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PEGYLATION: Novel Technology to Enhance Therapeutic Efficacy of Proteins and Peptides

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PEG 접합: 단백질 및 펩타이드 치료제의 약효를 증가시키는 새로운 기술

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ABSTRACT-Polyethylene glycol (PEG) is a water soluble, biocompatible, non-toxic polymer and PEGylation is a well established technique for the modification of therapeutic proteins and peptides. PEG-protein drugs have been extensively studies in relation to therapies for various diseases: cancer, inflammation and others. The covalent attachment of PEG to proteins and peptides prolonged plasma half-life, reduced antigenicity and immunogenicity, increased thermal and mechanical stability, and prevented degradation by enzymes. Several chemical groups for general and site specific conjugation have been exploited to activate PEG for amino group, carboxyl group, and cysteine groups. PEGylation of many proteins and peptides have been studied to enhance their properties for the potential uses. Also, the different positional isomers in several PEG-proteins have shown the difference *in vivo* stability and biological indicating that the site of PEG molecule attachment is one of the important factor to develop PEG-proteins as potential therapeutic agents.

Keywords-Polyethylene glycol, Conjugation, Therapeutic proteins and peptides, Activated PEG derivatives, Site-specific modification

The development of protein or peptide drugs for therapeutic purpose has generated a great deal of interest in methods of enhancing their delivery. In general, there are a number of problems to associate with the use of protein therapeutics. First, most parentally administered proteins are rapidly cleared from the blood circulation by the reticuloendothelial system (RES), kidney, spleen, or liver. The systemic clearance depends on the molecular size (relative to the cut-off for glomerular filtration), charge, attached carbohydrate chains, and the presence of cellular receptors for the protein. Metabolism by proteases/peptidases also leads to the rapid loss of biological activity and degradation. Second, proteins of bacterial origin are immunogenic, and even with recombinant human proteins immune responses are elicited after repeated use, which cause life-threatening hypersensitivity reactions.[1] Modification of proteins or peptides with polymers can provide many benefits for both in vivo and in vitro applications for alteration and control of biodistribution, pharmacokinetics and often toxicity of these compounds.[2] A variety of polymers has been attempted to achieve each of these objectives. The general requirements of any polymer used for this purpose are that it be water-soluble, biocompatible, non-immunogenic, and devoid of biological activity. Of the chemical modification technologies explored to date, PEG-modification appeared to be the most promising. Polyethylene glycol (PEG) is one of the most popular polymeric materials used for this purpose. PEG coupled to other molecules can be prepared in the various classes and their applications are listed in Table I.[3]

In this review, PEG coupling methods as well as site specific PEGylation methods will be described. Recent PEGylation studies of proteins and peptides investigated by several groups will be also reviewed.

Polyethylene Glycol Conjugates

PEG is FDA approved polymer for internal use in drugs, non-immunogenic, and a linear polymer based on the HO

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Table. I–*Various Classes of PEG Conjugates and Their Applications*

Conjugates of	Useful properties and applications
Drugs [6,7]	improved solubility, controlled permeability through biological barriers, longevity in blood stream, controlled release
Affinity ligands [5,8]	Used in aqueous two-phase partitioning systems for purification and analysis of biological macromolecules, cells
Cofactors[9-11]	bioreactors with continuous regeneration and recycling of macromolecular cofactors
Saccharides[12]	new biomaterials, drug carriers
Oligonucleotides and analogs[13]	improved solubility, resistance to nucleases, cell membrane permeability
Liposomes and particulates[14]	longevity in bloodstream, RES-evasion
Biomaterials[15]	reduced thrombogenicity, reduced protein and cell adherence

$(CH_2CH_2O)_n$- CH_2CH_2OH repeat unit, and is prepared by ring-opening polymerization of ethylene oxide.[4] Each ethylene oxide residue has a molecular weight of 44 Da, and n x 44 Da represents the number average MW of the PEG chain. Most forms of PEG useful in bioconjugate applications have molecular weights of 500-20,000 and the polydispersity (M_w/M_n) ratio) is low, typically no greater than 1.05 for molecular weights up to 10,000.[5] The polymer backbone of PEG is inert in biological environment as well as in most chemical reaction conditions under which the end groups of PEG can be subjected to chemical modification and/or conjugation reactions. The properties of PEG in solution are especially unusually frequently displaying amphiphilic tendencies, having the ability to solubilize both in aqueous layers and in hydrophobic membranes or organic phases. Carpenter *et al.* studied the biocompatibility (toxicity and bioaccumulation) of PEG (4,000 daltons) in dogs after intravenous injections. They found no detectable toxic or cumulative effects of PEG after repeated injections of dogs ranging up to 90 mg/kg per day and the major portion of the polymer excreted in the urine within 24 h after intravenous injection.[16]

