Research Report

Sensitive Measurement of Polyethylene Glycol-Modified Proteins

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ABSTRACT

An IgM monoclonal antibody (AGP3) against polyethylene glycol (PEG) was used to assay PEG-modified proteins by ELISA. PEG-modified β-glucuronidase could be measured at concentrations as low as 15 ng/mL, corresponding to 750 pg (1.8 fmol) of conjugate. This ELISA should be generally applicable to all PEG-modified proteins because AGP3 binds the backbone of the PEG chain independent of the linker used for PEG attachment.

INTRODUCTION

Recombinant proteins are increasingly being employed for the therapy of a wide variety of diseases. Many of the proteins in clinical development are modified by the covalent attachment of methoxypolyethylene glycol (1,3,13,17, 22), a flexible linear polymer containing repeating $O\text{-}CH_2\text{-}CH_2$ subunits (11). Polyethylene glycol (PEG)-modified proteins often exhibit prolonged circulation half-lives (6,7) and reduced proteolytic cleavage (4,27). PEG modification has also been shown to reduce the immunogenicity of enzymes (5), antibodies (21), toxins (31), and recombinant human proteins (19). Reduction of immunogenicity can be important for clinical applications because even recombinant human proteins can induce humoral immune responses (2,14).

Clinical development of PEG-modified proteins requires pharmacokinetic measurements in animals and patients. Ideally, the concentration of intact PEG-modified protein should be measured. Simple methods to measure intact PEG-protein conjugates, however, are not available. The concentration of the protein can be measured by immunological, biochemical, or functional assays, and the attachment of PEG can be verified by nuclear magnetic resonance (NMR) (18) or chemical (16,29) methods. These methods, however, cannot be employed for complex samples such as serum. Methods that directly measure PEG concentration are relatively insensitive (10,15,24).

We have developed a monoclonal antibody (mAb AGP3) that specifically binds to PEG (9). In this report, we investigated whether AGP3 could be used to measure the concentration of PEG-modified proteins by ELISA.

MATERIALS AND METHODS

Reagents

Methoxypolyethylene glycol succinimidyl propionate [molecular weight (MW) 5 kDa] was obtained from Fluka Chemie (Buchs, Switzerland). PEG-disuccinimidyl succinamide (MW 3.4 kDa) and methoxypolyethylene glycol succinimidyl propionate (MW 2 and 20 kDa) were purchased from Shearwater Polymers (Huntville, AL, USA). Sephadex® G-25 (Amersham Pharmacia Biotech Asia Pacific, Quarry Bay, Hong Kong) and Ultrogel AcA 22 gels as well as methoxypolyethylene glycol tresylate and chicken egg white lysozyme were from Sigma (St. Louis, MO, USA). A HiLoad 16/60 Superdex® 75 column was from Amersham Pharmacia Biotech Asia Pacific. Succinimidyl-6-(biotinamido) hexanoate (NHS-LC-biotin) was obtained from Pierce Chemical (Rockford, IL, USA).

Antibodies

1E8, an IgG_1 mAb against β-glucuronidase (βG), and AGP3, an IgM antibody against PEG, were generated as described (9). Streptavidin-horseradish peroxidase (HRP) was from Serotec (Oxford, UK). HRP-conjugated goat

anti-mouse IgM μ-chain antibody (HRP-GAM μ-chain) and HRP-conjugated goat anti-mouse Ig (HRP-GAM) were from Organon Teknika (Durham, NC, USA).

