Measurement of Poly(ethylene glycol) by Cell-Based Anti-poly(ethylene glycol) ELISA

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Poly(ethylene glycol) (PEG) is increasingly used in clinical and experimental medicine. However, quantification of PEG and PEGylated small molecules remains laborious and unsatisfactory. In this report, we stably expressed a functional anti-PEG antibody on the surface of BALB 3T3 cells (3T3/αPEG cells) to develop a competitive enzyme-linked immunosorbent assay (ELISA) for PEG quantification. The αPEG cell-coated plate bound biotinylated PEG$_{5K}$ (CH$_3$-PEG$_{5K}$-biotin) and CH$_3$-PEG$_{5K}$-^{131}I more effectively than did a traditional anti-PEG antibody-coated plate. Competitive binding between PEG (2, 5, 10, or 20 kDa) and a known amount of CH$_3$-PEG$_{5K}$-biotin allowed construction of a reproducible competition curve. The αPEG cell-based competition ELISA measured small molecules derivatized by PEG$_{2K}$, PEG$_{5K}$, PEG$_{10K}$, PEG$_{20K}$, and PEG$_{5K}$ at concentrations as low as 58.6, 14.6, 3.7, 3.7, and 14.6 ng/mL, respectively. Notably, the presence of serum or bovine serum albumin enhanced PEG measurement by the αPEG cell-based competition ELISA. Finally, we show here that the αPEG cell-based competition ELISA accurately delineated the pharmacokinetics of PEG$_{5K}$, comparable to those determined by direct measurement of radioactivity in blood after intravenous injection of CH$_3$-PEG$_{5K}$-^{131}I into mice. This quantitative strategy may provide a simple and sensitive method for quantifying PEG and PEGylated small molecules in vivo.

Poly(ethylene glycol) (PEG) is a water-soluble, nontoxic, low-immunogenic, and biocompatible polymer that has been approved by the Food and Drug Administration (FDA) for human use.[1] PEG is commonly employed as a protein-, drug-, and probe-modifying agent.[2−4] Upon conjugation, PEG can prolong circulation time in blood[5,6] and increase water solubility and reduce systemic toxicity.[7,8] In addition to therapeutic proteins (for example, interferon), increasing numbers of PEGylated small chemical molecules such as camptothecin,[9,10] SN-38,[11] paclitaxel,[12−14] and indomethacin[15] have been tested in animal models for improvement of their antitumor or anti-inflammatory effects. Furthermore, novel functions are continually being discovered for PEG. For example, PEG molecules are used directly in experimental medicine as therapeutic agents for neuronal injury,[16,17] as chemopreventive agents for chemical-induced colitis[18,19] and as tumor-

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suppressive agents for cancer,[20] though the detailed mechanisms of PEG action in these settings are not completely understood. New treatment modalities using PEG are likely to be developed in the future. Development of novel treatments using PEG and PEGylated small molecules requires thorough pharmacokinetic studies in animals and humans.

Sensitive detection of PEG should greatly facilitate pharmacokinetic studies. Methods such as colorimetric assays or high-performance liquid chromatography (HPLC) have been developed to measure PEG over the last three decades.[21-24] These methodologies are laborious and relatively insensitive (5-100 μg/mL range). Although the development of HPLC/tandem mass spectrometry (MS/MS) improves detection of PEG significantly, the requirement for expensive instruments and skillful laboratory personnel may limit its routine use. Furthermore, the presence of proteins in serum may impede these assays, therefore casting uncertainties on the appropriateness of using these methods for measuring PEG in pharmacokinetic studies. In our quest to develop accurate and reliable methods for measuring PEG-derivatized molecules, we have previously generated two monoclonal antibodies (AGP3 and E11) that bind the repeating (OCH_2CH_2) backbone of PEG.[25,26] These antibodies are very sensitive for detecting PEG-derivatized macromolecules by Western blotting, flow cytometry, and enzyme-linked immunosorbent assay (ELISA).[26] Despite our successes in measuring PEGylated macromolecules, sensitive and accurate quantification of PEG and PEGylated small molecules by conventional ELISA remains difficult.

Here we report a strategy for measuring PEG and PEGylated small molecules based on competitive binding with biotinylated PEG_{5K} (CH_3-PEG_{5K}-biotin) to a surface-expressed anti-PEG antibody (αPEG receptor) (Figure 1A). We generated the αPEG receptor by cloning the Fab fragment of the anti-PEG monoclonal antibody AGP3 and stably expressing it on the surface of BALB/3T3 fibroblasts. We tested the sensitivity of αPEG cell-based and AGP3 antibody-based sandwich ELISA for detection of PEG and PEGylated macromolecules. We also developed an αPEG cell-based competition ELISA for measuring PEG (2, 5, 10, or 20 kDa) and PEGylated small molecules. Finally, we studied the pharmacokinetics of PEG in mice using αPEG cell-based competition ELISA and compared the results with those obtained by measuring radioactivity in blood after intravenous injection of CH_3-PEG_{5K}-^{131}I to mice. Our data suggest that the αPEG cell-based competition ELISA may provide a useful tool for pharmacokinetic studies of PEG and PEGylated small molecules.

EXPERIMENTAL SECTION

Cells and Mice. BALB/3T3 cells (American Type Culture Collection, Manassas, VA) and GP2-293 cells (Clontech, Mountain View, CA) were grown in culture medium containing Dulbecco's modified Eagle's medium (DMEM; Sigma−Aldrich, St. Louis, MO), 10% heat-inactivated bovine calf serum (BCS, Sigma−Aldrich), and 100 units/mL penicillin and streptomycin (Invitrogen, Calsbad, CA), at 37 °C in a humidified atmosphere of 5% CO_2. Female BALB/c mice were purchased from the National Laboratory Animal Center, Taipei, Taiwan. All animal experiments were performed in accordance with institutional guidelines and were approved by the Animal Care and Use Committee of the Kaohsiung Medical University.

