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Technical Note

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Sensitivity of PEGylated Interferon Detection by Anti-Polyethylene Glycol (PEG) Antibodies Depends on PEG Length

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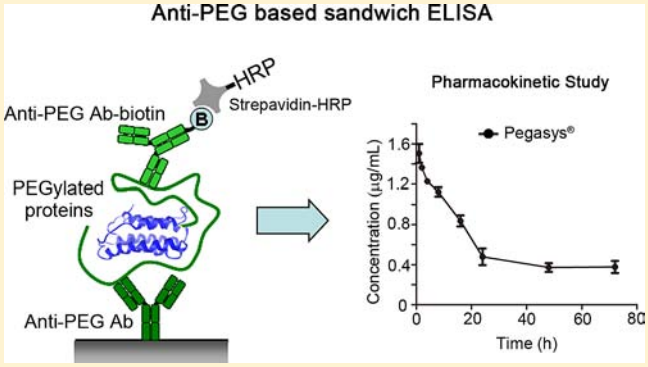
ABSTRACT: Attachment of poly(ethylene glycol) to proteins can mask immune epitopes to increase serum half-life, reduce immunogenicity, and enhance in vivo biological efficacy. However, PEGylation mediated epitope-masking may also limit sensitivity and accuracy of traditional ELISA. We previously described an anti-PEG-based sandwich ELISA for universal assay of PEGylated molecules. Here, we compared the quantitative assessment of PEGylated interferons by anti-PEG and traditional anti-interferon sandwich ELISA. The detection limits for PEG-Intron (12k-PEG) and Pegasys (40k-PEG) were 1.9 and 0.03 ng/mL for anti-PEG ELISA compared to 0.18 and 0.42 ng/mL for traditional anti-interferon sandwich ELISA. These results indicate that the anti-PEG sandwich ELISA was insensitive to PEGylation mediated epitope-masking and the sensitivity increased in proportion to the length of PEG. By contrast, PEG-masking interfered with detection by traditional anti-interferon sandwich ELISA. Human and mouse serum did not affect the sensitivity of anti-PEG ELISA but impeded traditional anti-interferon sandwich ELISA. The anti-PEG sandwich ELISA was comparable to anti-interferon sandwich ELISA and radioassay of ^{131}I-Pegasys in pharmacokinetic studies in mice. The anti-PEG sandwich ELISA provides a sensitive, accurate, and convenient quantitative measurement of PEGylated protein drugs.



INTRODUCTION

Polyethylene glycol (PEG) is a highly water-soluble and nontoxic polymer that is approved by the Food and Drug Administration (FDA) for human use.[1] Covalent linkage of PEG to proteins can shield binding sites, minimize proteolytic cleavage, and mask immunogenic sites, thereby prolonging the circulation time in the human body, improving therapeutic efficacy, and reducing the injection frequency to enhance patients' quality of life.[2−5] Based on these advantages, PEGylation has become one of the most useful pharmaceutical technologies to create protein drugs with markedly improved therapeutic properties compared with their unconjugated counter parts.[6,7] For example, the clinical treatment of chronic hepatitis B and C with interferon-α-2a (Roferon-A) has been limited by the short half-life of IFN-α in patients (less than 12 h), resulting in the need to administer repeated injections at least three times a week to achieve the desired therapeutic benefits. PEGylated interferons, including PEG-Intron (linear PEG_{12K}-interferon-α-2b) and Pegasys (branched PEG_{40K}-interferon-α-2a), display dramatically increased half-lives, allowing decreased dosing to once per week.[8] How to efficiently assess the pharmacokinetics of PEGylated drugs is an important issue with the ever increasing number of such compounds under development.

Measurement of drug concentrations in serum samples is important for clinical drug development and assessment of drug

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pharmacokinetics and bioavailability. However, attachment of PEG molecules to proteins may mask antibody-binding epitopes that interfere with detective sensitivity using protein-specific antibodies.[9,10] We previously reported that an anti-PEG based sandwich ELISA using anti-PEG monoclonal antibodies (AGP4 and 3.3) can detect a wide range of PEGylated molecules including Pegasys, PEG-Intron, Neulasta, and PEG-Lipo-Dox with ng/mL sensitivities.[10] Based on those results, we hypothesized that the anti-PEG sandwich ELISA is not affected by PEG generated epitope-masking.

