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Optimization of an Anti-poly(ethylene glycol) (anti-PEG) Cell-Based Capture System To Quantify PEG and PEGylated Molecules

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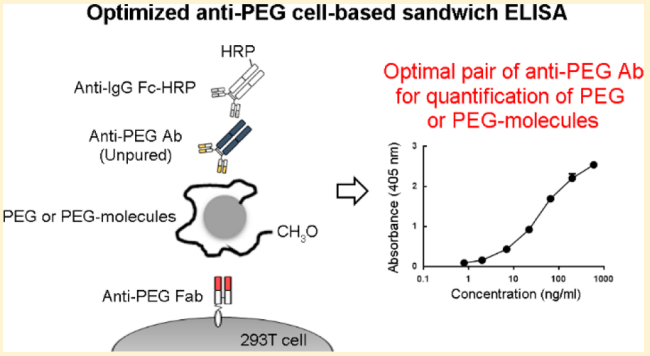
ABSTRACT: Sensitive determination of the pharmacokinetics of PEGylated molecules can accelerate the process of drug development. Here, we combined different anti-PEG Fab expressing 293T cells as capture cells (293T/3.3, 293T/6.3, and 293T/15−2b cells) with four detective anti-PEG antibodies (3.3, 6.3, 7A4, or 15−2b) to optimize an anti-PEG cell-based sandwich ELISA. Then, we quantified free PEG ($mPEG_{2K}$-NH_2 and $mPEG_{5K}$-NH_2) or PEG-conjugated small molecules ($mPEG_{5K}$-biotin and $mPEG_{5K}$-NIR797), proteins (PegIntron and Pegasys), and nanoparticles (Liposomal-Doxorubicin and quantum-dots). The combination of 293T/15−2b cells and the 7A4 detection antibody was best sensitivity for free PEG, PEG-like molecules, and PEGylated proteins with detection at ng mL^{-1} levels. On the other hand, 293T/3.3 cells combined with the 15−2b antibody had the highest sensitivity for quantifying Lipo-Dox at 2 ng mL^{-1}. All three types of anti-PEG cells combined with the 15−2b antibody had high sensitivity for quantum dot quantification down to 7 pM. These results suggest that the combination of 293T/15−2b cells and 7A4 detection antibody is the optimal pair for sensitive quantification of free PEG, PEG-like molecules, and PEGylated proteins, whereas the 293T/3.3 cells combined with 15−2b are more suitable for quantifying PEGylated nanoparticles. The optimized anti-PEG cell-based sandwich ELISA can provide a sensitive, precise, and convenient tool for the quantification of a range of PEGylated molecules.



Polyethylene glycol (PEG) is a synthetic biologically inert, nonimmunogenic linear polyether diol. It is nontoxic, produced in a large range of molecular weights, and has been approved by the Food and Drug Administration (FDA) for human use.[1−3] PEGylation confers molecules greater solubility in aqueous and organic media, increases serum half-life by reducing uptake by the reticuloendothelial system (RES),[4−6] shields drugs from enzymatic degradation,[7] decreases opsonization with serum proteins,[3,8,9] and reduces protein immunogenicity.[10] PEGs of various molecular weights have been used widely in biopharmaceuticals.[11] For example, the monomethoxylated form of PEG (mPEG) has been conjugated with small molecules such as 10-amino-7-ethyl camptothecin (CPT analogue),[12] amphotericin B (AMB),[13] silybin,[14] and zidovudine (AZT)[15] to increase water solubility, reduce systemic toxicity, and improve therapeutic efficacy. mPEG has also be covalently attached to proteins including PegIntron (linear PEG_{12K}-interferon-α-2b (IFNα-2b); Schering-Plough, U.S.A.),[16] Pegasys (branched PEG_{40K} interferon-α-2a (IFNα-2a); Hoffmann-La Roche, U.S.A.),[17,18] Neulasta (PEG_{20K} granulocyte colony-stimulating factor (G-CSF); Amgen, U.S.A.),[19] and Mircera (PEG_{30K} erythropoietin (EPO); Hoffmann-La Roche, U.S.A.)[20] to increase serum half-life. PEGylation is extensively applied in the nanoparticle field such as PEGylated liposomal-Doxorubicin (Lipo-Dox), Doxil (Johnson and Johnson, U.S.A.; Schering Plough, Europe),[21] and PEGylated liposomal-irinotecan, Onivyde (Merrimack, U.S.A.)[22] in cancer therapy. Several PEGylated nanoparticles are under development such as mPEG-gold nanoshells,[23] mPEG-superparamagnetic iron oxide (SPIO),[24] mPEG-microbubbles,[25,26] mPEG-solid lipid nanoparticles (SLN),[27] and mPEG-modified quantum dots (QDs).[28] Active research in the field of PEGylation and the vigorous development of the biopharmaceutical market suggests that the PEGylated drug market will continue to grow.[29]

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However, PEGylation alters the pharmacokinetic and pharmacodynamic properties of molecules.[30] The attached PEG molecules may also shield antibody-binding epitopes that interfere in the detection of protein-specific antibodies.[31,32] Therefore, how to accurately quantify and assess the pharmacokinetic parameters of these molecules is important for clinical drug development.

Current approaches for the quantitative measurement of PEG and PEGylated molecules include colorimetric methods, high-performance liquid chromatography (HPLC), radiolabeling, bioactivity assays, and enzyme-linked immunosorbent assays (ELISA).[30] The colorimetric approach is a simple but insensitive method for PEG quantification.[30] Erratic results are obtained for samples with plasma proteins and detergents.[1] HPLC, especially HPLC/tandem mass spectrometry (HPLC/MS/MS) or immunoaffinity purification (IAP) HPLC/MS/MS, are highly sensitive and reproducible methods for quantifying PEGylated molecules,[30,33] but multiple processing steps are usually required to reduce sample complexity before analysis due to the low tolerance for protein contamination.[34] Radiolabeling is a simple, highly specific, and sensitive method for PEGylated molecule assessment.[30] Radioisotopes are compatible with complex biological samples[30] and allow noninvasive imaging of compound biodistribution.[35,36] However, incorporation of radioisotopes may alter the pharmacokinetic properties of compounds.[37] Safety and disposal issues related to radioisotopes and the sophisticated equipment also restrict its universality.[30] Bioactivity assays are also used for detection for PEGylated enzymes and proteins. PEG, however, may affect interactions and catalytic activity of substrates and enzymes resulting in low detection sensitivity.[38] Our previous studies described antibody-based sandwich ELISA using pairs of monoclonal anti-PEG backbone antibodies (first generation, AGP3/IgM and E11/IgG;[39] second generation, AGP4/IgM and 3.3/IgG[40]) to assess PEGylated molecules in vitro and in vivo. However, the anti-PEG antibody-based sandwich ELISA could not detect most mPEG molecules (MW < 20000) or mPEGylated drugs.

