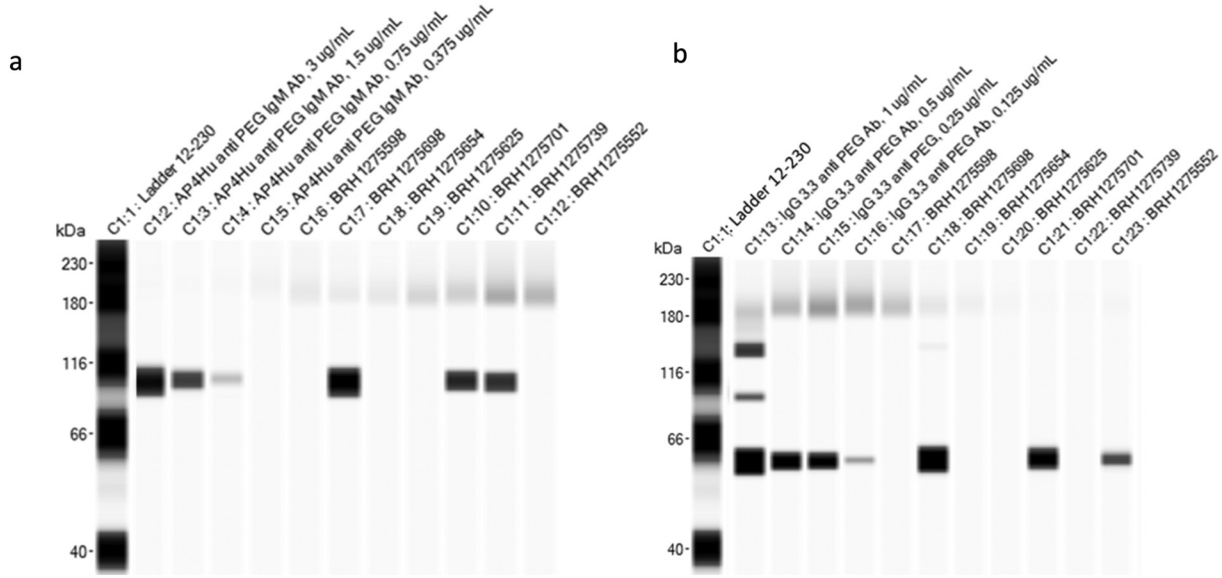

Fig. 8. Correlation Between Western Blot and ELISA: Lane 1 of both blots contains a molecular weight ladder (12 to 230 kDa) stained to show total protein. (a) Lanes 2 to 12 were probed with anti-human IgM-HRP. Lanes 2 to 5 show serial dilutions of the IgM anti-PEG positive control. Lanes 6 to 12 contain the anti-PEG immunoprecipitation fractions from 7 individual sera. (b) Lanes 2–12 were probed with anti-human IgG-HRP. Lanes 2 to 5 show serial dilutions of the IgG anti-PEG positive control. Lanes 6 to 12 contain the same samples as lanes 6–12 in (a).

Table 1
Comparison of results from the generic anti-PEG Elisa and western blot methods.

	ELISA (OD)			Western blot	
Individual serum	**IgM**	**IgG**	**IgG/IgM**	**IgM**	**IgG**
BRH1275598	0.219	1.072	0.452	–	–
BRH1275698	3.518	3.839	3.628	+ + + +	+ + + +
BRH1275654	0.251	0.865	0.354	–	–
BRH1275625	N/A	N/A	0.080	–	–
BRH1275701	2.918	3.827	3.555	+ + + +	+ + + +
BRH1275739	3.753	0.070	1.522	+ + + +	–
BRH1275552	0.151	3.292	1.691	–	+ +

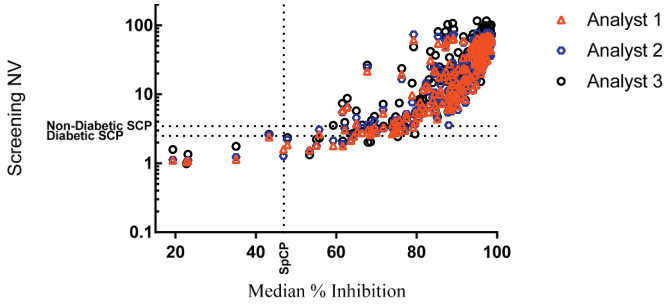

Fig. 9. Prevalence of Pre-existing Anti-PEG Antibodies and Generic Anti-PEG Screening ELISA Cutpoint. Three analysts used the anti-PEG specificity ELISA method to evaluate human serum samples from 100 normal healthy volunteers, 50 obese individuals, and 50 individuals with type 2 diabetes. Dotted horizontal and vertical lines indicate the assay screening and specificity cutpoints, respectively.