Here, we compared the measurement of PEGylated interferon by anti-PEG based and traditional anti-interferon based sandwich ELISAs. We first examined the detection limits of the anti-PEG and anti-interferon based sandwich ELISAs. We also investigated whether human or mouse serum affected detection sensitivities of the assays. Finally, we compared the measurement of ^{131}I labeled Pegasys (^{131}I-Pegasys) in the serum of mice by radioactive quantification or ELISA. The anti-PEG based sandwich ELISA displayed high sensitivity and was free from interference by serum proteins, suggesting that anti-PEG ELISA is particularly suitable as a standard method for measuring the pharmacokinetics of PEGylated protein drugs in preclinical and clinical study.

MATERIALS AND METHODS

Reagents and Animals. Maxisorp flat-bottom 96-well plates were purchased from Nunc, NY, USA. Skim milk was purchased from BD Difco, NJ, USA. Pegasys and Roferon-A were from Roche, NJ, USA. PEG-Intron was from Merck, NY, USA. Horseradish peroxidase-conjugated streptavidin were from Jackson ImmunoResearch Laboratories, PA, USA. 2,2′-Azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS) and 30% hydrogen peroxide were from Sigma-Aldrich, MO, USA. Five-week-old BALB/c female mice were purchased from the National Laboratory Animal Center, Taipei, Taiwan, and were bred in the specific pathogen-free facility at Kaohsiung Medical University.

Sandwich ELISA. In the anti-PEG sandwich ELISA,[10] Maxisorp flat-bottom 96 well plates were coated with 50 μL/well of AGP4 (20 μg/mL) in coating buffer (100 mM Na_2CO_3, pH 8.0) at 37 °C for 2 h and then at 4 °C for overnight. The wells were blocked with 5% skim milk in PBS at 37 °C for 2 h and then washed twice in phosphate-buffered saline (PBS). Nonmodifed interferon α-2a (Roferon-A), PEG_{12K}–interferon α-2b (PEG-Intron), and PEG_{40K}–interferon α-2a (Pegasys) were serially diluted in dilution buffer (2% skim milk in PBS), added to wells (50 μL/well) and incubated at room temperature for 2 h. After washing with PBS three times, the wells were sequentially incubated with 50 μL/well of biotinylated anti-PEG antibody (3.3-biotin, 5 μg/mL in dilution buffer) and 50 μL/well horseradish peroxidase (HRP)-conjugated streptavidin (1 μg/mL in dilution buffer). The wells were washed with PBS eight times and 100 μL of substrate solution (0.4 mg/mL ABTS, 0.01% hydrogen peroxide in 100 mM phosphate-citrate buffer, pH 4.0) was added and incubated in the dark at room temperature for 30 min. The absorbance in the wells was measured at 405 nm using a MRX microplate reader (Dynex Technologies, VA, USA).

Anti-interferon sandwich ELISA was performed using the VeriKine Human IFN-α ELISA Kit (catalog number 41100; Pestka Biomedical Laboratories, NJ, USA). All three interferons were serially diluted with dilution buffer and added to wells (100 μL/well) and incubated at room temperature for 1 h, following the manufacturer's guidelines. After washing with wash solution, the well was stained with 100 μL/well of diluted antibody concentrate at room temperature for 1 h followed by staining with 100 μL/well of diluted HRP conjugate. After the wells were washed four times, 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added to the wells. Color development was stopped by the addition of 100 μL of stop solution. The absorbance in the wells was measured at 450 nm using the MRX microplate reader.

Detection of Pegasys in Serum by ELISA. To investigate the influence of serum in sandwich ELISA, human serum was obtained from three healthy volunteers and mouse serum was collected from five BALB/c female mice. Endogenous anti-PEG antibodies were not detected in any of the serum samples. Pegasys in dilution buffer containing 0–20% human or murine serum was measured by the anti-PEG and the anti-interferon ELISAs as described above.

Analysis of Pegasys Stability in Human Serum. A 10 μg/mL aliquot of Pegasys was incubated with DEME buffer contain 50% human serum and 1% penicillin–streptomycin at 37 °C. Samples collected at different times (0, 24, 48, 72, and 96 h) were electrophoresed in a 7% sodium-dodecyl sulfate polyacrylamide gel under reducing conditions and then transferred onto a nitrocellulose paper (Millipore). After blocking in 5% skim milk, the blot was incubated with 5 μg/mL AGP4-biotin in PBS containing 2% skim milk for 1 h at room temperature. The blot was washed 3 times with PBS-T (PBS containing 0.05% Tween-20) and twice with PBS before incubation with streptavidin-HRP for 1 h at room temperature. 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, USA).

