Sensitive Quantification of PEGylated Compounds by Second-Generation Anti-Poly(ethylene glycol) Monoclonal Antibodies

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Poly(ethylene glycol) (PEG) is often attached to compounds to increase serum half-life, reduce immunogenicity, and enhance bioavailability. Accurate and sensitive quantification of PEG conjugates is critical for product development, pharmacokinetic measurements, and efficacy studies. However, PEGylated compounds can be difficult to quantify due to epitope masking by PEG. We previously generated two monoclonal antibodies to PEG (AGP3, IgM and E11, IgG) for quantitative detection of PEGylated proteins. We now report the identification of two second-generation mAbs to PEG (APG4, IgM and 3.3, IgG) that bind to the repeating subunits of the PEG backbone and facilitate more sensitive quantification of a wide range of PEGylated compounds. A sandwich ELISA in which APG4/3.3-biotin was employed as the capture/detection antibodies allowed quantification of PEG-Qdot 525 with 14-50-fold greater sensitivity than the original AGP3/E11 combination. Pegasys (PEG-interferon alpha-2a), PEG-Intron (PEG-interferon alpha-2b), Neulasta (PEG-G-CSF), and Lipo-Dox (PEGylated liposomal doxorubicin) could also be quantified with low ng/mL detection limits. The assay tolerated the presence of 50% human serum or 20% free PEG molecules. These new anti-PEG antibodies appear useful for qualitative and quantitative analysis of a wide range of PEGylated compounds.

INTRODUCTION

Covalent attachment of poly(ethylene glycol) (PEG) to peptides, proteins, and nanoparticles can enhance their stability, solubility, and circulation life. A wide range of PEGylated protein drugs is under development (1–7). More recently, PEG has been conjugated to nanoparticles, such as superparamagnetic iron oxide (SPIO) (8, 9), quantum dots (PEG-Qdot) (10, 11), and liposomes to enhance their biocompatibility and diminish uptake by the reticuloendothelial system. Several PEGylated compounds have been approved for clinical use by the FDA including Pegasys (PEG-interferon alpha-2a) (12, 13), PEG-Intron (PEG-interferon alpha-2b) (4), Somavert (PEG-human growth hormone receptor antagonist) (15), Cinzima (certolizumab pegol, pegylated anti-human TNF-alpha Fab') (16), Neulasta (pegfilgrastim, PEG-G-CSF) (17, 18), and Lipo-Dox (PEGylated liposomal doxorubicin) (19, 20). PEG modification will likely facilitate clinical utilization of more imaging reagents, therapeutic proteins, and small molecule drugs.

Although PEGylation is a promising technology to improve the pharmacokinetic properties of compounds, effective approaches to quantify PEGylated conjugates in complex samples are limited. Sandwich ELISA is commonly used to measure the protein component of PEGylated proteins, but shielding antibody epitopes with bulky PEG chains, which is advantageous for reducing protein immunogenicity, may also limit assay sensitivity. Chromatographic methods are complicated by peak broadening due to the polydispersity of PEG conjugates and strong peak tailing due to nonspecific adhesion to the stationary phase (21). LC-MS/MS approaches can be sensitive but usually require complex multistep sample processing steps to reduce sample complexity before analysis (22). Solid-phase extraction and multiple analyses are also typically required to measure total, free, and encapsulated drug concentrations in PEGylated liposomes due to the difficulty of directly assaying liposomes in serum samples (23). Therefore, the successful development of a sensitive anti-PEG sandwich ELISA may provide a simple and universal tool for quantifying PEGylated compounds. Previously, we described a sandwich ELISA system using monoclonal anti-PEG antibodies (APG3/IgM and E11/IgG) to measure PEGylated compounds in vitro and in vivo (24). We recently generated additional second-generation monoclonal antibodies against PEG. Here, we show that two of these antibodies (APG4 and 3.3) allow much more sensitive detection of a broader range of PEGylated compounds including PEG-interferon α (Pegasys and PEG-Intron), PEG-Qdot 525, and Lipo-Dox.

