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Selectivity of binding of PEGs and PEG-like oligomers to anti-PEG antibodies induced by methoxyPEG-proteins☆

Mark G.P. Saifer, L. David Williams, Monika A. Sobczyk, Shawnya J. Michaels, Merry R. Sherman*

Mountain View Pharmaceuticals, Inc., 3475 Edison Way, Suite S, Menlo Park, CA 94025-1821, United States

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ABSTRACT

The use of methoxypoly(ethylene glycol) (mPEG) in PEG conjugates of proteins and non-protein therapeutic agents has led to the recognition that the polymer components of such conjugates can induce anti-PEG antibodies (anti-PEGs) that may accelerate the clearance and reduce the efficacy of the conjugates. Others have classified anti-PEGs as "methoxy-specific" or "backbone-specific". The results of our previous research on anti-PEGs in the sera of rabbits immunized with mPEG or hydroxyPEG (HO-PEG) conjugates of three unrelated proteins were consistent with that classification (Sherman, M.R., et al., 2012. Bioconjug. Chem. 23, 485–499). Enzyme-linked immunosorbent assays (ELISAs) were performed on rabbit antisera and rabbit monoclonal anti-PEGs with competitors including 10 kDa mPEG, 10 kDa PEG diol and six linear or cyclic oligomers of oxyethylene (CH_2—CH_2—O), with molecular weights of ca. 150–264 Da. Our results demonstrate that (1) the binding affinities of anti-mPEGs depend more on the backbone lengths of the polymers and the hydrophobicities of their end-groups than on their resemblance to the methoxy terminus of the immunogenic polymer; (2) anti-PEGs raised against HO-PEG-proteins are not directed against the terminal hydroxy group, but against the backbone; (3) rabbit anti-PEGs bind to and distinguish among PEG-like oligomers with as few as three oxyethylene groups; and (4) none of the monoclonal or polyclonal anti-PEGs was *absolutely* "methoxy-specific" or "backbone-specific", but displayed distinct *relative* selectivities. If these results are relevant to human immune responses, the clinical use of stable conjugates of HO-PEG with proteins and non-protein therapeutic agents would be expected to produce fewer and less intense immune responses than those induced by conjugates with mPEG or PEGs with larger alkoxy groups.

1. Introduction

A growing recognition of the induction of antibodies against methoxypoly(ethylene glycol) (mPEG) has developed as a result of the increasing clinical and preclinical use of mPEG conjugates of proteins, peptides, aptamers, liposomes, red blood cells, viral vectors and other drug-delivery particles (reviewed in Garay et al., 2012; Sherman et al., 2012b). The development of anti-PEG antibodies (anti-PEGs) has been correlated with accelerated clearance or loss of responsiveness to therapy with PEGylated agents in only a few cases, but has a plausible role in other examples of suboptimal efficacy of such conjugates (Armstrong et al., 2007; Armstrong, 2009; Ganson et al., 2006; Garay et al., 2012; Sherman et al., 2012a; Sundy et al., 2007). These considerations motivated our investigation of other forms of PEG, especially hydroxyPEG (HO-PEG), as potentially less immunogenic and less antigenic alternatives to mPEG as the polymer components of long-acting biopharmaceuticals.

To illuminate some of these issues, we have investigated the following questions: (1) What properties of the immunogens,

Abbreviations: AAALAC, Association for the Assessment and Accreditation of Laboratory Animal Care; Albumin, human serum albumin; Anti-PEGs, anti-PEG antibodies; D_{50}, dilution of serum that corresponds to 50% of maximal binding in a direct ELISA; ELISA, enzyme-linked immunosorbent assay; H and L chains, heavy and light immunoglobulin chains; HO-PEG, hydroxyPEG; IACUC, Institutional Animal Care and Use Committee; IC_{50}, inhibitor concentration that reduces binding to 50% of maximal binding in a competitive ELISA; IFN-α, interferon-α; KLH, keyhole limpet hemocyanin; mAb, monoclonal antibody; mAU/min, milli-absorbance units per minute; mPEG, monomethoxypoly(ethylene glycol); mPEG-KLH, 5 kDa mPEG-KLH; NPC, *p*-nitrophenylcarbonate; PEG, poly(ethylene glycol); PEG20K-KLH, 20 kDa mPEG-KLH; pNPCOCl, *p*-nitrophenylchloroformate; RI, refractive index; RSD, relative standard deviation = s.d./mean; SOD, porcine Cu–Zn superoxide dismutase; UV, ultraviolet absorbance. Crown ether; EtO-TEG, mTEG; n-BuO-TEG, TEG diol and TetraEG are defined in Table 1.

* Corresponding author. Tel.: +1 650 365 5515x224; fax: +1 650 365 5525.
E-mail address: sherman@mvpharm.com (M.R. Sherman).

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Table 1
Structures of competitors used to assess anti-PEG antibody specificities.

Competitor	Abbreviation	Structure	M.W. Da
Tri(ethylene glycol)	TEG Diol	$HO—(CH_2—CH_2—O)_3—H$	150.2
Tri(ethylene glycol) monomethyl ether	mTEG	$CH_3—O—(CH_2—CH_2—O)_3—H$	164.2
Tri(ethylene glycol) monoethyl ether	EtO-TEG	$CH_3—CH_2—O—(CH_2—CH_2—O)_3—H$	178.2
Tri(ethylene glycol) n-butyl ether	n-BuO-TEG	$CH_3—(CH_2)_3—O—(CH_2—CH_2—O)_3—H$	206.2
Tetra(ethylene glycol)	TetraEG	$HO—(CH_2—CH_2—O)_4—H$	194.2
18-Crown-6-ether	Crown ether	$(CH_2—CH_2—O)_6$	264.3
10 kDa Methoxypoly(ethylene glycol)	10 kDa mPEG	$CH_3—O—(CH_2—CH_2—O)_{227}—H$	10,000
10 kDa Poly(ethylene glycol)	10 kDa PEG Diol	$HO—(CH_2—CH_2—O)_{227}—H$	10,000
20 kDa Poly(ethylene glycol)	20 kDa PEG Diol	$HO—(CH_2—CH_2—O)_{454}—H$	20,000

antigens and competitors contribute to the observed selectivities of anti-PEGs? (2) Do the properties of the protein component of an immunogen (including its intrinsic immunogenicity) influence the polymer-binding specificities of the resultant anti-PEGs? (3) Do the specificities of the tested monoclonal anti-PEGs (mAbs) differ from those of the tested antisera from rabbits immunized with mPEG or HO-PEG conjugates of various proteins? (4) What can be inferred about the sizes of the polymer-binding domains of anti-PEGs from their affinities for a cyclic oxyethylene ether (18-crown-6-ether)? (5) What is the smallest number of oxyethylene units ($CH_2—CH_2—O$) recognized by anti-PEGs? (6) Can the results of competitive enzyme-linked immunosorbent assays (ELISAs) with PEG-like oligomers account for the previously reported interference in assays of anti-PEGs by detergents such as Tween 20 and Tween 80 (Armstrong, 2009; Life Diagnostics; Meridian Life Science; Sherman et al., 2012b)?

These questions were addressed primarily by competitive ELISAs of antisera from rabbits immunized with mPEG or HO-PEG conjugates of three unrelated proteins and rabbit-derived mAbs that were described by the vendor as "methoxy-specific" or "backbone-specific" anti-PEGs, respectively. The competitors included six linear or cyclic PEG-like oligomers containing three, four or six oxyethylene groups, 10 kDa and 20 kDa PEGs (which contain ca. 227 and 454 oxyethylene groups, respectively) and PEG conjugates of human serum albumin containing ca. 20 molecules of 10 kDa mPEG or HO-PEG.

The results demonstrate that neither the polyclonal antisera nor the two mAbs that were analyzed are truly "methoxy-specific" or "backbone-specific". Both the "methoxy-specific" and "backbone-specific" anti-PEGs bind oligomers with more hydrophobic end-groups more tightly than a methoxy-terminated oligomer that more closely resembles the immunogenic polymer. If these results are relevant to human immune responses, the clinical use of stable conjugates of HO-PEG with proteins, peptides, liposomes or viral vectors is expected to induce fewer and less intense anti-PEG immune responses than would the use of analogous conjugates synthesized with mPEG or PEGs with larger alkoxy groups.

