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 (AGP4, IgM and 3.3, IgG) that bind to the repeating subunits of the PEG backbone and facilitate more sensitive quantification of a wider range of PEGylated compounds. A sandwich ELISA in which AGP4/3.3-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) (*14*), Somavert (PEG-human growth hormone receptor antagonist) (*15*), Cimzia (certolizumab pegol, pegylated antihuman 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 (AGP4 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}-NH_2, methoxy-PEG_{2000}-NH_2, hydroxy-PEG_{5000}-NH_2, methoxy-$PEG_{10,000}$-NH_2, methoxy-$PEG_{20,000}$-NH_2 (750, 2000, 5000, 10 000 and 20 000 Da, respectively), NH_2-PEG_{3000}-NH_2, methoxy-PEG_{5000}-COOH, and 4-arm poly(ethylene oxide)$_{10,000}$-NH_2 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

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10.1021/bc100067t

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). Pegasys 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 mAb against PEG), 1E8 (IgG_1 mAb against *E. coli* βG, eβG), E11, and 3.3 (IgG_1 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-eβG-PEG_{5000}, a conjugate formed between a murine antibody and PEG-derivatized eβG. The mice were i.p. injected with 100 μg RH1-eβG-PEG_{5000} one week later and then s.c. injected at weekly intervals with 50 μg of eβG-PEG_{5000} in complete Freund's adjuvant, 30 μg of eβG-PEG_{5000} in incomplete adjuvant, 30 μg of BSA-PEG_{5000} in incomplete adjuvant, and 10 μg of BSA-PEG_{5000} in incomplete adjuvant. Three days before fusion with FO myeloma cells, the mice were i.p. injected with 30 μg of BSA-PEG_{5000} 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-PEG_{5000}. 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 antimouse IgM μ-chain antibody, HRP-conjugated donkey antimouse IgG Fc, HRP-conjugated goat antimouse Ig, and HRP-conjugated streptavidin were from Jackson ImmunoResearch Laboratories (Westgrove, 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 CH_3-PEG_{750}-NH_2, CH_3-PEG_{2000}-NH_2, NH_2-PEG_{3000}-NH_2, CH_3-PEG_{5000}-NH_2, CH_3-$PEG_{10,000}$-NH_2, or CH_3-$PEG_{20,0000}$-NH_2 in 50 μL/well 0.1 M $NaHCO_3/Na_2CO_3$ (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 antimouse IgM μ chain (2 μg/mL) or HRP-conjugated donkey antimouse 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 H_2SO_4, 50 μL/well), the absorbance (450 nm) of wells was measured in a microplate reader (Molecular Device, Menlo Park, CA).

Immunoblotting. Samples of Pegasys (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 antimouse 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 $NaHCO_3/Na_2CO_3$ (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, Pegasys, 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-NH_2, 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% H_2O_2, 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 $p \leq 0.05$. 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, 3000, 5000, 10 000, and 20 000 Da) and branched PEG (4-arm poly(ethylene oxide), 10 000 Da) was examined by coating NH_2-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-NH_2, hydroxyl-PEG-NH_2, 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 CH_3-PEG_{750}-NH_2. 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 $\geq$ 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,

