analytical chemistry

Article

pubs.acs.org/ac

Measurement of Pre-Existing IgG and IgM Antibodies against Polyethylene Glycol in Healthy Individuals

Bing-Mae Chen,[†] Yu-Cheng Su,[†] Chia-Jung Chang,[†] Pierre-Alain Burnouf,[†] Kuo-Hsiang Chuang,[‡] Chien-Hsiun Chen,[†,§] Tian-Lu Cheng,[∥] Yuan-Tsong Chen,[†,⊥] Jer-Yuarn Wu,*[,†,§] and Steve R. Roffler*[,†,#]

[†]Institute of Biomedical Sciences, Academia Sinica, Taipei 11529, Taiwan

[‡]Graduate Institute of Pharmacognosy, Taipei Medical University, Taipei 11031, Taiwan

[§]School of Chinese Medicine, China Medical University, Taichung 40447, Taiwan

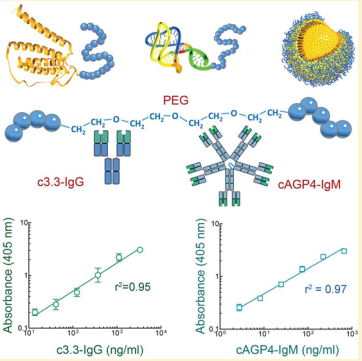
[∥]Department of Biomedical Science and Environmental Biology, Center for Biomarkers and Biotech Drugs, Kaohsiung Medical University, Kaohsiung 80708, Taiwan

[⊥]Department of Pediatrics, Duke University Medical Center, Durham, North Carolina 27710, United States

[#]Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung 80708, Taiwan

Supporting Information

ABSTRACT: Polyethylene glycol (PEG) is a biocompatible polymer that is often attached to therapeutic molecules to improve bioavailability and therapeutic efficacy. Although antibodies with specificity for PEG may compromise the safety and effectiveness of PEGylated medicines, the prevalence of pre-existing anti-PEG antibodies in healthy individuals is unclear. Chimeric human anti-PEG antibody standards were created to accurately measure anti-PEG IgM and IgG antibodies by direct ELISA with confirmation by a competition assay in the plasma of 1504 healthy Han Chinese donors residing in Taiwan. Anti-PEG antibodies were detected in 44.3% of healthy donors with a high prevalence of both anti-PEG IgM (27.1%) and anti-PEG IgG (25.7%). Anti-PEG IgM and IgG antibodies were significantly more common in females as compared to males (32.0% vs 22.2% for IgM, $p < 0.0001$ and 28.3% vs 23.0% for IgG, $p = 0.018$). The prevalence of anti-PEG IgG antibodies was higher in younger (up to 60% for 20 year olds) as opposed to older (20% for >50 years) male and female donors. Anti-PEG IgG concentrations were negatively associated with donor age in both females ($p = 0.0073$) and males ($p = 0.026$). Both anti-PEG IgM and IgG strongly bound PEGylated medicines. The described assay can assist in the elucidation of the impact of anti-PEG antibodies on the safety and therapeutic efficacy of PEGylated medicines.



Covalent attachment of polyethylene glycol (PEG) to small molecules, nucleotides, peptides, proteins, liposomes, and nanoparticles is widely used to improve their stability, solubility, and pharmacokinetic properties.[1] Several PEGylated drugs are approved for clinical use including Pegasys (PEG-interferon alpha-2a), Neulasta/pegfilgrastim (PEG-granulocyte colony-stimulating factor), Mircera (PEG-erythropoietin), Krystexxa/pegloticase (PEG-uricase), and Doxil/Caelyx/Lipodox (PEG-liposomal-doxorubicin).[2−5] Because of the beneficial properties within these successful examples, many new PEGylated drugs and nanomedicines are under preclinical and clinical development.[2,3,5]

Although PEG is assumed to be nonimmunogenic, anti-PEG antibodies can be generated in animals immunized with PEGylated proteins or liposomes and in patients treated with certain PEGylated drugs.[6−11] Anti-PEG antibodies have also been detected at frequencies ranging from 0.4% to as high as 36% in naive individuals,[6,11−16] possibly due to incidental exposure to PEG and PEG derivatives in consumer and medicinal products.[17]

Anti-PEG antibodies may hinder effective therapy by inducing formation of immune complexes which can be rapidly bound and cleared via Fc receptor-mediated phagocytosis by macrophages in the liver, thereby altering the pharmacokinetics and biodistribution of PEG-modified medicines.[7,9−11,18] Binding of anti-PEG antibodies to PEGylated drugs, nanoparticles, and liposomes may also activate the complement system, which could be a contributing factor to the development of infusion-related allergic reactions to PEGylated drugs.[11,15,19,20]

Accurate measurement of the prevalence and concentrations of anti-PEG IgG and IgM antibodies is important to understand the impact of anti-PEG antibodies on treatment efficacy and safety, factors predisposing individuals to the formation of anti-PEG antibodies, and how to effectively manage anti-PEG immunity.[17] Progress, however, is hampered by the dearth of sensitive assays and suitable standards to accurately measure and compare anti-PEG IgG and IgM antibodies among

Received: August 10, 2016
Accepted: October 11, 2016
Published: October 11, 2016

DOI: 10.1021/acs.analchem.6b03109
Anal. Chem. 2016, 88, 10661−10666

different studies.[21,22] Here, we generated chimeric human anti-PEG IgG and IgM antibodies based on the widely used 3.3 (IgG) and AGP4 (IgM) anti-PEG monoclonal antibodies.[23−30] A direct binding assay was developed to measure anti-PEG IgG and IgM in human plasma or serum samples, with relative antibody concentrations determined by comparison to the chimeric anti-PEG antibody standards. We further measured pre-existing IgG and IgM antibodies against PEG in 1504 healthy Han Chinese donors residing in Taiwan. Our results describe reference standards and methods to study anti-PEG responses as well as new information about the prevalence of pre-existing antibodies against PEG in naive individuals.

EXPERIMENTAL SECTION

Ethical Statement. The studies were approved by the Institutional Review Boards and Ethics Committees of Academia Sinica in Taiwan. Written informed consent was obtained from the subjects in accordance with institutional requirements and Declaration of Helsinki principles.

Plasma Sample Collection. Plasma samples of healthy Han Chinese subjects residing in Taiwan were enrolled from a prior project that had been collected, centrifuged, and stored at the National Center for Genome Medicine, Academia Sinica. All subjects of this study agreed to offer the remaining centrifugal plasma for other research in a blinded fashion.

Chimeric Antibody Production. Human chimeric anti-PEG antibodies (c3.3-IgG or cAGP4-IgM) were constructed from DNA isolated from 3.3 and AGP4 hybridoma cells and human peripheral blood mononuclear cells.[27] Stable producer cells were generated by retroviral transduction of 293FT cells. Recombinant c3.3-IgG was purified by protein A affinity chromatography. The concentrations of c3.3-IgG and cAGP4-IgM were measured by direct ELISA in comparison to human IgG and human IgM standards.

Human anti-PEG Assay. Maxisorp 96-well microplates (Nalge-Nunc International, Rochester, NY) were coated with 0.5 μg/well NH_2-$PEG_{10\,000}$-NH_2 in 50 μL/well 0.1 M $NaHCO_3/Na_2CO_3$ (adjusted to pH 9.5 with HCl) buffer overnight at 4 °C and then blocked with 200 μL/well 5% (w/v) skim milk powder (Difco) in Dulbecco's phosphate-buffered saline (PBS, Thermo Fisher Scientific) at room temperature for 2 h. Plates were washed once with PBS immediately before use. Human samples were diluted 25-fold in 2% (w/v) skim milk powder in PBS and then two additional 2-fold serial dilutions were made in dilution buffer (4% human reference serum, 2% (w/v) skim milk powder in PBS). Seven 3-fold serial dilutions of chimeric anti-PEG antibodies (c3.3-IgG or cAGP4-IgM) in dilution buffer were prepared starting at 2.5 or 2 μg/mL, respectively. Human plasma samples at dilutions of 25, 50, and 100-fold and serially diluted c3.3-IgG or cAGP4-IgM antibody standards (in duplicate) were added to separate plates at room temperature for 1 h. Unbound antibodies were removed by washing the plates twice with 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)/PBS and once with PBS. The 0.25 μg/mL horseradish peroxidase (HRP)-conjugated goat $F(ab')_2$ antihuman IgG Fc or HRP-conjugated goat $F(ab')_2$ antihuman IgM $Fc_{5\mu}$ in 50 μL PBS containing 2% (w/v) skim milk powder were added to the IgG or IgM detection plates, respectively, for 1 h at room temperature. The plates were washed as above before adding 100 μL/well ABTS substrate for 30 min at room temperature. The absorbance (405 nm) of wells was measured in a microplate reader (Molecular Devices). Positive responses were defined as samples with absorbance values at least 3 times greater than the mean background absorbance (dilution buffer, n = 10 for each plate). The relative concentrations of anti-PEG IgG or IgM in positive samples were calculated by comparison with c3.3-IgG or cAGP4-IgM standard curves, respectively.

All positive samples were further confirmed by a PEG competition assay. PEG-liposomes were diluted to 200 μg/mL in a final concentration of 2% (w/v) skim milk powder in PBS. The 50 μL/well of 2% (w/v) skim milk powder in PBS (no competition) or 200 μg/mL liposomes in 2% (w/v) skim milk powder (competition) were added for 30 min at room temperature to ELISA plates coated with NH_2-$PEG_{10\,000}$-NH_2. Positive plasma samples were diluted 25-fold in 2× dilution buffer (8% human reference serum, 4% (w/v) skim milk powder in PBS). c3.3-IgG and cAGP4-IgM were diluted to 1 μg/mL in 2× dilution buffer. The human plasma samples and c3.3-IgG and cAGP4-IgM control antibodies (50 μL) were added to the wells containing PEG-liposomes (competition) or no PEG-liposomes (no competition) at a 1:1 volume ratio at room temperature for 1 h. The plates were washed with 0.1% CHAPS/PBS two times and PBS one time, followed by 0.25 μg/mL HRP-conjugated goat $F(ab')_2$ antihuman IgG Fc or HRP-conjugated goat $F(ab')_2$ antihuman IgM $Fc_{5\mu}$ in 50 μL of PBS containing 2% (w/v) skim milk powder at room temperature for 1 h. After washing, the bound peroxidase activity was measured by adding 150 μL/well ABTS solution and measuring the absorbance (405 nm) of wells in a microplate reader. Samples were considered positive if the absorbance reading with the addition of PEG-liposomes was reduced by 35% as compared to the reading without addition of PEG-liposomes. Other experimental details can be found in the Supporting Information.

RESULTS AND DISCUSSION

Chimeric anti-PEG Antibodies. Accurate measurement of human anti-PEG antibodies in plasma samples can benefit from antibody reference standards. Our approach was to generate chimeric anti-PEG antibodies possessing binding sites from murine monoclonal antibodies and constant domains from human immunoglobulins. Chimeric IgG and IgM anti-PEG antibodies were based on the 3.3 and AGP4 antibodies, selected from a panel of IgG (E11, 3.3 and 6.3) and IgM (AGP3, AGP4, and rAGP6) monoclonal antibodies previously developed in our lab[7,24,27,31−33] based on their intermediate affinities (Figure S-1), which may better represent the spectrum of natural or induced anti-PEG antibodies in human subjects. Both 3.3 and AGP4 antibodies bind to the repeating ethylene oxide subunits of the PEG backbone (Figure S-2).

Recombinant chimeric human anti-PEG antibodies (c3.3-IgG and cAGP4-IgM) were generated by fusing the light and heavy chain variable regions of 3.3 and AGP4 monoclonal antibodies to human IgG or IgM constant regions, respectively (Figure 1a). Recombinant IgG and IgM antibodies with human heavy chains of the expected molecular weights were collected from the culture medium of stable mammalian producer cells (Figure 1b). Both c3.3-IgG and cAGP4-IgM bound to immobilized PEG molecules ranging in size from 2000 Da to 30 000 Da (Figure S-3). A strong log−log correlation between c3.3-IgG binding and PEG molecular weight was noted that was independent of PEG structure (Figure S-4). Additional control experiments confirmed that c3.3-IgG and cAGP4-IgM specifically bound to the PEG backbone (Figures S-5 and S-6).

DOI: 10.1021/acs.analchem.6b03109
Anal. Chem. 2016, 88, 10661−10666

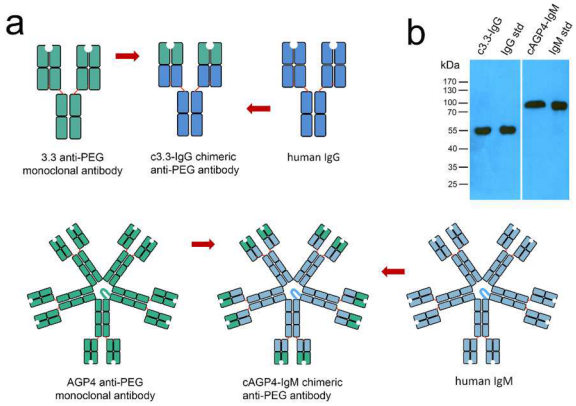


Figure 1. Chimeric ant-PEG antibodies. (a) Illustration of chimeric antibodies. The variable regions (antigen binding regions) of the anti-PEG monoclonal antibodies 3.3 (IgG) and AGP4 (IgM) were fused to the constant regions from human IgG or IgM to form c3.3-IgG and cAGP4-IgM. (b) 0.5 μg of chimeric antibodies or commercial human antibodies were separated on a reducing SDS PAGE, transferred to nitrocellulose paper and immunoblotted with antihuman IgG (left) or antihuman IgM (right) heavy chain specific secondary antibodies.

