Research Article

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Bioanalysis

Development and characterization of antibody reagents to assess anti-PEG IgG antibodies in clinical samples

Background: Polyethylene glycol (PEG) is a polymer that can be conjugated with therapeutic proteins. Monitoring anti-PEG antibodies in human subjects may be required as part of immunogenicity assessment. The lack of well-characterized anti-PEG reagents have limited our understanding of anti-PEG humoral response. **Results:** Antibodies reactive to PEG were engineered with a human IgG_1 Fc. Surface plasmon resonance and plate-based methods demonstrated that their binding was dependent on molecular weight (MW) of PEG. Specificity experiments using chemical analogs identified their specificity. **Conclusion:** Affinity, specificity and MW of PEG are critical characteristics that impact interactions of anti-PEG antibodies with PEG. These attributes especially MW of PEG and the assay formats may impact the ability to detect anti-PEG antibodies.

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Background

Polyethylene glycol (PEG) is a polymer that is commonly used as a covalent adduct to many biotherapeutic agents to increase their circulatory half-life; by increasing the hydrodynamic radius of the molecule, PEG reduces loss due to glomerular filtration leading to decreased renal clearance [1,2]. The PEG moiety on the therapeutic may vary in length (20–40 kD) and in branching. PEG is also known to be ubiquitously present in food and cosmetic products to which human exposure is believed to elicit anti-PEG antibodies [3–5]. The PEG in such products is generally smaller and simpler in structure than is linked to a protein therapeutic. Immunogenicity to biotherapeutics is known to influence their bioavailability, *in vivo* pharmacological potency and immune complex mediated adverse effects [6]. Although PEG has been considered to be of low immunogenic risk due to its simple subunit repeat structure and low charge density, there have been several reports documenting both pre-existing anti-PEG antibodies in humans as well as therapeutic-induced anti-PEG antibody response [3,7,8]. In some instances, drug-induced anti-PEG antibodies have been shown to increase the clearance of a PEGylated enzyme therapeutic [7]. Several groups have also raised both IgM and IgG antibodies against PEG [9–11] in mice.

Most immunogenicity assays developed for a PEGylated therapeutic protein describe anti-drug antibody (ADA) response to the whole drug with minimal to variable information on the specificity of the ADA toward the protein and PEG components of the therapeutic molecule. Some groups have addressed these questions by either using PEG as a competitor in the ADA assay [3,12] or by using a separate bridging assay that utilizes an IgM anti-PEG positive control; such an assay would solely detect an IgM response. That most pre-existing anti-PEG antibodies might be IgM might make such an assay relevant. However, it is conceivable that antibodies induced by sustained chronic exposure to a PEGylated therapeutic, or food and cosmetic additives might mature to an IgG isotype and would not be detected in an IgM bridging assay. Depending on the patient's immune status and the therapeutic indication, ADA of all isotypes specific to PEG may be relevant to drug safety in humans and therefore need monitoring. An ideal assay to

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Key terms

Polyethylene glycol: Polymer that may be linear or branched of varying lengths and is commonly used as a covalent adduct to some biotherapeutic agents. The structure of PEG is H-(O-CH_2-CH_2)$_n$-OH, attached to the protein by functionalized end group and terminating with a methyl cap group. All PEG molecules referred to in this study were single linear chain molecules with a single biotin attached at one end and a methyl cap at the other end. Molecular weight would be proportional to chain length and each preparation of PEG would be a heterogeneous mix with a normal distribution.

Immunogenicity: In the context used here, refers to the ability of a host to mount an immune response to a biotherapeutic.

Anti-drug antibodies: Antibodies capable of specifically binding to the drug molecule.

Specificity: Refers to the determination of any portion on the PEG molecule which is believed to mediate binding to anti-PEG antibodies.

Bridging assay: Assay format that uses the same unique reagent for both capture and detection of an analyte. Two molecules of a therapeutic each conjugated with a unique label could be bridged by an anti-therapeutic antibody.

Direct binding assay: Assay format that uses an immobilized target to capture an analyte specific to the target. In this study, biotinylated PEG is immobilized using streptavidin to capture anti-PEG antibodies which are detected via a ruthenylated antibody against Fc domain of the PEG-bound antibody.

detect such anti-PEG ADA would be capable of detecting anti-PEG antibodies of a wide range of affinities, with a broad specificity across all PEG sizes and shapes and a generic assay suitable for any PEGylated therapeutic drug regardless of the underlying protein portion of the drug and capable of identifying antibodies of all isotypes (IgM and IgG). We also realized that the multiplicity of the epitopes on the PEG backbone might preclude IgG anti-PEG ADA detection in the traditional bridging assay format by virtue of intrachain binding rather than interchain between molecules and therefore necessitate a direct assay format where the Fc in controls and samples will need to be of the same species. Such analytical requirements need a panel of well-characterized anti-PEG reagents. This manuscript describes our work toward generation, purification and characterization of anti-PEG antibodies that might be suitable for such an assay. Anti-PEG polyclonal and monoclonal antibodies engineered with a human Fc were generated for potential use in a direct assay format. To enable generic applications with these antibodies for multiple PEGylated therapeutics programs, biotin-conjugated PEG of varying molecular weights (MW) was used to characterize their binding to PEG using surface plasmon resonance (SPR) based methods on Biacore, and Meso Scale Diagnostics (MSD) electrochemiluminescence-based methods of direct binding to immobilized PEG as well as homogenous binding to PEG in solution. Since PEG is much smaller in MW in commercial cosmetic products and food additives than in therapeutics, preexisting and therapeutic-induced antibodies might be different. We therefore asked if MW of PEG had a bearing on backbone specific antibody binding. Our studies using PEG with MWs ranging from 350 Da to 40 kDa demonstrate the influence of epitope density of – $(CH_2CH_2O)_n$ per strand on the binding of these antibodies. Competition assays using methyl cap (mCap) mimicking analogs or PEG backbone structures with blocked mCaps on either ends were used to identify the specificity of these antibodies.

Our work shows the complexity of these anti-PEG antibodies in relationship to their affinity and avidity to the length of the PEG backbone and to the specific epitope that these antibodies recognize. The results underscore the heterogeneity of anti-PEG antibodies even to the same backbone structure and the limitations imposed by the selections of the PEG molecule and the assay format on the ability to detect anti-PEG antibodies with wide ranging specificity and varying affinities. We conclude robust assays capable of detecting the multitude of possible anti-PEG antibodies in humans require a diverse and well-characterized panel of reagents as described here.

Materials & methods

Custom-made monoclonal anti-PEG antibodies

Mice were immunized at Bristol–Myers Squibb (BMS), Redwood city with a panel of PEGylated BMS therapeutics and hybridomas were selected that showed binding to PEG coupled to unrelated proteins thus ensuring anti-PEG reactivity. The therapeutic proteins used as immunogens as well as PEGylated proteins used in the screening assays were chemically linked to a branched 40 kD PEG. Monoclonal hybridoma cell lines were derived and two antibodies were identified based on their specificity and affinity to PEG indirectly derived in direct binding assays. The variable region genes from these two cell lines were sequenced, subcloned into human IgG_1 Fc-bearing constructs, stably expressed in CHO cell lines and purified (sequence to be published in patent application). These are therefore referred to as chimeric anti-PEG antibodies PEG.1 and PEG.2 monoclonal antibodies (mAbs) since they retain their mouse Fab with a human IgG_1 Fc. Most of the assay format evaluations and characterization were done on the PEG.2 since it had slightly better binding than the PEG.1 chimeric antibody.