Since the first reports by Abuchowski and coworkers in 1977[17] concerning the alteration of immunological properties toward BSA that had been modified with PEG, the interest in PEG conjugation of biomolecules has grown almost exponentially. Many benefits of PEGylation for therapeutic proteins and peptides can be summarized as follows[1, 18-19] :

i) Significantly prolonged blood clearance in plasma
ii) Reduced antigenicity and immunogenicity including prevention of anaphylaxis
iii) Improved solubility
Increased stability including resistance to proteolysis as well as thermal and mechanical stability
iv) Improved bioavailability via reduced losses at injection sites

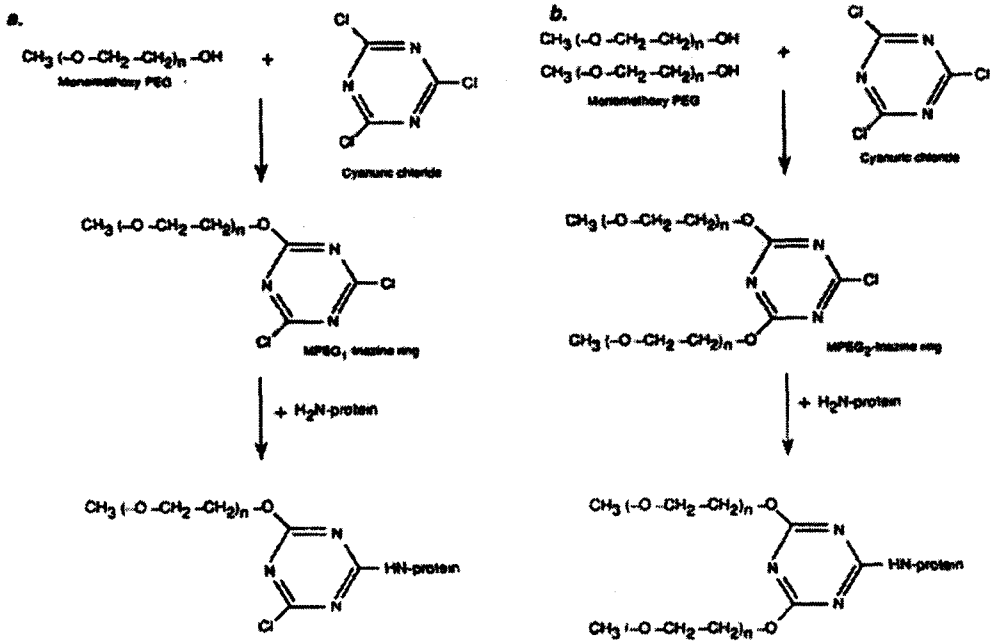
Polyethylene Glycol Derivatives

Activated PEG derivatives for amino group reaction

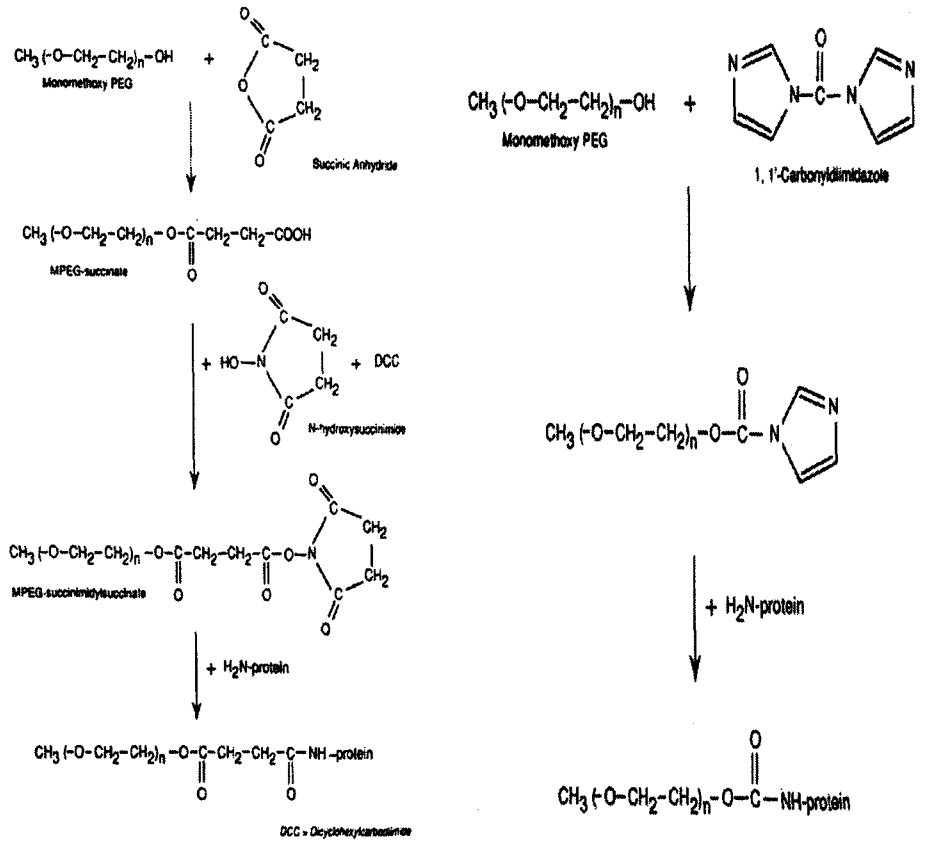
The most commonly used PEGylation reaction involves an electrophilically activated PEG and the εamino group of lysine or the N-terminal amino group of proteins.