Anti-PEG Antibody Assay. A direct ELISA was developed to measure both IgG and IgM anti-PEG antibodies in human plasma samples (Figure S-7). Plasma samples producing a positive reading, defined as a mean absorbance that was at least 3 times higher than the background absorbance, were further assayed in a competition ELISA. Reduction of at least 35% in the absorbance reading of wells in the presence of excess PEG-liposomes as compared to wells without addition of PEG-liposomes were considered positive. PEG-liposomes provided a good compromise to compete the binding of both anti-PEG IgG and especially IgM, which was more difficult to compete, likely due to multivalent binding of IgM to immobilized PEG molecules (Figure S-8). The relative concentrations of IgG or IgM anti-PEG antibodies in plasma samples were then determined by comparison to the c3.3-IgG or cAGP4-IgM standard curves, respectively.

Analysis of c3.3-IgG and cAGP4-IgM standard curves performed in eight assays performed over a period exceeding 2 months revealed good reproducibility (Figure S-9 and Table S-1) and linear relationships over two-orders of magnitude on log−log plots with correlation coefficients (r^2) of 0.95 and 0.97, respectively (Figure 2). PEG-liposomes effectively competed binding of both anti-PEG IgG and IgM in human samples, verifying anti-PEG assay specificity (Figure S-10).

Control experiments verified the specificity of secondary antihuman antibodies for human IgG and human IgM (Figure S-11), tolerance of the assay to the addition of 4% human reference plasma (pretested for lack of anti-PEG antibodies)

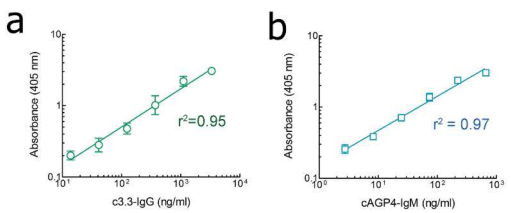


Figure 2. Chimeric antibody standard curves. Linear regression fit on log−log plots of 48 standard curves of c3.3-IgG (a) or cAGP4-IgM (b) performed on 8 separate days over a 2-month period. Bars, SD.

(Figure S-12), minimal cross competition between IgG and IgM anti-PEG antibodies (Figure S-13), insensitivity of assay response to PEG coating density (Figure S-14), and stability of immobilized PEG in assay plates (Figure S-15). PEG-like detergents (i.e., Tween 20) were replaced with CHAPS in all buffers to prevent inadvertent competition of anti-PEG antibody binding to immobilized PEG (Figure S-16).

Prevalence of anti-PEG Antibodies in Healthy Donors. Anti-PEG antibodies were analyzed in plasma samples obtained from 1504 healthy Han Chinese donors residing in Taiwan. The average age of the study population is 52.1 ± 17.4 years. Similar numbers of male (n = 756) and female (n = 748) donors were enrolled. A summary of the age and sex distribution is presented in Table 1. Of the 1504 healthy donors, 386 (25.7%) had detectable anti-PEG IgG antibodies and 407 (27.1%) had anti-PEG IgM antibodies (Figure 3a). In total, 8.4% of the population had both anti-PEG IgG and IgM antibodies. Reported values for the prevalence of pre-existing anti-PEG antibodies vary widely (Table S-2), likely due to difference in the study populations, variations in assay sensitivities, as well as measurement of limited numbers of samples in some studies. Our assay may help consolidate results from different studies.

The concentrations of anti-PEG IgG and IgM antibodies in the plasma of positive donors was estimated by comparison with c3.3-IgG and cAGP4-IgM standard curves, respectively. Anti-PEG IgG concentrations ranged from 238 to 0.3 μg/mL with a mean concentration of 5.76 ± 16.0 μg/mL and median concentration of 1.79 μg/mL (Figure 3b). Anti-PEG IgM concentrations ranged from 57.3 to 0.1 μg/mL with a mean concentration of 1.78 ± 3.54 μg/mL and median concentration of 0.96 μg/mL (Figure 3c). These antibody concentrations may be clinically important since peak serum concentrations of some PEGylated medicines such as PEG-Intron, Omontys, Mircera, Neulasta, and Pegasys are in the upper pg/mL to low μg/mL range.[34−38]

Anti-PEG Antibodies More Prevalent in Females. Investigation of possible gender-related differences in normal individuals revealed that the incidence of anti-PEG IgM was significantly greater in females as compared to males (32.0% vs 22.2%, p < 0.0001; Figure 4a). Likewise, female donors had a significantly higher incidence of anti-PEG IgG as compared to male donors (28.3% vs 23.0%, p = 0.018; Figure 4b). By contrast, there was no significant differences in the mean concentrations of anti-PEG IgM (1.98 μg/mL vs 1.49 μg/mL, p = 0.170; Figure 4c) or IgG (4.93 μg/mL vs 6.77 μg/mL, p = 0.262; Figure 4d) concentrations between female and male donors.

Anti-PEG IgG Prevalence and Concentration Are Inversely Correlated with Age. The frequency of female donors with positive anti-PEG IgG was around 20% for donors greater than 50 years of age but increased to around 60% for younger donors, following a one phase exponential decay model (Figure 5a). The frequency of IgG in males was less dependent on age but could also be fit with a one-phase exponential decay model (Figure 5b). The incidence of anti-PEG IgG in the overall population displayed a similar trend (Figure S-17). By contrast, the incidence of anti-PEG IgM was not significantly associated with age (Figure S-18).

There was also a significant trend of decreasing IgG concentrations with increasing donor age in both females (p = 0.0073; Figure 5c), males (p = 0.026; Figure 5d), and the overall population (p = 0.0005; Figure S-19). However, no

DOI: 10.1021/acs.analchem.6b03109
Anal. Chem. 2016, 88, 10661−10666

Table 1. Sample Population Characteristics

	age group (years)							
	total	20−29	30−39	40−49	50−59	60−69	70−79	≥80
	1504 (100%)	205 (13.6%)	206 (13.7%)	276 (18.4%)	255 (17.0%)	281 (18.7%)	203 (13.5%)	78 (5.2%)
male	756 (50.3%)	115 (56.1%)	97 (47.1%)	140 (50.7%)	123 (48.2%)	129 (45.9%)	116 (57.1%)	36 (46.2%)
female	748 (49.7%)	90 (43.9%)	109 (52.9%)	136 (49.3%)	132 (51.8%)	152 (54.1%)	87 (42.9%)	42 (53.8%)

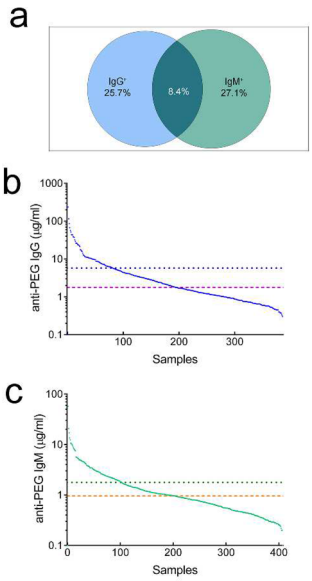


Figure 3. Anti-PEG IgG and IgM in normal individuals. (a) Venn diagram of anti-PEG antibody frequencies in 1504 normal donors. A total of 666 individuals (44.3%) had positive anti-PEG IgG or IgM. In total, 25.7% of the total population had anti-PEG IgG, 27.1% had anti-PEG IgM, and 8.4% had both anti-PEG IgG and IgM. The distribution of anti-PEG IgG (n = 386) (b) and anti-PEG IgM (n = 407) (c) concentrations are shown. Upper dotted lines indicate the mean antibody concentrations while the lower dashed lines indicate the median antibody concentrations.

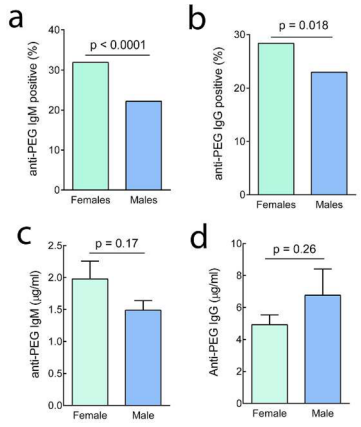


Figure 4. Anti-PEG antibodies are more prevalent in females than males. (a) The percentage of females (239 of 748) and males (168 of 756) with positive anti-PEG IgM. (b) The percentage of females (212 of 748) and males (174 of 756) with positive anti-PEG IgG. (c, d) The mean anti-PEG IgM (c) or anti-PEG IgG (d) concentrations in females and males among donors that were positive for anti-PEG IgM or anti-PEG IgG, respectively. Error bars, SEM.

significant associations were found between IgM concentrations and age (Figure S-20).

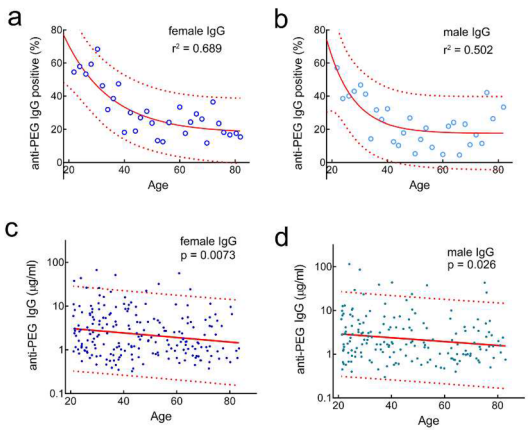


Figure 5. Anti-PEG IgG incidence decreases with age. The percentage of females (a) or males (b) with anti-PEG IgG is shown for 2-year age groups (for age groups with $n \geq 10$). One phase exponential decay models (female anti-PEG IgG positive (%) = 178 $e^{-0.061*Age}$ + 17.8, r^2 = 0.689) and (male anti-PEG IgG positive (%) = 378 $e^{-0.105*Age}$ + 17.6, r^2 = 0.502) are shown for female and male anti-PEG IgG frequencies versus age, respectively. The concentration of anti-PEG IgG in IgG-positive females (c) or males (d) is shown versus donor age. Linear regression lines are shown in red. p values indicate if the slope of regression lines are significantly different from zero. Dotted lines show 95% prediction intervals.

PEG as well as PEG derivatives are widely used in pharmaceutical and cosmetic products such as drug tablets, toothpaste, skin lotions, deodorant sticks, shaving creams, hand creams, face makeup, cream rouge, blush, mascara, lipstick, bath products, and hair care products.[39] Casual exposure to PEG compounds may induce anti-PEG antibodies,[10,15,40] possibly in concert with inflammatory responses at sites of dermal abrasion and inflammation.[17,41,42] The reason for the significantly higher prevalence of both IgG and IgM anti-PEG antibodies in females as compared to males is currently unknown but could be related to greater exposure of females to PEG and PEG derivatives in cosmetic products or to the tendency for women to have more autoimmune responses.[43] A higher prevalence of anti-PEG IgG in younger individuals might reflect diminished immune responsiveness in aged individuals, although additional studies are required to test this idea.[44]

Pre-Existing anti-PEG Antibodies Can Bind PEGylated Medicine. The ability of pre-existing anti-PEG IgM and IgG to bind to clinically relevant PEGylated medicines was examined. Serial dilutions of plasma samples from donors positive for anti-PEG IgG (G1, G4, G5, and G6), anti-PEG IgM (M1, M4, M5, and M6), or both anti-PEG IgG and IgM (GM2 and GM3) were added to ELISA plates coated with Pegasys (PEG-interferon alpha-2a) or Lipodox (PEG-liposomal-doxorubicin). Antibody binding was detected with antihuman IgG or IgM secondary antibodies. Plasma samples positive for anti-PEG IgG bound strongly to both Pegasys (Figure 6a) and Lipodox

DOI: 10.1021/acs.analchem.6b03109
Anal. Chem. 2016, 88, 10661−10666

(Figure 6c). Samples positive for anti-PEG IgM also bound to Pegasys (Figure 6b) and Lipodox (Figure 6d).

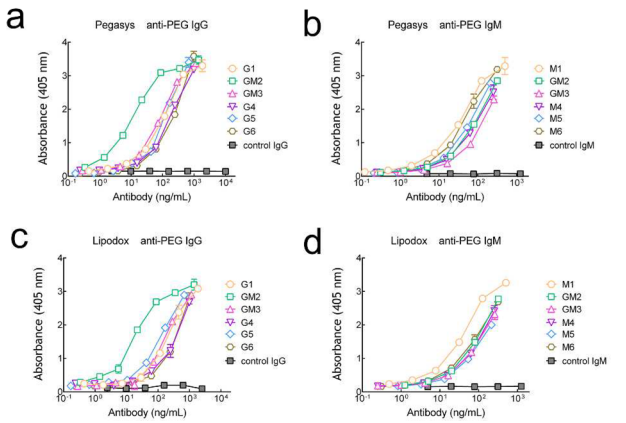


Figure 6. Pre-existing anti-PEG antibodies can bind to PEGylated medicines. Serial dilutions of plasma samples from donors that were positive for anti-PEG IgG (G1, G4, G5, and G6), anti-PEG IgM (M1, M4, M5, and M6), or both anti-PEG IgG and IgM (GM2 and GM3) were assayed for anti-PEG IgG (a, c) or anti-PEG IgM (b, d) binding to Pegasys (a, b) or Lipodox (c, d) in 96-well plates using antihuman IgG or IgM specific secondary antibodies. Control IgG and control IgM are negative control human IgG and IgM antibodies, respectively. Dilution buffer is PBS containing 4% human reference serum and 2% skim milk. (Bars, SE; n = 2).

Pre-existing anti-PEG antibodies may impact the clinical efficacy and safety of PEGylated medicines by altering their pharmacokinetics, biodistribution, or safety profiles. Animal studies have demonstrated that anti-PEG IgM can accelerate the clearance of PEGylated proteins and liposomes.[7,45−47] Importantly, even a relatively low affinity anti-PEG IgM antibody (AGP3) accelerated the clearance of PEGylated proteins from the circulation of mice.[7,45] Induced anti-PEG antibodies in human subjects receiving PEGylated drugs such as PEG-asparaginase and PEG-uricase are associated with rapid drug clearance and decreased therapeutic efficacy.[10,11,48] Recent studies in human patients have also observed a trend between pre-existing or induced anti-PEG antibodies and adverse allergic reactions during administration of PEGylated therapeutics.[11,15,20] We speculate that individuals with sufficiently high concentrations of pre-existing anti-PEG antibodies may respond poorly to some PEGylated medicines or display adverse responses during a first drug infusion. Our studies therefore emphasize the need to better understand the possible impact of pre-existing anti-PEG antibodies on the therapeutic efficacy and safety of PEGylated medicines.