Plasmid Construction. The V_L-Cκ and V_H-C_{H1} domains of the αPEG receptor were cloned from cDNA prepared from the AGP3 hybridoma[25] following a previously described method.[27] Primers used in the cloning of V_L-Cκ and V_H-C_{H1} were as follows: V_L-Cκ sense, 5′-tgctggggcccagccggccgatattgtgt-tgacgcaggct-3′; V_L-Cκ antisense, 5′-ccgctcgagacactcattcctgt-tgaagct-3′; V_H-C_{H1} sense, 5′-gaagatctgaagtgcagctggtggagtct-3′; and V_H-C_{H1} antisense, 5′-caggtcgacagctggaatgggcacatgcag-3′. The light- and heavy-chain sequences, joined by an internal ribosome entry site (IRES), were subcloned into a retroviral vector, pLNCX-eB7,[27] by use of SfiI and SalI restriction sites. The expression vector, pLNCX-αPEG-eB7, encodes an anti-PEG Fab fused to the immunoglobulin C2-type extracellular−transmembrane−cytosolic domains of the mouse B7-1 antigen (Figure 1A). A plasmid (pLNCX-αDNS-eB7) that encodes a membrane Fab with specificity for 5-(dimethylamino)naphthalene-1-sulfonyl chloride (DNS)[28] was constructed in an analogous fashion to act as a negative control.

Generation of αPEG Receptor-Expressing Cells by Retroviral Transduction. To produce pseudotyped retroviruses, pLNCX-αPEG-eB7 or pLNCX-αDNS-eB7 was cotransfected with pVSVG (Clontech) to GP2-293 cells by Lipofectamine 2000 (Invitrogen). Two days after transfection, the culture medium was filtered and mixed with 8 μg/mL Polybrene (Sigma−Aldrich), and the mixture was added to BALB/3T3 cells. Following retroviral transduction, cells were selected in G418-containing medium and sorted on a FACS Cantor (BD Biosciences, San Jose, CA) to generate 3T3/αPEG and 3T3/αDNS cells that expressed approximately equal levels of αPEG or αDNS receptors.

Fluorescence-Activated Cell Sorting Analysis of the αPEG Receptor. Surface expression of the receptors was measured by staining cells with 1 μg/mL mouse anti-hemagglutinin (anti-HA) antibody, followed by 1 μg/mL fluorescein isothiocyanate- (FITC-) conjugated goat anti-mouse IgG (Fc) (Jackson Immunoresearch Laboratories, West Grove, PA) in phosphate-buffered saline (PBS) containing 0.05% bovine serum albumin (BSA) on ice. PEG binding activity of the receptors was determined by incubating cells with 4 μM PEG quantum dots 525 (PEG-QDs; Invitrogen) in PBS containing 0.05% BSA on ice. After removal of unbound antibodies or PEG-QDs by extensive wash in cold PBS, the surface fluorescence of viable cells was measured on a FACScalibur flow

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cytometer (BD Biosciences) and analyzed with WinMDI version 2.5 (The Scripps Research Institute, San Diego, CA).

Synthesis of CH$_3$-PEG$_{5K}$-Biotin, CH$_3$-PEG$_{5K}$-FITC, CH$_3$−PEG$_{5K}$-NIR797, and CH$_3$-PEG$_{5K}$-^{131}I. To synthesize CH$_3$-PEG$_{5K}$-biotin, CH$_3$-PEG$_{5K}$-NH$_2$ (Sigma−Aldrich) was reacted with EZ-link sulfo-NHS-LC-biotin (Pierce, Rockford, IL) at a molar ratio of 1:5 in N,N-dimethylformamide (DMF) containing 0.1% triethylamine at room temperature for 1 h. To synthesize CH$_3$-PEG$_{5K}$-FITC, CH$_3$-PEG$_{5K}$-NH$_2$ was reacted with fluorescein isothiocyanate isomer I (Sigma−Aldrich) at a molar ratio of 1:5 in DMF containing 0.1% triethylamine at room temperature for 1 h. To synthesize CH$_3$-PEG$_{5K}$-NIR797, CH$_3$-PEG$_{5K}$-NH$_2$ was reacted with NIR797 isothiocyanate (Sigma−Aldrich) at a molar ratio of 1:3 in DMF containing 0.1% triethylamine at room temperature for 1 h. To synthesize CH$_3$-PEG$_{5K}$-SHPP, CH$_3$-PEG$_{5K}$-NH$_2$ was reacted with 3-(4-hydroxyphenyl)propionic acid N-hydroxysuccinimide ester (SHPP; Sigma−Aldrich) at a molar ratio of 1:10 in DMF containing 0.1% triethylamine at room temperature for 1 h. The presence of the biotin, FITC, NIR797, or SHPP group was verified by UV detection, whereas primary amine groups were detected by reaction with 2% ninhydrin solution (Sigma−Aldrich). The reaction mixture was separated on silica gel with dichloromethane−methanol (8:2 v/v) to obtain CH$_3$-PEG$_{5K}$-biotin, CH$_3$-PEG$_{5K}$-FITC, CH$_3$-PEG$_{5K}$-NIR797, or CH$_3$-PEG$_{5K}$-SHPP.

To synthesize CH$_3$-PEG$_{5K}$-^{131}I, CH$_3$-PEG$_{5K}$-SHPP was radioiodinated as previously described with modifications.[29] Briefly, 30.7 μg of CH$_3$-PEG$_{5K}$-SHPP in 100 μL of 0.2 M ammonium acetate buffer (pH = 3.3) was added to a 300 μL V-shaped vial containing 37 MBq of sodium [^{131}I]iodide (IBA Molecular, Louvain-La-Neuve, Belgium) and 10 μL of chloramine-T solution (45 mM) (Sigma−Aldrich). The reaction mixture was vortexed intermittently. After 10 min, 20 μL of 130 mM sodium thiosulfate and 50 μL of saturated sodium hydrogen carbonate were added to quench the reaction. The reaction mixture was purified on a G-25 Sephadex column (GE Healthcare, Piscataway, NJ). The radiochemical purity of CH$_3$-PEG$_{5K}$-^{131}I was determined by radio thin layer chromatography (TLC) on an aluminum sheet coated with reverse-phase silica gel powder (RP-18 F$_{254}$; Merck, Whitehouse Station, NJ) with ethanol as the mobile phase. The radiochemical yield was >70% and the radiochemical purity was >95% (data not shown). The specific activity of CH$_3$-PEG$_{5K}$-^{131}I was 0.939 MBq/μg.

