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Development of an Anti-Methoxy Poly(ethylene glycol) (α-mPEG) Cell-Based Capture System to Measure mPEG and mPEGylated Molecules

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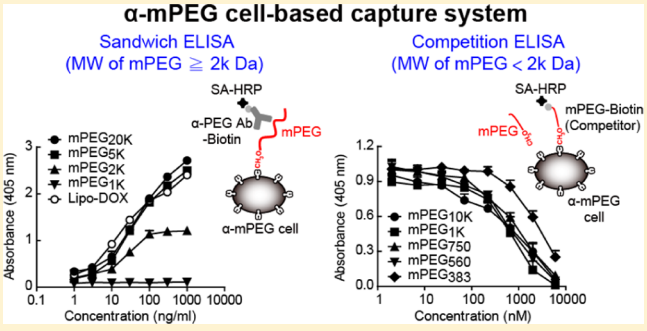
Supporting Information

ABSTRACT: Quantitative pharmacokinetic analysis of methoxy-poly(ethylene glycol) (mPEG) and mPEGylated molecules is important for clinical drug development. Here we developed sensitive sandwich and competitive ELISAs by expressing an anti-mPEG antibody on the surface of fibroblasts for effective capture of mPEG molecules in biological samples. α-mPEG sandwich ELISA could quantify the higher-molecular-weight of mPEG (2, 5, and 20 kDa) and mPEGylated molecules. α-mPEG cell-based competitive ELISA was developed to measure the lower-molecular-weight of mPEG molecules (559, 750, and 1000 Da) at nanomolar levels. In addition, α-mPEG cell-based ELISA was unaffected by the presence of 10% human serum or murine serum. We further demonstrate that the α-mPEG cell-based ELISA determined similar pharmacokinetics of $mPEG_{5K}$ as traditional gamma counting of ^{131}I-$mPEG_{5K}$. The α-mPEG cell-based ELISA may provide an accurate, high sensitivity and easy-to-use tool for directly measuring mPEG and mPEGylated molecules in complex biological samples to accelerate the clinical development of mPEG drugs.



INTRODUCTION

PEGylation is a FDA-approved technology for enhancing the bioavailability, safety, stability, and efficacy of a wide range of small molecules, proteins, liposomes, or nanoparticles.[1,2] The monomethoxylated form of PEG (mPEG) is widely used for clinical drug modification[3,4] because mPEG modification can reduce nonspecific binding and prevent recognition by the reticuloendothelial system (RES) *in vivo*.[5,6] Hence, a wide range

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of mPEGylated molecules are under development or in clinical use.[7,8] For example, mPEG has been attached to small chemical molecules such as 10-amino-7-ethyl camptothecin (CPT analogue),[9] amphotericin B (AMB),[10] silybin[11] and zidovudine (AZT)[12] to increase water solubility, reduce systemic toxicity, and improve the therapeutic index. mPEG-modified liposomal doxorubicin (Lipo-DOX) has been approved by the FDA for the clinical treatment of breast and ovarian carcinomas and Kaposi's sarcoma[13,14] and mPEG-derivatized micelles (Genexol-PM) are currently undergoing phase I/II clinical trials.[15] mPEG-proteins, mPEG-gold nanoshell,[16] mPEG-superparamagnetic iron oxide,[17] mPEG-microbubbles,[18,19] mPEG-solid lipid nanoparticles (SLN)[20] and mPEG-modified quantum dots[21] also display improved biocompatibility and reduced receptor-mediated uptake by the RES.[22] Development of an accurate method to quantify mPEG molecules and mPEGylated nanoparticles, proteins, and drugs in complex biological samples is important for drug development and clinical trials.

Current approaches for studying the pharmacokinetics of mPEG and mPEGylated molecules in animals and patients have room for improvement. Radiolabeling of mPEG offers high sensitivity and specificity but safety concerns and special operating requirements makes this approach inconvenient. In addition, because mPEG is difficult to label directly, it is necessary to use other functional groups at the end of mPEG molecules for radiolabeling.[23] Thus, radiolabeled mPEG indirectly measures the pharmacokinetics of mPEG. Radioactivity can also be released from compounds (ie., deiodination) in living bodies, resulting in inaccurate measurement of mPEGylated conjugates *in vivo*.[23] High performance liquid chromatography (HPLC), especially HPLC/tandem mass spectrometry (HPLC/MS/MS), can separate and quantify PEG in a mixture of compounds at low ng/mL concentrations.[24] However, the requirements of multiple processing steps to reduce sample complexity before analysis and sophisticated equipment can limit its routine use. In particular, detection methods may be restricted by protein contamination.[25] We previously described a sandwich ELISA system using the pairing of monoclonal anti-PEG antibodies (first generation, AGP3/IgM and E11/IgG; second generation, AGP4/IgM and 3.3/IgG) to measure PEGylated molecules *in vitro* and *in vivo*.[26,27] However, the anti-PEG antibody-based sandwich ELISA cannot measure most mPEG molecules (<20k Da) or mPEGylated drugs. The limitations of the currently available systems reveal a need for an accurate and convenient method for pharmacokinetic studies of mPEG and mPEGylated molecules for both experimental laboratories and pharmaceutical factories.

In this study, we report a novel method to measure mPEG and mPEGylated molecules by expressing an anti-mPEG antibody (α-mPEG Ab; 15−2b) on the surface of fibroblasts for effective capture of mPEG molecules. A mPEG-quantitative sandwich ELISA was created by pairing α-mPEG cells for capture with a biotin-conjugated anti-PEG antibody (AGP4-biotin) for detection. In addition, we developed a competition ELISA using $mPEG_{5K}$-biotin to compete binding of mPEG to α-mPEG Ab on α-mPEG cells (Figure 1). We investigated the sensitivity of α-mPEG cell-based ELISA to measure various mPEG and mPEGylated molecules. Serum interference of the α-mPEG cell-based ELISA was also assessed. Finally, we use the α-mPEG cell-based ELISA to study the pharmacokinetics of mPEG in mice and compared the results with those obtained by measuring radioactivity in blood after intravenous injection of ^{131}I-$mPEG_{5K}$ to mice. Our results demonstrate that the α-mPEG cell-based ELISA may provide a potent tool for the pharmacokinetic study of mPEG and mPEGylate molecules.

