Cross-Reactivity of Select PEG-Binding Antibodies to Other Polymers Containing a C-C-O Backbone

Justin McCallen, †,# John Prybylski, †,# Qi Yang, † and Samuel K. Lai*, †,‡,§

† Division of Pharmacoengineering and Molecular Pharmaceutics, Eshelman School of Pharmacy; ‡ UNC/NCSU Joint Department of Biomedical Engineering; and § Department of Microbiology & Immunology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States

Supporting Information

ABSTRACT: Polyethylene glycol (PEG), a flexible and relatively hydrophilic polymer, is widely used not only in medicine but also in numerous everyday hygiene, food, and skincare products. Recent animal and human studies have shown that antibodies (Abs) that bind PEG can be induced, leading to markedly reduced therapeutic efficacy of PEGylated therapeutics as well as possibly resulting in acute anaphylaxis and hypersensitivity reactions. Because humans are exposed to numerous other synthetic polymers, we sought to investigate whether such “anti-PEG” antibodies may also bind other synthetic polymers, particularly those with structural similarities to PEG. In a screen of six commercially available and two recombinantly produced anti-PEG IgG and IgM antibodies, we found five antibodies (3 IgG and 2 IgM) that readily bind polypropylene glycol (PPG), polytetramethylene ether glycol (PTMEG), and poly-1,4-butylene adipate (PBA). In contrast, none of the eight antibodies bound dextran (DEX) or polyepoxysuccinic acid (PES), and only two exhibited detectable affinity to polyethylenimine (PEI), suggesting that these PEG-binding antibodies likely possibly recognizable accessible C-C-O groups in the polymer backbone. We also observed similar cross-reactivity in plasma of human subjects with high titers of PEG-binding IgG and IgM. These results directly demonstrate potential cross-reactivity of select PEG-binding antibodies, which represents a new category of antidrug antibodies whereby an adverse immune response can be elicited as a result of prior exposures to PEG or other synthetic PEG-like polymers.

KEYWORDS: anti-PEG, polyethylene glycol, antibody cross-reactivity, polypropylene glycol, polymer immunogenicity

1. INTRODUCTION

Polyethylene glycol (PEG) is a flexible and uncharged polymer with high water solubility, and it is routinely used in nanoparticle and protein therapeutics to improve the stability and circulation kinetics of protein drugs and nanocarriers. 1,2 Specifically, PEG grafting has been shown to markedly reduce RES clearance of proteins as well as polymeric nanoparticles and liposomes by decreasing the adsorption of plasma proteins that can act as opsonins. 2-4 Interestingly, PEG is frequently used to reduce the immunogenicity of the parent compound or drug delivery vehicle. 5,6 As a reflection of the popularity of the PEGylation strategy, at least a dozen PEG-modified therapeutics are currently approved by the FDA, and many more are in clinical development.

Over the past two decades, however, there are increasing reports irrevocably demonstrating that PEG-binding antibodies (Abs) can be elicited in humans, including in individuals who likely have not received PEGylated therapeutics administered systemically. 8 The broad medicinal and consumer use of PEG, which is found in numerous industrial and consumer products, including hygiene products, skin creams, stool softeners, and food additives, implies the vast majority of the population has most likely been consistently exposed to PEG. Regardless of the route of exposure and the specific immunological mechanism leading to immunological memory, the presence of both plasma anti-PEG IgG and IgM antibodies can lead to accelerated blood clearance (ABC) and consequently reduce the efficacy of PEG-modified therapeutics. 9 Indeed, since the first report of anti-
PEG antibodies induced by PEG-modified proteins, subsequent research has shown that repeated dosing of otherwise long-circulating nanocarriers modified with PEG or PEG-containing molecules frequently results in rapid clearance by mononuclear phagocyte system (MPS) cells in numerous animal models. In humans, the presence of anti-PEG antibodies has been associated with rapid clearance of various PEGylated proteins in clinical trials. Recent reports also suggest patients with substantial quantities of preexisting anti-PEG antibodies could potentially experience acute anaphylaxis and hypersensitivity reactions to PEGylated therapeutics.

Despite these potential drawbacks, PEG remains the most widely used stealth polymer in biomedical applications, a consequence of its desirable physiochemical properties that stem from its chemical structure. It is not surprising then that a wide range of polymers used for medical and consumer applications share similar chemical structures with PEG, most notably a polymer backbone that includes C-C-O groups. For example, polypropylene glycol (PPG) is often used along with PEG in the form of pluronics, an excipient often found in gel and cream based products. PPG is also used on its own in select nanomedicines. Similarly, polytetramethylene ether glycol (PTMEG) can be found in adhesives for skin products, transdermal drug delivery, and nanoparticle-based chemotherapeutics delivery systems. Extending beyond C-C-O to C-C-X chemistries (where X denotes a hydrophilic atom), polyethylenimine (PEI) is a popular cationic polymer with a C-C-N backbone used in formulating polyplexes for DNA and siRNA therapy.