Pharmacokinetic Analysis of ^{131}I-Pegasys in Mice. For comparison of anti-PEG sandwich ELISA with existing methods, the pharmacokinetics of Pegasys in mice was analyzed by the anti-PEG and the anti-interferon ELISAs and a traditional radioassay as a reference method. ^{131}I-Pegasys were synthesized by Hsin-Ell Wang, Ph.D. (Department of Medical Radiation Technology and Institute of Radiological Sciences, National Yang-Ming University, Taiwan). BALB/c mice were intravenously (i.v.) injected with 740 kBq of ^{131}I-Pegasys (containing 5 μg of Pegasys). Serum was isolated from venous blood of the mice at the indicated time points after injection (1, 2, 4, 8, 16, 24, 48, and 72 h) and analyzed by the sandwich ELISAs described above. The radioactivity of ^{131}I-Pegasys in the blood was independently determined by Wallac 1470 Wizard gamma counter (Perkin-Elmer, MA, USA). Results (mean ± SD) are expressed as the concentration of Pegasys in serum (μg/mL).

Statistical Analysis. To determine whether serum affected the measurement of ELISA, statistical comparisons were made using or one-way ANOVA followed by Dunnett's multiple comparison tests between control group (0%) and test groups (2%, 5%, and 20%). Statistical significance was defined as $p < 0.05$.

RESULTS

Quantification of PEGylated Protein Drugs by anti-PEG and Anti-Interferon Sandwich ELISAs. To evaluate the utility of anti-PEG and anti-interferon sandwich ELISAs, defined concentrations of a non-PEGylated interferon (Roferon-A), a 12K-PEG modified interferon (PEG-Intron), and a

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40K-PEG modified interferon (Pegasys) were measured by AGP4/3.3 anti-PEG sandwich ELISA[10] and a commercial VeriKine Human IFN-α ELISA Kit. Figure 1A shows that the

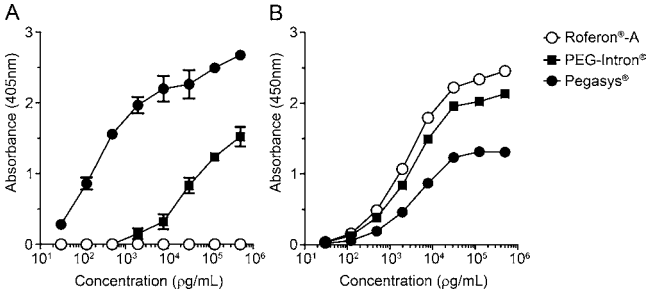

Figure 1. Comparison of anti-PEG and anti-IFN sandwich ELISAs for detection of nonmodified and PEGylated interferon. Serially diluted Roferon-A (○), PEG-Intron (■), and Pegasys (●) were detected by anti-PEG sandwich ELISA (A) or anti-IFN sandwich ELISA (B), respectively. Representative data from three independent experiments are shown. Bars, SD.

detection limits of the anti-PEG sandwich ELISA were 1.9 ng/mL for PEG-Intron and 0.03 ng/mL for Pegasys. As expected, Roferon-A was not detected by the anti-PEG sandwich ELISA since Roferon-A does is not modified with PEG molecules. The detected limits of Roferon-A, PEG-Intron, and Pegasys were 0.12, 0.18, and 0.42 ng/mL as measured by the VeriKine Human IFN-α ELISA Kit (Figure 1B). Thus, the detection sensitivity of the anti-PEG sandwich ELISA increased as the size of the PEG chains on interferon increased, whereas the anti-interferon sandwich ELISA showed the opposite trend.