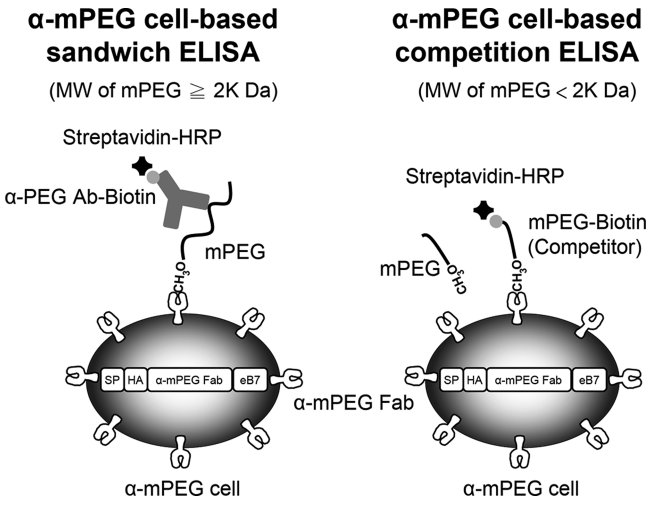

Figure 1. α-mPEG cell-based ELISA systems. Schematic representation of the α-mPEG cell. The α-mPEG cell is derived from the BALB/c 3T3 cell stably expressing α-mPEG receptor on the cell membrane. The receptor gene includes, from N to C terminus, an immunoglobulin signal peptide (SP), a HA epitope, the α-mPEG Fab fragment, and immunoglobulin C2-type extracellular-transmembrane-cytosolic domain of the murine B7-1 antigen (eB7). The sandwich ELISA is generated by α-mPEG cell and the detective anti-PEG antibody, which can estimate the higher-molecular-weight (≥2k Da) mPEG. The α-mPEG cell-based competition ELISA can detect the lower-molecular-weight (<2k Da) mPEG molecules.

EXPERIMENTAL METHODS

Reagents and Animals. BALB/3T3 mouse fibroblast cells (American Type Culture Collection, Manassas, VA) and GP2−293 retrovirus packaging cells (Clontech, Mountain View, CA) were cultured in Dulbecco's modified Eagle's medium (DMEM ; Sigma-Aldrich, St Louis, MO) containing 10% (v/v) heat-inactivated bovine calf serum (BCS; Sigma-Aldrich) and 100 units ml^{-1} penicillin and streptomycin (Invitrogen, Calsbad, CA), at 37 °C in a humidified atmosphere of 5% (v/v) CO_2. 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. $mPEG_{5K}$-biotin, $mPEG_{5K}$-FITC, $mPEG_{5K}$-NIR797, $mPEG_{5K}$-SHPP, and $mPEG_{5K}$-SHPP-^{131}I were synthesized as previously described.[28]

Characterization of the Anti-mPEG Antibody. Maxisorp 96-well microplates (Nalge- Nunc International, Roskilde, Denmark) were coated with 0.5 μg $well^{-1}$ of $mPEG_{2K}$-NH_2, OH-PEG_{3K}-NH_2, NH_2-PEG_{3K}-NH_2, $mPEG_{750}$-NH_2, $mPEG_{560}$-NH_2, and $mPEG_{383}$-NH_2 in 50 μL $well^{-1}$ of 0.1 M $NaHCO_3$ (pH 9.0) for 2 h at 37 °C and then blocked with 200 μL $well^{-1}$ of dilution buffer (5% (wt/vol) skim milk in PBS) overnight at 4 °C. A 20 μg mL^{-1} sample of anti-PEG antibody (AGP4) or the graded concentrations of anti-mPEG antibody (α-mPEG Ab; 15−2b) in 50 μL 2% (wt/vol) skim milk were added to the plates for 1 h at RT. The plates were washed with PBST (PBS containing 0.05% (v/v) Tween-20) three times and with PBS once. HRP conjugated goat anti mouse IgM μ chain (2 μg mL^{-1}) or HRP conjugated goat anti mouse IgG Fc (2 μg mL^{-1}) in 50 μL dilution buffer were added for 1 h at room temperature. The plates were washed with PBS and bound peroxidase activity was measured by adding 150 μL $well^{-1}$ ABTS solution [0.4 mg mL^{-1}, 2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich), 0.003% (v/v) H_2O_2, and 100 mM phosphate-citrate, pH 4.0] for 30 min at room

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temperature. Color development was measured at 405 nm on a microplate reader (Molecular Device, Menlo Park, CA).

Plasmid Construction. The V_L-C_κ and V_H-C_{H1} domains of the 15−2b anti-mPEG antibody were cloned from cDNA prepared from the 15−2b hybridoma following a previously described method.[29] Primers used in the cloning of V_L-C_κ and V_H-C_{H1} were as follows: V_L-C_κ sense, 5′-tgctggggcccagccggccgatattgtgatgacccag-3′; V_L-C_κ antisense, 5′-tgtttgttttactggtgctc gttttgctcgctcgagacactcattcctgtt-3′; V_H-C_{H1} sense, 5′-gaagatctgaggttaagctggaggag-3′; and V_H-C_{H1} antisense, 5′-tagtcaggtcgacaagtttttttgtccaccgtgg-3′. The V_L-C_κ and V_H-C_{H1} genes, joined by a composite furin-2A protease cleavage site,[30] were cloned into the pLNCX-eB7 retroviral vector[29] by using *Sfi*I and *Sal*I restriction sites. The expression vector, pLNCX-α-mPEG-eB7, encodes the 15−2b α-mPEG Fab fused to the immunoglobulin C2-type extracellular-transmembrane-cytosolic domains of the mouse B7-1 antigen (Figure 1). A plasmid (pLNCX-α-DNS-eB7) that encodes a membrane Fab with specificity for 5-(dimethylamino) naphthalene-1-sulfonyl chloride (DNS) was constructed in an analogous fashion to act as a negative control.[28]

Generation of α-mPEG Expressing Cells by Retroviral Transduction. To produce pseudotyped retroviruses, pLNCX-α-mPEG-eB7 or pLNCX-α-DNS-eB7 were 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^{-1} of 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 α-mPEG and α-DNS cells that stably expressed approximately equal levels of α-mPEG or α-DNS antibodies on their surface.

