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Analytical Measurement of PEGylated Molecules

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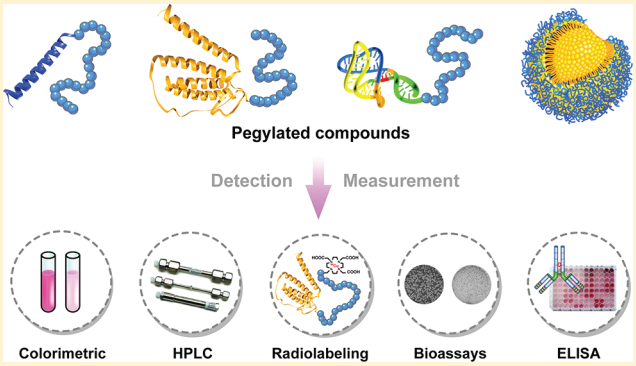
ABSTRACT: Attachment of poly(ethylene glycol) (PEG) to proteins, peptides, liposomes, drugs, and nanoparticles can improve pharmaceutical pharmacokinetic properties and enhance in vivo biological efficacy. Since the first PEGylated product was approved by the Food and Drug Administration in 1990, increasing numbers of PEGylated compounds have entered clinical use. Successful clinical development of PEGylated pharmaceuticals requires accurate methods for the qualitative and quantitative analysis of intact PEG conjugates in biological fluids. In this article, we review assay methods that can be utilized for the detection and measurement of PEGylated pharmaceuticals in complex biological samples for determination of biodistribution and pharmacokinetic properties. In particular, we describe relevant colorimetric, chromatographic, radiolabeled, biological, and enzyme-linked immunosorbent assays for the pharmacokinetic study of PEGylated molecules.



INTRODUCTION

PEG is a water-soluble, nontoxic, non-antigenic, biocompatible polymer that has been approved by the Food and Drug Administration for human intravenous, oral, and dermal applications.[1,2] Attachment of PEG (PEGylation) to drugs, peptides, proteins, nanoparticles, micelles, and liposomes is a mature technology for enhancing the bioavailability, stability, safety, and efficacy of a wide range of therapeutic agents. Therefore, PEGylated molecules are increasingly employed as mainstream therapeutic and diagnostic agents, which in turn have created great demand for methods facilitating the qualitative and quantitative analysis of PEG-derivatized molecules for both drug development and clinical applications.

PEGylation of peptides and proteins can reduce immunogenicity,[3] minimize proteolytic cleavage,[4] and increase serum half-life,[5] which in turn can increase drug efficacy and reduce injection frequency to enhance patient compliance and quality of life. Several PEGylated protein conjugates are now commercially available including Pegasys (PEG-interferon alpha-2a)[6,7] and PEG-Intron (PEG-interferon alpha-2b)[8] for hepatitis, Somavert (PEG-human growth hormone receptor antagonist) for acromegaly,[9] Cimzia (certolizumab pegol, PEGylated anti-human TNF-alpha Fab′) for rheumatoid arthritis,[10] Neulasta (pegfilgrastim, PEG-recombinant human granulocyte colony-stimulating factor analogue) for neutropenia associated with cancer chemotherapy,[11,12] PEG-erythropoietin (EPO) for anemia,[13] Adagen (PEG-adenosine deaminase) for immunodeficiency,[14] PEG-Hirudin for thrombosis,[15] and Oncaspar (PEG-asparaginase) for cancer treatment.[16] PEGylation of protein drugs has become routine and there is a trend of PEGylating established protein drugs for new uses.

PEGylation of polymeric nanoparticles, liposomes, and micelles can improve drug bioavailability and efficacy by reducing unintended uptake in normal tissues, decreasing systemic toxicity, prolonging circulation time in the blood, and enhancing tumor accumulation.[17−21] For example, PEG-modified liposomal doxorubicin (Doxil), approved by the FDA for the clinical treatment of ovarian and breast carcinomas and Kaposi's sarcoma,[22,23] displays comparable efficacy to doxorubicin but with significantly reduced cardiotoxicity, myelosuppression, vomiting, and alopecia.[24,25] Several polymeric micelle formulations (paclitaxel (Genexol) and cisplatin) are currently undergoing phase I/II clinical trials, and have shown improved antitumor efficacy and reduced systemic toxicity.[26] PEGylation of nanoparticles such as superparamagnetic iron oxide (PEG-SPIO/phase III, MRI contrast agent),[27] PEG-microbubbles,[28] ultrasound contrast agents, and quantum dots[29] improves their biocompatibility and reduces receptor-mediated uptake by the reticuloendothelial system,[30] but can also influence their imaging contrast properties.[31,32] These nanoparticles have been extensively studied in cells and animal models for cell trafficking studies,[33,34] tumor targeting,[35,36] and diagnostics.[37,38] PEGylated nanoparticles are being increasingly employed for clinical applications.

PEG has also been employed to prolong the circulation and increase the water solubility of small molecules such as camptothecin (PEG-CPT/phase II),[39] SN-38,[40] and paclitaxel.[41−43] In addition, PEGylation of indomethacin has been examined in animal models for improved anti-inflammatory effects.[44] Novel

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functions are also continually being discovered for PEG. For example, PEG molecules have been investigated as therapeutic agents for neuronal injury,[45] as chemopreventive agents for chemically induced colitis,[46,47] and as tumor suppressive agents for cancer.[47,48] New treatment modalities using PEG are likely to be developed in the future.

All trends indicate that PEG will be increasingly employed to improve the properties of therapeutic or diagnostic agents as more proteins, macromolecular drugs, and nanoparticles enter clinical trials in the 21st century. The successful translation of PEGylated molecules to the clinic depends on accurate methods for the quantitative analysis of pharmacokinetic parameters in animals and patients. Measurement of drug exposure and tissue distribution is critical for interpretation of preclinical and clinical therapeutic efficacy and toxicity. Sensitive in vivo quantification is also important for assessing stability, metabolism, and bioavailability of PEGylated compounds. Here, we provide an overview of analytical methods for the quantitative measurement of PEGylated molecules in biological mixtures.

COLORIMETRIC METHODS

Colorimetric methods have been employed for more than 60 years to detect PEG and PEGylated molecules (Table 1). Shaffer and colleagues developed the first and most commonly cited method for quantification of PEG molecules based on the formation of insoluble complexes between PEG and heteropoly inorganic acids (phosphomolybdic and silicotungstic acid) in the presence of barium chloride. The method can detect 0.05 to 1 mg of PEG at concentrations as low as 0.01 mg/mL.[49] Treatment with barium and iodine to form a barium−iodide complex produced a linear response at 535 nm from 1.25 to 7.5 μg/mL of PEG4000.[50,51] This approach can also measure the concentration of distearoylphosphatidylethanolamine-PEG (DSPE−PEG), used for the formation of PEGylated liposomes, at concentrations as low as 25 μg/mL.[52] These methods, however, are technically demanding and time-consuming due to the need to collect and wash precipitates and are therefore not ideal for rapid quantification of PEG in multiple, complex biological samples. Ingham and colleagues developed a more convenient assay for determination of PEG based on the nephelometric quantitation of turbidity which appears when PEG is added to Nessler's solution (an alkaline solution of potassium mercuric iodide).[53] However, plasma proteins drastically diminish the scattering intensity; as little as 1 μg/mL albumin in the final assay mixture destroys the quantitative relationship.

Two-phase systems can be employed to detect PEG and PEGylated compounds in the presence of proteins. Guermant and colleagues measured PEG indirectly by determining the influence of PEG on the partitioning of a fluorescein dye between water and aqueous ammonium sulfate phases.[54] A more general assay developed by Nag and colleagues is based on the partitioning of colored PEG-Fe(SCN)$_3$ complexes from an aqueous ammonium ferrothiocyanate phase to chloroform (Figure 1). The visible absorbance at 510 nm in the chloroform phase is linearly dependent on PEG concentration over a range of 5−100 μg PEG5000 (PEG with a molecular weight of 5000 Da).[55] This assay can be employed for estimation of PEG in plasma samples containing as much as 5 mg plasma proteins in a 50 μL sample. Amphipathic compounds, such as Triton X-100, which possesses repeating ethylene oxide units like PEG, also influence the partitioning of the chromophore and thus may interfere with the assay. In addition, PEGylated compounds that partition poorly into chloroform are difficult

Table 1. Colorimetric Methods for Measuring PEG and PEGylated Molecules

PEGylated compound	PEG length	method	possible interference	sensitivity	ref
Free PEG	1000−6000	Formation of insoluble complexes between PEG and heteropoly inorganic acids in the presence of barium chloride	Plasma, Urine	10 μg/mL	49
Free PEG	400−6000	Nessler's solution	Proteins	20 μg/mL for PEG4000 500 μg/mL for PEG400	53
Free PEG	1000−5000	Spectrophotometric measurement of fluorescein dye absorbance in an aqueous two-phase system containing PEG in the upper phase and ammonium sulfate in the lower phase	Triton X-100 CTAB	5 μg/mL for PEG5000	54
Free PEG PEG-protein	750−15000	Measurement of absorbance in the chloroform phase in an aqueous ammonium ferrothiocyanate and chloroform two-phase system	Triton X-100 dioxan	5 μg	55
PEG-protein	5000	Alkaline hydrolysis of PEG-protein before analysis in an aqueous ammonium ferrothiocyanate/chloroform biphasic system	Triton X-100 dioxan	1 μg/mL	56
PEG-liposome	2000	Phospholipase C treated PEG-liposome are analyzed in an ammonium ferrothiocyanate and chloroform biphasic system	Triton X-100 dioxan	1 μg/mL	57
PEG-liposome	5000	Measurement of the absorbance at 234 nm of PEG-dichlorotriazine in liposomes	None reported	250 μg/mL	194