The functional and physiochemical similarities between these polymers and PEG beg an obvious question: if antibodies can bind specifically to the PEG backbone, would the same antibodies potentially also bind other polymers that are structurally similar to PEG, and thereby lead to reduced efficacy and/or adverse outcomes and safety concerns in humans? To address this question, we selected a group of common polymers with varying structural similarities to PEG, including those possessing C-C-O groups in their backbone, and evaluated by quantitative competitive ELISA whether anti-PEG IgG and IgM antibodies would bind any of the selected polymers. Despite the high specificity of most antibody—antigen interactions, we found that antibodies raised against PEG in vivo can potentially bind a series of other non-PEG polymers, raising concerns about potential complications associated with anti-PEG antibodies beyond PEGylated therapeutics.

2. MATERIALS AND METHODS

2.1. Anti-PEG Antibodies and Polymers. The anti-PEG IgG and IgM antibodies used here include mouse monoclonal IgG CH2074 and CH2076 (Silver Lake Research; lot no. N0716 and K0868, respectively), rabbit polyclonal IgG PEGPAb-01 (Life Diagnostics; cat. no. C-I3011A-1), rabbit monoclonal IgG RM105 (Abcam; cat. no. ab190652), mouse IgM AGP4 (IBMS), rabbit IgM PEG-2-128

Table 1. Structures and Properties of Polymers Included in the Study

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>Total Molecular Weight (M&lt;sub&gt;a&lt;/sub&gt;)</th>
<th>Subunit Molecular Weight (g/mol)</th>
<th>Hydrophobicity Of Subunit (LogP)&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyethylene glycol (PEG)</td>
<td></td>
<td>8000</td>
<td>44.1</td>
<td>0.06</td>
</tr>
<tr>
<td>Polypropylene glycol (PPG)</td>
<td></td>
<td>4000</td>
<td>60.1</td>
<td>0.42</td>
</tr>
<tr>
<td>Polytetramethylene ether glycol (PTMEG)</td>
<td></td>
<td>2900</td>
<td>74.1</td>
<td>1.12</td>
</tr>
<tr>
<td>Poly(1,4-butylene adipate) (PBA)</td>
<td></td>
<td>12000</td>
<td>201.3</td>
<td>1.15</td>
</tr>
<tr>
<td>Polyethyleneimine, linear (PEI)</td>
<td></td>
<td>2500</td>
<td>43.1</td>
<td>-0.51</td>
</tr>
<tr>
<td>Polyeopxy succinic acid (PES)</td>
<td></td>
<td>11280</td>
<td>134.1</td>
<td>-1.57</td>
</tr>
<tr>
<td>Dextran, linear (DEX)</td>
<td></td>
<td>40000</td>
<td>180.2</td>
<td>1.82</td>
</tr>
</tbody>
</table>

<sup>1</sup>LogP values of polymer subunit calculated by Molinspiration online software.
To capture PEG-binding antibodies, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-methoxy PEG₉₆ (DSPE-PEG; Nanocs, New York, NY, USA) was coated onto medium binding half-area 96-well Costar plates (Corning) at 50 μg/mL in Dulbecco's phosphate buffered saline (DPBS) overnight at 4 °C. After blocking plates with 50 μL of 5% dry nonfat milk solids in DPBS, 20 μL of polymer at various concentrations in 1% milk was added per well, followed by 20 μL of each test antibody (2 μg/mL). The final antibody concentration in each well was 1 μg/mL, and polymer concentrations ranged from 2 mg/mL to 15.6 μg/mL. All plates were incubated for 1 h of shaking at 250 rpm. Antibodies bound to the DSPE-PEG-coat were detected using one of the following detection antibodies, together with a 1-Step Ultra TMB (ThermoFisher): goat antimuskl IgG HRP (Santa Cruz cat. no. sc-2005), goat antimuskl IgM HRP (Invitrogen cat. no. 626820), goat antirabbit IgG (Invitrogen cat. no. 656120), goat antirabbit IgM (Abcam cat. no. ab97195), or goat antirat IgM (Abcam cat. no. ab97180). All secondary antibodies were diluted 1:10,000 in 1% milk. After stopping the TMB conversion with 2 N sulfuric acid, the absorbance at 450 nm was measured using a SpectraMax M2 plate reader ( Molecular Devices). All wash and incubation steps were performed using DPBS without any surfactant, as commonly used surfactants such as Tween contain PEG and could thus artificially alter ELISA measurements. For analysis across multiple plates, all results were normalized to the average A450 measured in the wells without free PEG competition.

2.3. Validation of Competitive ELISA Assays. To verify that high concentrations of polymers do not nonspecifically interfere with antibody–antigen binding, high-affinity half-area 96-well Costar plates (Corning) were coated with 50 μL of unlabeled RSV (10 μg/mL) and then incubated overnight at 4 °C. After blocking plates with 5% nonfat milk in DPBS, 50 μL of anti-RSV IgG (Synagis; MedImmune NDC 605774-4114-1) at 1 μg/mL in 1% milk was added to each well. To block the anti-RSV antibody, either PEG, PPG, PTMEG, PBA, or PEI was added to each well at 2.0 mg/mL. Antibodies bound to virus were detected with a goat F(ab′)2 antihuman IgG Fc (Rockland cat. no. 709-1317) diluted 1:10,000 in 1% milk. The plates were developed, quenched, and measured as previously described. All wash and incubation steps were performed using DPBS (Figure S1A).