PEG Modification of βG

Recombinant *E. coli*-derived βG was produced and purified as described (7). βG or lysozyme were passed through a 2.6 × 30-cm Sephadex G-25 column equilibrated with 0.1 M borate buffer, pH 8.0, and concentrated by ultrafiltration to 3 mg/mL. Methoxypolyethylene glycol succinimidyl propionate [at w/w ratios (PEG:βG) of 40:1, 20:1, and 4:1 for PEG with MW of 20, 5, and 2 kDa, respectively] in 0.1 M borate buffer, pH 8.0 (150 mg/mL) was added to βG and mixed for 2 h at room temperature. βG-PEG refers to the preparation of βG modified with 5-kDa PEG chains. Lysozyme was similarly modified, except that 1:20 (PEG: lysozyme) molar ratios were employed to obtain lysozymes modified with only one PEG chain. One-tenth volume of a saturated solution of glycine was added to stop the reaction. Unreacted PEG was removed by gel filtration on a 2.5× 100-cm Ultrogel AcA 22 column or a HiLoad 16/60 Superdex 75 column. Columns were eluted with PBS at 15 mL/h. Relevant fractions containing PEG-modified proteins were pooled, concentrated by ultrafiltration to 1 mg/mL, and stored at -80°C. βG was cross-linked with PEG-di-succinimidyl succinamide (3.3 mg PEG/mg βG) in a similar manner to produce βG-PEG-βG. Protein concentrations were determined by the bicinchoninic acid assay (Pierce Chemical) with bovine serum albumin (BSA) used as the reference protein. PEG groups were estimated by measuring amine groups before and after PEG modification with fluorescamine (29).

Purification of AGP3

AGP3 ascites obtained from pristane-primed eight-week-old BALB/c female mice was centrifuged at 5000× *g* for 10 min to remove debris. Ascites fluid (5 mL) was passed through a 5-mL Sephadex G-25 column equilibrated with PBS and then purified by gel filtration on a 2.5 × 100-cm Ultrogel AcA 22 column eluted with PBS at 15 mL/h. Fractions containing AGP3 were collected, concentrated by ultrafiltration to 1–2 mg/mL, and stored at -80°C. Yields of 5–6 mg AGP3/mL ascites were typically achieved.

Biotinylation of AGP3

AGP3 (1 mg/mL) in 0.1 M borate buffer, pH 8.0, was mixed with a 25-fold molar excess of NHS-LC-biotin for 1 h at room temperature to produce AGP3-biotin. One-tenth volume of a saturated solution of glycine was added to stop the reaction before aliquots were stored at -80°C. The antigen-binding activity of AGP3-biotin was determined by ELISA in 96-well microplates coated overnight with 1 μg/well βG-tPEG (20 mg methoxypolyethylene glycol tresylate/mg βG, prepared as described in Reference 9). Plates were blocked 1 h at 37°C with 2% skim milk in PBS before 50 μL/well samples of AGP3 or AGP3-biotin in dilution buffer (0.5% BSA, 0.05% Tween® 20 in PBS) were added to the wells for 1 h at room temperature. Plates were washed six times with PBS-TB (0.05% BSA, 0.1% Tween 20 in PBS), and 50 μL/well of either streptavidin-HRP (1:1000) or HRP-GAM μ-chain (1:1000) in dilution buffer were added for 1 h at room temperature. The plates were washed six times, and 100 μL/well ABTS substrate [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] were added for 30 min at room temperature. Absorbance (405 nm) of wells was measured in a Molecular Devices (Sunnyvale, CA, USA) microplate reader.

AGP3 Immunoblot

βG (0.5 μg), βG-PEG (0.5 μg), and βG-PEG-βG (2 μg) were electrophoresed on a 3%–12.5% gradient SDS polyacrylamide gel and transferred to nitrocellulose paper as described (9). The paper was blocked for 1 h with 5% skim milk in PBS and then incubated for 2 h at room temperature with 0.5 μg/mL mAb 1E8 or AGP3 in dilution buffer. Blots were washed three times with PBS-TM and incubated for 1 h at room temperature with HRP-GAM

(1:1000 in dilution buffer). Blots were washed three times before bands were visualized by ECL® detection according to the manufacturer's instructions (Pierce Chemical).