3.5.3. Sensitivity

To determine the ELISA sensitivity, two-fold dilutions of the anti-PEG positive control samples were prepared in the immunoglobulin depleted negative control serum pool prior to analysis. The assay sensitivity, as defined as the lowest concentration of positive control samples that generated results consistently greater than the screening cutpoint, was 100 ng/mL for c3.3 (IgG) and 800 ng/mL for cAGP4 (IgM) (Fig. 10).

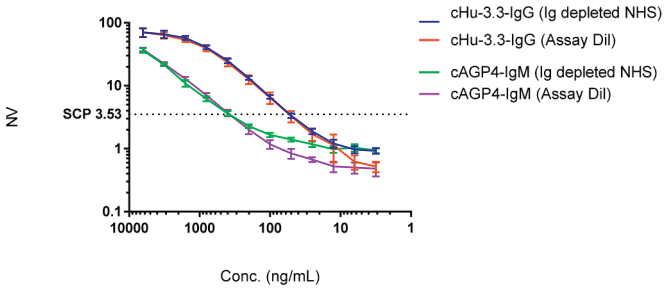

Fig. 10. Sensitivity and Dilutability: Anti-PEG positive control samples c3.3 (IgG) and cAGP4 (IgM) were prepared in Ig depleted human serum then two-fold serially diluted in either Ig depleted human serum or assay diluent. Diluted control samples were then analyzed in the anti-PEG ELISA. Sensitivity was defined by the lowest concentration of positive control that generated consistency positive results relative to the screening cutpoint.

3.5.4. Dilutability

In the absence of matrix effects, sample titers were determined by comparing the results generated from serial dilutions of positive control samples prepared in the immunoglobulin depleted negative control serum pool and in assay diluent. For all results greater than the assay cut point the serum matrix had no impact on anti-PEG antibodies c3.3 (IgG) and cAGP4 (IgM) signal (Fig. 10). Similarly, the titers of five of the positive donor sera were determined two times by three analysts and the variability of the titer values was calculated (Fig. 11). Based on these results, a 4-fold or greater change in titer was determined to represent a significant difference.

3.5.5. Drug Tolerance

In theory, circulating PEGylated protein could interfere with the detection of anti-PEG antibodies, so experiments were performed to quantify the amount of PEGylated protein that could be tolerated. It is common knowledge that ADA assays for protein therapeutics show a direct correlation between increased ADA concentration and increased

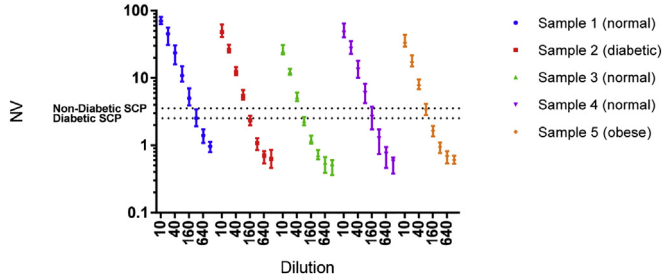

Fig. 11. Positive Sample Titration: Five positive samples were two-fold serially diluted in assay diluent twice by three analysts. The data points are depicted as the mean with the range.