CONCLUSIONS

We developed chimeric human anti-PEG IgG and IgM antibody standards to expedite assay of anti-PEG IgG and IgM antibodies in human plasma samples. The chimeric antibodies have fully human H and L chain constant regions that can be specifically detected with antihuman IgG or IgM secondary antibodies for direct comparison of antibody standard curves and donor samples. A direct ELISA was developed to measure anti-PEG responses in samples containing up to 4% human plasma with linear log−log responses from 15 to 4000 ng/mL for c3.3-IgG and 3 to 1000 ng/mL for cAGP4-IgM. Assay of plasma samples from 1504 healthy Han Chinese donors residing in Taiwan revealed a high prevalence of both anti-PEG IgM (27.1%) and IgG (25.7%) antibodies. We report for the first time significantly increased prevalence of anti-PEG antibodies in females as compared to males as well as higher prevalence and concentrations of pre-existing anti-PEG IgG antibodies in younger (20−40 years) as opposed to older (>50 years) males and females. Our study provides important information on the widespread prevalence of pre-existing anti-PEG antibodies and suggests that additional studies on the clinical impact of pre-existing anti-PEG antibodies on the therapeutic efficacy and safety of PEGylated medicines are warranted. In particular, it is important to determine the relationships between natural anti-PEG antibody levels and antibody class on PEGylated drug bioactivity, pharmacokinetics, and biodistribution with attention to the influence of drug dose, differences in PEGylation levels, and PEG chain length on therapeutic safety and efficacy.[11]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b03109.

Additional information on methods and results (PDF)

AUTHOR INFORMATION

Corresponding Authors

*Phone: (886)-2-2789-9075. E-mail: jywu@ibms.sinica.edu.tw.
*Phone: (886)-2-2652-3079. E-mail: sroff@ibms.sinica.edu.tw.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by intramural funding from Academia Sinica and a grant from the Academia Sinica Research Program on Nanoscience and Nanotechnology. We thank the Proteomics Core Facility of the Institute of Biomedical Sciences, Academia Sinica, for MALDI-TOF measurements. We appreciate the technical help of Ms. Show-Ling Yang and Mr. Talal Qaisi. Lentivirus vectors were provided by the National RNAi Core Facility, Institute of Molecular Biology/Genomic Research Center, at Academia Sinica, Taipei, Taiwan. We gratefully acknowledge the members of the Translational Resource Center (Grant NSC102-2325-B-001-023) of the National Research Program for Biopharmaceuticals and the National Center for Genome Medicine (Grant NSC102-2319-B-001-001) of the National Core Facility Program for Biotechnology, National Science Council, at Academia Sinica, for their support in project management, subject recruitment, data coordination, genotyping performance, and statistical analysis.

REFERENCES

(1) Harris, J. M.; Chess, R. B. *Nat. Rev. Drug Discovery* **2003**, *2*, 214−221.
(2) Banerjee, S. S.; Aher, N.; Patil, R.; Khandare, J. *J. Drug Delivery* **2012**, *2012*, 103973.
(3) Wang, A. Z.; Langer, R.; Farokhzad, O. C. *Annu. Rev. Med.* **2012**, *63*, 185−198.
(4) Alconcel, S. N. S.; Baas, A. S.; Maynard, H. D. *Polym. Chem.* **2011**, *2*, 1442−1448.
(5) Swierczewska, M.; Lee, K. C.; Lee, S. *Expert Opin. Emerging Drugs* **2015**, *20*, 531−536.

DOI: 10.1021/acs.analchem.6b03109
Anal. Chem. 2016, 88, 10661−10666

(6) Richter, A.; Åkerblom, E. *Int. Arch. Allergy Immunol.* **2004**, *74*, 36−39.
(7) Cheng, T. L.; Wu, P. Y.; Wu, M. F.; Chern, J. W.; Roffler, S. R. *Bioconjugate Chem.* **1999**, *10*, 520−528.
(8) Wang, X.; Ishida, T.; Kiwada, H. *J. Controlled Release* **2007**, *119*, 236−244.
(9) Suzuki, T.; Ichihara, M.; Hyodo, K.; Yamamoto, E.; Ishida, T.; Kiwada, H.; Ishihara, H.; Kikuchi, H. *Int. J. Pharm.* **2012**, *436*, 636−643.
(10) Armstrong, J. K.; Hempel, G.; Koling, S.; Chan, L. S.; Fisher, T.; Meiselman, H. J.; Garratty, G. *Cancer* **2007**, *110*, 103−111.
(11) Hershfield, M. S.; Ganson, N. J.; Kelly, S. J.; Scarlett, E. L.; Jaggers, D. A.; Sundy, J. S. *Arthritis Res. Ther.* **2014**, *16*, R63.
(12) Garratty, G. *Transfus Med. Rev.* **2004**, *18*, 245−256.
(13) Sundy, J. S.; Ganson, N. J.; Kelly, S. J.; Scarlett, E. L.; Rehrig, C. D.; Huang, W.; Hershfield, M. S. *Arthritis Rheum.* **2007**, *56*, 1021−1028.
(14) Liu, Y.; Reidler, H.; Pan, J.; Milunic, D.; Qin, D.; Chen, D.; Vallejo, Y. R.; Yin, R. *J. Pharmacol Toxicol Methods* **2011**, *64*, 238−245.
(15) Ganson, N. J.; Povsic, T. J.; Sullenger, B. A.; Alexander, J. H.; Zelenkofske, S. L.; Sailstad, J. M.; Rusconi, C. P.; Hershfield, M. S. *J. Allergy Clin. Immunol.* **2016**, *137*, 1610.
(16) Myler, H.; Hruska, M. W.; Srinivasan, S.; Cooney, E.; Kong, G.; Dodge, R.; Krishna, M.; Zhu, J.; Felix, T.; Gleason, C. *Bioanalysis* **2015**, *7*, 1093−1106.
(17) Yang, Q.; Lai, S. K. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology* **2015**, *7*, 655−677.
(18) Sundy, J. S.; Baraf, H. S.; Yood, R. A.; Edwards, N. L.; Gutierrez-Urena, S. R.; Treadwell, E. L.; Vázquez-Mellado, J.; White, W. B.; Lipsky, P. E.; Horowitz, Z. *J. Am. Med. Assoc.* **2011**, *306*, 711−720.
(19) Szebeni, J.; Storm, G. *Biochem. Biophys. Res. Commun.* **2015**, *468*, 490−497.
(20) Lipsky, P. E.; Calabrese, L. H.; Kavanaugh, A.; Sundy, J. S.; Wright, D.; Wolfson, M.; Becker, M. A. *Arthritis Res. Ther.* **2014**, *16*, R60.
(21) Wiberg, M.; Wang, D.; Yang, L.; Kamerud, J. In *Pharmaceutical Sciences World Congress (PSWC)*, New Orleans, LA, November 14−18, 2010; p T3292.
(22) Schellekens, H.; Hennink, W. E.; Brinks, V. *Pharm. Res.* **2013**, *30*, 1729−1734.
(23) Bailon, P.; Palleroni, A.; Schaffer, C. A.; Spence, C. L.; Fung, W. J.; Porter, J. E.; Ehrlich, G. K.; Pan, W.; Xu, Z. X.; Modi, M. W.; Farid, A.; Berthold, W.; Graves, M. *Bioconjugate Chem.* **2001**, *12*, 195−202.
(24) Cheng, T. L.; Cheng, C. M.; Chen, B. M.; Tsao, D. A.; Chuang, K. H.; Hsiao, S. W.; Lin, Y. H.; Roffler, S. R. *Bioconjugate Chem.* **2005**, *16*, 1225−1231.
(25) Myler, H. A.; McVay, S.; Kratzsch, J. *J. Pharmacol. Toxicol. Methods* **2010**, *61*, 92−97.
(26) Bailon, P.; Won, C.-Y. *Expert Opin. Drug Delivery* **2009**, *6*, 1−16.
(27) Su, Y.-C.; Chen, B.-M.; Chuang, K.-H.; Cheng, T.-L.; Roffler, S. R. *Bioconjugate Chem.* **2010**, *21*, 1264−1270.
(28) Hyotyla, J. T.; Deng, J.; Lim, R. Y. *ACS Nano* **2011**, *5*, 5180−5187.
(29) Cheng, T.-L.; Chuang, K.-H.; Chen, B.-M.; Roffler, S. R. *Bioconjugate Chem.* **2012**, *23*, 881−899.
(30) de Dios, K.; Manibusan, A.; Marsden, R.; Pinkstaff, J. *J. Immunol. Methods* **2013**, *396*, 1−7.
(31) Tsai, N.-m.; Cheng, T.-L.; Roffler, S. R. *Biotechniques* **2001**, *30*, 396−402.
(32) Kao, C.-H.; Wang, J.-Y.; Chuang, K.-H.; Chuang, C.-H.; Cheng, T.-C.; Hsieh, Y.-C.; Tseng, Y.-l.; Chen, B.-M.; Roffler, S. R.; Cheng, T.-L. *Biomaterials* **2014**, *35*, 9930−9940.
(33) Tung, H.-Y.; Su, Y.-C.; Chen, B.-M.; Burnouf, P.-A.; Huang, W.-C.; Chuang, K.-H.; Yan, Y.-T.; Cheng, T.-L.; Roffler, S. R. *Mol. Cancer Ther.* **2015**, *14*, 1317−1326.
(34) Glue, P.; Fang, J. W.; Rouzier-Panis, R.; Raffanel, C.; Sabo, R.; Gupta, S. K.; Salfi, M.; Jacobs, S. *Clin. Pharmacol. Ther.* **2000**, *68*, 556−567.
(35) Zeuzem, S.; Diago, M.; Gane, E.; Reddy, K. R.; Pockros, P.; Prati, D.; Shiffman, M.; Farci, P.; Gitlin, N.; O'Brien, C. B. *Gastroenterology* **2004**, *127*, 1724−1732.
(36) Macdougall, I. C.; Robson, R.; Opatrna, S.; Liogier, X.; Pannier, A.; Jordan, P.; Dougherty, F. C.; Reigner, B. *Clin. J. Am. Soc. Nephrol.* **2006**, *1*, 1211−1215.
(37) Holmes, F.; Jones, S.; O'shaughnessy, J.; Vukelja, S.; George, T.; Savin, M.; Richards, D.; Glaspy, J.; Meza, L.; Cohen, G. *Ann. Oncol.* **2002**, *13*, 903−909.
(38) Hu, X.; Cui, Y.; White, J.; Zhu, Y.; Deykin, A.; Nestorov, I.; Hung, S. *British journal of clinical pharmacology* **2015**, *79*, 514−522.
(39) Fruijtier-Pölloth, C. *Toxicology* **2005**, *214*, 1−38.
(40) Garay, R. P.; El-Gewely, R.; Armstrong, J. K.; Garratty, G.; Richette, P. *Expert Opin. Drug Delivery* **2012**, *9*, 1319−1323.
(41) Cerutti, A.; Cols, M.; Puga, I. *Nat. Rev. Immunol.* **2013**, *13*, 118−132.
(42) Vinuesa, C. G.; Chang, P.-P. *Nat. Immunol.* **2013**, *14*, 119−126.
(43) Paavonen, T. *Ann. Med.* **1994**, *26*, 255−258.
(44) Linton, P. J.; Dorshkind, K. *Nat. Immunol.* **2004**, *5*, 133−139.
(45) Cheng, T.-L.; Chen, B.-M.; Chern, J.-W.; Wu, M.-F.; Roffler, S. R. *Bioconjugate Chem.* **2000**, *11*, 258−266.
(46) Ishihara, T.; Takeda, M.; Sakamoto, H.; Kimoto, A.; Kobayashi, C.; Takasaki, N.; Yuki, K.; Tanaka, K.; Takenaga, M.; Igarashi, R.; Maeda, T.; Yamakawa, N.; Okamoto, Y.; Otsuka, M.; Ishida, T.; Kiwada, H.; Mizushima, Y.; Mizushima, T. *Pharm. Res.* **2009**, *26*, 1792−1800.
(47) Mima, Y.; Hashimoto, Y.; Shimizu, T.; Kiwada, H.; Ishida, T. *Mol. Pharmaceutics* **2015**, *12*, 2429−2435.
(48) Ganson, N. J.; Kelly, S. J.; Scarlett, E.; Sundy, J. S.; Hershfield, M. S. *Arthritis Res. Ther.* **2006**, *8*, R12.

DOI: 10.1021/acs.analchem.6b03109
Anal. Chem. 2016, 88, 10661−10666

Supporting Information

Measurement of pre-existing IgG and IgM antibodies against polyethylene glycol in healthy individuals

Bing-Mae Chen[1], Yu-Cheng Su[1], Chia-Jung Chang[1], Pierre-Alain Burnouf[1], Kuo-Hsiang Chuang[2], Chien-Hsiun Chen[1,3], Tian-Lu Cheng[4], Yuan-Tsong Chen[1,5], Jer-Yuarn Wu[1,3*] and Steve R. Roffler[1,6,*]

[1]Institute of Biomedical Sciences, Academia Sinica, Taipei 11529, Taiwan.