2.4. Anti-PEG Antibody Quantification and Human Plasma Samples. As described by Yang et al., frozen plasma samples from individual healthy subjects were previously purchased from ProMedDX (Norton, MA, USA), and the levels of human anti-PEG IgG and IgM were quantified using recombinant anti-PEG standards 6.3 IgG₉ and AGP3 IgM. Plasma samples from seven individual healthy subjects (Table 2) found to possess high anti-PEG IgG and/or IgM levels (samples with ≥1 μg/mL anti-PEG IgG and/or ≥0.1 μg/mL IgM) were included in this study. To assess the potential cross-reactivity of PEG-binding antibodies in these plasma samples, a quantitative competitive ELISA was performed as described above. Due to the limited volume of the specimens available, we were only able to perform competition using a single concentration of polymers. For the initial antibody incubation step, solutions of each of the seven polymers were made at 4 mg/mL in 20 μL of 1% nonfat dry milk solids and added to respective wells. Human samples that contained anti-PEG IgG and/or IgM were diluted 5–10X in 20 μL of 1% nonfat dry milk solids and added to respective wells. The addition of anti-PEG plasma samples yielded a final polymer concentration of 2 mg/mL. We verified that this polymer concentration did not nonspecifically alter the binding of a human antibody (Synagis) against its antigen (respiratory syncytial virus) (Figure S1A). Antibody binding was detected by IgM antihuman IgM HRP (Rockland Immunochemicals, cat. no. 609-1307, 1:25,000 dilution) or antihuman IgG HRP (Rockland Immunochemicals, cat. no. 709-1317, 1:10,000 dilution), followed by addition of 1-Step Ultra TMB, quenching with 2 N sulfuric acid, and measurement of absorbance at 450 nm. All wash and incubation steps were performed using DPBS without any surfactant.

2.5. Data Analysis of Competition ELISAs. The following three-parameter Hill equation was used to calculate the nonlinear fit parameters that describe polymer inhibition of PEG-binding antibodies: 

\[
Y = Y_{\text{c}} - \frac{\text{Antibody concentration}}{K_{i} + \text{Antibody concentration}}
\]

where \(Y\) represents the normalized sample absorbance, \(X\) the concentration of competing polymer, \(K_i\) is the calculated inhibition constant, and \(h\) is the Hill equation coefficient.

For antibody–polymer competition combinations that yielded \(h\) within a 95% confidence interval of 1 and \(K_i > 0\), inhibition parameters are described in Tables S1 and S2. For competitive inhibition analysis of the various polymers and human samples containing PEG-binding antibodies, the sample absorbance values were normalized to the average A450 measured in the wells with no PEG competition. Using Tukey’s multiple comparison test, the difference in antibody cross-reactivity of each plasma sample was examined. Additionally, the cross-reactivity of PEG-binding Abs in each plasma sample was assessed by two-way ANOVA to determine an overall difference in normalized absorbance with respect to polymer and sample variance. Data and results were processed using GraphPad Prism 6.

2.6. Direct ELISA with PS-PEG Bead Competition. To further confirm the binding of the commercial anti-PEG antibodies to PEG-like polymers, we used a direct ELISA format whereby hydroxyl end-group PEG, PPG, and PTMEG, as well as PEI, was coated onto high-affinity half-area 96-well Costar plates (Corning) at 100 μg/mL in DPBS overnight at 4 °C. After blocking plates with 50 μL of 5% dry nonfat milk solids in DPBS, 50 μL of each test antibody (1 μg/mL) was added to each well with or without 5 μg/mL of methoxy PEG 5K amine (GenKem Technology cat. no. M-NH2-5000) conjugated to 100 nm carbosyl (COOH) polystyrene (PS) beads (Invitrogen; cat. no. F8803) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) chemistry described by Yang et al. All plates were incubated for 1 h of shaking at 250 rpm and washed 4 times with PBS, and respective secondary antibodies were added at 1:10,000 dilution. After incubating the secondary antibodies for 1 h, the ELISA plates were washed 4 times with PBS and developed as described above.

2.7. Polymer Conjugation of Polystyrene Nanoparticles. The following polymers with hydroxyl end-groups were oxidized with potassium permanganate (Sigma cat. no. 223468): PEG, 8000 Da;
PPG, 4000 Da; PTMEG, 2900 Da; PBA, 12000 Da. Twenty-five milliliters of 100 mM KMnO₄ was mixed with 25 mL of each polymer solution (50 mg/mL in 1 N sodium hydroxide) in an Erlenmeyer flask with a stir bar at 500 rpm. After an overnight reaction, the solutions were spun at 4000 g for 10 min to remove manganese oxide. The polymer solutions were dialyzed in water to remove any remaining potassium permanganate. The carboxylic end-group polymers (PEG-COOH, PPG-COOH, PTMEG-COOH, or PBA-COOH) were then conjugated to 200 nm amine polystyrene (PS) beads (Invitrogen; cat. no. F8763) through EDC chemistry using a previously described protocol.