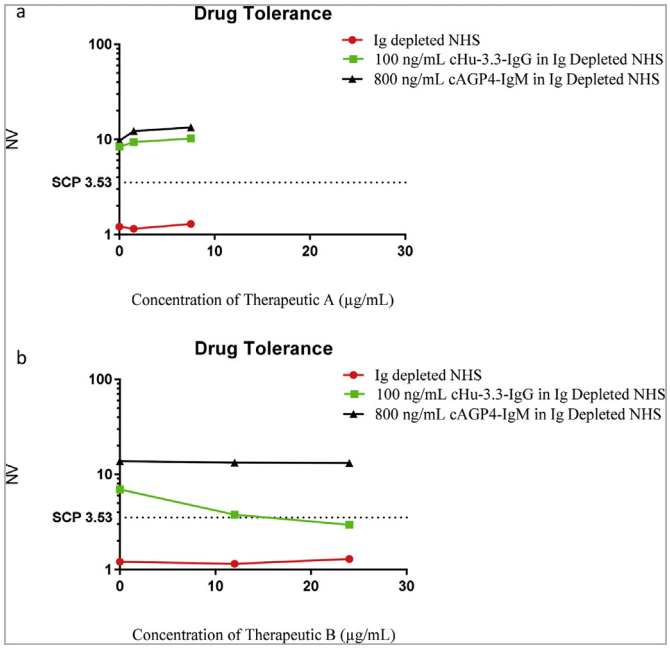

Fig. 12. Drug Tolerance: c3.3 (IgG), cAGP4 (IgM) and Ig depleted human serum prepared in the absence and presence of Therapeutic A (a) or Therapeutic B (b) then screened in the generic anti-PEG ELISA. Data points represent the mean of duplicates with < 20% CV.

drug tolerance. We chose ADA concentrations near the LOD because this demonstrated drug tolerance under the challenging conditions, and because these concentrations align with FDA's recommendation that clinical ADA assays should be sensitive to 100 ng/mL. Positive control samples were prepared near the limit of detection (100 ng/mL c3.3 and 800 ng/mL cAGP4) in Ig depleted serum spiked with various concentrations of PEGylated therapeutics A and B, then analyzed in the ELISA. Interference was defined by the concentration of PEGylated protein associated with a false negative test result. Both positive control antibodies remained detectable in the presence of 7.5 µg/mL therapeutic A (higher concentrations were not tested; Fig. 12a). The signal from cAGP4 was unaffected by 24 µg/mL of therapeutic B (higher concentrations were not tested) whereas antibody c3.3-IgG was detected at 12 µg/mL but not at 24 µg/mL of therapeutic B (Fig. 12b). The drug tolerance for therapeutic A and B was sufficient for testing the samples within each study so there was no need to further improve upon it using procedures such as acid treatment.

4. Discussion

Scientists have been measuring anti-PEG antibodies for more than 40 years. As anti-PEG assays have improved the reported incidence of antibodies to PEG has gradually increased (Schellekens et al., 2013; Yang et al., 2016). Because these improvements were instituted haphazardly across labs over several decades it is difficult to determine the effect these changes have had on the measured incidence of antibodies to PEG, but it seemed obvious to us that combining all improvements into the design of one anti-PEG assay might profoundly change our ability to detect anti-PEG antibodies. For the purpose of detecting antibodies to PEGylated-biopharmaceutical proteins we proposed a two-part bioanalysis strategy in which detection of antibodies to a PEGylated protein would be separated into an anti-PEG and an anti-protein ADA assay. There were two key benefits to this approach. First, when anti-PEG and anti-protein assay conditions were incompatible they could be optimized separately. Second, it avoided the problem of protein epitopes being masked by PEG, as observed by others (Lamppa et al., 2011: Pochechueva et al., 2014; Gefen et al., 2013).

It is well established (but not universally recognized) that surfactant competition has an important influence on anti-PEG assays (Liu et al., 2011; Sherman et al., 2012). Many common surfactants used in immunoassays contain repeating ethylene glycol subunits, and use of those PEG-like surfactants in an anti-PEG immunoassay creates unintended competition between the surfactant and the PEGylated antigen leading to low sensitivity and/or false negative results. Our results show that at a high surfactant concentration of 0.5%, non-PEG-like surfactants permitted detection of anti-PEG antibodies whereas PEG-like surfactants reduced sensitivity and led to false negative results. Even a lesser concentration of the most commonly used PEG-like surfactant (Tween® 20) to 0.05% (a concentration universally used in immunoassays) our assay remained significantly less sensitive than the when using a high concentration of a non-PEG-like surfactants such as DDM. Using an Octet kinetic assay format, we showed that the amount of Tween® 20 present in most immunoassay wash buffers was sufficient to rapidly displace antibodies to a PEGylated antigen. We performed a more comprehensive comparison of commonly available surfactants than we could find in past literature and noted there are good alternatives to DDM. One disadvantage of DDM is cost, so although we choose to use DDM in the present version of our assay, this may change in a future iteration.