EXPERIMENTAL PROCEDURES

Reagents and Animals. Methoxy-PEG(750)-NH₂, methoxy-PEG(2000)-NH₂, hydroxy-PEG(5000)-NH₂, methoxy-PEG(10,000)-NH₂, methoxy-PEG(20,000)-NH₂ (750, 2000, 5000, 10 000 and 20 000 Da, respectively), NH₂-PEG(3000)-NH₂, methoxy-PEG(2000)-COOH, and 4-arm poly(ethylene oxide) (2000)-NH₂ were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). EZ-link NHS-LC-Biotin was purchased from Pierce (Rockford, IL). PEG-Qdot 525 (Qdot 525 ITK amino (PEG) quantum dots), a nanocrystal semiconductor material coated with multiple linear
2 kDa amino-PEG molecules, was purchased from Invitrogen (Carlsbad, CA). Lipo-Dox was from Taiwan Tung Yang Biopharm (TTY Biopharm Company Ltd., Taipei, Taiwan). Pegasy and PEG-Intron were from Roche (Nutley, NJ, CA) and Schering-Plough (Kenilworth, NJ, CA), respectively. Neulasta (pegylated G-CSF) was a gift from Amgen (Thousand Oaks, CA). Specific pathogen-free BALB/c mice were obtained from the National Laboratory Animal Center, Taipei, Taiwan.

**Antibodies.** Hybridomas secreting AGP3, AGP4, IgM (IgM mAb against PEG), E18 (IgG; mAb against E. coli βG, eβG), E11, and 3.3 (IgG1 mAbs against PEG) were generated by immunizing female BALB/c mice with PEG-derived proteins as described previously (25). Briefly, mice were i.v. injected with 200 µg of RH1-e/βG-PEG5000, a conjugate formed between a murine antibody and PEG-derivatized eβG. The plates were i.p. injected with 100 µg RH1-eβG-PEG5000 one week later and then s.c. injected at weekly intervals with 50 µg of eβG-PEG5000 in complete Freund’s adjuvant, 30 µg of eβG-PEG5000 in incomplete adjuvant, 30 µg of BSA-PEG5000 in incomplete adjuvant, and 10 µg of BSA-PEG5000 in incomplete adjuvant. Three days before fusion with FO myeloma cells, the mice were i.p. injected with 30 µg of BSA-PEG5000 in PBS. Hybridomas were generated by fusing spleen cells with FO myeloma cells and then screening culture media by ELISA in 96-well microtiter plates coated with 1 µg/well βG or βG-PEG5000. Hybridomas were cloned 3 times by limiting dilution in 96-well microtiter plates containing thymocyte feeder cells in HT medium supplemented with 15% fetal calf serum. Anti-PEG rabbit mAb PEG-B-47 was from Epitomics (Burlingame, CA). B139 M and B141 M mouse anti-PEG mAbs were from Meridian Life Science, Inc. (Saco, ME). Horseradish peroxidase (HRP)-conjugated goat antiantigen IgM μ-chain antibody, HRP-conjugated donkey antiantigen IgG Fc, HRP-conjugated antiantigen Ig, and HRP-conjugated streptavidin were from Jackson ImmunoResearch Laboratories (West-grove, PA).

**Antibody Biotinylation.** Antibodies (3.3, E11, AGP3, and AGP4) in PBS at 2 mg/mL were mixed with a 25-fold molar excess of EZ-link NHS-LC-Biotin (dissolved in DMSO) for 2 h at room temperature to produce 3.3-biotin, E11-biotin, AGP3-biotin, and AGP4-biotin. One-tenth volume of a saturated glycine solution was added to stop the reaction. Free biotin was removed by dialysis in PBS at 4 °C. Antibody concentration was evaluated by the bicinchoninic acid assay (Pierce, Rockford, IL) with bovine serum albumin used as the reference protein.