Antibody-Based and Cell-Based Sandwich ELISA. In all ELISA experiments (sandwich or competition ELISA), DMEM containing 2.5% bovine calf serum was used as the diluent or wash buffer unless otherwise indicated. 3T3/αPEG and 3T3/αDNS cells (2 × 10^5 cells/well) were seeded overnight in 96-well plates (Nalge Nunc International, Roskilde, Denmark) in culture medium. Graded concentrations of CH$_3$-PEG$_{2K}$-NH$_2$, CH$_3$-PEG$_{5K}$-NH$_2$, CH$_3$-PEG$_{10K}$-NH$_2$, or CH$_3$-PEG$_{20K}$-NH$_2$ (Sigma−Aldrich) or PEG-QDs were added to the wells (50 μL/well) at room temperature for 1 h. After being washed, the cells were sequentially incubated with biotinylated AGP3 (0.25 μg/well)[25] and streptavidin-conjugated horseradish peroxidase (streptavidin−HRP, 50 ng/well). The plates were washed with PBS,

and bound peroxidase was measured by adding 150 μL/well ABTS solution [0.4 mg/mL, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma−Aldrich), 0.003% H$_2$O$_2$, and 100 mM phosphate−citrate, pH = 4.0] for 30 min at room temperature. For the antibody-based sandwich ELISA, unmodified AGP3 (1 μg/well)[25] in coating buffer (0.1 M NaHCO$_3$, pH = 8) was added to Maxisorp 96-well microplates (Nalge Nunc International) for 3 h at 37 °C. Plates were blocked with 5% skim milk in PBS overnight at 4 °C. Standard sandwich ELISA was then performed by sequential addition of PEG-QDs or PEG, biotinylated AGP3, streptavidin−HRP, and ABTS. Color development was measured at 405 nm on a microplate reader. All the readings were background-adjusted by subtracting absorbance of a blank control in the ELISA procedures. The detection limits of all ELISA experiments (sandwich or competition ELISA) were defined by using the independent t-test to compare the statistical significance of differences between a control and examined samples (PEG, PEGylated macromolecules, and PEGylated small molecules). Data were considered significant at $p \leq 0.05$.

Standard Curve of CH$_3$-PEG$_{5K}$-Biotin. 3T3/αPEG and 3T3/αDNS cells (2 × 10^5 cells/well) were seeded in 96-well plates as above. CH$_3$-PEG$_{5K}$-biotin was serially diluted and added to the cells at room temperature for 1 h. After washing, the cells were sequentially exposed to streptavidin−HRP and ABTS. Color development was measured at 405 nm on a microplate reader.

αPEG Cell-Based Competition ELISA. 3T3/αPEG cells (2 × 10^5 cells/well) were seeded in 96-well plates as above. Twofold serially diluted CH$_3$-PEG$_{5K}$-NH$_2$ was prepared and mixed 1:1 (v/v) with 250 ng/mL CH$_3$-PEG$_{5K}$-biotin (thus the final concentration of CH$_3$-PEG$_{5K}$-biotin was 125 ng/mL), and then the mixture was added to the cells at room temperature for 1 h. Following washing, cells were sequentially incubated with streptavidin−HRP and ABTS at room temperature. Color development (optical density) was measured at 405 nm on a microplate reader. In all subsequent experiments, we arbitrarily chose to use 250 ng/mL CH$_3$-PEG$_{5K}$-biotin in the αPEG cellbased competition ELISA.

To determine the effects of length of PEG, CH$_3$-PEG$_{2K}$-NH$_2$, CH$_3$-PEG$_{5K}$-NH$_2$, CH$_3$-PEG$_{10K}$-NH$_2$, and CH$_3$-PEG$_{20K}$-NH$_2$ were 2-fold serially diluted and mixed 1:1 (v/v) with CH$_3$-PEG$_{5K}$-biotin, and then the mixture was added to the cells at 37 °C for 1 h, followed by ELISA procedures as above. To determine whether the presence of proteins affects the assay, DMEM alone or DMEM containing 2.5%, 10%, or 40% bovine calf serum or 0.05% bovine serum albumin (BSA; Sigma−Aldrich) were used as diluents for CH$_3$-PEG$_{5K}$-NH$_2$. αPEG cell-based competition ELISA was then performed as described above; DMEM containing 2.5% BCS was used for the wash buffer and diluent for secondary and tertiary reagents.

Competition curves were derived by fitting the optical density against the concentration of CH$_3$-PEG$_{5K}$-NH$_2$ to a four-parameter logistic model, using Prism 4 software (GraphPad Software, San Diego, CA). To validate the accuracy of the competition curve, back-fitted concentration (BC) at each concentration level was deduced from the curves and then compared to the nominal concentration (NC). Difference in back-fitted concen-

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tration and nominal concentration is expressed as percent relative error (% RE) as follows:

$$\% \text{ RE} = [(\text{BC} - \text{NC})/\text{NC}] \times 100$$

Pharmacokinetics of PEG in Mice. CH_3-PEG_{5K}-NH_2 was intravenously injected into female BALB/c mice (5 mg/mouse, $n = 8$). Blood was withdrawn at different time points by use of heparinized capillary tubes. Plasma was 50- and 100-fold diluted, and the competition ELISA was then performed as described above. Concentrations of PEG in serum were deduced by fitting optical density to the competition curve obtained from PEG standards. The serum half-life of CH_3-PEG_{5K}-NH_2 was estimated by fitting the data to a one-phase exponential decay model with Prism 4 software.

To validate pharmacokinetics of PEG, female BALB/c mice ($n = 5$) were intravenously injected with 740 μBq of CH_3-PEG_{5K}-^{131}I (containing 5 mg of CH_3-PEG_{5K}-SHPP). Blood was withdrawn at different time points by use of heparinized capillary tubes. The radioactivity of serum samples was counted on a Wallac 1470 Wizard γ counter (Perkin-Elmer, Inc., Waltham, MA). Results (mean ± SD) are expressed as the concentration of CH_3-PEG_{5K}-SHPP in serum (nanograms per milliliter).