[2]Graduate Institute of Pharmacognosy, Taipei Medical University, Taipei 11031, Taiwan

[3]School of Chinese Medicine, China Medical University, Taichung 40447, Taiwan

[4]Department of Biomedical Science and Environmental Biology, Center for Biomarkers and Biotech Drugs, Kaohsiung Medical University, Kaohsiung 80708, Taiwan

[5]Department of Pediatrics, Duke University Medical Center, Durham, North Carolina, United States

[6]Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung 80708, Taiwan

*Corresponding authors

Dr. Steve Roffler, Room N232, Institute of Biomedical Sciences, Academia Sinica, Academia Road, Section 2, No. 128, Taipei 11529, Taiwan
Tel: (886)-2-2652-3079
Email: sroff@ibms.sinica.edu.tw

Dr. Jer-Yuarn Wu, Room N520, Institute of Biomedical Sciences, Academia Sinica, Academia Road, Section 2, No. 128, Taipei 11529, Taiwan
Tel: (886)-2- 2789-9075
Email: jywu@ibms.sinica.edu.tw

Table of Contents:

Experimental section

Antibodies

Peroxidase AffiniPure donkey anti-mouse IgG (H+L) (715-035-150), peroxidase-AffiniPure goat anti-mouse IgM, μ-chain specific (115-035-020), AffiniPure goat anti-human IgA + IgG + IgM (H+L) (109-005-064), peroxidase AffiniPure $F(ab')_2$ fragment goat anti-human IgG, Fcγ fragment specific (109-036-098) and peroxidase AffiniPure $F(ab')_2$ fragment goat anti-human IgM, $Fc_{5\mu}$ fragment specific (109-036-129) were from Jackson ImmunoResearch Laboratories (West Grove, PA). Standard human IgG_1 (ab90283) was from Abcam (Cambridge, United Kingdom) and standard human IgM (009-0107-0001) was from Rockland (Limerick, PA).

Cell lines and reagents

Human 293FT (Thermo Fisher Scientific, Waltham, MA), GP293 cells (Clontech, Mountain View, CA) and 293T cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium supplemented with 2.98 g/L HEPES, 2 g/L $NaHCO_3$, 10% fetal calf serum (GE Healthcare Life Sciences, Logan, UT), 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air. $mPEG_{750}$-NH_2, $mPEG_{2000}$-NH_2, PEG_{5000}-NH_2, $mPEG_{10,000}$-NH_2, $mPEG_{20,000}$-NH_2, $mPEG_{30,000}$-NH_2 (750, 2000, 5000, 10,000, 20,000 and 30,000 Da, respectively), NH_2-PEG_{3000}-NH_2, 4-arm $PEG_{10,000}$-NH_2, $mPEG_{5000}$ activated with cyanuric chloride, $mPEG_{5000}$ p-nitrophenyl carbonate, $mPEG_{5000}$ tresylate and $mPEG_{5000}$ succinate *N*-hydroxysuccinimide were from Sigma-Aldrich Chemical Co. (St. Louis, MO). NH_2-$PEG_{10,000}$-NH_2 was from Iris Biotech GmbH (Marktredwitz, Germany). $mPEG_{2000}$ succinimidyl propionic acid was from Nektar Therapeutics (San Francisco,

CA). Distearoyl phosphatidylcholine (DSPC), 1,2-distearoyl-sn- glycero-3-phosphoethanolamine -N-[methoxy(polyethylene glycol)-2000 (DSPE-PEG_{2000}) and cholesterol were from Avanti Polar Lipids (Alabaster, AL). Lipodox was from Taiwan Tung Yang Biopharm (TTY Biopharm Company Ltd., Taipei, Taiwan). Pegasys (PEG-interferon alpha-2a) and PEG-Intron (peginterferon alpha-2b) were from Roche (Nutley, NJ) and Schering-Plough (Kenilworth, NJ), respectively.

Chimeric antibody DNA plasmids

To generate human chimeric anti-PEG antibodies (c3.3-IgG or cAGP4-IgM), the DNA sequences of the V_L and V_H domains of the 3.3 and AGP4 monoclonal antibodies were determined by 5' rapid amplification of cDNA ends (5' RACE)[1] and cloned from cDNA prepared from the 3.3 and AGP4 hybridoma cells. Human IgG_1 Cκ, CH_1-CH_2-CH_3 and IgM CH_1-CH_2-CH_3-CH_4 constant domains were cloned from cDNA prepared from human peripheral blood mononuclear cells. 3.3V_L-Cκ, AGP4V_L-Cκ, 3.3V_H-CH_1-CH_2-CH_3 and AGP4 V_H-CH_1-CH_2-CH_3-CH_4 domains were assembled by overlap PCR from mouse 3.3V_L, AGP4V_L, 3.3V_H and AGP4V_H and human Cκ, CH_1-CH_2-CH_3 and CH_1-CH_2-CH_3-CH_4 fragments, respectively. The light and heavy chains of 3.3 and AGP4 were joined by a IRES bicistronic expression linker[2] by overlap PCR and inserted into the pLNCX2 plasmid (Clontech, Mountain View, CA) to generate pLNCX2-c3.3-IgG and pLNCX2-cAGP4-IgM plasmids. The human J chain gene was cloned from cDNA prepared from human peripheral blood mononuclear cells and subcloned via EcoRI and PmeI sites into the lentiviral expression vector pLKO_AS3w.Ppuro, which contain a CMV early enhancer/chicken β actin (CAG) promoter (obtained from the National RNAi Core Facility, Institute of Molecular Biology, Genomic Research Center, Academia Sinica, Taiwan), to

generate the pLKO_AS3w.Ppuro-J chain plasmid.

Lentiviral transduction of the J chain gene into 293T cells

Recombinant lentiviral particles were packaged as described.[3] Briefly, 7.5 μg pLKO_AS3w.Ppuro-J chain plasmid, 6.75 μg pCMVΔR8.91 packaging plasmid[4] and 0.75 μg pMD.G VSV-G envelope plasmid[5] (obtained from the National RNAi Core Facility, Institute of Molecular Biology, Genomic Research Center, Academia Sinica, Taiwan) were co-transfected in 293FT cells using 45 μL TransIT-LT1 transfection reagent (Mirus Bio) in a 10 cm culture dish (90% confluency). After 48 h, lentiviral particles were harvested and concentrated by ultracentrifugation at 50 000xg for 1.5 h at 4°C. Lentiviral particles were suspended in complete culture medium containing 5 μg/mL polybrene, filtered through a 0.45 μm filter and added to the 293T cells in 6-well plates. The cells were selected in complete medium containing puromycin (5 μg/mL) to generate stable 293T/J chain cells.

Chimeric anti-PEG antibody production

To generate chimeric anti-PEG IgG and IgM producer cells, pLNCX2-c3.3-IgG or pLNCX2-cAGP4-IgM DNA were co-transfected with pVSVG into GP293 cells to produce recombinant retroviral particles. At 2 days after transfection, the culture medium was added to 293T (for IgG) or 293T/J chain (for IgM) cells in the presence of 8 μg/mL polybrene. Stable cell lines were selected in medium containing 0.5 mg/mL G418 (Merck Millipore, Billerica, MA). Recombinant c3.3-IgG was purified by protein A Sepharose 4 Fast Flow chromatography (GE Healthcare, Little Chalfont, United Kingdom). The concentrations of c3.3-IgG and cAGP4-IgM were measured by direct ELISA. 0.5 μg/well of affiniPure goat anti-human IgA + IgG + IgM

(H+L) was coated in ELISA plates overnight at 4^{o}C. The plates were blocked with 5% (w/v) skim milk powder in PBS for 2 h and then 3-fold serial dilutions of purified human IgG_1 or human IgM starting at 2 μg/mL along with serially diluted c3.3-IgG or cAGP4-IgM were added for 2 h before the plates were washed and binding detected with HRP-conjugated anti-human IgG or IgM secondary antibody. The concentrations of c3.3-IgG and cAGP4-IgM were calculated from the human IgG and human IgM standard curves.

Immunoblotting

0.25 μg human IgG, human IgM, c3.3-IgG or cAGP4-IgM were electrophoresed in a 10% SDS-PAGE gel under reducing conditions before overnight transfer to nitrocellulose paper by capillary diffusion in blotting buffer (50 mM NaCl, 2 mM EDTA, 0.5 mM 2-mercaptoethanol, 10 mM Tris-HCl, pH 7.5). Blots were blocked for 1 h with 5% (w/v) skim milk powder in PBS and incubated for 1 h at RT with HRP-conjugated goat $F(ab')_2$ anti-human IgG Fc (for human IgG and c3.3-IgG) or HRP-conjugated goat $F(ab')_2$ anti-human IgM $Fc_{5\mu}$ (for human IgM and cAGP4-IgM) for 1 h at RT. After washing three times with PBS-T and twice with PBS, specific bands were visualized by ECL detection according to the manufacturer's instructions (Pierce, Rockford, IL), then detected by a LAS-3000 mini Fujifilm imaging system (FujiFilm, Tokyo, Japan).

Preparation of PEGylated proteins

Aliquots of 3 mg BSA in 0.5 mL borate buffer (100 mM sodium borate, 50 mM NaCl, 1 mM EDTA, pH 8.2) were reacted for 2 hours at room temperature with 6.8 mg $mPEG_{5000}$ activated with cyanuric chloride (M11), $mPEG_{5000}$ p-nitrophenyl carbonate (M13), $mPEG_{5000}$

tresylate (M18) or $mPEG_{2000}$ succinimidyl propionic acid (M20). Samples were dialyzed against 100 mM Tris-HCl, pH 8.0. PEGylated BSA was purified by ion-exchange chromatography on DEAE Sepharose. Removal of free PEG from PEGylated BSA was confirmed by measuring residual free PEG as described.[6] The average number of PEG chains attached per BSA, as determined by matrix-assisted laser desorption/ionization coupled with a time-of-flight mass spectrometer (MALDI-TOF), was 1.6 for M11, 2.4 for M13, 2.1 for M18 and 10 for M20. PEG was also attached to E. coli beta-glucuronidase[7] or BSA to act as competitors in confirmatory ELISAs. 6 mg of BSA (3 mg/mL) or E. coli beta-glucuronidase (3 mg/mL) in borate buffer (100 mM sodium borate, 50 mM NaCl, 1 mM EDTA, pH 8.2) were reacted with a 100-fold molar excess of $mPEG_{5000}$ succinate *N*-hydroxysuccinimide for 2 h at room temperature. The reaction was stopped by addition of 1 M Tris-HCl.

Characterization of antibody binding to PEG

Maxisorp 96-well microplates (Nalge-Nunc International, Penfield, NY) were coated with 0.5 μg/well $mPEG_{750}$-NH_2, $mPEG_{2000}$-NH_2, NH_2-PEG_{2000}-NH_2, $mPEG_{3000}$-NH_2, NH_2-PEG_{3000}-NH_2, PEG_{5000}-NH_2, branched 4-arm $mPEG_{10,000}$-NH_2, NH_2-$PEG_{10,000}$-NH_2, $mPEG_{20,000}$-NH_2, $mPEG_{30,000}$-NH_2 or PEGylated BSA in 50 μL/well 0.1 M $NaHCO_3/Na_2CO_3$ (adjusted to pH 9.5 with HCl) buffer overnight at 4°C and then blocked with 200 μL/well 5% (w/v) skim milk powder in PBS at room temperature for 2 h. Amine-terminated mPEG was used to coat microtiter plates in all assays because the amine group facilitates stable attachment to the plates.[8] Serial dilutions of monoclonal anti-PEG antibodies or chimeric anti-PEG antibodies diluted in PBS containing 2% (w/v) skim milk powder were added to the plates at room temperature for 1 h. Unbound antibodies were removed by washing twice with 0.1 % CHAPS/PBS and once with

PBS. Antibody binding was detected with peroxidase donkey anti-mouse IgG (H+L) or peroxidase goat anti-mouse IgM for IgG or IgM monoclonal antibodies or with HRP-conjugated goat $F(ab')_2$ anti-human IgG Fc or HRP-conjugated goat $F(ab')_2$ anti-human IgM $Fc_{5\mu}$ for chimeric antibodies. The plates were washed as described above. The bound peroxidase activity was measured by adding 150 μL/well ABTS solution [0.4 mg/mL, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), 0.003% H_2O_2, and 100 mM phosphate-citrate, pH 4.0) for 30 min at room temperature. The absorbance (405 nm) of wells was measured in a microplate reader (Molecular Device).

Preparation of PEG-liposomes

Distearoyl phosphatidylcholine (DSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000 (DSPE-PEG_{2000}) and cholesterol were dissolved in chloroform at a 65:5:30 molar ratio, respectively. A dried lipid film was formed at 65˚C by rotary evaporation (Buchi, Rotavapor RII) and rehydrated in Tris-buffered saline (TBS, 50 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.4) at 65˚C to a final lipid concentration of 20 mg/mL. The liposomal suspension was submitted to 10 freeze/thaw cycles in liquid nitrogen and a heated water bath at 80˚C, followed by 21 extrusions at 75˚C through 400, 200, and 100 nm polycarbonate membranes each using a mini-extruder (Avanti Polar Lipids, Inc.). The final lipid concentration was measured by Bartlett's assay[9] and adjusted to 4 mg/mL with TBS before use.

Detection of anti-human antibody binding to PEGylated medicines

For detection of human anti-PEG antibody binding to Pegasys, Maxisorp 96-well microplates were coated with 0.25 μg/well Pegasys in 50 μL/well 0.1 M $NaHCO_3/Na_2CO_3$

(adjusted to pH 9.5 with HCl) buffer overnight at 4°C and then blocked with 200 µL/well 5% (w/v) skim milk powder in PBS at room temperature for 2 h. The plates were washed once before addition of test plasma. For detection of anti-PEG antibodies to Lipodox, Maxisorp 96-well microplates were first coated with 0.25 µg/well AGP4 mouse anti-PEG antibody in 50 µL/well 0.1 M $NaHCO_3/Na_2CO_3$ (adjusted to pH 8.0 with HCl) buffer overnight at 4°C and then blocked with 200 µL/well 5% (w/v) skim milk powder in PBS at room temperature for 2 h. After washing once with PBS, 1 µg/well Lipodox in 2% (w/v) skim milk powder/PBS was added for 2 h at room temperature. The plates were washed three times with PBS before addition of human plasma samples. Binding of chimeric standard anti-PEG antibodies or human plasma samples were measured as described for the human anti-PEG ELISA.