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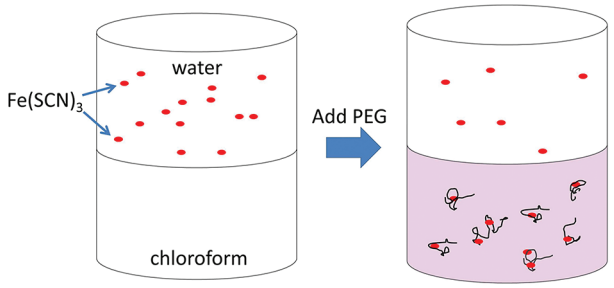

Figure 1. Two-phase system for PEG determination. Partitioning of ferrothiocyanate−PEG complexes into the chloroform phase produces a visible purple−pink coloration in proportion to PEG concentration that can be detected at 510 nm.

to assay. However, sample hydrolysis can extend analysis to PEGylated compounds that do not partition well into the organic phase. For example, alkaline hydrolysis of a chloroform-insoluble PEGylated protein prior to measurement of the released PEG allowed determination of the amounts of free and coupled PEG in the biphasic system.[56] Likewise, phospholipase-C digestion of PEG-conjugated distearoylphosphatidylethanolamine (PEG-DSPE) stealth liposomes to remove interfering phospholipids before analysis in the biphasic system allowed measurement of PEG2000 in amounts as low as 1 μg/mL with a linear response over a range of 1−100 μg.[57,58]

Colorimetric assays are simple and utilize commonly available lab equipment for analysis and detection (Table 2). On the other hand, colorimetric assays are sensitive to interference from proteins and detergents and are therefore most applicable to monitoring PEGylation reactions in relatively defined samples rather than for accurate determination of PEGylated compound concentrations in complex biological fluids.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC is a powerful analytical technique that is widely used for the measurement of PEG, PEGylated small drugs, and PEGylated liposomes and micelles (Table 3). HPLC analysis of clinical samples usually requires pretreatment steps to reduce sample complexity. Typically, proteins are first removed by precipitation with chloroform,[59,60] acetonitrile,[61] 5-sulfosalicylic acid,[62] trifluoroacetic acid,[63] $ZnSO_4$/acetone,[64] or $ZnSO_4$−methanol.[65] Acetonitrile and chloroform are preferred precipitation agents because nonvolatile salts can precipitate in analytical columns.

PEG molecules separated by HPLC can be directly measured with a refractive index detector, which has been commonly employed to measure PEG concentrations in urine samples during studies examining intestinal permeability. Early methods suffered from poor sensitivity with detection limits ranging from 0.2 to 5 mg/mL for PEG400.[66−68] Assay sensitivity can be improved by more extensive sample preparation to remove substances that interfere with refractive index measurements. Utilization of sized regenerated cellulose membranes and mixed ion exchange resin for sample preparation combined with size-exclusion HPLC allowed measurement of as little as 5 μg/mL PEG3350 and 50 μg/mL PEG400 in urine.[69] Size exclusion HPLC with refractive index detection was also used to measure residual unconjugated PEG in a PEGylated protein preparation with a limit of detection of 10 μg/mL by utilizing two tandem Shodex Protein KW803 and KW804 columns.[70] Refractive index, however, suffers from low sensitivity and is sensitive to changes in ambient temperature, pressure, and flow rate. In general, refractive index detection is only suitable when relatively noncomplex samples, such as urine, contain at least 10−100 μg of PEG per injection.

PEG can also be detected by UV absorption at wavelengths shorter than 200 nm, but sensitivity is only comparable to that obtained with an refractive index detector.[71] The limit of detection can be improved by modification of the PEG chain. For example, attachment of a dibenzoate group at the terminal end of PEG600 allowed UV detection at 230 nm, thereby greatly improving the limit of detection to 1 μg per injection.[60] However, the hydrophobic properties of the benzoate group may alter the pharmacokinetic behavior of PEG, limiting the general utility of this method.

High-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) allows sensitive quantitation of free PEG in biological fluid samples. Pelham and colleagues used HPLC-MS/MS for the determination of PEG3350 in human urine and plasma with quantification limits of 30 ng/mL and 100 ng/mL, respectively. Samples were treated with metaphosphoric acid to precipitate proteins, extracted with chloroform, and separated on a SUPELCO TSK gel column.[72]

HPLC is an excellent choice for the quantification of PEGylated drugs and small molecules that can be detected on UV or fluorescence detectors. Liu and colleagues measured PEGylated puerarin (PEG-PUE), an isoflavone C-glycoside, in rat plasma samples by gradient elution of PEG-PUE on a CAPCELL PAK C18 column with 0.2% aqueous phosphoric acid and acetonitrile as the mobile phase and detection at 250 nm with a limit of quantitation of 80 ng/mL.[73] PEGylated SN38, a camptothecin derivative, was measured in tumor-bearing mice after precipitation of proteins with acetonitrile, separation on a C18 reversed-phase column, and fluorescence detection with a limit of detection of 10 ng/mL.[63] Posey and colleagues also applied this method in a phase 1 pharmacokinetic study of weekly pegamotecan (polyethylene glycol-camptothecin, PEG-CPT) in patients with advanced solid tumors and lymphomas.[74] Although generally useful, the HPLC conditions and detection method must be optimized for each compound based on the chromatographic and spectral properties of the conjugated drug or small molecule.

HPLC approaches are also useful to measure encapsulated drug concentrations in PEGylated liposomes and micelles. Typically, lysosomes are disrupted to release free drug which can then be assayed by traditional HPLC methods. For example, Wei and colleagues measured micelle-encapsulated or liposome-encapsulated doxorubicin formulations in rat plasma with a lower limit of quantitation of 5 ng/mL by precipitating proteins with acetonitrile, evaporation and sample reconstitution in mobile phase before separation on a C18 reversed-phase column, and measurement of doxorubicin fluorescence.[61] UV−visible detection can help avoid potential interfering species commonly observed when using fluorescent detection; doxorubicin and PEGylated liposomal doxorubicin were separated on a C_{16} reversed-phase column and then were determined by measuring UV absorption at 487 nm, giving a linear response in the range 10−25 μg/mL.[62]

Sophisticated HPLC and detection combinations have been developed for detailed characterization of PEG and PEGylated proteins, peptides, and nanoparticles including accurate determination of conjugate molecular weight, polymer mass distribution, degree and sites of PEG modification, positional isomers, and other physiochemical properties.[75−91] LC-MS/MS, however, has been more difficult to apply for the quantitative analysis of PEGylated proteins because of the generation of broad continuous mass spectrum due to the size and charge

Table 2. Advantages and Disadvantages of PEG-Quantification Methods

	advantages	disadvantages
Colorimetric method	1 Simple assays based on turbidity or chromophore formation between chemical agents and PEG 2 Suitable for monitoring PEGylation reactions	1 Relatively insensitive and may produce erratic results in the presence of plasma proteins or detergents 2 Not generally applicable for analysis of pharmacokinetic samples 3 Sample pretreatment required for PEGylated compounds that partition poorly into organic phase
HPLC	1 High reproducibility and sensitivity 2 Appropriate for PEGylated drug-containing liposomes and drug conjugates 3 LC-MS/MS measure peptide-PEG and protein-PEG conjugates at low ng/mL concentrations	1 Usually requires multiple processing steps to reduce sample complexity before analysis 2 Limited throughput compared to multiplex methods 3 Extensive method development required for PEGylated peptides and proteins 4 Sophisticated equipment may be required
Radiolabeling	1 Highly specific and sensitive 2 Simple analysis and high throughput 3 Compatible with complex biological samples 4 May allow noninvasive imaging of compound biodistribution	1 Safety and disposal issues 2 Radioactivity may be released from compounds (ie., deiodination) 3 Pharmacokinetic properties of compounds may be altered
Bioactivity assay	1 Can directly quantify PEGylated molecules in complex biological samples 2 Can assess the bioactivity and function of PEGylated molecules	1 PEG can affect interactions and catalytic activity of substrates and enzymes, resulting in lower detection sensitivity 2 Requires an appropriate and sensitive bioassay 3 Assays using a complex biological system may introduce experimental uncertainty 4 Endogenous compounds may interfere with the assay
Protein ELISA	1 Can directly quantify PEGylated molecules in serum samples 2 Simple analysis and high throughput 3 Can assess the binding activity of PEGylated antibodies or the native state of PEGylated proteins	1 PEG chains usually mask the epitopes of proteins, which can decrease detection sensitivity 2 ELISA assay using antiprotein antibodies cannot differentiate between PEGylated and non-PEGylated molecules
Anti-PEG ELISA	1 Can directly quantify PEGylated molecules in serum sample 2 Simple analysis and high throughput 3 No need to develop specific antibodies against the compound of interest such as peptides, proteins or drugs 4 Provide a universal assay format for nearly all PEGylated compounds	1 Cannot recognize the integrity of PEG-compounds, if the compounds were hydrolyzed or decomposed 2 Assay sensitivity is decreased for molecules containing a single short PEG molecule

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Table 3. HPLC Quantitation of PEG and PEGylated Molecules