RESULTS AND DISCUSSION

Surface Expression of a Functional αPEG Receptor. We cloned the coding sequences of the Fab fragment of an anti-PEG antibody (AGP3) in an engineered retroviral vector, pLNCX, that contains a HA epitope and the immunoglobulin C2-type extracellular−transmembrane−cytosolic domains of the mouse B7-1 receptor (eB7). In pLNCX-αPEG-eB7, the light and heavy chains are separated by an internal ribosome entry site (IRES) (Figure 1A), which allows "cap-independent" protein translation of the second cistron on a bicistronic transcript. Upon protein translation, the light chain can pair with the heavy chain in the endoplasmic reticulum through disulfide bond formation to form an αPEG receptor on the cell surface.

The expression and function of the surface αPEG receptor was confirmed by flow cytometry, by use of anti-HA antibodies (Figure 1B) and PEG quantum dots (PEG-QDs) (Figure 1C). Though αPEG and the control αDNS receptors were expressed at similar levels on the cell surface (Figure 1B), only 3T3/αPEG cells specifically bound PEG-QDs (Figure 1C). These results indicate that surface-expressed αPEG receptors maintained the PEG binding activity of the native AGP3 antibody.

Measuring PEG and PEG-QDs by AGP3 Antibody-Based and αPEG Cell-Based Sandwich ELISA. PEG does not contain a chromophore or other resonance structure, which renders its direct photometric detection difficult. Over the past few decades, several techniques have been developed for measuring PEG, including colorimetric determination after complex formation with barium and iodine,[30] partitioning of a chromophore in aqueous ammonium ferrothiocyanate reagent,[24] or salting-out of a fluorophore into a two-phase system.[31] Others have developed chromatographic techniques such as HPLC−refractive index or gas

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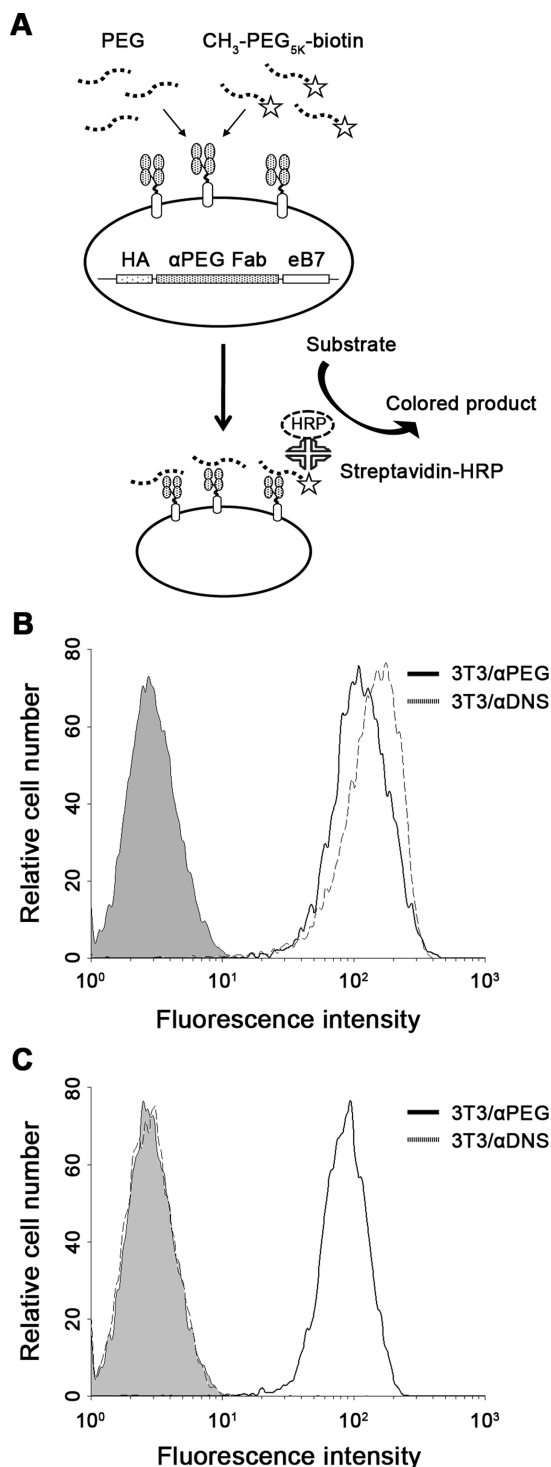
Figure 1. Surface display of functional αPEG receptors. (A) Schematic representation of the αPEG receptor. The receptor gene includes, from N to C terminus, an immunoglobulin signal peptide, a HA epitope, the αPEG Fab fragment (composed of light chain, IRES, and heavy chain), and the immunoglobulin C2-type extracellular−transmembrane−cytosolic domains of the murine B7-1 antigen (eB7). (B, C) 3T3/αPEG (−), 3T3/αDNS (---), and 3T3 (shaded area) cells were analyzed by flow cytometry: (B) a specific antibody to the HA epitope was used to assess surface expression or (C) staining with PEG quantum dots was used to assess the PEG-binding activity of the receptors.

chromatography to measure PEG.[23,32,33] Each of these methods, however, possesses drawbacks and disadvantages. For example, the sensitivity of most of these techniques is only 5−100 μg/mL.

On the other hand, chromatographic techniques require sophisticated and expensive instruments. The refractive index measurement is sensitive to ambient temperature, while gas chromatography requires laborious sample pretreatment.[33,34] In addition, gas chromatography also requires that samples are volatile, thus limiting the length of PEG detectable by this method to less than ~1000.