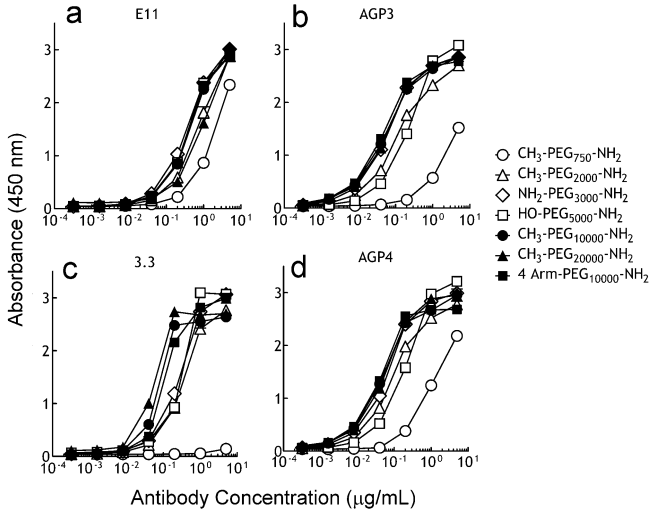

Figure 1. Antibody binding to adsorbed PEG molecules. Graded concentrations of E11 (a), AGP3 (b), 3.3 (c), or AGP4 (d) were added to microplate wells coated with the indicated amino-PEG molecules. After 1 h, the wells were washed and antibody binding was determined by adding HRP-conjugated goat antimouse IgM μ chain or HRP-conjugated donkey antimouse IgG Fc antibodies, followed by adding TMB substrate. The mean absorbance values (450 nm) of triplicate determinations are shown. Bars, SD.

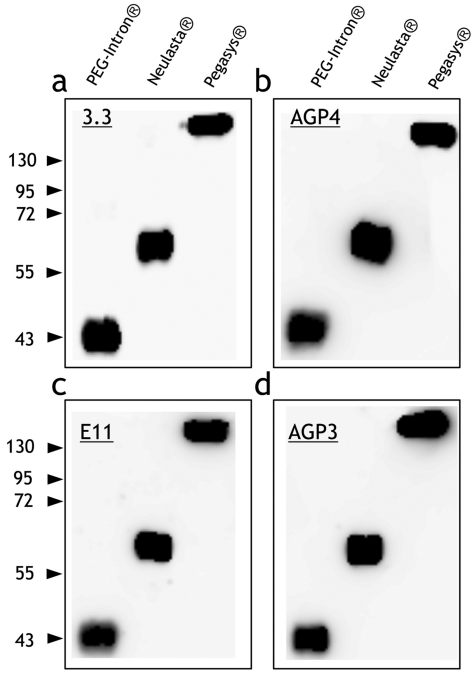

Figure 2. Immunoblot of PEGylated proteins. PEG-Intron, Neulasta, and Pegasys were electrophoresed on a 10% reduced SDS-PAGE gel, transferred to nitrocellulose paper, and probed with mAb 3.3 (a), AGP4 (b), E11 (c), or AGP3 (d) antibodies as described in Experimental Procedures. Lane 1, PEG-INTRON; lane 2, Neulasta; lane 3, Pegasys, molecular mass in thousands.

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-$PEG_{12\ kDa}$-interferon alpha-$2b_{19\ kDa}$), Neulasta (methoxy-$PEG_{20\ kDa}$-G-$CSF_{19\ kDa}$), 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*).

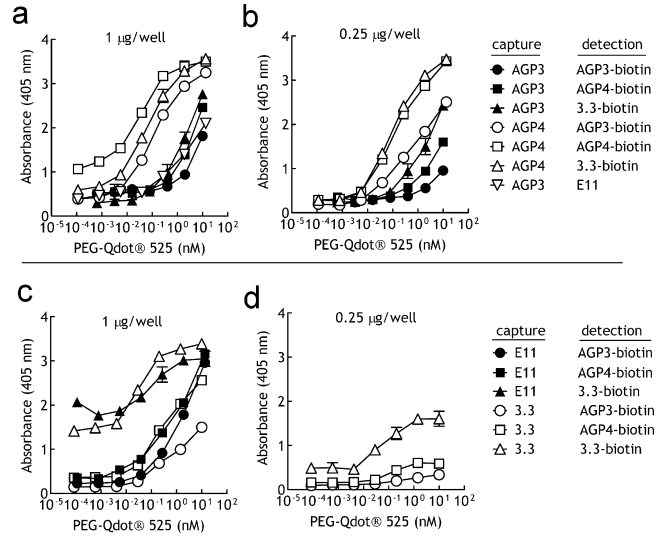

Figure 3. Comparison of first- and second-generation anti-PEG antibodies for detection of PEG-Qdot 525 by sandwich ELISA. Graded concentrations of PEG-Qdot 525 were assayed in triplicate in a sandwich ELISA in which 1 μg/well (left panels) or 0.25 μg/well (right panels) anti-PEG IgG (3.3 and E11) or IgM (AGP3 and AGP4) antibodies were coated in wells as capture antibodies. PEG-Qdot 525 was detected with 0.25 μg/well E11 followed by anti-IgG-HRP or by 0.25 μg/well AGP3-biotin, AGP4-biotin, or 3.3-biotin followed by streptavidin-HRP. Bars, SD.