HPLC type	sample preparation	separation column	mobile phase	analytes	sensitivity	ref.
Refractive index HPLC (RI-HPLC)	Lyophilization and chloroform extraction	Styrene divinylbenzene beads, 5 μm particles (Polymer Laboratories, Amherst, MA)	Water/methanol	PEG 400 in urine	4 mg/mL	66
RI-HPLC	0.22 μm filtration	Styrene divinylbenzene 250 mm × 4.6 mm, 5 μm particles (Polymer Laboratories, Amherst, MA)	Water/methanol	PEG 400 in urine	0.2 mg/mL	67
RI-HPLC	Size-based filtration, concentration, mixed ion-exchange (for PEG400)	TSK3000PW, TSK2000PW and TSK2000SW size-exclusion gel filtration columns (Tosoh Bioscience)	Water	PEG3350 and PEG400 in urine	5 μg/mL for PEG3350 50 μg/mL for PEG400	69
RI-HPLC	None	Protein KW803 300 mm × 8 mm, 5 μm particles and KW804 300 mm × 8 mm, 7 μm particles size-exclusion columns in tandem (Shodex)	20 mM HEPES, pH 6.5	PEG43,000 and PEGylated protein	5 μg/mL	70
RI-HPLC	Saturated with ammonium sulfate, dichloromethane extraction	μBondapak C18 reversed-phase 300 mm × 3.9 mm, 10 μm particles (Waters Corporation)	Water/methanol	PEG400 in urine	0.25 mg/mL	68
UV based HPLC	Introduce dibenzoate group to PEG, chloroform extraction	Nucleosil 10 C18 reversed-phase 250 mm × 4.6 mm, 10 μm particles (Macherey-Nagel) or Spherisorb 10 ODS reversed-phase 250 mm × 4.6 mm, 10 μm particles (Waters Corporation)	Water/methanol or water/acetonitrile gradient	PEG600 in urine	1 μg per injection	60
UV based HPLC	4-hydroxybenzaldehyde precipitation, methanol extraction	CAPCELL PAK C18 reversed-phase 250 mm × 4.6 mm, 5 μm particles (Shiseido Fine Chemicals)	H_3PO_4/water/acetonitrile gradient	PEG4700-PUE	80 ng/mL	73
UV based HPLC	Triton X-100 extraction, 5-sulfosalicylic acid precipitation	Discovery RP-amide C16 reversed-phase 250 mm × 3 mm, 5 μm particles (Supelco)	0.05 M sodium acetate/acetonitrile	Doxorubicin and PEG2000 liposomal doxorubicin	10 ng/mL	62
Fluorescence based HPLC	Trifluoroacetic acid precipitation, evaporative concentration	Juniper C18 reversed-phase 150 mm × 2 mm, 5 μm particles (Phenomenex)	Ammonium acetate/acetonitrile gradient	PEG40K-SN38, SN38 or CPT-11 in human serum	10 ng/mL	63,74
Fluorescence based HPLC	Acetonitrile precipitation, evaporative concentration.	Diamonsil C18 reversed-phase 200 mm × 4.6 mm, 5 μm particles (Dikma)	Formic acid/ammonia/water/methanol	PEG2000 liposomal doxorubicin and micelle doxorubicin	5 ng/mL	61
HPLC/MS/MS	Meta-phosphoric acid precipitation, chloroform extraction	TSK-GEL 300 mm × 7.8 mm, 6 μm particles (Supelco)	Ammonium acetate/acetonitrile	PEG3350 in urea or plasma	30 ng/mL in urine, 100 ng/mL in plasma	72
LC-MS/MS	Proteins were denatured and alkylated, enriched on 96 well SPE C18 plate, filtered, trypsin digested, cleaned up on 96-well Oasis MCX plate.	ACE C8 reversed-phase 50 mm × 2.8 mm, 5 μm particles (Advanced Chromatography Technologies)	Formic acid/water/acetonitrile gradient	Pegasys	3.6 ng/mL	92
LC-MS/MS	Acidic isopropanol precipitation, extraction, trypsin digestion	Zorbax Eclipse XDB C8 column reversed-phase 500 mm × 2.1 mm, 3.5 μm particles (Agilent)	Formic acid/water/methanol gradient	PEGylated protein	10 ng/mL	195
LC-MS/MS	Plasma proteins precipitated with PEG6000, immunoaffinity purification, filtration, concentration, trpsin digestion	Wide-pore Zorbax 300SB C18 reversed-phase 50 mm × 1.0 mm, 3.5 μm particles (Agilent)	Water/acetonitrile/formic acid gradient	Mircera	0.3 ng/mL	94
LC-MS/MS	Extraction on a 96-well SPE plate, gas-phase dePEGylation by in-source collision-induced dissociation	Acquity UPLC BEH C18 reversed-phase 50 mm × 2.1 mm, 1.7 μm particles (Waters)	Formic acid/acetonitrile/water gradient	human calcitonin peptide receptor antagonist conjugated with PEG20000	5 ng/mL	95

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distributions of PEG and proteins. However, utilization of clever pretreatment steps before analysis to reduce sample complexity is allowing utilization of LC-MS/MS for analysis of larger PEGylated compounds. For example, measurement of Pegasys in human serum samples was accomplished by denaturing and alkylating proteins, enriching Pegasys on 96-well solid-phase extraction C18 plates, enzymatically digesting the eluants and concentrating proteins on a 96-well Oasis MCX plate before separation on an ACE C8 column and analysis by LC-MS/MS of a signature peptide derived from the target protein. The quantitation limit of this assay was 3.6 ng/mL.[92] Similarly, the concentration of an investigational peptide linked to a 40 kDa PEG in monkey plasma was accomplished by acidic isopropanol precipitation and tryptic digestion to produce a unique tryptic peptide arising from the PEGylated therapeutic protein for detection by LC-MS/MS with a lower limit of quantitation of 10 ng/mL on a Zorbax Eclipse XDB C8 column.[93] Mircera (C.E.R.A., a continuous erythropoietin receptor activator conjugated with a single 30 kDa PEG chain) could be detected in horse plasma at a concentration of 0.3 ng/mL by an optimized process entailing pretreatment of equine plasma with PEG 6000 to precipitate proteins, immunoaffinity collection of Mircera on magnetic beads coated with specific antibodies, buffer exchange, tryptic digestion, separation on a wide-pore Zorbax 300SB C18 column under gradient elution conditions and MS/MS analysis of a signature tryptic peptide.[94] The concentration of an investigational human calcitonin peptide receptor antagonist conjugated with PEG20,000 in cynomolgus monkey serum was also determined with a lower limit of quantitation of 5 ng/mL by performing solid phase extraction on a 96-well solid phase extraction plate before gas phase dePEGylation by in-source collision-induced dissociation to generate surrogate peptide fragments for quantitative analysis of the peptide by LC-MS/MS.[95]

A drawback of LC-MS/MS for PEGylated protein and peptide determination is the need to develop appropriate conditions for cleanup and analysis for each protein (Table 2). In general, protein precipitation in an organic solvent, solid phase extraction or affinity purification can be employed to deplete serum proteins and concentrate PEGylated proteins before enzymatic digestion and analysis. Methods for sample cleanup and preparation for HPLC analysis have been recently reviewed.[96−98] Besides the need for sample cleanup, the presence of PEG chains may also sterically hinder tryptic digestion and quantitative generation of small peptides.[94] In addition, LC-MS/MS may overestimate PEGylated protein concentrations due to detection of intact surrogate peptides from dePEGylated peptides as well as other truncated metabolite species present in clinical samples.[99]

RADIOLABELING

Radiolabeling offers a straightforward and sensitive method to determine the pharmacokinetics of PEG and PEGylated compounds. Typically, a radioisotope (a radionuclide tracer atom) is included in the chemical composition of PEG or PEGylated molecules, and is administrated in small amounts to minimize interference with the experimental system. The radionuclide atom continually emits radiation which can be detected and quantified by a gamma counter to monitor X-ray and gamma-ray emissions or by a scintillation counter (also known as an α/β counter) to monitor α and β particle emissions. A variety of radioisotopes have been employed to measure PEG and PEGylated compound pharmacokinetics (Table 4).

Because methoxy-PEG is difficult to directly label, it is usually necessary to use other functional end groups for radiolabeling.

Table 4. Radioisotopes Commonly Used to Measure PEG and PEGylated Compound Pharmacokinetic Properties

radioisotope	emission type	half-life	detector
^{18}F	gamma-ray	109.8 min	gamma counter
^{32}P	β particle	14.3 days	liquid scintillation counter
^{59}Fe	β particle gamma-ray	44.5 days	liquid scintillation counter gamma counter
^{64}Cu	β particle gamma-ray	12.7 days	liquid scintillation counter gamma counter
^{99m}Tc	gamma-ray	6 h	gamma counter
^{111}In	gamma-ray	2.8 days	gamma counter
^{123}I	gamma-ray	13.3 h	gamma counter
^{125}I	gamma-ray	59.4 days	gamma counter
^{186}Re	gamma-ray	3.7 days	gamma counter