Custom-made polyclonal bovine antibodies

Human IgG transchromosomal cows (bovine-tc) at Sanford Applied Biosciences, Sioux Falls, South Dakota [13] were immunized with either keyhole limpet hemocyanin (KLH) chemically conjugated to 5 kD PEG or with a mix of PEGylated therapeutic molecules. While the KLH-conjugated PEG had a far higher number of PEG molecules coupled to each KLH, the therapeutic molecules typically had a single 20–30 kD PEG attached to a protein. The bovine-tc model predominantly produces IgG molecules bearing the human heavy chain and light chain and a very minor population having bovine kappa light chains. This system enabled generation of PEG-specific polyclonal antibodies (pAbs) similar to the monoclonal chimeric antibodies, but bearing both the Fc and Fab from human IgG. Cows were immunized at monthly intervals and sera were tested for both anti-PEG and anti-protein reactivity. Animals with the highest anti-PEG titers as determined in a direct binding assay to a PEGylated therapeutic were selected for plasmapheresis. Anti-PEG reactivity was tested in a generic assay format using the crude serum, purified total IgG and PEG affinity purified IgG. Of the three cows used in the immunization protocol, plasma from one cow named #2026 was selected based on its titer to two PEGylated therapeutic molecules for further affinity purification and further characterization of the anti-PEG pAb.

Purification of anti-PEG polyclonal antibodies

Peg affinity resin preparation

5 kD PEG - $CH_3O(CH_2CH_2O)_n(CH_2)_2SH$ (Sunbright® ME-050SH, NOF America Corporation, NY, USA) was solubilized in water to 100 mg/ml with 5 mM TCEP (tris[2-carboxyethyl]phosphine). PD-10 desalting column (GE Healthcare Bio-Sciences Corp., NJ, USA) was used to exchange to phosphate buffered saline (PBS) buffer, pH 7.5 and remove TCEP right before conjugating to SulfoLink coupling resin (Thermo Fisher Scientific Inc., IL, USA). Conjugation was allowed to occur at ambient temperature for 1 h. The ratio of 5 kD PEG to resin was about 1 mg PEG per ml resin. Higher amount of PEG per ml resin did not provide higher binding capacity in purifying anti-PEG pAbs. The PEG coupled resin was incubated with excess amount of L-cysteine solution for 1 h to block unused iodoacetyl groups.

Purification of anti-peg pAbs

500 ml of bovine plasma was first passed over MabSelect Protein A Affinity resin (GE Healthcare Bio-Sciences Corp.) which yielded approximately 3.5 g of total IgG antibodies and was neutralized to pH 7. Anti-PEG pAbs typically had mild-to-moderate binding to PEG affinity resin. Multiple batch purification processes were needed to completely recover anti-PEG antibody from total antibody. Since batch purification enabled longer contact time, the PEG affinity resin was incubated with total IgG with gentle stirring at ambient temperature for 1 h. The suspension was poured into an appropriate Econo-column (Bio-Rad, CA, USA) and flow through (FT) was carefully collected. The resin was washed thoroughly by PBS buffer and the antibodies eluted by low pH solution of 50 mM sodium acetate, pH 2.7 and immediately neutralized by 1 M Tris, pH 8. The binding capacity of the resin to anti-PEG pAbs was around 2.5–3.0 mg/ml resin. FT was evaluated for any residual anti-PEG activity and repeatedly passed over the column until most of the reactivity in the FT was eliminated. Around 80 mg of anti-PEG pAb were obtained by PEG affinity purification. Higher MW aggregated antibody, which accounted for about 40% of total IgG, was removed by Superdex 200 sizing-exclusion column (GE Healthcare Bio-Sciences Corp.). The remaining 40 mg of monomeric antibody was purified from a size exclusion column in PBS buffer, and concentrated to 3 mg/ml, aliquoted and frozen in -80°C. Unlike typical chromatographic purifications of anti-protein antibodies multiple batch purifications were needed to overcome

the poorer affinity and to maximize extraction of anti-PEG antibodies from total antibodies.

Commercial anti-PEG antibodies

Rabbit monoclonal anti-PEG IgG (B-47) was purchased from Epitomics, (an Abcam Company, CA, USA) and mouse monoclonal anti-PEG IgG (6.3) and IgM (AGP4) were purchased from Academia Sinica (Taipei, Taiwan). These were used in comparison with our custom antibodies for specificity testing. Product specification information from the providers indicated mCap specificity for the B-47 and backbone specificity for the 6.3 clone.

Biotin-conjugated PEG

Biotin was conjugated to 40 kD branched PEG at BMS using maleimide-thiol chemistry. 40 kD-branched-PEG-Mal (NOF #GL2–400MA01, MW = 42 kD) was prepared at 13.5 mg/ml in 20 mM sodium phosphate pH 7.0/150 mM NaCl. *N*-biotinyl-Cysteine (Carbosynth #FB154460, MW = 347) was prepared at 10 mg/ml in DMSO. A 21-fold excess of this cys-biotin was added to 40 kD-PEG-Mal and mixed for 2 h at room temperature. The sample was brought to 5 ml with phosphate buffer and then dialyzed twice against 2 l of Dulbecco's Phosphate-Buffered Saline (DPBS) to remove free cys-biotin. To verify conjugation, the sample pre- and post-biotin reaction was run on nonreduced SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), blotted onto nitrocellulose membrane, blocked with 1% BSA-PBS-Tween (1% BSA in 1× PBS and 0.05% Tween 20 from Teknova #D5120), probed with Streptavidin-HRP (Pierce antibody products #21126 Thermo Fisher Scientific Inc.) at 1 µg/ml, and reacted with hP substrate (Kit #1721064 from BioRad) [14]. A band in the post-reaction sample of the same MW as the barium chloride–iodine stained PEG (70 kD) reacted with the Streptavidin-HRP while the preconjugate PEG-Mal and cysteine-conjugated control did not react with Streptavidin-HRP. PEG molecules of various MW conjugated to biotin were purchased from Nanocs [15]. These varied from 550 daltons to 40 kD PEG (Cat #PGI-BN xxx, where xxx varied with size).

SPR analysis of anti-PEG binding to PEG

Neutravidin was purchased from Thermo Scientific (Rockford, IL, USA). Ethanolamine hydrochloride, *N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were purchased from Sigma-Aldrich (MO, USA). The regeneration buffers (10 mM glycine-HCl at pH 3.0, 2.5, 2.0 and 1.5), the HBS-N running buffer (10 mM HEPES, pH 7.4, 150 mM NaCl), the immobilization buffer (10 mM sodium acetate buffer, pH 5.0) and the series S CM5 sensor chips used for SPR experiments were purchased from GE Healthcare (NJ, USA). A Biacore T200 SPR instrument (GE Healthcare, NJ, USA) was used to study the binding interactions between the biotinylated PEGs and the anti-PEG antibodies.

Immobilization of Biotin-PEG onto a Neutravidin-coated sensor

Immobilization of neutravidin onto the CM5 sensor was achieved using the wizard template method in the Biacore T200 control software (version 1.0) with a target immobilization level of 5000 response units (RUs). Typically, two flow cells of a CM5 sensor were pretreated with an injection of 50 mM NaOH (30 µl at 30 µl/min) to remove any nonspecifically bound substances. A freshly prepared mixture of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (400 mM) and *N*-hydroxysuccinimide (100 mM) was injected (10 µl/min) onto both flow cells for 7 min to activate the carboxyl groups on the surface. Neutravidin (50 µg/ml in 10 mM sodium acetate buffer at pH 5.0) was then injected onto both flow cells at short pulses (12 s to 6 min at 5 µl/min) to ensure the target immobilization level was reached. Finally, excess active groups on both flow cells were blocked with an injection (10 µl/min) of 1 M ethanolamine, pH 8.0, for 7 min. The surface was conditioned with three injections of 50 mM NaOH/1 M NaCl followed by another three injections of 10 mM glycine-HCl at pH 1.5 (10 µl of each injection at 10 µl/min) prior to the immobilization of the biotin-PEG. Biotin-PEG was immobilized onto one flow cell by multiple short injections (18 s per injection) of the biotin-PEG at 5 µl/min to achieve the target immobilization level.