We previously developed an anti-mPEG antibody (15−2b) and generated a cell-based ELISA system combined with the AGP4 antibody to measure mPEG (MW < 2000) and mPEGylated molecules.[14] In the present study, we attempted to identify the optimal pair of anti-PEG antibodies for the anti-PEG cell-based ELISA. We expressed the Fab fragment of three types of anti-PEG antibodies [antishort PEG backbone antibody (MW ≥ 750), 6.3; antilong PEG backbone antibody (MW ≥ 2000), 3.3; and anti-mPEG antibody (CH_3O-end PEG), 15−2b] on the surface of 293T cells (293T/3.3, 293T/6.3 and 293T/15−2b), respectively, as PEG capture reagents. The function and membrane expression levels of the anti-PEG Fab fragments were analyzed by flow cytometry. We paired the three anti-PEG capture cells with different anti-PEG antibody detection antibodies (3.3, 6.3, 7A4, or 15−2b) to measure small PEG molecules ($mPEG_{2K}$-NH_2 and $mPEG_{5K}$-NH_2), PEG-like molecules ($mPEG_{5K}$-biotin and $mPEG_{5K}$-NIR797), PEGylated proteins ($mPEG_{12K}$-IFNα-2b, PegIntron; and $mPEG_{40K}$-IFNα-2a, Pegasys), and PEGylated nanoparticles (Liposomal-Doxorubicin, Lipo-Dox; and quantum-dots, QDs; Figure 1). We believe that the best pairings of anti-PEG cell capture and anti-PEG detection antibody may provide a sensitive, precise, and universal tool for the study of the pharmacokinetics of mPEG and mPEGylated molecules in basic research laboratories and pharmaceutical companies.

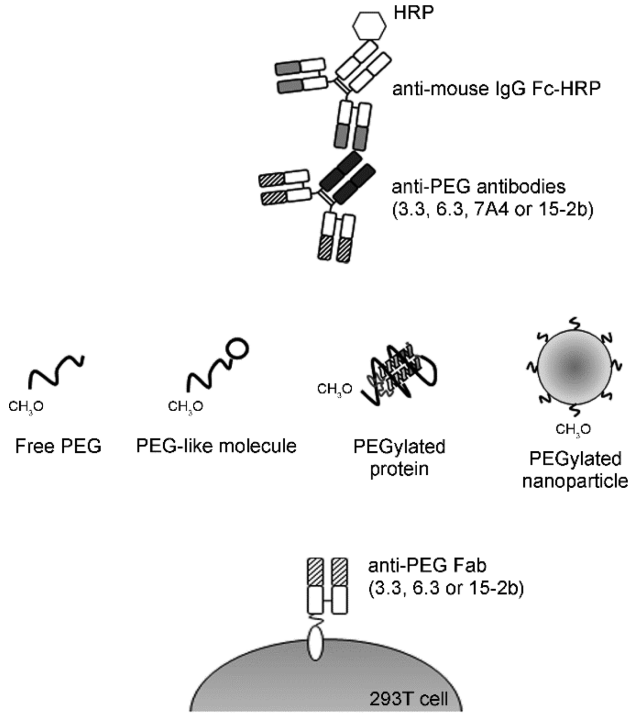

Figure 1. Optimization of anti-PEG cell-based sandwich ELISA for the quantification of free PEG and PEGylated molecules. To optimize the pairs of PEG capture (anti-PEG cells) and detection antibodies (anti-PEG antibodies) for quantification of free PEG and PEGylated molecules, we expressed anti-PEG Fab on the surface membrane of 293T cells (293T/3.3, 293T/6.3, and 293T/15−2b cells). The serially diluted free PEG molecules ($mPEG_{2K}$-NH_2 and $mPEG_{5K}$-NH_2), PEG-like molecules ($mPEG_{5K}$-Biotin and $mPEG_{5K}$-NIR797), PEGylated proteins (PegIntron and Pegasy), and PEGylated nanoparticles (Liposomal-Doxorubicin, Lipo-Dox; and quantum-dots, QDs) were detected by cell-based sandwich ELISA using anti-PEG antibodies (3.3, 6.3, 7A4, or 15−2b) and horseradish peroxidase (HRP) conjugated-antimouse IgG Fc secondary antibody.

EXPERIMENTAL SECTION

Reagents. $mPEG_{750}$-NH_2 (average M_n 750), $mPEG_{1K}$-NH_2 (average M_n 1000), $mPEG_{2K}$-NH_2 (average M_n 2000), $mPEG_{5K}$-NH_2 (average M_n 5000) were purchased from Fluka Chemie (Buchs, Switzerland) and NH_2-$PEG_{3.45K}$-NH_2 (average M_n 3400) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). $mPEG_{5K}$-biotin was purchased from Jenkem Technology (Allen, TX, U.S.A.). $mPEG_{5K}$-NIR797 was synthesized as previously described.[41] Pegasys and PegIntron were from Roche (Nutley, NJ, CA). PEG-QDs 565 (Qtracker 565 Vascular Labels), a nanocrystal semiconductor material coated with multiple linear 2 kDa mPEG molecules, was purchased from Invitrogen (Carlsbad, CA, U.S.A.). Lipo-Dox was from Taiwan Tung Yang Biopharm (TTY Biopharm Company, Taipei, Taiwan). A commercial methoxy-PEG ELISA kit was purchased from Life Diagnostics Inc. (West Chester, PA, U.S.A.).

Cells and Animals. 293T human embryonic kidney epithelial cells (American Type Culture Collection, Manassas, VA, U.S.A.) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St Louis, MO, U.S.A.) containing 10% (v/v) heat-inactivated bovine calf serum (BCS; Thermo, Waltham, MA, U.S.A.) and 100 units mL^{-1} penicillin and streptomycin (Invitrogen, Calsbad, CA, U.S.A.), at 37 °C in a humidified atmosphere of 5% (v/v) CO_2. Specific pathogen-free BALB/c mice were purchased from the National Laboratory

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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 Kaohsiung Medical University.

Antibodies. Hybridomas secreting 3.3, 6.3 (IgG_1 mAbs against PEG) and 15−2b (IgG_{2b} mAbs against mPEG) have been described.[14,40,41] A hybridoma secreting 7A4 (IgG_1 mAbs against PEG) was generated by immunizing female BALB/c mice with PEG-derived proteins as described previously.[41] Briefly, mice were *i.v.* injected with 200 μg of RH1-eβG-PEG_{5K}, a conjugate formed between a murine antibody and PEG-derivatized *E. coli* β-glucuronidase. The mice were *i.p.* injected with 100 μg RH1-eβG-PEG_{5K} 1 week later and then *s.c.* injected at weekly intervals with 50 μg of eβG-PEG_{5K} in complete Freund's adjuvant, 30 μg of eβG-PEG_{5K} in incomplete adjuvant, 30 μg of BSA-PEG_{5K} in incomplete adjuvant, and 10 μg of BSA-PEG_{5K} in incomplete adjuvant. Three days before fusion with FO myeloma cells, the mice were *i.p.* injected with 30 μg of BSA-PEG_{5K} in PBS. Hybridomas were generated by fusing spleen cells with FO myeloma cells and then screening culture media by ELISA in 96-well microtiter plates coated with 1 μg/well βG-PEG_{5K}. Hybridomas were cloned three times by limiting dilution in 96-well microtiter plates containing thymocyte feeder cells in HT medium supplemented with 15% fetal calf serum. Anti-PEG antibody-secreting hybridoma cell lines were injected into the abdomens of 7-week-old BALB/c mice (2×10^6 cells for each mouse) 7 days after *i.p.* injection of 400 μL of Freund's incomplete adjuvant. Monoclonal antibodies were purified from the ascites fluid of mice by affinity chromatography on Protein G Sepharose (GE Healthcare, Little Chalfont, U.K.) in high-salt buffer. Horseradish peroxidase (HRP)-conjugated goat antimouse IgG Fc antibody and fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG $F(ab')_2$ antibody were from Jackson ImmunoResearch Laboratories (Westgrove, PA, U.S.A.).