2.8. Dot Blot for Direct Binding Assay. To examine the direct binding of PEG-binding antibodies to various polymers, 2 µL of polymer-conjugated PS beads or unmodified amine PS beads were coated onto a nitrocellulose membrane (ThermoFisher cat. no. LC2001) and left to dry for 15 min at RT. After blocking the nitrocellulose strip with 5 mL of 5% nonfat dry milk solids in DPBS for 1 h, 5 mL of 1 µg/mL of each PEG-binding antibody in 1% milk was added to the blot, and all blots were incubated for 1 h of shaking at 100 rpm. After washing the blot, antibodies bound to polymer-coated PS beads were detected using the respective detection antibodies (as described in section 2.2) and ECL substrate (Bio-Rad cat. no. 170-...
5061). All secondary antibodies were diluted 1:25,000 in 1% milk, incubated for 1 h of shaking at 100 rpm. All wash and incubation steps were performed using PBBS. Blots were imaged for chemiluminescence using the Bio-Rad ChemiDoc MP system. To validate the specificity of the dot blot assay, we measured anti-PEG antibody binding to a “non-cross-reactive” polymer, PEI. We incubated PEI with 100 nm COOH-PS beads at ten molar excess for 1 h, washed with distilled water 3×, and measured the zeta potential using a Malvern Instruments ZetaSizer Nano-ZS (Table S3). PS-COOH conjugated with methoxy PEG 5K amine, PEI associated PS-COOH beads, or unmodified PS-COOH beads was coated onto a nitrocellulose membrane and left to dry for 15 min at RT. Blots were incubated with primary and secondary antibodies and then developed using the procedure described above.

2.9. Food and Drug Administration-Approved Products Containing PEG, PPG, and PEG/PPG Copolymers. The significance of this study’s results is underscored by the numerous pharmaceutical and consumer products that contain polymers such as PEG and PPG. To determine the relative distribution of these polymers in FDA-approved products, a query of the openFDA drug labeling database was performed for products indexed as containing polymers in FDA-approved products, a query of the openFDA drug labeling database was performed for products indexed as containing polymers in FDA-approved products, a query of the openFDA drug labeling database was performed for products indexed as containing polymers in FDA-approved products, a query of the openFDA drug labeling database was performed for products indexed as containing polymers in FDA-approved products, a query of the openFDA drug labeling database was performed for products indexed as containing polymers in FDA-approved products. Although many products are properly labeled in the labeling database, we also screened other anti-PEG IgGs. With PEGPAB-01, a polyclonal rabbit IgG preparation, we were able to detect cross-reactivity to both PPG and PTMEG, but not PBA (Figure 1C). Unlike CH2074 and CH2076, PEGPAB-01 possessed the greatest affinity to PEG: the Ki values of PEGPAB-01 were 37 ± 23, 240 ± 65, and 230 ± 83 μM for PEG, PPG, and PTMEG, respectively (Figure 2). Finally, we also tested 6.3, a monoclonal IgG that has undergone extensive affinity maturation and binds PEG with less than ten nanomolar affinity. We detected no appreciable binding of 6.3 to any of the polymers included in our screening polymer panel other than PEG (Figure 1D), underscoring the highly variable nature of the potential cross-reactivity. 

3. RESULTS

3.1. Competitive ELISA with Anti-PEG IgG Antibodies. Using a quantitative competitive ELISA format that we previously established to quantify specific anti-PEG IgG and IgM in humans, we first evaluated potential polymer cross-reactivity with two mouse monoclonal anti-PEG IgGs, CH2074, and CH2076, which binds the PEG polymer backbone irrespective of the polymer end group. Because the molecular weights of the polymers vary, we compared the competitive inhibition not only as a function of the molar concentration of the polymers but also their monomer molar concentrations. With both antibodies, we found detectable cross-reactivity to PPG, PTMEG, and PBA, in addition to the expected reactivity to PEG, based on the ability of these polymers to specifically block the binding of the antibodies to the DSPE-PEG coat on the ELISA plates (Figure 1A–B) while not blocking the binding of RM105, an antibody against the methoxy-PEG terminus (Figure S1B). Surprisingly, PPG appeared to possess greater inhibition potency than even PEG, suggesting potentially a higher affinity between both antibodies and PPG. Indeed, the inhibitory constant (Ki) values for PPG, which reflect the binding affinity (lower Ki implies higher affinity), were 2.9 ± 0.3 and 2.4 ± 0.4 μM of PPG polymer for CH2074 and CH2076, respectively, which is significantly lower than the respective Ki values for PEG of 8.3 ± 3.2 and 6.8 ± 1.3 μM (Figure 2 and Table S1A–B). The affinities of CH2074 and CH2076 to PTMEG appeared comparable to those for PEG, with Ki values of 14 ± 4.9 and 12 ± 2.1 μM, respectively (Figure 2 and Table S1). Among the polymers that exhibited cross-reactivity to both antibodies, PBA was least able to inhibit binding of the two antibodies to PEG, with Ki values of 62 ± 22 μM and 239 ± 104 μM for CH2074 and CH2076, respectively. Since previous work has shown that antibodies that bind the PEG backbone specifically recognize a small number of repeated units of ethylene glycol monomers, we also measured inhibition with respect to the molar concentration of monomers (Figures 1–2, and Figure S1B). We observed largely identical results to analysis based on molar concentrations of the polymers, with the primary difference being PTMEG possessing greater affinity than PEG to both antibodies when quantified in monomer units. We next tested competition inhibition with PEI, a linear polymer that replaces the oxygen in the C-C-O polymer backbone with nitrogen; PES, a linear C-C-O polymer with carboxylic acid groups anchored to both carbons; and DEX, a linear polysaccharide polymer rich in carbons and oxygen groups. None of these three polymers exhibited any appreciable affinity to either antibody.

