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Optimized ELISA for Anti-PEG Antibody Detection in Healthy Donors and Patients Treated with PEGylated Liposomal Doxorubicin

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Abstract

There is considerable interest in quantifying anti-PEG antibodies, given their potential involvement in accelerated clearance, complement activation, neutralization, and acute reactions associated with drug delivery systems. Published and commercially available anti-PEG enzyme-linked immunosorbent assay (ELISA) differ significantly in terms of reagents and conditions, which could be confusing to users who want to perform in-house measurements. Here, we optimize the ELISA protocol for specific detection of anti-PEG IgG and IgM in sera from healthy donors and in plasma from cancer patients administered with PEGylated liposomal doxorubicin. The criterion of specificity is the ability of free PEG or PEGylated liposomes to inhibit the ELISA signals. We found that coating high-binding plates with monoamine methoxy-PEG_{5000}, as opposed to bovine serum albumin-PEG_{20000}, and blocking with 1% milk, as opposed to albumin or lysozyme, significantly improves the specificity, with over 95% of the signal being blocked by competition. Despite inherent between-assay variability, setting the cutoff value of the optical density at the 80^{th} percentile consistently identified the same subjects. Using the optimized assay, we longitudinally measured levels of anti-PEG IgG/IgM in cancer patients before and after the PEGylated liposomal doxorubicin chemotherapy cycle (1 month apart, 3 cycles total). Antibody titers did not show any increase but rather a decrease between treatment cycles, and up to 90%

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of antibodies were bound to the infused drug. This report is a step toward harmonizing anti-PEG assays in human subjects, emphasizing the cost-effectiveness and optimized specificity.

Keywords

PEG; ELISA; Doxil; PEGylated liposomal doxorubicin; patients; specific

1. INTRODUCTION

PEGylation is the critical pharmaceutical technology for improving colloidal stability, biocompatibility, and stealth properties of biomaterials, biologics, and nanopharmaceuticals for therapy, imaging, and vaccination purposes [1, 2]. Anti-PEG antibodies in humans were reported over four decades ago [3]. Anti-PEG antibodies have been suggested to promote complement activation, premature clearance, and adverse reactions in animals, with circumstantial evidence linking similar effects in humans, as extensively reviewed [4]. Many groups have demonstrated boosted levels of anti-PEG antibodies due to PEGylated drug treatments in animal models [5, 6] and PEGylated mRNA lipid nanoparticle (LNP) vaccines in humans [7, 8]. The literature indicates a significant increase in seropositive donors over the years, likely due to exposure to cosmetics and hygiene products [9, 10]. While several groups attempted to standardize anti-PEG enzyme-linked immunosorbent assay (ELISA) [11, 12], the available protocols exhibit significant diversity in selecting coating agents, blocking agents, washing buffers, and competition reagents (summarized in Table 1).

Theoretically, anti-PEG ELISA assays should incorporate the presence of free PEG or PEGylated agents to derive the “true” anti-PEG antibody titers [13]. As shown in Table 1, some investigators do not report competition with PEGylated reagents, and therefore, the specificity is not clear. These technical discrepancies are confusing to researchers who are new to the field and who are “shopping” for a simple and specific assay. Furthermore, the lack of specificity could be one of the reasons for the wide variability in the prevalence of anti-PEG antibodies, ranging from 20% to 99% [11, 12, 14–16], and calls for re-evaluation and protocol optimizations. While we did not aim to evaluate multiple conditions and protocols since this was done previously [11, 15], here we summarize our efforts to compare some of the reported conditions, including coating, blocking, and washing reagents, with the overall goal of optimizing assay conditions for longitudinal measurements in anti-PEG titers in ovarian cancer patients treated with multiple cycles of PEGylated liposomal doxorubicin (hereafter PLD).

2. MATERIALS AND METHODS

2.1 Materials:

Purified human serum albumin (Cat# 800-125P) was from Gemini Bio-Products. Recombinant bovine serum albumin (molecular biology grade, Cat#76285-184) was from VWR. Purified bovine serum albumin fraction V (Cat# BP1600), Greiner high binding ELISA plates (Cat# 07-000-102), and lysozyme (Cat# 89833) were from ThermoFisher. Purified bovine serum albumin fraction V (Cat# 160069) was from MP Biomedicals (Solon,

OH, USA). IgG/IgM depleted heat-inactivated serum (Cat# 34020-1) was from Pel-Freez Biological. Instant non-fat milk (Cat# A614-1005) was from Foothold USA (Landover, MD). 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (Cat# 555214) was from BD. Peroxidase AffiniPure™ Goat Anti-Mouse IgG (Cat# 115-035-003), goat anti-human IgG Fc specific (Cat# 109-001-008), and goat-anti-human IgM Fc5μ specific (Cat# 109-035-129) were from Jackson ImmunoResearch Laboratories, Inc. Mouse monoclonal anti-PEG IgG (Cat# 9B5-6-25-7; methoxy, and Cat# 5D6-3; backbone) were from Life Diagnostics, Inc. Monoamine-mPEG$_{5000}$ (Cat# MPEG-NH2-5000) and succinimidyl valerate SVA-mPEG$_{20000}$ (Cat# MPEG-SVA-20K) were from Laysan Bio (Arab, AL). For conjugation, BSA in PBS (1 mL, 10 mg/mL) was combined with a 16-fold excess of SVA-mPEG$_{20000}$. The reaction mixture was incubated at 4°C for 12h and purified using an Amicon Ultra Centrifugal Filter, 50 kDa MWCO (Thermo Fisher). Whatman Nucleopore Track-Etch Membranes (Cat# 800309, 100nm) were from Cytiva. Egg phosphatidylethanolamine (Cat# 840051C) was from Avanti Polar Lipids (Alabaster, AL, USA), and distearoyl phosphatidylethanolamine (DSPE)-mPEG$_{2000}$ (Cat # SUNBRIGHT DSPE-020CN) was from NOF America Corporation. Lipids were kept in chloroform (32.5mM EPC and 10mM DSPE-mPEG$_{2000}$) at −20ºC. PEGylated liposomal doxorubicin (DOXOrubicin Hydrochloride Liposome Injection) was obtained from the University of Colorado Cancer Center pharmacy as leftovers after infusion in sterile vials.

2.2 Liposome preparation:

Egg PC/DSPE-mPEG$_{2000}$ (95:5 molar ratio) was dried under a nitrogen stream. The dry lipid cake was resuspended in PBS for a total lipid concentration of 17 mM (0.855 mM of DSPE-mPEG$_{2000}$) and incubated at 37°C for 30 minutes. The solution was then vortexed for 2 min, and lipids were extruded by a syringe extruder (Avanti Polar Lipids) through 100nm Nucleopore Track-Etch membranes (13-15 times). Liposome size was confirmed to be below 120 nm with ZetaSizer Nano (Malvern, UK). Liposomes were stored at 4°C before use.