2. Materials and methods

2.1. Reagents and supplies

Unless otherwise indicated, reagents were from the sources identified by Sherman et al. (2012b). The structures of the PEG-like oligomers that were used as competitors in analyses of the binding specificities of anti-PEGs are shown in Table 1. Tri(ethylene glycol) (TEG diol), tri(ethylene glycol) monomethyl ether (mTEG) and tetra(ethylene glycol) (TetraEG) were from Sigma–Aldrich Chemical Co. (St. Louis, MO). The n-butyl ether of TEG diol (n-BuO-TEG) and the monoethyl ether of TEG diol (EtO-TEG) were from TCI America (Portland, OR), as was 18-crown-6-ether (1,4,7,10,13,16-hexaoxacyclooctadecane). A rabbit "methoxy-specific" monoclonal anti-PEG antibody ("methoxy-specific" mAb) induced by 5 kDa mPEG-keyhole limpet hemocyanin (mPEG-KLH) and a rabbit "backbone-specific" mAb induced by 20 kDa mPEG-KLH (PEG20K-KLH) were from Epitomics (Burlingame, CA; clones PEG-B-47 and PEG-2-128-7, respectively) (Epitomics, Catalog #s 2061-1 and 3104-1). Peroxidase-linked goat anti-rabbit IgG (H and L chains) was from EMD-Calbiochem (Billerica, MA) and peroxidase-linked goat anti-rabbit IgM (mu chain-specific) was from Rockland Immunochemicals (Gilbertsville, PA).

2.2. Chromatographic and synthetic methods

Except as indicated below, the columns, chromatographic methods and analyses of the numbers of polymer molecules coupled per protein molecule or subunit were those used by Sherman et al. (2012b). Synthesis, purification and analysis of conjugates of mPEG and HO-PEG with porcine uricase, human recombinant interferon-α (IFN-α), human serum albumin and superoxide dismutase (SOD) were described by Sherman et al. (2012b). The reactive forms of PEG used for synthesis of the IFN-α conjugates were 20 kDa mPEG and HO-PEG monopropionaldehydes. The reactive forms of PEG used for synthesis of conjugates of uricase, albumin and SOD were mono-*p*-nitrophenylcarbonate derivatives of 10 kDa mPEG and PEG diol.

An SOD conjugate of tri(ethylene glycol) monomethyl ether (mTEG) was prepared as follows. Para-nitrophenyl chloroformate (pNPCOCl, 1 g, 5 mmol) was dissolved in 10 mL acetonitrile; mTEG (530 mg, 3.2 mmol) was added, followed by 1 mL (13 mmol) pyridine. After 2 days at room temperature, a 2-mL portion of the reaction was mixed with 18 mL cold 10 mM HCl and the white precipitate of dinitrophenyl carbonate was removed by filtration. Part of the clear supernatant (12 mL) was loaded on a 4.6 mm × 150 mm Jupiter C4 300 Å column from Phenomenex (Torrance, CA) and washed through with 20% (v/v) acetonitrile containing 1 mM HCl. Aliquots of the load solution and of 5-mL fractions of the eluate were analyzed by reversed-phase chromatography on the same column, at 1 mL/min, with a 20–50% (v/v) gradient of acetonitrile containing 1 mM HCl, monitored by absorbance at 271 nm. The fraction with the highest absorbance was calculated to contain 0.3 mM mTEG-*p*-nitrophenyl carbonate (mTEG-NPC). A 0.5-mL portion was concentrated to 0.13 mL in a Savant SpeedVac® concentrator (Holbrook, NY).

Porcine liver SOD (Sigma S8429; 1.9 mL of 20 mg/mL) was fractionated on a Superdex 200 HiLoad 16/60 column in 20 mM carbonate buffer, pH 10.1, containing 150 mM NaCl, to remove lower-molecular-weight contaminants. The two 5-mL fractions that were eluted between 85 and 95 mL were pooled and a 0.95-mL portion was mixed with 0.13 mL of concentrated mTEG-NPC solution (ca. 1.2 mM). After incubation for 1 day at 4 °C, the reaction mixture was dialyzed in a SpectraPor3 dialysis sack against two 400-mL changes of 20 mM sodium carbonate buffer, pH 10.

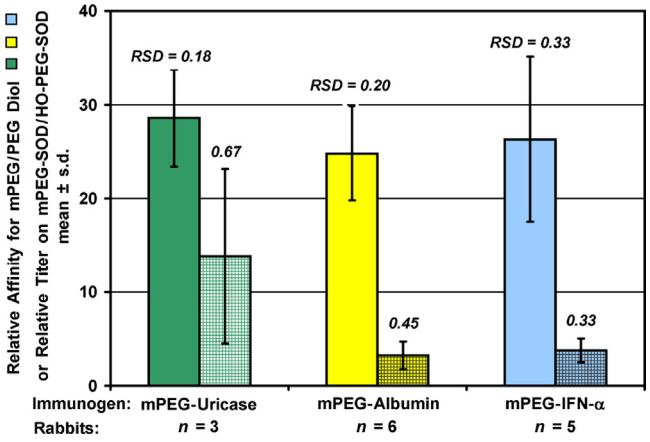

Fig. 1. Relative affinities (solid bars) provide a more sensitive measure of anti-PEG antibody selectivities than relative titers (hatched bars). In antisera from groups of rabbits immunized with mPEG conjugates of the indicated proteins, the relative affinities for 10 kDa mPEG versus 10 kDa PEG diol were measured by competitive ELISAs and the relative titers were measured by direct ELISAs with mPEG-SOD versus HO-PEG-SOD as the antigen. Values of the relative standard deviations (RSDs) are indicated.

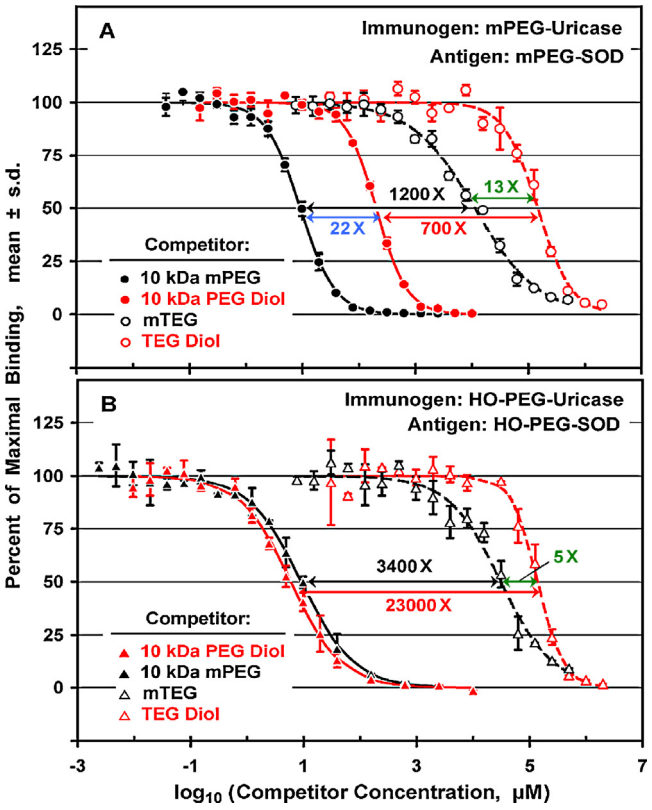

Fig. 2. The backbone lengths of the competitors have smaller effects on the affinities of polyclonal anti-PEGs induced by mPEG-uricase (A) than on affinities of anti-PEGs induced by HO-PEG-uricase (B). The assay plates were coated with mPEG-SOD (A) or HO-PEG-SOD (B). Sera from each rabbit were diluted 1/1000. The secondary antibody (goat anti-rabbit IgG H and L chains) was diluted 1/15,000. Arrows indicate the ratios of the affinities of the respective anti-PEGs for 10 kDa mPEG to mTEG (black), for mTEG to TEG diol (green), for 10 kDa mPEG to 10 kDa PEG diol (blue) and for 10 kDa PEG diol to TEG diol (red).

The concentrations of free amino groups in samples of the dialyzed conjugate and unmodified SOD were assayed by their reaction with *o*-phthalaldehyde thioglycolic acid (Lochmann et al., 2004). The reacted samples were analyzed by size-exclusion chromatography on a Superdex 75 HR 10/30 column and the eluate was monitored by refractive index (RI) and absorbance at 340 nm (UV). A comparison of the UV/RI ratio of the peak containing SOD indicated the presence of about half as many free amino groups in the mTEG-SOD sample as in unmodified SOD. Assay plates for immunoassays were coated with an amount of mTEG-SOD corresponding to 1 μg SOD per well, as was used when mPEG-SOD or HO-PEG-SOD was the antigen (Sherman et al., 2012b).

2.3. Animals and immunizations

This report is based on assays of sera from 25 rabbits immunized with mPEG or HO-PEG conjugates of porcine uricase, human recombinant IFN-α or human serum albumin, prepared and analyzed as described by Sherman et al. (2012b). The uricase conjugates contained ca. 2.3 molecules of 10 kDa mPEG or HO-PEG per uricase subunit. The IFN-α conjugates contained either one or two molecules of 20 kDa mPEG or HO-PEG per molecule of IFN-α (PEG_1- or PEG_2-IFN-α). The albumin conjugates contained ca. 17 molecules of 10 kDa mPEG or HO-PEG. The immunization and bleeding schedules and method of serum preparation were described by Sherman et al. (2012b). All rabbit procedures were performed at Lampire Biological Laboratories (Pipersville, PA) by AAALAC-certified technicians in accordance with IACUC-approved protocols.