Our previous data demonstrated that AGP3 specifically binds to the PEG backbone.[25] AGP3 is useful for measuring PEGylated macromolecules such as PEG-QDs (Figure 2A and unpublished data), but attempts to develop a sandwich ELISA for PEG (2, 5, 10, and 20 kDa) were not successful (Figure 2B). We therefore sought to test whether 3T3/αPEG cells could be used to detect PEG. PEG (2, 5, 10, and 20 kDa) was added to 3T3/αPEG cells that had been coated in 96-well plates. Captured PEG molecules were then quantified by sequential addition of biotinylated AGP3 antibody, streptavidin-conjugated horseradish peroxidase (streptavidin−HRP) and ABTS substrate. As shown in Figure 2C, 3T3/αPEG cells detected PEG molecules with a sensitivity that depended on the length of the PEG chain; CH_3-PEG_{20K}-NH_2 was most effectively detected, whereas CH_3-PEG_{5K}-NH_2 and CH_3-PEG_{10K}-NH_2 were clearly detected but with a low overall reading by the pairing of surface-expressed αPEG receptors and biotinylated AGP3. In contrast, control 3T3/αDNS cells did not capture noticeable amounts of PEG, regardless of the lengths of the PEG molecules (data not shown). Both the AGP3 antibody and the αPEG receptor (derived from the AGP3 antibody) recognizes the $(OCH_2CH_2)_n$ backbone of PEG. In our previous studies,[26] AGP3 was found to specifically bind PEG molecules (>1.5 kDa) and the sensitivity positively correlated to the length of the PEG chains. In addition, Wahab et al.[35] have indicated that the (OCH_2CH_2) subunits can form hydrogen bonds with H_2O, resulting in various structures of PEG in aqueous solutions. It is likely that, in aqueous solutions, longer PEG molecules may more readily form particular structures (epitopes) recognized by AGP3 (or αPEG receptors). On the contrary, it is also reasonable that shorter PEG molecules contain fewer epitopes, thus limiting the detection sensitivity of the αPEG cell-based sandwich ELISA. These results suggest that the αPEG cell-based sandwich ELISA can detect PEG, though further optimization of the experimental conditions is required.

Binding of PEG in AGP3 Antibody-Based and αPEG Cell-Based ELISA. The biotinylated APG3 used as a detection antibody in Figure 2C might be unable to bind to shorter PEG molecules (2, 5, and 10 kDa species) after they are bound by 3T3/αPEG cells, thus yielding unsatisfying results. We reasoned that the detection sensitivity of the αPEG cell-based ELISA to shorter PEG could be improved by eliminating the use of biotinylated AGP3 in the assay. To test this hypothesis, biotinylated PEG_{5K} (CH_3-PEG_{5K}-biotin) was added to 3T3/αPEG cells or to AGP3 precoated on 96-well plates. Streptavidin−HRP and ABTS were sequentially added to the plates to quantify the captured CH_3-PEG_{5K}-biotin. Using this

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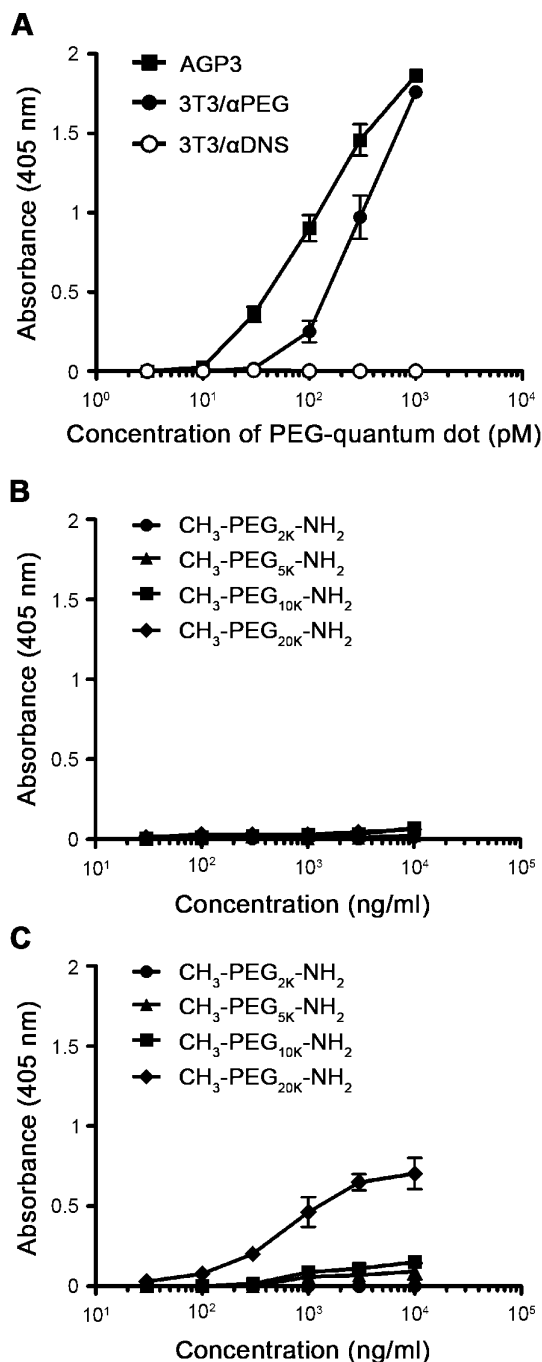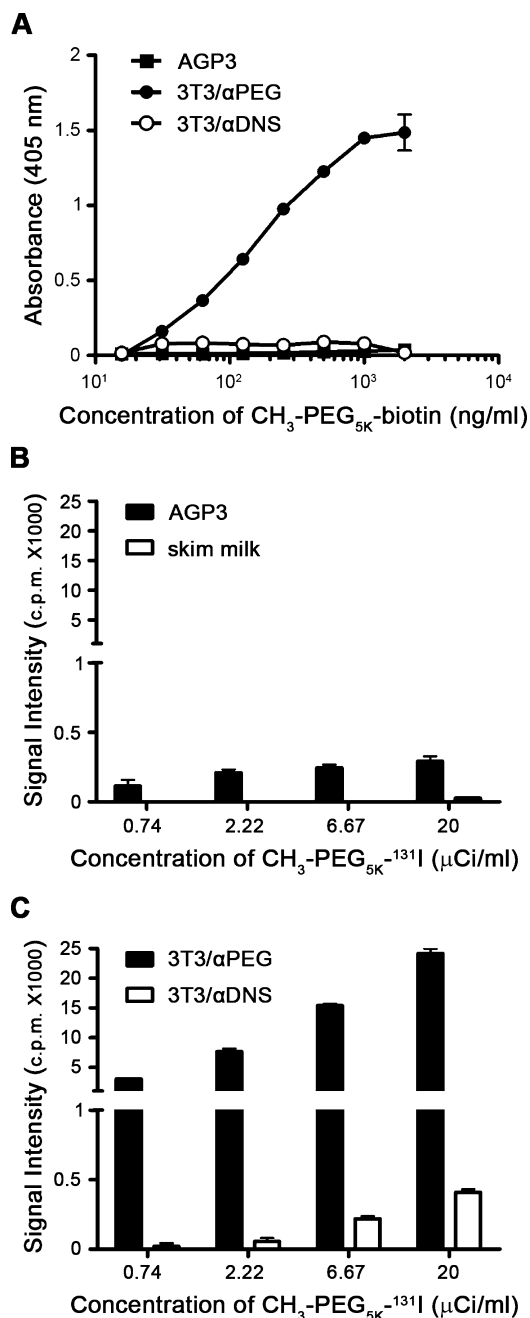
Figure 2. Binding of PEGylated macromolecules or PEG in antibody-based or cell-based sandwich ELISA. (A) Sandwich ELISA, with AGP3 antibody (■), 3T3/αPEG cells (●), or 3T3/αDNS cells (○) as the capture reagent, was used to measure the concentration of PEG-quantum dots. (B, C) Graded concentrations of CH_3-PEG_{2K}-NH_2 (●), CH_3-PEG_{5K}-NH_2 (▲), CH_3-PEG_{10K}-NH_2 (■), and CH_3-PEG_{20K}-NH_2 (◆) were measured by (B) AGP3 antibody-based or (C) αPEG cell-based sandwich ELISA. Mean ± SD values are shown.