Characterization of Anti-PEG Antibodies. Maxisorp 96-well microplates (Nalge Nunc International, Roskilde, Denmark) were coated with 20 μg $well^{-1}$ of $mPEG_{750}$-NH_2, $mPEG_{1K}$-NH_2, $mPEG_{2K}$-NH_2 and NH_2-$PEG_{3.45K}$-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 2.5 μg/mL anti-PEG antibody (3.3, 6.3, 7A4, and 15−2b) in 50 μL of 2% (wt/vol) skim milk were added to the plates for 1 h at room temperature (RT). The plates were washed with PBST (PBS containing 0.05% (v/v) Tween-20) three times and with PBS once. HRP-conjugated goat antimouse IgG Fc (2 μg mL^{-1}) in 50 μL of dilution buffer were added for 1 h at RT. The plates were washed with PBS, and bound peroxidase activity was measured by adding 150 μL $well^{-1}$ of ABTS solution [0.4 mg mL^{-1}, 2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich, St. Louis, MO, U.S.A.), 0.003% (v/v) H_2O_2, and 100 mM phosphate-citrate, pH 4.0] for 30 min at RT. Color development was measured at 405 nm on a microplate reader (Molecular Devices, Menlo Park, CA, U.S.A.).

Plasmid Construction. The V_L-C_κ and V_H-C_{H1} domains of the 3.3, 6.3, and 15−2b anti-PEG antibodies were cloned from cDNA prepared from the 3.3, 6.3, or 15−2b hybridoma cells, respectively, following a previously described method.[42] 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 gttttgctcgctcgagacact-cattcctgtt-3′; V_H-C_{H1} sense, 5′-gaagatctgaggttaagctggaggag-3′; and V_H-C_{H1} antisense, 5′-tagtcaggtcgacaagtttttgtccaccgtgg-3′. The V_L-C_κ and V_H-C_{H1} genes, joined by a composite furin-2A protease cleavage site,[43] were cloned upstream of eB7 in the pLKO_AS3 lentiviral vector by using NheI and SalI restriction sites. The expression vector, pLKO_AS3-anti-PEG-eB7, encodes the 3.3, 6.3 or 15−2b anti-PEG Fab fragment fused to the immunoglobulin C2-type extracellular-transmembrane-cytosolic domains of the mouse B7−1 antigen (Figure 2).

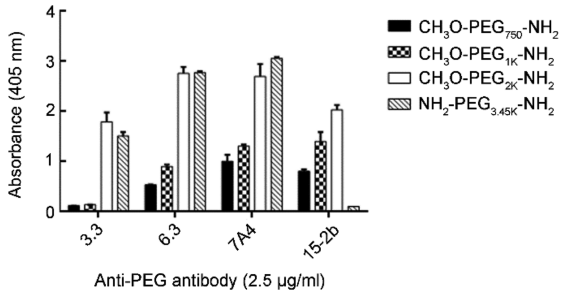

Figure 2. Characterization of anti-PEG antibodies. Coating of PEG molecules in microtiter plates was confirmed by direct ELISA with four anti-PEG antibodies, 3.3, 6.3, 7A4, or 15−2b. Bars, SD. Anti-PEG antibodies (2.5 μg/mL) were added to microtiter plate wells coated with PEG molecules with different molecular weights, $mPEG_{750}$-NH_2 (black), $mPEG_{1K}$-NH_2 (plaid), $mPEG_{2K}$-NH_2 (white), or different terminal functional groups, NH_2-$PEG_{3.45K}$-NH_2 (slashed). Binding of each anti-PEG antibody was determined by measuring absorbance at 405 nm after staining with goat antimouse IgG Fc-HRP and ABTS. The mean absorbance values of triplicate determinations are shown. Bars, SD.

Generation of Anti-PEG Expressing Cells by Lentiviral Transduction. To produce pseudotyped lentiviruses, pLKO_AS3−3.3-eB7, pLKO_AS3−6.3-eB7, or pLKO_AS3−15−2b-eB7 were cotransfected with pCMVΔR8.91 and pMD.G (Academia Sinica, Taipei, Taiwan) in 293T cells by PureFection (System Biosciences, Palo Alto, CA, U.S.A.). Two days after transfection, the culture medium was filtered and mixed with 8 μg mL^{-1} of Polybrene (Sigma-Aldrich, St. Louis, MO, U.S.A.), and the mixture was added to 293T cells. Following lentiviral transduction, cells were selected in 2 μg mL^{-1} puromycin-containing medium and sorted on a FACS Cantor (Beckman Coulter, Brea, CA, U.S.A.) to generate anti-PEG cells that stably expressed approximately equal levels of anti-PEG antibodies (3.3, 6.3, or 15−2b) on their surface.

Fluorescence-Activated Cell Sorting Analysis of the anti-PEG Expressing Cells. Surface expression of the anti-PEG Fab were measured by staining 5×10^5 cells with 3.75 μg mL^{-1} of FITC-conjugated goat antimouse IgG $F(ab')_2$ (Jackson Immunoresearch Laboratories, Westgrove, PA, U.S.A.) in 200 μL of PBS containing 0.05% (wt/vol) BSA on ice. The PEG binding activities of the membrane-anchored anti-PEG Fab were determined by incubating 5×10^5 cells with 4 nmol L^{-1} PEG-QDs 565 in 200 μL of PBS containing 0.05% (wt/vol) BSA on ice. After removal of unbound antibodies or PEG-QDs 565 by extensive washing in cold PBS containing 0.05% (wt/vol) BSA, the surface fluorescence of viable cells was measured on a FACScan flow cytometer (BD Biosciences, San Jose, CA, U.S.A.).