AGP3 ELISA

Maxisorp™ 96-well microplates (Nalge Nunc International, Roskilde, Denmark) were coated with 50 µL/well of AGP3 (200 µg/mL) in PBS for 3 h at 37°C or in 35 mM $NaHCO_3$, 15 mM Na_2CO_3, pH 9.3, overnight at 4°C. Plates were blocked with 2% skim milk in PBS overnight and washed twice with PBST (0.05% Tween 20 in PBS) before serial dilutions of βG or βG-PEG (50 µL in dilution buffer) were added to wells for 1.5 h at room temperature. Plates were washed six times with PBST, and 50 µL/well AGP3-biotin (20 µg/mL in dilution buffer) were added for 1.5 h at room temperature. Plates were again washed with PBST, and 50 µL/well streptavidin-HRP (1:1000) were added for 1 h at room temperature. Plates were washed before 100 µL/well ABTS substrate was added for 5–30 min at room temperature. Absorbance (405 nm) of wells was measured in a microplate reader. The effect of serum on the AGP3 ELISA was examined by performing the assay in the presence of 10% serum isolated from healthy BALB/c mice.

RESULTS

Methoxypolyethylene glycol succinimidyl propionate (MW 5 kDa) was covalently attached to an enzyme (βG) to form βG-PEG. An average of 6.8 PEG chains was attached to each 70-kDa subunit of the βG tetramer. The electrophoretic mobility of βG-PEG (Figure 1A, lane 3) was slower than unmodified βG (Figure 1A, lane 2) as determined by SDS PAGE followed by immunoblotting with a mAb against βG, demonstrating successful conjugation of PEG to βG. βG was also successfully cross-linked with PEG to form βG-PEG-βG (Figure 1A, lane 1). βG-PEG-βG does not contain a terminal methoxy group on the PEG chain. AGP3 bound βG-PEG (Figure 1B, lane 3) and βG-PEG-βG (Figure 1B, lane 1) but not unmodified βG (Figure 1B, lane 2). Binding of AGP3 to βG-PEG-βG shows that AGP3 binds to the backbone of PEG rather than the methoxy group present at the terminal of methoxypolyethylene glycol.

AGP3 was examined as the basis of an ELISA for the detection of PEG-modified proteins. AGP3 was modified with a 25-fold molar excess of NHS-LC-biotin to produce AGP3-biotin. AGP3-biotin retained the same antigen-binding activity to βG-tPEG as AGP3 in ELISA using HRP-goat anti-mouse IgM µ-chain antibody for detection (Figure 2). Detection of antibody binding to βG-PEG with streptavidin-HRP showed that AGP3-biotin, but not AGP3, could be detected (Figure 2), demonstrating that biotin was indeed incorporated into AGP3-biotin.

A sandwich ELISA was developed in which AGP3 was used for the capture antibody and AGP3-biotin was used as the detection antibody. Figure 3 shows that this assay could detect βG-PEG at concentrations as low as 15 ng/mL, corresponding to 750 pg conjugate in a 50-µL sample. The assay only detected PEG-modified protein as shown by the lack of signal when unmodified βG was assayed (Figure 3). The effect of serum on the ELISA was examined by adding 10% mouse serum to samples before the assay. The sensitivity of the assay was about the same as without serum (Figure 3).

To investigate the influence of PEG chain length on the sensitivity of the ELISA, PEG with nominal molecular weights of 20, 5, and 2 kDa was covalently attached to βG. The average degree of modification was 5.1, 7.1, and 11.4 PEG chains per subunit of the βG tetramer for PEG chains with MW of 20, 5, and 2 kDa, respectively. Figure 4A shows that all the βG conjugates were modified with PEG as demonstrated by the reduced mobility of the conjugates. Measurement of conjugate concentrations by ELISA revealed that assay sensitivity increased for longer PEG chains (Figure 4B). Assay of βG modified with 20-kDa PEG chains was about eight times more sensitive than βG modified with 5-kDa PEG chains, which in turn was about four times more sensitive than βG modified with 2-kDa PEG chains.