To evaluate whether the polymer cross-reactivity observed with CH2074 and CH2076, including greater affinity to select PEG-like polymers, was an isolated occurrence, we also screened other anti-PEG IgGs. With PEGPAB-01, a polyclonal rabbit IgG preparation, we were able to detect cross-reactivity to both PPG and PTMEG, but not PBA (Figure 1C). Unlike CH2074 and CH2076, PEGPAB-01 possessed the greatest affinity to PEG: the Ki values of PEGPAB-01 were 37 ± 23, 240 ± 65, and 230 ± 83 μM for PEG, PPG, and PTMEG, respectively (Figure 2). Finally, we also tested 6.3, a monoclonal IgG that has undergone extensive affinity maturation and binds PEG with less than ten nanomolar affinity. We detected no appreciable binding of 6.3 to any of the polymers included in our screening polymer panel other than PEG (Figure 1D), underscoring the highly variable nature of the potential cross-reactivity.

3.2. Competitive ELISA with Anti-PEG IgM Antibodies. In addition to examining the cross-reactivity of anti-PEG IgG,
we also evaluated the cross-reactivity of commercially available PEG-binding IgM antibodies. IgM antibodies typically arise first in the humoral immune response; due to more limited affinity maturation compared to IgG, the Fab domains on IgM generally possess lower affinity to antigen, but a higher overall avidity due to its pentameric structure comprising 10 Fab arms. The presence of anti-PEG IgM has also been correlated to ABC of PEGylated drugs in numerous rodent studies. Thus, we hypothesized that anti-PEG IgM antibodies may exhibit similar, if not potentially greater, cross-reactivity than anti-PEG IgG antibodies. Of the four IgMs tested, two IgM—AGP3 (raised against PEGylated β-glucuronidase in mouse) and 26A04 (raised against 5 kDa PEG conjugated to keyhole limpet hemocyanin in a rat)—exhibited detectable cross-reactivity. In particular, on a monomer molar concentration basis, PPG and PTMEG all appeared to limit AGP3 binding to DSPE-PEG to the same extent as free PEG. In contrast, PBA limited 26A04 binding to DSPE-PEG to the same if not greater extent than free PEG (Figure 3 and Table S2).

### 3.3. Assaying Direct Binding of Anti-PEG Antibodies to Different Polymers via Dot Blot and Direct ELISA

To complement qualitative competitive ELISA assays, we sought to verify direct binding of PEG-binding monoclonal to select polymers with C-C-O groups in their backbone via a dot blot assay. To do so, we first functionalized the end group of PEG, PPG, PTMEG, and PBA polymers with carboxyl groups, which allowed us to conjugate them to amine-modified PS beads. In good agreement with the competitive ELISA results, we observed direct binding of CH2074, CH2076, and PEGPAB-01 to all polymer-conjugated beads (Figure 4). Interestingly, with 6.3, we observed detectable direct binding to PPG- and PBA-coated beads, suggesting that the antibody possesses low

---

**Figure 3.** Cross-reactivity of purified anti-PEG IgMs to a library of polymers via competition ELISA: (A) AGP4, (B) AGP3, (C) PEG-2-128, and (D) 26A04. Error bars represent the standard deviation of n = 4 with duplicates for each run. Solid, colored curve fits represent the nonlinear fit of the Hill equation shown in section 2. Grey shading represents the 95% confidence interval of the nonlinear fit. Left panels represent measured absorbance with respect to the molar concentration of the polymers, while the right panels represent measured absorbance with respect to the molar concentrations of monomers. If the nonlinear fit is not shown, the linear fit did not converge and did not substantially compete with DSPE-PEG.
affinity to both polymers, and the affinity was not sufficient to inhibit 6.3 binding to DSPE-PEG in the competitive ELISA experiments. The IgM antibodies AGP4 and PEG-2-128 bound only PEG-conjugated PS-beads, whereas AGP3 and 26A04 showed detectable binding to PPG-, PTMEG-, and PBA-conjugated beads. We detected no binding by either control mouse IgG or control mouse IgM to different polymer-coated beads, as well as a lack of binding of anti-PEG antibodies to PEI-coated beads, verifying the specificity of the PEG-binding Abs to the polymers tested. Altogether, the dot blot experiments support the earlier observations that select PEG-binding antibodies can exhibit reactivity to other PEG-like polymers.