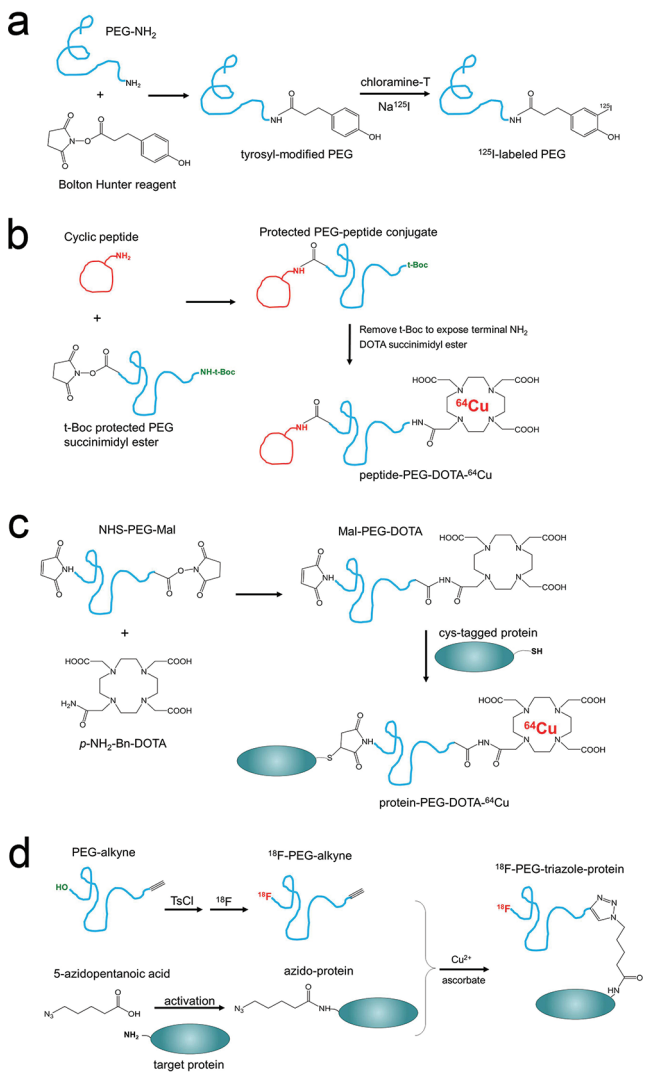

Figure 2. Radiolabeling of PEG for pharmacokinetic analysis. (a) Amino-PEG is reacted with Bolton-Hunter reagent to introduce a tyrosyl moiety for subsequent iodination by the chloramine-T procedure. (b) A t-Boc protected PEG succinimidyl ester is reacted with the primary amine group of a cyclic peptide. Removal of the t-Boc protective group allows reaction of DOTA succinimydyl ester to the terminal amine group on PEG to form a peptide-PEG-DOTA conjugate for ^{64}Cu labeling. (c) 2-(4-Aminobenzyl)-DOTA is reacted with NHS-PEG-Mal, which is then reacted to the cysteine-tag in the protein for chelation of ^{64}Cu. (d) A PEG-alkane labeled with ^{18}F and a azido-derivatized protein are combined in a click reaction to form a ^{18}F-PEG-triazole-protein conjugate.

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Table 5. Radiolabeling Methods for the Quantitative Measurement of PEG and PEGylated Molecules

isotopes	PEG compounds	labeling method	measurement	ref.
^{125}I	PEG 3−190KDa	Conjugate tyramine to terminal hydroxyl groups of PEG molecules and then label ^{125}I to the tyramine residues by the chloramine T method	Directly detect by a gamma counter	101
^{125}I	PEG 5−20KDa	Conjugate Bolton Hunter reagent to terminal amine groups of PEG molecules and then label ^{125}I by the chloramine T method	Directly detect by a gamma counter	100
^{125}I	PEG2K-RGD peptide	Directly label ^{125}I to the tyrosine residue of RGD peptide or PEG-RGD peptide by the chloramine T method	Directly detect by a gamma counter	102
^{123}I ^{127}I	PEG10K- cholecystokinin	Directly label ^{123}I or ^{127}I to the tyrosine residue of cholecystokinin by the chloramine T method	Directly detect by a gamma counter	108
^{125}I	PEG-superoxide dismutase (PEG-SOD)	Directly label ^{125}I to the tyrosine residue of PEG-SOD by the chloramine T method	Directly detect by a gamma counter	103
^{125}I	Somavert (B2036-PEG)	Directly label ^{125}I to the tyrosine residue of B2036-PEG by the chloramine T method	Radioimmunoassay & gamma counter	158
^{111}In	PEG-LLP2A peptide	Conjugate DOTA to the amine group of PEG-LLP2A peptide and label with ^{111}In	Directly detect by a gamma counter	196
^{111}In	PEG2000-anti-EGFR antibody (PEG-528MAb)	Labeling 528Mab with PEG2000 and DTPA, followed by labeling with ^{111}In	Directly detect by a gamma counter	109
^{111}In	PEG3400-anti-EGFR antibody (PEG-C225)	A heterofunctional PEG with one end attached to DTPA, and the other end attached to C225, followed by labeling with ^{111}In	Directly detect by a gamma counter	114
^{111}In	PEG-immunoliposome	Incorporate ^{111}In-DTPA in PEGylated liposomes	Directly detect by a gamma counter	121
^{111}In	Lymphoma-binding peptide-PEG-DOTA	Peptide bearing a branched chain amine-terminated PEG chain was reacted with *p*-isothiocyanatobenzyl-DOTA to allow labeling with ^{111}In	Directly detect by a gamma counter	113
^{64}Cu	RGD-PEG3400-DOTA	React DOTA sulfosuccinimydyl ester to the terminal amine group of NH_2−PEG attached to a cyclic RGD peptide to allow labeling with ^{64}Cu	Directly detect by a gamma counter	111
^{64}Cu	EGF-PEG5000-DOTA	React 2-(4-aminobenzyl)-DOTA to NHS-PEG5000-Mal, react DOTA-PEG5000-Mal to a cysteine-tag in EGF for chelation of ^{64}Cu	Directly detect by a gamma counter	115
^{64}Cu	scVEGF-PEG5000-DOTA	Label 2-(4-aminobenzyl)-DOTA to NHS-PEG5000-Mal, react DOTA-PEG5000-Mal with the cysteine-tag in scVEGF for chelation of ^{64}Cu	Directly detect by a gamma counter	116
^{64}Cu	PEG-liposome	A ^{64}Cu specific chelator, BAT, was conjugated to an artificial lipid to form a BAT-PEG-lipid	Directly detect by a gamma counter	124
^{64}Cu	PEG-liposome	Conjugate bifunctional ^{64}Cu chelators, TETA-PDP and CB-TE2A-PDEA, to liposomes via attachment to a maleimide lipid	Directly detect by a gamma counter	125
^{64}Cu	SPIO core coated with DOTA-PEG-DSPE	NHS-DOTA reacted with NH_2−PEG-DSPE on SPIO cores to allow labeling with ^{64}Cu	Directly detect by a gamma counter	128
^{99m}Tc	PEG-liposome	Label ^{99m}Tc to the HYNIC derivative of DSPE and incorporate into PEG-liposomes	Directly detect by a gamma counter	123
^{99m}Tc	PEG-liposomal doxorubicin (Doxil)	Use ^{99m}Tc-BMEDA complex loading to label Doxil	Directly detect by a gamma counter	127
^{186}Re	Doxil	Use an ammonium sulfate gradient to load ^{186}Re-BMEDA complexes into Doxil	Directly detect by a gamma counter	120
^{18}F	RGD dimer peptide with 4 arm PEG linkers	^{18}F-fluoropropionate reacted with NH_2−PEG-peptide conjugate	Directly detect by a gamma counter	112
^{18}F	Fluoro-PEG-Triazole-$E(RGDyK)_2$	Cu(I)-catalyzed Huisgen cycloaddition to label RGD peptides with ^{18}F by forming 1,2,3-triazoles	Directly detect by a gamma counter	118
^{18}F	PEG-liposome	Incorporate 3-[^{18}F]fluoro-1,2-dipalmitoylglycerol into PEG-liposomes	Directly detect by a gamma counter	122
^{32}P	PEI(-PEG)/siRNA complex	siRNA was [^{32}P] end-labeled using T4 polynucleotide kinase	Directly detect by a scintillation counter	129
^{3}H	PEG-liposome	[^{3}H]cholesteryl hexadecyl ether was incorporated in liposomes	Directly detect by a scintillation counter	197

Amine-PEG can be conveniently labeled with ^{125}I using Bolton Hunter reagent (Figure 2a).[100] Similarly, a tyramine group can be introduced to the terminal hydroxyl group of PEG to allow radiolabeling with ^{125}I by the chloramine T method (Table 5).[101]

For the pharmacokinetic study of PEGylated peptides, radioiodine can be conveniently incorporated if tyrosine residues are present in the polypeptide backbone. A RGD peptide conjugated with PEG2000 was labeled with ^{125}I by the chloramine T method for pharmacokinetic and biodistribution studies.[102] Likewise, plasma and brain levels of PEG-conjugated superoxide dismutase (PEG-SOD), which is being explored as an agent to reduce oxygen radical-mediated damage following brain injury in normal and fluid percussion rats, were determined by gamma counter detection of ^{125}I-labeled PEG-SOD.[103] Similar experimental procedures were also used for the pharmacokinetic study of ^{125}I-labeled PEG-salmon calcitonin,[104] ^{125}I-labeled PEG-recombinant human growth hormone,[105] ^{125}I-labeled PEG-recombinant human interleukin-6,[93] and ^{125}I-labeled PEG-recombinant tissue plasminogen activator.[106] Because radioiodinated peptides are susceptible to deiodiniation, complementary methods such as HPLC should be employed to confirm that radioactivity remains associated with the PEGylated peptide.[107,108]

Labeling of PEGylated proteins that do not possess accessible tyrosine residues can be accomplished by introducing a chelating agent into the protein for subsequent binding of radiometals such as ^{111}In, ^{99m}Tc, ^{186}Re, or ^{59}Fe. Reactive derivatives of chelating agents such as 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (DOTA) and diethylenetriaminepentaacetic acid (DTPA) are commercially available for labeling the primary amine group (−NH_2) in lysine or the thiol