2.4. Direct and competitive ELISAs and data analyses

All immunoassay procedures and analyses of the data were performed as described by Sherman et al. (2012b). It is important to note that no PEG-containing detergent (e.g., Tween 20 or Tween 80) was used in any step of the ELISAs. Direct ELISAs were performed in the absence of competitors with the assay plates coated with either mPEG-SOD or HO-PEG-SOD and with the serum dilution as the independent variable. The serum dilutions corresponding to 50% of the maximal binding (D_{50}) were calculated for each antigen and the *relative titer* was calculated as the ratio of D_{50} on mPEG-SOD to D_{50} on HO-PEG-SOD (Sherman et al., 2012b). Competitive ELISAs were performed at a fixed concentration of serum or dilution of a mAb, with mPEG-SOD, HO-PEG-SOD or mTEG-SOD as the antigen and the concentrations of the competitors (or of the oxyethylene groups in those competitors) as the independent variables. The concentration of each competitor that inhibited 50% of the maximal binding (IC_{50}) was calculated as described by Sherman et al. (2012b) and the *relative affinity* was calculated as the ratio of IC_{50} for the lower-affinity competitor to IC_{50} for the higher-affinity competitor. When the competitors were multi-PEGylated conjugates of serum albumin, the concentrations of the competitors were expressed as the concentrations of PEG in those competitors. Since there were no consistent differences between results obtained for antisera from rabbits immunized with $mPEG_1$- or $mPEG_2$-IFN-α, data for all rabbits immunized with either of those immunogens were combined. For the same reason, data from rabbits immunized with $HO\text{-}PEG_1$- or $HO\text{-}PEG_2$-IFN-α were combined.

The data for the ELISAs were normalized to Percent of Maximal Binding using the mean value of the maximal rate of color production under a given combination of experimental conditions. The maximal rates, in mAU/min, depend on factors including: the choice and dilution of serum or mAb, the polymer component of the SOD conjugate used to coat the assay plates (mPEG, HO-PEG or mTEG) and the choice and dilution of the peroxidase-linked secondary antibody. For the data in Figs. 2–7, the maximal values of mAU/min were in the range of 41–163 (see Table S1 of the Supplementary Data). Within this range, the rates of color production were linear during the 6-min period of monitoring, with correlation coefficients generally >0.99, and the differences between the values of IC_{50} for pairs of competitive binding curves (and hence

the calculated relative affinities) were not altered by increasing or decreasing the maximal value, e.g., by decreasing or increasing the dilution of the secondary antibody, respectively.

Among seven rabbits immunized with $mPEG_{17}$-albumin, one anomalous rabbit showed no appreciable preference for 10 kDa mPEG versus 10 kDa PEG diol when its sera from any of three monthly bleeds were assayed by either direct or competitive ELISAs. The tested competitors included multi-PEGylated conjugates of albumin, for which the affinities for mPEG-albumin versus HO-PEG-albumin in sera from 14 other rabbits immunized with mPEG conjugates of any of three proteins differed by a factor of >1000 (Sherman et al., 2012a,b). Therefore, data from this anomalous rabbit were excluded from all analyses of the results in this report.

3. Results

3.1. Overview

The questions raised in Section 1 were addressed primarily by competitive ELISAs with antisera from rabbits immunized with mPEG or HO-PEG conjugates of three unrelated proteins – human serum albumin, recombinant human IFN-α and porcine uricase. The results obtained with the unfractionated polyclonal antibody preparations (diluted rabbit sera) were compared with those obtained with two of the growing number of commercially available mAbs that were described by the vendor as "methoxy-specific" or "backbone-specific" anti-PEGs, respectively (Epitomics Catalog #s 2061-1 and 3104-1). The competitors included six PEG-like oligomers that ranged in size from tri(ethylene glycol) (TEG diol) to 18-crown-6-ether (1,4,7,10,13,16-hexaoxacyclooctadecane) (Table 1). The variables investigated in this research and the corresponding figure numbers are summarized in Table 2.

3.2. Competitive ELISAs as sensitive measures of anti-mPEG antibody selectivity

Relative titers were calculated from the results of direct ELISAs and *relative affinities* were calculated from the results of competitive ELISAs at a constant serum dilution, as described in Section 2.4. The results for sera from 14 rabbits immunized with mPEG conjugates of each of three unrelated proteins are compared in Fig. 1. Results for the *relative titers* varied considerably within groups of rabbits immunized with the same immunogen, e.g., the mean values from replicate assays of the relative titers of sera from three rabbits immunized with the same preparation of mPEG-uricase were ca. 3, 18 and 20, respectively. In contrast, the mean values of the *relative affinities* of sera from the same three rabbits were ca. 23, 30 and 33, respectively.

For each of the immunogens used to induce the anti-PEGs compared in Fig. 1, the mean value of the relative affinity measured by competitive ELISA was between twice and eight times the mean value of the relative titer measured by direct ELISA. For the antisera raised against mPEG conjugates of uricase and human serum

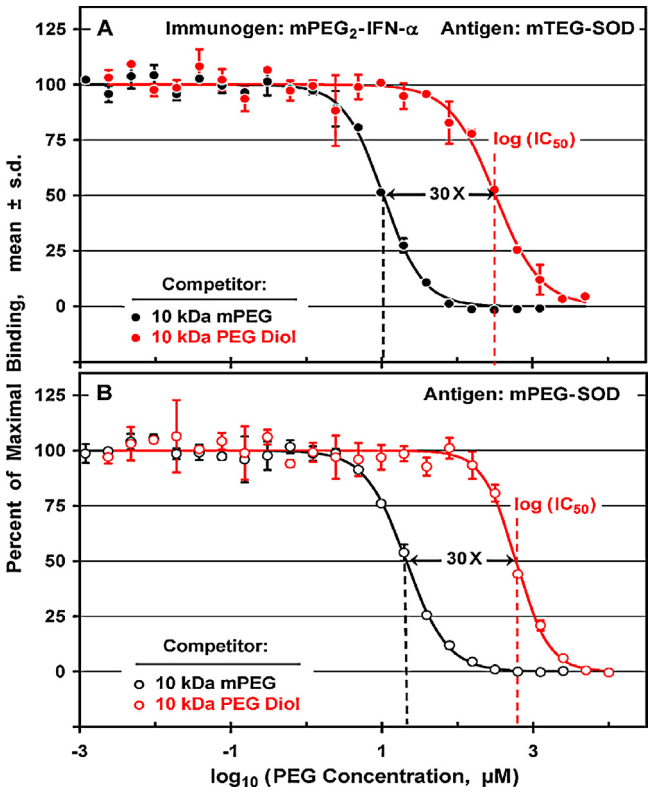

Fig. 3. The lengths of the polymer backbones of the antigens have no measurable effect on the relative affinities for 10 kDa mPEG and 10 kDa PEG diol of polyclonal anti-PEGs induced by $mPEG_2$-IFN-α. The same dilution (1/300) of serum from a rabbit was used in ELISAs with serial dilutions of 10 kDa mPEG (black symbols and curves) or 10 kDa PEG diol (red symbols and curves) as competitors (A and B). The secondary antibody was diluted 1/10,000 (A) or 1/15,000 (B). The PEG concentrations corresponding to 50% inhibition of maximal binding (IC_{50}) were calculated for each competitor on each antigen; values of log (IC_{50}) are indicated by dashed vertical lines.

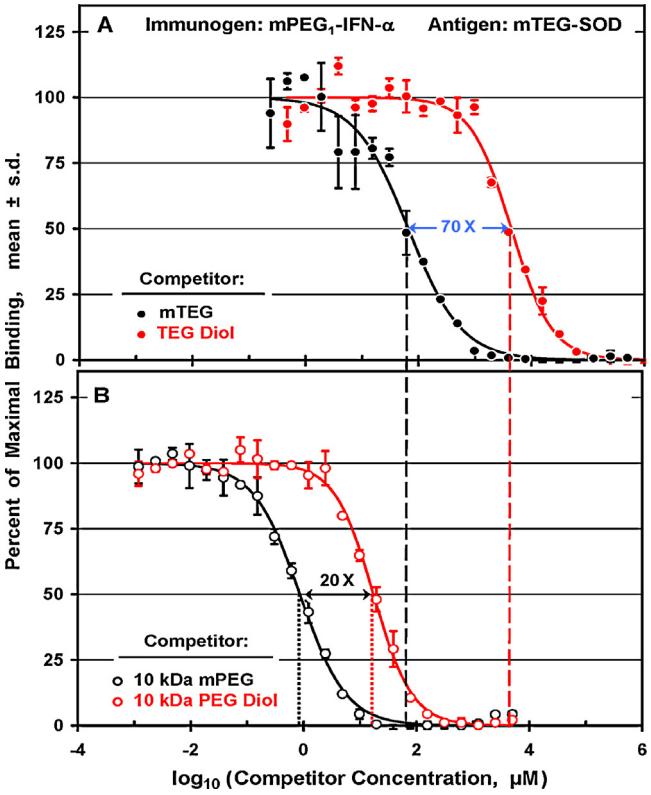

Fig. 4. The effects of the backbone length of the competitors on the relative affinities of polyclonal anti-mPEGs are amplified when the antigen contains an mPEG-like oligomer (mTEG). Affinities for mTEG versus TEG diol (A) are compared with the affinities of the same antibodies for 10 kDa mPEG versus 10 kDa PEG diol (B). Serum from a rabbit that was immunized with 20 kDa $mPEG_1$-IFN-α, was diluted 1/200, and the secondary antibody was diluted 1/10,000 (A and B). Horizontal arrows indicate the effects of the terminal methoxy group on the relative affinities of the oligomeric competitors (blue) and the 10 kDa PEGs (black). Dashed vertical lines facilitate comparisons of the values of IC_{50} of mTEG (black) and TEG diol (red) with those of the 10 kDa PEGs (dotted lines).