Curve fitting

c3.3-IgG and cAGP4-IgM standard curves transformed by plotting the ELISA absorbance values versus the logarithm of antibody concentrations were fit with a sigmoidal dose response curve with variable slope using GraphPad Prism 5 software (GraphPad, San Diego, CA). EC_{50} values (the concentration of antibody producing 50% absorbance readings) were calculated using interpolation of the sigmoidal dose response curves using GraphPad Prism 5. Standard curves transformed by plotting the logarithm of absorbance versus the logarithm of antibody concentration were fit with a straight line by linear regression analysis using GraphPad Prism 5. The goodness of fit was estimated as the correlation coefficient r^2. The frequencies of anti-PEG IgG or IgM positive males and females were calculated for two year periods (20-21, 22-23 etc) to provide sufficient numbers of individuals in each age cohort. The data for groups with at least 10 individuals was fit with a one-phase exponential decay equation. The annual population

frequency of anti-PEG IgG or IgM was fit to a one-phase exponential decay equation for groups with at least 15 individuals.

Statistical Analysis

Statistical analysis was assessed with GraphPad Prism 5 software. Significance of differences between mean values were estimated using the two-tailed unpaired Student's t-test. Significance of differences in positive IgG and IgM frequencies among males and females was calculated using the z score for two population proportions. Cook's D was used to identify outlier data points which might unduly influence linear correlations. One data point was excluded from the linear regression analyses anti-PEG IgG concentration in males and the IgM concentration in females as a function of age, respectively. Figures were generated using GraphPad Prism 5. The statistical significance was set at $p < 0.05$.

Results

Comparison of IgG monoclonal antibody binding to immobilized $mPEG_{2000}$ (methoxyPEG with a molecular weight of 2000) revealed relative affinities of 6.3 > 3.3 > E11 (**Fig. S-1a**). The same rank order was observed for binding of the IgG monoclonal antibodies to long mPEG molecules (20,000 Da) (**Fig. S-1b**). Comparison of the binding of IgM monoclonal antibodies to short (**Fig. S-1c**) and long (**Fig. S-1d**) mPEG molecules demonstrated relative binding avidities of rAGP6 > AGP4 >> AGP3. Anti-PEG monoclonal antibodies 3.3 and AGP4 were selected to construct chimeric human anti-PEG antibodies due to their intermediate affinity, which may be more reflective of anti-PEG antibodies in patient samples.

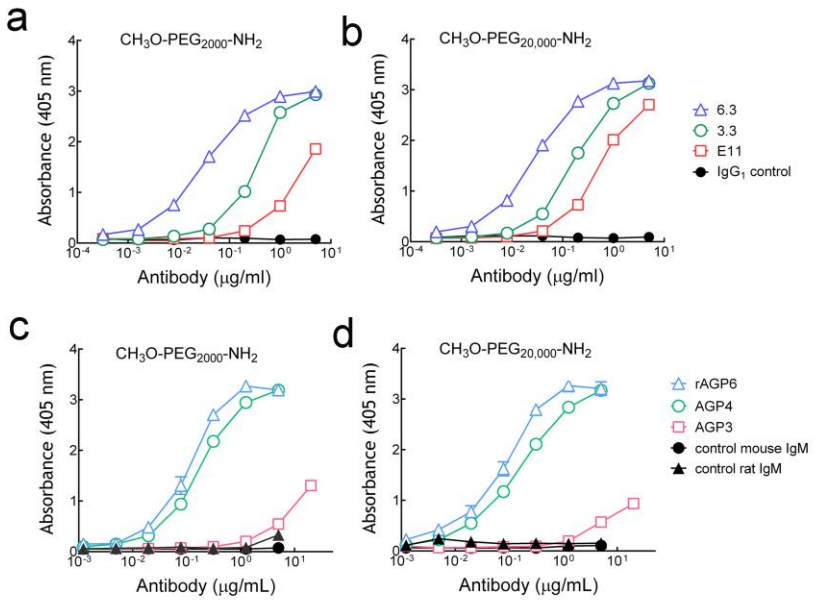


Figure S-1. Comparison of the binding of anti-PEG monoclonal antibodies to immobilized PEG molecules. **a, b**) Anti-PEG mouse monoclonal IgG antibodies (6.3, 3.3 and E11) or an isotype-matched negative control IgG_1 antibody were assayed for binding to ELISA plates coated with (**a**) short (CH_3O-PEG_{2000}-NH_2) or (**b**) long (CH_3O-$PEG_{20,000}$-NH_2) mPEG molecules. **c, b**) Anti-PEG rat monoclonal IgM (rAGP6), anti-PEG mouse monoclonal IgM antibodies (AGP4 and AGP3) or negative control IgM antibodies were assayed for binding to ELISA plates coated with (**c**) short (CH_3O-PEG_{2000}-NH_2) or (**d**) long (CH_3O-$PEG_{20,000}$-NH_2) mPEG molecules.

Anti-PEG antibodies can be divided into those that bind the PEG backbone or to the terminus of mPEG. These antibody classes can be distinguished by their differential binding to NH_2-PEG_{3000}-NH_2, in which both amine groups can be immobilized to surfaces, thus allowing binding of backbone-specific antibodies but not mPEG-specific antibodies. Thus, the control anti-mPEG specific antibody 15-2b[10] bound strongly to immobilized $mPEG_{2000}$ (**Fig. S-2a**) but not to NH_2-PEG_{3000}-NH_2 (**Fig. S-2b**), which lacks a terminal methoxy group. By contrast, 3.3 and AGP4 bound to both immobilized $mPEG_{2000}$ and NH_2-PEG_{3000}-NH_2, demonstrating that the antibodies bind to the repeating ethylene oxide subunits of the PEG backbone.

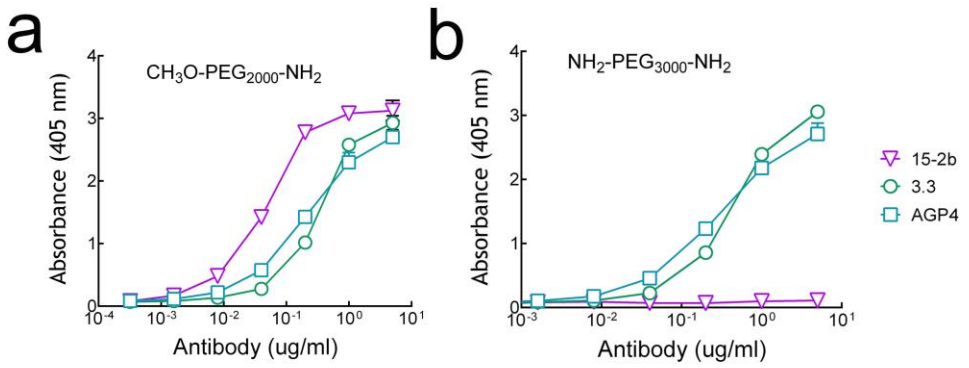


Figure S-2. Binding of monoclonal antibodies to immobilized PEG and mPEG molecules. Anti-mPEG mouse monoclonal antibody (15-2b) or anti-PEG mouse monoclonal antibodies (3.3 and AGP4) were assayed for binding to ELISA plates coated with (**a**) mPEG (CH_3O-PEG_{2000}-NH_2) or (**b**) diaminePEG (NH_2-PEG_{3000}-NH_2) molecules. (Bars, SD, n=2).

Analysis of the binding of the chimeric antibodies demonstrated that c3.3-IgG bound to immobilized mPEG molecules ranging in size from 30,000 Da to 2000 Da, with better binding observed for longer chain mPEG (**Fig. S-3a**). cAGP4-IgM binding to mPEG was less sensitive to mPEG size (**Fig. S-3b**).

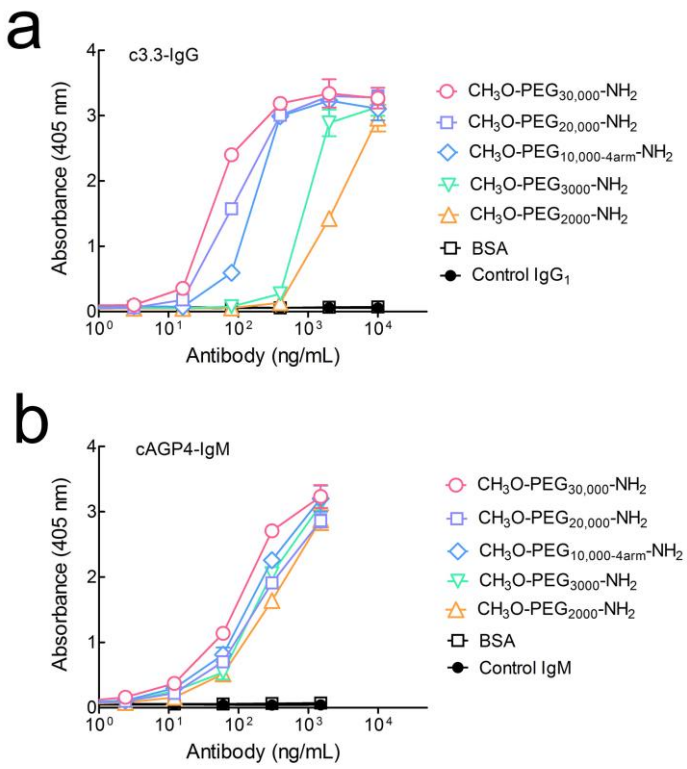


Figure S-3. Chimeric anti-PEG antibodies can bind a large range of immobilized PEG molecules. Serial dilutions of c3.3-IgG (**a**) or cAGP4-IgM (**b**) were added in duplicate to ELISA plates coated with the indicated mPEG molecules. BSA coated plates and control human IgG or IgM were used as negative control antigen and antibodies, respectively. Antibody binding was detected with HRP-labeled anti-human IgG (for c3.3-IgG) or anti-human IgM (for cAGP4-IgM) followed by

The relationship between chimeric anti-PEG antibody binding and PEG length was analyzed by determining the concentration of antibody that produced 50% of the maximum signal by direct ELISA in plates coated with different sizes of PEG. A strong log-log correlation was observed between c3.3-IgG binding to immobilized PEG molecules and PEG molecular weight (**Fig. S-4a**). Of note, c3.3-IgG displayed almost identical binding to a linear PEG molecule of 10,000 Da and a branched chain methoxy PEG of 10,000 Da, further supporting the notion that PEG size is the primary determinant of c3.3-IgG binding. Binding of cAGP4-IgM weakly depended on PEG molecular size (**Fig. S-4b**).

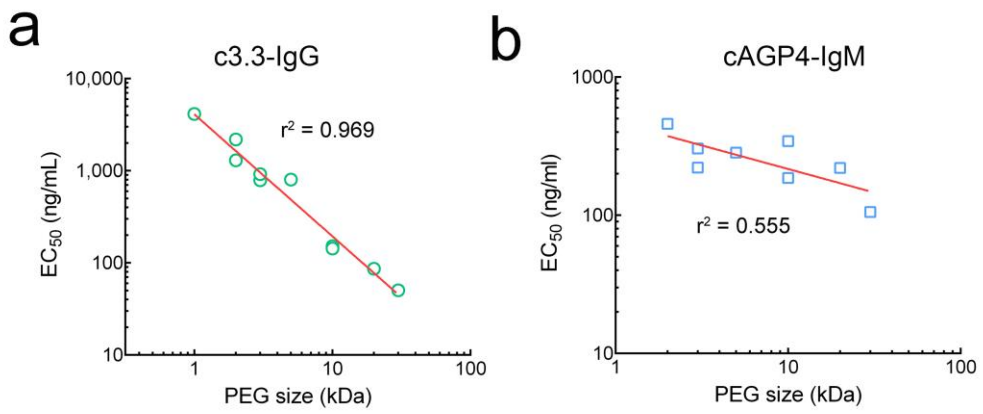


Figure S-4. Relationship between PEG size and anti-PEG antibody binding. Serial dilutions of c3.3-IgG and cAGP4-IgM were assayed by direct ELISA for binding to different sizes of immobilized PEG ($mPEG_{1000}$, $mPEG_{2000}$, NH_2-PEG_{2000}, NH_2-PEG_{3000}, $mPEG_{3000}$, PEG_{5000}, NH_2-$PEG_{10,000}$, branched $mPEG_{10,000}$, $mPEG_{20,000}$ and $mPEG_{30,000}$). The concentration of chimeric antibody producing 50% absorbance responses (EC_{50}) were calculated by interpolation of sigmoidal dose response curves plotted against the log of antibody concentration (correlation coefficients r^2 ranging from 0.9925 to 0.9994). Results show the linear regression curves for (**a**) c3.3-IgG EC_{50} versus PEG size and (**b**) cAGP4-IgM EC_{50} versus PEG size on log-log plots.

To further examine the specificity of chimeric antibodies, plates were coated with PEG and diamine PEG molecules, which do not possess terminal methoxy functionalities. Both c3.3-IgG and cAGP4-IgM bound to all the immobilized PEG molecules (**Fig. S-5**), further confirming that the chimeric antibodies bind to the repeating ethylene oxide backbone of PEG.

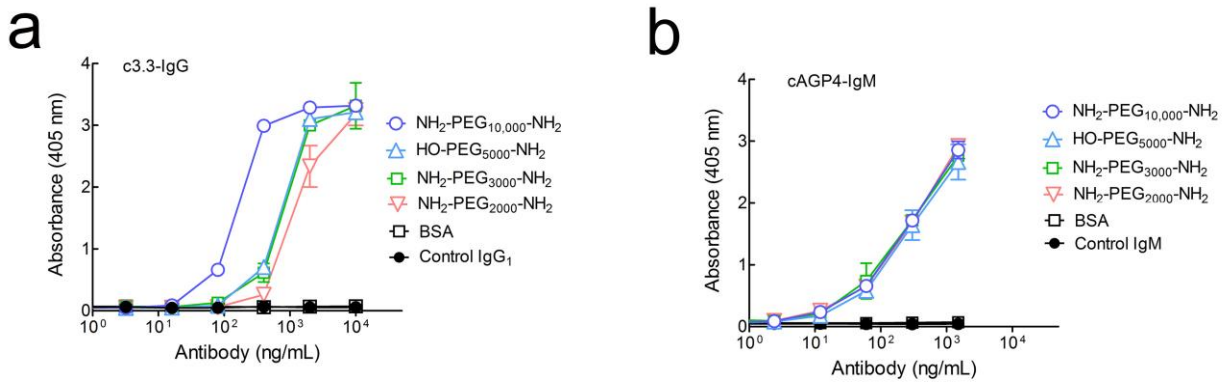


Figure S-5. Chimeric anti-PEG antibodies can bind immobilized PEG and amino-PEG molecules. Serial dilutions of c3.3-IgG (**a**) or cAGP4-IgM (**b**) were added in duplicate to ELISA plates coated with the indicated diamine and PEG molecules. BSA coated plates and control human IgG or IgM were used as negative control antigen and antibodies, respectively. Antibody binding was detected with HRP-labeled anti-human IgG (for c3.3-IgG) or anti-human IgM (for cAGP4-IgM) followed by addition of HRP substrate. Results show mean values. (Error bars, SD; n = 2).