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

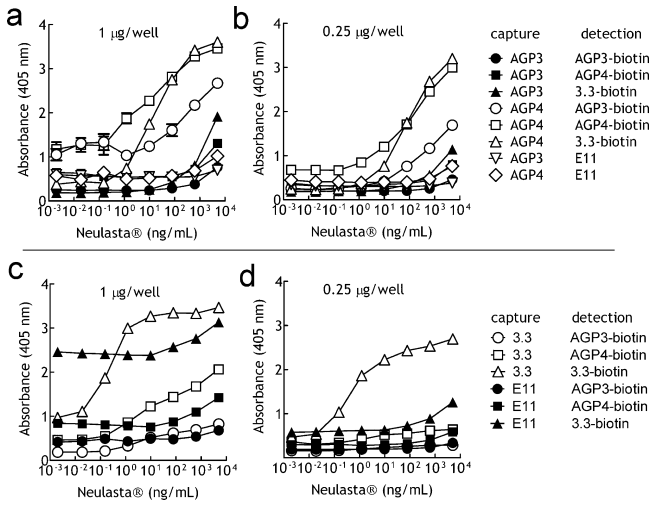

Figure 4. Comparison of first- and second-generation anti-PEG antibodies for detection of Neulasta by sandwich ELISA. Graded concentrations of Neulasta were assayed in triplicate in a sandwich ELISA in which 1 μg/well (left panels) or 0.25 μg/well (right panels) anti-PEG IgG (3.3 and E11) or IgM (AGP3 and AGP4) antibodies were coated in wells as capture antibodies. Neulasta was detected with 0.25 μg/well E11 followed by anti-IgG-HRP or by 0.25 μg/well AGP3-biotin, AGP4-biotin, or 3.3-biotin followed by streptavidin-HRP. Bars, SD.