To determine whether the ELISA assay could detect proteins modified with a single chain of PEG, lysozyme was modified with one PEG molecule with molecular weights of 2, 5, or 20 kDa. Purified lysozyme-PEG conjugates appeared as single bands on SDS polyacrylamide gels (Figure 5A), indicating that the conjugates were homogeneous 1:1 conjugates. The AGP3 ELISA could assay lysozyme modified with one molecule of 20-kDa PEG (Figure 5). No signal was detected, however,

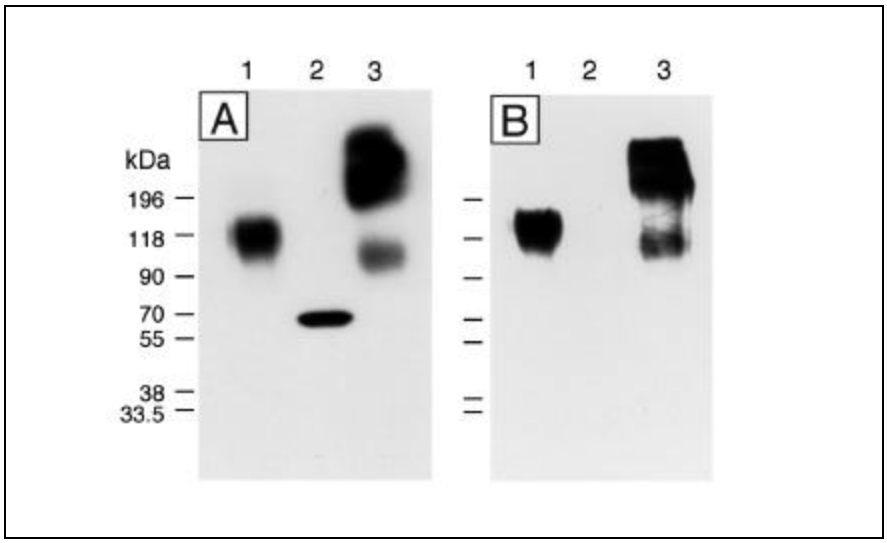

Figure 1. Immunoblot of PEG-modified proteins. Samples were electrophoresed on a 3%–12.5% reduced SDS polyacrylamide gel, transferred to nitrocellulose paper, and probed with mAb 1E8 (A) or AGP3 (B) as described in Materials and Methods. Lane 1, βG-PEG-βG; lane 2, βG; lane 3, βG-PEG. kDa, molecular mass in thousands.

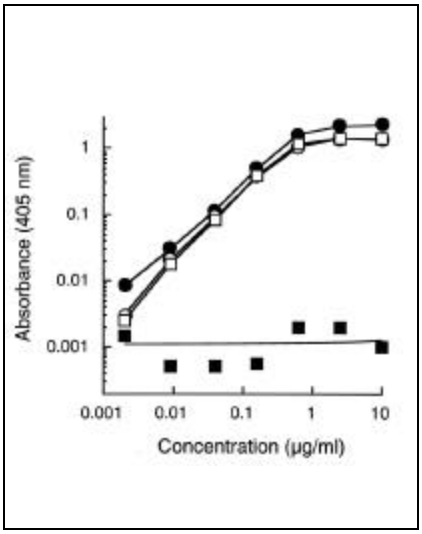

Figure 2. Activity of biotinylated AGP3. Serial dilutions of AGP3 (squares) or AGP3-biotin (circles) were assayed for binding to βG-tPEG by ELISA. Antibody binding was detected with HRP-GAM μ-chain (open symbols) or HRP-streptavidin (closed symbols). The average absorbance values of duplicate determinations are shown.

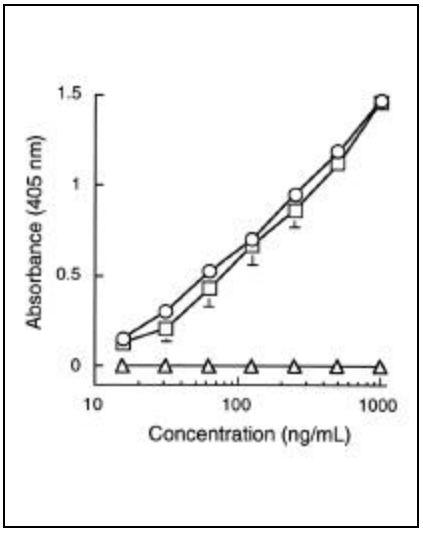

Figure 3. AGP3 ELISA. Serial dilutions of βG (Δ) and βG-PEG in PBS (O) or PBS containing 10% mouse serum (□) were assayed by AGP3 ELISA as described in Materials and Methods. The mean absorbance values of triplicate determinations, read 30 min after addition of substrate, are shown. Bars, standard error.

when lysozyme modified with a single chain of 2- or 5-kDa PEG was assayed (results not shown).