PEG-Cyanuric chloride[20,22] (Scheme 1) – One of the first methods developed used PEG-cyanuric chloride. The treatment of mPEG-OH with trichloro-s-triazine (cyanuric chloride) was used by Davis and co-workers as a method for attachment of reactive dichlorotriazine residue to the polymer end group. The dichlorotriazine can react with nucleophilic functional group (usually amines but also hydroxyls and sulfhydryls), which results in displacement of one of the chlorides. The lack of selectivity resulted in marked loss of biological activity of some protein conjugates.[23] This speculation was supported by the observation that an attempted mPEG-dichlorotriazine modification of L-Asparaginase, an enzyme known to be inactivated by tyrosine-modifying reagents, produced a conjugate retaining only 7% of its original activity.[24] This method uses unphysiological conditions (0.1 M sodium tetraborate pH 9.2) for coupling to protein; it probably destroys rather more biological activity than the other major methods.[17] The Improved procedure for synthesis of mPEG2-chlorotriazine involved reflux in benzene of cyanuric chloride and mPEG-OH in a 1:2 molar ratio in the presence of zinc oxide and the modification of a variety of proteins has been demonstrated by Inada and co-workers.[25]

PEG-hydroxysuccinimide (Scheme 2) – Carboxylate groups activated with NHS esters are highly reactive toward amine



Scheme 1-Activation of mPEG with cyanuric chloride and reaction with amino group a. mPEG1-triazine, b. mPEG2-triazine.



Scheme 2–Succinimidyl active ester methods.

nucleophiles, which was introduced by Lomant and co-workers in the mid-1970s as reactive ends of cross-linking reagents.[26] The excellent reactivity at physiological pH rapidly established NHS esters as the major amine coupling reagent in

the bioconjugation reactions. Either PEG or mPEG maybe acylated with anhydrides to yield ester derivatives terminating in free carboxylate groups. Modification of PEG with succinic anhydride or glutaric anhydride gives bis-modified products having carboxylates at both ends. PEG-N-succinimidyl succinate has been used to conjugate asparaginase,[27] adenosine deaminase[28] and uricase.[29] PEG-N-succinimidyl glutarate has been used to conjugate IL-2.[30] Unlike the cyanuric chloride method, these methods yield activated PEGs that do not inactivate SH-dependent enzymes.[31] However, main deficiency of these activation procedures is the potential for hydrolysis of the ester bond formed by acylation of the hydroxyl groups of PEG. To avoid the hydrolytic cleavage between PEG and protein molecules, the stable carbamate (aliphatic urethane) bonds with mPEG-chloroformate compound, reactive intermediate, were introduced by Zalipsky et al.[32] This linkage is identical to that obtained through carbonyldiimidazole activation of hydroxyl groups with subsequent coupling of amines. Since the reactivity of the succinimidyl carbonate (SC) is much greater than that of the imidazole carbamate formed, succinimidyl carbonate method of PEG activation and coupling has become the chemical reaction of choice for attaching the polymer to amine containing proteins and other molecules.