The importance of removing Tween® 20 from anti-PEG immunoassays has at times been overlooked. One reason for this may relate to the investigator's choice of positive control. For example, the anti-PEG Mab c3.3 (and 3.3, the parental form from which it was engineered) are common control samples. However, the manufacturer of 3.3/c3.3 has indicated that the parental MAB was selected to be resistant to the effects of Tween® 20. We confirmed that a low concentration of Tween® 20 had little effect on the ability of our anti-PEG assay to detect c3.3, while the same low concentration of Tween® 20 on another control Mab (cAGP-4) and several anti-PEG positive human sera was to greatly or entirely eliminate anti-PEG detection. We conclude that past assays that incorporated Tween® 20 (or other PEG-like surfactants) and based detection upon 3.3 or c3.3, may have grossly under reported the prevalence of anti-PEG antibodies.

There are unique considerations related to the format of anti-PEG assays. For example, anti-PEG antibodies seem to be of lower affinity than many anti-protein antibodies, a situation that generally favors assays in which antibody-antigen binding occurs on a solid surface rather than in solution. Also, the bridging assay format most frequently used to detect ADA to pharmaceutical antibodies can be remarkably insensitive to anti-PEG antibodies especially for high molecular weight PEGs (unpublished observations). We suspect the reason for this is that a high local concentration of repeating ethylene glycol subunits causes anti-PEG antibodies to favor intra-molecule binding instead of inter-molecule bridging. This may also explain why some anti-PEG assays formats favor detection of either IgG (dimeric binding) or IgM (di- tri- or pentameric binding) anti-PEG antibodies and tend to be less impacted by short chain PEGs. To circumvent these disadvantages of the bridging format we focused on a sandwich format in which antibodies to PEG were in turn detected via anti-human IgG + M detection

reagents. For the purpose of detecting human antibodies to PEG, this approach became practical only recently due to the commercial introduction of chimeric human anti-PEG IgG and IgM Mabs (such as c3.3-IgG and cAGP4-IgM). We noted that optimal assay conditions to detect IgG were less than optimal to detect IgM and vice versa and so we settled on conditions that detect both isotypes well.

The repeating structure of unbranched linear PEG simplified our assay design. Our capture antigen was BSA PEGylated 1:5 with 20 kDa linear PEG. As demonstrated by our confirmatory assay, the anti-PEG antibodies we detected showed essentially equal specificity to PEGylated protein conjugates containing a 20 kDa linear PEG, a 40 kDa PEG and a 10 kDa PEG. This allowed us to design a generic anti-PEG assay that could be applied to many PEGylated biopharmaceuticals.

It is likely that most humans are frequently exposed to PEG or PEG-like chemicals (variously described as polysorbate or tween on the labels of such products as toothpaste, shampoo, cosmetics, processed foods, OTC drugs). Hence, it should not be surprising to find a high incidence of anti-PEG antibodies among the general population regardless of exposure to PEGylated prescription pharmaceuticals. Pre-existing antibodies can create challenges for assay developers because matrix from supposedly naive individuals may generate a range of specific signals and non-specific background. With this in mind, our approach was to determine whether pre-existing signals might be due to anti-PEG antibodies before changing the assay in ways that would eliminate potential noise. We examined our pre-existing ELISA "background" signal in orthogonal methods, including; competitive binding in the same ELISA, antibody depletion, an Octet kinetic binding system, and a PAGE-Western blot assay. In this way we confirmed that optimization of our generic anti-PEG ELISA eliminated background without eliminating specific antibodies to PEG. Although our ELISA was more sensitive than other methods, which made it difficult to correlate the weaker signals, we maintained very good agreement between; competitive binding and antibody depletion coupled with analysis in the same anti-PEG ELISA, an Octet binding assay, and PAGE western analysis.