Binding analysis

Typically, 120 µl of anti-PEG antibody at three different concentrations (100, 300 and 1000 nM) in the HBS-N running buffer was injected onto both flow cells at a flow rate of 30 µl/min. At the end of the sample injection, the running buffer was allowed to flow over the sensor surface for 10 min to allow dissociation. After dissociation, the sensor surface was regenerated for the next sample concentration by injecting 15 µl of 10 mM glycine-HCl, pH 1.5, at 30 µl/min. Anti-PEG antibody at 100 mM was injected twice to ensure reproducibility. Blank (running buffer) injections were performed in intervals after several sample injections for the purpose of double referencing calculation during data analysis. The antibody solutions were run over the control flow cell and the PEG-immobilized flow cell in sequence, and the responses were monitored as a function of time at 25°C. Binding RUs of each antibody to each PEG molecule at the end of the association phase were reported in the Biacore T200 control software (version 1.0).

Direct binding assay on MSD platform

Biotin-PEG of varying MW was coated on Streptavidin Gold plates (MSD, Rockville, MD, catalog #L15SA-1) at a concentration of 2 µg/ml in assay buffer for an hour at 22°C. The assay buffer was commercially obtained Starting Block™ (PBS Buffer without Tween 20 from Thermo Scientific, catalog #37538). Plates were washed with PBS (Sigma-Aldrich, MO, USA, catalog #P4417–100TAB) and blocked with StartingBlock™ buffer before adding samples containing anti-PEG antibodies. Alternate blocking buffers were not evaluated since background was not an issue in the absence of biological samples and having the same buffer across all steps potentially minimized day-to-day variability in assay performance. Calibrators and quality control samples were prepared in assay buffer using either PEG.2 mAb or #2026 pAb. Antibody samples were allowed to incubate on the plates shaking for 2 h at 22°C before washing five-times with PBS containing no Tween 20. The bound anti-PEG antibodies were detected using ruthenylated tagged mouse anti-human IgG mAb R10Z8E9 (licensed from the University of Birmingham, UK) and called ST-R10. Conjugation of R10 antibody with the sulfo-tag label was done using standard protocols provided by MSD and a single lot was used for comparative purposes across all experiments. Following incubation with ST-R10 brought up in assay buffer to 0.25 µg/ml for 30 min, the plates were washed as in the earlier step. MSD Read Buffer T (4×) with Surfactant (catalog #R92TC-2 from MSD) prepared fresh at a 1× concentration was added to the wells and read on Sector Imager 2000.

Semihomogenous binding assay on MSD platform

Biotin-PEG at a fixed optimized concentration was incubated with a titration of anti-PEG antibodies in solution allowing the antibody binding with the PEG molecule to occur in solution. Following an incubation of the mix for 2 h on a shaking platform, a portion of the mix was added to pre-blocked Streptavidin Gold MSD plates. Unbound material was washed away and the bound complexes on the plate were detected using ST- R10. The plates were read thereafter on a Sector Imager 2000. Ratio and concentration of the labeled materials were optimized depending on the MW of the PEG. The assay was developed using 2 µg/ml of biotin tagged PEG and 1 µg/ml of the ST-R10 antibody. The titrations for both the PEG.2 mAb and #2026 pAb were compared at the same range.

Specificity determination on MSD platform

A competition assay format with inhibitors containing specific epitopes was chosen to differentiate the anti-PEG antibodies (commercially available and BMS generated). The monomethyl ether of triethylene glycol (mTEG) from Sigma Aldrich Corp (Atlanta, GA, catalog #90450–250ML) with linear formula $CH_3(OCH_2CH_2)_3OH$ and MW 164.20 (Figure 1B) was utilized to determine specificity to the mCap termini of PEG including only a few ethylene oxide subunits. Amine-PEG-amine (aPEGa) from Creative PEGWorks (Winston Salem, NC, catalog #PSB-335) with the linear formula $NH_2(CH_2)_2O(CH_2CH_2O)_n(CH_2)_2NH_2$ and MW 20,000 has like any PEG molecule a normal distribution around a mean of 454 ethylene oxide subunits (Figure 1B) and was utilized to determine specificity to the PEG backbone. Differential inhibition by both competitors was used to indicate whether an antibody binding to the ethylene oxide subunit was dependent on the secondary structure of the PEG backbone. The binding was tested in two experimental formats: the direct binding format where biotin-PEG was first immobilized on a streptavidin plate followed by antibodies to bind and the semihomogenous format where biotin PEG was incubated with the antibodies in buffer and then the complex was captured on a streptavidin plate. Four antibodies (PEG.2 mAb, #2026 pAb, B-47 mAb and 6.3 mAb) were titrated in buffer with no competitor and buffer with five concentrations of a competitor and the mix was allowed to incubate on a shaker for 1 h before adding to the wells.

Direct binding format on MSD platform

In the direct binding competition assay, biotin-PEG at 20kD MW was coated on Streptavidin Gold plates (MSD, MD, USA, catalog #L15SA-1) at a concentration of 2 µg/ml in Dulbecco's PBS (Lonza BioWhittaker®, MD, USA, catalog #17–512Q). Plates were washed with PBS (Sigma-Aldrich, MO, catalog #P4417–100TAB) and blocked with StartingBlock™ Buffer. Anti-PEG antibody (PEG.2 mAb, #2026 pAb, B47 mAb and 6.3 mAb) samples were prepared in StartingBlock buffer with and without individual competitors and allowed to incubate for 1 h at room temperature with shaking for binding to competitors to occur. The blocked plate was washed five times with PBS, after which the samples were allowed to incubate on the plates for 1.5 h followed by washing five times with PBS. The bound anti-PEG antibodies were detected using the appropriate ruthenylated anti-species antibody (mouse anti-human IgG mAb R10Z8E9, goat anti-mouse or goat anti-rabbit pAbs from MSD) during 1 h incubation, followed by washing five times with PBS. MSD Read Buffer T (4×) with Surfactant prepared fresh at a 1× concentration was added to the plates and read on the Sector Imager 2000.

Semihomogenous format on MSD platform

Biotin-PEG (20 kD MW) at 2 µg/ml was incubated with a titration of an anti-PEG antibody (PEG.2 mAb,