2.3 Serum samples:

Blood (3–5 mL) from anonymous healthy donors was collected in 2018-2019 (16 males, 13 females, ages 23 to 79 years) at the University of Colorado Blood Donor Center, according to the Colorado Multiple Institutional Review Board (COMIRB) protocol for anonymous collection as part of blood donation. Blood was collected in Vacutainer® Z (no additives). Blood from consented ovarian cancer patients was collected between 2022 and 2023 according to the COMIRB protocol 22-0083. Blood was collected in lipirudin tubes before the infusion (3-5 mL) and immediately after the infusion (3-5 mL) of PLD. Blood from the BALB/c mouse strain was collected without anticoagulant at the University of Colorado Anschutz Medical Campus animal facility. Serum or plasma was prepared and handled according to the protocol described previously [17]. Aliquots were stored at −80ºC and were subjected to no more than three freeze-thaw cycles.

2.4 Human anti-PEG IgG and IgM ELISA:

High-binding 96-well microplates were coated with 100 μL of 0.02 mg/mL NH_2-mPEG$_{5000}$ in PBS overnight at room temperature. Wells were blocked with 300 μL of 1% (w/v)

milk/PBS at room temperature for 1 h. Serum samples were diluted in 1% w/v milk/PBS in the Eppendorf microtubes. For competition control, 5 μL of the competitor (EPC/DSPE-$mPEG_{2000}$, PLD, or diol-PEG_{3350}) or PBS was added to 100 μL of diluted serum and incubated at room temperature for 20 min before adding to the plate. Samples were incubated in triplicates on the plate for 1 h at room temperature. For washing, the plate was inverted and tapped gently against clean paper towels; the wells were washed with 400 μL of 1xPBS three times. IgG or IgM was detected by corresponding HRP conjugated antibodies diluted in 1% milk/PBS to 1:5,000. Antibody (100 μL) was added per well and incubated at room temperature for 1 h. Wells were washed in 1xPBS (400 μL) three times. The signal was developed by adding 100 μL TMBS in the dark for 10 min; the reaction was stopped with 50 μL of 2N H_2SO_4. The values (optical density, O.D.) were determined by subtracting OD570 from OD450 and plotted with Prism v.10. The 80th percentile (a value where 80% of the values are lower and 20% of the values are higher than the O.D. values in the group) was calculated with Prism.

3. RESULTS

ELISA for the detection of anti-PEG antibodies in human serum (Table 1) is based on coating high-binding plates with PEG, blocking with albumin or milk, and detection with anti-human specific antibody linked to horseradish peroxidase (HRP). There are a wide variety of reported reagents used for blocking, coating, washing, and inhibition (Table 1). We explored combinations of coating and blocking reagents based on their availability in the laboratory. Since purified human and mouse bovine serum albumin could contain some immunoglobulin that can be recognized by the HRP-conjugated antibodies, we first tested the binding of the detection antibodies to microwell plates coated with different albumins. Purified BSA from 2 sources (ThermoFisher and MP Biomedicals) and recombinant BSA did not show appreciable binding of anti-human IgG-HRP, whereas purified HSA showed some non-specific binding (Fig. 1A). Therefore, we used BSA rather than HSA in the subsequent experiments. For the plate coating, we prepared multi-$mPEG_{20000}$-BSA or used commercially available monoamine-$mPEG_{5000}$. For blocking, we compared 1% w/v BSA, 1% w/v lysozyme, and 1% w/v fat-free milk. The serum was diluted 100x in 1% milk/PBS. Because anti-PEG ELISA is negatively affected by PEG-based detergents (e.g., Tween-20) [15], we used 1xPBS without detergent in all washing steps, as reported by the Lai group [18]. In sera from 3 healthy donors, a combination of monoamine-$mPEG_{5000}$ for coating and 1% w/v milk for blocking resulted in the best specificity (Fig. 1B), with over 98% of the signal inhibited by PLD (95 μg/mL doxorubicin, corresponding to 108 μg/mL $mPEG_{2000}$) and multi-mPEG20k-BSA (240 μg/mL albumin, approximately 600-800 μg/mL PEG). Lysozyme blocking showed lower signals in all conditions, suggesting that it induces some changes in PEG accessibility. Coating with multi-mPEG20k-BSA resulted in the least specificity and a higher residual signal after the inhibition (Fig. 1C). While the reason is not clear, it could be due to less efficient plate coating by “stealthy” PEGylated BSA, leaving more gaps for nonspecific antibody binding.

To compare the specificity of anti-PEG IgG vs. anti-PEG IgM ELISA, we incubated the serum of the same donor with different concentrations of PLD or diol-PEG_{3350} (as an example of soluble PEG) for 1h. Then, we added serum to ELISA plates coated with

monoamine-mPEG$_{5000}$ and blocked with 1% milk (final 1:100 dilution in 1% w/v milk/PBS). The signal was reduced by >95% by 24 μg/mL PLD (27.1 μg/mL mPEG$_{2000}$) for both IgG and IgM (Fig. 2A). Diol-PEG$_{3350}$ also inhibited the signal but was less efficient, requiring 0.48 mg/mL to reduce the IgG signal by 93%, and 1.19 mg/mL to reduce the IgM signal by 97% (Fig. 2B).

Next, we employed commercially available mouse monoclonal anti-methoxy PEG (mPEG) and anti-backbone IgG antibodies to establish linearity. These antibodies were diluted in 1% milk and detected with anti-mouse IgG-HRP. The assay produced a linear signal in the range of 78–625 ng/mL for both antibodies and a complete inhibition by PLD (Fig. 3A). To establish the repeatability, we repeated the ELISA of anti-PEG IgG and IgM 9 times using sera from the same donor. The assay showed a coefficient of variation of 17.5% for IgG and 22.8% for IgM (Fig. 3B). Given the variability, in the subsequent experiments, we included a common serum sample in all plates to normalize the values.

Next, we measured anti-PEG antibody levels in 29 healthy donors using PEGylated liposomes or diol-PEG$_{3350}$ added to every serum sample. While PLD showed robust inhibition, it is expensive and hazardous. Instead, we prepared 100 nm doxorubicin-free PEGylated liposomes (EPC/DSPE-mPEG$_{2000}$; 95:5 mole ratio) as a cheaper and commonly available alternative. PEGylated liposomes (corresponding to 83 μg/mL mPEG$_{2000}$) completely inhibited the IgG and IgM signals (Fig. 4A), whereas diol-PEG$_{3350}$ (4.7 mg/mL) did not completely inhibit the IgM signals in some donors (Fig. 4B). Upon subtraction of the O.D. values of the inhibitor, we observed similar trends using both inhibitors, with 76% and 64% of donors having above-baseline IgG (Fig. 5A) and IgM (Fig. 5B) levels, respectively. There were some relative differences between donors in both assays. However, the donors in the 80th percentile of O.D. values (e.g., have a value greater than 80% of the data points within the dataset) were the same individuals (Fig. 5A–B, arrows), suggesting that setting the cutoff values is useful to mitigate the inherent variability of the assay.