Influence of Serum on Anti-PEG and Anti-Interferon Sandwich ELISAs. To investigate the effect of serum on detection sensitivity, defined concentrations of Pegasys were incubated in 0%, 5%, or 20% human or mouse serum before assay by anti-PEG sandwich ELISA or the VeriKine Human IFN-α ELISA. The presence of 5% or 20% mouse (Figure 2A) or human (Figure 2C) serum did not affect the sensitivity of the anti-PEG ELISA. By contrast, 20% mouse (Figure 2B) or human (Figure 2D) serum significantly ($p < 0.05$) decreased the sensitivity of the IFN-α ELISA. To assess the stability of the linkage between PEG and interferon in serum, Pegasys (10 μg/mL) was incubated with 50% human serum for 0, 24, 48, 72, and 96 h. The stability of Pegasys in human serum was detected by Western blot using biotinylated anti-PEG antibody (AGP4-Biotin). Figure 2E showed that Pegasys did not dissociate in human serum even after 96 h, indicating that the linkage between PEG and interferon is very stable in serum. These results indicate that the presence of serum does not hamper detection of PEGylated proteins by anti-PEG sandwich ELISA.

Pharmacokinetics of ^{131}I-Pegasys Determined by Anti-PEG and Anti-Interferon Sandwich ELISAs. To investigate the feasibility of using anti-PEG sandwich ELISA to measure PEGylated interferon in pharmacokinetic studies, 740 kBq (5 μg) of ^{131}I-labeled Pegasys was i.v. injected into BALB/c mice. Venous sera were collected at the indicated times and the concentrations of ^{131}I-Pegasys were determined by anti-PEG ELISA, VeriKine Human IFN-α ELISA, and a gamma counter which directly determined levels of radioactivity in the sera. Figure 3 shows that the pharmacokinetics of ^{131}I-Pegasys determined using the anti-PEG based ELISA ($t_{1/2}$ = 13.14 h) was similar to the half-life measured by the Human IFN-α ELISA Kit ($t_{1/2}$ = 12.44 h). Lower concentrations and a

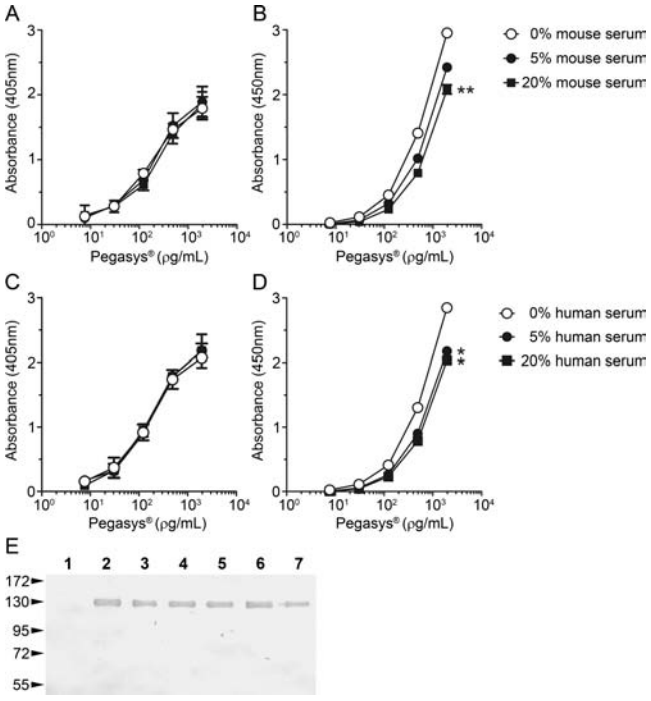

Figure 2. Serum interference in anti-PEG and anti-IFN sandwich ELISAs. Pegasys serially diluted in 0% (○), 5% (●), or 20% (■) mouse or human serum were detected by the anti-PEG sandwich ELISA (A, C) or anti-IFN sandwich ELISA (B, D). Bars, SD *, $p < 0.05$; **, $p < 0.01$, Dunnett's multiple comparison tests between control group (0%) and other groups. (E) Stability of the linkage between PEG and interferon in serum. Pegasys was incubated in 50% human serum for 0 h (lane 2), 24 h (lane 3), 48 h (lane 4), 72 h (lane 5), and 96 h (lane 6). Samples were electrophoresed on a 7% reduced SDS-PAGE gel, transferred to nitrocellulose paper, and probed with AGP4-biotin as described in Materials and Methods. Lane 1, 50% serum as blank control; Lane 7, Pegasys as a positive control. Representative data from three independent experiments are shown.