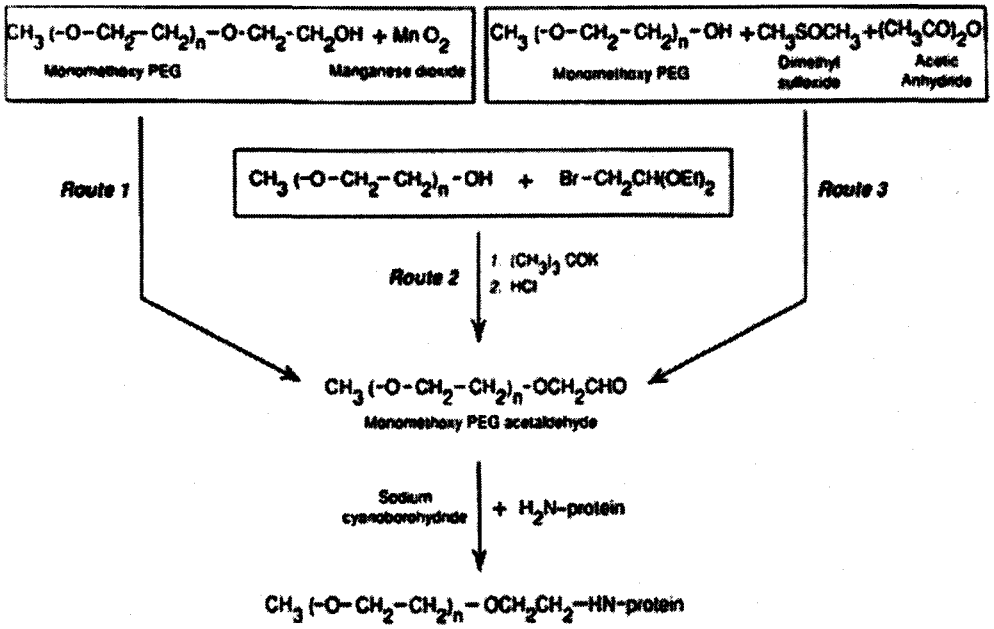
PEG-Aldehyde[33] (Scheme 3) – The introduction of aldehyde groups at the PEG terminals makes the polymer suitable for conjugation via reductive amination reaction. The advantage of PEG-aldehyde derivatives is that the active function will not readily degrade before the coupling reaction is initiated. Attachment of mPEG-acetaldehyde, prepared by DMSO/acetic anhydride oxidation, to 1,3-diol groups on poly_vinyl alcohol) via acid-catalyzed cyclic acetal formation was described.[34]

PEG-Tresylate (Scheme 4) – Nilsson and Mosbach had used tresyl chloride (2,2,2-trifluoroethanesulfonyl chloride) to activate agarose and other solid supports carrying method are that coupling to proteins takes place quickly and under very mild conditions (e.g.pH 7.5 phosphate buffer at room temperature) and PEG is attached to the protein directly through a highly stable amine linkage.

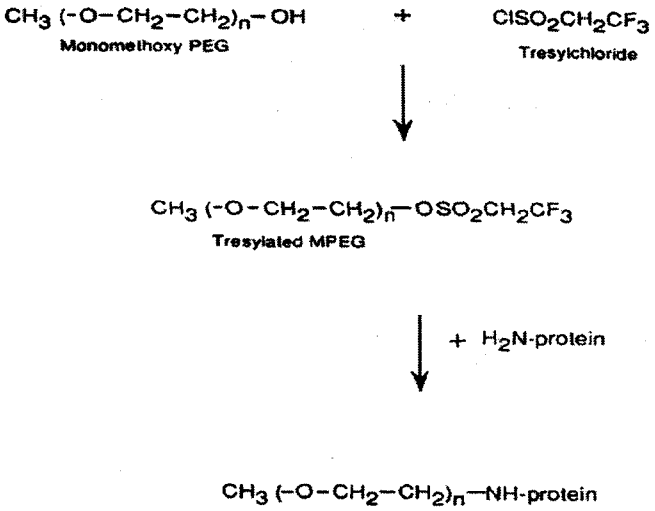
Activated PEG derivatives for other functional groups

In addition to the attachment of PEG via amino groups which often produces heterogeneous PEG conjugates, the site-directed PEGylation of proteins can be achieved via other functional groups on the protein surface.

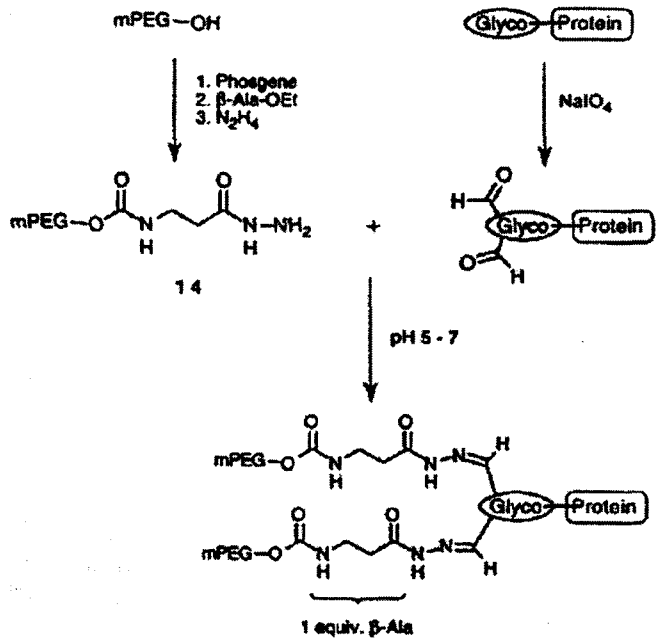
Carboxylate or carbonyl residues (Scheme 5) – In the case of glycoproteins it is possible to utilize the reactivity of oligosaccharide residues for attachment of mPEG chains, without affecting the polypeptide portions of the molecule.