While numerous animal experiments and human studies concluded that anti-PEG antibodies are boosted in response to injection of PEGylated carriers, studies in animals suggested that clinical doses of PLD do not cause an increase in the anti-PEG titers [19, 20], which was explained by the cytotoxic effect of PLD on immunoglobulin-producing B-cells in the spleen [4, 21]. Pharmacokinetic studies did not find evidence for accelerated clearance of PLD in patients upon repeated dosing [22]. However, to the best of our knowledge, measurements of anti-PEG antibodies in patients at different treatment cycles have not been performed. We collected plasma from ovarian cancer patients (Table 2) who were administered with PLD (40mg/m^2). Each patient had the blood drawn prior to the infusion and right after the infusion (~40 min). For all patients (n=9), we collected pre-infusion and post-infusion plasma for at least one treatment cycle, whereas 4 patients had their plasma collected in 3 cycles (1 month apart), and 3 patients had their plasma collected in 2 cycles. We used the optimized ELISA to measure the levels of anti-PEG IgG and IgM in plasma and used PLD (95 μg/mL doxorubicin, corresponding to 108 μg/mL mPEG$_{2000}$) as the inhibitor for all pre-infusion samples (Fig. 6A). We used 1:10 dilution since all patients had low levels of antibodies at 1:100 dilutions. According to Fig. 6B–C, none of the patients showed an increase in the titer of anti-PEG IgG and IgM during the treatment, suggesting that anti-PEG

antibodies are not boosted. Based on the inhibition of pre-infusion plasma signals with PLD, patient 4 and patient 6 were clearly seropositive for IgG, and patient 4 was seropositive for IgM (Fig. 6B–C, arrows), whereas others showed some inhibition of signals, but the pre-infusion values were close to the background to begin with. Interestingly, in patient 6, who showed elevated levels of IgG in the preinfusion plasma, there was a decrease in the anti-PEG IgG levels upon subsequent cycles. Furthermore, a large fraction of the signal was inhibited after the infusion, and over 95% of the signal was inhibited by PLD in vitro, confirming both the specificity of the assay and the interaction between infused PLD and anti-PEG antibodies in vivo.

4. DISCUSSION

Standardization and specificity are critical to using anti-PEG antibodies as biomarkers of toxicological responses and nano/bio-pharmaceutical biocompatibility. While this study did not attempt to test multiple conditions and reagents as was done previously [11, 15], we propose a straightforward and economical protocol, utilizing readily available commercial reagents without expensive detergents. Using monoamine $mPEG_{5000}$ for plate coating and 1% milk for blocking achieved the best specificity (e.g., over 95% inhibition with PEGylated liposomes). Other lengths of PEG and other concentrations of milk can be explored (Table 1), but it is beyond the scope of this study. We deem confirming specificity through competitive inhibition by PEGylated reagents to be essential, and our data suggest that PEGylated liposomes are more efficient competitors than diol-PEG_{3350}. Using PEGylated liposomes is especially relevant for our study as we aimed to detect anti-PEG antibodies that interact with PLD, and PLD and EPC/DSPE-$mPEG_{2000}$ are interchangeable. We suggest that the choice of competition reagent should preferentially be the same type as the PEGylated drug used (Table 1).

We measured longitudinal titers of anti-PEG IgG and IgM in patients treated with PLD. As was suggested in animal studies [21], our data for the first time show no boosting of antibodies in patients at clinical doxorubicin doses (40 mg/m^2). Rather, we demonstrate a decrease in titers in seropositive patients, which is in line with the hypothesis of the induced damage to the anti-PEG antibody-producing immune compartment [19]. Furthermore, as a validation of the assay specificity, we demonstrate that the infused PLD depletes the antibody, representing the ideal case of the competition experiment.

Despite the specificity of the assay, there was certain between-experimental variability in O.D. values, likely due to differences in the coating, incubation times, and washings/readouts. Chimeric human-mouse monoclonal antibodies [11, 12] can be used to standardize between experiments and reduce variability across different labs. We addressed this problem by including a common serum sample with a high O.D. signal for IgG and IgM in every plate for normalization. Furthermore, the serum contains different clones and isoforms of anti-PEG antibodies with varying degrees of glycosylation and various affinities and specificities toward different PEGylation types, densities, architectures, and different complement fixation efficiencies [12, 23–25]. This raises the question of whether the absolute quantification of antibody concentration using standards is necessary. Notably, taking an example from measuring antiphospholipid antibodies in the antiphospholipid

syndrome, there is a known variability between laboratories despite the availability of commercial kits and standard chimeric antiphospholipid antibodies [26, 27]. The current guidelines for APL antibodies as a diagnostic biomarker consider > 99th percentile of historical O.D. values to improve the specificity of the test [28]. We suggest that setting a high cutoff O.D. value can address the variability of anti-PEG assays. Understanding pathophysiological correlates of the "risk" of complement activation, clearance, toxicities, and loss of efficacy is critical to setting clinically meaningful cutoff levels for anti-PEG and other nanoparticle-binding antibodies in patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

5. ACKNOWLEDGEMENTS

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6. REFERENCES

[1]. Gao Y, Joshi M, Zhao Z, Mitragotri S, PEGylated therapeutics in the clinic, Bioengineering & Translational Medicine, n/a (2023) 1–28.

[2]. Shi D, Beasock D, Fessler A, Szebeni J, Ljubimova JY, Afonin KA, Dobrovolskaia MA, To PEGylate or not to PEGylate: Immunological properties of nanomedicine's most popular component, polyethylene glycol and its alternatives, Adv Drug Deliv Rev, 180 (2022) 114079. [PubMed: 34902516]

[3]. Richter AW, Akerblom E, Polyethylene glycol reactive antibodies in man: titer distribution in allergic patients treated with monomethoxy polyethylene glycol modified allergens or placebo, and in healthy blood donors, Int Arch Allergy Appl Immunol, 74 (1984) 36–39. [PubMed: 6706424]

[4]. Chen BM, Cheng TL, Roffler SR, Polyethylene Glycol Immunogenicity: Theoretical, Clinical, and Practical Aspects of Anti-Polyethylene Glycol Antibodies, ACS Nano, 15 (2021) 14022–14048. [PubMed: 34469112]

[5]. Suzuki T, Ichihara M, Hyodo K, Yamamoto E, Ishida T, Kiwada H, Ishihara H, Kikuchi H, Accelerated blood clearance of PEGylated liposomes containing doxorubicin upon repeated administration to dogs, Int J Pharmaceut, 436 (2012) 636–643.