Finally, to confirm that cross-reactive anti-PEG antibodies not only can directly bind to PEG-like polymers but also be inhibited by PEG grafted on nanoparticles, we performed a direct ELISA with plates coated with different polymers and quantified the extent to which the measured absorbance was reduced by PEG-grafted nanoparticles. In agreement with the competitive ELISA data based on DSPE-PEG-coated plates, we observed the greatest extent of cross-reactivity for CH2074 and CH2076, as indicated by OD values that were substantially reduced in the presence of PEG-coated nanoparticles (Figure S2). Unlike with the dot blot assay above, we observed relatively little direct binding of PEGPAB-01 to other polymers; this may be a consequence of relatively lower affinity of the antibody to different polymers, exacerbated by inadequate polymer coating on ELISA plates relative to covalent grafting of the polymers on the surface of nanoparticles (used in the dot blot assays). Similar to the dot blot experiments, we observed binding of 6.3 to the PPG-coated surface, which could be inhibited by PEG-coated nanoparticles.

3.4. Human Plasma Samples. All commercially available or recombinantly produced anti-PEG antibodies were derived from rodents or rabbits, and each has a different polymer cross-reactivity profile. Therefore, we sought to assess whether the observed cross-reactivity of these purified PEG-binding antibodies may also extend to endogenous circulating anti-PEG antibodies produced by humans. To do so, we used human plasma samples previously found to have high titers of anti-PEG antibodies (Table 2), and performed competitive ELISAs with the different polymers. In general, plasmas containing high titers of PEG-binding-specific IgG, as determined by inhibition of binding to DSPE-PEG by free PEG, were also inhibited by free PPG to nearly the same extent, and by free PTMEG to a lesser extent (Figure 5A). The detectable cross-reactivity, primarily to PPG and PTMEG, agrees well with the competitive ELISA results obtained with commercially available or recombinant, animal-derived PEG-binding IgGs. Similar to the variations we observed with individual antibodies, there

Figure 4. Dot blot to qualitatively examine direct binding of PEG-binding antibodies to amine-terminated polystyrene (PS-NH₂) nanoparticles coated with different polymers. For each dot blot in the left panel, with membranes were blotted with (as indicated in the top row diagram), PEG−PS, PPG−PS, PTMEG−PS, PBA−PS, and unmodified PS-NH₂ beads, respectively. The right panel verified that PEG-binding antibodies do not bind the polymer PEI: left to right, PEG−PS prepared from carboxylated polystyrene (PS-COOH) beads, PEI coated PS-COOH beads, and unmodified PS-COOH beads. All particles were coated onto a nitrocellulose membrane, incubated with designated PEG-binding antibody, and detected using appropriate secondary antibody.
were substantial variations in detectable cross-reactivity to different polymers between various plasma samples \((p < 0.05, \text{Tukey's multiple comparison test; Tables S4 and S5})\), highlighting the unpredictable nature of the cross-reactivity. For instance, PEG-binding IgG in sample #039 bound only PPG among the various polymers tested; those in sample #357 also bound PPG and PTMEG; and those in sample #134 actually bound PTMEG more tightly than PPG and even possessed modest binding to PEI, which we did not observe previously. On average, PEG and PPG appear to possess the greatest afinity to PEG-binding IgMs in human plasma \((\text{Figure 5B})\), with virtually no detectable afinity to any of the other polymers. The apparent cross-reactivity of both PEG-binding IgG and IgM antibodies, as well as a diverse cross-reactivity profile among human subjects, underscores the importance of quantitatively assessing polymer-binding antibodies in actual clinical studies.

4. DISCUSSION

In living systems, the immune system is designed to generate antibodies that recognize and bind specific chemical patterns; for example, prior exposure to a particular influenza strain will likely result in binding antibodies against a different strain of influenza if the two strains share sufficient structural homology. It is therefore hardly surprising that the incorporation of polymer components in a diverse array of drug and consumer products will most likely induce the formation of “cross-reactive” antibodies that can bind other polymers with sufficient structural similarities. Here, using commercially available and recombinant PEG-binding antibodies originating from rodents or rabbits, as well as human plasma possessing PEG-binding IgG and IgM, we demonstrate for the first time the existence of such cross-reactive antibodies against different polymers. Importantly, in the case of humans, such cross-reactive antibodies are pre-existing (i.e. are present even in the absence of acute stimulation from parenteral administration of PEGylated therapeutics). Thus, while PEG-binding antibodies are commonly referred to as “anti-PEG”, our finding would argue that these antibodies should perhaps be more aptly named as anti-[C-C-O] antibodies to better reflect the precise binding motif and the potential for binding to a multitude of chemically distinct polymers.