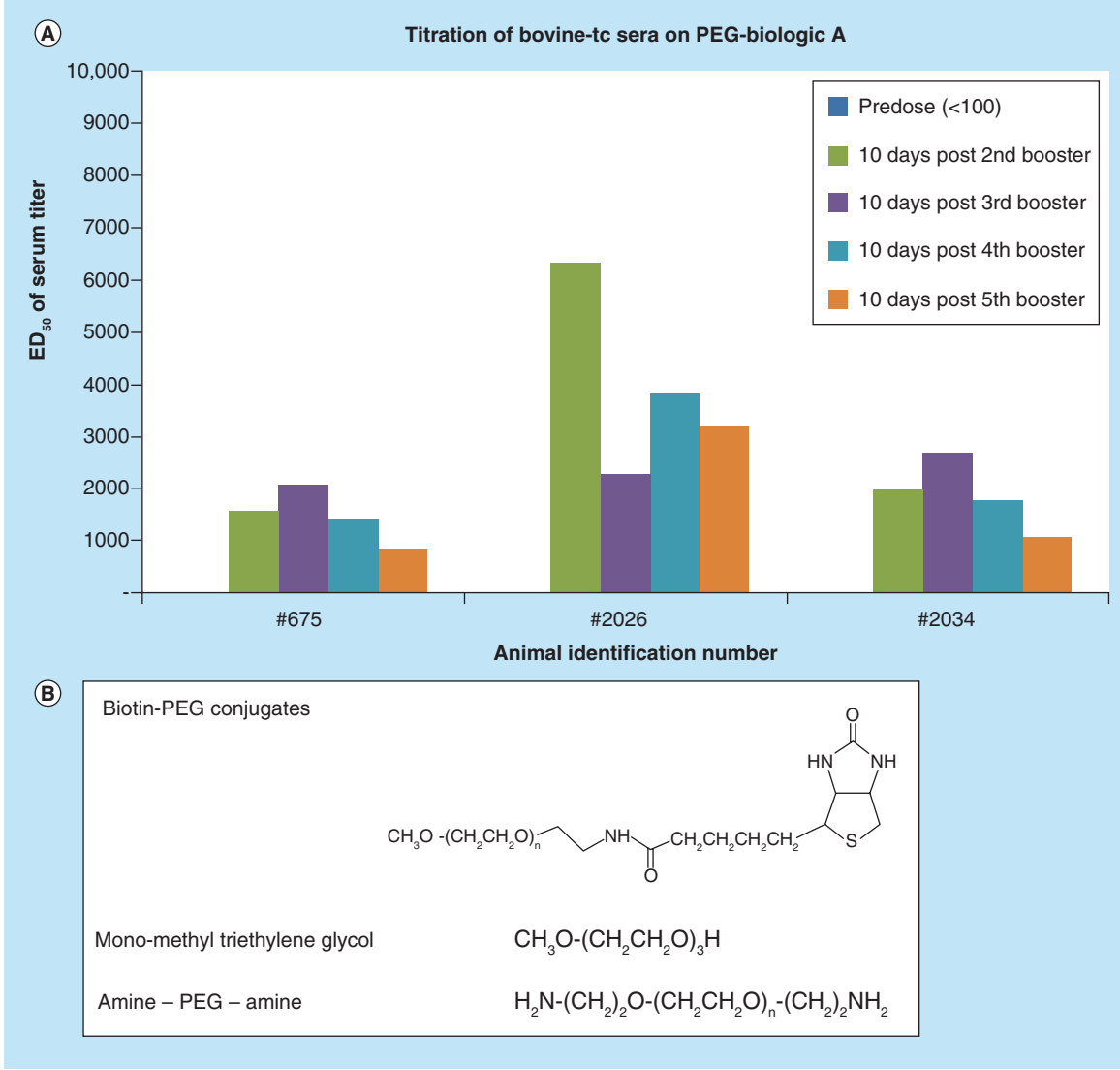

Figure 1. Titration of immunized transchromosomal bovine sera against a PEGylated Bristol–Myers Squibb drug demonstrating polyethylene glycol specific reactivity. (A) Titration of immunized transchromosomal bovine sera against a PEGylated Bristol–Myers Squibb (BMS) drug demonstrating PEG specific reactivity. Serum samples from human IgG transchromosmal bovines immunized with a PEG–keyhole limpet hemocyanin conjugate were collected after every booster and serially diluted in an ELISA on plates coated with a PEGylated BMS drug. ED_{50} values representing the reciprocal of serum dilution with 50% of its maximal binding are compared across three bovines. All predose samples were <100 electrochemiluminescent units. **(B)** Materials and their chemical structure. ED_{50}: Serum dilution where 50% of its maximal binding is observed; PEG: Polyethylene glycol.

#2026 pAb, B47 mAb and 6.3 mAb) and a competitor in solution allowing the antibody to bind with the PEG molecule or the competitor in solution. Following a 2 h incubation of the mix on a shaking platform at room temperature, a portion of the mix was added to pre-blocked Streptavidin Gold plates. Unbound material was washed away with PBS and the bound complexes on the plate were detected using 1 µg/ml of the appropriate ruthenylated anti-species antibody (mouse anti-human IgG mAb R10Z8E9, goat anti-mouse or goat anti-rabbit polyclonal antibodies from MSD). Following five washes with PBS, MSD Read Buffer T (4×) with Surfactant prepared fresh at a 1× concentration was added to the plates and read on the Sector Imager 2000.

Results

Generation of custom-made anti-PEG IgG reagents

Immunization using PEG-conjugated therapeutic molecules or with KLH-PEG successfully resulted in the formation of PEG reactive polyclonal IgG in the animals. Figure 1A shows markedly different seroreactivity from three different tc-bovines. These results were also confirmed by binding experiments using a sec-

ond PEGylated therapeutic (Supplementary Figure 1). Titers in general peaked after 2–3 boosters discounting any merit in further boosters unlike standard immunization regimes for protein specific antibodies. We also observed better titers in response to KLH-conjugated PEG than to PEGylated therapeutic molecule immunizations (data not shown). This could be attributed to the availability of far higher amounts of PEG on a molar basis in the KLH preparations than in the one PEG to one protein conjugates with therapeutic molecules. Additionally re-engineering the two mAbs with a human IgG Fc did not affect their reactivity to PEG; both the chimeric and native hybridoma IgG had similar binding to PEG (data not shown).

Purification of anti-PEG polyclonal antibodies

Purification of the polyclonal anti-PEG antibodies using PEG affinity chromatography resulted in a relatively lower yield of purified IgG than what would have been expected from an immunogenic protein. Antibody sample was stable for at least 4 weeks in 4°C following one freeze thaw cycle. Preliminary analysis by mass spectrometry indicated approximately 10 different antibodies in the polyclonal mixture at detectable levels (data not shown). The purified polyclonal antibodies also had a high content of aggregates that had to be separated and thus lowering the final yield.

Binding of anti-PEG antibodies to PEG in SPR binding experiments

During the experiment, short injections (18 s) of the biotin-PEG were performed to ensure that similar RU were achieved across the PEGs with different MW. Therefore, similar mass of ligand was achieved across the 10 PEGs studied in this work. It should be noted that the absolute moles of PEG immobilized were inversely proportional to the MW of the PEG molecule. However, the immobilization level was similar in terms of the total number of ethylene oxide subunits.

Three concentrations of each anti-PEG antibody (1000, 300 and 100 nM) were used to study the binding interaction with the PEG at each MW. Anti-PEG antibody at 100 mM was injected twice and used to evaluate binding reproducibility of the assay. Figure 2A & B illustrate the binding sensorgrams of each antibody to the PEG molecule at five different MW. A complete list of the sensorgrams of the 10 PEGs is published in the supplemental figures (Supplementary Figure 4). The overlaid sensorgrams obtained from the duplicate runs demonstrate good binding reproducibility. Figure 2C illustrates the binding RU of each antibody (1000 nM) to each PEG molecule at the end of the association phase. For better comparison of the binding across the 10 PEGs, the RUs were normalized assuming that an immobilization level of 100 RU was achieved for each biotin-PEG. As shown in Figure 2C, the PEG.2 mAb showed the highest normalized binding RU to the 20 kD PEG, while the #2026 pAb shows the highest RU to the 1 kD PEG. This result was consistent with the binding pattern observed from the plate-based assays where the PEG.2 mAb had a preferential binding for the larger sized PEG and the #2026 pAb preferred the small to intermediate sized PEG. It should be noted that the RU observed for the #2026 pAb binding to each PEG was higher than the one observed for the PEG.2 mAb, again consistent with the plate-based assays. Assuming a 1:1 binding interaction between the PEG and the anti-PEG antibody, none of the binding RUs observed for the polyclonal antibody exceeded the binding capacity of each PEG immobilized on the sensor surface. The unexpected low binding RU observed for PEG.2 indicated that the monoclonal antibody had very limited access to the PEG molecule immobilized on the sensor surface and its binding may require a secondary structure of the PEG which may not be available after immobilization. Both the monoclonal and the polyclonal antibodies were identified to have fast off rates to the PEG molecules from the binding sensorgrams shown in Figure 2. This was apparent for the 20 kD PEG where more than 50% of both bound antibodies dissociated from the immobilized PEG molecule at the end of the 10 min dissociation phase. Significant negative binding RUs were observed for the interaction between the 40 kD PEG and the polyclonal antibody at the three concentrations studied in this work. Examination of the original binding responses on the reference and the PEG-immobilized flow cells showed significant higher binding of the polyclonal antibody on the reference flow cell without the 40 kD PEG immobilization. This indicates that the #2026 pAb does not bind the 40 kD PEG and the observed negative binding RUs were due to the nonspecific binding of the polyclonal antibody to the reference flow cell.