[6]. Abu Lila AS, Shimizu T, Ishida T, 3 - PEGylation and anti-PEG antibodies, in: Parambath A (Ed.) Engineering of Biomaterials for Drug Delivery Systems, Woodhead Publishing, 2018, pp. 51–68.

[7]. Bavli Y, Chen BM, Gross G, Hershko A, Turjeman K, Roffler S, Barenholz Y, Anti-PEG antibodies before and after a first dose of Comirnaty? (mRNA-LNP-based SARS-CoV-2 vaccine), Journal of Controlled Release, 354 (2023) 316–322. [PubMed: 36549393]

[8]. Ju Y, Lee WS, Pilkington EH, Kelly HG, Li S, Selva KJ, Wragg KM, Subbarao K, Nguyen THO, Rowntree LC, Allen LF, Bond K, Williamson DA, Truong NP, Plebanski M, Kedzierska K, Mahanty S, Chung AW, Caruso F, Wheatley AK, Juno JA, Kent SJ, Anti-PEG Antibodies Boosted in Humans by SARS-CoV-2 Lipid Nanoparticle mRNA Vaccine, ACS Nano, 16 (2022) 11769–11780. [PubMed: 35758934]

[9]. Armstrong JK, Hempel G, Koling S, Chan LS, Fisher T, Meiselman HJ, Garratty G, Antibody against poly(ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients, Cancer, 110 (2007) 103–111. [PubMed: 17516438]

[10]. Yang Q, Lai SK, Anti-PEG immunity: emergence, characteristics, and unaddressed questions, Wiley Interdiscip Rev Nanomed Nanobiotechnol, 7 (2015) 655–677. [PubMed: 25707913]

[11]. Chen BM, Su YC, Chang CJ, Burnouf PA, Chuang KH, Chen CH, Cheng TL, Chen YT, Wu JY, Roffler SR, Measurement of Pre-Existing IgG and IgM Antibodies against Polyethylene Glycol in Healthy Individuals, Anal Chem, 88 (2016) 10661–10666. [PubMed: 27726379]

[12]. Yang Q, Jacobs TM, McCallen JD, Moore DT, Huckaby JT, Edelstein JN, Lai SK, Analysis of Pre-existing IgG and IgM Antibodies against Polyethylene Glycol (PEG) in the General Population, Anal Chem, 88 (2016) 11804–11812. [PubMed: 27804292]

[13]. Deuker MFS, Mailander V, Morsbach S, Landfester K, Anti-PEG antibodies enriched in the protein corona of PEGylated nanocarriers impact the cell uptake, Nanoscale Horiz, 8 (2023) 1377–1385. [PubMed: 37591816]

[14]. Neun BW, Barenholz Y, Szebeni J, Dobrovolskaia MA, Understanding the Role of Anti-PEG Antibodies in the Complement Activation by Doxil in Vitro, Molecules, 23 (2018). [PubMed: 30577607]

[15]. Ehlinger C, Spear N, Doddareddy R, Shankar G, Schantz A, A generic method for the detection of polyethylene glycol specific IgG and IgM antibodies in human serum, J Immunol Methods, 474 (2019) 112669. [PubMed: 31614128]

[16]. Kozma GT, Meszaros T, Berenyi P, Facsko R, Patko Z, Olah CZ, Nagy A, Fulop TG, Glatter KA, Radovits T, Merkely B, Szebeni J, Role of anti-polyethylene glycol (PEG) antibodies in the allergic reactions to PEG-containing Covid-19 vaccines: Evidence for immunogenicity of PEG, Vaccine, 41 (2023) 4561–4570. [PubMed: 37330369]

[17]. Chen F, Wang G, Griffin JI, Brenneman B, Banda NK, Holers VM, Backos DS, Wu L, Moghimi SM, Simberg D, Complement proteins bind to nanoparticle protein corona and undergo dynamic exchange in vivo, Nat Nanotechnol, 12 (2017) 387–393. [PubMed: 27992410]

[18]. Li Z, Ma A, Miller I, Starnes R, Talkington A, Stone CA, Phillips EJ, Choudhary SK, Commins SP, Lai SK, Development of anti-PEG IgG/IgM/IgE ELISA assays for profiling anti-PEG immunoglobulin response in PEG-sensitized individuals and patients with alpha-gal allergy, J Control Release, 366 (2024) 342–348. [PubMed: 38182056]

[19]. Ishida T, Atobe K, Wang X, Kiwada H, Accelerated blood clearance of PEGylated liposomes upon repeated injections: effect of doxorubicin-encapsulation and high-dose first injection, J Control Release, 115 (2006) 251–258. [PubMed: 17045355]

[20]. Suzuki T, Ichihara M, Hyodo K, Yamamoto E, Ishida T, Kiwada H, Ishihara H, Kikuchi H, Accelerated blood clearance of PEGylated liposomes containing doxorubicin upon repeated administration to dogs, Int J Pharm, 436 (2012) 636–643. [PubMed: 22850293]

[21]. Ishida T, Kiwada H, Anti-polyethyleneglycol antibody response to PEGylated substances, Biol Pharm Bull, 36 (2013) 889–891. [PubMed: 23727911]

[22]. La-Beck NM, Zamboni BA, Gabizon A, Schmeeda H, Amantea M, Gehrig PA, Zamboni WC, Factors affecting the pharmacokinetics of pegylated liposomal doxorubicin in patients, Cancer Chemother Pharmacol, 69 (2012) 43–50. [PubMed: 21590446]

[23]. Schroeder HW Jr., Cavacini L, Structure and function of immunoglobulins, J Allergy Clin Immunol, 125 (2010) S41–52. [PubMed: 20176268]

[24]. Oskam N, Damelang T, Streutker M, Ooijevaar-de Heer P, Nouta J, Koeleman C, Van Coillie J, Wuhrer M, Vidarsson G, Rispens T, Factors affecting IgG4-mediated complement activation, Front Immunol, 14 (2023) 1087532. [PubMed: 36776883]

[25]. Lin YC, Chen BM, Tran TTM, Chang TC, Al-Qaisi TS, Roffler SR, Accelerated clearance by antibodies against methoxy PEG depends on pegylation architecture, J Control Release, 354 (2023) 354–367. [PubMed: 36641121]

[26]. Gardiner C, Hills J, Machin SJ, Cohen H, Diagnosis of antiphospholipid syndrome in routine clinical practice, Lupus, 22 (2013) 18–25. [PubMed: 22988029]