Anti-PEG Cell-Based Sandwich ELISA and Commercial Methoxy-PEG ELISA. In all sandwich ELISA experiments, PBS containing 2% (wt/vol) skim milk was used as the sample dilution buffer and PBS was used as the wash buffer. The 3.3, 6.3, or

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15−2b anti-PEG cells (2×10^5 cells well^{-1}) were seeded overnight in 96-well plates (Nalge Nunc International, Roskilde, Denmark) coated with 50 μg mL^{-1} poly-D-lysine (Corning, New York, U.S.A.) in culture medium. After extensive washing, the cells were fixed with 2% (wt/vol) paraformaldehyde for 5 min at room temperature (RT). Fixation was stopped by addition of 0.1 M glycine for 30 min at RT. The plates were blocked with 5% (wt/vol) skim milk in PBS for 2 h at 37 °C. Graded concentrations of $mPEG_{750}$-NH_2, $mPEG_{2K}$-NH_2, $mPEG_{5K}$-NH_2, $mPEG_{5K}$-biotin, $mPEG_{5K}$-NIR797, Pegasys (Roche, Nutley, NJ, U.S.A.), PEG-Intron (Schering-Plough, Kenilworth, NJ, U.S.A.), Lipo-Dox (TTY Biopharm Company, Taipei, Taiwan) or PEG-QDs 565 (Invitrogen, Carlsbad, CA, USA) were added to the wells (50 μL well^{-1}) at RT for 1 h. After washing, the cells were sequentially incubated with ascites containing 3.3, 6.3, 7A4, or 15−2b anti-PEG antibody and horseradish peroxidase (HRP)-conjugated goat antimouse IgG Fc (20 ng well^{-1}). The plates were washed with PBS and bound peroxidase activity was measured by adding 150 μL well^{-1} ABTS solution for 1 h at RT. Color development was measured at 405 nm on a microplate reader (Molecular Devices, Menlo Park, CA, U.S.A.). The commercial Methoxy-PEG ELISA was performed according to the manufacturer's instructions.

ELISA Data Analysis. All the readings were background-adjusted by automatically subtracting the absorbance value of a blank control in the ELISA procedures. Statistical significance of differences between controls and samples (PEG and PEGylated molecules) in all ELISA experiments (PEG-based or cell-based sandwich ELISA) were defined by using an independent *t* test. Data were considered significant at a *P* value of less than 0.05.

RESULTS AND DISCUSSION

Characterization of Anti-PEG Antibodies. To investigate the binding specificity of the anti-PEG monoclonal antibodies (3.3, 6.3, 7A4, and 15−2b), PEG molecules with different molecular weights ($mPEG_{750}$-NH_2, $mPEG_{1K}$-NH_2, and $mPEG_{2K}$-NH_2) and different terminal functional groups (NH_2-$PEG_{3.45K}$-NH_2) were coated in 96-well plates and then the binding of anti-PEG monoclonal antibodies (3.3, 6.3, 7A4, or 15−2b) was determined by direct ELISA. Figure 2 shows that 3.3 can bind $mPEG_{2K}$-NH_2 and NH_2-$PEG_{3.45K}$-NH_2, but not $mPEG_{750}$-NH_2 and $mPEG_{1K}$-NH_2, indicating that the binding epitope of 3.3 has at least 43 OCH_2CH_2 subunits (the number of subunits in PEG_{2K}); 6.3 and 7A4 bound PEG molecules consisting of at least 16 OCH_2CH_2 subunits (the number of subunits in PEG_{750}); 15−2b selectively bound to all PEG molecules that possessed a terminal methoxy group but not to NH_2-$PEG_{3.45K}$-NH_2. Our previous work showed that 15−2b bound mPEG molecule with at least 12 OCH_2CH_2 subunits (the number of subunits in PEG_{560}).[14] These results suggest that 6.3 and 7A4 recognize short backbones of PEG molecules (MW ≥ 750), 3.3 binds to long backbones of PEG molecules (MW ≥ 2000), and 15−2b selectively binds to short backbone PEG molecules with a methoxy group (CH_3O-end PEG MW ≥ 560) (Table 1).

Display of Functional Anti-PEG Antibodies on 293T Cells. We inserted the coding sequences of the Fab fragment of three anti-PEG antibodies (3.3, 6.3, or 15−2b) in an engineered lentiviral vector, pLKO_AS3, that contains a myc tag and the immunoglobulin C2-type extracellular-transmembrane-cytosolic domains of the mouse B7−1 receptor (eB7). In pLKO_AS3-anti-PEG-eB7, the light (V_L-C_κ) and heavy chains (V_H-C_{H1}) are separated by a furin cleavage site and 2A peptide (Figure 3A),

Table 1. Recognition Pattern of Anti-PEG Antibodies

antibody	subtype	PEG average molecule weight	epitope
6.3	IgG1	≥750	$(OCH_2CH_2)_{n=16}$
7A4	IgG1	≥750	$(OCH_2CH_2)_{n=16}$
3.3	IgG1	≥2000	$(OCH_2CH_2)_{n=43}$
15−2b	IgG2b	CH_3O-end PEG ≥ 560	CH_3-$(OCH_2CH_2)_{n=12}$

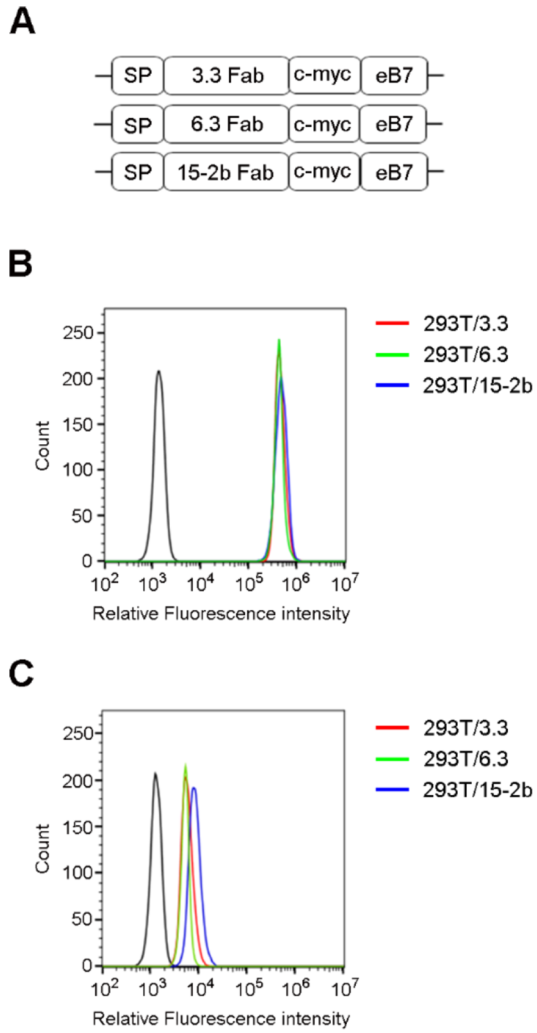

Figure 3. Surface display of functional anti-PEG Fab on 293T cells. 293T/3.3, 293T/6.3, and 293T/15−2b cells were generated by stably expressing (A) the receptor gene including (from N to C terminus), an immunoglobulin signal peptide (SP), the anti-PEG Fab (3.3, 6.3 or 15−2b) fragment, a c-myc epitope, and immunoglobulin C2-type extracellular-transmembrane-cytosolic domains of the murine B7−1 antigen (eB7). 293T/3.3 cells (red lines), 293T/6.3 cells (green lines), and 293T/15−2b cells (blue lines) were analyzed by flow cytometry using (B) a specific antibody to the mouse IgG $F(ab')_2$ to assess surface expression or (C) staining with $mPEG_{2K}$-quantum dots (QDs) to assess the PEG-binding activity of the three anti-PEG cells. The black lines on the graphs show mock staining with PBS containing 0.05% (wt/vol) BSA.