Scheme 3–Production of PEG-aldehyde and coupling to a protein.[33]



Scheme 4–Activation of mPEG with tresyl chloride.



Scheme 5–mPEG-hydrazide derivative for carbohydrate group.

Hydrazides in general are good nucleophiles, yet relatively weak bases (pKa ~3) and often more reactive than amine under mildly acidic conditions maintained in aqueous buffers. There are two principal modes of reactivity of hydrazides both of which are highly selective and useful for conjugation with peptides, proteins, and glycoproteins: A) Reaction with carbonyl group; relatively stable hydrazone linkages that do not require further stabilization by reduction, and B) Reaction with carboxyl group; readily activated with water-soluble carbodiimide at mild acidic pH followed up the reaction with hydrazide.[36]

Cysteine residues (Scheme 6)–The thiol groups in the proteins are also attempted to conjugate with PEG as a new strategy.[37] This method uses a PEG-maleimide produced by reaction of PEG-amine with N-maleimido-6-amino-caproyl ester of 1-hydroxy-2-nitro-4-benzene sulfonic acid. Although it has been applied to produce biologically active PEG-cys-rIL-2, its use will be limited to proteins carrying cysteine residue not involved in sulfhydryl bridges. Other PEG derivatives used for cysteine residue such as PEG-Vinyl sulfone,[38] PEG-Iodoacetamide,[39] PEG-orthopyridyl disulfide[40] have been also developed.

Modification of a single amino acid residue in a protein can be effected either as a reflection of the unique reactivity of the residue, usually associated with some functional aspect of the protein e.g. the modification of active histidine residues[41] by peptidyl chloromethanes in serine protease, which takes advantage of high affinity binding of the reagent to produce specific modifications in high yield.

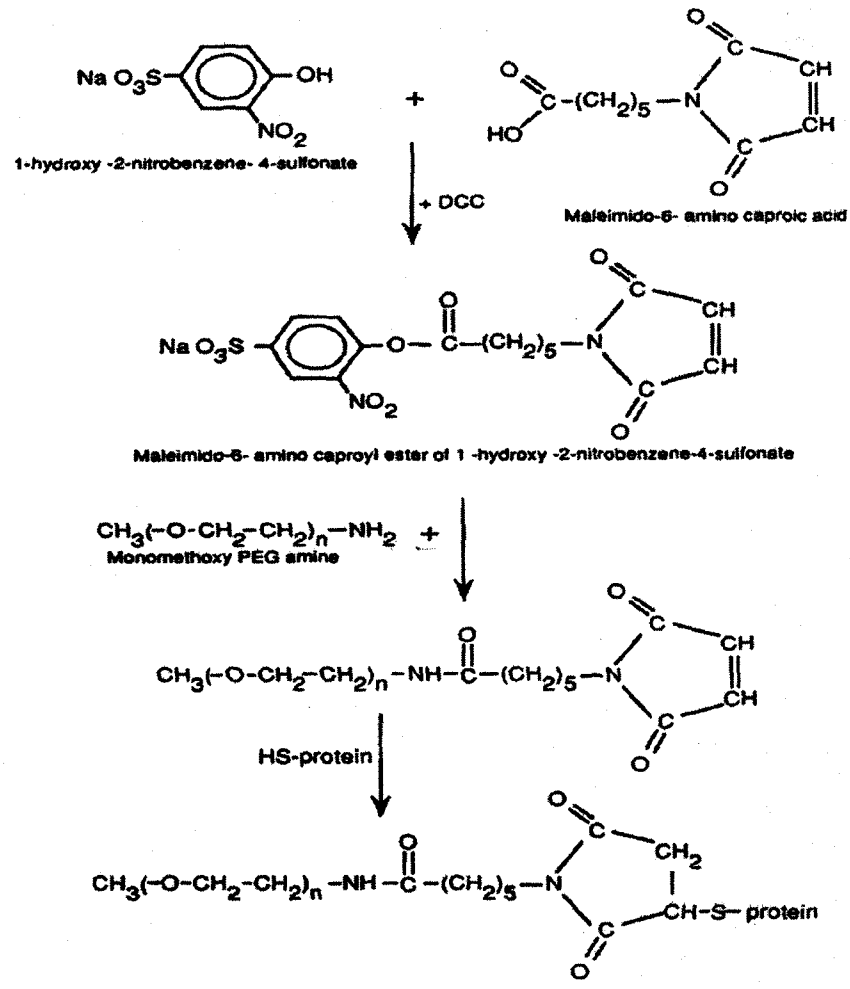
Polyethylene glycol-Proteins and Peptides

Over 40 proteins have now been studied (Table II) and a clear pattern has emerged.

PEG-modification has generally extended the plasma half-life of numerous proteins by a factor of 3 to 486-fold shown in Table III. Several mechanisms account for this, including the increased size of proteins normally below the limit for glomerular filtration, interference with the interaction of carbohydrate chains with their specific receptors, masking specific amino acid sequences for which there are cellular receptors, and reduced proteolysis and antigenicity.