[27]. Pengo V, Biasiolo A, Bison E, Chantarangkul V, Tripodi A, Italian C Federation of Anticoagulation, Antiphospholipid antibody ELISAs: survey on the performance of clinical laboratories assessed by using lyophilized affinity-purified IgG with anticardiolipin and anti-beta2-Glycoprotein I activity, Thromb Res, 120 (2007) 127–133. [PubMed: 17020782]

[28]. Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, Derksen RH, PG DEG, Koike T, Meroni PL, Reber G, Shoenfeld Y, Tincani A, Vlachoyiannopoulos PG, Krilis

SA, International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS), J Thromb Haemost, 4 (2006) 295–306. [PubMed: 16420554]

[29]. Cheng TL, Wu PY, Wu MF, Chern JW, Roffler SR, Accelerated clearance of polyethylene glycol-modified proteins by anti-polyethylene glycol IgM, Bioconjug Chem, 10 (1999) 520–528. [PubMed: 10346886]

[30]. Estape Senti M, de Jongh CA, Dijkxhoorn K, Verhoef JJF, Szebeni J, Storm G, Hack CE, Schiffelers RM, Fens MH, Boross P, Anti-PEG antibodies compromise the integrity of PEGylated lipid-based nanoparticles via complement, J Control Release, 341 (2022) 475–486. [PubMed: 34890719]

[31]. Moreno A, Pitoc GA, Ganson NJ, Layzer JM, Hershfield MS, Tarantal AF, Sullenger BA, Anti-PEG Antibodies Inhibit the Anticoagulant Activity of PEGylated Aptamers, Cell Chem Biol, 26 (2019) 634–644 e633. [PubMed: 30827937]

[32]. Kloos R, van der Sluis IM, Mastrobattista E, Hennink W, Pieters R, Verhoef JJ, Acute lymphoblastic leukaemia patients treated with PEGasparaginase develop antibodies to PEG and the succinate linker, Br J Haematol, 189 (2020) 442–451. [PubMed: 31883112]

[33]. Chen E, Chen BM, Su YC, Chang YC, Cheng TL, Barenholz Y, Roffler SR, Premature Drug Release from Polyethylene Glycol (PEG)-Coated Liposomal Doxorubicin via Formation of the Membrane Attack Complex, ACS Nano, 14 (2020) 7808–7822. [PubMed: 32142248]

[34]. Bavli Y, Chen BM, Gross G, Hershko A, Turjeman K, Roffler S, Barenholz Y, Anti-PEG antibodies before and after a first dose of Comirnaty(R) (mRNA-LNP-based SARS-CoV-2 vaccine), J Control Release, 354 (2023) 316–322. [PubMed: 36549393]

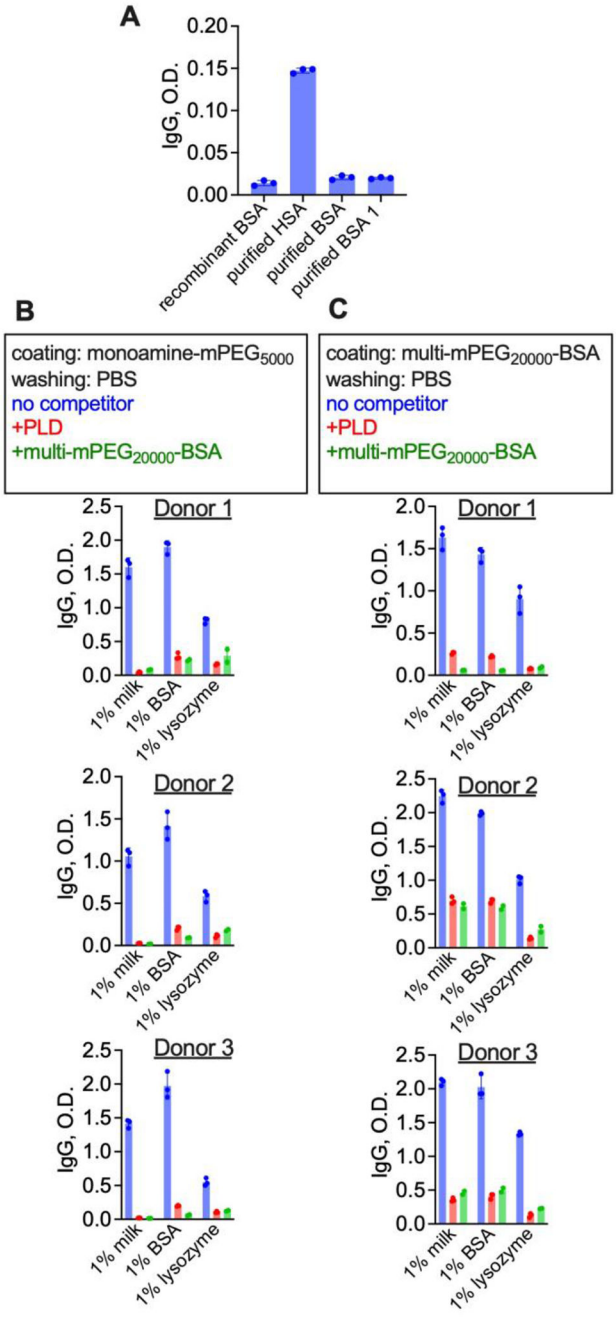

Fig. 1. Optimization of coating, blocking, and competition reagents for detection of human anti-PEG IgG.

A) There is very minimal non-specific binding of anti-human IgG-HRP to high-binding plates coated with different albumin types. HSA showed elevated binding and was excluded from subsequent experiments. Data show the average and SD of 3 technical replicates. B) Coating with monoamine-mPEG$_{5000}$. **C)** Coating with multi-mPEG$_{20000}$-BSA. Monoamine-mPEG$_{5000}$ and blocking with 1% w/v milk produce better specificity of the assay. Data show the average and SD of 3 technical replicates.