which allows antibody expression from a single open reading frame.[43] Human embryonic kidney epithelial 293T cells were infected with recombinant lentivirus and selected in medium containing puromycin to obtain 293T/3.3, 293T/6.3, and 293T/15−2b cells. The expression and function of the anti-PEG Fab on 293T/3.3, 293T/6.3, and 293T/15−2b cells were

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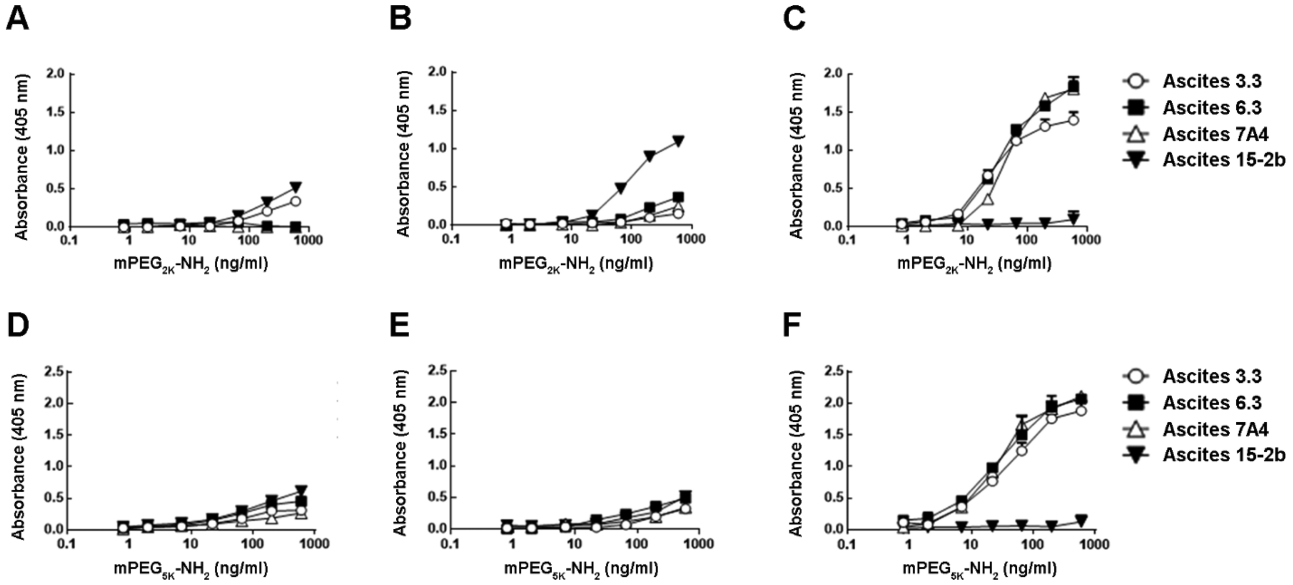

Figure 4. Detection of free PEG molecules by anti-PEG cell-based sandwich ELISA. Sandwich ELISA with (A, D) 293T/3.3 cells, (B, E) 293T/6.3 cells, or (C, F) 293T/15−2b cells as the capture reagents and the ascites of anti-PEG antibodies (3.3 (○), 6.3 (■), 7A4 (△), or 15−2b (▼)) as the detection antibodies were used to measure the concentrations of (A−C) $mPEG_{2K}$-NH_2 or (D−F) $mPEG_{5K}$-NH_2. The mean absorbance values (405 nm) of triplicate determinations are shown. Bars, SD.

confirmed by flow cytometry after directly staining the cells with FITC-conjugated antimouse IgG $F(ab')_2$ antibodies and $mPEG_{2K}$-quantum dots (QDs) to detect functional antibody binding. Figure 3B shows that 3.3, 6.3, and 15−2b Fab fragments were expressed at similar levels on 293T/3.3, 293T/6.3, and 293T/15−2b cells, respectively. All three anti-PEG Fabs expressed on cells specifically bound $mPEG_{2K}$-QDs (Figure 3C), indicating that surface displayed anti-PEG Fab maintained PEG binding activity.

Quantification of Free PEG and PEG-Like Molecules by Anti-PEG Cell-Based Sandwich ELISA. To investigate the optimal pair of anti-PEG antibodies in the cell-based ELISA system to quantify free PEG and PEG-like molecules, we coated 293T/3.3, 293T/6.3, and 293T/15−2b cells in 96-well plates, respectively, followed by addition of graded concentrations of free PEG ($mPEG_{2K}$-NH_2 and $mPEG_{5K}$-NH_2) and PEG-like molecules ($mPEG_{5K}$-biotin and $mPEG_{5K}$-NIR797). Captured PEG molecules were then quantified by sequential addition of anti-PEG ascites (3.3, 6.3, 7A4, or 15−2b), horseradish peroxidase (HRP)-conjugated goat antimouse IgG Fc antibody, and ABTS substrate. Figure 4 shows that 293T/15−2b cell-based sandwich ELISA could detect free PEG larger than 2000 at concentration as low as 7 to 2 ng mL^{-1}, whereas the 293T/3.3 and 293T/6.3 cells detected free PEG with low sensitivity. The PEG detection efficiency of three of four anti-PEG antibodies (3.3, 6.3, and 7A4) were not significantly different. There was no signal detected using 15−2b antibody, because its binding epitope (CH_3O-end PEG ≥ 560) was occupied by the capture of 293T/15−2b cells. A similar result was observed in quantification of PEG-like molecules (Figure 5). 293T/15−2b cells could sensitively detect $mPEG_{5K}$-biotin and $mPEG_{5K}$-NIR797 at concentrations of 7 ng mL^{-1}, whereas 293T/3.3 and 293T/6.3 cells performed poorly with all detection anti-PEG antibodies (3.3, 6.3, and 7A4). Of particular note, 293T/15−2b cells combined with anti-PEG antibody 7A4 had the best capacity to quantify PEG-like molecules. This result revealed that conjugation of diverse compounds to PEG molecules did not interfere with the detection ability in the 293T/15−2b cell-based sandwich ELISA. However, none of the three cell-based sandwich ELISAs could detect $mPEG_{750}$-NH_2 (data not shown), likely due to epitope competition by anti-PEG Fab on the cells and the detection anti-PEG antibodies for short PEG molecules. Taken together, 293T/15−2b cells combined with 7A4 detection antibody are most suitable for the quantification of small PEG molecules in the cell-based sandwich ELISA.