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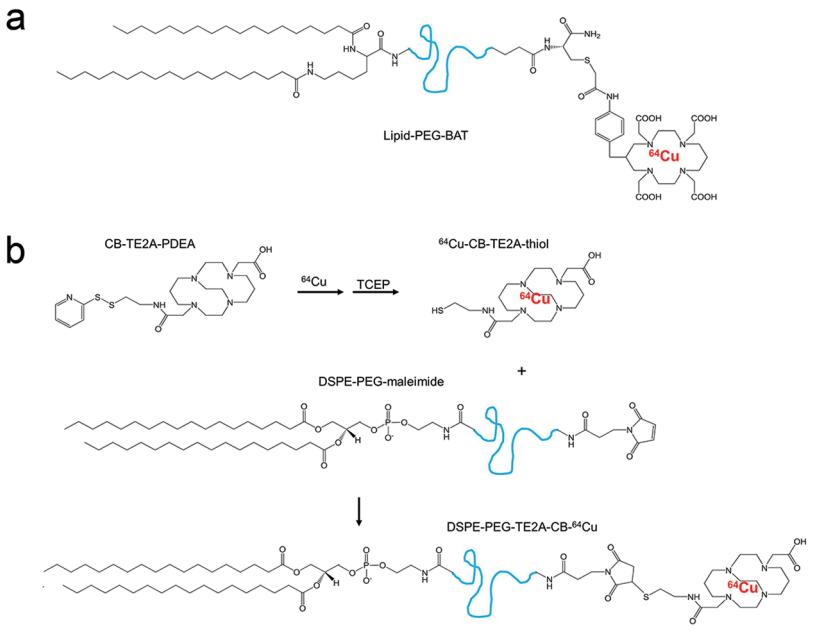

Figure 3. Surface labeling of PEGylated liposomes. (a) 6-[*p*-(Bromoacetamido)benzyl]-1,4,8,11-tetraazacyclotetradecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (BAT) is attached to stearic acid via a PEG spacer to allow anchorage of ^{64}Cu on liposomes for PET imaging. (b) CB-TE2A-PDEA is loaded with ^{64}Cu at elevated temperature and then reduced to generate a sulfhydryl group for reaction with maleimide-PEG2000-DSPE to generate DSPE-PEG-TE2A-CB−^{64}Cu for liposome labeling.

group (−SH) in cysteine. For example, Lee and colleagues sequentially modified an anti-EGFR antibody (528 mAb) with PEG2000 and DTPA dianhydride to allow labeling with ^{111}In for pharmacokinetic analysis of the PEGylated antibody.[109] ^{111}In conjugates prepared with DTPA anhydride, however, can produce intermolecular and intramolecular cross-linking and alter in vivo pharmacokinetics.[109,110]

Chelating agents can also be directly introduced to one end of a bifunctional PEG molecule to allow chelation of an isotope and covalent attachment to the target protein or peptide. To facilitate positron emission tomography (PET) imaging of brain tumor $\alpha_v\beta_3$ integrin expression, Chen and colleagues first reacted an amine-PEG-succinimidyl ester with a cyclic peptide followed by reaction of DOTA sulfosuccinimydyl ester to the exposed terminal amine group of NH_2−PEG for labeling with ^{64}Cu (Figure 2b).[111] Peptides possessing amine-terminated PEG chains can be reacted with *p*-isothiocyanatobenzyl-DOTA to allow labeling with ^{111}In or with *N*-succinimidyl 4-[^{18}F]fluorobenzoate to incorporate ^{18}F.[112,113]

Heterofunctional PEG molecules have also been synthesized with one end attached to the radiometal chelator^{111}In-DTPA and the other to a protected S-acetylthioacetate group. Deprotection of the S-acetylthioacetate allowed reaction of the resulting DTPA-PEG-SH to maleimide-activated anti-EGFR antibody (C225) for labeling with ^{111}In.[114] Site directed radiolabeling of proteins can be achieved by first introducing a cysteine tag into the target protein; Conjugates formed by reaction of 2-(4-aminobenzyl)-DOTA to the amine-reactive NHS group of NHS-PEG5000-Mal can be selectively reacted via the thiol-reactive maleimide to the cysteine-tag in the protein for chelation of radioactive metals such as ^{64}Cu (Figure 2c).[115,116]

Recently, the Cu(I)-catalyzed azide−alkyne cycloaddition of organo azides with terminal alkynes to form 1,2,3-triazoles, also known as the click reaction, is finding increasing use for labeling PEGylated peptides,[117] typified by utilization of a ^{18}F-labeled PEG-aldehyde to label a dimeric RGD peptide, resulting in favorable pharmacokinetic properties for tumor imaging (Figure 2d).[118] A variety of alkyne-PEG and azide-PEG derivatives are now commercially available for use in click reactions (i.e., Nanocs, Creative PEGWorks, Iris Biotech GMBH).

Radiolabeling of PEGylated liposomes and micelles is useful for biodistribution and pharmacokinetic studies.[119−121] In general, radioisotopes can either be attached to the surface of a liposome or trapped inside the liposome. Two major approaches have been employed to label the surface of liposomes: membrane-soluble chelating agents can be used to anchor metallic radioisotopes on the liposome surface, or radiolabeled chelating agents can be covalently linked to the surface of liposomes. Torchilin and colleagues anchored ^{111}In-DTPA-sterylamide in PEGylated liposomes to study in vivo half-life and accumulation in infracted rabbit myocardium.[121] Incorporation of 3-[^{18}F]fluoro-1,2-dipalmitoylglycerol into PEGylated liposomes allowed PET imaging and determination of liposome biodistribution in rats.[122] Laverman and colleagues incorporated a hydrazine nicotinyl (chelator) derivative of distearoylphosphatidyl-ethanolamine (DSPE) into PEGylated liposomes for subsequent labeling with ^{99m}Tc.[123]

Besides lipid insertion methods, PEGylated liposomes can also be labeled by covalent attachment of a chelator to the liposome surface. 6-[*p*-(Bromoacetamido)benzyl]-1,4,8,11-tetraazacyclotetradecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (BAT) was attached to stearic acid via a PEG spacer to allow anchorage of ^{64}Cu on liposomes for PET imaging (Figure 3a).[124] The longer half-life of ^{64}Cu (12.7 h) as compared to ^{18}F (110 min) can facilitate longer biodistribution studies. ^{64}Cu can also be linked to liposomes via the disulfide protected chelators (6-(6-(3-(2-pyridyldithio)propionamido)hexanamido)benzyl)-1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA-PDP) or 4-(2-(2-pyridyldithioethyl)ethanamido)-11-carboxymethyl-1,4,8,11-tetraazabicyclo(6.6.2)hexadecane (CB-TE2A-PDEA).

These chelators require loading of ^{64}Cu at elevated temperature before the disulfide group is reduced and reacted with the maleimide group on the commercially available maleimide-PEG2000-DSPE (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine) lipid on the surface of the liposomes (Figure 3b).[125]

A disadvantage of surface labeling of liposomes is the possibility that the surface modifications may change the in vivo behavior of the liposomes, and it may be argued as to whether these surface-altered liposomes can represent the clinically used liposomal therapeutic drugs. In fact, it has been observed that the location of a ^{64}Cu-chelator complex can alter liposome uptake in the liver and spleen.[124] To reduce the surface effects of radiolabeling liposomes, radioisotopes can be introduced inside liposomes by remote loading in which a chemical gradient is formed between the inner and outer space of the liposome. A commonly employed labeling method utilizes glutathione encapsulated liposomes to load and trap ^{99m}Tc-*N*,*N*-bis(2-mercaptoethyl)-*N*′,*N*″-diethyl-ethylenediamine (^{99m}Tc-BMEDA) with good efficiency and stability (Figure 4).[126]

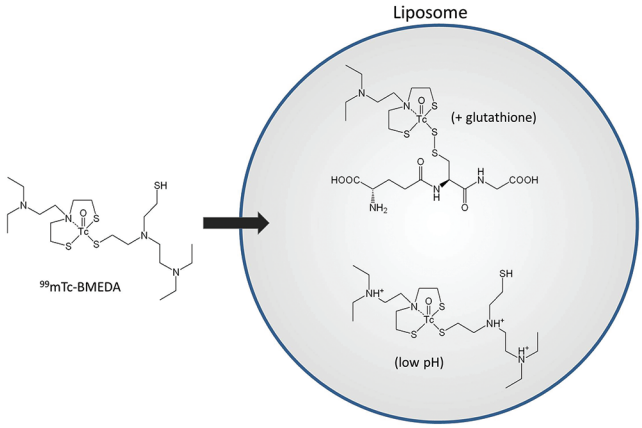

Figure 4. Internal labeling of liposomes. Lipophilic BMEDA can shuttle radioactive metals (^{99m}Tc, ^{86}Re) into liposomes. Radioactive BMEDA complexes are retained inside liposome by conversion of BMEDA to a more hydrophilic species, either by reaction with preloaded glutathione or by protonization by a low pH environment inside liposomes.

Tc-BMEDA and ^{186}Re-BMEDA can also be directly loaded into the commercially available PEGylated liposomal doxorubicin, Doxil,[120,127] allowing determination of liposome pharmacokinetics and biodistribution.

Other PEGylated compounds can also be radiolabeled for pharmacokinetic studies. Superparametric iron oxide nanoparticles coated with amine-PEG-DSPE were reacted with DOTA-NHS-ester to append the chelating agent at the amine group at the PEG termini for binding of ^{64}Cu. The biodistribution of these nanoparticles in mice could be followed by both PET and magnetic resonance imaging (MRI).[128] PEGylated nucleotides, such as small interfering RNAs (siRNA), can be conveniently end-labeled with ^{32}P using T4 polynucleotide kinase and γ-[^{32}P]ATP to allow measurement of siRNA-PEG biodistribution and pharmacokinetics.[129]

Radiolabeling offers highly sensitive and specific measurement of PEGylated compounds in vivo (Table 2). In addition, they offer the advantage of allowing noninvasive visualization of biodistribution by multiple imaging methods. The major disadvantages include possible alterations of the physical properties of the pharmaceutical and additional safety and cost issues associated with radioisotope handling and disposal.