A long-standing assumption in the field of antidrug antibodies (ADA) is that they are directly induced by prior exposure to the particular drug compound of interest. This implies that patients should not possess high titer antibodies to a drug that the patient never received \(a \text{ priori}\), and that the use of one drug should not result in formation of ADA against another drug, especially if the second drug is substantially different in its pharmacological nature. PEG-binding antibodies represent an essentially new class of ADA that have important polypharmacy implications. Polypharmacy refers to the potentially adverse effects of taking multiple medications concurrently. Unlike traditional ADA, which occurs only after repeated use of that particular drug, PEG-binding antibodies can render two PEGylated drugs that otherwise share few structural similarities (e.g., PEG–protein conjugate vs liposome with PEG corona) non efficacious or even unsafe. Evidence of such polypharmacy ADA is already beginning to emerge: serious adverse reactions were reported for two patients in a...
clinical trial for PEGylated phenylalanine ammonia lyase who received unrelated intramuscular injections of contraceptives containing PEG as an excipient.\(^5\) Now, in light of the potential polymer cross-reactivity reported here, the list of potentially incompatible medications would become substantially longer: it is entirely possible that polymer binding antibodies elicited from one drug may induce cross-reactive antibodies that render a second drug containing a different polymer less efficacious or, worse, trigger an adverse immune response.

To begin to gain an appreciation of the magnitude of this potential overlap, we searched the openFDA drug label database for entries listing PEG, PPG, or PEG/PPG copolymers as inactive ingredients. Despite the limitation that this search only included labels with properly indexed inactive ingredients in the database, we found 12,578 entries that included PEG, with the majority being topically or orally administered products (Figure 6A–B). PSORB, a copolymer containing a large number of oxyethylene repeats and which is notably used as an emulsifier for essential oils, yielded 4,676 product results. Tween, the trademarked name for PSORB, yielded another 4,624 results. PXM, an ABA hydrophilic copolymer of PEG and PPG, yielded 838 results, whereas Brij, a nonionic surfactant containing PEG, yielded 10 product results (Figure 6A). PPG shares a high degree of structural similarity to PEG, and not surprisingly possesses chemical and physical properties suitable for use in pharmaceutical and consumer products. In the drug label search of PPG, we found 2,943 entries, most of which are topically administered. For both PEG and PPG, the vast majority of the drugs were available over the counter (Figure 6C–D). Given the large number of products that include PEG and PPG, it is possible, if not likely, that the use of such over-the-counter (OTC) products may stimulate acute production of PEG-binding antibodies, which in turn could impact the efficacy and safety of systemically or mucosally dosed PEGylated therapeutics. Indeed, we have recently shown that PEG-binding antibodies in mucus can directly alter the mucus penetration and local biodistribution of PEGylated nanoparticles dosed vaginally.\(^33\) Likewise, recent use of systemically dosed PEGylated therapeutics may also induce high titers of PEG-binding antibodies that in turn may compromise the safe use of many over-the-counter products. The widespread inclusion of PEG and PPG in over-the-counter products will no doubt create a polypharmacy challenge that cannot be easily resolved simply by raising physician or pharmacist awareness of potential anti-PEG immunity.

For many decades, PEG was regarded as non-immunogenic, and had even been used to reduce the immunogenicity of protein antigens\(^5,6\) until evidence emerged demonstrating that both animals and humans can generate antibodies that specifically bind PEG domains of various PEG conjugates.\(^56–58\) It has been speculated that anti-PEG immunity may be gaining in prevalence, based on a comparison of the results from Richter and Akerblom in 1984 (detectable anti-PEG antibodies in 4.9% of healthy donors\(^59\)) to later results such as those of Armstrong et al. in 2003 (27%–28% of their healthy donors\(^60\)). Indeed, in our most recent study based on quantitative competitive ELISA with recombinant anti-PEG antibody standards, we detected the presence of PEG-binding antibodies in nearly 74% of the population, with 37% of samples containing >100 ng/mL PEG-binding antibody. While the same study did not find evidence supporting the emergence of PEG-binding antibodies over time, the surprising prevalence indicates that polymer-specific ADA is likely of potential concern to the majority of the population. Given the growing number of PEGylated protein and drug delivery systems that are in development, in clinical trials, and on the market,\(^61\) the presence of PEG-binding antibodies must be carefully evaluated in order to avoid adverse side effects such as hypersensitivity and anaphylactic reactions. The same screening process should also extend to consumer and pharmaceutical products containing PPG or other PEG-like polymers.