Fluorescence-Activated Cell Sorting Analysis of the α-mPEG Expressing Cells. Surface expression of the α-mPEG Fab and α-DNS Fab were measured by staining cells with 1 μg mL^{-1} mouse antihemagglutinin (anti-HA) antibody, followed by 1 μg mL^{-1} of fluorescein isothiocyanate- (FITC−) conjugated goat antimouse IgG (Fc) (Jackson Immunoresearch Laboratories, Westgrove, PA) in PBS containing 0.05% (wt/vol) bovine serum albumin (BSA) on ice. The mPEG binding activities of the α-mPEG Fab and α-DNS Fab were determined by incubating cells with 10 nmol L^{-1} $mPEG_{5K}$-FITC in PBS containing 0.05% (wt/vol) BSA on ice. After removal of unbound antibodies or $mPEG_{5K}$-FITC by extensive wash in cold PBS containing 0.05% (wt/vol) BSA, the surface fluorescence of viable cells was measured on a FACScan flow cytometer (BD Biosciences) and fluorescence intensities were analyzed with FlowJo7.6.1 software (Tree Star, Inc., San Carlos, CA).

Confocal Analysis of α-mPEG Cells. The α-mPEG or α-DNS cells were fixed with 1% (wt/vol) paraformaldehyde and were then washed with TBS containing 3% (wt/vol) BSA (Sigma-Aldrich). These cells were sequentially incubated with 1 μg mL^{-1} of mouse antihemagglutinin (anti-HA) antibody, 2 μg mL^{-1} of PE conjugated goat antimouse IgG Fc (Jackson Immunoresearch Laboratories), 10 nmol L^{-1} of $mPEG_{5K}$-FITC and DAPI. The cells were washed with TBS, mounted with fluorescence mounting medium (DakoCytomation), and viewed under a digital fluorescence confocal microscope (LSM 510 META NLO DuoScan, Carl Zeiss).

α-mPEG Antibody-Based, α-mPEG Cell-Based Sandwich ELISA and Commercial ELISA Kit. In all ELISA experiments (sandwich or competition ELISA), PBS containing 2% (wt/vol) skim milk was used as the sample dilution buffer and PBS was used as the wash buffer. The 15−2b α-mPEG and α-DNS cells (2×10^5 cells $well^{-1}$) were seeded overnight in 96-well plates (Nalge Nunc International, Roskilde, Denmark) in culture medium. After extensive washing, the cells were fixed with 1% (wt/vol) paraformaldehyde for 3 min. The plates were blocked with 5% (wt/vol) skim milk in PBS for 2 h at 37 °C. Graded concentrations of $mPEG_{1K}$-NH_2, $mPEG_{2K}$-NH_2, $mPEG_{5K}$-NH_2, $mPEG_{20K}$-NH_2 (Sigma-Aldrich), $mPEG_{5K}$-SHPP, $mPEG_{5K}$-FITC, $mPEG_{5K}$-NIR797, Lipo-Dox (TTY Biopharm Company Ltd.), PEG-Intron (Schering-Plough, Kenilworth, NJ, U.S.A.) or Pegasys (Roche, Nutley, NJ) were added to the wells (50 μL $well^{-1}$) at room temperature for 1 h. After washing, the cells were sequentially incubated with biotinylated AGP4 (0.25 μg $well^{-1}$) and streptavidin-conjugated horseradish peroxidase (streptavidin-HRP, 50 ng $well^{-1}$). The plates were washed with PBS and bound peroxidase activity was measured by adding 150 μL $well^{-1}$ ABTS solution [0.4 mg mL^{-1}, 2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich), 0.003% (v/v) H_2O_2, and 100 mM phosphate-citrate, pH 4.0] for 30 min at room temperature. For the antibody-based sandwich ELISA, unmodified 15−2b antibody (1 μg $well^{-1}$) in coating buffer (0.1 M $NaHCO_3$, pH = 9) was added to Maxisorp 96-well microplates (Nalge Nunc International) for 2 h at 37 °C. The plates were blocked with 5% (wt/vol) skim milk in PBS overnight at 4 °C. Standard sandwich ELISA was then performed by sequential addition of Lipo-Dox or mPEG, biotinylated AGP4, streptavidin-HRP, and ABTS. Color development was measured at 405 nm on a microplate reader. For the commercial mPEG ELISA kit (Cat. No. MP-0001; Life Diagnostics, Inc., PA, U.S.A.), α-mPEG Ab-conjugated HRP (100 μL $well^{-1}$) was first added to the α-PEG backbone Ab coated plate, and then the graded concentrations of $mPEG_{1K}$-NH_2, $mPEG_{2K}$-NH_2, $mPEG_{5K}$-NH_2, $mPEG_{20K}$-NH_2, or Lipo-Dox were dispensed into each well (100 μL $well^{-1}$) at room temperature for 1 h. After washing, TMB reagent was added and gently mixed into the wells for 20 min. Stop solution was added to the plates to stop the reaction, and the color development was measured at 450 nm within 5 min.

α-mPEG Cell-Based Competition ELISA. The α-mPEG cells (2×10^5 cells $well^{-1}$) were prepared in 96-well plates as above. $mPEG_{383}$-NH_2, $mPEG_{560}$-NH_2, $mPEG_{750}$-NH_2, $mPEG_{1K}$-NH_2, or $mPEG_{10K}$-NH_2 were 2-fold serially diluted and mixed 1:1 (v/v) with 250 ng mL^{-1} $mPEG_{5K}$-biotin (thus the final concentration of $mPEG_{5K}$-biotin was 125 ng mL^{-1}), and then the mixture was added to microtiter plate wells coated with α-mPEG cells at room temperature for 1 h. After extensive washing, the cells were sequentially incubated with streptavidin-HRP and ABTS. Color development was measured at 405 nm on a microplate reader.

ELISA Data Analysis. 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 controls and samples (mPEG and mPEGylated molecules). Data were considered significant at $p \leq 0.05$.

Assessment of the Serum Interference in the α-mPEG Cell-Based Sandwich ELISA. PBS, PBS containing 2% (wt/vol) skim milk or PBS containing 2.5% (v/v) or 10% (v/v) mouse or human serum were used as diluents for $mPEG_{5K}$-NH_2. The 15−2b α-mPEG cell-based sandwich ELISA was then performed as described above; PBS was used as the wash buffer and PBS containing 2% (wt/vol) skim milk was used as diluents for secondary and tertiary reagents.