BIOACTIVITY ASSAYS

Bioactivity assays can be employed for quantitation of PEGylated molecules in serum samples if a suitable and sensitive bioassay is available. PEGylated enzymes are particularly suited to this approach since a wide variety of detectable substrates are commercially available. For example, Hoffmann and colleagues measured PEG-hirudin (thrombin inhibitor) in blood by a chromogenic method based on inhibition of thrombin hydrolysis of the substrate S-2238.[130,131] Similarly, Hempel and colleagues measured the pharmacokinetics of PEG-asparaginase based on the generation of indooxine for photometric detection at 710 nm.[132] Enzyme assays have also been employed to measure PEG-uricase,[133,134] antibody-β-glucuronidase-PEG conjugates,[5,135] PEG-organophosphorus hydrolase,[136] PEG-recombinant human acetylcholinesterase hypolysine variants,[137] and PEG-arginine deiminase[138] in serum samples.

PEGylated cytokines often can be assayed in clinical samples by established bioassays. The proliferation of an IL-2 dependent HT2 murine cell line was used to measure the serum pharmacokinetics of PEGylated-IL-2.[139] The pharmacokinetic properties of PEGylated IL-2 have also been measured using the cytopathic effect assay (CPE assay) in which the protective effect of IL-2 against vesicular stomatitis virus challenged MDBK cells are determined.[140] Likewise, PEGylated human IFN-β-1a pharmacokinetics in mice, rats, and monkeys was determined by assessing the antiviral activity of samples against A549 cells exposed to encephalomyocarditis (EMC) virus.[141] The pharmacokinetics of PEGylated GM-CSF in Sprague–Dawley rats were determined by measuring the proliferative effects of serum samples on C3H/He mouse bone-marrow derived cells.[142] François and colleagues developed an interesting bioassay for the measurement of PEGylated interferon based on the IFN-α induced activation of a MxA promoter-chloramphenicol acetyltransferase reporter gene.[143]

There are several considerations when utilizing bioassays for pharmacokinetic studies. An appropriate and sensitive bioassay is required for the compound of interest. The assay should provide a specific readout for the PEGylated compound without interference by related compounds. For measurement of human proteins such as enzymes and cytokines, one must ensure that endogenous and related compounds are not detected by the assay.[143] Bioassays tend to lack high reproducibility due to the reliance on a complex biological system for the readout. Attachment of PEG chains to proteins and peptides can also reduce their biological activity and hinder receptor binding,[144,145] which can decrease assay sensitivity. For example, PEG chain on proteins can decrease their apparent association rate when assayed by surface plasmon resonance.[146] Also, Pegasys displayed only 7% of wild-type activity in a cytopathic effect assay measuring antiviral activity.[147] Likewise, PEG-modified granulocyte colony stimulating factor displayed greatly reduced activity in a cell proliferation assay.[148] In some cases, PEGylation can even alter the spectrum of a proteins biological activity.[149,150] Finally, biological assays assume that the PEGylated compound displays constant activity in vivo. Alterations in biological activity with time (i.e., dePEGylation, interaction with inhibitors, degradation) may affect biological activity and provide false results.[151] These caveats suggest that bioassays are most suitable for in vitro determination of the biological activity of PEGylated compounds and are generally less desirable for in vivo pharmacokinetic studies if alternative analytical approaches are available.

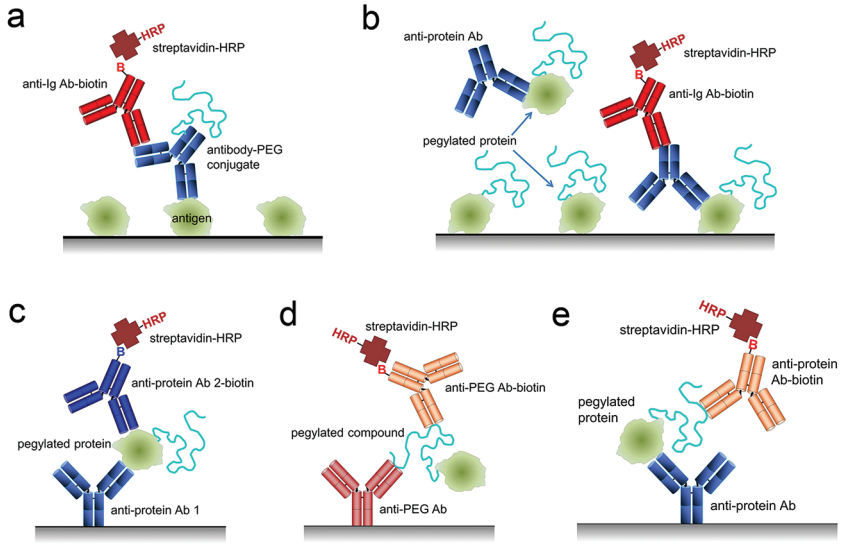

Figure 5. ELISA detection of PEGylated compounds. (a) In direct ELISA, PEGylated antibody binding to antigen-coated microtiter wells is detected with biotin-labeled anti-immunoglobulin antibody and streptavidin-HRP. (b) In competition ELISA, PEGylated protein (or any PEGylated compound) is coated in wells of microtiter plates. The concentration of PEGylated compound in samples is determined by measuring how completely the binding of antiprotein antibodies to the coated antigen is blocked (competed) by the soluble protein. (c) In sandwich ELISA, antibodies that bind distinct epitopes on the PEGylated protein are employed to capture and detect the PEGylated protein. (d) In sandwich PEG ELISA, anti-PEG antibodies are employed for both capture and detection of the PEG portion of the PEGylated compound. Note that this assay can use the same antibody for both capture and detection. (e) In combination sandwich ELISA, the PEGylated protein is captured by an antiprotein antibody coated in microtiter plate wells and detected by a biotin-conjugated anti-PEG antibody.

■ DIRECT ELISA

Direct enzyme-linked immunosorbent assay (direct ELISA) is useful for measuring the concentration of PEGylated antibodies in serum samples. In this approach, a specific antigen is coated in the wells of microtiter plates for capture of PEGylated antibodies in the sample (Figure 5a). The amount of antibody bound to the antigen is then determined by adding a detection antibody, typically a horseradish peroxidase (HRP) conjugate. For example, a PEGylated humanized antitumor necrosis factor (TNF) Fab fragment (CDP870) developed for treatment of rheumatoid arthritis (RA) was captured in wells coated with recombinant human TNF-α and then detected with HRP-conjugated goat antihuman κ light chain antibody followed by addition of tetramethyl benzidine (TMB) substrate.[152] Direct ELISA employing antigen-coated microtiter plates were also used to measure the pharmacokinetics of a PEGylated single chain Fv fragment in monkeys[153] and the pharmacokinetics in rats of a PEGylated Fab′ fragment of an antibody against LINGO-1.[154]

Direct ELISA can also be employed to measure the concentration of other PEGylated compounds by using a competition assay format. PEGylated compound is typically coated in the wells of a microtiter plate. The degree that serum samples block binding of a specific antibody to plate-coated PEGylated compound provides a measure of the serum concentration of the PEGylated compound (Figure 5b). Using this format, Song and colleagues measured serum PEG-hirudin at concentrations as low as 87 ng/mL by coating PEG-hirudin in microtiter plates and then determining the ability of serum PEG-hirudin to compete the binding of rabbit antihirudin to the immobilized antigen.[155] A similar ELISA was used to measure serum levels of PEGylated asparaginase with a limit of detection of 0.004 IU/mL[156,157] and PEGylated consensus interferon with a limit of quantification of 8.4 ng/mL in rhesus monkey serum.[151] The competition assay can also be performed by coating a specific antibody in the microtiter plate and then measuring competition of serum samples for radiolabeled or tagged antigen. Using this approach, Muto and colleagues detected serum concentrations of Somavert with a competitive binding radioimmunoassay in which serum Somavert competed with trace amounts of radiolabeled antigen (^{125}I-Somavert) for binding to rabbit anti-Somavert antibodies immobilized in microtiter plates.[158] A competitive ELISA using ^{3}H-radiolabeled peptide antigen was also employed to measure the pharmacokinetics of a PEGylated thromobopoietin peptide in rats and monkeys.[159] Although direct ELISA possesses the advantage of requiring only one specific antibody for compound detection, they may not be able to distinguish between PEGylated and dePEGylated metabolites.[151] Direct ELISA may also produce anomalous results if neutralizing antibodies are present in the sample. For example, the development of neutralizing antibodies in monkeys blocked binding of a PEGylated scaffold protein to antigen coated in microtiter plates, resulting in underestimation of serum levels of the PEGylated protein.[160]

■ SANDWICH ELISA

Sandwich ELISA is a powerful approach to measure the concentration of PEGylated conjugates in complex biological samples. Sandwich ELISA employs two antibodies, one to capture the analyte on a solid surface and a second to detect the concentration of the captured analyte (Figure 5c). The analyte must possess at least two antigen-binding sites, usually distinct binding epitopes on the analyte. However, the same epitope can be used for multimeric analytes since the capture and detection antibodies can still bind to the same molecule. Sandwich ELISA is usually more sensitive (ng/mL to pg/mL) and more specific (when using two distinct antibodies) than direct and competitive ELISA and is therefore a good choice when very low concentrations of analyte must be determined.