**Antibody ELISA.** Maxisorp 96-well microplates (Nalge-Nunc International, Roskilde, Denmark) were coated with 0.5 µg/well CH2-PEG15000-NH2, CH2-PEG5000-NH2, NH2-PEG5000-NH2, CH2-PEG20000-NH2, CH2-PEG50000-NH2, CH2-PEG10000-NH2, or CH1-PEG20000-NH2 in 50 µL/well 0.1 M NaHCO3/Na2CO3 (adjusted to pH 8.0 with HCl) for 3 h at 37 °C and then blocked with 200 µL/well dilution buffer (2% skim milk in PBS) at 4 °C overnight. Graded concentrations of antibodies in 50 µL 2% skim milk were added to the plates at RT for 1 h. The plates were washed with PBS-T (PBS containing 0.05% Tween-20) three times and with PBS two times. HRP-conjugated goat antiantigen IgM μ chain (2 µg/mL) or HRP-conjugated donkey antiantigen IgG Fc (2 µg/mL) in 50 µL dilution buffer were added for 1 h at room temperature. The plates were washed as described above, and bound peroxidase activity was measured by adding 100 µL/well TMB substrate solution (BioLegend, San Diego, CA) for 30 min at room temperature. After adding stop buffer (2 N H2SO4, 50 µL/well), the absorbance (450 nm) of wells was measured in a microplate reader (Molecular Device, Menlo Park, CA).

**Immunoblotting.** Samples of Pegasy (0.5 µg), PEG-Intron (0.5 µg) or Neulasta (0.5 µg) were electrophoresed in a 10% SDS-PAGE gel under reducing conditions before overnight transfer to nitrocellulose paper by capillary diffusion in blotting buffer (50 mM NaCl, 2 mM EDTA, 0.5 mM 2-mercaptoethanol, 10 mM Tris-HCl, pH 7.5). Blots were blocked for 1 h with 5% skim milk in PBS and incubated for 1 h at RT with 1 µg/mL AGP3, AGP4, 3.3, or E11 in dilution buffer. The blots were washed 3 times with PBS-T and twice with PBS before incubation with goat antiantigen Ig-HRP for 1 h at RT. After washing three times with PBS-T and twice with PBS, specific bands were visualized by ECL detection according to the manufacturer’s instructions (Pierce, Rockford, IL), then detected by a LAS-3000 mini Fujifilm imaging system (Fujifilm, Tokyo, Japan).

**Sandwich ELISA.** Maxisorp 96-well microplates were coated with 50 µL/well of AGP3, AGP4, E11, 3.3, B139M, B141 M, or PEG-B-47 (5 or 20 µg/mL) in 0.1 M NaHCO3/Na2CO3 (adjusted to pH 8.0 with HCl) for 4 h at 37 °C and then at 4 °C overnight. The plates were blocked with 200 µL/well 2% skim milk in PBS for 2 h at room temperature and then washed with PBS three times. Graded concentrations of PEG-Qdot 525, Lipo-Dox, Pegasy, PEG-Intron, or Neulasta in dilution buffer were added to wells for 2 h at room temperature. Defined amounts of human serum or free PEG molecules (methoxy-PEG-NH2, 2000 or 10 000 Da) were added with samples in some experiments. After washing with PBS-T three times and PBS twice (unless B139 M or B141 M antibodies were assayed, in which case Tween-20 was omitted from all washing steps to prevent competition of the antibodies with detergent), the plates were sequentially stained with 50 µL/well detection antibody (5 µg/mL 3.3-biotin, AGP4-biotin, AGP3-biotin, B141M-biotin, or PEG-B-47-biotin) and 1 µg/mL HRP-conjugated streptavidin. The plates were washed with PBS-T six times and with PBS two times and 100 µL/well ABTS solution (0.4 mg/mL 2,2’-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid), 0.003% H2O2, 100 mM phosphate citrate, pH 4.0) was added for 30 min at room temperature. The absorbance of the wells at 405 nm was measured on a microplate reader.

**Statistical Analysis.** The detection limit in ELISAs was defined as the lowest concentration of PEGylated compound that produced a statistically higher signal than the blank. Statistical significance was calculated using Graphpad Prism 5.0 with the t-test. Data were considered significant at p ≤ 0.05. The relative sensitivities of antibody combinations in ELISA were also compared by interpolating the concentration of analyte that produced an absorbance reading of 1.5 units.