Having confidence that our ELISA detected the intended analyte, we investigated the prevalence of anti-PEG antibodies in humans. We purchased sera from 100 healthy blood donors, 50 obese but otherwise healthy blood donors and 50 blood donors with type 2 diabetes. Review of the scientific literature indicates that over the years the reported incidence of anti-PEG antibodies among the general population has increased from 0.2% to 72%. Early on it was widely believed that PEG was not immunogenic. In 1983 it was typical for the reported incidence to be near 0.2% (Richter and Akerblom, 1984). By 2016 there was a refined understanding of the factors that influence detection of anti-PEG antibodies and reports at that time spanned a wide range that placed the incidence in the general population between 23% and 72% (Chen et al., 2016; Lubich et al., 2016; Yang et al., 2016). Using a modern anti-PEG assay, Yang et al. (2016) examined a repository of human sera frozen between 1970 and 1999 and found an incidence of 59% in older samples compared to 72% in the most contemporary samples. Although one might question the stability of serum antibodies stored frozen up to 46 years, their results suggest that despite the reported incidence generally being < 1% at the time those older historical samples were obtained, the actual incidence of anti-PEG antibodies has risen only modestly since 1970 while the measured incidence has risen dramatically due to the use of improved anti-PEG assays. We believe our assay represents a further level of refinement, none the less, we were surprised to find the incidence of pre-existing anti-PEG antibodies to be 97.5%, however, having used multiple methods to verify assay performance during the development process we conclude that a high incidence of anti-PEG antibodies aligns with our modern environment where there is repeated and sometimes daily exposure to PEG and PEG-like chemicals.

Knowing that our conclusion may surprise others as it did us, we devised one additional challenge. While we know that immunoassay conditions must be optimized to account for species differences, we frequently find that immunoassay conditions can be virtually interchangeably between human and non-human primates. We therefore used our assay to test for anti-PEG antibodies in commercially available sera from 10 cynomolgus monkeys. Our premise was that non-human primates might represent a truly naive population that is not exposed to PEG or PEG-like additives (the vendor did not respond to our requests for confirmation). The same assay that detected a nearly 100% incidence in humans detected no anti-PEG antibodies among the 10 monkey sera.

A key application for anti-PEG antibody analysis is to understand the clinical impact these antibodies may have. Reports of severe allergic reactions to PEG (Sanders, 2019) are exceedingly rare. Tween 80 is included in the formulation of a great number of pharmaceuticals with no severe acute reactions, indicating no obvious link between pre-existing antibodies to PEG and severe reactions in patients. Less serious consequences of anti-PEG antibodies may have been overlooked, for example, manageable allergic reactions to injectable drugs are not uncommon and the mechanism can be unclear. There have been retrospective attempts to find correlations (Zhang et al., 2016) but it is our opinion that differences between anti-PEG assays, especially data obtained using older methods, prevents a robust retrospective analysis. Instead we should look to future studies supported by modern methods. Besides a long history of PEG and PEG-like additives being used in drug formulations, approved drugs have more recently included PEGylated active ingredients. Based upon the molecules in pharmaceutical pipelines this trend is likely to increase. Yet in our experience, many labs seem to a lack an awareness of the unique requirements of anti-PEG assays. As we've demonstrated, modern assays can detect antibodies to PEG in 23 to 97.5% of the general population, however some labs fail to detect anti-PEG antibodies in any patient sera before or after treatment with PEGylated drugs. Before drugs are approved one would expect such disparities to be resolved, and this is true in at least some instances. One lab found that 25% of healthy blood donors had anti-PEG antibodies and subsequently reported that in clinical trials rapid clearance of PEG-asparaginase was closely associated with antibodies to PEG (Armstrong et al., 2007). In a phase 1 study of PEGylated phenylalanine ammonia lyase it was reported that 16% of subjects had pre-existing antibodies to PEG and that after one dose 100% of subjects had antibodies to PEG but that no clear correlation could be made between anti-PEG antibodies and the adverse events observed (Longo et al., 2014). In another example, the prescribing information for Pegloticase indicates 42% of patients developed anti-PEG antibodies that were associated with treatment failure (Krystexxa (pegloticase) prescribing information, 2016). This was confirmed by an independent lab that found antibodies to PEG, including pre-existing anti-PEG, were associated with rapid drug clearance, loss of efficacy, and infusion reactions after treatment with Pegloticase (Hershfield et al., 2014). During a chronic dosing study 100% of patients developed antibodies to a pegylated bacterial lyase with most patients developing anti-PEG as well, however safety and efficacy were said to be acceptable (Gupta et al., 2018). Clearly the clinical impact of anti-PEG antibodies varies and must therefore be assessed on a case by case basis. Also, if modern anti-PEG assays can be made so sensitive that the clinical impact cannot be determined, then incidence will be less important than titer or perhaps some yet to be determined property of anti-PEG antibodies, such as affinity, or isotype.