modification, we substantially increased the detection limit of CH_3-PEG_{5K}-biotin to 15.6 ng/mL in the αPEG cell-based ELISA (Figure 3A). On the contrary, AGP3 antibody-based ELISA still failed to detect the CH_3-PEG_{5K}-biotin.

To further investigate the potential mechanism that dictates the differential ability in detecting PEG by the surface-expressed αPEG receptor and coated AGP3 antibody, we compared direct

Figure 3. Binding of biotinylated PEG and CH_3-PEG_{5K}-^{131}I by the AGP3 antibody-based and αPEG cell-based ELISA. (A) Graded concentrations of CH_3-PEG_{5K}-biotin were added to plate-coated AGP3 antibody (■), 3T3/αPEG cells (●), or 3T3/αDNS cells (○), followed by detection with streptavidin−HRP and ABTS. (B) Direct binding of CH_3-PEG_{5K}-^{131}I by AGP3 antibody-coated plates (solid bars) or skim milk-coated plates (open bars). (C) Direct binding of CH_3-PEG_{5K}-^{131}I by 3T3/αPEG cell-coated plates (solid bars) or 3T3/αDNS cell-coated plates (open bars). Mean ± SD values are shown.

binding of CH_3-PEG_{5K}-^{131}I to AGP3 antibody (Figure 3B) and 3T3/αPEG cells (Figure 3C) precoated in 96-well plates. We found 3T3/αPEG cells captured significantly more CH_3-PEG_{5K}-^{131}I than did coated AGP3 antibody. On average, signal intensity per well was 26.5-fold (3026 vs 114, $p < 0.0001$), 36.4-fold (7635 vs 210, $p < 0.0001$), 62.7-fold (15 380 vs 245, $p < 0.0001$), and 82.3-fold (24 125 vs 293, $p < 0.0001$) greater for the αPEG cell-coated plate than for the AGP3 antibody-coated plate after addition of 0.74, 2.22, 6.67, and 20 μCi/mL CH_3-PEG_{5K}-^{131}I. Lack of direct

binding of CH_3-PEG_{5K}-^{131}I by AGP3 antibody-coated plate is surprising and unaccounted for at present, as AGP3 is a monoclonal antibody specific to the PEG backbone.[36] It is unlikely that insufficient AGP3 was coated on microtiter plates to bind CH_3-PEG_{5K}-^{131}I, as up to 1 μg of the antibody/well was coated in the plate and this amount of antibody can detect PEGylated macromolecules with high sensitivity (as shown in Figure 2A and previous results[36]). Alternatively, antibodies randomly attached to the plate may be in a disorganized orientation, which could "pull away" among themselves for binding of PEG (which is theoretically a flexible and linear structure), and ultimately decrease avidity. On the contrary, surface αPEG receptor should display only one organization (outward-pointing) after coating the 3T3/αPEG cells to the plate. In addition, 3T3/αPEG cells may be considered to be analogous to microbeads, which have been shown to increase immobilization of capture antibodies in assay plates (ELISA plates)[37,38] due to a larger surface area as compared to a flat-bed well, thus enhancing assay sensitivity. The combination of unidirectional organization and larger surface area on which the αPEG receptors are presented may explain better binding of free PEG by a mechanism analogous to the "cooperative binding model".[39]

Development of a Sensitive αPEG Cell-Based Competition ELISA for Measuring PEG and PEGylated Small Molecules. We reasoned that PEG could compete with CH_3-PEG_{5K}-biotin for binding sites on 3T3/αPEG cells. We therefore modified the assay described in Figure 3A as the basis of developing an αPEG cell-based competition ELISA for measuring PEG. Graded concentrations of CH_3-PEG_{5K}-NH_2 were mixed with a fixed amount of CH_3-PEG_{5K}-biotin prior to addition to 3T3/αPEG cells. As shown in Figure 4A, increasing amounts of CH_3-PEG_{5K}-NH_2 indeed competed with the CH_3-PEG_{5K}-biotin for binding sites on 3T3/αPEG cells as demonstrated by the proportionally decreased absorbance at 405 nm. The prediction power and accuracy of a competition curve can be represented by low percent relative error (% RE) between back-fitted concentration and nominal concentration.[40] To validate the accuracy of the αPEG cell-based competition ELISA, back-fitted concentrations at different concentration levels were deduced from the competition curves and then compared to the nominal concentration. As shown in Table 1, % RE was low (≤10%) at each concentration level, indicating that the αPEG cell-based competition ELISA is a reliable method for measuring PEG.