To rule out the possibility that c3.3-IgG and cAGP4-IgM bind to amino groups present in immobilized PEG molecules or to chemical linkers present in some PEG molecules, binding of these antibodies was measured against PEG that was covalently attached to bovine serum albumin via four different linkers (cyanuric chloride, p-nitrophenyl carbonate, tresylate or succinimydyl propionic acid). Both chimeric antibodies bound equally well to the four PEGylated BSA molecules (**Fig. S-6**), demonstrating that the anti-PEG chimeric antibodies do not bind to amino groups in immobilized PEG or to the linker present in PEG molecules.

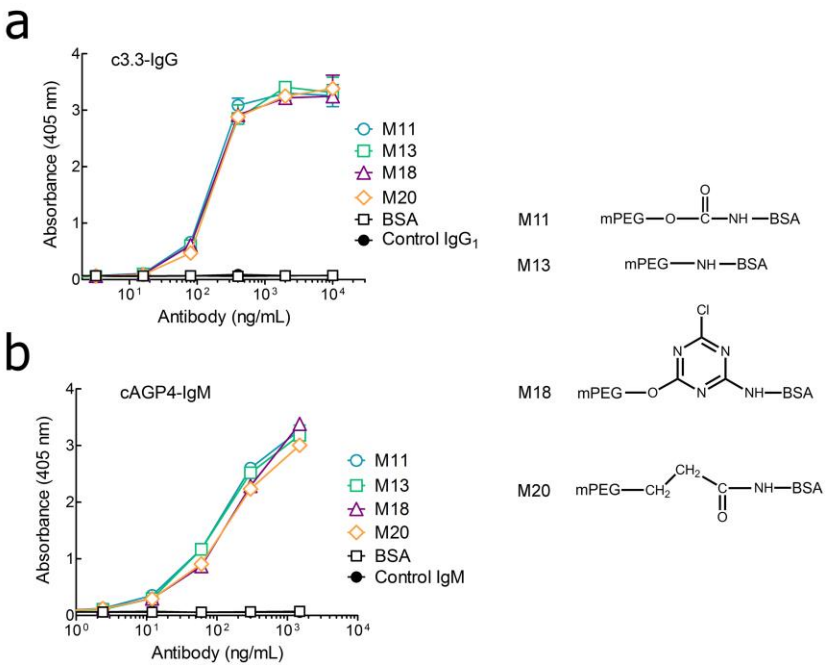


Figure S-6. Chimeric anti-PEG antibodies bind to PEGylated proteins independently of the attachment linker. Serial dilutions of c3.3-IgG (**a**) or cAGP4-IgM (**b**) were added to ELISA plates coated with BSA or PEGylated BSA formed with the indicated spacers. Negative control human IgG or IgM (solid circles) were also assayed. Antibody binding was detected with HRP-labeled anti-human IgG (for c3.3-IgG) or anti-human IgM (for cAGP4-IgM) followed by addition of HRP substrate. Results show mean values of duplicate determinations. Error bars, SD.

To assay anti-PEG antibodies, human plasma samples are first diluted 25-fold in PBS/2% (w/v) skim milk powder and then 50-fold and 100-fold dilutions are prepared in PBS containing 4% human reference serum and 2% (w/v) skim milk powder (dilution buffer) (**Fig. S-7**). Serial dilutions of c3.3-IgG or cAGP4-IgM are also made in dilution buffer. Plasma samples and duplicates of the serially diluted c3.3-IgG or cAGP4-IgM standards are assayed on plates coated with NH_2-$PEG_{10,000}$-NH_2. Samples producing a positive reading, defined as a mean absorbance that was at least three times higher than the background absorbance, were further assayed in a competition ELISA. Reduction of at least 35% in the absorbance reading of wells in the presence of excess PEG-liposomes as compared to wells without addition of PEG-liposomes were considered to be positive. The relative concentrations of IgG or IgM anti-PEG antibodies in plasma samples were then determined by comparison to the c3.3-IgG or cAGP4-IgM standard curves, respectively.

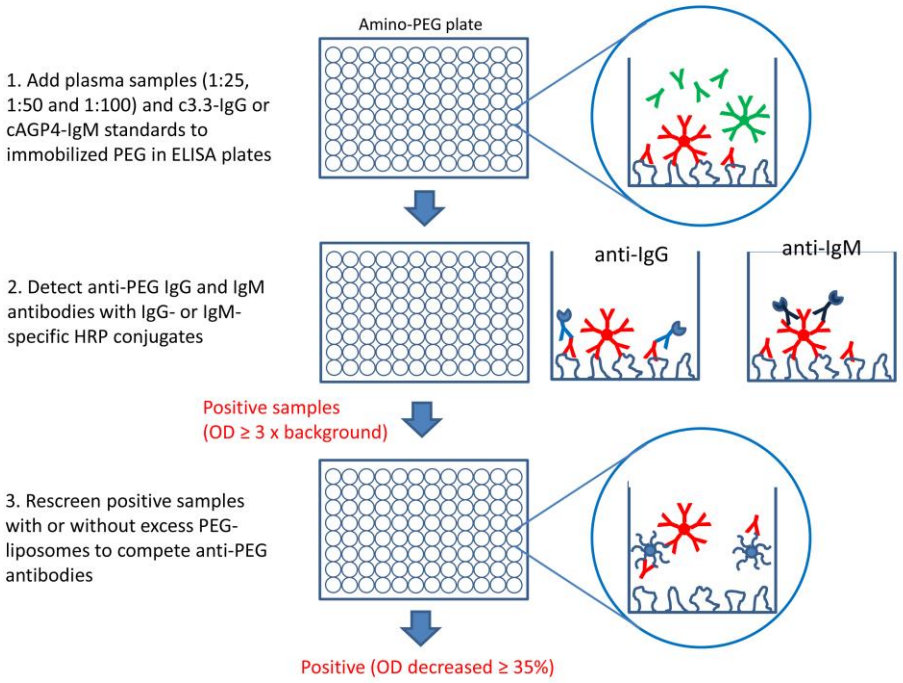


Figure S-7. Outline of anti-PEG antibody assay. Serial dilutions of plasma samples and c3.3-IgG or cAGP4-IgM chimeric antibody standards are assayed for binding to immobilized NH_2-$PEG_{10,000}$-NH_2 in 96-well plates. Samples that produce absorbance readings at least three times higher than background absorbance (dilution buffer) are assayed again with or without the addition of a PEG competitor (PEG-liposomes). Samples that display at least a 35% reduction in absorbance in the presence of the PEG competitor are considered positive.

An additional competition assay was used to confirm the specificity of anti-PEG antibodies and minimize false positives. Toward this aim, the ability of PEG or PEGylated compounds to compete binding of c3.3-IgG and cAGP4-IgM to immobilized PEG was examined. All the tested compounds effectively reduced c3.3-IgG binding by at least 35% (**Fig. S-8a**), which was arbitrarily set as the threshold value for confirmation of specific binding to PEG. By contrast, only PEG-liposomes and PEGylated beta-glucuronidase effectively competed the binding of cAGP4-IgM to immobilized NH_2-$PEG_{10,000}$-NH_2 (**Fig. S-8b**). For consistency, we selected PEG-liposomes (200X) in competition assays to confirm anti-PEG antibody binding to PEG.

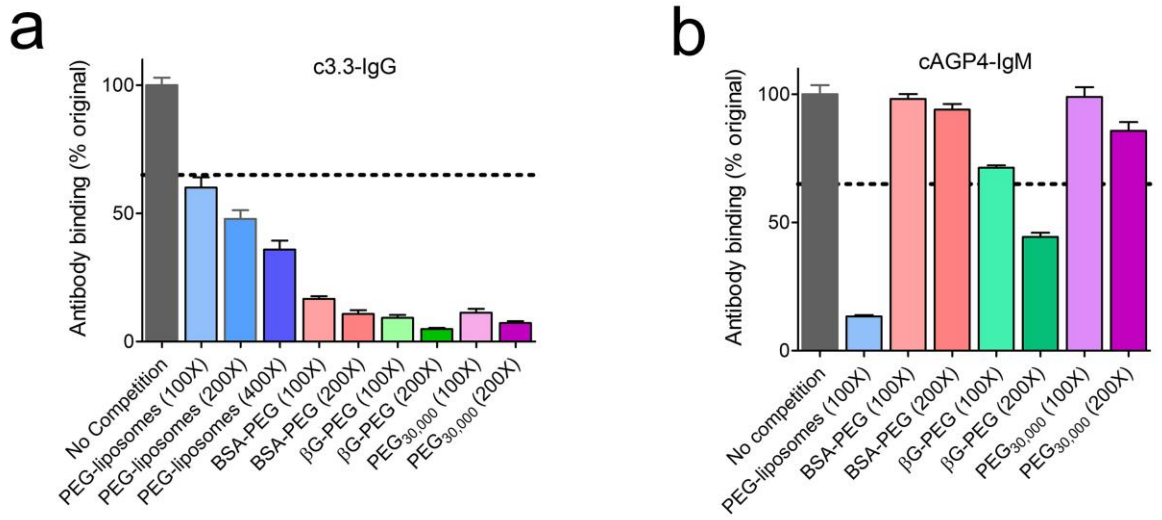


Figure S-8. Competition of chimeric human anti-PEG antibody binding to immobilized PEG. The ability of the indicated PEG compounds to compete the binding of 1 μg/mL c3.3-IgG (**a**) or 1 μg/mL cAGP4-IgM (**b**) is shown in comparison to the chimeric antibodies without competitor (black bars). PEG competitors were added at 100, 200 or 400 (100X, 200X and 400X) fold molar ratios to the respective chimeric antibodies. Results show mean values ± SD of triplicate determinations. The dashed lines indicate a 35% reduction in binding as compare with no competition.

Semi-logarithmic plots of c3.3-IgG (**Fig. S-9a**) and cAGP4-IgM (**Fig. S-9b**) were well represented by a sigmoidal dose response curve with variable slope with correlation coefficients (r^2) of 0.98 and 0.99, respectively. The mean EC_{50} (concentration of antibody producing 50% response in the ELISA) were 194.5 ± 1.03 ng/mL for c3.3-IgG and 117.0 ± 1.03 ng/mL for cAGP4-IgM (**Table S-1**).

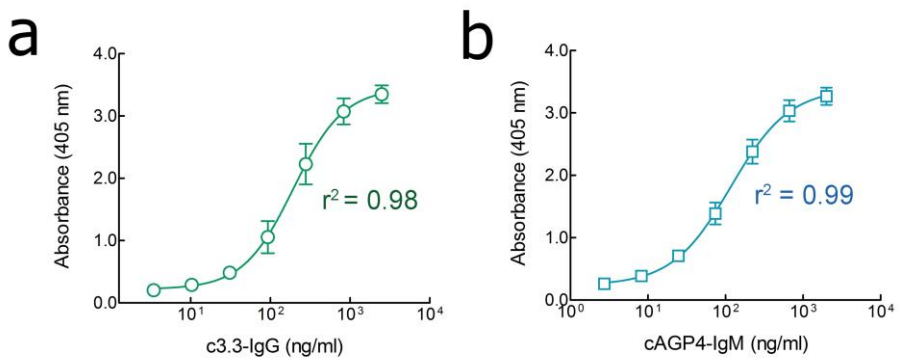


Figure S-9. Chimeric antibody standard curves. Sigmoidal dose response curve fit of a semi-log plot of forty-eight c3.3-IgG (**a**) or cAGP4-IgM (**b**) standard curves performed on eight separate days over a two-month period. Bars, SD.

Table S-1. Summary of reference antibody standard curves

Parameter	c3.3-IgG	cAGP4-IgM
Goodness of fit R^2	0.9777	0.9872
EC_{50} ± SE	194.6 ± 1.03 ng/mL	116.9 ± 1.03 ng/mL
95% confidence interval EC_{50}	183.6 – 206.2 ng/mL	111.1 – 122.9 ng/mL
Hill Slope ± SE	1.377 ± 0.0519	1.152 ± 0.0339
95% confidence interval Hill slope	1.275 – 1.479	1.085 – 1.218

Results are from eight independent assays, each with 8 replicates, performed over a period of more than two months. Each standard curve included seven 3-fold serial dilutions starting from 2500 ng/mL for c3.3-IgG and 2000 ng/mL for cAGP4-IgM. The data were transformed by X = Log (concentration) and fit to a variable slope sigmoidal dose-response curve in Graphpad Prism.

The specificity of anti-PEG antibodies was examined by performing a competition assay in which plasma samples were mixed with excess PEG-liposomes to specifically remove anti-PEG antibodies from plasma samples. PEG-liposomes effectively competed binding of both anti-PEG IgG (**Fig. S-10a**) and IgM (**Fig. S-10b**) in human samples, verifying the specificity of anti-PEG antibodies in plasma samples.