Adenosine Deaminase (ADA)

Severe combined immunodeficiency disease(SCID) associated with an inherited deficiency of adenosine deaminase is fatal unless affected children are kept in protective isolation. David *et al.*[43] reported that coupling of PEG to ADA resulted in a loss of immunogenicity in a mouse model system with the circulatory half-life significantly extended. Hershfield *et al.*[27] demonstrated the clinical usefulness of PEG-ADA in two children with ADA deficiency. PEG-ADA was approved by FDA as an orphan drug for children with SCID in 1990.



Scheme 6–Activation of PEG-amine for SH group.

Table II–*Peptides and Proteins Modified by PEG Molecules*[42]

Enzymes	Adenosine Deaminase, Alkaline phosphatase, Arginase, Asparaginase, Calase, Chimotrypsin, Cytochrome c, Elastase, Galactosidase, Gluconato oxydase, β-Glucuronidase, Glutaminase-asparaginase, Kallicrein, Lipase, Peroxidase, Phenylalanine ammonialyase, Purine nucleoside phosphorylase, Streptokinase, Superoxide dismutase, Thermolysin, Tissue plasminogen activator, Trypsin, Tryptophanase, Urokinase, Uricase
Polypeptides	Albumin, Antigen E, Erythropoietin, Factor VIII, G-CSF, GM-CSF, Hb, hGrowth hormone, aHirudin, Honeybee venom, IgG, Immunotoxin, Insulin, Interferons, IL-2, IL-6, Melanin, α-Proteinase inhibitor, Somatostatin, TNF, Calcitonin

Table III–*Circulating Half Lives of PEG-Proteins*

Proteins	Host	$t_{1/2}$, native (hr)	$t_{1/2}$, PEG-proteins (hr)
Adenosine Deaminase[43]	mouse	0.5	28
Asparaginase[44]	rat	2.9	56
Catalase[45]	mouse	0.15	>4
Superoxide Dismutase[46]	mouse	0.06	17
Streptokinase[47]	mouse	0.07	0.33
Interleukin-2[48]	rat	0.05	0.33
Bovine Albumin[17]	rabbit	93	96
Interferon-α[49]	rat	0.17	12.1
Uricase[50]	human	< 3	8

Asparaginase

PEG-Asparaginase was approved by FDA in 1994 as an antitumor agent which is specific for the treatment of acute lymphocytic leukemia (ALL). Asparaginase is commonly used in free form, but some patients develop an immune based resistance that overcomes by the PEGylated form. Park *et al.*[26] demonstrated that PEG adduct of *E. coli* L-asparaginase was well tolerated in patients with chemotherapy-refractory cancer. PEG-asparaginase is distributed in a volume larger than the plasma compartment, giving clinically useful enzyme levels in pleural and peritoneal spaces. One unit of PEG-asparaginase was about as effective as five units of aspara- ginase against the L5178Y tumor in mice. Mice inoculated with l5178Y cells and treated with PEG-L-glutaminase-L-asparaginase on alternate days for 11 days survived 18-22 days while mice similarly injected with the unmodified enzme survived 11-15 days and control animals 9-11 days.[51] Fuertges *et al.* demonstrated that PEG-asparaginase (70% modification) showed minimal toxicity when given to a group of four patients with lymphoma or small cell carcinoma of the lung, however, there were no episodes of hyperglycemia or hypocalcemia and no neurological toxicity in contrast to *Achromobacter* asparaginase.[44]

Superoxide Dismutase (SOD)

PEG-SOD has been used as novel anti-inflammatory protein drugs capable of alleviating many of the inflammatory symptoms. Kawasaki *et al.*[52] demonstrated that PEG-SOD suppressed the ischemia-reperfusion injury to skeletal muscle in which lipid peroxidation by superoxide radicals produced by xanthine oxidase is contributed. PEG-SOD has been also assessed for improving the survival of skin flaps by Huang *et al.*[53] Control flaps were found to have a survival rate of 40%, native SOD (20000 U/kg, i.v.) improved this to 52%, whereas PEG-SOD achieved 80% survival. Interarticular injection of unmodified SOD into arthritic joints is sometimes followed by swelling and local irritation, whereas PEG-SOD does not elicit these reactions.[44]

Recombinant Interleukin-2 (rIL-2)

Modification of rIL-2, glycosylated lymphokine, with PEG enhanced the solubility of rIL-2, prolonged its plasma clearance and increased its systemic exposure. PEG-rIL-2 was approximately 60 times more potent than native rIL-2 as shown by the fact that 0.4 ug of PEG-rIL-2 had effects comparable to 25 ug of unmodified material in murine tumor model.[29] Over 40% modification of lysine residues in the rIL-2 with PEG, however, caused a marked reduction in the bioactivity of the protein. Site-directed pegylation of mutant rIL-2 was carried out successfully by Goodson et al. and the PEGylted rIL-2 had full bioactivity relative to the unmodified molecule and showed an increase its systemic exposure.[36]