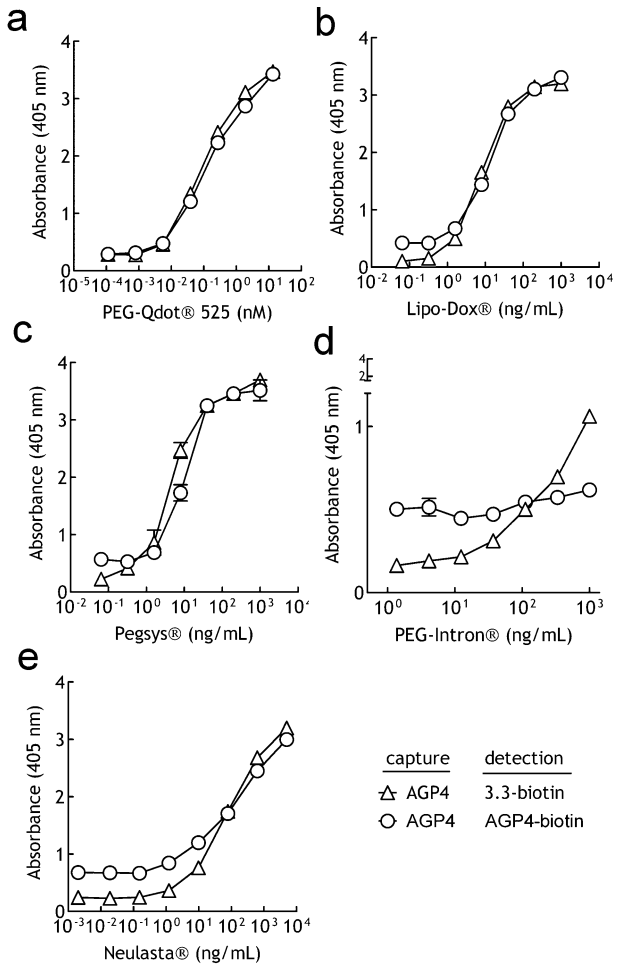

Figure 5. Quantitative analysis of PEGylated compounds by sandwich ELISA. Results of a sandwich ELISA in which 0.25 μg/well AGP4 was used as a capture antibody to measure the concentrations of PEG-Qdot 525 (a), Lio-Dox (b), Pegasys (c), PEG-Intron (d), and Neulasta (e), respectively, with 0.25 μg/well AGP4-biotin (○) or 3.3-biotin (Δ) as detection antibodies. Results show mean values of triplicate determination. Bars, SD.

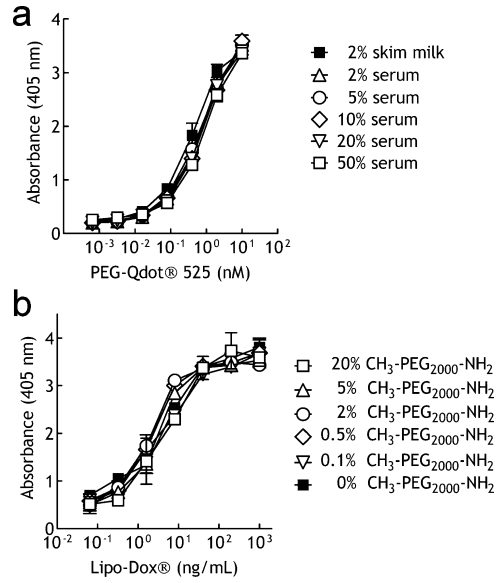

Figure 6. The influence of human serum and free PEG in the AGP4/3.3-biotin sandwich ELISA. (a) Sandwich ELISA in which AGP4/3.3-biotin were employed as the capture/detection antibodies to measure PEG-Qdot 525 in the presence of 2% skim milk or 2−50% human serum. (b) Lipo-Dox was spiked with the indicated amounts of free CH_3-PEG2000-NH_2 before serial dilutions were measured by a sandwich ELISA in which AGP4/3.3-biotin were used as capture/detection antibodies, respectively. Bars, SD.

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 CH_3-PEG_{2000}-NH_2 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 (OCH_2CH_2) 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

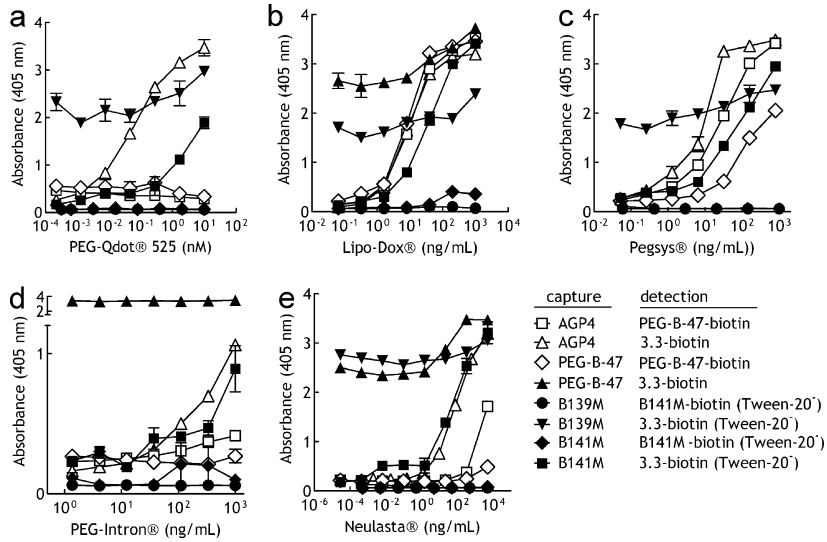

Figure 7. Comparison of anti-PEG antibodies for sandwich ELISA of PEGylated compounds. Graded concentrations of PEG-Qdot 525 (a), Lipo-Dox (b), Pegasys (c), PEG-Intron, and Neulasta were assayed in a sandwich ELISA in which 0.25 μg/well AGP4, PEG-B-47, B139 M, or B141 M were used as capture antibodies and 0.25 μg/well 3.3-biotin, PEG-B-47-biotin, or B141M-biotin were employed as detection antibodies. Bars, SD.