Table 2
Variables studied and corresponding figures.

Variable	Examples	Figures
Types of ELISAs (independent variable)	Direct (serum dilution); Competitive (molar concentration of competitor)	1 and 10 2–9
Antigens used to coat the assay plates	10 kDa mPEG-superoxide dismutase (SOD); 10 kDa HO-PEG-SOD; mTEG-SOD	2, 3 and 5
Competitors	10 kDa mPEG; 10 kDa and 20 kDa PEG diol; 10 kDa mPEG-albumin conjugates 10 kDa HO-PEG-albumin conjugates PEG-like oligomers	1–9
Protein components of the immunogens	Porcine uricase; human serum albumin; human interferon-α; keyhole limpet hemocyanin	1, 8–10
Polymer components of the immunogens	5 kDa, 10 kDa and 20 kDa mPEG; 10 kDa and 20 kDa HO-PEG	2, 5 and 7
Activation chemistries of the polymers	*p*-Nitrophenylcarbonate; propionaldehyde; maleimide	8 and 9
Classifications of the anti-PEGs	Polyclonal, monoclonal; IgG, IgM	8–10

albumin, the relative standard deviations (RSDs) of the relative affinities for 10 kDa mPEG versus 10 kDa PEG diol were less than half of the RSDs of the relative titers. Therefore, the analyses of anti-PEG specificities in the rest of this report were based on results from competitive ELISAs, rather than direct ELISAs.

3.3. *Effects of polymer size and the methoxy group of competitors*

The affinities of polyclonal anti-PEGs induced by mPEG-proteins are influenced by the length of the polymer backbone, as well as the presence of a methoxy group in the competitor, as exemplified by anti-PEGs induced by mPEG-uricase (Fig. 2A). On a molar basis, 10 kDa mPEG, which has an average of ca. 227 oxyethylene groups (CH_2-CH_2-O), was bound ca. 1200 times more tightly than an mPEG-like oligomer containing only three oxyethylene groups (mTEG; see Table 1). These anti-mPEGs also had ca. 700 times higher affinity for 10 kDa PEG diol than for TEG diol, illustrating the importance of the length of the polymer backbone.

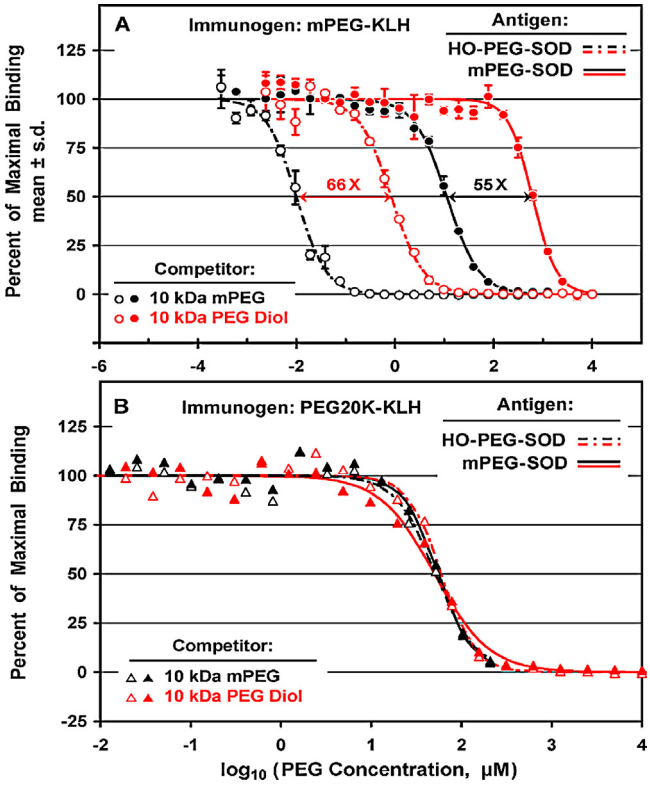

Fig. 5. Rabbit anti-PEG mAbs that are "methoxy-specific" (A) or "backbone-specific" (B) have markedly different patterns of competitive binding to 10 kDa mPEG (black) and 10 kDa PEG diol (red). The antigens were HO-PEG-SOD (open symbols, dashed curves) or mPEG-SOD (filled symbols, solid curves). (A) A rabbit IgG mAb (Epitomics Clone ID: PEG-B-47) was used at a final protein concentration of 485 ng/mL. Red and black horizontal arrows indicate the effects of the terminal methoxy group on the relative affinities of the 10 kDa PEGs when tested on HO-PEG-SOD or mPEG-SOD as the antigen, respectively. (B) A rabbit IgM mAb (Epitomics Clone ID: PEG-2-128-7) was used at a final protein concentration of 460 ng/mL. The secondary antibody (anti-rabbit IgG H and L chains) was diluted 1/15,000 (A and B).

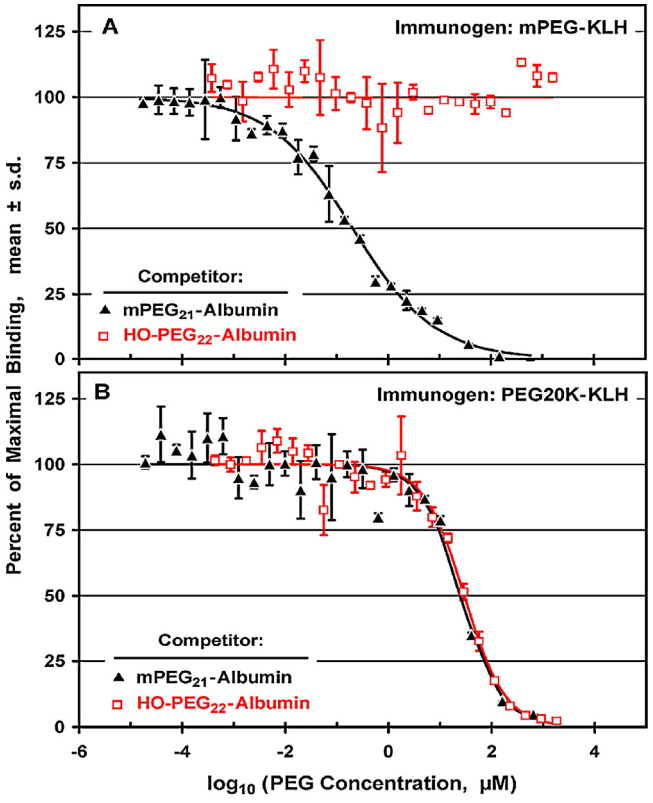

Fig. 6. Binding of a "methoxy-specific" mAb to mPEG-SOD is inhibited by multi-PEGylated albumin conjugates made with 10 kDa mPEG, but not by similar conjugates made with 10 kDa HO-PEG (A); binding of a "backbone-specific" mAb to mPEG-SOD is inhibited to the same extent by mPEG and HO-PEG conjugates of albumin (B). (A) A rabbit IgG mAb (Epitomics Clone ID: PEG-B-47) was used at a final protein concentration of 485 ng/mL. (B) A rabbit IgM mAb (Epitomics Clone ID: PEG-2-128-7) was used at a final protein concentration of 1150 ng/mL. The competitors were dilutions of human serum albumin coupled to ca. 21 molecules of 10 kDa mPEG (filled black triangles) or ca. 22 molecules of 10 kDa HO-PEG (open red squares). The secondary antibody (anti-rabbit IgG H and L chains) was diluted 1/15,000 (A) or 1/10,000 (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