Limited sensitivity is the major problem in traditional anti-PEG antibody-based sandwich ELISA for quantifying free PEG molecules. We previously demonstrated that anti-PEG antibody (AGP3)-based sandwich ELISA could sensitively quantify PEGylated macromolecules but not small molecules such as free PEG molecules (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).[44] We also previously demonstrated that anti-PEG cell-based sandwich ELISA was several orders of magnitude more sensitive for low molecular weight PEG than the traditional ELISA using anti-PEG antibody-coated plates.[14,44] The anti-PEG Fab expressed on the membrane of mammalian cells may enhance its surface area for PEG molecule detection.[14,45] In addition, surface anti-PEG Fab can display unidirectional organization (outward organization) after coating anti-PEG cells on the plate, which prevents the multidirectional organization in traditional antibody-coating ELISA and increases PEG detection efficiency.[14,46] In our cell-based sandwich ELISA system, despite different anti-PEG antibodies displaying the same orientation, anti-mPEG cell-based ELISA (293T/15−2b) exhibits higher detection sensitivity for PEG molecules than anti-PEG backbone cell-based ELISA (293T/3.3 and 293T/6.3). The mPEG molecules bound by 15−2b Ab may expose more epitopes for the detection anti-PEG antibody as compared to cell-anchored anti-PEG backbone Fab since the binding epitope of the 15−2b antibody (CH_3O-end PEG ≥ 560) is smaller than 3.3 (MW ≥ 2000) or 6.3 (MW ≥ 750) Ab (Table 1). However, the anti-mPEG cell-based ELISA still cannot detect short mPEG molecules with an average molecular weight of less than 2000. To overcome this limitation, we previously developed an anti-mPEG cell-based competition ELISA, which could effectively detect the concentration

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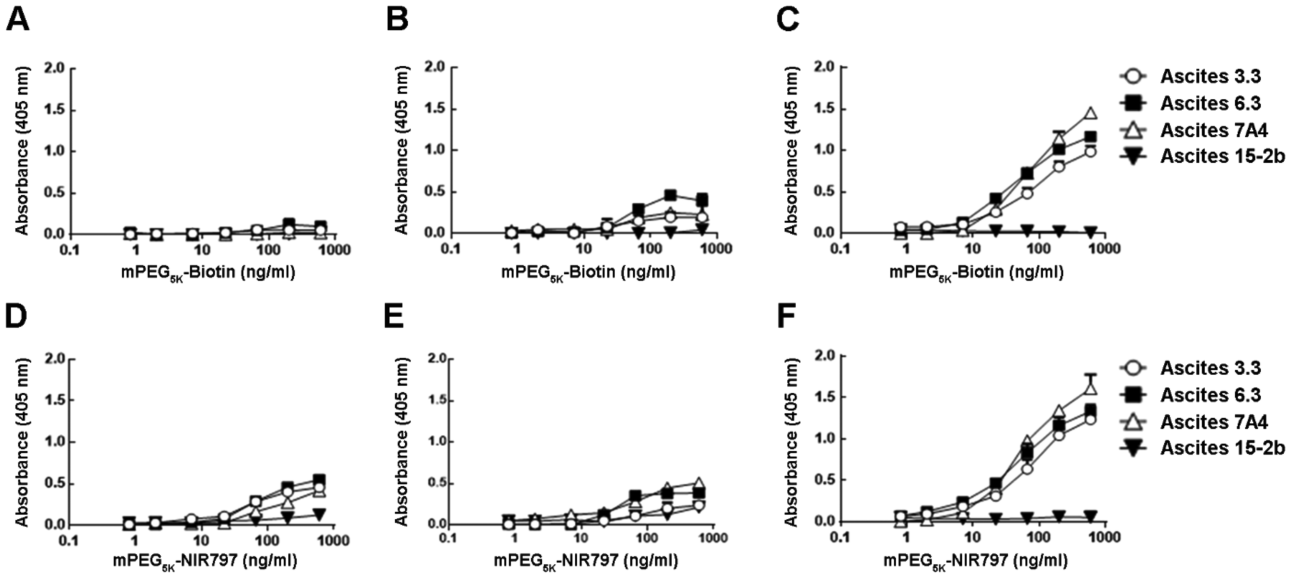

Figure 5. Detection of PEG-like molecules by anti-PEG cell-based sandwich ELISA. Sandwich ELISA with (A, D) 293T/3.3 cells, (B, E) 293T/6.3 cells, or (C, F) 293T/15−2b cells as the capture reagents and the ascites of anti-PEG antibodies (3.3 (○), 6.3 (■), 7A4 (△), or 15−2b (▼)) as the detection antibodies were used to measure the concentrations of (A−C) $mPEG_{5K}$-biotin or (D−F) $mPEG_{5K}$-NIR797. The mean absorbance values (405 nm) of triplicate determinations are shown. Bars, SD.