**RESULTS**

**Comparison of Antibody Binding to Immobilized PEG.** The binding specificity of anti-PEG antibodies to different lengths of free linear PEG (MW 750, 2000, 5000, 10 000, and 20 000 Da) and branched PEG (4-arm poly(ethylene oxide), 10 000 Da) was examined by coating NH2-PEG molecules on microtiter plates and then performing ELISA. Both first-generation (E11, AGP3) and second-generation (3.3 and AGP4) antibodies bound to methoxy-PEG-NH2, hydroxyl-PEG-NH2, and diamino PEG demonstrated that the antibodies did not bind to terminal methoxy or hydroxyl groups in PEG (Figure 1). E11, AGP3, and AGP4 bound to both large and small PEG molecules. mAb 3.3, on the other hand, bound PEG molecules longer than 2000 Da but did not bind well to CH2-PEG5000-NH2. Longer PEG chains resulted in enhanced binding of all antibodies, although E11 binding displayed less sensitivity to PEG size. AGP3, AGP4, and 3.3 bound to long PEG chain (MW ≥ 10 000 Da) with similar apparent avidities, whereas E11 bound with ~10-fold lower avidity.

**Immunoblot Analysis of PEG and PEGylated Proteins.** The ability of the anti-PEG antibodies to recognize various PEGylated compounds in immunoblots was determined. AGP3,
AGP4, E11, and 3.3 bound to PEGylated proteins, including PEG-Intron (lane 1), Neulasta (lane 2), and Pegasys (lane 3) (Figure 2). The predicted molecular weights of PEG-Intron (methoxy-PEG12 kDa-interferon alpha-2b19 kDa), Neulasta (methoxy-PEG20 kDa-G-CSF19 kDa), and Pegasys (methoxy-PEG 43 kDa-interferon alpha-2a 19 kDa) are 31 kDa, 39 kDa, and 62 kDa. However, PEG-Intron, Neulasta, and Pegasys migrated with apparent molecule weights of 43 kDa, 60 kDa, and 170 kDa. The higher apparent molecule weights are likely due to the large hydrodynamic volume of PEG in SDS-PAGE gels (26).

Comparison of First- and Second-Generation Antibodies for Sandwich ELISA. We compared different combinations of the first- and second-generation anti-PEG antibodies (capture/detection antibodies) to measure PEGylated compounds by sandwich ELISA. High (1 µg/well) and low (0.25 µg/well) amounts of capture antibodies were examined. Assay sensitivities were compared by determining the concentration of analyte (PEG-Qdot 525) that produced an optical absorbance reading of 1.5 units. The best combinations of first generation antibodies (1 µg/well E11 for capture and AGP3-biotin for detection or 1 µg/well AGP3 for capture and E11 for detection) produced a 1.5 absorbance reading at 1.4 nM and 3.5 nM PEG-Qdot 525, respectively (Figure 3a and c). E11-biotin could not be used in assays because biotinylation destroyed its activity. By contrast, lower concentrations of the second-generation antibodies (AGP4 for capture (0.25 µg/well) with AGP4-biotin or 3.3-biotin for detection) produced absorbance readings at 0.1 and 0.07 nM PEG-Qdot 525, respectively (Figure 3b). Coating higher concentrations of AGP4 produced excessive background readings (Figure 3a). 3.3 performed relatively poorly as a capture antibody for PEG-Qdot 525 (Figure 3c,d). By contrast, the combination of 3.3/3.3-biotin for capture/detection showed the best sensitivity for Neulasta detection (Figure 4c and d). However, the AGP4/3.3-biotin combination produced well-behaved dose-response curves with better sensitivity than when AGP3 or E11 were used as capture antibodies (Figure 4a and b). The combination of 0.25 µg AGP4 for capture and 3.3-biotin for detection appeared to represent a good choice for sensitive detection of PEGylated compounds.

Quantitative Sandwich ELISA of PEGylated Compounds. To further investigate the utility of AGP4/3.3-biotin and AGP4/AGP4-biotin sandwich ELISAs for the detection of PEGylated compounds, a range of PEGylated compounds were analyzed. Both formats allowed detection of a wide range of PEGylated compounds, although AGP4/3.3-biotin tended to produce lower background levels with good detection limits of 0.79 pM for PEG-Qdot 525, 0.32 ng/mL for Lipo-Dox, 0.32 ng/mL for Pegasys, 4.12 ng/mL for PEG-Intron, and 1.22 ng/mL for...
Neulasta, respectively (Figure 5). AGP4/3.3-biotin was the only combination that allowed quantification of PEG-Intron (Figure 5d).