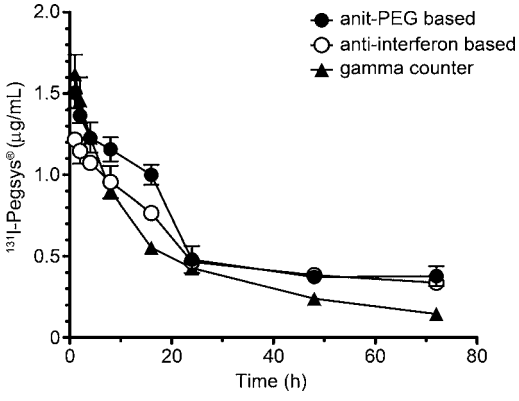

Figure 3. Serum concentration−time curve of ^{131}I-Pegasys i.v. injected into mice. Sera of BALB/c mice (n = 4) were collected at the indicated times (1, 2, 4, 8, 16, 24, 48, 72 h) after i.v. injection of ^{131}I-Pegasys. The concentration of ^{131}I-Pegasys was determined by anti-PEG sandwich ELISA (●), anti-IFN sandwich ELISA (○), or gamma counter (▲). Representative data from three independent experiments are shown. Bars, SD.

shorter half-life ($t_{1/2}$ = 7.64 h) were determined by measuring the radioactivity in serum samples. Based on these results, we conclude that the anti-PEG based sandwich ELISA is a highly

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efficient method to measure the pharmacokinetics of PEGylated protein drugs.

DISCUSSION

The shielding effect of PEG can mask antibody-binding epitopes on proteins, resulting in weaker interactions with detection and capture antibodies during ELISA, thereby decreasing assay sensitivity. We demonstrated that an anti-PEG sandwich ELISA to detect PEGylated interferon is superior to a commercial anti-interferon sandwich ELISA. The anti-PEG sandwich ELISA displayed high sensitivity and was not inhibited by PEG-mediated epitope masking or the presence of serum in samples. The sensitivity of the anti-PEG sandwich ELISA increased with the length of PEG chains attached to interferon. The concentration of ^{131}I-Pegasys in serum samples measured by anti-PEG sandwich ELISA was comparable to a commercial anti-interferon sandwich ELISA and to direct measurement of serum radioactivity. These data indicate that the anti-PEG sandwich ELISA may reliably measure PEGylated proteins for drug development or in clinical trials.

PEGylation of proteins and peptides is a Food & Drug Administration (FDA) approved technology to increase half-life and therapeutic efficacy.[5,11−13] Many PEGylated protein drugs are now available for clinical use, including PEGylated antitumor necrosis factor α antibody (PEG-anti-TNFα),[14] PEGylated anti-interleukin-8 (PEG-anti-IL8),[15] PEGylated Toll like receptor 7 ligands,[5] and PEGylated granulocyte colony stimulating factor (PEG-G-CSF).[16] The anti-PEG sandwich ELISA employs anti-PEG antibodies for PEG-dependent/target molecule-independent quantization of any PEGylated compound. Our previous results showed that PEG-G-CSF (Neulasta) and PEGylated nanoparticles such as PEG-Qdot 525 and Lipo-Dox were also detected by the anti-PEG sandwich ELISA.[10,17] In this study, we used the anti-PEG sandwich ELISA to quantify two types of PEGylated interferons (PEG-Intron and Pegasys). Taken together, our results indicate that anti-PEG sandwich ELISA can be used for quantification of a variety of PEGylated substances.

Site-specific monoPEGylation is accepted as good manufacturing practice (GMP) for clinical use.[18,19] Protein drugs are usually conjugated with single high molecular weight (40 kDa) PEG molecule to achieve homogeneous modification and improved therapeutic efficacy.[7,16] For example, attachment of a branched PEG polymer with an average molecular mass of 40 000 to interferon increased its serum half-life by 330-fold in rats.[20] HCV patients treated once per week with Pegasys experienced greater decreases in liver inflammation than patients treated with standard three-times-weekly interferon,[7] thereby increasing patient compliance and quality of life. However, long chain PEG molecules may mask antibody-binding epitopes on proteins[9,10,21] and decrease the sensitivity of traditional protein-specific ELISA (Figure 1B). We used anti-PEG antibodies to generate a quantitative platform for PEGylated drugs in which PEGylated proteins are quantified by surface-linked PEG molecules. Our previous results demonstrated that a single long chain PEG chain contains multiple epitopes for binding by anti-PEG antibodies.[10,22,23] Detection sensitivity is proportional to the length of the PEG chain. The anti-PEG sandwich ELISA is therefore particularly suited for measuring protein drugs modified with long chain PEG molecules.