DISCUSSION

AGP3 is a murine IgM mAb that was developed by immunizing mice with βG-PEG (9). We have previously shown that AGP3 can accelerate the clearance of PEG-modified immunoenzymes for antibody-directed enzyme prodrug therapy (8,9). Previous studies also demonstrated that AGP3 binds to PEG rather than the linker used for conjugation (9). It was possible, however, that AGP3 bound to the terminal methoxy group of PEG. We found in the current study that AGP3 bound βG-PEG-βG, a conjugate that does not contain a terminal methoxy group, showing that AGP3 binds to the repeating subunits of the PEG backbone. This finding indicates that multi-

ple AGP3 mAbs can bind to a single PEG chain and prompted us to examine whether AGP3 could be used in a sandwich ELISA for the detection of PEG-modified proteins.

An ELISA was developed in which AGP3 was employed for both the capture and detection of protein-PEG conjugates. AGP3 could be modified with a 25-fold molar excess of biotin without loss of antigen-binding activity. The ELISA detected a PEG-modified protein (βG-PEG) with a detection limit of around 15 ng/mL. This corresponds to 1.8 fmol βG-PEG [βG tetramer modified with 27 PEG (5 kDa) molecules with an overall MW of 415 kDa], indicating that the detection sensitivity should be adequate for most PEG-modified proteins. The sensitivity of the assay depended on the length of the PEG chains attached to βG; longer PEG chains resulted in increased assay sensitivity. This is likely due to the binding of multiple AGP3 antibodies to the PEG backbone, allowing amplification of the signal as PEG chain length increases. The ability of the ELISA assay to detect lysozyme modified with a single 20-kDa PEG molecule must also rely on the capability of both the capture and detection AGP3 antibodies to bind to the same PEG chain. The assay could not detect lysozyme modified with a single PEG molecule of 2 or 5 kDa, which is likely due to steric hindrance between the capture and detecting antibodies. The ELISA was insensitive to 10% serum, suggesting that this method can be used to quantify PEG-conjugate concentrations in serum samples for pharmacological and pharmacokinetic studies. It should be noted that the assay might overestimate the plasma concentration of heterogeneous conjugate preparations since conjugates containing fewer PEG chains will clear faster (30). This effect is expected to be more pronounced for small conjugates that contain few PEG chains.

There are currently no methods to directly measure low concentrations of intact PEG conjugates for pharmacological studies. Conjugates can be indirectly measured by first radiolabeling the protein (7,32) or PEG (23), but radioisotopes pose safety concerns and require special handling. Functional assays can be used to measure the concentration of the protein component of conjugates (9,12), but no information is provided about the stability of the attached PEG chains. Methods that measure the number of PEG molecules attached to a protein (16,29) require that purified conjugates be employed, which is difficult to achieve in pharmacokinetic studies. Methods that directly measure the concentration of PEG are relatively insensitive. Colorimetric methods based on complex formation between barium-iodide and PEG require that proteins are first removed and have detection limits of 1–5 µg PEG (10). A colorimetric method based on partitioning of a chromophore present in aqueous ammonium ferrothiocyanate reagent can be employed for complex protein mixtures but has a detection limit of 1–5 µg PEG (24,25). HPLC can quantify PEG with a detection limit of 1–5 µg/mL (20,28). Phase partitioning can be used to measure PEG, but the assay sensitivity is about 1 µg PEG (15). Finally, polyclonal antibodies against PEG can detect the presence of 1 µg/mL PEG in PEG-modified proteins (26). The newly developed ELISA can measure approximately 250 pg PEG in PEG-βG [27 PEG (5 kDa) chains per βG tetramer with an overall MW of 415 kDa], several orders of magnitude more sensitive than previous methods. In addition, the AGP3 ELISA does not require prior separation of PEG conjugates from complex mixtures, simplifying application to pharmacokinetic studies.