Pharmacokinetics of mPEG in Mice. $mPEG_{5K}$-SHPP was intravenously injected into female BALB/c mice (5 mg $mouse^{-1}$, n=8). Blood was withdrawn at different times by use of heparinized capillary tubes. Plasma was 50- and 200-fold diluted, and the α-mPEG cell-based sandwich or competition ELISA was then performed as described above. Concentrations of $mPEG_{5K}$-SHPP in serum were deduced by fitting optical density values to the standard curve obtained from serially diluted $mPEG_{5K}$-SHPP. The serum half-life of $mPEG_{5K}$-SHPP was estimated by fitting the data to a two-phase exponential decay model with Prism 4 software (GraphPad Software, San Diego, CA).

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

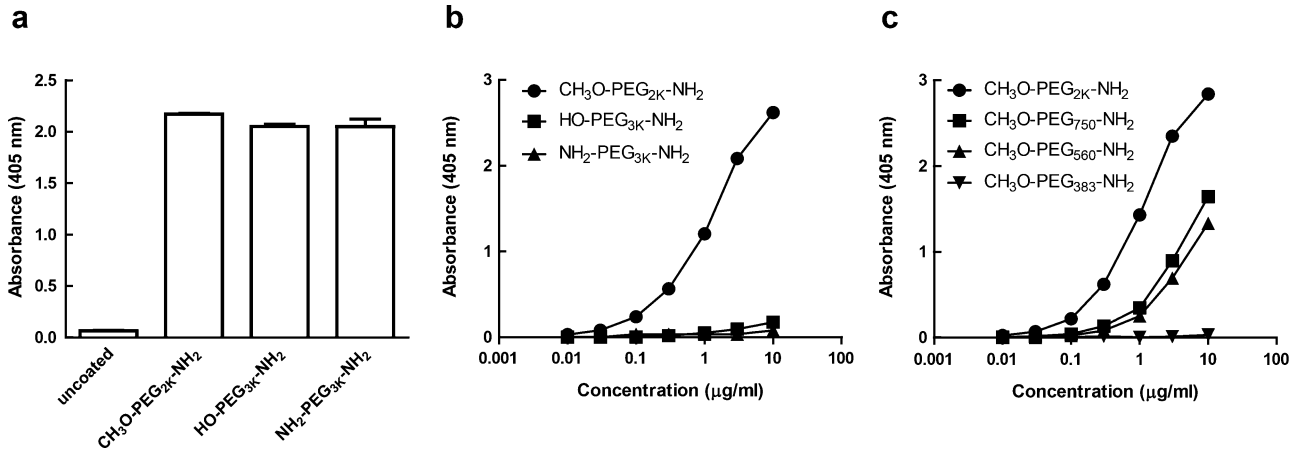

Figure 2. Characterization of the anti-mPEG antibody. (a) Coating of PEG molecules in microtiter plates was confirmed by direct ELISA with the anti-PEG antibody AGP4. Bars, SD. Graded concentrations of anti-mPEG antibody (α-mPEG Ab; 15−2b) were added to microtiter plate wells coated with (b) PEG molecules which contain different terminal functional groups, including $mPEG_{2K}$-NH_2 (●), OH-PEG_{3K}-NH_2 (■), and NH_2-PEG_{3K}-NH_2 (▲), or (c) mPEG molecules with different molecular weight, including $mPEG_{2K}$-NH_2 (●), $mPEG_{750}$-NH_2 (■), $mPEG_{560}$-NH_2 (▲), and $mPEG_{383}$-NH_2 (▼). Binding of 15−2b was determined by measuring absorbance at 405 nm after staining with goat antimouse IgG Fc-HRP and ABTS. The means absorbance values of triplicate determinations are shown. Bars, SD.

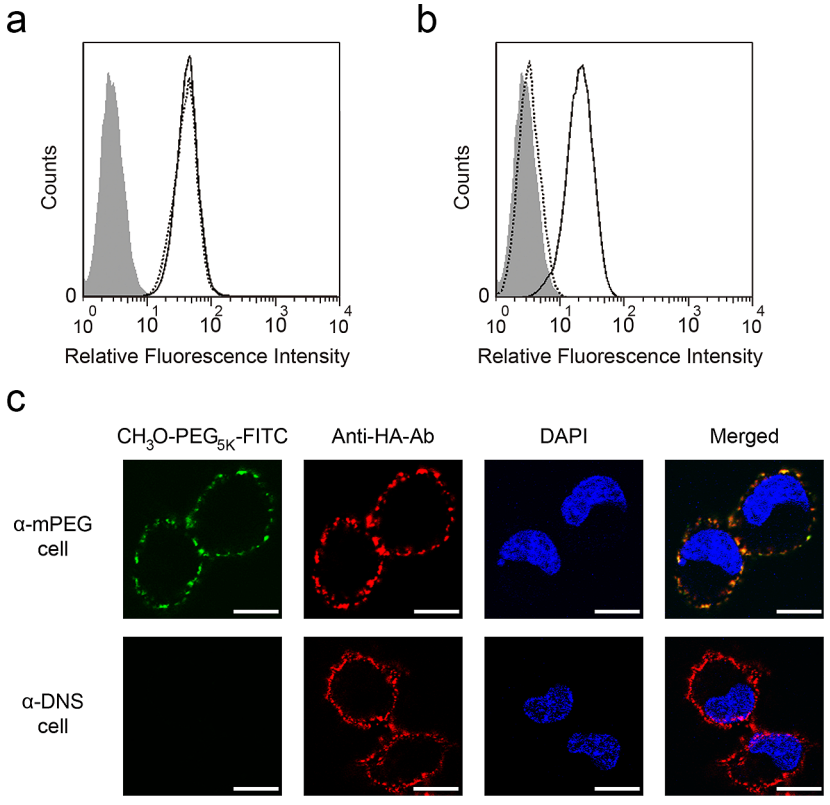

Figure 3. Surface display of functional α-mPEG Fab. α-mPEG cells (solid lines) and α-DNS cells (dashed lines) were analyzed by flow cytometry using (a) a specific antibody to the HA epitope to assess surface expression or (b) staining with $mPEG_{5K}$-FITC to assess the PEG-binding activity of the α-mPEG Fab and α-DNS cells. Shaded areas of graphs show mock staining with PBS containing 0.05% (wt/vol) BSA. (c) α-mPEG cells (top row) and α-DNS cells (bottom row) were stained with α-HA antibody (red fluorescence) and $mPEG_{5K}$-FITC (green fluorescence). DAPI was used to confirm the location of nuclei. Cells were observed with a digital confocal microscope. Merged images are shown. Scale bars in this figure correspond to 10 μm.