Binding of anti-PEG antibodies to various size PEG molecules on the MSD platform

PEG.2 mAb and #2026 pAb were selected from a panel of antibody preps that we screened for binding to PEG for further characterization. PEG in solution was tested in addition to immobilized PEG coated on a plate to rule out any possible binding artifacts with coated PEG due to altered shape or epitope masking. Furthermore we also asked whether the molecular size of the linear PEG molecule influenced the binding. Our initial binding data was confirmed using 40 kD PEG, the size used in some of our PEGylated biotherapeutics and followed by PEG <5 kD, a size commonly present in food and cosmetic products. Initial screening experiments indicated a profound divergence in

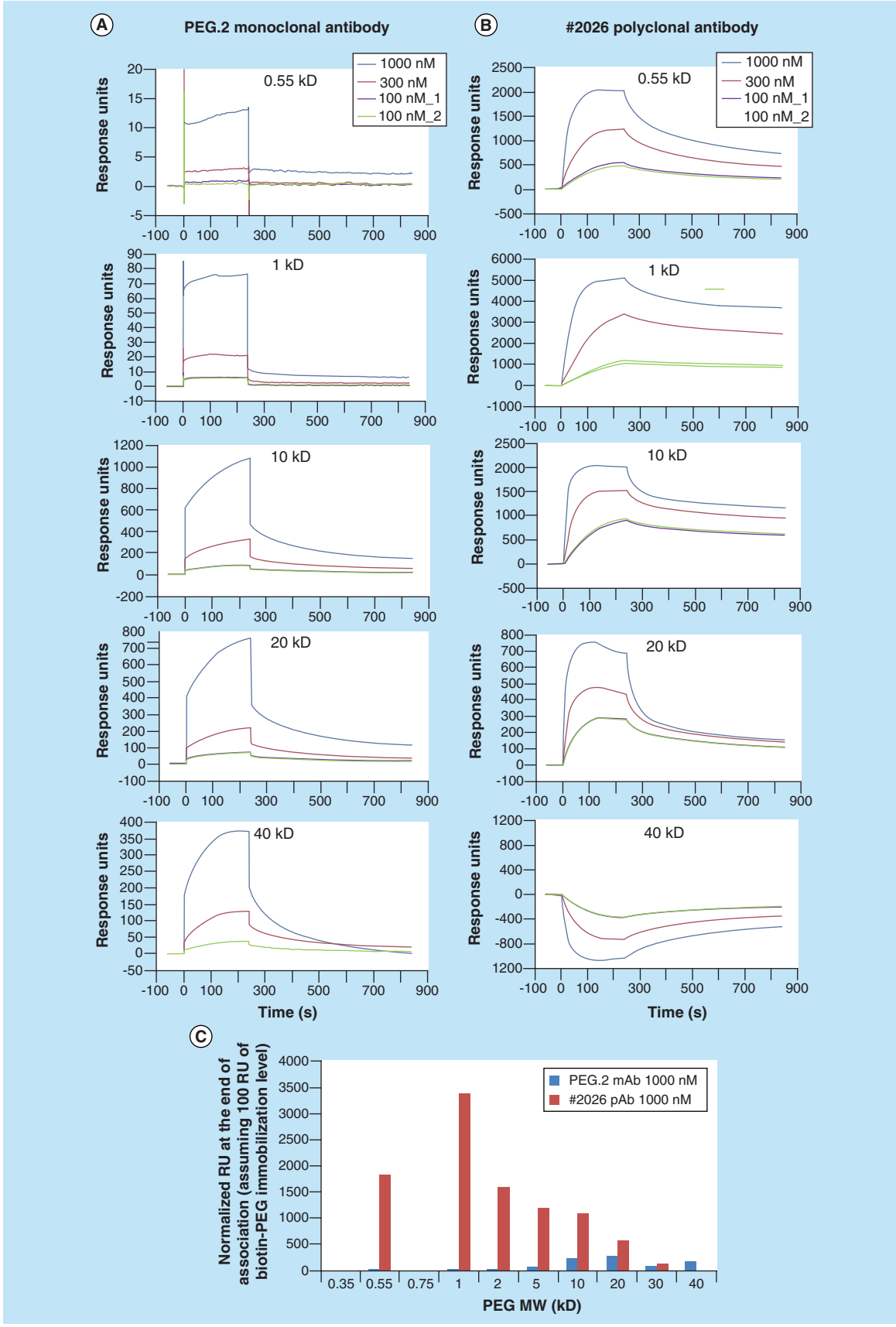
A
PEG.2 monoclonal antibody
B
#2026 polyclonal antibody
1000 nM
300 nM
100 nM_1
100 nM_2
0.55 kD
1 kD
10 kD
20 kD
40 kD
Response units
Time (s)
C
Normalized RU at the end of association (assuming 100 RU of biotin-PEG immobilization level)
PEG.2 mAb 1000 nM
#2026 pAb 1000 nM
0.35
0.55
0.75
1
2
5
10
20
30
40
PEG MW (kD)

Figure 2. Antibody binding to immobilized polyethylene glycol on sensor by surface plasmon resonance measurements to show differential on and off profiles and effect of PEG MW on antibody binding (see facing page).
Association phase: 0–240 s. Dissociation phase: 240–840 s. **(A)** Biacore binding sensorgrams of PEG.2 monoclonal antibody. **(B)** Biacore binding sensorgrams of #2026 polyclonal antibody. **(C)** Differential effect of PEG MW on binding by two anti-PEG antibodies.
MW: Molecular weight; PEG: Polyethylene glycol; RU: Response units.

the binding profiles of the antibodies to PEG of 40 kD and 550 daltons sizes. This led us to explore binding to PEG molecules of intermediate sizes as well. Figure 3 summarizes the titration data from five selected PEG molecules of different MW using the two anti-PEG antibodies. Binding to all PEG molecules that were also tested are shown in Supplementary Figure 2. Both direct (Figure 3A) and solution phase binding (Figure 3B) for any given antibody demonstrated a consistent binding pattern to PEG molecules of varying MW. Overall the data indicated a preference by the PEG.2 mAb for the larger sized PEG and by the #2026 pAb for a smaller to intermediate size PEG. The comparative binding data of these two antibodies to various PEG molecules are shown in Figure 3C. The 0.35 kD and 0.75 kD PEG molecules did not show any binding to either antibody; this might have been due to a problem with the PEG itself and was not investigated further (Supplementary Figure 2). The comparison was done using two different concentrations of the two antibodies to account for the profound differences in their binding signal intensity as seen on the PEG molecules. We next investigated whether the differential profiles reflected differences in antibody specificity to binding epitopes on the PEG.