Bruno and colleagues analyzed the pharmacokinetics of Pegasys and PEG-Intron with a quantitative sandwich ELISA

Table 6. Quantitation of PEGylated Molecules by Protein-Specific Antibody-Based ELISA

PEGylated proteins	PEG length	ELISA type	Capture/detection layers	sensitivity	ref
Pegasys	40-kDa branched PEG	Sandwich ELISA	Mouse monoclonal antihuman IFN-α antibodies/HRP-conjugated antihuman-IFN-α antibody	125 pg/mL	161,162
PEG-Intron	12 kDa	Sandwich ELISA	Mouse antihuman interferon-alpha monoclonal antibody/ HRP-conjugated monoclonal anti-interferon-alpha antibody	100 pg/mL	163
Neulasta	20 kDa	Sandwich ELISA	Mouse monoclonal anti-G-CSF antibody/HRP-conjugated polyclonal anti-G-CSF antibody	166 pg/mL	165
Mircera	30 kDa	Sandwich ELISA	Mouse monoclonal anti-EPO antibody/HRP-conjugated rabbit anti-EPO polyclonal antibody	500 pg/mL	166
Cimzia	40 kDa	Direct ELISA	Recombinant human TNF-α/ HRP-conjugated goat antihuman κ light chain antibody	Not reported	152
NNC126−0083 (PEGylated recombinant Human Growth Hormone)	43 kDa	Sandwich ELISA	Mouse monoclonal anti-NNC126−0083 antibody/biotin-labeled rhGH-specific monoclonal antibody	Not reported	198
PEG-EPO	5 kDa	Sandwich ELISA	Mouse monoclonal anti-EPO antibody/HRP-conjugated rabbit anti-EPO polyclonal antibody	500 pg/mL	199
PEG-Hirudin	5 kDa	Competition ELISA	PEG-hirudin/rabbit IgG antihirudin antibody	87 ng/mL	155
PEG-asparaginase	5 kDa	Competition ELISA	Asparaginase/Rabbit anti-asparaginase serum + biotinylated goat antirabbit IgG	0.004 IU/mL	156,157

using two mouse monoclonal antihuman IFN-α antibodies that recognize different epitopes of IFN-α with a sensitivity of 125 pg/mL (Table 6).[161] Motzer and colleagues also applied this method in a phase II trial of Pegasys in patients with advanced renal cell carcinoma with an analytical sensitivity of about 125 pg/mL.[162] Similar sandwich ELISA approaches were used to measure PEG-Intron,[163] Neulasta,[164,165] and Mircera.[166] Eggermont and colleagues measured serum PEG-Intron concentrations in melanoma patient serum samples with a validated and highly sensitive electrochemiluminescent immunoassay with a lower limit of quantitation (LLOQ) of 40 pg/mL. PEG-Intron was captured on streptavidin paramagnetic beads via a biotinylated sheep polyclonal antibody against PEG-Intron and detected with an anti-PEG-Intron mouse monoclonal antibody labeled with ruthenium trisbipyridine chelate, which produces an electrochemiluminescent signal in the presence of tripropylamine in an ORIGIN analyzer.[167]

ELISA approaches possess important advantages over other analytical approaches including the ability to detect compounds in their native state (i.e., no need to radiolabel or otherwise modify the analyte), superb specificity, and sensitivity and compatibility with complex samples such as serum without the need to perform sample cleanup. However, ELISA assays using antiprotein antibodies generally cannot differentiate between PEGylated and non-PEGylated molecules and therefore assume that measurements represent the PEGylated compound. This can be solved in some cases by separating PEGylated from non-PEGylated proteins. For example, the pharmacokinetics of recombinant human leptin modified with a single branched 42 kDa PEG molecule were measured in human blood samples by sandwich ELISA using an antileptin antibody after size exclusion chromatography to remove unmodified leptin.[168] However, this greatly complicates assay of multiple samples. In addition, protein-specific antibody-based ELISA can be affected by the presence of PEG chains. PEG is well-known for its ability to decrease protein antigenicity, principally by shielding antigenic epitopes on the surface of proteins from antibody binding.[77] The shielding effect of PEG can also mask antibody-binding epitopes during ELISA, resulting in weaker interactions with capture and detection antibodies, thereby decreasing assay sensitivity. For example, attachment of branched 40 kDa PEG to IFN-α 2b significantly inhibited monoclonal antibody binding to IFN.[169] Antibody binding to PEGylated asparaginase was also significantly reduced by attachment of multiple linear 10 kDa PEG molecules and even more dramatically reduced by attachment of multiple 10 kDa branched PEG chains.[170] Generally, large, branched PEG molecules and attachment of multiple PEG chains to proteins more effectively shields antibody-binding epitopes, reducing assay sensitivity. For maximal accuracy, purified PEG-conjugate should be used for construction of the standard curve.

■ ANTI-PEG ELISA

The development of antibodies that specifically bind to PEG has allowed the development of highly sensitive and accurate ELISAs for PEGylated molecules. Richter and Akerblom were the first to generate antibodies against PEG by immunizing rabbits with PEG linked to ovalbumin, bovine superoxide dismutase, and ragweed pollen in Freund's complete adjuvant.[171] These polyclonal antibodies allowed detection of PEGylated proteins down to about 1 μg/mL. To overcome the inherent limitations of polyclonal antibodies, we[135,172−174] and others[175] generated mouse monoclonal antibodies that can specifically bind to PEG molecules (Table 7). Anti-PEG antibodies were generated by attaching PEG to highly immunogenic carrier proteins and immunizing mice multiple times to overcome the very low immunogenicity of PEG. For example, we developed two mouse monoclonal anti-PEG antibodies (AGP3/IgM and E11/IgG1) by sequentially immunizing mice with an antibody-PEG-β-glucuronidase conjugate.[135,173] Both antibodies bind the repeating subunits of the PEG backbone and can detect PEG-modified proteins by ELISA, immunoblotting, and flow cytometry.

Anti-PEG sandwich ELISA allows detection of PEGylated compounds by direct binding to PEG independent of the attached compound (Figure 5d). A sandwich ELISA in which E11/AGP3 were employed as the capture/detection antibodies was developed to detect PEG-modified proteins at concentrations as low as 1.2 ng/mL. The ELISA could also quantify, in the presence of 10% fetal bovine serum, PEG2000-quantum dots and PEG2000-liposomes at concentrations as low as 1.4 ng/mL (3.1 pM) and 2.4 ng/mL (3.13 nM phospholipids), respectively (Table 8). AGP3 and E11 have been widely used in proprietary assays throughout the pharmaceutical and biotechnology industries for pharmacokinetic measurements of PEGylated compounds.[176,177] An advantage of anti-PEG ELISA is general application to the detection of a wide variety of PEGylated compounds since the antibodies bind to PEG.

Table 7. Anti-PEG Antibodies

name	species	specificity	biotin version?	applications[a]	source[b]	refs
PEG-B-47	Rabbit IgG_1	Terminal methoxy group of PEG	Yes	ELISA, WB, FC	Epitomics	99,160,174,184,186
PEG-2−128−7	Rabbit IgM	PEG backbone	No	ELISA, WB	Epitomics	
AGP3	Mouse IgM	PEG backbone	Yes	ELISA, WB, FC, IHC	Academia Sinica	135,147,172−174,176−182,185,187,189−191
E11	Mouse IgG_1	PEG backbone	No	ELISA, WB, FC, IHC	Academia Sinica	135,172−174,176,177,188,192
AGP4	Mouse IgM	PEG backbone	Yes	ELISA, WB, FC, IHC	Academia Sinica	174,200
3.3	Mouse IgG_1	PEG backbone	Yes	ELISA, WB, FC, IHC	Academia Sinica	174
B139M	Mouse IgG_1	PEG backbone[c]	No	ELISA	Acris Antibodies	174
B141M	Mouse IgG_1	PEG backbone[c]	No	ELISA	Acris Antibodies	174

[a]ELISA, Enzyme-linked immunosorbent assay; WB, Western blot; FC, flow cytometry, IHC, immunohistochemistry. [b]Supplier Addresses: Epitomics, Inc., 863 Mitten Road, Suite 103, Burlingame, California; Academia Sinica, Academia Road, Section 2, No. 128, Taipei 11529, Taiwan; Acris Antibodies, Inc., 6815 Flanders Drive, Suite 140, San Diego, CA 92121. [c]Antibody binding is blocked by detergents containing PEG-like structures such as Tween-20.