RESULTS

Characterization of Anti-mPEG Antibodies. To investigate the binding specificity of the anti-mPEG monoclonal antibody (α-mPEG Ab; 15−2b), PEG molecules with different terminal functional groups ($mPEG_{2K}$-NH_2, OH-PEG_{3K}-NH_2, and NH_2-PEG_{3K}-NH_2) were coated in 96-wells plates and then 15−2b binding to the coated wells was determined by direct ELISA. Figure 2a shows that similar amounts of $mPEG_{2K}$-NH_2, OH-PEG_{3K}-NH_2 and NH_2-PEG_{3K}-NH_2 were coated in the 96-well plates as determined by direct ELISA using an anti-PEG antibody (AGP4) which can specifically bind to the PEG backbone.[26] 15−2b selectively bound to $mPEG_{2K}$-NH_2 but not

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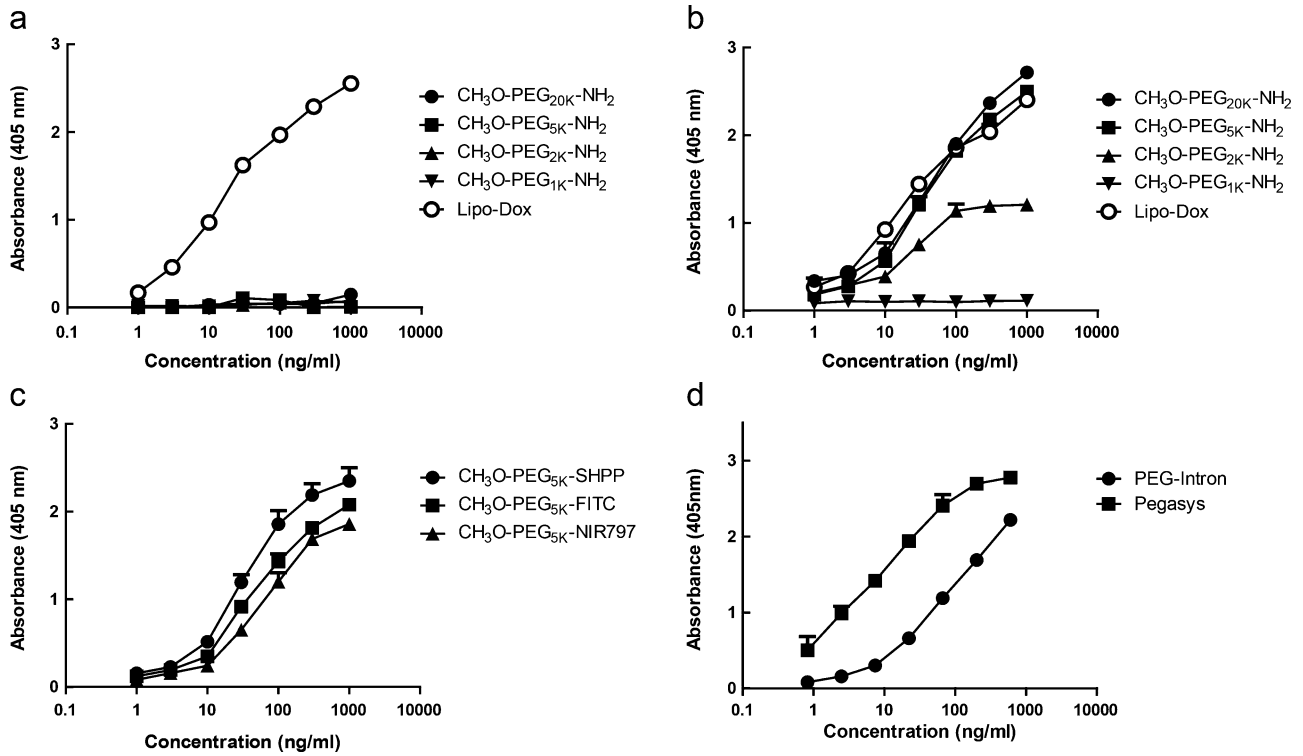

Figure 4. Detection of mPEG or mPEGylated molecules by α-mPEG antibody-based or α-mPEG cell-based sandwich ELISA. Sandwich ELISA with (a) α-mPEG Ab (15−2b), or (b) α-mPEG cells as the capture reagents and AGP4-biotin as the detection antibody was used to measure the concentration of $mPEG_{20K}$-NH_2 (●), $mPEG_{5K}$-NH_2 (■), $mPEG_{2K}$-NH_2 (▲), $mPEG_{1K}$-NH_2 (▼), and Lipo-Dox (○). (c) Graded concentrations of $mPEG_{5K}$-SHPP (●), $mPEG_{5K}$-FITC (■) and $mPEG_{5K}$-NIR797 (▲) were measured by α-mPEG cell-based sandwich ELISA. (d) Graded concentrations of PEG-Intron (●) and Pegasys (■) were measured by α-mPEG cell-based sandwich ELISA. The means absorbance values (405 nm) of triplicate determinations are shown. Bars, SD.

OH-PEG_{3K}-NH_2 and NH_2-PEG_{3K}-NH_2 (Figure 2b), indicating that 15−2b binds to the methoxy terminus of mPEG. To examine the binding epitope of 15−2b, different molecular weights of mPEG-NH_2 (MW 2k, 750, 560, and 383 Da) were coating in 96 well plates and then 15−2b binding was determined by direct ELISA. Figure 2c shows that 15−2b bound $mPEG_{2K}$-NH_2, $mPEG_{750}$-NH_2, and $mPEG_{560}$-NH_2 but not $mPEG_{383}$-NH_2, suggesting that the binding epitope of 15−2b consists of the terminal methoxy group and at least 12 additional OCH_2CH_2 subunits (the number of subunits in PEG_{560}).

Surface Display of Functional Anti-mPEG Antibodies on Fibroblasts. We constructed a retroviral vector, pLNCX-α-mPEG-eB7, to directly express and anchor the Fab fragment of 15−2b (α-mPEG Ab) on cells. The α-mPEG and control α-DNS Fab fragments were constructed by joining the light chain (V_L-C_K) through a furin cleavage site and 2A peptide[30] to the heavy chain (V_H-CH_1) domain followed by the C-like extracellular, transmembrane and cytosolic domains of the mouse B7-1 antigen (eB7). Mouse BALB/c 3T3 fibroblasts were infected with recombinant retrovirus and selected in G418 to obtain α-mPEG or control α-DNS cells. The expression and function of the α-mPEG Fab on α-mPEG cells were confirmed by flow cytometry and confocal microscopy after staining the cells with anti-HA antibodies to detect the HA epitope tag on the Fab fragments and $mPEG_{5K}$-FITC to detect functional antibody binding. Figure 3a shows that α-mPEG and control α-DNS Fab fragments were expressed at similar levels on α-mPEG and α-DNS cells, respectively, but that only α-mPEG cells specifically bound $mPEG_{5K}$-FITC (Figure 3, parts b and c), indicating that surface displayed α-mPEG Fab maintained mPEG binding activity.