Effect of Serum and Free PEG on Anti-PEG Sandwich ELISA.
Compared with the control, addition of up to 50% human serum did not affect the quantitative assay of PEG-Qdot 525 (Figure 6a). Likewise, spiking samples with up to 20% free CH3-PEG2000-NH2 did not interfere with the detection of Lipo-Dox (Figure 6b).

Comparison of Commercial Antibodies for Anti-PEG Sandwich ELISA.
Several anti-PEG antibodies were compared for detection of five PEGylated compounds by sandwich ELISA (Figure 7). An assay using B141 M for capture and 3.3-biotin for detection successfully quantified four of five PEGylated compounds. The combination of B141M/B141M-biotin for capture/detection, on the other hand, did not produce detectable signals for any of the compounds (Table 1). B139 M did not produce useful signals when employed as a capture antibody for any of the tested substances (Figure 7a–e). PEG-B-47 worked well as a capture antibody when combined with PEG-B-47-biotin for quantification of Lipo-Dox, but was relatively insensitive for the detection of the other four PEGylated compounds. Use of AGP4 for capture in combination with PEG-B-47-biotin for detection extended the utility of the assay to both Lipo-Dox and Pegasys (Figure 7b,c). The combination of AGP4/3.3-biotin for capture/detection, respectively, allowed sensitive detection of all five PEGylated compounds (Table 1).

**DISCUSSION**
In this report, we describe two new second-generation anti-PEG monoclonal antibodies, 3.3 and AGP4, that specifically bind to the repeating (OCH2CH2) subunits of PEG and allow more sensitive detection of PEGylated compounds as compared to our first-generation E11 and AGP3 antibodies (24). Utilization of AGP4 and 3.3-biotin in a sandwich ELISA as capture and detection antibodies, respectively, also allowed more sensitive detection of Pegasys, PEG-Intron, Neulasta, PEG-Qdot 525, and Lipo-Dox as compared to commercially available anti-PEG...
Improved Quantification of PEGylated Compounds

A plethora of PEGylated compounds is under development for a variety of medical applications. For leukemia (27–29), Macugen (PEG-anti VEGF aptamer) for age-related macular degeneration (30), and Adagen (PEG-adenosine deaminase) for severe combined immunodeficiency disease (SCID) (31, 32), PEGylation may also improve the properties of novel agents. The quantification of PEGylated compounds is crucial for clinical studies to determine their pharmacokinetic properties. Sandwich ELISA is a well-established technique to quantify analytes in serum samples and biological fluids due to its high sensitivity and specificity.

In this study, we examined the detection of five commercially available PEG conjugates. PEG-Qdot 525 is a nanocrystal semiconductor material coated with multiple linear 2 kDa aminopeg molecules. Lipo-Dox is liposomal doxorubicin decorated with multiple linear 2 kDa methoxy-PEG-distearoyl phosphatidyl ethanolamine molecules (37). Pegasys is an interferon alpha-2a conjugated with a single 12 kDa methoxy PEG (39), and Neulasta is a G-CSF conjugated with a single 20 kDa monomethoxy PEG (40).

Our results demonstrated that AGP4 and 3.3 can both serve as capture/detection antibodies to quantify all five PEGylated compounds. Because AGP4/3.3-biotin conjugates have been shown to be 50-fold enhanced sensitivity (Figures 3 and 4). The combination of AGP4/3.3-biotin was preferable to 3.3/AGP4-biotin for capture/detection because this combination produced equal detection sensitivity with lower background readings (Figure 5). When compared with first-generation anti-PEG antibodies (E11 and AGP3), one-fourth the amount of AGP4 (0.25 µg/well) was preferable to 3.3/AGP4-biotin for capture/detection because this combination produced equal detection sensitivity with lower background readings (Figure 5). When compared with first-generation anti-PEG antibodies (E11 and AGP3), one-fourth the amount of AGP4 (0.25 µg/well) was preferable to 3.3/AGP4-biotin for capture/detection because this combination produced equal detection sensitivity with lower background readings (Figure 5).