Specificity

Specificity of most commercial anti-PEG antibodies are classified either to methyl group capping the PEG (mCap) or to the backbone composed of ethylene oxide subunits; this was the basis for investigating specificity of our in-house antibodies [11]. Tween 20 in the wash and assay buffers interfered with the binding of antibodies that were specific to the backbone indicating cross reactive epitopes. Thus, we eliminated this detergent from all experimental steps.

Since our direct binding results suggested that the size of the PEG backbone might influence specificity to the backbone, we used two competitors. The first consisted of an mCap forming a methyl ether bond with just three ethylene oxide subunits and the second competitor a 20 kD MW PEG that had no terminal methyl ether bonds but instead the terminal hydroxyls on both ends were capped by an amine group (see Figure 1B). In order to differentiate the influence of long polymerized subunits from smaller stretches of subunits on the binding, the number of ethylene oxide subunits was normalized to one another for comparative purposes at each inhibitor concentration. The competitors were tested at 10-fold lower, and 10-, 100- and 1000-fold higher ethylene oxide subunits present in biotin-PEG. Inhibition of binding in the presence of competitors was compared and used to ascertain whether an antibody showed specificity to the mCap (inhibition with mTEG but no inhibition with amine-PEG-amine at any ratio) the mCap and adjacent linear ethylene oxide subunits (stronger inhibition with mTEG and some inhibition with amine-PEG-amine), the ethylene oxide subunits in the backbone independent of secondary structure (similar inhibition with mTEG and with amine-PEG-amine) or the ethylene oxide subunits in the backbone but dependent on some secondary structure (stronger inhibition with amine-PEG-amine than with mTEG).

Two commercial antibodies with specificities provided by the manufacturer were used to test the validity of our approach to specificity determination. A rabbit mAb B-47 described as having mCap specificity and a mouse mAb 6.3 with high affinity for the PEG backbone were evaluated. There was stronger binding and better resolution of inhibition during competitor titration in the direct assay format than in the semihomogenous format. This was probably due to the epitopes on PEG being in closer proximity on solid surface which would help binding to antibodies with inherently faster off rates by limiting their diffusion. While inhibition of the mAb B-47 was far greater with mTEG than amine-PEG-amine as expected from its mCap specificity, it was interesting to note some cross reactivity to amine-PEG-amine at higher concentrations in the direct format assays suggesting the antibody probably also recognizes some backbone subunits most likely adjacent to the mCap. The conclusion that B-47 mAb is purely mCap specific was therefore not consistent with our direct binding assay data. For the mAb 6.3, there was far greater inhibition with amine-PEG-amine than mTEG at equivalent subunits; while inhibition by amine-PEG-amine was observed starting at an equivalent number of subunits of competitor to biotin-PEG level, mTEG competitor needed to have at least 100-fold excess subunits over biotin-PEG. This indicated that mAb 6.3 clone is backbone subunit specific and most likely dependent on secondary structure. The differences in the two competitors at various amounts were better evident in the direct (Figure 4A) than in the semihomogenous format (Supplementary Figure 4).

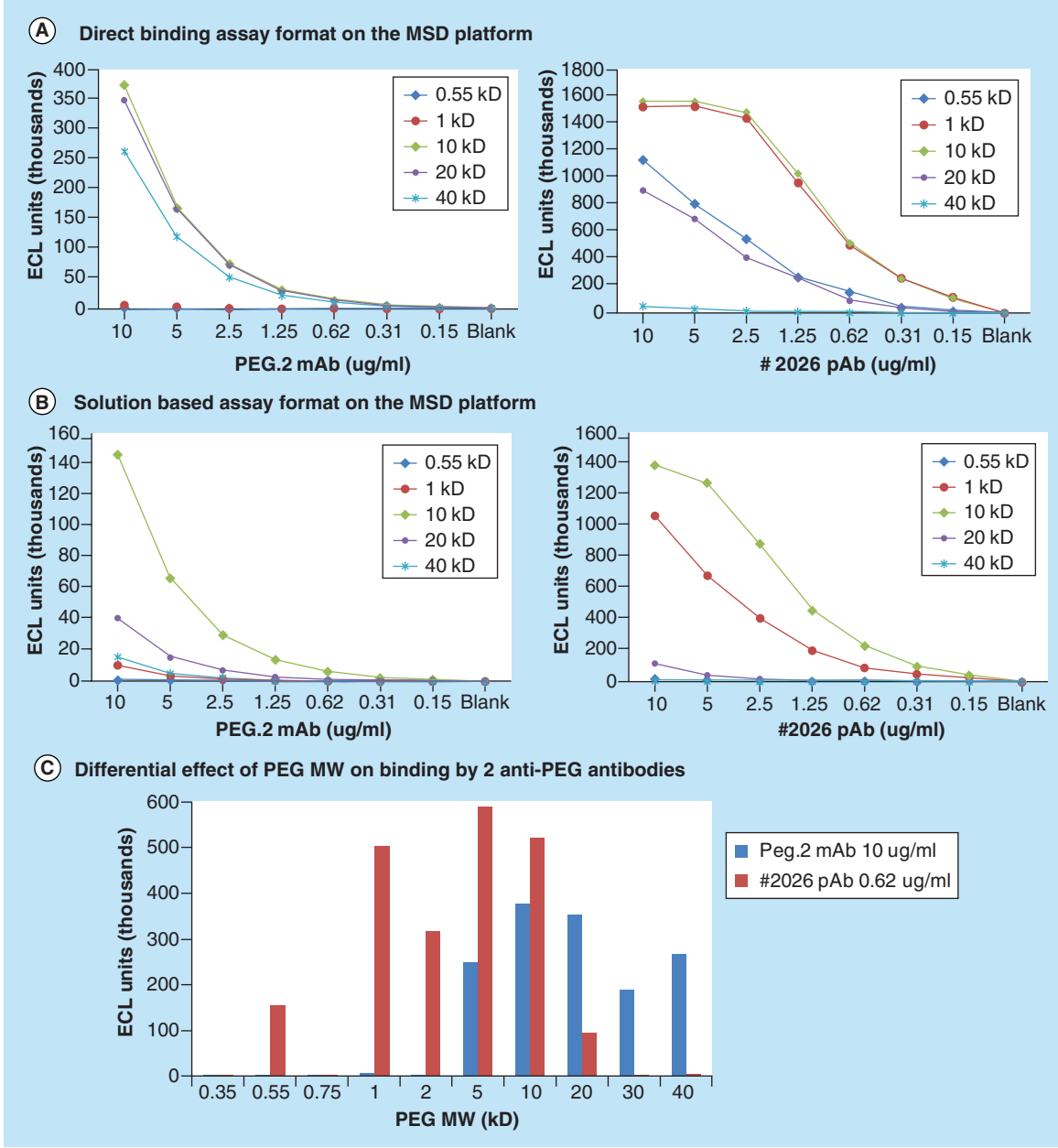

Figure 3. Titration of PEG.2 monoclonal antibody and #2026 polyclonal antibody anti-PEG antibodies in buffer using multiple assay formats showing influence of PEG molecular weight on antibody binding. (A) Direct binding assay format on the MSD platform. **(B)** Solution-based assay format on the MSD platform. **(C)** Differential effect of PEG MW on binding by two anti-PEG antibodies.
ECL: Electrochemiluminescent; mAb: Monoclonal antibody; MSD: Meso Scale Diagnostics; MW: Molecular weight; pAb: Polyclonal antibody; PEG: Polyethylene glycol.