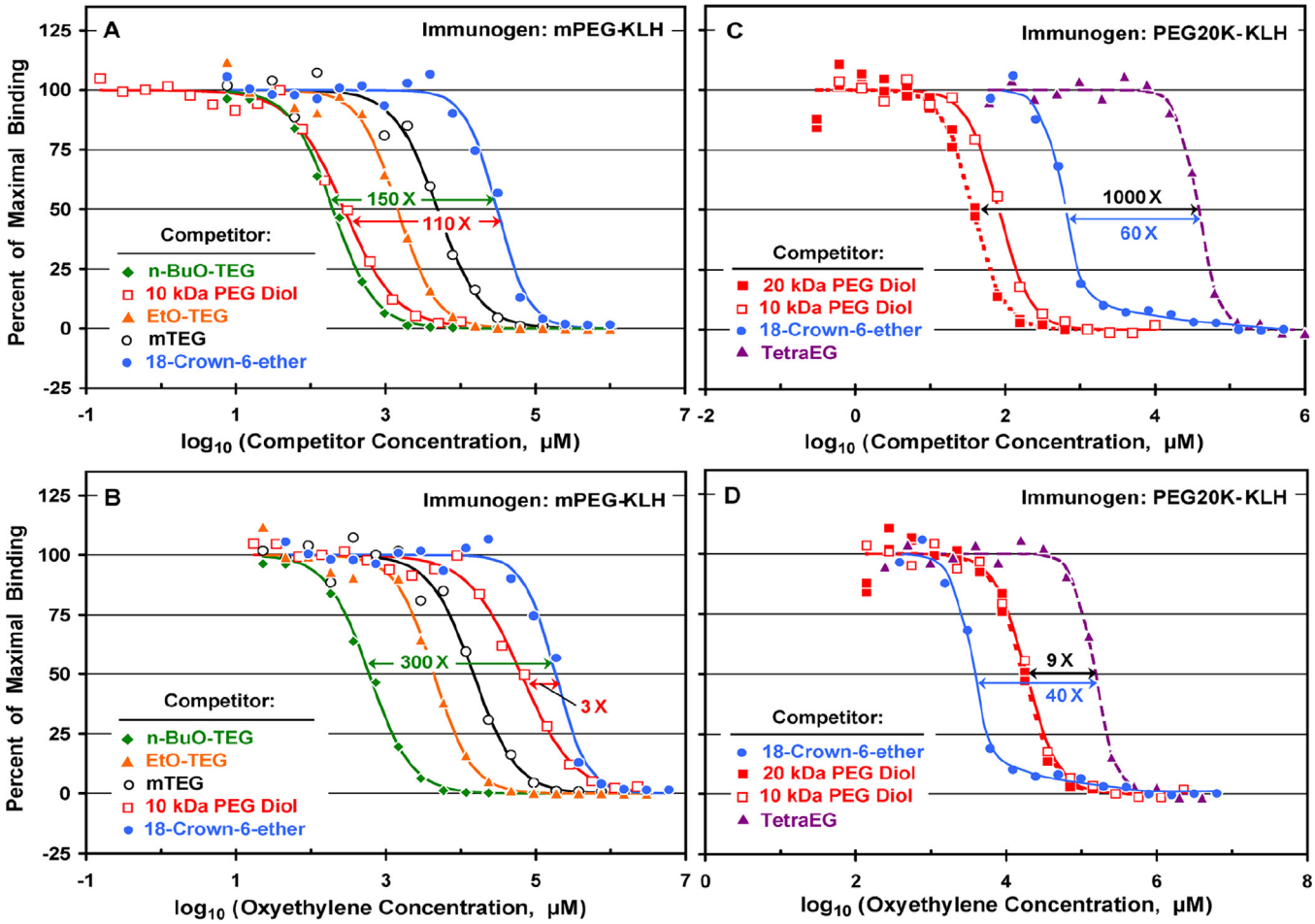

Fig. 7. The binding selectivities of a "methoxy-specific" IgG mAb (A and B) and a "backbone-specific" IgM mAb (C and D) are distinguished by competitive ELISAs with 10 kDa and 20 kDa PEG diols and PEG-like oligomers. The antigen was mPEG-SOD. (A and B) Epitomics Clone ID: PEG-B-47 was used at a final protein concentration of 97 ng/mL, with a 1/15,000 dilution of the secondary antibody. (C and D) Epitomics Clone ID: PEG-2-128-7 was used at a concentration of 1150 ng/mL, with a 1/10,000 dilution of the secondary antibody. The percentages of maximal binding are shown as a function of the concentrations of the competitors (A and C) or of oxyethylene units in the respective competitors (B and D). Green and red arrows indicate the relative affinities of the "methoxy-specific" mAb for n-BuO-TEG and 10 kDa PEG diol, respectively, each relative to that of 18-crown-6-ether (A and B). Black and blue arrows indicate the affinities of the "backbone-specific" mAb for 20 kDa PEG diol and 18-crown-6-ether, respectively, each relative to that of TetraEG (C and D). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

For anti-PEG antibodies induced by HO-PEG-uricase, the length of the polymer backbone was an even more important determinant of the affinities for various polymers, based on their molar concentrations (Fig. 2B). The affinities for mTEG and TEG diol were ca. 3400-fold and ca. 23,000-fold lower, respectively, than for the corresponding 10 kDa PEGs, while the affinities for 10 kDa PEG diol and 10 kDa mPEG did not differ appreciably from each other, as shown by Sherman et al. (2012b). Thus, the length of the polymer backbone had more pronounced effects on the relative affinities of anti-HO-PEG antibodies than on those of anti-mPEG antibodies.

3.4. Minimal effect of backbone length of the antigen on affinities for large PEGs

The relative affinities of polyclonal antibodies induced by $mPEG_2$-IFN-α for 10 kDa mPEG versus 10 kDa PEG diol are not affected appreciably by using an antigen (mTEG-SOD) in which the polymer backbone has only three oxyethylene groups, instead of an average of ca. 227 oxyethylene groups, as in 10 kDa mPEG-SOD (Fig. 3). These results confirm that in assays of the relative affinities of anti-mPEGs, the presence of a terminal methoxy group in the competitors is more important than the length of the polymer backbone of the antigen.

3.5. Modulation of impact of a methoxy group by competitor size

When the assay plates are coated with a protein conjugate of an mPEG-like molecule of low molecular weight (mTEG-SOD), the observed selectivity of antibodies induced by $mPEG_1$-IFN-α for mTEG versus TEG diol is greater than that detected for 10 kDa mPEG versus PEG diol as the competitors (Fig. 4). As indicated by the higher value of IC_{50} for mTEG than for either of the 10 kDa PEGs, the high-molecular-weight competitors are bound more tightly than the low-molecular-weight competitors. On the other hand, the relative affinities for the methoxy-containing competitors, compared with the non-methoxy competitors of similar molecular weights, are more than three times as high for the oligomeric competitors (70×) compared with the 10 kDa PEGs (20×). Thus, end-group selectivities are detected with greater sensitivity in comparisons of PEG-like oligomers than in comparisons of larger polymers, in which binding to the polymer backbone partially obscures differences between the affinities for the end-groups.

3.6. Selectivities of anti-PEG mAbs among oligomeric, polymeric and multi-PEGylated competitors

A "methoxy-specific" rabbit mAb induced by mPEG-KLH (Epitomics Clone ID: PEG-B-47) has measurable affinity for 10 kDa PEG