Declaration of Competing Interests

None.

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References

Abuchowski, A., McCoy, J.R., Palczuk, N.C., van Es, T., Davis, F.F., 1977. Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. J. Biol. Chem. 252, 3582–3586.

Armstrong, J.K., Hempel, G., Koling, S., Chan, L.S., Fisher, T., Meiselman, H.J., Garratty, G., 2007. Antibody against poly(ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients. Cancer 110, 103–111.

Chen, B.M., Su, Y.C., Chang, C.J., Burnouf, P.A., Chuang, K.H., Chen, C.H., Cheng, T.L., Chen, Y.T., Wu, J.Y., Roffler, S.R., 2016. Measurement of pre-existing IgG and IgM antibodies against polyethylene glycol in healthy individuals. Anal. Chem. 88, 10661–10666.

Cheng, T.L., Chen, B.M., Chern, J.W., Wu, M.F., Roffler, S.R., 2000. Efficient clearance of Ply(ethylene glycol)-modified immunoenzyme with anti-peg monoclonal antibody for prodrug cancer therapy. Bioconjug. Chem. 11, 258–266.

FDA IM assay guidance, 2019. https://www.fda.gov/media/119788/download.

Fruijtier-Polloth, C., 2005. Safety assessment on polyethylene glycols (PEGs) and their derivatives as used in cosmetic products. Toxicology 214, 1–38.

Gefen, T., Vaya, J., Khatib, S., Harkevich, N., Artoul, F., Heller, E.D., Pitcovski, J., Aizenshtein, E., 2013. The impact of PEGylation on protein immunogenicity. Int. Immunopharmacol. 15, 254–259.

Gupta, S., Lau, K., Harding, C.O., Shepherd, G., Boyer, R., Atkinson, J.P., Knight, V., Olbertz, J., Larimore, K., Gu, Z., Li, M., Rosen, O., Zoog, S.J., Weng, H.H., Schweighardt, B., 2018. Association of immune response with efficacy and safety outcomes in adults with phenylketonuria administered pegvaliase in phase 3 clinical trials. EBioMedicine 37, 366–373.

Hamidi, M., Azadi, A., Rafiei, P., 2006. Pharmacokinetic consequences of pegylation. Drug Deliv. 13, 399–409.

Hershfield, M.S., Ganson, N.J., Kelly, S.J., Scarlett, E.L., Jaggers, D.A., Sundy, J.S., 2014. Induced and pre-existing anti-polyethylene glycol antibody in a trial of every 3-week dosing of pegloticase for refractory gout, including in organ transplant recipients. Arthritis Res.Ther. 16, R63.

Ishida, T., Maeda, R., Ichihara, M., Irimura, K., Kiwada, H., 2003. Accelerated clearance of PEGylated liposomes in rats after repeated injections. J. Control. Release 88, 35–42.

Krystexxa (pegloticase) prescribing information, 2016. https://www.accessdata.fda.gov/drugsatfda_docs/label/2016/125293s085lbl.pdf Reference ID:3929005.

Lamppa, J.W., Ackerman, M.E., Lai, J.I., Scanlon, T.C., Griswold, K.E., 2011. Genetically engineered alginate lyase-PEG conjugates exhibit enhanced catalytic function and reduced immunoreactivity. PloS one 6, e17042.

Laverman, P., Carstens, M.G., Boerman, O.C., Dams, E.T., Oyen, W.J., van Rooijen, N., Corstens, F.H., Storm, G., 2001. Factors affecting the accelerated blood clearance of polyethylene glycol-liposomes upon repeated injection. J. Pharmacol. Exp. Ther. 298, 607–612.