The PEG.2 mAb that had shown preferential binding to larger MW PEG showed greater inhibition with amine-PEG-amine than mTEG implying backbone subunit specificity but more dependent on some secondary structure present in the 20 kD amine-PEG-amine inhibitor. In contrast, the #2026 pAb that had showed preferential binding to smaller to intermediate MW of PEG showed similar inhibition by mTEG and amine-PEG-amine indicative of its recognition of backbone subunit that does not appear to be dependent on any possible secondary shapes. The competitors thus showed some backbone specific antibodies like PEG.2 mAb might require an unknown secondary structure in addition to primary repeating units for binding. Results from the semihomogenous assay (Supplementary Figure 3) corroborated the direct binding assay data (Figure 4A) although the direct format demonstrated the inhibition more robustly and at lower

competitor levels than the semihomogenous format. Figure 4B shows the contrasting inhibition patterns of the two antibodies in the same panel with similar levels of competitor supporting the same conclusion.

In both assay formats, the amount of inhibition increases with the molarity of competitors. At the two highest levels of competitor, there is complete suppression of binding for both antibodies suggesting the need to carefully monitor the levels of the PEG reagent in any assay designed to pick up anti-PEG antibodies.

Discussion

PEGylation of biotherapeutics is a commonly used approach in biotechnology to improve pharmacological bioavailability of biotherapeutic products [1,2]. Most therapeutic molecules tend to have PEG conjugates with sizes exceeding 10–20 kD MW. Investigations on the immunogenicity of biotherapeutics have brought the role of PEG into focus. While commercial anti-PEG IgM antibodies have been used for ADA assay development, there are no well-characterized reagents nor robust methods available to confirm or refute presence and kinetics of an anti-PEG IgG response. Making interpretation of any such assay is rendered more complex by the presence of endogenously occurring anti-PEG antibodies that are elicited in response to exposure to ubiquitously present PEG in many commercial products as well as in food [5]. It is unclear whether such pre-existing antibodies play any role in an immune response to the PEG portion of a biotherapeutic and if there are any downstream consequences on safety and efficacy [4]. Lack of well-characterized reagents and reproducible methods for reliable detection of anti-PEG IgG in human clinical samples underly our inability to make conclusions on their clinical relevance.

The generation and affinity purification of anti-PEG IgG pAb was challenging unlike similar processes to raise pAbs to protein or peptide immunogens. Their binding affinity to PEG is relatively weak and required the use of several booster doses, stronger adjuvants and the use of stoichiometric excess of PEG on KLH conjugates to elicit strong reactive anti-PEG antibody titer. Furthermore anti-PEG pAb formed a fairly significant amount of aggregation post elution from the affinity column and required additional preparative size exclusion chromatography steps for isolation of the monomeric IgG species. Antibody aggregation affected stability in buffer at 4°C, as well as increased instability of freeze–thaw samples. The higher signal intensity from the polyclonal bovine antibody could be accounted for by the high dose of KLH-conjugated PEG and the high molar ratio of PEG in the conjugate used in immunizations; in contrast the monoclonal PEG.2 clone was identified from mice immunized with a PEGylated therapeutic molecule having one PEG molecule per therapeutic molecule. However given the unusual genetic background of the human IgG expressing Tc bovine models, this observation may need to be confirmed in other larger mammals typically used for raising polyclonal antibodies. Several groups that have raised antibodies to PEG in rabbits have reported the immunogenic importance of the methoxy group in eliciting anti-PEG antibodies [16,17]. The polyclonal antibodies from the bovines used in this study did not appear to have a preferential reactivity to the methoxy group; instead our specificity data suggests strong backbone specificity. Saifer *et al.* [17] reported several factors including the backbone length influencing the selectivity of their anti-PEG antibodies; our data is in alignment with the reported effect of PEG MW on antibody binding. Use of Tween based detergents significantly impacted our assays due to their cross reactivity with the PEG backbone and relates to such observations from other groups [17].

Key term

Semihomogenous assay: Assay format that uses solution phase binding to capture an analyte specific to the target. Biotinylated PEG is allowed to bind to anti-PEG antibodies in solution before immobilizing the complex on a plate. These are detected via a ruthenylated antibody against Fc domain of the PEG-bound antibody.

Recognition of PEG by anti-PEG antibody is influenced by the size of the PEG molecule

Our current understanding of the immunogenic domains in a PEG molecule has probably oversimplified the role of the chain length in recognition to backbone subunits. There are perhaps other secondary structural constraints native to the full length of the PEG molecule that influences recognition of different antibodies binding to the same subunit. While the monoclonal PEG.2 clearly preferred the larger size PEG and was unable to recognize the same subunits on a smaller version of the PEG, the polyclonal did not exhibit any such bias. These antibodies show markedly different binding and specificity profiles to PEG independent of the platform and assay structure implying that the selection of PEG in an assay can clearly influence its ability to detect anti-PEG antibodies (Table 1). Moreover, differences between the direct and semihomogenous formats show the advantage of using direct binding assays to obtain stronger signals; directly immobilized PEG was a better way to capture anti-PEG antibodies than PEG used in solution. This is in contrast to most conventional immunogenicity assays where labeled therapeutic molecules are used in solution to detect ADAs recognizing epitopes on a protein structure of the therapeutic.

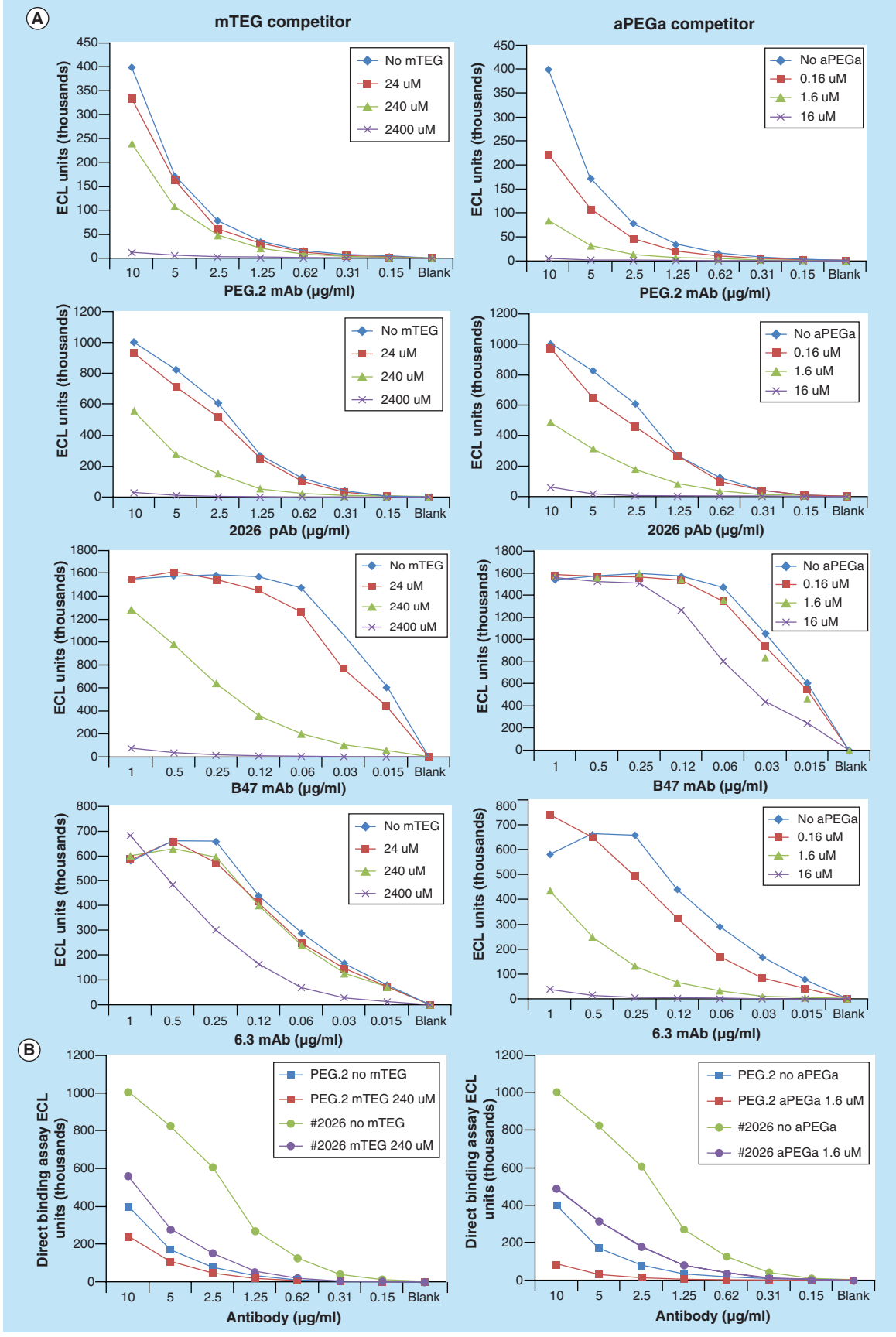
A
mTEG competitor
aPEGa competitor
ECL units (thousands)
No mTEG
24 uM
240 uM
2400 uM
No aPEGa
0.16 uM
1.6 uM
16 uM
PEG.2 mAb (µg/ml)
2026 pAb (µg/ml)
B47 mAb (µg/ml)
6.3 mAb (µg/ml)
10 5 2.5 1.25 0.62 0.31 0.15 Blank
1 0.5 0.25 0.12 0.06 0.03 0.015 Blank
B
Direct binding assay ECL units (thousands)
PEG.2 no mTEG
PEG.2 mTEG 240 uM
#2026 no mTEG
#2026 mTEG 240 uM
PEG.2 no aPEGa
PEG.2 aPEGa 1.6 uM
#2026 no aPEGa
#2026 aPEGa 1.6 uM
Antibody (µg/ml)