### Table 1. Comparison of Anti-PEG Antibodies for the Quantification of PEGylated Compounds by Sandwich ELISA

<table>
<thead>
<tr>
<th>capture antibody (0.25 µg/well)</th>
<th>detection antibody (0.25 µg/well)</th>
<th>PEG-Qdot 525 (pM)</th>
<th>Lipo-Dox (ng/mL)</th>
<th>Pegasys (ng/mL)</th>
<th>PEG-Intron (ng/mL)</th>
<th>Neulasta (ng/mL)</th>
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<tr>
<td>AGP4 3.3-biotin</td>
<td></td>
<td>40</td>
<td>7.2</td>
<td>8.1</td>
<td>1800</td>
<td>61.2</td>
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<tr>
<td>AGP4 PEG-B-47-biotin</td>
<td></td>
<td>&gt;10 000</td>
<td>7.6</td>
<td>20.6</td>
<td>&gt;2000</td>
<td>4300</td>
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<td>PEG-B-47 3.3-biotin</td>
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<td>&gt;10 000</td>
<td>NM</td>
<td>&gt;10 000</td>
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<td>NM</td>
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<tr>
<td>PEG-B-47 PEG-B-47-biotin</td>
<td></td>
<td>6.5</td>
<td>170</td>
<td>&gt;2000</td>
<td>&gt;10 000</td>
<td></td>
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<td>NM</td>
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<tr>
<td>B139M B141M-biotin</td>
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<td>29.4</td>
<td>59.2</td>
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<td>&gt;2000</td>
<td>&gt;10 000</td>
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</table>

*Results show the concentration of PEGylated compound that can produce an absorbance reading (A<sub>360</sub>) of 1.5 in a sandwich ELISA using the indicated capture and detection antibodies. NM, not meaningful, high background reading at all concentrations. ND, not determined.*

Antibodies. AGP4 and 3.3 antibodies should be useful for the quantification of a wide range of PEG conjugates.

Figure 7. Comparison of anti-PEG antibodies for sandwich ELISA of PEGylated compounds. Graded concentrations of PEG-Qdot 525 (a), Lipo-Dox (b), Pegasys (c), PEG-Intron, and Neulasta were assayed in a sandwich ELISA in which 0.25 µg/well AGP4, PEG-B-47, B139 M, or B141 M were used as capture antibodies and 0.25 µg/well 3.3-biotin, PEG-B-47-biotin, or B141M-biotin were employed as detection antibodies. Bars, SD.
a single PEG molecule, although detection sensitivity was greatly enhanced for conjugates containing a longer PEG chain (Neulasta vs PEG-Intron) or a branched-chain PEG (Pegasy). Conjugates with multiple PEG chains (PEG-Qdot 525 and Lipo-Dox) afforded the most sensitive detection by anti-PEG sandwich ELISA (Table 1).

AGP4 and 3.3-biotin appeared to offer advantages compared with other anti-PEG antibodies. PEG-B-47 is a rabbit monoclonal antibody that binds to terminal methoxy-PEG moieties. This explains why only conjugates containing branched-chain methoxy-PEG (Pegasy, Figure 7e) or multiple methoxy-PEG molecules (Lipo-Dox, Figure 7b) could be detected by PEG-B-47/PEG-B-47-biotin in sandwich ELISA format. Mouse monoclonal anti-PEG antibodies (B139 M and B141 M) bound amino-PEG molecules that were immobilized on plastic (data not shown). Binding of both these antibodies to PEG was blocked by Tween 20 (results not shown), indicating that B139 M and B141 M can recognize the short repeating (OCH2CH2) subunits present in Tween-20. Even though all assays with these antibodies were performed without Tween-20 in the wash buffers, B139 M did not produce usable signals in sandwich ELISAs. On the other hand, B141 M in combination with 3.3-biotin was able to quantify PEG-Qdot 525, Lipo-Dox, Pegasy, and Neulasta, although generally with less sensitivity than AGP4/3.3-biotin (Figure 7 and Table 1).

In summary, the combination of AGP4 for capture and 3.3-biotin for detection in a sandwich ELISA format allowed sensitive measurement of PEGylated proteins, nanoparticles, and liposomes with minimal interference from free PEG or human serum. These characteristics should allow universal and simple measurement of the increasing numbers of novel PEG-conjugates for pharmacological and pharmacokinetic studies. Although AGP4/3.3-biotin provided versatile detection of PEGylated compounds, the plastic antibody format for ELISA may vary from compound to compound.

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LITERATURE CITED


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