Liu, Y., Reidler, H., Pan, J., Milunic, D., Qin, D., Chen, D., Vallejo, Y.R., Yin, R., 2011. A double antigen bridging immunogenicity ELISA for the detection of antibodies to polyethylene glycol polymers. J. Pharmacol. Toxicol. Methods 64, 238–245.

Longo, N., Harding, C.O., Burton, B.K., Grange, D.K., Vockley, J., Wasserstein, M., Rice, G.M., Dorenbaum, A., Neuenburg, J.K., Musson, D.G., Gu, Z., Sile, S., 2014. Single-dose, subcutaneous recombinant phenylalanine ammonia lyase conjugated with polyethylene glycol in adult patients with phenylketonuria: an open-label, multicentre, phase 1 dose-escalation trial. Lancet 384, 37–44.

Lubich, C., Allacher, P., de la Rosa, M., Bauer, A., Prenninger, T., Horling, F.M., Siekmann, J., Oldenburg, J., Scheiflinger, F., Reipert, B.M., 2016. The mystery of antibodies against polyethylene glycol (PEG) - what do we know? Pharm. Res. 33, 2239–2249.

Pasut, G., Veronese, F.M., 2012. State of the art in PEGylation: the great versatility achieved after forty years of research. J. Control. Release 161, 461–472.

Pochechueva, T., Chinarev, A., Bovin, N., Fedier, A., Jacob, F., Heinzelmann-Schwarz, V., 2014. PEGylation of microbead surfaces reduces unspecific antibody binding in glycan-based suspension array. J. Immunol. Methods 412, 42–52.

Povsic, T.J., Lawrence, M.G., Lincoff, A.M., Mehran, R., Rusconi, C.P., Zelenkofske, S.L., Huang, Z., Sailstad, J., Armstrong, P.W., Steg, P.G., Bode, C., Becker, R.C., Alexander, J.H., Adkinson, N.F., Levinson, A.I., 2016. Pre-existing anti-PEG antibodies are associated with severe immediate allergic reactions to pegnivacogin, a PEGylated aptamer. J. Allergy Clin. Immunol. 138, 1712–1715.

Richter, A.W., Akerblom, E., 1984. Polyethylene glycol reactive antibodies in man: titer distribution in allergic patients treated with monomethoxy polyethylene glycol modified allergens or placebo, and in healthy blood donors. Int. Archiv. Allerg. Appl. Immunol. 74, 36–39.

Sanders, L., 2019. He'd never had allergies, but suddenly he had two episodes that nearly killed him. Why? The New York Times Magazine. Retrieved from. https://www.nytimes.com/2019/01/17/magazine/hed-never-had-allergies-but-suddenly-he-had-two-episodes-that-nearly-killed-him-why.html.

Schellekens, H., Hennink, W.E., Brinks, V., 2013. The immunogenicity of polyethylene glycol: facts and fiction. Pharm. Res. 30, 1729–1734.

Sherman, M.R., Williams, L.D., Sobczyk, M.A., Michaels, S.J., Saifer, M.G., 2012. Role of the methoxy group in immune responses to mPEG-protein conjugates. Bioconjug. Chem. 23, 485–499.

Shimizu, T., Abu Lila, A.S., Awata, M., Kubo, Y., Mima, Y., Hashimoto, Y., Ando, H., Okuhira, K., Ishima, Y., Ishida, T., 2018. A cell assay for detecting anti-PEG immune response against PEG-modified therapeutics. Pharm. Res. 35, 223.

Wenande, E., Garvey, L.H., 2016. Immediate-type hypersensitivity to polyethylene glycols: a review. Clin. Exp. Allergy 46, 907–922.

Yang, Q., Jacobs, T.M., McCallen, J.D., Moore, D.T., Huckaby, J.T., Edelstein, J.N., Lai, S.K., 2016. Analysis of pre-existing IgG and IgM antibodies against polyethylene glycol (PEG) in the general population. Anal. Chem. 88, 11804–11812.

Zhang, P., Sun, F., Liu, S., Jiang, S., 2016. Anti-PEG antibodies in the clinic: current issues and beyond PEGylation. J. Controll. Rel. 244, 184–193.