Quantification of mPEG and mPEGylated Molecules by α-mPEG Cell-Based or Antibody-Based Sandwich ELISA. The sensitivities of α-mPEG cell-based sandwich ELISA, α-mPEG Ab-based sandwich ELISA, and a commercial mPEG ELISA kit (Life Diagnostics, Inc.) were compared for measuring mPEG and mPEGylated molecules by adding mPEG (1, 2, 5, and 20 kDa) or Lipo-Dox (liposomal doxorubicin conjugated with $mPEG_{2K}$) to 96-well plates coated with α-mPEG cells, α-mPEG Ab or α-PEG backbone Ab (commercial kit), respectively. Captured mPEG molecules were then quantified by sequential addition of biotinylated AGP4 antibody, streptavidin-conjugated horseradish peroxidase (streptavidin-HRP) and ABTS substrate. For the commercial mPEG ELISA kit, captured mPEG molecules were quantified by sequential addition of α-mPEG Ab-conjugated HRP and TMB reagent. Parts a and b of Figure 4 show that the α-mPEG cell-based and α-mPEG Ab-based sandwich ELISA possessed similar detection sensitivities for Lipo-DOX (mPEGylate nanoparticles). By contrast, the α-mPEG cell-based sandwich ELISA could detect mPEG molecules larger than 2000 Da at concentrations as low as 1 ng mL^{-1} whereas the α-mPEG Ab sandwich ELISA could not detect any mPEG molecule. The commercial mPEG ELISA kit could detect mPEG (1, 2, 5, and 20 kDa) and Lipo-DOX (Figure S1, Supporting Information). However, the detection sensitivity of the commercial kit was much lower than that of the α-mPEG cell-based sandwich ELISA. In addition, the α-mPEG cell-based sandwich ELISA could also detect $mPEG_{5K}$-SHPP, $mPEG_{5K}$-FITC, and

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$mPEG_{5K}$-NIR797 at concentrations as low as 1 ng mL^{-1} (Figure 4c). And mPEGylated proteins such as PEG-Intron ($mPEG_{12K}$-interferon α-2b) and Pegasys ($mPEG_{40K}$-interferon α-2a) also can be detected by α-mPEG cell-based sandwich ELISA. The detection sensitivity increased as the length of the mPEG chain on the proteins increased (Figure 4d). These results indicate that the α-mPEG cell-based sandwich ELISA is a highly sensitive method for measuring mPEGylated molecules (nanoparticles, small molecules and proteins) and mPEG molecules larger than 2000 Da. Importantly, conjugation of diverse compounds to mPEG did not interfere in the α-mPEG cell-based sandwich ELISA.

Measurement of Short mPEG Molecules by α-mPEG Cell-Based Competition ELISA. The α-mPEG cell-based sandwich ELISA performed relatively poorly for mPEG molecules smaller than about 2000 Da. We therefore developed a α-mPEG cell-based competition ELISA that facilitated detection of mPEG molecules smaller than 2000 Da. Graded concentrations of mPEG (10k, 1k, 750, 560, and 383 Da) were mixed with a fixed amount of $mPEG_{5K}$-biotin (mPEG competitor) prior to addition to wells coated with α-mPEG cells. After extensive washing, bound mPEG competitor was detected with adding streptavidin-HRP and substrate. As shown in Figure 5, concentrations of $mPEG_{10K}$-NH_2, $mPEG_{1K}$-NH_2, $mPEG_{750}$-NH_2, and $mPEG_{560}$-NH_2 as low as 70 nM effectively competed with $mPEG_{5K}$-biotin for binding to α-mPEG cells, indicating that the α-mPEG cell-based competition ELISA is suitable for measuring mPEG molecules as small as 560 Da. In addition, the competitive efficacy of mPEG (560 to 10k) with $mPEG_{5K}$-biotin for the binding site of α-mPEG receptors depends on the number of the terminal methoxy group but not the molecular weight as well as the length of PEG backbone.

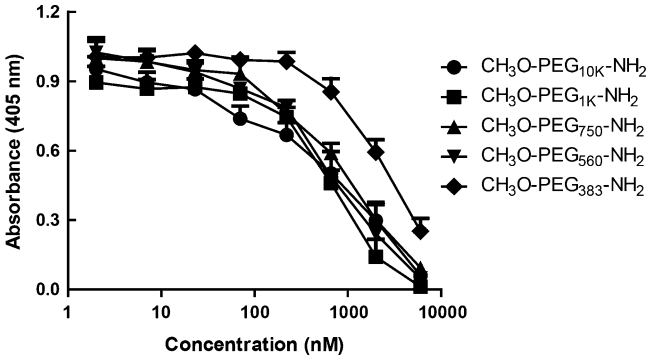

Figure 5. Detection of mPEG by α-mPEG cell-based competition ELISA. Graded concentrations of $mPEG_{10K}$-NH_2 (●), $mPEG_{1K}$-NH2 (■), $mPEG_{750}$-NH_2 (▲) and $mPEG_{560}$-NH_2 (▼), and $mPEG_{383}$-NH_2 (♦) were measured by α-mPEG cell-based competition ELISA. The mean absorbance values (405 nm) of triplicate determinations are shown. Bars, SD.

Effect of Serum on the Sensitivity of α-mPEG Cell-Based ELISA. The presence of serum on the sensitivity of the α-mPEG cell-based ELISA was examined by detecting $mPEG_{5K}$ in the presence of 2.5% (v/v) and 10% (v/v) human or mouse serum. Inclusion of human (Figure 6a) or mouse (Figure 6b) serum produced similar assay sensitivities as inclusion of 2% (wt/vol) skim milk. By contrast, assay of mPEG in PBS alone substantially reduced detection sensitivity. A similar phenomenon was also observed in the α-mPEG Ab-based ELISA for measuring Pegasys (Figure S2, Supporting Information). We conclude that inclusion of a carrier protein or serum enhances detection sensitivity, indicating that the assay is suitable for the pharmacokinetic study of mPEG and mPEG-like molecules.