We also tested the effect of PEG length (2, 5, 10, and 20 kDa PEG) in the αPEG cell-based competition ELISA. We found that longer lengths of unlabeled PEG produced better competition, as indicated by the leftward shift of the competition curves (Figure 4B). CH_3-PEG_{2K}-NH_2, CH_3-PEG_{5K}-NH_2, CH_3-PEG_{10K}-NH_2, and CH_3-PEG_{20K}-NH_2, at concentrations as low as 58.6, 14.6, 3.7, and 3.7 ng/mL, respectively, could compete with CH_3-PEG_{5K}-biotin, indicating that αPEG cell-based competition ELISA is a

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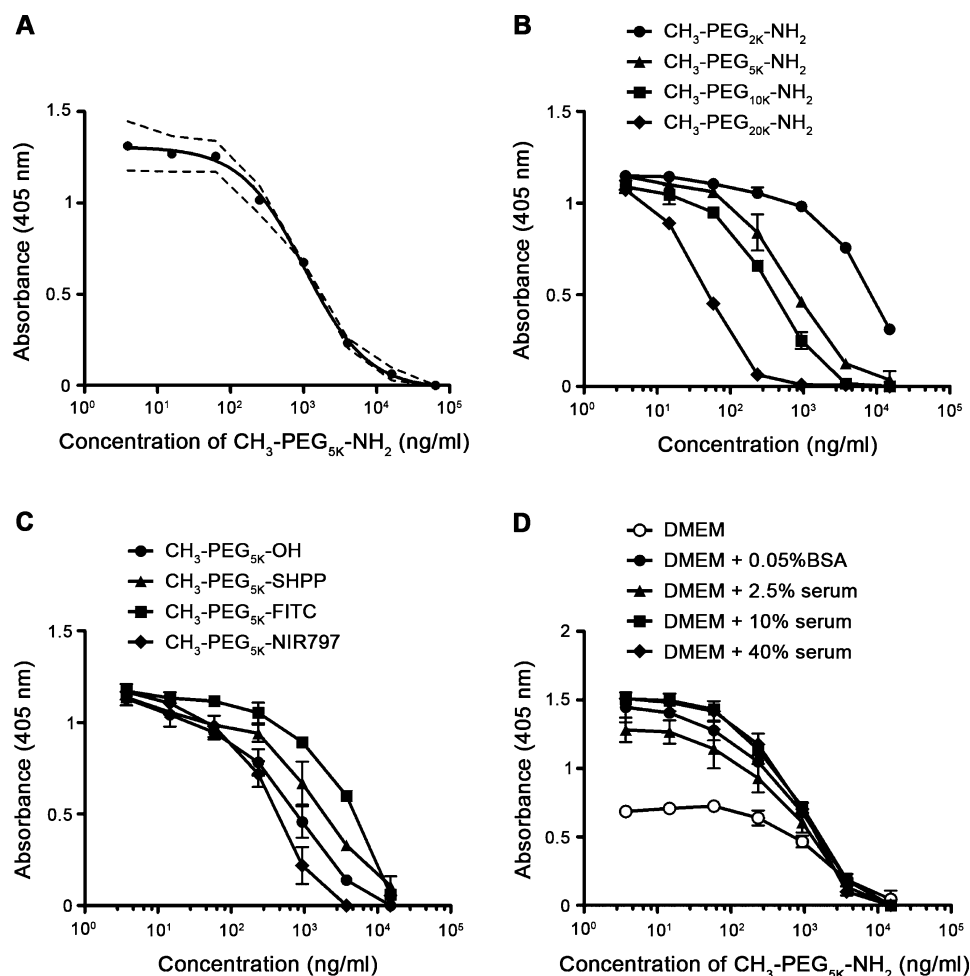(40) Findlay, J. W. A.; Dillard, R. F. *AAPS J.* **2007**, *9*, E260−E267.

Figure 4. Sensitive αPEG cell-based competition ELISA. (A) Standard competition curve of CH_3-PEG_{5K}-NH_2 against CH_3-PEG_{5K}-biotin. Dashed line: 95% confidence interval. (B) Competition curves of CH_3-PEG_{2K}-NH_2 (●), CH_3-PEG_{5K}-NH_2 (▲), CH_3-PEG_{10K}-NH_2 (■), and CH_3-PEG_{20K}-NH_2 (◆) against CH_3-PEG_{5K}-biotin. (C) Competition curves of CH_3-PEG_{5K}-OH (●), CH_3-PEG_{5K}-SHPP (▲), CH_3-PEG_{5K}-FITC (■), and CH_3-PEG_{5K}-NIR797 (◆) against CH_3-PEG_{5K}-biotin. (D) Effect of serum proteins on the competition assay. DMEM alone (○) or DMEM containing 0.05% BSA (●), 2.5% serum (▲), 10% serum (■), or 40% serum (◆), respectively, was used as diluent of PEG in the competition ELISA. Mean ± SD values are shown.

Table 1. Accuracy of αPEG Cell-Based Competition ELISA: Difference in Back-Fitted and Nominal Concentrations

NC^a (ng/mL)	BC^b (ng/mL)	% RE^c
62.5	68.2 ± 16.8^d	9.09
250	268 ± 64.5	7.19
1000	1026 ± 172.7	2.62
4000	4041 ± 194.8	1.03

[a] Nominal concentration. [b] Back-fitted concentration. [c] Percent relative error. [d] Results represent the mean ± SD ($n = 6$).

highly sensitive method for measuring PEG. Since longer PEG may contain more epitopes recognized by the αPEG receptor, it is probably not surprising that the longer PEG competes better with CH_3-PEG_{5K}-biotin in our assay. In addition, the αPEG cell-based competition ELISA could detect CH_3-PEG_{5K}-OH, CH_3-PEG_{5K}-SHPP, CH_3-PEG_{5K}-FITC, and CH_3-PEG_{5K}-NIR797 at concentrations as low as 14.6 ng/mL (Figure 4C), indicating that αPEG cell-based competition ELISA can also measure PEGylated small molecules with good sensitivity.