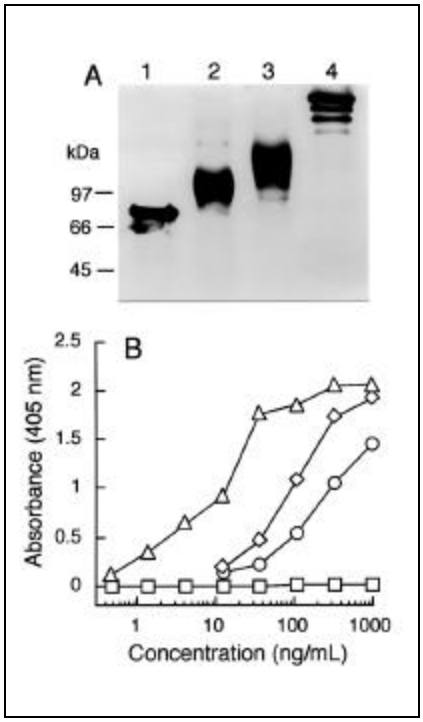

Figure 4. Effect of PEG chain length on ELISA sensitivity. (A) βG (lane 1) or βG modified with PEG possessing average molecular weights of 2 (lane 2), 5 (lane 3), or 20 (lane 4) kDa were electrophoresed on a reducing 3%–12.5% gradient SDS polyacrylamide gel and stained with Coomassie® Blue R-250. kDa, molecular mass in thousands. (B) Serial dilutions of βG (□) and βG modified with PEG possessing average molecular weights of 2 (O), 5 (◇), or 20 (Δ) kDa were assayed by AGP3 ELISA as described in Materials and Methods. The mean absorbance values of triplicate determinations, read 5 min after addition of substrate, are shown. Bars, standard error.

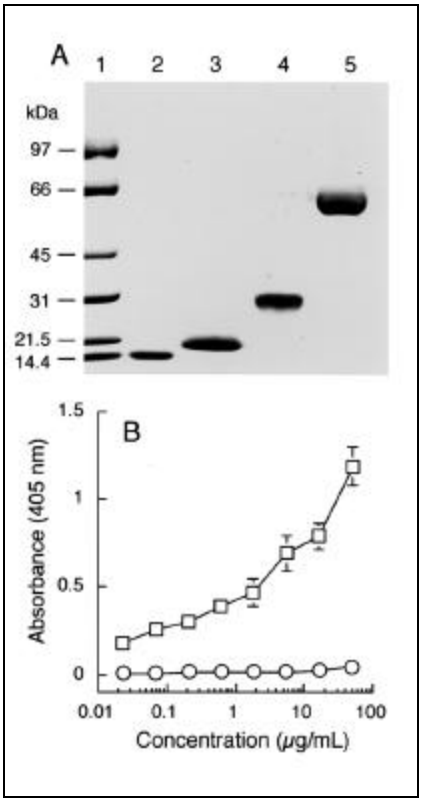

Figure 5. AGP3 ELISA of proteins with a single PEG chain. (A) Lysosyme (lane 2) or lysosyme modified with a single PEG chain possessing an average molecular weight of 2 (lane 3), 5 (lane 4), or 20 (lane 4) kDa were electrophoresed on a reducing 10% SDS polyacrylamide gel and stained with Coomassie Blue R-250. Lane 1 shows molecular weight standards. kDa, molecular mass in thousands. (B) Serial dilutions of lysosyme (O) or lysosyme modified with a PEG chain of 20 kDa (□) were assayed by AGP3 ELISA as described in Materials and Methods. The mean absorbance values of triplicate determinations, read 5 min after addition of substrate, are shown. Bars, standard error.

Given that the assay measures PEG rather than protein, the newly developed ELISA should be applicable to the measurement of any PEG-modified protein in complex mixtures. In addition, AGP3 can be used to probe the presence and size distribution of PEG conjugates on immunoblots. The sensitivity of the AGP3 ELISA should allow convenient measurement of PEG-conjugate pharmacokinetics for preclinical and clinical studies.

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