Comparison of Radiolabeling and α-mPEG Cell-Based ELISA for Determination of mPEG Pharmacokinetics in Mice. The α-mPEG cell-based sandwich ELISA and cell-based competitive ELISA were compared with the direct radiolabeling method to measure the pharmacokinetics of a mPEG molecule *in vivo*. mPEG in blood samples periodically collected from BALB/c mice after intravenous administration of 5 mg $mPEG_{5K}$-SHPP or $mPEG_{5K}$-SHPP-^{131}I were measured by the α-mPEG cell-based sandwich or cell-based competition ELISA or by directly measuring radioactivity, respectively. The pharmacokinetics of $mPEG_{5K}$-SHPP measured by the α-mPEG cell-based sandwich ELISA and cell-based competition ELISA were similar but displayed slightly slower elimination as compared to direct measurement of radiolabeled mPEG, with initial and terminal half-lives of 7.6 and 220.9 min, 7.5 and 218.5 min, and 6.9 and 156.5 min, respectively (Figure 7).

■ DISCUSSION

We have successfully developed a α-mPEG cell-based sandwich ELISA and a α-mPEG cell-based competition ELISA to quantify mPEG and mPEGylated molecules in complex biological samples for pharmacokinetic studies. The sandwich ELISA, which employs α-mPEG cells for capture and an anti-PEG backbone antibody for detection, is suitable for measurement of higher-molecular-weight (≥2k Da) mPEG molecules. The α-mPEG cell-based competition ELISA can detect lower-molecular-weight (<2k Da) mPEG molecules at nanomolar levels. Importantly, the presence of mouse or human serum did not affect the sensitivity of the α-mPEG cell-based ELISA. In addition, the α-mPEG cell-based ELISA could be used to determine the pharmacokinetics of a mPEG molecule in mice without the need for radiolabeling. Our study suggests that the α-mPEG cell-based ELISA and competitive ELISA are useful tools to directly quantify mPEG and mPEGylated molecules for biological and pharmacokinetic studies.

Many small molecular, peptide, protein and nanoparticle drugs are modified with mPEG, an established and FDA-approved method to improve the pharmacokinetic and biodistribution of therapeutic agents. A general method to directly quantify mPEG or mPEGylated molecules would be very useful for academic, biotechnology and pharmaceutical applications. However, quantification of mPEGylated drugs has traditionally relied on diverse methods that assay for the attached drug molecules. For example, the pharmacokinetics of Caelyx (mPEGylated liposomal doxorubicin), paclitaxel-loaded mPEG−PLA NPs (Methoxypoly(ethylene glycol)−polylactide nanoparticles) or mPEG-zidovudine (AZT) were determined by measuring the concentration of doxorubicin, paclitaxel or AZT, respectively.[31−34] Likewise, ELISA measurement of mPEGylated molecules typically requires the development of specific antibodies for each assay system and is usually limited to PEGylated proteins and peptides, but not mPEG and mPEGylated small molecules.[23] Covalent attachment of mPEG to proteins and peptides can also mask antibody-binding epitopes, thereby decreasing assay sensitivity.[35,36] In the current study, we demonstrated that α-mPEG cell-based ELISAs can sensitively quantify mPEG and mPEGylated molecules by directly binding to mPEG. Modification of compounds with mPEG does not interfere with detection because α-mPEG cells can bind to the terminal ends of mPEG. Moreover, specifically

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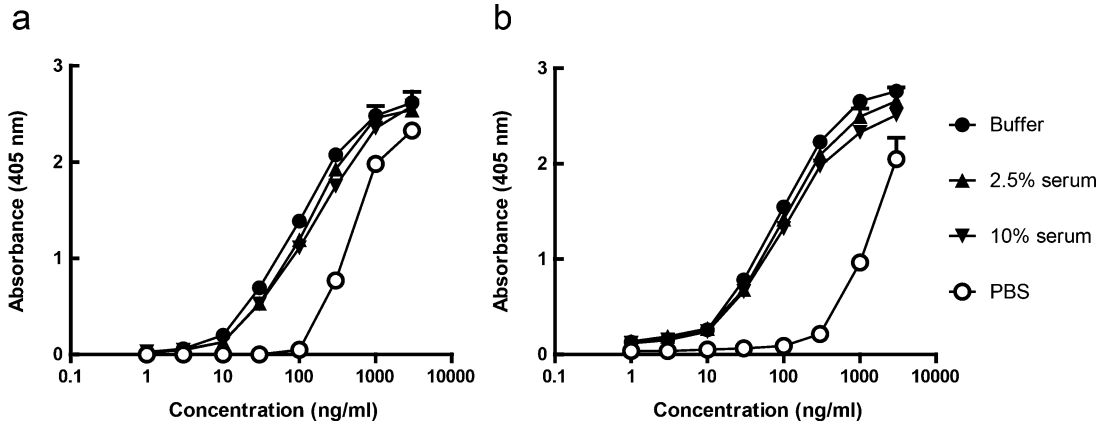

Figure 6. Effect of serum proteins in the α-mPEG cell-based sandwich ELISA. Sandwich ELISA in which α-mPEG cells/AGP4-biotin were employed as the capture/detection reagents to measure $mPEG_{5K}$-NH_2 in the presence of PBS (○), PBS containing 2% (wt/vol) skim milk (●) or PBS containing 2.5% (v/v) serum (▲), or 10% (v/v) serum (▼), respectively. Human serum (a) and mouse serum (b) were investigated. The means absorbance values (405 nm) of triplicate determinations are shown. Bars, SD.