Development of a quantitative method that is insensitive to the presence of serum in samples is important for in vivo pharmacokinetic studies of drugs. The half-life of a drug in blood is a key factor in pharmacokinetic analysis and is related to absorption and distribution of the drug after administration. However, serum interference is an often encountered obstacle in ELISA assay development.[24,25] For example, some serum proteins, such as heterophilic antibodies, can cause high nonspecific binding in virus antibody ELISAs[26,27] and may hamper detection of proteins by epitope-masking.[28] In addition, serum can seriously lower the detection sensitivity of traditional ELISA.[29] We also observed here that serum impeded the detection sensitivity of the anti-interferon sandwich ELISA. By contrast, the presence of mouse or human serum in samples[10,22,23] did not decrease the sensitivity of the anti-PEG sandwich ELISA.

The covalent linkage between PEG and interferon in Pegasys is very stable in human serum (Figure 2E). The lower concentration of ^{131}I-Pegasys in serum determined by measuring radioactivity is unlikely due to dissociation of the linkage between PEG and interferon, but rather likely resulted from deiodination of ^{131}I from interferon. Iodine can be hydrolyzed from radiolabeled proteins by iodotyrosine deiodinase, resulting in underestimation of protein concentrations.[30,31] This may explain the higher interferon concentrations detected by the anti-PEG and anti-interferon sandwich ELISAs (Figure 3). In our study, anti-IFN sandwich ELISA was used as a standard assay kit to compare with anti-PEG sandwich ELISA to determine the pharmacokinetics of Pegasys in mice. However, the anti-IFN sandwich ELISA cannot sensitively detect PEG-modified interferon, especially Pegasys (Figure 1B), and the presence of mouse serum also interfered in the sensitivity of anti-IFN sandwich ELISA (Figure 2B). Similar to previous studies,[20] we used a high dose of Pegasys (5 μg per mouse) to allow detection at later time points by anti-IFN sandwich ELISA. By contrast, the anti-PEG sandwich ELISA can detect PEGylated interferon very sensitively with a detection limit of 0.03 ng/mL for Pegasys (Figure 1A). In addition, the presence of 20% of mouse serum did not affect the sensitivity of anti-PEG sandwich ELISA (Figure 2A). Another potential advantage of the anti-PEG ELISA is that endogenous cytokines in sera will not be detected (as shown for Roferon-A in Figure 1A). By contrast, traditional cytokine ELISAs can also detect circulating cytokines, thereby overestimating PEGylated cytokine concentrations.[32,33] Thus, anti-PEG sandwich ELISA may more precisely measure PEGylated drug concentrations as compared to traditional methods.

In summary, we demonstrate that the anti-PEG sandwich ELISA can be used to quantify PEGylated protein drugs. The advantages of anti-PEG sandwich ELISA are (1) it is insensitive to PEG-mediated epitope masking, (2) it is insensitive to serum interference, and (3) it can quantify any PEGylated molecule. The sensitivity of detection is proportional to the length of the PEG conjugated on the protein drug, so the ELISA is most suitable for proteins that contain a single long PEG molecule or multiple short PEG molecules.[10,22,23] The anti-PEG sandwich ELISA may also be superior when endogenous compounds can be recognized by traditional ELISA. The universality and convenience of the anti-PEG sandwich ELISA suggests that this assay offers a standard method for quantification of PEGylated proteins in preclinical and clinical pharmacokinetic studies.

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Author Contributions

Ta-Chun Cheng and Kuo-Hsiang Chuang contributed equally.

Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Fu, J., Fiegel, J., Krauland, E., and Hanes, J. (2002) New polymeric carriers for controlled drug delivery following inhalation or injection. *Biomaterials 23*, 4425−4433.

(2) Pisal, D. S., Kosloski, M. P., and Balu Iyer, S. V. (2010) Delivery of therapeutic proteins. *J. Pharm. Sci. 99*, 2557−2575.

(3) Jev evar, S., Kunstelj, M., and Porekar, V. G. (2010) PEGylation of therapeutic proteins. *Biotechnol. J. 5*, 113−128.

(4) Werle, M., and Bernkop-Schnurch, A. (2006) Strategies to improve plasma half life time of peptide and protein drugs. *Amino Acids 30*, 351−367.