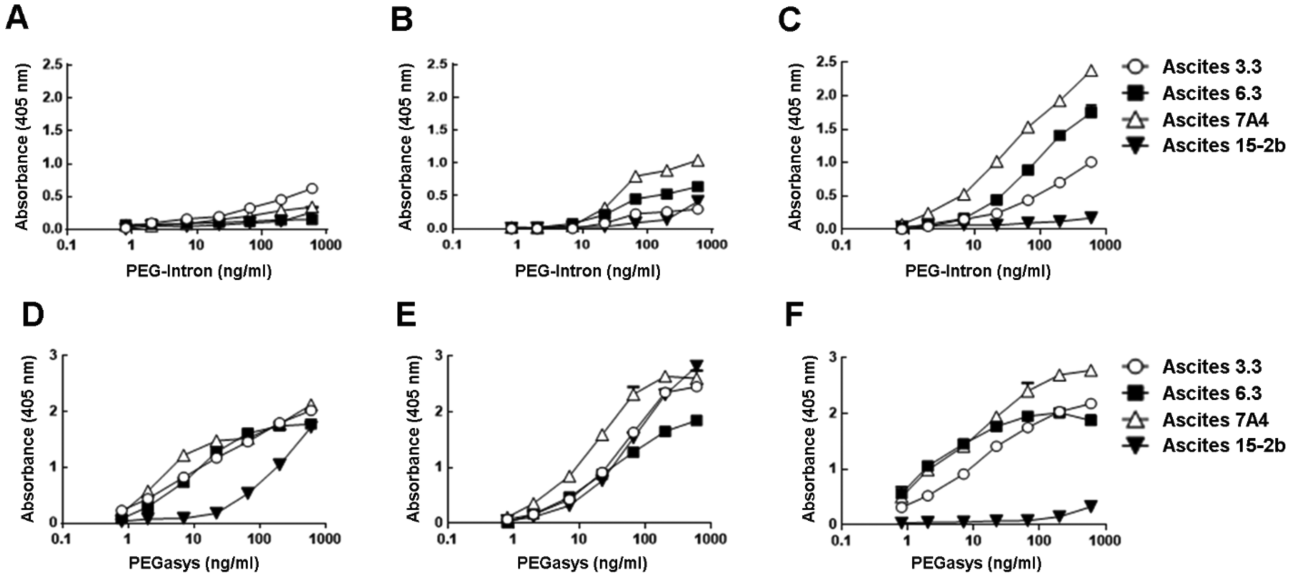

Figure 6. Detection of PEGylated proteins by anti-PEG cell-based sandwich ELISA. Sandwich ELISA with (A, D) 293T/3.3 cells, (B, E) 293T/6.3 cells, or (C, F) 293T/15−2b cells as the capture reagents and anti-PEG detection antibodies (3.3 (○), 6.3 (■), 7A4 (△), or 15−2b (▼)) were used to measure serial dilutions of (A−C) PegIntron ($mPEG_{12K}$-IFNα-2b) or (D−F) Pegasys ($mPEG_{40K}$-IFNα-2a). The mean absorbance values (405 nm) of triplicate determinations are shown. Bars, SD.

of mPEG molecules smaller than 2000 at concentrations as low as 70 nM.[14] Thus, we suggest that anti-mPEG cell-based sandwich ELISA is more suitable for quantifying small PEG molecules.

Quantification of PEGylated Proteins by Anti-PEG Cell-Based Sandwich ELISA. We also investigated using the anti-PEG cell-based sandwich ELISA to detect PegIntron ($mPEG_{12K}$-IFNα-2b) and Pegasys ($mPEG_{40K}$-IFNα-2a) using a previously described procedure, as a test of the quantification of PEGylated proteins. As shown in Figure 6, similar results to those of the small molecule quantification were obtained: the sensitivity of the 293T/15−2b cells for detecting PEGylated proteins was better than 293T/3.3 and 293T/6.3 cells; and the combination of 293T/15−2b cells and 7A4 had the best sensitivity for PEGylated protein quantification at 2−0.8 ng mL^{-1}. As we showed in previous studies, the sensitivity of the anti-PEG cell-based sandwich ELISA increased with the length of PEG chains attached to protein drugs.[32] We believe that the longer PEG molecules exposes more epitopes to the anti-PEG antibody, which leads to high detection sensitivity. Together, these results suggest that the combination of 293T/15−2b cell and 7A4 detection antibody may be the optimal pair for sensitively quantifying PEGylated proteins by cell-based sandwich ELISA.

Currently, site-specific monoPEGylation has been accepted as good manufacturing practice (GMP) for clinical use.[47,48]

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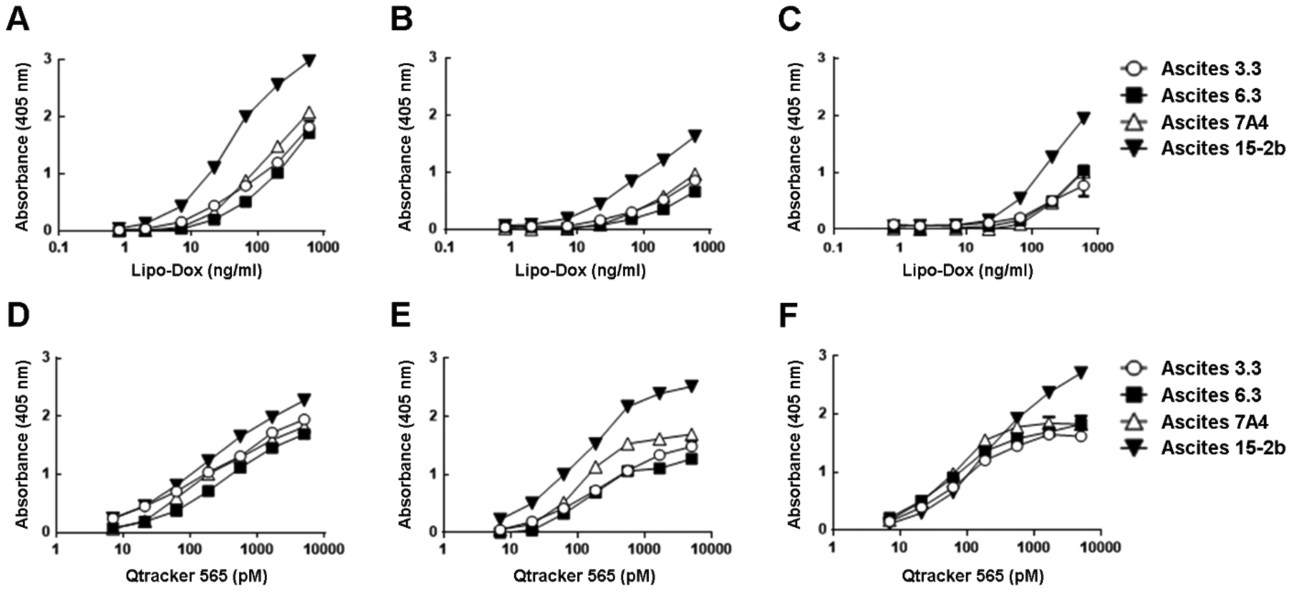

Figure 7. Detection of PEGylated nanoparticles by anti-PEG cell-based sandwich ELISA. Sandwich ELISA with (A, D) 293T/3.3 cells, (B, E) 293T/6.3 cells, or (C, F) 293T/15−2b cells as the capture reagents and anti-PEG detection antibodies (3.3 (○), 6.3 (■), 7A4 (△), or 15−2b (▼)) were used to measure serial dilutions of (A−C) Lipo-Dox or (D−F) Qtracker 565. The mean absorbance values (405 nm) of triplicate determinations are shown. Bars, SD.

Protein drugs are usually conjugated with a single high molecular weight PEG molecule to avoid poor coverage,[49,50] achieve homogeneous modification and improved therapeutic efficacy.[51,52] PEGs with various molecular weights have been widely used in biopharmaceuticals. For example, methoxy PEG-20K ($mPEG_{20K}$) covalently conjugated on recombinant human granulocyte colony-stimulating factor analogue (Neulasta) for neutropenia associated with cancer chemotherapy;[19] $mPEG_{30K}$ is also used for erythropoietin (Mircera) for anemia;[20] and $mPEG_{40K}$ conjugated to IFNα-2a (Pegasys) is used for treating chronic hepatitis C.[17] Thus, it is important to develop a universal tool for the quantification of PEGylated proteins with different lengths of PEG. Our results show that the combination of anti-mPEG cells (293T/15−2b) with an anti-PEG backbone Ab (7A4) produces the best quantification sensitivity for PEGylated proteins (PegIntron [$mPEG_{12K}$-IFNα-2b] and Pegasys [$mPEG_{40K}$-IFNα-2a]) and the detection sensitivity of anti-PEG cells increased in a PEG length-dependent manner (Figure 6). We think that this optimal pair can properly quantify different lengths of PEG molecules on PEGylated proteins and, therefore, accelerate the development of all types of PEGylated drugs in research laboratories and in industry.