Figure 4. (A)Specificity determination by titration of anti-polyethylene glycol antibody in buffer using direct assay format showing influence of competitors and (B) comparative differences between two custom-made antibodies' inhibition with competitors in a direct binding assay format (see facing page).
aPEGa: Amine–polyethylene glycol–amine; ECL: Electrochemiluminescent; mAb: Monoclonal antibody; mTEG: Mono-methyl triethylene glycol; pAb: Polyclonal antibody; PEG: Polyethylene glycol

While it would be prudent to use a similar size PEG as used in the therapeutic to detect any induced antibodies, alternate low MW forms might be needed for detection of pre-existing antibodies that are believed to be triggered by low MW PEG products in naturally occurring products. A heterogeneous mixture of PEG molecules of varying lengths can be used in assays to pick up a far more diverse range of anti-PEG antibodies. Most antibodies probably have some degree of reactivity to the backbone subunit; even the commercial rabbit antibody B-47 believed to be mCap specific showed some dependence on subunits. Our results underscore the heterogeneity of anti-PEG antibodies and the limitations imposed by the selections of the PEG molecule and the assay format on the ability to detect all species of anti-PEG antibodies in a clinical sample whether pre-existing or formed as a result of exposure to a therapeutic. In order to develop robust assays capable of detecting the multitude of possible anti-PEG antibodies in humans, the reagents used to develop any anti-PEG antibody immunogenicity assay will need to be thoroughly characterized in terms of their specificity, dependence on MW of PEG and their affinity before any subject data can be meaningfully interpreted [18].

Conclusion & future perspective

Many PEGylated products have been approved by health authorities. Over time there has been little evidence of a sustained anti-PEG immunogenic response with any link to the product's efficacy or safety. This might be related to the lack of robust and reliable methods and reagents to demonstrate evidence (or lack thereof) of anti-PEG immunogenicity. PEG tends to be immunologically inert and is widely believed to elicit low affinity antibodies, typically IgM; based on our experience, we assume the inherent difficulties in generating and purifying polyclonal anti-PEG IgG might have constrained reagent availability and impeded the development of methods to detect IgG anti-PEG antibodies. Unlike immunogenic responses to proteins that can be driven to affinity maturation following chronic exposure, the immunogenicity to PEG could span a wide spectrum ranging from low affinity interactions to moderately high. Pre-existing antibodies that arise following long-term exposure to PEG in nutritional and cosmetic products may also influence maturation of IgG antibodies. The present classification of anti-PEG antibodies as being either anti-backbone or anti-mCap or anti-linker does not fully represent their wide diversity. The MW or length and branching structure of the PEG utilized in any assay to identify anti-PEG antibodies could skew their detection. The analytical challenge is to develop assays designed to detect IgG response to PEG and reflect the innate diversity of anti-PEG antibodies in human subjects. Such assays should be capable of detecting low affinity antibodies, any boost in the titer of pre-existing antibodies and antibodies induced by the PEG conjugate specific to the therapeutic product. These anti-

Table 1. Characteristics of evaluated anti-polyethylene glycol IgG antibodies.

Attributes of anti-PEG antibodies	PEG.1 (BMS)	PEG.2 (BMS)	#2026 (BMS)	B-47 (commercial)	6.3 (commercial)
Immunized animal species	Mouse	Mouse	Transchromosomal Bovine	Rabbit	Mouse
Type	Monoclonal chimeric IgG	Monoclonal chimeric IgG	Polyclonal human IgG	Monoclonal rabbit IgG	Monoclonal mouse IgG
Heavy chain	Human IgG1	Human IgG1	Human	Rabbit	Mouse
Light chain	Mouse	Mouse	Mostly bovine	Rabbit	Mouse
Specificity	NA	Backbone subunit dependent on secondary length	Backbone subunit independent of secondary length	Methyl cap with adjoining subunits	Backbone subunit independent of secondary length
Affinity	NA	Varies with PEG MW	Varies with PEG MW	High	NA
MW of PEG giving preferential binding	NA	Moderate to larger	Smaller to moderate	NA	NA

BMS: Bristol–Myers Squibb; MW: Molecular weight; NA: Not available; PEG: Polyethylene glycol.

bodies might differentially bind to the backbone subunit of PEG based on its shape, length and the assay format. The different IgG responses are likely to have very different analytical outcomes in any one assay. Unlike the conventional approaches to developing materials and methods for a typical anti-protein therapeutic IgG assay the PEG poses new questions and compels us to think about a need for a wider array of well-characterized reagents whose analytical behavior in an immunogenicity assay should be reflecting those found in the human subjects. Since PEG is a commonly used adduct with several therapeutic molecules, the methods and reagents should be ideally made available for standardization across different PEGylated platforms amenable for comparative and cooperative research to confidently rule in or out the role of PEG in immune mediated aberrations on the pharmacological activity of the therapeutic.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/full/10.4155/bio.15.112

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- The ubiquitous presence of polyethylene glycol (PEG) in cosmetic and nutritional products and its use as a PK enhancer for biotherapeutics necessitates addressing anti-PEG antibodies as part of immunogenicity risk assessment of the therapeutic program.
- Variable length and branching of PEG molecules coupled with its repeating subunit structure present analytical challenges to detect a broad range of antibodies especially IgG that bind to PEG.
- Contrary to having well-established robust methods for making and characterizing anti-protein custom reagents for any therapeutic program, there are technical challenges for anti-PEG antibodies.
- Human Fc-bearing IgG anti-PEG positive control antibodies can be designed and potentially be used in alternative assay formats to detect anti-PEG IgG in clinical samples.
- Classification of anti-PEG IgG antibody specificity to methyl cap and backbone domains might need further characterization since the length of the PEG clearly has an influence on antibody binding.

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