(5) Chan, M., Hayashi, T., Mathewson, R. D., Yao, S., Gray, C., Tawatao, R. I., Kalenian, K., Zhang, Y., Hayashi, Y., Lao, F. S., Cottam, H. B., and Carson, D. A. (2011) Synthesis and characterization of PEGylated toll like receptor 7 ligands. *Bioconjugate Chem. 22*, 445−454.

(6) Jo, Y. W., Youn, Y. S., Lee, S. H., Kim, B. M., Kang, S. H., Yoo, M., Choi, E. C., and Lee, K. C. (2006) Long-acting interferon-alpha 2a modified with a trimer-structured polyethylene glycol: preparation, in vitro bioactivity, in vivo stability and pharmacokinetics. *Int. J. Pharm. 309*, 87−93.

(7) Rajender Reddy, K., Modi, M. W., and Pedder, S. (2002) Use of peginterferon alfa-2a (40 KD) (Pegasys) for the treatment of hepatitis C. *Adv. Drug Delivery Rev. 54*, 571−586.

(8) Matthews, S. J., and McCoy, C. (2004) Peginterferon alfa-2a: a review of approved and investigational uses. *Clin. Ther. 26*, 991−1025.

(9) Hershfield, M. S., Chaffee, S., Koro-Johnson, L., Mary, A., Smith, A. A., and Short, S. A. (1991) Use of site-directed mutagenesis to enhance the epitope-shielding effect of covalent modification of proteins with polyethylene glycol. *Proc. Natl. Acad. Sci. U. S. A. 88*, 7185−7189.

(10) Su, Y. C., Chen, B. M., Chuang, K. H., Cheng, T. L., and Roffler, S. R. (2010) Sensitive quantification of PEGylated compounds by second-generation anti-poly(ethylene glycol) monoclonal antibodies. *Bioconjugate Chem. 21*, 1264−1270.

(11) Harris, J. M., and Chess, R. B. (2003) Effect of pegylation on pharmaceuticals. *Nat. Rev. Drug Discovery 2*, 214−221.

(12) Alconcel, S. N. S., Baas, A. S., and Maynard, H. D. (2011) FDA-approved poly(ethylene glycol)-protein conjugate drugs. *Polym. Chem. 2*, 1442−1448.

(13) Kim, T. H., Youn, Y. S., Jiang, H. H., Lee, S., Chen, X., and Lee, K. C. (2011) PEGylated TNF-related apoptosis-inducing ligand (TRAIL) analogues: pharmacokinetics and antitumor effects. *Bioconjugate Chem. 22*, 1631−1637.

(14) Yang, K., Basu, A., Wang, M., Chintala, R., Hsieh, M. C., Liu, S., Hua, J., Zhang, Z., Zhou, J., Li, M., Phyu, H., Petti, G., Mendez, M., Janjua, H., Peng, P., Longley, C., Borowski, V., Mehlig, M., and Filpula, D. (2003) Tailoring structure-function and pharmacokinetic properties of single-chain Fv proteins by site-specific PEGylation. *Protein Eng. 16*, 761−770.

(15) Koumenis, I. L., Shahrokh, Z., Leong, S., Hsei, V., Deforge, L., and Zapata, G. (2000) Modulating pharmacokinetics of an anti-interleukin-8 F(ab')(2) by amine-specific PEGylation with preserved bioactivity. *Int. J. Pharm. 198*, 83−95.

(16) Kinstler, O., Molineux, G., Treuheit, M., Ladd, D., and Gegg, C. (2002) Mono-N-terminal poly(ethylene glycol)-protein conjugates. *Adv. Drug Delivery Rev. 54*, 477−485.

(17) Cheng, T. L., Cheng, C. M., Chen, B. M., Tsao, D. A., Chuang, K. H., Hsiao, S. W., Lin, Y. H., and Roffler, S. R. (2005) Monoclonal antibody-based quantitation of poly(ethylene glycol)-derivatized proteins, liposomes, and nanoparticles. *Bioconjugate Chem. 16*, 1225−1231.

(18) Chapman, A. P., Antoniw, P., Spitali, M., West, S., Stephens, S., and King, D. J. (1999) Therapeutic antibody fragments with prolonged in vivo half-lives. *Nat. Biotechnol. 17*, 780−783.

(19) Natarajan, A., Xiong, C. Y., Albrecht, H., DeNardo, G. L., and DeNardo, S. J. (2005) Characterization of site-specific ScFv PEGylation for tumor-targeting pharmaceuticals. *Bioconjugate Chem. 16*, 113−121.