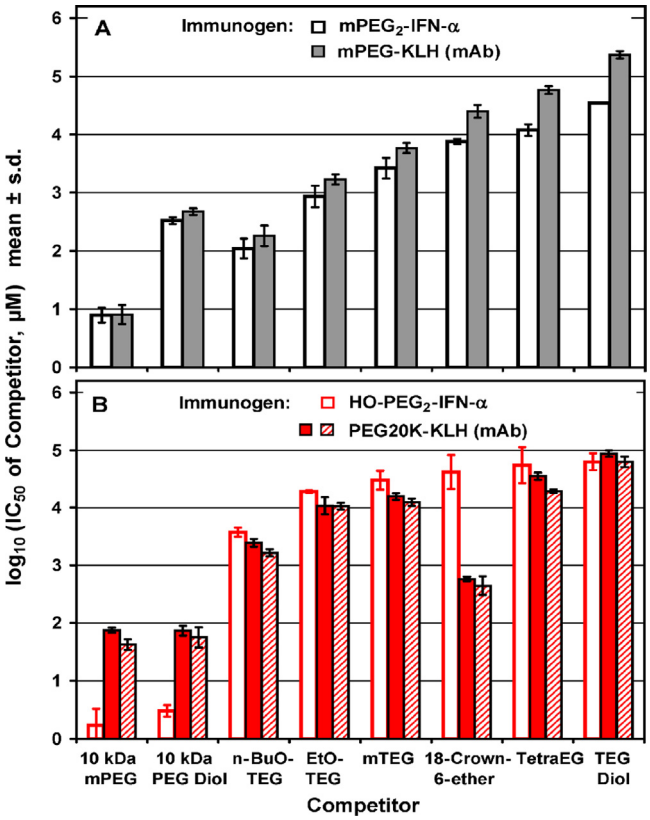

Fig. 8. Potencies of 10 kDa mPEG and PEG diol and six PEG-like oligomers are compared as inhibitors of the binding to mPEG-SOD of rabbit antisera or mAbs that are "methoxy-specific" (A) or "backbone-specific" (B). (A) Competitive ELISAs were performed with a 1/1000 dilution of serum from a rabbit immunized with $mPEG_2$-IFN-α (white bars) or with a "methoxy-specific" mAb (Epitomics Clone ID: PEG-B-47), diluted to 97 ng/mL (gray); the secondary antibody was diluted 1/15,000. (B) Competitive ELISAs were performed with a 1/300 dilution of serum from a rabbit immunized with HO-PEG_2-IFN-α (white) or with a "backbone-specific" mAb (Epitomics Clone ID: PEG-2-128-7) diluted to either 1150 or 1530 ng/mL (solid red). The secondary antibody (anti-rabbit IgG H and L chains) was diluted to between 1/5000 and 1/15,000 in various experiments (solid red). The same "backbone-specific" mAb was assayed at a protein concentration of 590 ng/mL, with the secondary anti-rabbit IgM antibody diluted 1/3000 (hatched red). The six PEG-like oligomers are arranged in order of decreasing affinities (increasing values of IC_{50}) for binding to "methoxy-specific" anti-PEGs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

diol (ca. 2% that of 10 kDa mPEG), whether assayed on mPEG-SOD or HO-PEG-SOD as the antigen (Fig. 5A). In contrast, a "backbone-specific" rabbit mAb induced by a 20 kDa mPEG conjugate of KLH (Epitomics Clone ID: PEG-2-128-7) exhibits no preferential binding to mPEG-SOD versus HO-PEG-SOD as the antigen or to 10 kDa mPEG versus 10 kDa PEG diol as the competitor (Fig. 5B). This indicates that the "backbone-specific" mAb does not recognize the terminal hydroxy group(s) on either the antigen (HO-PEG-SOD) or the competitor (PEG diol). Although the mAb used in Fig. 5B is of the IgM subtype, the secondary antibody raised against the H and L chains of IgG gave a much higher signal than the secondary antibody raised against the mu chains of rabbit IgM, which was used in certain other experiments (see Figs. 8B and 10).

The "methoxy-specific" mAb has significant affinity for multi-PEGylated conjugates of albumin made with 10 kDa mPEG, but no measurable affinity for multi-PEGylated conjugates of albumin made with 10 kDa HO-PEG (Fig. 6A). Similar results were obtained in analyses of anti-PEGs in sera from rabbits immunized with mPEG conjugates of uricase, albumin or IFN-α (Sherman et al., 2012a,b). The "backbone-specific" mAb has indistinguishable affinities for multi-PEGylated conjugates of albumin containing 10 kDa mPEG or 10 kDa HO-PEG (Fig. 6B). Similarly, polyclonal anti-PEGs induced by HO-PEG conjugates of uricase, IFN-α or serum albumin exhibit no appreciable differences in their affinities for the same multi-PEGylated competitors (Sherman et al., 2012a,b). Thus, neither the "backbone-specific" mAb nor the polyclonal anti-HO-PEGs exhibit(s) appreciable preference for a hydroxy group versus a methoxy group at the remote terminus of large protein-linked polymers.

3.7. Unexpected selectivities of "methoxy-specific" and "backbone-specific" anti-PEG mAbs

The end-group selectivities of the "methoxy-specific" and "backbone-specific" mAbs for competitors of various sizes and structures were analyzed with respect to the molar concentrations of the competitors, themselves, and of the oxyethylene groups (CH_2—CH_2—O) in the respective competitors (Fig. 7). Although the "methoxy-specific" mAb was induced by an mPEG conjugate of KLH, it is noteworthy that the rank order of the affinities among the alkyl ethers of TEG is n-BuO-TEG > EtO-TEG > mTEG. This antibody binds n-BuO-TEG much more tightly than 18-crown-6-ether, whether the relative affinities are measured with respect to the molar concentrations of the competitors (150-fold) or of the oxyethylene groups in the respective competitors (300-fold). The weak binding of this crown ether by the "methoxy-specific" mAb (Fig. 7A and B) is in marked contrast with the relatively high affinity of the "backbone-specific" mAb for the crown ether (Fig. 7C and D).

The affinity of the "backbone-specific" mAb for 20 kDa PEG diol is about twice that for 10 kDa PEG diol on a molar basis, but the affinities of this mAb for the large polymers are indistinguishable based on their respective concentrations of oxyethylene groups (Fig. 7C and D). The affinity of this "backbone-specific" mAb for 20 kDa PEG diol is ca. 1000 times higher than that for TetraEG on a molar basis, but is only nine times higher on the basis of the respective concentrations of oxyethylene groups. Based on the latter concentrations, 18-crown-6-ether is the most potent competitor among the tested PEG-like oligomers for the "backbone-specific" mAb (Fig. 7D), but the least potent competitor for the "methoxy-specific" mAb (Fig. 7B).

In contrast with the results shown in Fig. 7, no inhibition of binding of anti-interferon-α antibodies in the sera of rabbits immunized with either $mPEG_2$-IFN-α or HO-PEG_2-IFN-α was detected, even with the highest tested concentrations of mTEG, n-BuO-TEG, EtO-TEG or 18-crown-6-ether. The results are shown in Fig. S2 of the Supplementary Data.

3.8. Rank orders of affinities of polyclonal and monoclonal anti-PEGs

The rank orders of the affinities of an anti-mPEG antiserum and a "methoxy-specific" mAb for two large polymers and six oligomeric competitors are the same (Fig. 8A). Among three tested alkyl ethers of TEG diol, the relative affinities were n-BuO-TEG > EtO-TEG > mTEG, all of which are higher than the affinities for TetraEG or TEG diol. A "backbone-specific" mAb and an anti-HO-PEG serum are similar with respect to their relative affinities for n-BuO-TEG > EtO-TEG > mTEG > TetraEG > TEG diol (Fig. 8B). However, the rank order of affinities of the tested anti-HO-PEG serum for three competitors was 10 kDa PEG diol ≫ n-BuO-TEG > 18-crown-6-ether, while the rank order of affinities of the "backbone-specific" mAb was 10 kDa PEG diol > 18-crown-6-ether > n-BuO-TEG. In these comparisons, the symbol "≫" designates a difference in affinity of more than a factor of 100.

Another difference between the polyclonal anti-HO-PEGs and the "backbone-specific" mAb is that the polyclonal anti-HO-PEGs exhibit >10-fold higher affinities than the "backbone-specific" mAb

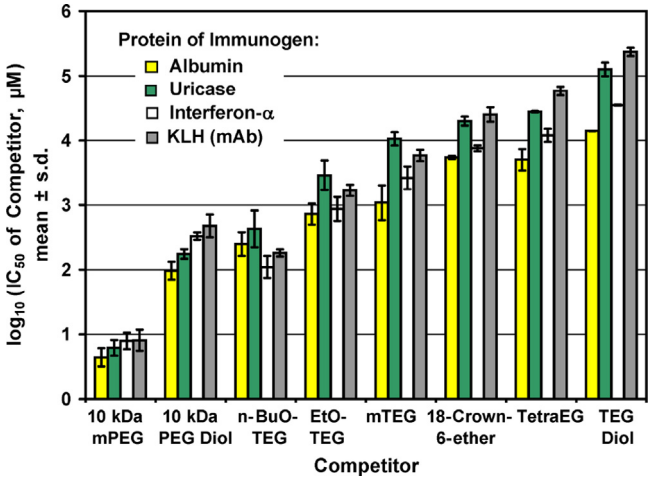

Fig. 9. Potencies of 10 kDa mPEG, 10 kDa PEG diol and six PEG-like oligomers are compared as inhibitors of the binding to mPEG-SOD by polyclonal antibodies induced in rabbits by mPEG conjugates of three unrelated proteins and a "methoxy-specific" mAb induced by an mPEG conjugate of another unrelated protein. ELISAs were performed with the same competitors as in Fig. 8 on sera from individual rabbits immunized with either $mPEG_{17}$-albumin (yellow bars), mPEG-uricase (green) or $mPEG_2$-IFN-α (white), each diluted 1/1000. A "methoxy-specific" rabbit IgG mAb (Epitomics Clone ID: PEG-B-47) was used at a final protein concentration of 97 ng/mL (gray). The secondary antibody (anti-rabbit IgG H and L chains) was diluted 1/15,000. The results for log (IC_{50}) for the eight competitors are arranged in the same order as in Fig. 8. To facilitate comparisons among data for anti-mPEGs induced by all four immunogens, the results from Fig. 8A, for serum from a rabbit immunized with $mPEG_2$-IFN-α and for a "methoxy-specific" mAb, are repeated here (white and gray bars, respectively).