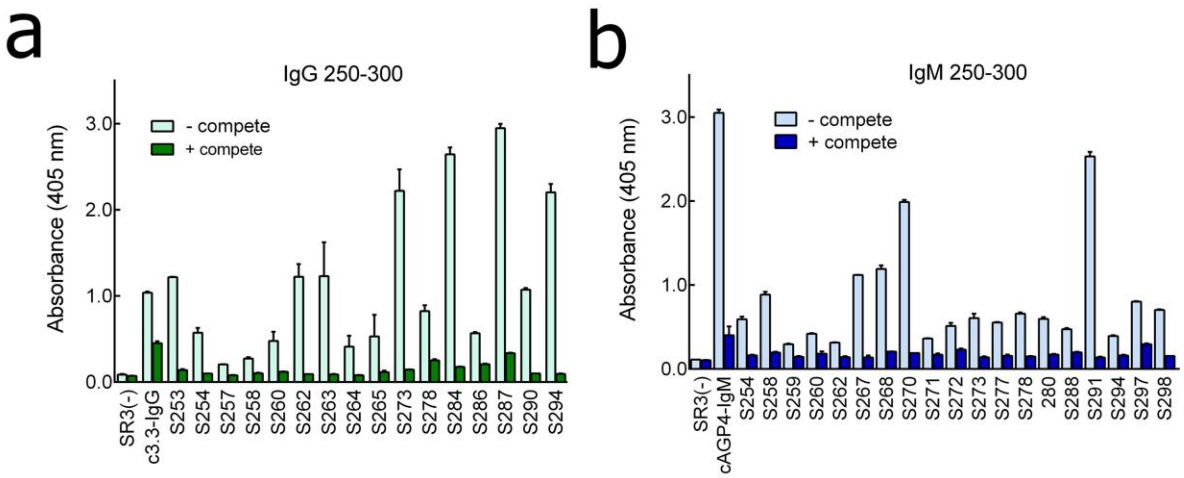


Figure S-10. Competition of anti-PEG antibodies. Plasma samples from donors 250 to 300 that tested positive for anti-PEG IgG (**a**) or anti-PEG IgM (**b**) were assayed without (- compete) or with (+ compete) addition of a 200-fold molar excess of PEG-liposomes to compete binding of the antibodies to NH_2-$PEG_{10,000}$-NH_2 in 96-well plates. SR3 represents human reference serum. (Bars, SE; n=2).

The specificity of secondary anti-human antibodies used to detect binding of anti-PEG antibodies to PEG-coated plates showed that HRP-labeled anti-IgM did not cross react with c3.3-IgG (**Fig. S-11a**) and HRP-labeled anti-IgG did not recognize cAGP4-IgM (**Fig. S-11b**), confirming specificity for the detection of IgM and IgG antibodies.

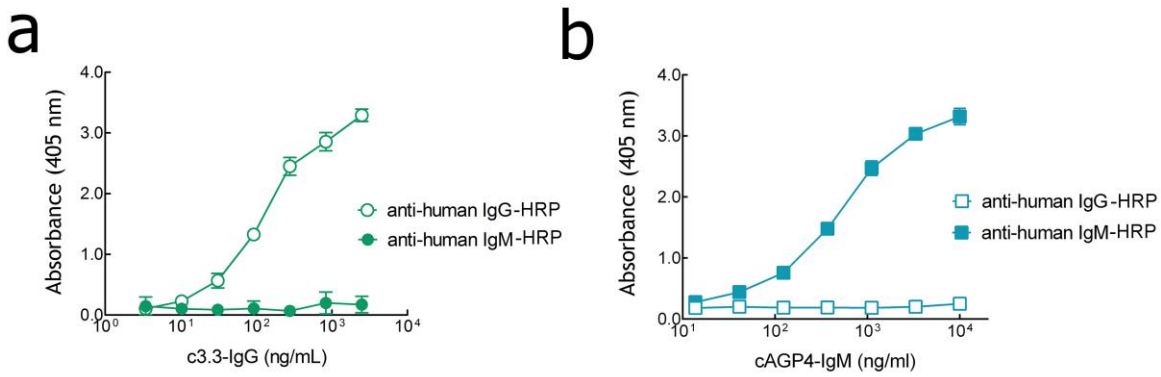


Figure S-11. Specificity of secondary antibodies. c3.3-IgG (**a**) and AGP4-IgM (**b**) binding to ELISA plates coated with NH_2-$PEG_{10,000}$-NH_2 was detected with HRP-conjugated anti-human IgG (open circles) or anti-human IgM (solid circles) secondary antibodies (Bars, SD; n=3).

The effect of human plasma on the anti-PEG assay was examined. Addition of 4% human reference plasma (pretested for the absence of anti-PEG antibodies) did not alter the binding of c3.3-IgG (**Fig. S-12a**) or cAGP4-IgG (**Fig. S-12b**) to immobilized NH_2-$PEG_{10,000}$-NH_2. Human plasma samples were therefore diluted 25 fold (corresponding to 4% plasma). The c3.3-IgG and cAGP4-IgM antibody standards and further dilutions of plasma samples were performed in buffers supplemented with 4% human reference serum to reduce the impact of matrix effects.

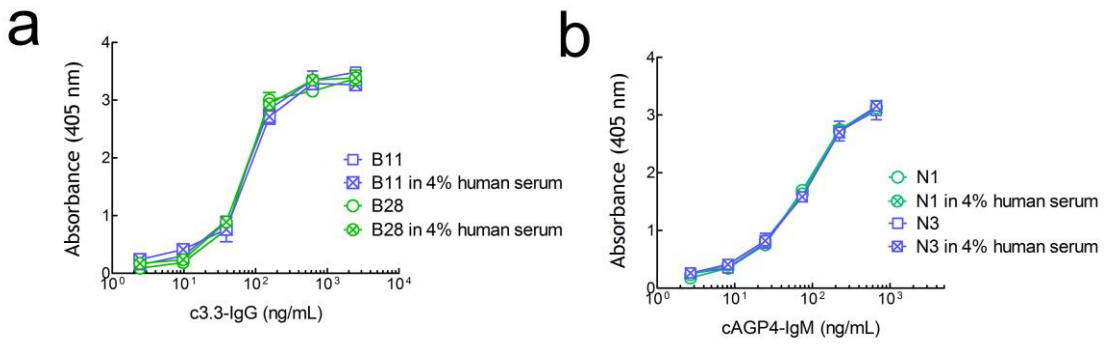


Figure S-12. Effect of human plasma on chimeric anti-PEG antibody binding to immobilized PEG. Two batches of (**a**) c3.3-IgG (B11 and B28) and two batches of (**b**) cAGP4-IgM (N1 and N3) were assayed for binding to NH_2-$PEG_{10,000}$-NH_2 coated in 96-well ELISA plates in the absence or presence of 4% human reference serum (Bars, SD; n=3).

Since samples may contain both anti-PEG IgG and IgM antibodies, we investigated possible cross competition of anti-PEG IgG and IgM antibodies in the assay. Addition of increasing concentrations of cAGP4-IgM to a fixed amount of c3.3-IgG did not alter the c3.3-IgG ELISA reading (**Fig. S-13a**). Addition of a 10-fold excess of c3.3-IgG to a fixed amount of cAGP4-IgM marginally decreased the cAGP4-IgM ELISA reading by 8% (**Fig. S-13b**). We conclude that the amount of PEG coating is sufficient to capture mixtures of anti-PEG IgG and IgM with limited competition effects, allowing assay of samples containing both anti-PEG IgG and IgM antibodies.

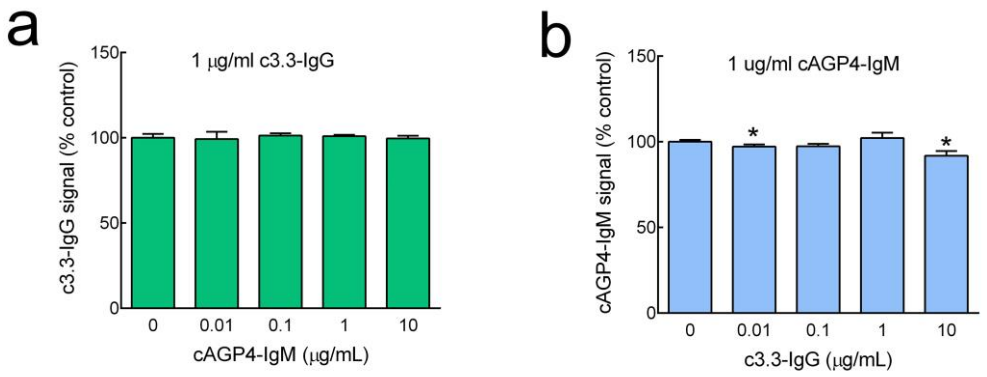


Figure S-13. Examination of competition between chimeric human anti-PEG IgG and IgM antibodies. The mean binding of 1 μg/mL c3.3-IgG in the presence of the indicated concentrations of cAGP4-IgM (**a**) or 1 μg/mL cAGP4-IgM in the presence of the indicated concentrations of c3.3-IgG (**b**) to immobilized NH_2-$PEG_{10,000}$-NH_2 is shown in comparison to no competition (n=3; bars, SD; *, $p < 0.05$ in comparison to no competition).

To investigate if PEG coating density in microtiter plates can strongly influence assay results, the binding of c3.3-IgG and cAGP4-IgM to plates coated with a 20-fold range of PEG concentrations was examined. Both c3.3-IgG and cAGP4-IgM binding was insensitive to the coating density of the PEG molecules (**Fig. S-14**).

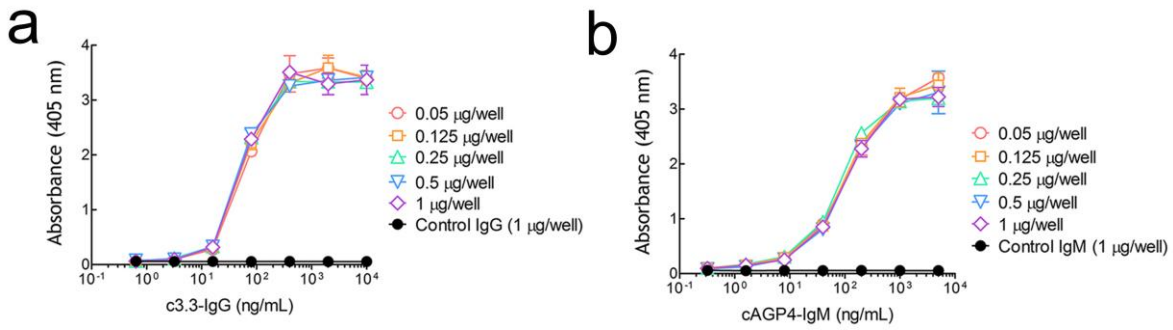


Figure S-14. Effect of PEG density on the binding of chimeric anti-PEG antibodies. Serial dilutions of c3.3-IgG (**a**) or cAGP4-IgM (**b**) were added in duplicate to ELISA plates coated with the indicated amounts of $mPEG_{20,000}$. Negative control human IgG or IgM were also assayed. Antibody binding was detected with HRP-labeled anti-human IgG (for c3.3-IgG) or anti-human IgM (for cAGP4-IgM) followed by addition of HRP substrate. Results show mean values, Error bars, SD.

The stability of immobilized PEG in microtiter plates was examined by repeatedly washing PEG-coated plates with PBS and then measuring c3.3-IgG and cAGP4-IgM binding. Similar dose-response curves were measured for plates washed once, ten times or thirty times (**Fig. S-15**). We conclude that NH_2-$PEG_{10,000}$-NH_2 is stably immobilized on microtiter plates.

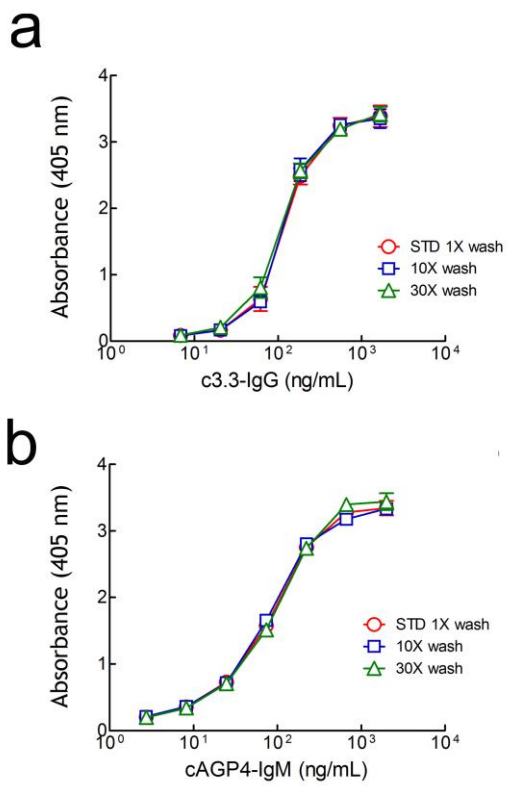


Figure S-15. Stability of immobilized PEG in ELISA plates. ELISA plates were coated with NH_2-$PEG_{10,000}$-NH_2 and blocked with skim milk. The plates were washed 1X, 10X or 30X with PBS before serial dilutions of c3.3-IgG (**a**) or cAGP4-IgM (**b**) were added in duplicate. Antibody binding was detected with HRP-labeled anti-human IgG (for c3.3-IgG) or anti-human IgM (for cAGP4-IgM) followed by addition of HRP substrate. Results show mean values. (Error bars, SD; n = 2).

Detergents with ethylene oxide repeats, such as Tween 20, can compete anti-PEG antibody binding to PEGylated compounds.[11] We therefore examined if the presence of Tween 20 in wash buffers influences measurement of anti-PEG antibodies in human plasma samples. Indeed, 0.05% Tween 20 in the wash buffers greatly decreased the detection of both anti-PEG IgG and IgM antibodies in human plasma samples as compared to the same ELISA using 0.1% CHAPS in the wash buffers (**Fig. S-16**). CHAPS detergent is therefore used in all wash buffers for the anti-PEG antibody assay.