(20) Ramon, J., Saez, V., Baez, R., Aldana, R., and Hardy, E. (2005) PEGylated interferon-alpha2b: a branched 40K polyethylene glycol derivative. *Pharm. Res. 22*, 1374−1386.

(21) Vugmeyster, Y., Entrican, C. A., Joyce, A. P., Lawrence-Henderson, R. F., Leary, B. A., Mahoney, C. S., Patel, H. K., Raso, S. W., Olland, S. H., Hegen, M., and Xu, X. (2012) Pharmacokinetic, biodistribution, and biophysical profiles of TNF nanobodies conjugated to linear or branched poly(ethylene glycol). *Bioconjugate Chem. 23*, 1452−1462.

(22) Tsai, N. M., Cheng, T. L., and Roffler, S. R. (2001) Sensitive measurement of polyethylene glycol-modified proteins. *Biotechniques 30*, 396−402.

(23) Chuang, K. H., Tzou, S. C., Cheng, T. C., Kao, C. H., Tseng, W. L., Shiea, J., Liao, K. W., Wang, Y. M., Chang, Y. C., Huang, B. J., Wu, C. J., Chu, P. Y., Roffler, S. R., and Cheng, T. L. (2010) Measurement of poly(ethylene glycol) by cell-based anti-poly(ethylene glycol) ELISA. *Anal. Chem. 82*, 2355−2362.

(24) Nielsen, K., Kelly, L., Gall, D., Smith, P., Bosse, J., Nicoletti, P., and Kelly, W. (1994) The use of divalent cation chelating agents (EDTA/EGTA) to reduce non-specific serum protein interaction in enzyme immunoassay. *Vet. Res. Commun. 18*, 433−437.

(25) Stahnke, H., Kittlaus, S., Kempe, G., and Alder, L. (2012) Reduction of matrix effects in liquid chromatography-electrospray ionization-mass spectrometry by dilution of the sample extracts: how much dilution is needed? *Anal. Chem. 84*, 1474−1482.

(26) Kricka, L. J. (1999) Human anti-animal antibody interferences in immunological assays. *Clin. Chem. 45*, 942−956.

(27) Martins, T. B., Pasi, B. M., Litwin, C. M., and Hill, H. R. (2004) Heterophile antibody interference in a multiplexed fluorescent microsphere immunoassay for quantitation of cytokines in human serum. *Clin. Diagn. Lab. Immunol. 11*, 325−329.

(28) Urbonaviciute, V., Furnrohr, B. G., Weber, C., Haslbeck, M., Wilhelm, S., Herrmann, M., and Voll, R. E. (2007) Factors masking HMGB1 in human serum and plasma. *J. Leukocyte Biol. 81*, 67−74.

(29) DeForge, L. E., Shih, D. H., Kennedy, D., Totpal, K., Chuntharapai, A., Bennett, G. L., Drummond, J. H., Siguenza, P., and Wong, W. L. (2007) Species-dependent serum interference in a sandwich ELISA for Apo2L/TRAIL. *J. Immunol. Methods 320*, 58−69.

(30) Neal, J. C., Stolnik, S., Schacht, E., Kenawy, E. R., Garnett, M. C., Davis, S. S., and Illum, L. (1998) In vitro displacement by rat serum of adsorbed radiolabeled poloxamer and poloxamine copolymers from model and biodegradable nanospheres. *J. Pharm. Sci. 87*, 1242−1248.

(31) Friedman, J. E., Watson, J. A., Jr., Lam, D. W., and Rokita, S. E. (2006) Iodotyrosine deiodinase is the first mammalian member of the

NADH oxidase/flavin reductase superfamily. *J. Biol. Chem. 281*, 2812−2819.

(32) Grassi, J., Roberge, C. J., Frobert, Y., Pradelles, P., and Poubelle, P. E. (1991) Determination of IL1 alpha, IL1 beta and IL2 in biological media using specific enzyme immunometric assays. *Immunol. Rev. 119*, 125−145.

(33) Liao, K., Sikkema, D., Wang, C., Chen, K., DeWall, S., and Lee, T. N. (2012) Development and validation of a cell-based SEAP reporter assay for the detection of neutralizing antibodies against an anti-IL-13 therapeutic antibody. *J. Immunol. Methods 375*, 258−263.