Quantification of PEGylated Nanoparticles by Anti-PEG Cell-Based Sandwich ELISA. Next, to find the optimal combination of anti-PEG antibodies for quantifying PEGylated nanoparticles by anti-PEG cell-based sandwich ELISA, we detected Lipo-Dox and quantum-dots (QDs) by anti-PEG cell-based sandwich ELISA. As shown in Figure 7, 293T/3.3, 293T/6.3, and 293T/15−2b cells were all able to detect Lipo-Dox with different sensitivities. 293T/3.3 cells combined with 15−2b had the highest Lipo-Dox detection sensitivity of 2 ng mL^{-1}. On the other hand, combined with 15−2b, all three anti-PEG cells were able to sensitively quantify QDs at concentrations as low as 7 pM. However, there was no significant difference between each pair of anti-PEG cells (293T/3.3, 293T/6.3, and 293T/15−2b) and detection antibodies. We suggest that the combination of 293T/3.3 cells and 15−2b detection antibody gives the highest sensitivity for quantification of PEGylated nanoparticles by cell-based sandwich ELISA.

Simplification of the manufacturing process for PEG quantification assays can reduce the cost and accelerate the timeline of PEGylated drug development. In traditional quantitative ELISA, a purified, biotin-labeled detection antibody is needed for detection of antigens. The detection signal can be amplified by staining with avidin-peroxidase or avidin-fluorescence.[53,54] The complex process of biotin labeling may add time and cost and also affect the antibody's ability to bind to the target protein. In our anti-PEG cell-based sandwich ELISA, we expressed the Fab region of anti-PEG antibodies on the 293T cell membrane as a PEG capture reagent and directly monitored PEG molecules with an unpurified solution containing anti-PEG antibodies without additional biotin labeling, followed by using HRP-conjugated antimouse Fc secondary antibody for color development. The use of unpurified antibodies such as culture medium with anti-PEG antibodies is expected to significantly reduce the complexity of the assay process in this cell-based sandwich ELISA. Additionally, 293T cell clones that express high levels of membrane-anchored anti-PEG Fab can provide a stable and high quality source of PEG capture cells. Moreover, the anti-PEG cell-coated plates can be lyophilized and then stored by cryopreservation for 6 months without affecting the quantification ability, which meets the required shelf life specifications for commercial ELISA kits. We also compared the quantitative sensitivity of free PEG ($mPEG_{5K}$-NH_2) and PEGylated molecules ($mPEG_{5K}$-NIR797, Pegasys, and Lipo-Dox) between our optimized anti-PEG cell-based sandwich ELISA (Figure 8A) and a commercial anti-PEG ELISA kit (Figure 8B). The result shows that the detective sensitivity of the commercial anti-PEG ELISA kit was much lower than our anti-PEG cell-based sandwich ELISA. Unfortunately, there is currently no well-established production line for commercialization of cell-based sandwich ELISA kits. However, we expect that a commercialized anti-PEG cell-based sandwich ELISA kit will be able to provide a precise, sensitive, low-cost, and simple

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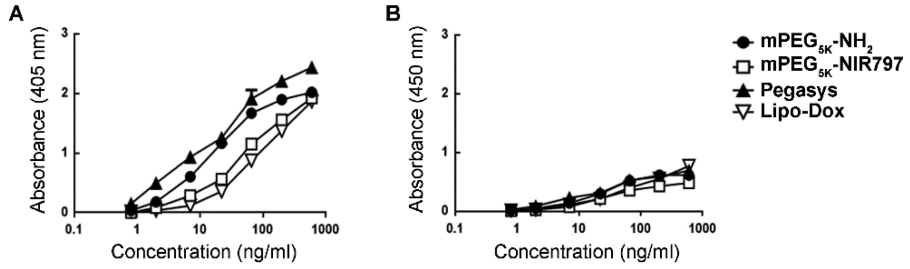

Figure 8. Comparison of the sensitivity for free PEG and PEGylated molecules between optimized anti-PEG cell-based sandwich ELISA and a commercial anti-PEG ELISA kit. (A) Sandwich ELISA with 293T/15−2b cells as the capture reagent and the ascites of anti-PEG antibody, 7A4, as the detection antibody was used to measure serial dilutions of $mPEG_{5K}$-NH_2 (●), $mPEG_{5K}$-NIR797 (□), and Pegasys (▲), and 293T/3.3 cells as the capture reagent and the ascites of anti-mPEG antibody, 15−2b, was used to quantify serial dilutions of Lipo-Dox (▽). (B) Commercial anti-PEG ELISA kit was used to measure $mPEG_{5K}$-NH_2 (●), $mPEG_{5K}$-NIR797 (□), Pegasys (▲), and Lipo-Dox (▽) following the manufacturer's instructions. The mean absorbance values (405 or 450 nm) of triplicate determinations are shown. Bars, SD.

method for analyzing the pharmacokinetics of free PEG and PEGylated molecules.

CONCLUSIONS

We compared the detection sensitivity of three different anti-PEG cells (293T/3.3, recognizing long backbones of PEG; 293T/6.3, recognizing short backbones of PEG; 293T/15−2b, recognizing the terminal methoxyl group of mPEG) to quantify free PEG and PEGylated molecules by cell-based sandwich ELISA. The results showed that a combination of 293T/15−2b cells and 7A4 ascites was the optimal pair for sensitively quantifying free PEG, PEG-like molecules and PEGylated proteins. On the other hand, 293T/3.3 cells combined with 15−2b anti-mPEG antibody were more suitable for quantifying PEGylated nanoparticles. We suggest that this optimized anti-PEG cell-based sandwich ELISA has the following advantages: (1) sensitive quantification of each type of free PEG and PEGylated molecules by using different pairs of anti-PEG capture cells and detection antibodies; (2) cloned 293T cells that express high levels of membrane-tethered anti-PEG Fab can provide a stable and high quality source of PEG capture cells; and (3) the simplified and convenient manufacturing process of the anti-PEG cell-based sandwich ELISA can reduce the cost as there is no need for purification and additional biotin labeling of detection antibodies. According to these advantages, we believe that the anti-PEG cell-based sandwich ELISA provides a sensitive, precise, and universal tool that may be used in basic research laboratories and pharmaceutical companies to study the pharmacokinetics of PEG and PEGylated molecules.

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S.R.R. and T.L.C. designed the research; W.W.L. and Y.C.H. performed experiments; W.W.L., Y.C.H., S.R.R., T.L.C., Y.A.C., and K.H.C. analyzed and interpreted the data; W.W.L. wrote the paper; C.C.H., C.H.C., I.J.C., K.W.C., Y.C.L., T.C.C., and Y.T.W. assisted in eliminating problems during experiments and provided suggestions and corrections for the manuscript; all authors approved of the final manuscript.

Author Contributions

‡These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

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