Our current study is beginning to shed light on how PEG-binding antibodies specifically recognize PEG. It has long been recognized that most PEG-binding antibodies specifically bind the polymeric backbone, with estimates ranging from three to seven repeated units of C-C-O.\(^10,37\) Nevertheless, the nature and relative importance of the interactions with each atom of the repeated unit remain unclear. From analysis of CH2074, CH2076, PEGPAB-01, AGP3, and 26A04, it appears the precise number of carbons in the polymer backbone subunit (i.e., the distance between each C-C-O repeat) is not critical, and that the interface where the Fab domain binds PEG readily tolerates at least two extra carbon atoms (PTMEG), or possibly more (PBA). It also appears that maintaining the hydrophobicity of the two carbons adjacent to the oxygen is essential to antibody specificity. Indeed, we speculate that the addition of a hydrophobic group to the carbon atom (PPG) could further stabilize Fab–polymer interactions, whereas the introduction of hydrophilic moieties to the carbon atom (PEG) could further destabilize Fab–polymer interactions. Finally, the oxygen atom along the polymer backbone appeared essential for the high affinity to PEG-binding antibodies; swapping oxygen with another electrophilic atom (N) effectively suppressed the association of Fab to the polymer. The lack of association of PEG-binding antibodies to PEI is consistent with the observations from Kierstead et al., which found that clearance of polyethoxylazoline liposomes in mice after induction of PEG-binding antibodies was not altered compared to clearance in naïve mice.\(^34\) These findings support the conclusion that the binding of anti-PEG antibodies to PEG is likely based on highly specific hydrogen bond interactions with the oxygen moiety.

Finally, it is important to highlight the variable nature of the polymer cross-reactivity: different monoclonal PEG-binding antibodies possess different cross-reactive profiles, ranging from substantial (e.g., CH2074, CH2076, AGP3, and 26A04) to negligible (e.g., 6.3 and AGP4) cross-reactivity. Indeed, many commercially available anti-PEG antibodies are frequently considered poor antibodies due to their nonspecific binding to many compounds, including proteins. While it is obvious that less cross-reactive antibodies such as 6.3 and AGP4 represent superior research reagents, the immune system always generates a polyclonal response resulting in a diverse repertoire of antibodies against any antigen of interest, and substantial diversity is retained even upon extensive affinity maturation. For instance, in individuals who can naturally suppress HIV viremia in the absence of antiretroviral therapy due to production of broadly neutralizing antibodies (bnAb) against HIV, these bnAb generally represent only a fraction of the overall antibody repertoire against HIV present in the systemic circulation.\(^52\) Given that all commercially available anti-PEG antibodies are induced in animals and that we observed polymer cross-reactivity among PEG-binding IgG and IgM in human plasma, it is likely that PEG-binding antibodies that are cross-reactive to other PEG-like polymers are frequently induced.
5. CONCLUSION
PEG has been used for decades to improve the therapeutic efficacy and safety of many proteins and nanoparticle-based therapeutics, but recently it has faced substantial scrutiny due to emerging reports showing that antibodies can be induced to bind PEG. Here, we add to the potential concerns with PEG-binding antibodies by demonstrating that, in addition to PEG, select PEG-binding antibodies can also bind other polymers containing repeating C-C-O units. This discovery has important ramifications in the context of efficacious and safe use of a diverse spectrum of medicines, given the ubiquitous presence of PEG and other C-C-O backbone containing polymers in various therapeutics. Overcoming the polypharmacy nature of polymer-specific ADA will no doubt require the concerted effort of scientists, physicians, pharmacists, and other healthcare practitioners.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsbiomater.7b00147.

Verification of the competitive ELISAs that were performed in Figures 1 and 3, examination of the direct binding of PEG-binding antibodies to PEG and PEG-like polymers, all calculated inhibition constants, PEI coating of PS-COOH nanoparticles, and in depth statistical examination of PEG-binding antibodies in human plasma to PEG and PEG-like polymers (PDF)

AUTHOR INFORMATION

Corresponding Author
*(Samuel K. Lai) Address: Division of Molecular Pharmaceutics, University of North Carolina at Chapel Hill, Marsico 4213, 125 Mason Farm Road, Chapel Hill, NC 27599. Phone: (+001)-919-966-3024. E-mail: lai@unc.edu. Homepage: http://www.lailab.com.

ORCID®
Samuel K. Lai: 0000-0003-4721-528X

Author Contributions
#J.M. and J.P. contributed equally to this work.

Funding
Financial support was provided by National Institutes of Health (R21EB017938, S.K.L.), UNC Research Opportunities Initiative grant in Pharmacoeengineering (S.K.L.), The David and Lucile Packard Foundation (2013-39274, S.K.L.), startup funds from the Eshelman School of Pharmacy and Lineberger Comprehensive Cancer Center (S.K.L.), a PhRMA Foundation Predoctoral Fellowship (Q.Y.), and a UNC Dissertation Completion Fellowship (Q.Y.).

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors are grateful to the Yisong Wan lab for the use of their Bio-Rad ChemiDoc MP system and also Loretta Hing for technical assistance.

REFERENCES
(14) Hershfield, M. S.; Ganson, N. J.; Kelly, S. J.; Scarlett, E. L.; Jaggers, D. A.; Sundj, J. S. Induced and pre-existing anti-polyethylene glycol antibody in a trial of every 3-week dosing of pegloticase for refractory gout, including in organ transplant recipients. Arthritis research & therapy 2014, 16 (2), R63.
(19) Nooead, P.; Li, W.; Roether, J. A.; Mourino, V.; Goudouri, O. M.; Schubert, D. W.; Boccaccini, A. R. Development of bioactive glass based scaffolds for controlled antibiotic release in bone tissue