Hemoglobin (Hb)

PEG-hemoglobin is showing promise as a blood substitute because the conjugate retains two essential functions, maintenance of blood volume and tissue oxygenation.[54] PEG ylated hemoglobin (Phase II by Enzon) for blood transfusion appears to be superior to stroma free or dextran conjugated hemoglobin.[55] The conjugation, performed with PEG of 5 kDa activated as succinimidyl carbonate, gave a product with a molecular weight in the range of 120-130 kDa. PEG-Hb was also found to be efficient in the oxygenation of solid tumors in rats even after three days after injection.[56] This property is considered useful for the therapy of many solid tumors since it was proved that the effectiveness of many antineoplastic agents may depend on the level of cellular oxygenation.

Colony Stimulating Factor (CSF)/Macrophage (M)-CSF

CSF-1 that mainly stimulates macrophage colonies in bone marrow cells, has been PEGylated to increase its systemic exposure.[57] In order to retain most of its biological activity, only 1-2 PEGs per CSF-1 could be attached. Therefore, the conjugate obtained with PEG of size 10 kDa had significantly improved pharmacokinetics.

Interferon (IFN)

PEGylated IFN-α as a therapeutic agent for the treatment of hepatitis B has been finished its clinical study recently and extended its application to hepatitis C . Since the number of PEG molecules per IFN affected the biological activity significantly, only one PEG attached IFN (1PEG-IFN) was developed as a clinical material.[48] Monkrsh *et al* prepared positional isomers of monoPEGylated IFN-α and measured the biological activity of each isomer.[56]

IFN-β and IFN-γ also have been conjugated with PEG and systemic increases were observed.[59,60]

Salmon Calcitonin

Salmon Calcitonin (sCT) has been PEGylated with succinimidyl carbonate monomethoxy PEG (12 kDa) and characterized by HPLC and MALDI-TOF mass spectrometry by Lee *et al.*[61] PEGylated sCTs showed substantially improved stability in rat liver homogenates vs. native sCT. In clearance studies in rat, PEGylated sCTs had significantly longer half lives than intact sCT (11.2 min and 54 min for mono- and di-PEG-sCT, respectively, vs. 4.7 min for intact sCT) shown in Figure 1. Also, three different positional isomers of mono-PEGylated sCTs were separated by reversed phase HPLC (Figure 2) and identified to be N-terminus, Lys18-, and Lys-11 by tryptic digestion (Figure 3) and MALDI-TOF mass spectrometry. The degradation half-lives of these three isomers in rat kidney homogenates significantly increased in the order of N-terminus (125.5 min), Lys11-(157.3 min), and Lys18-modified mono-PEG-sCT (281.5 min) over the native sCT (4.8 min) in Figure 4. These studies demonstrated that *in vivo* stability of PEGylated sCTs is highly dependent on the site of PEG molecule attachment.[62]

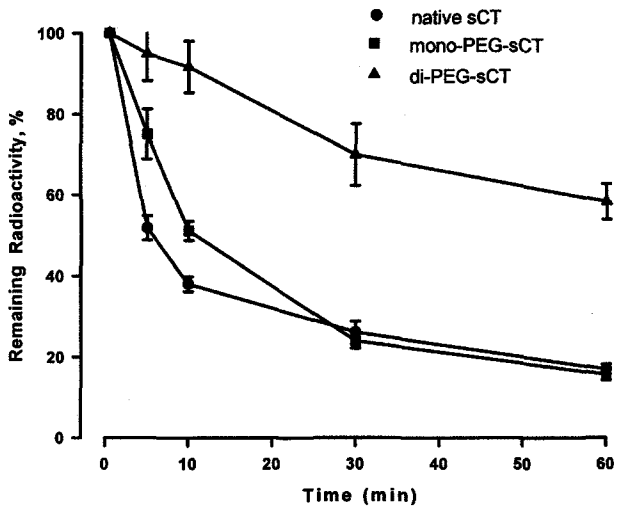


Figure 1–Blood clearance of PEGylated sCTs in the rat.