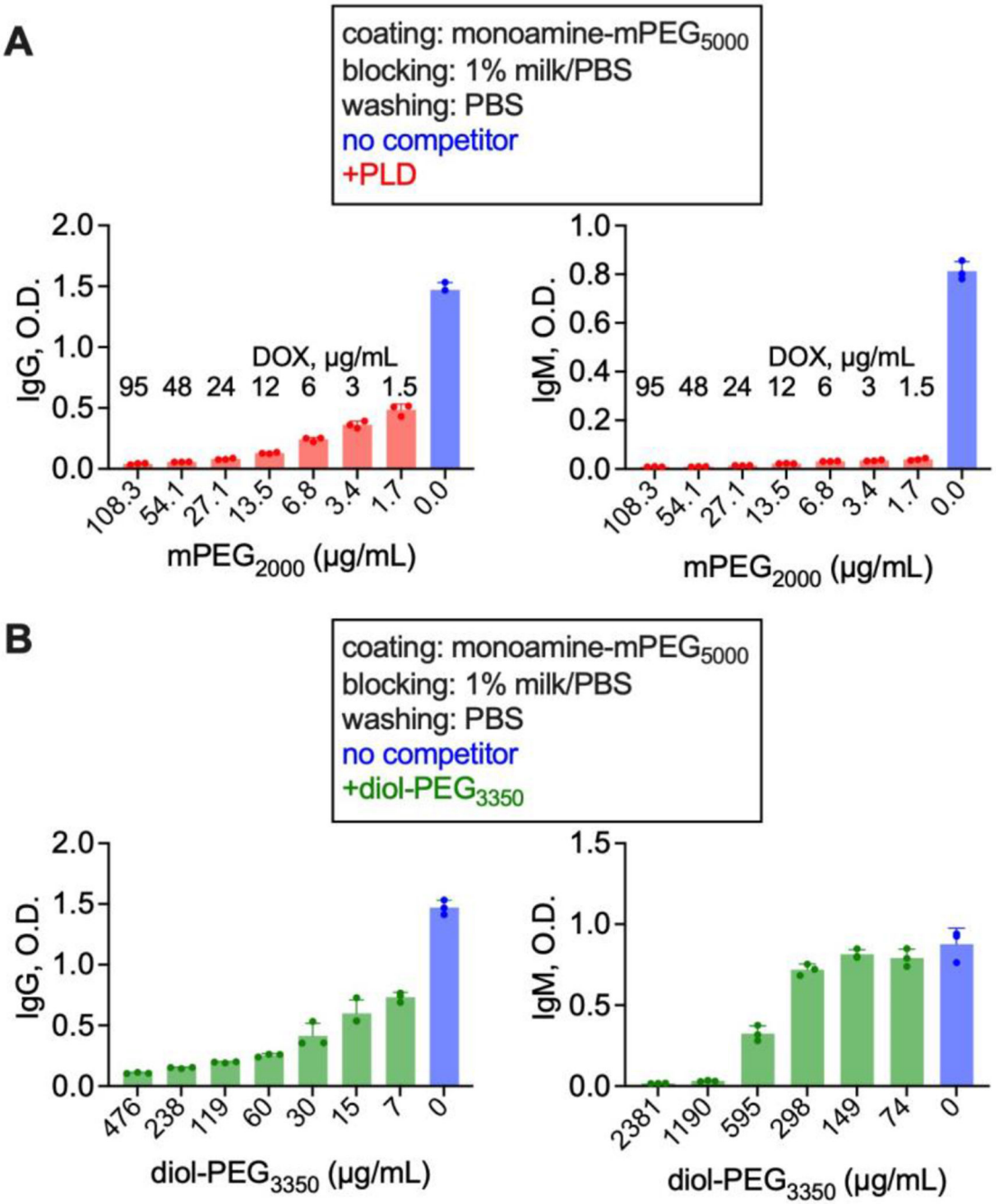

Fig. 2. Competition with PLD (A) and diol-PEG$_{3350}$ (B).

Serum from the same donor was used. PLD is a more efficient inhibitor than diol-PEG3350. Each bar is the average and SD of 3 technical replicates.

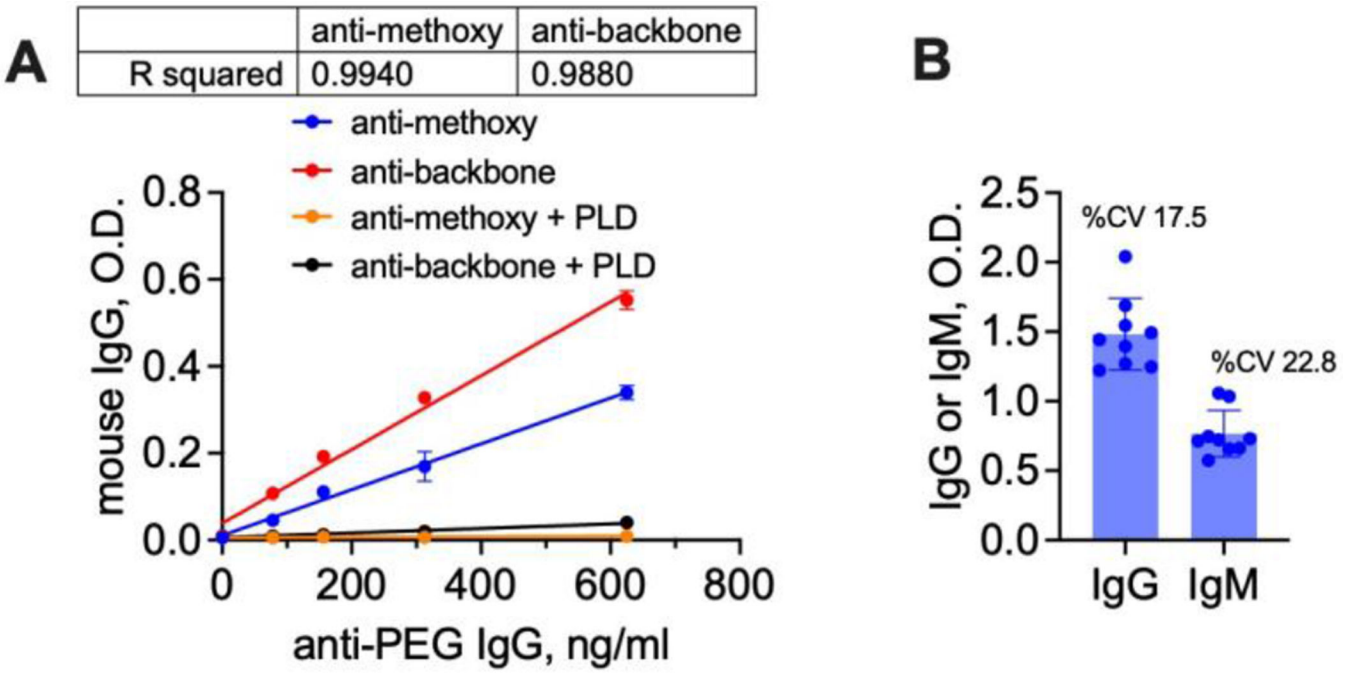

Fig. 3. The assay linearity and repeatability.

Coating: monoamine-mPEG$_{5000}$ and blocking with 1% w/v milk. **A**) Mouse monoclonal antibodies diluted in 1% milk were used to construct the dilution curve, and PLD was used for blocking. **B**) The assay was repeated 9 times using serum from the donor in Fig. 2. Coefficient of variation (%) is shown. Each dot is the average of 3 technical replicates.