Table 8. Anti-PEG Antibody-Based ELISA

PEGylated proteins	PEG length	capture/detection layers	sensitivity	ref
Pegylated quantum dots	2000	E11 anti-PEG antibody/AGP3 anti-PEG antibody and HRP-conjugated goat antimouse IgM μ chain antibody	1.4 ng/mL	173
Lipodox	2000	E11 anti-PEG antibody/AGP3 anti-PEG antibody and HRP-conjugated goat antimouse IgM μ chain antibody	2.4 ng/mL	173
PEG-β-glucuronidase	3400	E11 anti-PEG antibody/AGP3 anti-PEG antibody and HRP-conjugated goat antimouse IgM μ chain antibody	1.2 ng/mL	173
Pegylated thrombopoietin peptide	20 kDa	Rabbit antipeptide antibody/AGP3 anti-PEG antibody and HRP-conjugated antimouse immunoglobulin antibody	6.2 ng/mL	178,179
Mircera	30 kDa	Rabbit antierythropoietin antibody/AGP3 anti-PEG antibody and HRP-conjugated goat antimouse IgM μ chain antibody	150 pg/mL	180,181
Mircera	30 kDa	Rabbit antierythropoietin antibody/digoxigenin-labeled AGP3 anti-PEG antibody and HRP-conjugated antidigoxigenin Fab	30 pg/mL	181
PEG-human growth hormone	20 kDa	AGP3 anti-PEG antibody/sulfo-tag labeled goat anti-hGH antibody	0.5 ng/mL	182
PEG-human growth hormone	43 kDa	Anti-PEG antibody/anti-hGH antibody	Not specified	183
Lipodox	2000	AGP4 anti-PEG antibody/biotin-labeled 3.3 anti-PEG antibody and streptavidin-HRP	0.32 ng/mL	174
PEG-quantum dots	2000	AGP4 anti-PEG antibody/biotin-labeled 3.3 anti-PEG antibody and streptavidin-HRP	0.36 ng/mL	174
Pegasys	43 kDa branched	AGP4 anti-PEG antibody/biotin-labeled 3.3 anti-PEG antibody and streptavidin-HRP	0.32 ng/mL	174
PEG-Intron	12 kDa	AGP4 anti-PEG antibody/biotin-labeled 3.3 anti-PEG antibody and streptavidin-HRP	4.1 ng/mL	174
Neulasta	20 kDa	AGP4 anti-PEG antibody/biotin-labeled 3.3 anti-PEG antibody and streptavidin-HRP	1.2 ng/mL	174
PEG-peptide	40 kDa branched	Rabbit antipeptide antibody/10F05 anti-PEG antibody and HRP-labeled goat antimouse antibody	0.1 ng/mL	175

Anti-PEG antibodies can also be used in combination with antiprotein antibodies in sandwich ELISA format (Figure 5e). For example, a sandwich ELISA was used to measure a PEGylated thrombopoietin mimetic peptide (PEG-TPOm, RWJ-800088) in human serum samples by using an anti-thrombopoietin antibody for capture and AGP3 anti-PEG antibody for detection with a limit of detection of 6.25 ng/mL.[178,179] Mircera in human serum samples was measured by adding biotin-labeled rabbit polyclonal antibodies against human erythropoietin in commercially available microtiter plates precoated with streptavidin, adding serum samples, and then detecting with AGP3 anti-PEG antibody followed by peroxidase-conjugated goat anti-mouse IgM.[180,181] The assay allowed measurement of serum Mircera concentrations over the range 150−4000 pg/mL. Modification of the assay by using digoxigenin-labeled AGP3 followed by detection with peroxidase-conjugated polyclonal antidigoxigenin Fab fragment increased the dynamic detection range of the assay to 30−1000 pg/mL.[181] Myler and colleagues detected PEGylated human growth hormone (PEG-hGH) in growth hormone deficient patients in a sandwich electrochemiluminescent immunosorbent assay. Biotinylated AGP3 was bound to streptavidin-coated microtiter plates as the capture layer, whereas goat anti-hGH polyclonal antibody labeled with Sulfo Tag succinimidyl ester was used for detection by measuring electrochemiluminescence.[182] Interestingly, acid treatment of samples before analysis greatly increased assay specificity, presumably by dissociating PEG-hGH from serum resident growth hormone binding protein (GHBP), thereby reducing detection of endogenous GHBP by goat anti-hGB secondary antibody.

The pharmacokinetics of NNC126−0083, rhGH with a 43 kDa PEG molecule attached at glutamine 141, were determined in a validated sandwich ELISA using an anti-PEG antibody for capture and a biotin-labeled hGH-specific monoclonal antibody for detection.[183] Combined use of anticompound and anti-PEG antibodies allows highly specific detection of PEGylated compounds in complex biological samples.

We recently generated two second-generation monoclonal antibodies to PEG (AGP4/IgM and 3.3/IgG) that also bind to the repeating subunits of the PEG backbone but with greater affinity than AGP3 and E11.[174] A sandwich ELISA in which AGP4 was employed as the capture antibody and biotinylated 3.3 (3.3-biotin) was used as the detection antibody allowed quantification with detection limits of 0.79 pM for PEG-Qdot 525, 0.32 ng/mL for Lipo-Dox, 0.32 ng/mL for Pegasys, 4.1 ng/mL for PEG-Intron, and 1.2 ng/mL for Neulasta, respectively. 3.3-Biotin was also found to allow detection of several PEGylated compounds in combination with other commercially available monoclonal antibodies.[174]

Comparison of anti-PEG antibody binding to different sizes of PEG indicates that E11, AGP3, and AGP4 can bind PEG750 (~16 repeating oxyethylene repeat units) but binding increased for longer chain PEG molecules.[174] By contrast, the anti-PEG antibody 3.3 bound PEG2000 but did not bind to PEG750, indicating that 3.3 recognizes a larger antigen-binding epitope than the other antibodies. Both AGP4 capture and 3.3-biotin detection antibodies could bind to the same PEG chain as shown by the ability to detect PEG-Intron (possessing a single 12 kDa methoxy PEG), Neulasta (incorporating a single 20 kDa monomethoxy PEG), and Pegasys (with a single 40 kDa branched-chain methoxy PEG). Detection sensitivity was greater for proteins possessing longer PEG chains (Pegasys > Nuelasta ≫ PEG-Intron). Nanoparticles and liposomes decorated with multiple short PEG chains provided the highest assay sensitivities, likely due to binding of multiple anti-PEG antibodies to the surface of the particles. The anti-PEG ELISA was insensitive to the presence of 50% human serum, demonstrating suitability for analysis of human samples.

Wunderlich and colleagues also developed a mouse monoclonal anti-PEG antibody (10F05) by immunizing mice with keyhole limpet hemocynanin (KLH) conjugated with PEG-2000. 10F05 could recognize a PEGylated peptide conjugated with a branched 40 kDa PEG in serum at concentrations lower than 100 pg/mL.[175] Xu and colleagues quantified a 38 amino acid therapeutic peptide (MK-2662) modified with a single branched 40 kDa PEG in human plasma by an immunoaffinity purification method (IAP) using streptavidin-coated magnetic beads decorated with biotinylated rabbit monoclonal antibody PEG-B-47, which binds to the terminal methoxy group of mPEG along with 16 oxyethylene repeat units,[184] to concentrate PEGylated peptide followed by tryptic digestion and measurement of surrogate peptide concentration by LC-MS/MS with a dynamic range of 2−200 nM.[99] Preconcentration of pepylated peptides or proteins by anti-PEG antibody immunopurification is a promising method to replace precipitation and solid-phase extraction methods for LC-MS/MS analysis.

In addition to ELISA, anti-PEG antibodies are useful to characterize PEGylated compounds and for detection of PEG or PEGylated compounds in tissues. Anti-PEG antibodies can visualize PEGylated proteins and compounds on immunoblots.[173,174,185] For example, Bailon and colleagues characterized the integrity and predominant mono-PEGylation of Pegasys by immunoblotting using AGP3 anti-PEG antibody for detection.[147] Anti-PEG antibodies can also help visualize PEGylated compound in tissue sections.[186−188] Ton and colleagues used AGP3 anti-PEG antibody for immunohistochemical detection of tissue localization of CDP791, a PEGylated di-Fab′ conjugate that binds VEGFR-2, in a phase I clinical trial in patients with hemangioma.[187] White and colleagues studied the localization of PEGLA, a PEGylated leukemia inhibitory factor antagonist which can block the action of leukemia inhibitory factor, in the uterus by immunohistochemistry using E11 anti-PEG antibody for detection.[188]

For reasons not clearly understood, anti-PEG antibodies can strongly bind PEG molecules immobilized on a surface such as a protein, liposome, nanoparticle, or microtiter plate but bind less effectively to unconjugated PEG molecules in solution. Therefore, sandwich ELISA using anti-PEG antibodies cannot readily detect unconjugated PEG molecules.[174] By contrast, recombinant AGP3 anti-PEG Fab fragments expressed on the surface of 3T3 fibroblasts effectively bound both conjugated and unconjugated PEG molecules.[189−191] An anti-PEG cell-based competition ELISA allowed PEG quantification by measuring the ability of PEG to compete binding of biotinylated PEG5000 (CH_3-PEG5000-biotin) to the cells. The αPEG cell-based competition ELISA could measure the concentrations of PEG and small compounds linked to PEG2000, PEG5000, PEG10000, and PEG20000 with detection limits ranging from 3 to 60 ng/mL. The αPEG cell-based competition ELISA accurately delineated the pharmacokinetics of PEG5000, comparable to those determined by direct measurement of radioactivity in blood after intravenous injection of CH_3−PEG5K−^{131}I into mice. This quantitative strategy may provide a simple and sensitive method for quantifying PEG and PEGylated small molecules in vivo.

■ SUMMARY

Increasing numbers of pegylated proteins and small peptides are expected to reach preclinical and clinical trials in the near future. A multitude of nanoparticle PEGylated therapeutic and diagnostic agents are also under active development, indicating a continued demand for accurate, robust, and high throughput methods for the quantitative measurement of PEGylated compounds. Improved HPLC methods are expected to play an important role in the characterization of PEGylated compounds and increasingly be used for defining pharmacokinetic parameters of new PEGylated compounds. Anti-PEG ELISA or combined anticompound/anti-PEG ELISA should continue to play an important role in pharmacokinetic studies due to the high throughput, ability to assay complex biological samples, and good sensitivity and specificity of these immunological methods. Combination of technologies should facilitate creation of powerful assays as typified by the use of anti-PEG antibodies to capture PEGylated compounds for subsequent analysis by LC-MS/MS[99] or the combination of immunological and nanoscale technologies.[192] Adoption of established technologies and development of new methods will accelerate clinical translation of novel PEGylated agents such as carbon nanotubes, nanodiamonds, microbubbles, dendrimers, and other nanoparticles.[193]

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