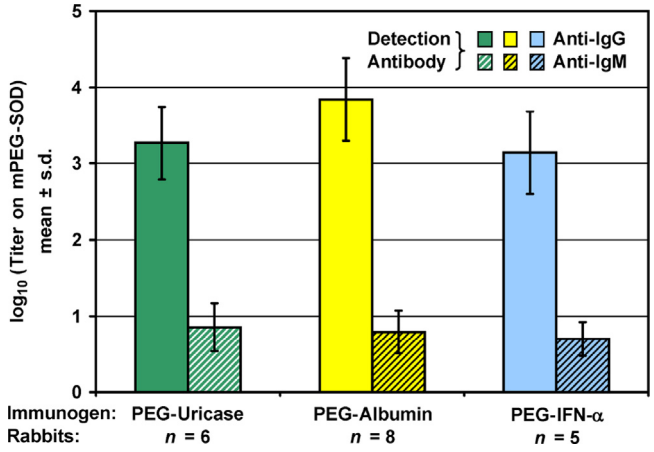

Fig. 10. Measurement of the titers of IgG and IgM isotypes of anti-PEG antibodies in sera from the indicated numbers of rabbits immunized with PEG conjugates of uricase, albumin or IFN-α. Direct ELISAs were performed with mPEG-SOD as the antigen. The detection antibodies were either peroxidase-linked goat anti-rabbit IgG (H and L chains), at a 1/15,000 dilution, or peroxidase-linked goat anti-rabbit IgM at a 1/3000 dilution. These dilutions provided similar maximal absorbance signals in the peroxidase assays. The titers were calculated as the concentrations of sera corresponding to 50% of the maximal signal (D_{50}), as described in Section 2.4.

for both of the 10 kDa PEGs under these assay conditions. Finally, the rank orders of the affinities of the "backbone-specific" mAb for the eight competitors are the same, regardless of whether the secondary antibody used for detection of binding was directed against rabbit IgM or against the H and L chains of rabbit IgG (Fig. 8B), which was the secondary antibody used in most of the other experiments in this report.

3.9. Properties of immunogens that do not affect the relative affinities of anti-mPEGs

The rank order of affinities of antibodies induced by mPEG conjugates of four unrelated proteins for 10 kDa mPEG and PEG diol and six PEG-like oligomers is the same regardless of: (1) the nature of the protein in the immunogen; (2) the coupling chemistry; (3) the size of the PEG in the immunogen; (4) the ratio of PEG-to-protein in the immunogen; and (5) whether the tested anti-PEGs were polyclonal or monoclonal (Fig. 9). The protein components of the four immunogens are indicated in Fig. 9. The coupling chemistries included reductive alkylation with 20 kDa mPEG propionaldehyde to make conjugates of IFN-α and KLH (for preparation of the "backbone-specific" mAb); the use of a *p*-nitrophenylcarbonate derivative of 10 kDa mPEG to form urethane bonds with serum albumin and uricase; and the use of 5 kDa mPEG maleimide to form thioether bonds with sulfhydryl groups in KLH (for preparation of the "methoxy-specific" mAb). Information about the sizes and coupling chemistries of the mPEGs used to induce the mAbs was provided by Dr. Helen (Hua) Zhong, a senior scientist at Epitomics (personal communication). Thus, the sizes of the mPEGs in the immunogens used to generate the data in Fig. 9 ranged from 5 kDa to 20 kDa. The average numbers of molecules of PEG per molecule of protein were one or two for the IFN-α conjugates, ca. 2.3 per uricase subunit and ca. 17 for serum albumin. The wide range of PEG-to-protein ratios of the immunogens (on a molar or a mass basis) did not appear to alter the selectivities of the resultant anti-PEGs.

3.10. Unusual specificity profile of a "backbone-specific" anti-PEG mAb

In view of the striking difference between the relative affinities of the tested polyclonal anti-HO-PEG antiserum and the "backbone-specific" mAb for the 10 kDa PEGs and 18-crown-6-ether (Fig. 8B), the selectivities of polyclonal anti-HO-PEGs among four competitors – 10 kDa mPEG, the crown ether, mTEG and TetraEG – were assayed in sera from 11 rabbits (Fig. S1 of Supplementary Data). The sera were from three rabbits immunized with HO-PEG-uricase, four immunized with HO-PEG-albumin and two each immunized with HO-PEG_1-IFN-α or HO-PEG_2-IFN-α. The results show that the mean affinities of the polyclonal anti-HO-PEGs for the crown ether, mTEG and TetraEG, relative to that for 10 kDa mPEG, differed by less than a factor of ten among the sera from groups of rabbits immunized with HO-PEG conjugates of all three proteins. However, the latter pattern of relative affinities was distinct from that of the "backbone-specific" mAb. In particular, the mAb showed a strong preference for the crown ether > mTEG > TetraEG, and the affinities of the mAb for the PEG-like oligomers were much closer to its affinity for 10 kDa mPEG (and 10 kDa PEG diol, as shown in Fig. 8B) than were the affinities of the anti-HO-PEG sera for the same ligands.

3.11. Much lower titers of IgM than of IgG anti-PEGs

Since all polyclonal antibodies described here were in sera collected two weeks after the sixth immunization, it was expected that the isotype of the antibodies would be predominantly IgG, rather than IgM (Durandy, 2003). This expectation was borne out by the results of replicate direct ELISAs performed with peroxidase-linked goat anti-rabbit IgG (H and L chains) or with peroxidase-linked goat anti-rabbit IgM as the detection antibody (Fig. 10). The magnitudes of the titers detected in sera from rabbits immunized with mPEG versus HO-PEG conjugates of the same protein (Fig. 1) differed much less than the titers detected with anti-IgG versus anti-IgM (Fig. 10). Therefore, data for three rabbits each immunized with mPEG or HO-PEG uricase were combined for the analyses in Fig. 10, as were data for four rabbits each immunized with mPEG or HO-PEG-albumin and for two rabbits immunized with mPEG-IFN-α and three rabbits immunized with HO-PEG-IFN-α. For each group of rabbits immunized with PEG conjugates of uricase, albumin or

Table 3
Major and minor determinants of anti-PEG selectivities.

Anti-PEG classification	Major determinant	Minor determinant(s)	Relative affinities
"Methoxy-specific"			
Polyclonal	End-group hydrophobicity	Backbone length	10 kDa mPEG > 10 kDa PEG diol; n-BuO-TEG > EtO-TEG > mTEG > Crown ether > TetraEG > TEG diol
Monoclonal (Epitomics PEG-B-47)	End-group hydrophobicity	Backbone length	10 kDa mPEG > 10 kDa PEG diol; n-BuO-TEG > EtO-TEG > mTEG > Crown ether > TetraEG > TEG diol
"Backbone-specific"			
Polyclonal	Backbone length	End-group hydrophobicity	10 kDa mPEG ≈ 10 kDa PEG diol; n-BuO-TEG > EtO-TEG > mTEG ≈ Crown ether ≈ TetraEG ≈ TEG diol
Monoclonal (Epitomics PEG-2-128-7)	Backbone length	End-group hydrophobicity; size of polymer-binding domain	20 kDa PEG diol > 10 kDa PEG diol ≈ 10 kDa mPEG; Crown ether > n-BuO-TEG > EtO-TEG ≈ mTEG > TetraEG > TEG diol

IFN-α, the mean titer of IgG antibodies was at least 100-fold higher than the mean titer of IgM antibodies.

4. Discussion and conclusions

4.1. Factors that influence the selectivities of anti-PEG antibodies

The induction of anti-PEG antibodies after immunization of laboratory animals or patients with mPEG conjugates of various proteins, red blood cells, liposomes or adenoviruses has been documented by several independent research groups (Armstrong, 2009; Cheng et al., 2012; Ganson et al., 2006; Richter and Åkerblom, 1983; Sherman et al., 2012a,b; Shimizu et al., 2012). We have reported previously that most of the polyclonal anti-PEGs induced in rabbits by mPEG-protein conjugates bind preferentially to mPEGs compared to PEG diols of similar molecular weights, under the immunization and assay conditions used in our research (Martinez et al., 2012; Sherman et al., 2012a,b). This report confirms that such preferential binding occurs and provides additional insights into several factors that contribute to the observed selectivities of anti-PEGs. These factors include (1) the hydrophobicities of the end-groups of polymers or oligomers used as competitors; (2) the backbone lengths of the polymeric or oligomeric competitors; and (3) the backbone lengths of the polymers or oligomers in the protein conjugates with which the assay plates are coated (Table 3). An interesting question to be addressed in future research is the temporal relationship between the inductions of the "methoxy-specific" anti-PEGs and the "backbone-specific" anti-PEGs. All of the data reported here were obtained with sera obtained after six immunizations of the rabbits with the PEGylated proteins (see Sherman et al., 2012b).