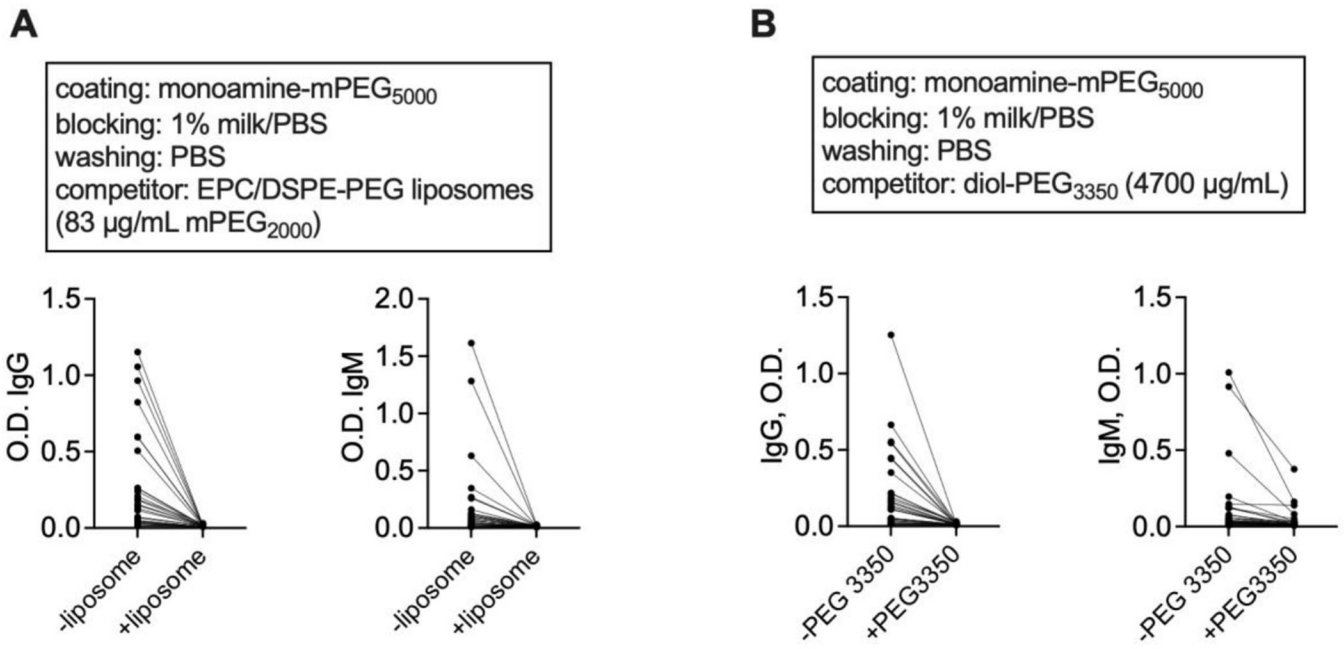

Fig. 4. Raw anti-PEG IgG and IgM values in 29 healthy donors under optimized conditions with inhibitors.

A) PEGylated liposomes (EPC/DSPE-mPEG$_{2000}$) and **B**) diol-PEG$_{3350}$ show almost complete inhibition of IgG signals. PEGylated liposomes show better inhibition of IgM signals than PEG$_{3350}$. Each dot is the average of 3 technical replicates.

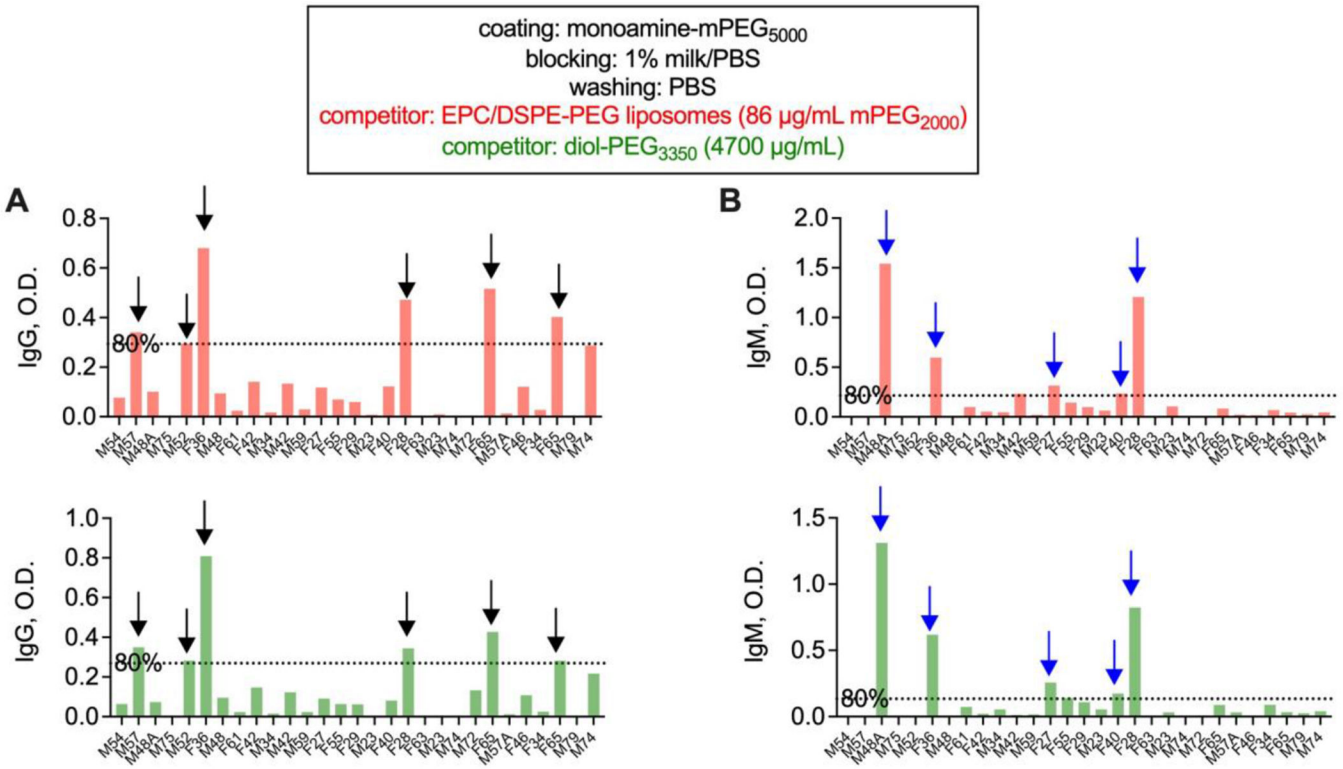

Fig. 5. Normalized subtracted anti-PEG IgG and IgM signals in 29 healthy donors using PEGylated liposomes or diol-PEG_{3350} for competition. The data were normalized to a common serum sample included in all plates, and the inhibitor signal was subtracted. **A**) IgG; **B**) IgM. Despite some level of experimental variability, the 80^{th} percentile O.D. identifies the same subjects (black arrows - IgG; blue arrows – IgM). Each dot is the average of 3 technical replicates.