We further investigated whether the presence of serum hampers the assay. Inclusion of serum or bovine serum albumin

(BSA) seemed to enhance substantially color development and to extend the concentration at which the competition curve reached a plateau (Figure 4D). The underlying mechanism by which proteins enhance color development is presently unknown. Proteins may stabilize αPEG receptors (thus better anti-PEG activity), as BSA is commonly used as a stabilizer for restriction enzymes or antibodies.[41,42] Alternatively, serum proteins may be required for proper formation of the antigenic structure of PEG recognizable by the αPEG receptor, as we originally obtained AGP3 (the parental anti-PEG antibody) by immunizing mice with PEG conjugated to a recombinant protein.

In contrast to previously reported methods, the new αPEG cell-based competition ELISA is based on the specific binding of an αPEG receptor to PEG. This αPEG receptor was derived from a specific anti-PEG antibody AGP3 and showed very sensitive and specific detection of PEG and PEGylated small molecules with detection limitations of 58.6 ng/mL (PEG_{2K}), 14.6 ng/mL (PEG_{5K}), and 3.7 ng/mL (PEG_{10K} and PEG_{20K}), respectively, which is nearly 1000-fold more sensitive than previously

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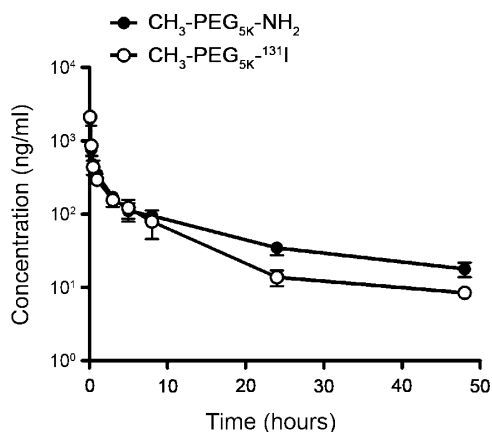(42) Kauffmann, R. H.; Van Es, L. A.; Daha, M. R. *J. Immunol. Methods* **1979**, *31*, 11–22.

Figure 5. Pharmacokinetics of PEG. BALB/c mice ($n = 8$) were intravenously injected with 5 mg of CH_3-PEG_{5K}-NH_2. The concentration of CH_3-PEG_{5K}-NH_2 in serum was measured by αPEG cell-based competition ELISA (●). For the serum half-life of CH_3-PEG_{5K}-^{131}I, BALB/c mice ($n = 5$) were intravenously injected with 740 μBq of CH_3-PEG_{5K}-^{131}I (containing 5 mg of CH_3-PEG_{5K}-SHPP). The radioactivity in serum samples were directly measured by a γ counter (○). Mean ± SD values are shown.

published methodologies. Our method does not require expensive equipment or radioisotope labeling of the PEG standard, and more importantly, the presence of serum does not interfere with the assay, a favorable advantage over other techniques. Indeed, the merit of this assay can be fully appreciated by in vivo assay of PEG pharmacokinetics (see below).

Pharmacokinetics of PEG in Mice. The pharmacokinetic behavior of PEG is largely unknown, despite the increasing use of PEG or PEGylated small molecules (such as PEGylated SN-38) in vivo. To determine whether the αPEG cell-based competition ELISA could detect PEG in blood, BALB/c mice were intravenously injected with 5 mg of CH_3-PEG_{5K}-NH_2. Blood samples were periodically collected and the concentration of CH_3-PEG_{5K}-NH_2 in serum samples was measured by the αPEG cell-based competition ELISA. As a control, we intravenously injected CH_3-PEG_{5K}-^{131}I to mice and measured radioactivity in the blood directly by a γ counter. Figure 5 shows that the pharmacokinetics of PEG_{5K} measured by αPEG cell-based competition ELISA was similar to that obtained by direct measurement of radioactivity in the blood, with estimated terminal half-lives of 7.6 and 8 min, respectively. Radioactive iodine requires special handling and proper disposal. In addition, attachment of radioactive iodine to PEG requires specialized techniques with an adaptor such as 3-(4-hydroxyphenyl)propionic acid N-hydroxysuccinimide ester (SHPP) to the end of PEG, which could affect its metabolism in vivo. Finally, radioactive iodine may be cleaved (hydrolyzed) from PEG once in serum,[43] which may result in underestimation at late time points.

Pelham et al.[21] used HPLC/MS/MS to study the pharmacokinetics of PEG-3350 in normal subjects who orally ingested PEG. While this method improved detection of PEG significantly (to 30 ng/mL), some issues remained. First, removal of serum proteins is required prior to HPLC. In a large-scale clinical trial, the task of removing serum proteins from all of the participants' blood samples can be daunting. Second, HPLC analyzes only one sample at a time, resulting in prolonged experimental effort to complete measurement of PEG concentration in large-scale clinical trials. Third, HPLC/MS/MS is expensive and requires skillful laboratory personnel for proper operation. It may be impractical to install multiple HPLC/MS/MS stations for speeding up PEG measurements in a large-scale clinical trial. HPLC/MS/MS may be more suitable for trials involving small number of participants. On the contrary, the αPEG cell-based competition assay is fast and affordable. A 96-well plate can accommodate at least 39 samples, allowing a single person to complete hundreds of PEG measurements in just a few hours. High efficiency and low operational cost make the new assay a very attractive tool for pharmacokinetics studies of PEG and PEGylated small molecules.

CONCLUSIONS

In this report, we present a major technical breakthrough in measuring PEG concentration. We developed a novel ELISA based on competitive binding of PEG and biotinylated PEG (CH_3-PEG_{5K}-biotin) to the binding sites on cells that express αPEG receptors. This method detects PEG at concentrations nearly 1000-fold lower than previously reported techniques and is easier and less complicated than HPLC/MS/MS. Our method possesses several advantages: (1) it displays high sensitivity and high specificity for PEG and PEGylated small molecules; (2) no sophisticated instruments are required to allow affordable laboratory tests; and (3) the presence of serum does not interfere with the assay, rendering it suitable for the quantification of PEG in blood and/or other biological samples. On the basis of these benefits, we believe that the αPEG cell-based competition ELISA should provide an easy-to-use and affordable tool for measuring PEG and chemical−PEG concentration, aiding in the pharmacokinetic study of PEG and PEGylated small molecules.

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