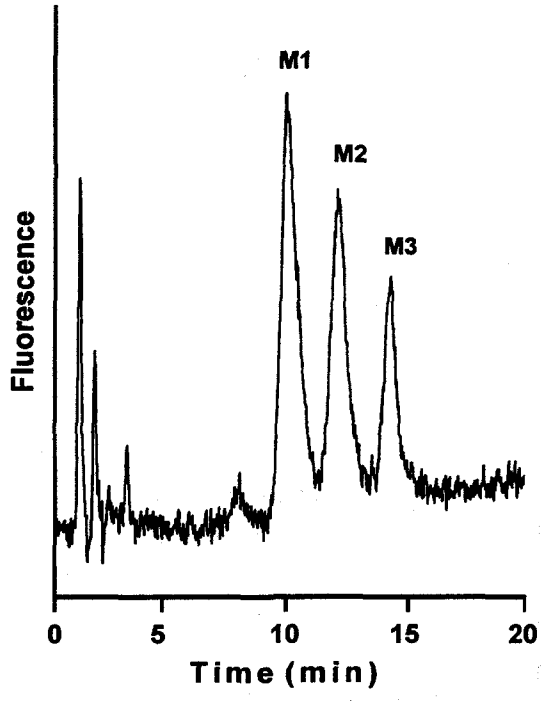


Figure 2–Reversed-phase gradient HPLC separation of 3 positional isomers of mono-PEGylated sCTs.

Others

The modification of human growth hormone with PEG has suggested that proteins other than enzymes may be effectively modified to extend in vivo half-life.[63] While the activity of human growth hormone is significantly decreased by this modification, reflecting reduced binding affinity to the cell-

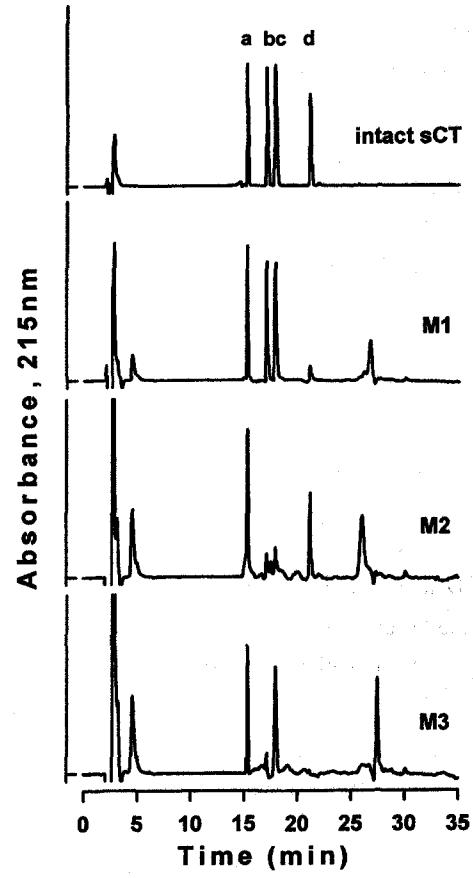


Figure 3–Reversed-phase gradient HPLC of tryptic digested sCT and 3 isomers of mono-PEGylated sCTs. M1: N-terminus-mono-PEG-sCT, M2: Lys^{18}-mono-PEG-sCT, M3: Lys^{11}-mono-PEG-sCT.

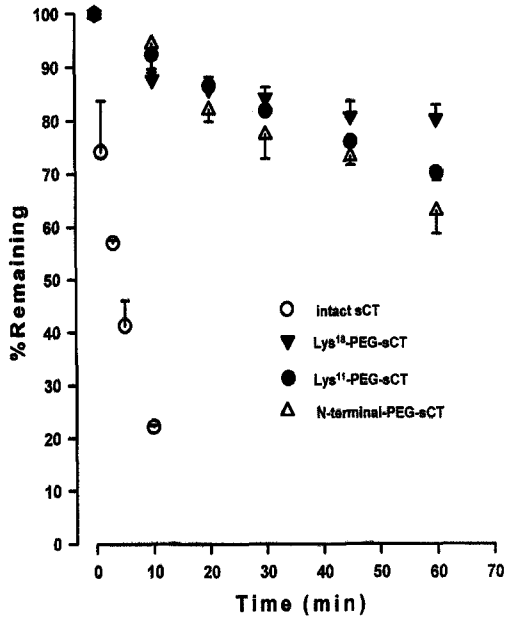


Figure 4--Stability of mono-PEGylated sCTs in rat kidney homogenates.

surface receptor, the extension of circulatory half-life more than compensated for this reduction in activity.

Site specific modification of Insulin with PEG was prepared and showed pronouncedly increased resistance to fibrillation whereas nonspecific insulin conjugates (7 possible derivatives) showed decreased potency in rats.[64]

Also, Arginine Deiminase,[65] β-glucosidase,[66] α-galactosidase,[66] uricase,[50] urokinase,[67] and streptokinase[47] were conjugated with PEG to enhance their efficacy as therapeutic agents.

Conclusions

PEG-modification of proteins and peptides represents a significant advance in protein pharmaceuticals. The benefits of PEG-modification, improved half-life and bioavailability, resistance to proteolysis, and reduced immunogenicity and antigenicity, received the significant attention to develop the therapeutic protein drugs. Also site -specific PEGylation of proteins can be another main area to develop in order to produce the homogeneous products reproducibly.

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