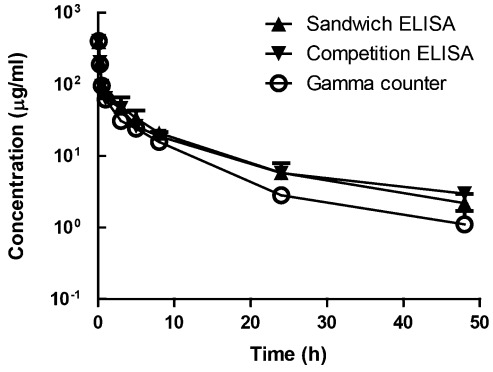

Figure 7. Pharmacokinetics of mPEG. BALB/c mice (n = 5) were intravenously injected with 5 mg $mPEG_{5K}$-SHPP or 740 μBq of $mPEG_{5K}$-SHPP-^{131}I containing 5 mg of $mPEG_{5K}$-SHPP. The concentration of $mPEG_{5K}$-SHPP in serum samples was measured by the α-mPEG cell-based sandwich ELISA (▲) or cell-based competition ELISA (▼). The radioactivity of $mPEG_{5K}$-SHPP−^{131}I in serum samples was directly measured by a gamma counter (○). Bars, SD.

recognizing the CH_3O− end of mPEG is also considered as a key point of the α-mPEG capture to increase mPEG detection efficacy. Because the epitopes of capture Ab (anti-mPEG) and detection Ab (anti-PEG backbond) are different, they will not block the binding site to each other. Indeed, we found that the detection sensitivity of α-mPEG cell-based ELISA increased with the length of mPEG. On the basis of these benefits, α-mPEG cell-based ELISAs appear to represent universal and convenient tools for quantification of mPEG and mPEGylated molecules directly and for pharmacokinetic studies.

An important feature of the α-mPEG cell-based ELISA is maintenance of sensitivity and accuracy in the presence of serum, which is critical for pharmacokinetic studies of mPEG and mPEGylated molecules. Although HPLC is a widely used technique for the measurement of mPEG, mPEGylated small drugs, and mPEGylated micelles and liposomes, proteins must first be removed from clinical samples to reduce the sample complexity. Pretreatment steps can cause unreproducible loss of mPEG, resulting in systemic errors that are difficult to estimate accurately.[23] Radiolabeled mPEG can be used in pharmacokinetic studies without sample cleanup, but hydrolysis of the radiolabel by enzymes in serum or organs, such as deiodinase, can affect assay accuracy.[37] Endogenous proteins or clotting factors in serum can also affect the accuracy of traditional antiprotein ELISA.[38−40] Interestingly, we found that serum did not interfere with the detection sensitivity of α-mPEG cell-based ELISA. Rather, the addition of either a specific protein such as skim milk or a mixture of proteins naturally found in serum drastically enhanced the sensitivity of the assay for mPEG and mPEGylated molecules (Figure 6). It is possible that serum proteins can stabilize α-mPEG antibodies on 3T3 fibroblasts, as BSA is commonly used as a stabilizer for antibodies and restriction enzymes.[41] Determination of mPEG pharmacokinetics in mice by α-mPEG cell-based ELISA or radioisotope labeling produced similar results, further verifying that the α-mPEG capture-based ELISA is unaffected by serum and is suitable to quantify the pharmacokinetics of mPEG and mPEGylated molecules in biological samples.

An interesting finding of our study was that the α-mPEG cell-based ELISA was orders of magnitude more sensitive for the low molecular weight of mPEG than a traditional ELISA using α-mPEG Ab-coated plates (Figure 4). Previous studies have demonstrated that the sensitivity of ELISA can be enhanced by increasing the antibody-coated surface area. For example, the detection sensitivity of antibody-coated gold nanoparticles was 10 times higher than the same antibody coated in microtiter plate wells.[42] Moreover, Kumada and colleagues found that organizing the displaying direction of capture antibodies can also enhance ELISA sensitivity. They coated polystyrene-binding peptide (PS-tag)-conjugated capture Ab on PS microwell plate with the same orientation, which increased the detection sensitivity than the random orientation of capture Ab in traditional ELISA.[43] As mentioned above, coating Ab on the larger surface area with unidirectional direction will enhance the detection efficacy and sensitivity of ELISA. In our study, the α-mPEG cells can be considered as a microparticle which can provide the larger surface area and the more steric space for the display of capture antibody than a flat-bed well. Surface α-mPEG receptors can display unidirectional organization (outward organization) after coating α-mPEG cells on the plate, which increase mPEG detection efficacy than traditional Ab-coating ELISA. Together, the larger surface area and the more steric space for α-mPEG receptor expression, and unidirectional orientation of the α-mPEG receptor allow α-mPEG cells efficient trapping mPEG and mPEGylated

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molecules in ELISA plates, thus enhancing the detection sensitivity down to nanomolar level.

Simplifying the manufacturing process of drug quantitative ELISA will make this method more potential to be developed. The traditional ELISA need to use the high purity of antibodies for sample detection. Therefore, ascites method had been developed for producing high yields of antibodies. However, ascites method results in animal suffering, which has been prohibited by US and European markets. Many nonanimal-based methods have been generated, for example, inoculating hybridoma cells in bioreactor systems. Owing to the fragile hybridoma cells, most of bioreactor systems are not easy to set up without technical expertise in the beginning.[44] Besides, all of methods as mentioned above need to process antibody purification after production. In order to simplify the manufacturing process of drug quantitative ELISA, we establish a α-mPEG cell-based ELISA which is made of α-mPEG cell. The α-mPEG cell is derived from the BALB/c 3T3 cell stably expressing α-mPEG Fab on the cell membrane, which can self-proliferation in the serum-free culture condition. Notably, large amount of α-mPEG cell can easily obtain without additional purification. Therefore, this method can be easy manipulated in many laboratories which contain the fundamental techniques of cell culture. Moreover, the α-mPEG cell-coated plates can be lyophilized and then kept in cryopreservation for 6 months, which meets the required shelf life of commercial ELISA kits. The detection sensitivity of the α-mPEG cell-based ELISA after 6 month of cryopreservation to mPEG molecules is stable (data not shown). To sum up, the α-mPEG cell reveals the characteristics of the fast growth and ease-to-obtain without additional purification, which may efficiently reduce the manufacturing complexity of mPEG quantitative ELISA and allow it to be easily adopted in general laboratories and industries.

■ CONCLUSIONS

In summary, α-mPEG cell-based ELISAs possess potential advantages for quantification of mPEG and mPEGylated molecules, including: (1) direct quantification of any mPEG and mPEGylated molecules, (2) maintenance of sensitivity and accuracy in the presence of serum, and (3) simple manufacturing process due to no need for antibody purification. According to those benefits, we believe that the α-mPEG capture-based ELISA may provide a universal tool for research laboratories and pharmaceutical companies to study the pharmacokinetics of mPEG and mPEGylated molecules.

■ ASSOCIATED CONTENT

Supporting Information

Figures showing detection of mPEG or mPEGylated molecules by commercial mPEG ELISA kit and effect of serum proteins in the α-mPEG antibody-based sandwich ELISA. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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