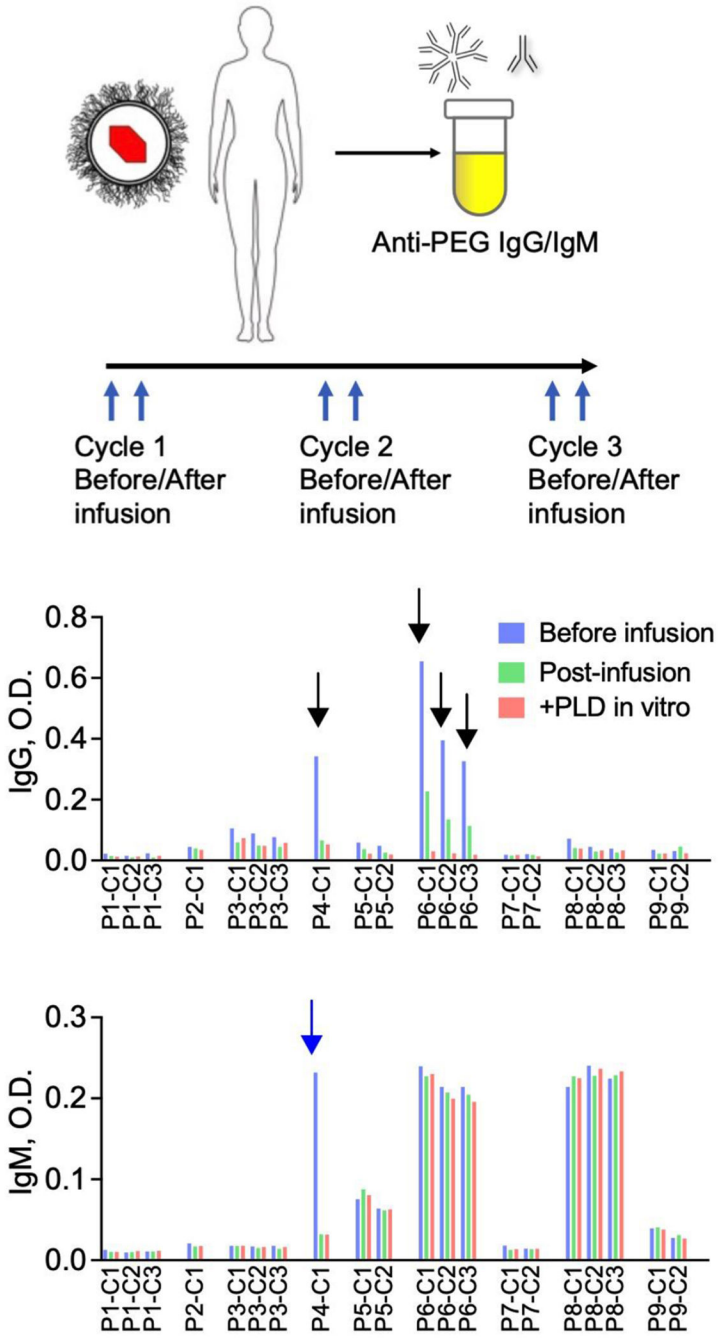

Fig. 6. Longitudinal monitoring of anti-PEG antibodies in patients infused with PLD.

A) Each consented patient had the anticoagulated blood drawn before the infusion and right after the infusion (up to 3 chemotherapy cycles, 1 month apart). At the completion of the study, all the plasma samples were tested for anti-PEG IgG (**B**) and IgM (**C**) at 1:10 plasma dilution. As an inhibitor control, each preinfusion sample was incubated with PLD, as described above. Notably, none of the patients in this cohort showed an increase in the anti-PEG levels.

Table 1.

Comparison of several published anti-PEG ELISA protocols.

Analyte species	Plate Coating	Blocking	Washing	Competition	Reference
Dog, Rat	DSPE-mPEG$_{2000}$	1% w/v BSA	0.05% TBS-T	PEGylated liposomes	[5]
Mouse	beta-glucoronidase-PEG, BSA-PEG	2% w/v skim milk	0.05% PBS-T	N/M	[29]
Human	8-arm amino PEG$_{40000}$	5% w/v skim milk	0.1% CHAPS	N/M	[8]
Human	DSPE-mPEG$_{5000}$	5% w/v nonfat milk	PBS	diol-PEG$_{8000}$	[12]
Human	PEG-filgrastim	2% w/v BSA	0.05% CHAPS	mPEG$_{5000}$	[30]
Human	multi PEG-BSA	assay diluent	0.5% n-dodecyl-beta-maltoside	multi-PEG-BSA	[15]
Human, Mouse	Diol-PEG$_{2000}$, mPEG$_{2000}$, or PEGylated liposomes Doxebo	5% w/v nonfat milk	0.1% CHAPS	N/M	[14]
Human, Rhesus monkey	PEGylated aptamers, PEGylated adenosine deaminase	1% w/v BSA	1% BSA	N/M	[31]
Human	PEG-asparaginase, PEG-filgrastim	2–5% w/v BSA	0.05% CHAPS	mPEG 5000, PEG-asparaginase	[32]
Human	NH_2-PEG$_{10000}$-NH_2	5% w/v skim milk	N/M	N/M	[33]
Human	NH_2-PEG$_{10000}$-NH_2	5% w/v skim milk	0.1% CHAPS	PEGylated liposomes	[34]
Human	NH_2-PEG$_{10000}$-NH_2	5% w/v skim milk	0.1% CHAPS	PEGylated liposomes	[11]
Human	DSPE-mPEG$_{5000}$	5% non-fat milk	PBS	mPEG10000	[18]

N.M, not mentioned; TBS-T, Tris-buffered saline-Tween 20; CHAPS, (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate)

Table 2.

Patients treated with PLD and enrolled in the study.

Study ID	Sex	Race	Ethnicity	Age	Diagnosis
P1	Female	White	non-hispanic	78	stage III recurrent ovarian cancer
P2	Female	White	non-hispanic	61	recurrent stage IIIC high-grade serous ovarian cancer
P3	Female	White	non-hispanic	78	recurrent higih grade serous ovarian cancer
P4	Female	Asian	non-hispanic	54	recurrent ovarian cancer
P5	Female	White	non-hispanic	70	recurrent clear cell carcinoma of the ovary
P6	Female	White	non-hispanic	61	recurrent endometrial cancer
P7	Female	White	non-hispanic	77	recurrent high-grade serous primary peritoneal carcinoma
P8	Female	White	non-hispanic	68	stage IVB adenocarcinoma of Gyn origin
P9	Female	White	non-hispanic	77	recurrent mixed serous and clear cell ovarian cancer,