4.2. Factors with no detected effects on the selectivities of anti-mPEGs

The rank orders of the affinities for eight competitors that ranged in size from TEG diol (150 Da) to 10 kDa mPEG are indistinguishable for polyclonal and monoclonal anti-PEGs induced in rabbits by mPEG conjugates of four unrelated proteins (Fig. 9). The protein components of the immunogens included KLH and porcine uricase, both of which are known to be highly immunogenic (Harris and Markl, 1999; Sherman et al., 2008), as well as human IFN-α and human serum albumin. The distinctive properties of the proteins to which the PEGs were coupled in the immunogens have no effect on the selectivities of the anti-mPEGs induced and detected under the present experimental conditions. Furthermore, the selectivities of the anti-mPEGs appear not to be affected by the molecular weights of the mPEGs in the immunogens (5 kDa, 10 kDa or 20 kDa) or by the chemical linkages between the polymers and the proteins of the immunogens (thioethers, urethane bonds or secondary amines).

4.3. Importance of the hydrophobicity of PEG-like oligomers

For each of three tested anti-mPEG antisera and a "methoxy-specific" mAb, 10 kDa mPEG is the most potent and TEG diol was the least potent, on a molar basis, of the eight competitors tested (Fig. 9 and Table 3). Among the alkyl ethers of TEG, the relative competitive potencies are: n-BuO-TEG > EtO-TEG > mTEG, suggesting that the hydrophobicity of the competitor, rather than its resemblance to the terminus of the polymer component of the immunogen (which was a methoxy group), is the dominant factor determining the affinities of the antibodies. This conclusion argues against the equivalence of C_1 through C_4 alkoxy PEGs in the production of protein conjugates, as was suggested in several families of patents (Bailon, 2007; Burg et al., 2011; Papadimitriou, 2007; Saifer et al., 1994).

4.4. Anti-PEG binding of PEG-like oligomers with only three oxyethylene units

The results of several types of experiments indicate that polyclonal and monoclonal anti-PEGs recognize PEG-like oligomers containing only three oxyethylene groups, both as competitors and as antigens (Figs. 2, 4A, 7–9). Previous investigators have inferred that the smallest numbers of oxyethylene units that could be recognized by the anti-PEG antibodies that they studied were either 4–5 units (Armstrong, 2009), 6–7 units (Richter and Åkerblom, 1983) or about 16 units (Cheng et al., 2005). In fact, mTEG is bound with sufficient affinity to enable its use as the polymer component of the antigen (mTEG-SOD) in competitive ELISAs of sera from all of the tested rabbits immunized with mPEG-proteins (e.g., Figs. 3A and 4).

4.5. Potential interference of PEG-like detergents in anti-PEG assays

The demonstration that PEG-like oligomers with as few as three oxyethylene groups can compete effectively with the binding of antibodies induced by conjugates of four unrelated proteins with either mPEG or HO-PEG reinforces previous warnings against the use of detergents such as Tween 20 or Tween 80 in assays of anti-PEGs (Armstrong, 2009; Life Diagnostics; Meridian Life Science; Sherman et al., 2012b). Similar caution should be taken with other detergents containing oxyethylene groups, e.g., Triton X-100. Tweens contain three HO-PEGs and one HO-PEG ester, each with an average of five oxyethylene groups (Sherman et al., 2012b) while

Triton X-100 contains one HO-PEG chain with about nine oxyethylene groups (Sigma–Aldrich). Interestingly, Su et al., 2010 reported that different murine anti-PEG mAbs varied in their sensitivities to inhibition by Tween 20, as observed by Sherman et al. (2012b) for polyclonal rabbit anti-PEGs raised against mPEG-uricase vs. HO-PEG-uricase.

4.6. Competitive potencies of multi-PEGylated albumin with mPEG vs. HO-PEG

The ability of sufficiently high concentrations of 10 kDa PEG diol to completely inhibit the binding of the "methoxy-specific" mAb to mPEG-SOD (Fig. 5A) appears to be inconsistent with the inability of an albumin conjugate of 10 kDa HO-PEG (HO-PEG$_{22}$-albumin) to inhibit the same binding at the tested concentrations (Fig. 6A). However, these results are consistent with the interpretation that 10 kDa PEG diol that is free in solution is more accessible to the polymer-binding domains of anti-mPEGs than are molecules of 10 kDa HO-PEG that are coupled to serum albumin or, in principle, to other large carriers. In contrast, albumin-bound mPEG (Fig. 6A) is ca. 50-fold more effective as a competitor than unconjugated mPEG (Fig. 5A), presumably because of the enhanced avidity that arises from the cooperative binding of the sterically accessible terminal methoxy groups to the mAb. Such cooperativity was documented for polyclonal anti-mPEG antibodies by Sherman et al. (2012b). In Fig. 7A and B of that report, the affinities of polyclonal antibodies raised against mPEG-IFN-α and mPEG-uricase for 10 kDa mPEG conjugated to albumin were between 10-fold and 20-fold higher than their respective affinities for free 10 kDa mPEG in solution. In contrast, anti-PEGs raised against HO-PEG-uricase had similar affinities for free 10 kDa PEG diol in solution and for 10 kDa HO-PEG in albumin conjugates (Fig. 7C of Sherman et al., 2012b).

4.7. Tight binding of 18-crown-6-ether by a "backbone-specific" anti-PEG mAb

The most striking difference in selectivities between the "backbone-specific" mAb and antisera from 11 rabbits immunized with HO-PEG conjugates of three proteins is between the relatively high affinity of the mAb for 18-crown-6-ether and the relatively low affinities of the polyclonal anti-HO-PEGs for the same crown ether (Fig. 8B and Fig. S1 of Supplementary Data). Analyses of the most stable conformations of the crown ether (Al-Jallal et al., 2005; Dunitz and Seiler, 1974; Leuwerink and Briels, 1995) suggest that an anti-PEG would need relatively large binding domains to accommodate this ligand. Such large binding pockets might also accommodate folded segments of the backbone of a large PEG, although the orientations of the adjacent oxyethylene units are more constrained in the cyclic oligomer. We infer that the "backbone-specific" IgM mAb has larger binding domains than the polyclonal anti-HO-PEGs, as well as the polyclonal and monoclonal anti-mPEGs (Figs. 8 and 9 and Table 3). The apparently high affinity of the rabbit IgM mAb for the crown ether was not an artifact due to the use of a secondary antibody that was directed against the H and L chains of rabbit IgG, since the same result was obtained with an anti-rabbit IgM as the secondary antibody (Fig. 8B).

4.8. Potential clinical implications

There are obvious risks in attempting to extrapolate to human clinical experience from the present results, which are based primarily on studies of anti-PEGs induced in hyperimmune rabbits, whose initial immunization included complete Freund's adjuvant (Sherman et al., 2012b). The ensemble of data in this report and previous publications by Sherman et al. (2012a,b) indicates that (1) sera from all except one of 15 rabbits immunized with an mPEG-protein bound 10 kDa mPEG with about 20-fold to 30-fold higher affinity than 10 kDa PEG diol (Fig. 1) and (2) PEG-like oligomers with more hydrophobic end-groups, e.g., ethoxy or butoxy groups, are bound more tightly than a similar oligomer containing a methoxy group, as was present in the immunogens (Figs. 8 and 9 and Table 3). These results suggest that the induction of treatment-limiting anti-PEG antibodies in response to mPEG-protein conjugates, like those in current clinical use, will not be mitigated by replacing mPEG with alkoxy-PEGs containing larger and more hydrophobic end-groups. On the other hand, less frequent and less intense immune responses to PEGylated proteins (and, by analogy, PEGylated red blood cells, liposomes, viral vectors and other drug-delivery vehicles) may be achieved by coupling them to HO-PEG, instead of mPEG. Testing of this hypothesis will benefit from future studies of the occurrence of accelerated clearance of analogous mPEG and HO-PEG conjugates of the same protein following initial and subsequent exposures of laboratory animals and, eventually, human patients.

Acknowledgements

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Appendix A. Supplementary data

Potencies as inhibitors of the binding to mPEG-SOD by a "backbone-specific" mAb and polyclonal anti-HO-PEG antibodies in sera from 11 rabbits are compared for three PEG-like oligomers, each relative to that of 10 kDa mPEG, in Fig. S1. Evidence that anti-interferon-α (anti-IFN-α) antibodies are not inhibited by concentrations of 10 kDa mPEG and PEG-like oligomers that inhibit the binding of anti-PEG antibodies raised against mPEG or HO-PEG conjugates of IFN-α and other proteins is provided in Fig. S2. Values of the maximal binding, in units of the kinetic colorimetric assays, which were used to normalize the data in Figs. 2–7, are shown in Table S1.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2013.07.014.

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