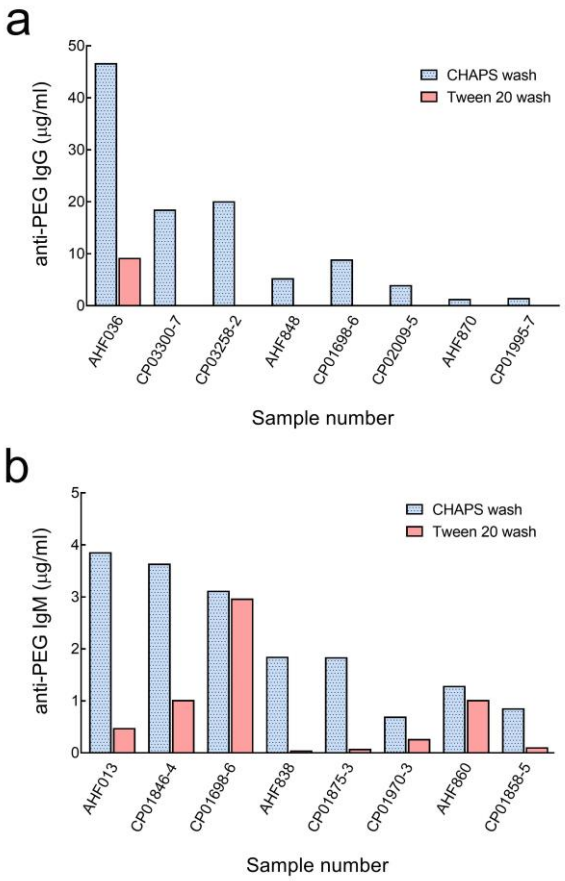


Figure S-16. Tween 20 can interfere with the measurement of anti-PEG antibodies. Selected plasma samples from donors that previously tested positive for anti-PEG IgG (**a**) or anti-PEG IgM (**b**) antibodies were assayed in the standard anti-PEG assay (using 0.1 % CHAPS detergent in all wash buffers) or in an analogous anti-PEG assay that used 0.05% Tween 20 in the wash buffers. Results show the calculated anti-PEG IgG or IgM antibody concentrations in each sample.

Table S-2. Comparison of the prevalence of anti-PEG antibodies among different studies.

Year	Sample population	Sample number	Females/ males	Anti-PEG antibody positive	Anti-PEG IgM positive	Anti-PEG IgG positive	Assay method	Citation
1984	Naive healthy donors	453	NR	0.2%	NR	NR	Hemagglutination	[12]
1984	Naive allergy patients	92	NR	3.3%	NR	NR	Hemagglutination	[12]
2004	Naive healthy donors	250	NR	25%	14%	18%	Haemagglutination	[13]
2007	Gout patients	24	4/20	NR	NR	8.3%	Direct ELISA against 10-kDa mPEG-glycine	[14]
2011	Naive healthy donors	350	NR	4.3%	NR	NR	Bridging assay using hapten-$PEG_{40,000}$	[15]
2014	Naive severe gout patients	30	8/22	19%	NR	NR	Direct ELISA against 10-kDa mPEG-glycine + competition ELISA	[16]
2015	Naive acute coronary syndrome patients	354	NR	36%	NR	NR	Direct ELISA against 10-kDa mPEG-nitrophenyl carbonate + competition ELISA	[17]
2015	Naive chronic hepatitis B infected HBeAg+ subjects	32	NR	6.3%	NR	NR	Bridge assay using PEG-IFN or direct ELISA	[18]
2016	Naive healthy donors	1504	748/756	44.3%	27.1%	25.7%	Direct ELISA against 10-kDa NH_2-PEG-NH_2 + competition ELISA	Present study

NR, not reported

The incidence of anti-PEG IgG antibodies in the overall population as a function of age was calculated for years in which 15 individuals were assayed. There was trend of increased frequency of anti-PEG IgG antibodies in younger individuals (**Fig. S-17**). The relationship between anti-PEG IgG frequency and age was fit with a one phase exponential decay model (anti-PEG IgG positive (%) = $201e^{-0.073*Age} + 17.0$, $r^2 = 0.621$).

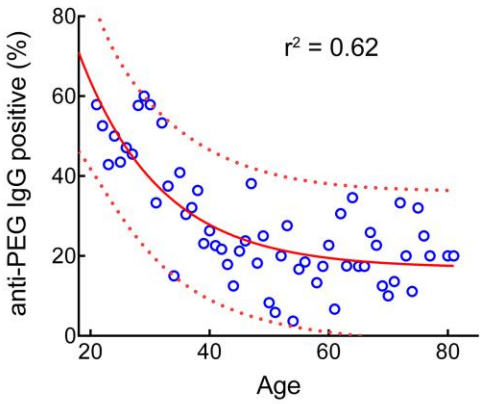


Figure S-17. Anti-PEG IgG incidence decreases with age. The percentage of donors with anti-PEG IgG is shown for each yearly age group (for age groups with $n \geq 15$). A one phase exponential decay model is shown for anti-PEG IgG frequencies versus age. Dotted lines show 95% prediction intervals.

The incidence of anti-PEG IgM antibodies in females and males was calculated for two year intervals in groups that had at least 10 donors. Two year intervals were used to accrue sufficient donors in each interval. The incidence of anti-PEG IgM antibodies in the overall population as a function of age was also calculated for years in which at least 15 individuals were assayed. There were no significant or obvious relationships between the frequency of anti-PEG IgM antibodies and age in any of the groups (**Fig. S-18**).

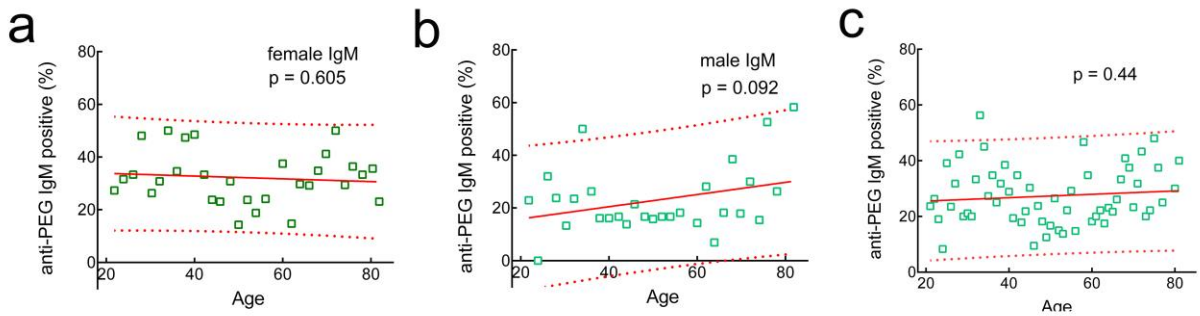


Figure S-18. Anti-PEG IgM incidence is not associated with age. The percentage of females (**a**) or males (**b**) with anti-PEG IgM is shown for two-year age groups (for age groups with n ≥ 10). (**c**) The percentage of all donors with anti-PEG IgM is shown for each yearly age group (for age groups with n ≥ 15) is shown. p values indicate if the slope of the regression line is significantly different from zero. Dotted lines show 95% prediction intervals.

The concentration of anti-PEG IgG antibodies in the plasma of donors that were positive for anti-PEG IgG was examined. A significant trend of decreasing IgG concentrations with increasing donor age was noted in the overall population ($p = 0.0005$; **Fig. S-19**).

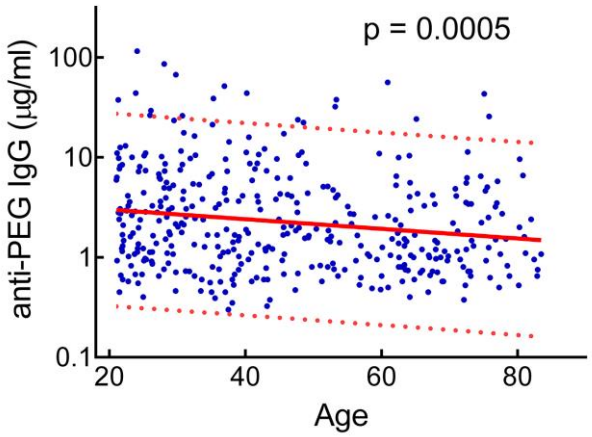


Figure S-19. Anti-PEG IgG incidence decreases with age. The concentration of anti-PEG IgG in positive donors is shown versus donor age. Linear regression lines are shown in red. p values indicate if the slope of the regression line is significantly different from zero. Dotted lines show 95% prediction intervals.

The concentration of anti-PEG IgM antibodies in the plasma of donors that were positive for anti-PEG IgM was examined. However, no significant associations were found between IgM concentrations and age in females (p = 0.303), males (p = 0.227) or in the overall population (p = 0.92) (**Fig. S-20**).

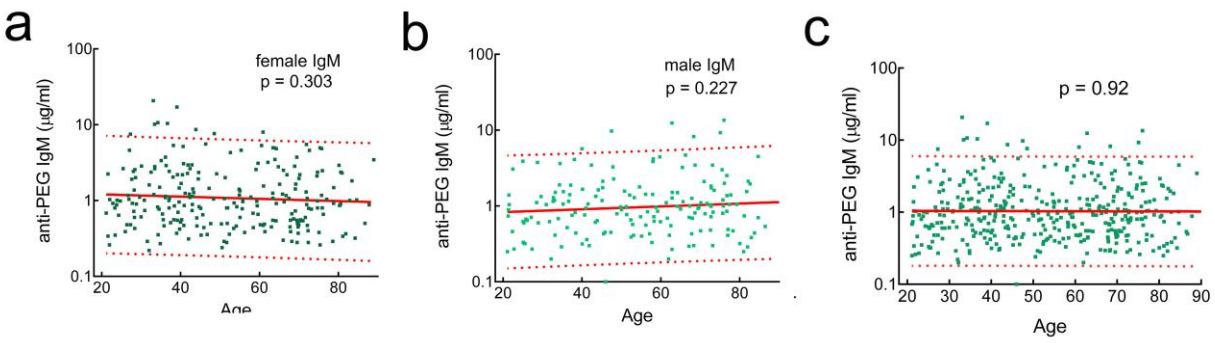


Figure S-20. Anti-PEG IgM concentration is independent of donor age. The concentration of anti-PEG IgM in IgM-positive females (**a**), males (**b**) and the overall population (**c**) is shown versus donor age. Linear regression lines are shown in red. p values indicate if the slope of regression lines are significantly different from zero. Dotted lines show 95% prediction intervals.

References

(1) Gilliland, L. K.; Norris, N. A.; Marquardt, H.; Tsu, T. T.; Hayden, M. S.; Neubauer, M. G.; Yelton, D. E.; Mittler, R. S.; Ledbetter, J. A. *Tissue Antigens* **1996**, *47*, 1-20.

(2) Fang, J.; Qian, J. J.; Yi, S.; Harding, T. C.; Tu, G. H.; VanRoey, M.; Jooss, K. *Nat Biotechnol* **2005**, *23*, 584-590.

(3) Su, Y. C.; Al-Qaisi, T. S.; Tung, H. Y.; Cheng, T. L.; Chuang, K. H.; Chen, B. M.; Roffler, S. R. *MAbs* **2014**, *6*, 1069-1083.

(4) Zufferey, R.; Nagy, D.; Mandel, R. J.; Naldini, L.; Trono, D. *Nat Biotechnol* **1997**, *15*, 871-875.

(5) Kahl, C. A.; Marsh, J.; Fyffe, J.; Sanders, D. A.; Cornetta, K. *J Virol* **2004**, *78*, 1421-1430.

(6) Chung, T.-W.; Chung, C.-H.; Lue, Y.-F. *Anal Biochem* **2000**, *285*, 264-267.

(7) Cheng, T.-L.; Chen, B.-M.; Chan, L.-Y.; Wu, P.-Y.; Chern, J.-W.; Roffler, S. R. *Cancer Immunol Immunother* **1997**, *44*, 305-315.

(8) Su, Y.-C.; Chen, B.-M.; Chuang, K.-H.; Cheng, T.-L.; Roffler, S. R. *Bioconjugate Chem.* **2010**, *21*, 1264-1270.

(9) Bartlett, G. R. *J Biol Chem* **1959**, *234*, 466-468.

(10) Kao, C.-H.; Wang, J.-Y.; Chuang, K.-H.; Chuang, C.-H.; Cheng, T.-C.; Hsieh, Y.-C.; Tseng, Y.-l.; Chen, B.-M.; Roffler, S. R.; Cheng, T.-L. *Biomaterials* **2014**, *35*, 9930-9940.

(11) Sherman, M. R.; Williams, L. D.; Sobczyk, M. A.; Michaels, S. J.; Saifer, M. G. *Bioconjugate Chem.* **2012**, *23*, 485-499.

(12) Richter, A.; Åkerblom, E. *Int. Arch. Allergy Immunol.* **1984**, *74*, 36-39.

(13) Garratty, G. *Transfus Med Rev* **2004**, *18*, 245-256.

(14) Sundy, J. S.; Ganson, N. J.; Kelly, S. J.; Scarlett, E. L.; Rehrig, C. D.; Huang, W.; Hershfield, M. S. *Arth. Rheum.* **2007**, *56*, 1021-1028.

(15) Liu, Y.; Reidler, H.; Pan, J.; Milunic, D.; Qin, D.; Chen, D.; Vallejo, Y. R.; Yin, R. *J Pharmacol Toxicol Methods* **2011**, *64*.

(16) Hershfield, M. S.; Ganson, N. J.; Kelly, S. J.; Scarlett, E. L.; Jaggers, D. A.; Sundy, J. S. *Arthritis Res. Ther.* **2014**, *16*, R63.

(17) Ganson, N. J.; Povsic, T. J.; Sullenger, B. A.; Alexander, J. H.; Zelenkofske, S. L.; Sailstad, J. M.; Rusconi, C. P.; Hershfield, M. S. *J. Allergy Clin. Immunol.* **2015**.

(18) Myler, H.; Hruska, M. W.; Srinivasan, S.; Cooney, E.; Kong, G.; Dodge, R.; Krishna, M.; Zhu, J.; Felix, T.; Gleason, C. *Bioanalysis* **2015**, *7*, 1093-1106.