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

		assay sensitivity (analyte concentration that produces an absorbance value of 1.5 units)				
capture antibody (0.25 μg/well)	detection antibody (0.25 μg/well)	PEG-Qdot 525 (pM)	Lipo-Dox (ng/mL)	Pegasys (ng/mL)	PEG-Intron (ng/mL)	Neulasta (ng/mL)
AGP4	3.3-biotin	40	7.2	8.1	1800	61.2
AGP4	PEG-B-47-biotin	>10 000	7.6	20.6	>2000	4300
PEG-B-47	3.3-biotin	>10 000	NM	>10 000	NM	NM
PEG-B-47	PEG-B-47-biotin	>10 000	6.5	170	>2000	>10 000
B139M	3.3-biotin	NM[b]	NM	NM	ND[c]	NM
B139M	B141M-biotin	>10 000	>10 000	>10 000	>2000	>10 000
B141M	3.3-biotin	5800	29.4	59.2	>2000	54.6
B141M	B141M-biotin	>10 000	>10 000	>10 000	>2000	>10 000

[a] Results show the concentration of PEGylated compound that can produce an absorbance reading (A_{280}) of 1.5 in a sandwich ELISA using the indicated capture and detection antibodies. [b] NM, not meaningful, high background reading at all concentrations. [c] ND, not determined.

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

A plethora of PEGylated compounds is under development or already approved for medical use such as Oncaspar (PEG-asparaginase) 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 entering clinical testing such as PEGylated polyethylenimine (PEI) for in vivo DNA delivery (*33*), PEGylaed Zn-protoporphyrin for photodynamic tumor therapy (*34*), PEGylated SWENTs (single-walled carbon nanotubes) with ultralong blood circulation for drug delivery (*35*), and PEGylated aptamers that act as agonists or antagonists (*36*). The large number of PEG-conjugates under development will require qualitative and quantitative detection in preclinical and clinical studies to determinate 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 amino-PEG 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 40 kDa branched-chain methoxy PEG (*38*), PEG-Intron is an interferon alpha-2b 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 investigated. 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 vs 1 μg/well for first-generation antibodies) allowed effective capture of PEGylated compounds in sandwich ELISA assays with 14−50-fold enhanced sensitivity (Figures 3 and 4). The sandwich ELISA using AGP4/3.3-biotin also allowed sensitive quantification of Lipo-Dox in the presence of free PEG molecules (Figure 6). For reasons not well understood, anti-PEG antibodies bind best when PEG is linked to a molecule (i.e., protein, liposome, nanoparticles) or adsorbed on a surface (i.e., nitrocellulose paper in immunoblots or plastic in direct ELISA). Free PEG therefore had little effect on the assay of PEGylated compounds because of the much greater sensitivity of the sandwich ELISA for PEG-conjugates as compared to free PEG. Interestingly, membrane-tethered anti-PEG antibodies expressed on 3T3 fibroblasts can bind to soluble PEG molecules much more effectively than anti-PEG antibodies coated in 96-well microtiter plates (*41*). Likewise, human serum did not affect the assay of PEG-Qdot 525. Of note, the sandwich ELISA could detect conjugates possessing

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 (Pegasys). 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 compounds containing branched-chain methoxy-PEG (Pegasys, 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 B141M) bound amino-PEG molecules that were immobilized on plastic (data not show). 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 (OCH_2CH_2) 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, Pegasys, 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 ideal antibody format for ELISA may vary from compound to compound.

ACKNOWLEDGMENT

This study was supported by a grant from the Academia Sinica, Taipei, Taiwan (AS-98-TP-B09).

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BC100067T