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INTRODUCTION

With the rapid advances in the field of biotechnology during the last decade, many peptides and proteins have been produced and evaluated for therapy of various diseases, including cancer. However, rapid clearance and the possibility of immunogenicity after the in vivo administration of these biotechnology-driven products have impeded their marketing. To circumvent these problems, synthetic and natural polymers such as polyethylene glycol (PEG) and dextrans, respectively, have been covalently attached to proteins, and some of these protein-polymer conjugates have shown promising therapeutic results. The conjugation of proteins with polymers usually causes a reduction in the recognition of the protein by the immune system, resulting in a decrease in protein clearance and immunogenicity. Most of the protein-polymer conjugates retain the pharmacologic activity of the protein, although to a lesser extent than the native protein. Additionally, in most of the examples in the literature, a significant increase in the plasma half life of the protein more than compensates for any reduction in the pharmacologic effects of the polymer-protein conjugates. Therefore, polymer conjugation in most cases would result in a net increase in the pharmacologic activity of the protein.

The intent of this article is to review the pharmacokinetics and pharmacodynamics of proteins conjugated to PEG which is one of the most widely used synthetic polymers for protein conjugation.

PHYSIOCHEMICAL PROPERTIES

Polyethylene glycol (PEG) is a polymer with the structure $(–CH_2CH_2O–)_n$ that is synthesized normally by ring opening polymerization of ethylene oxide. The polymer is usually linear at molecular weights (MWs) ≤ 10 kD. However, the higher MW PEGs may have some degree of branching. Polyethylene glycols of different MWs have already been used in pharmaceutical products for different reasons (e.g., increase in solubility of drugs). Therefore, from the regulatory standpoint, they are very attractive for further development as drug or protein carriers.

For coupling proteins to PEG, usually monomethoxy PEG $[CH_3 (–O–CH_2–CH_2)_n–OH]$ is first activated by means of cyanuric chloride, 1,1'-carbonyldiimidazole, phenylchloroformate, or succidinimidyl active ester (1) before the addition of the protein. In most cases, the activating agent acts as a linker between PEG and the protein, and several PEG molecules may be attached to one molecule of protein as depicted in Figure 1. Therefore, pharmacokinetics and pharmacodynamics of the PEG-protein conjugates are dependent on the MW of the PEG used for conjugation, the number of PEG molecules per each molecule of protein, and the nature of the bond between the protein and the linker. Interested readers

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are referred to a comprehensive review of the PEG-protein coupling methods by Deluged et al. (1).

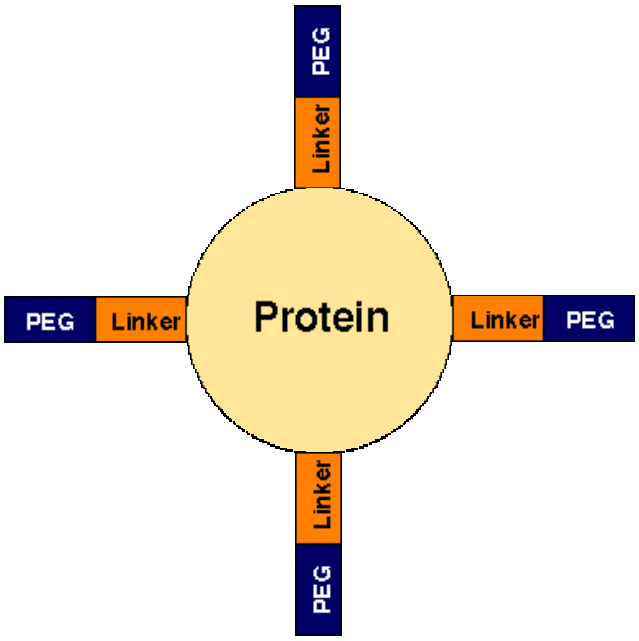

Figure 1. Schematic presentation of a protein-PEG conjugate. The number of PEG molecules per each protein molecule varies for different conjugates.

IN VIVO DISPOSITION OF PEG BACKBONE

It is believed that the kinetics of proteins attached to polymers are substantially affected by the kinetics of the polymer itself. Therefore, before reviewing specific PEG-protein conjugates, an analysis of the plasma kinetics and tissue distribution of PEGs is necessary.

The plasma kinetics of PEGs are reported (2, 3) to be dependent on both the MW of the polymer and the site of injection. Yamaoka et al. (2) investigated the disposition of radiolabeled PEGs with MWs of 6 kD (PEG-6), 20 kD (PEG-20), 50 kD (PEG-50), and 170 kD (PEG-170) after iv administration to mice. Similar to other polymers such as dextrans (4, 5), the plasma concentrations (Fig. 2) and area under the plasma concentration-time curves (AUCs) (Table 1) of higher MW PEGs were substantially greater than those of the lower MW polymers. Additionally, the half life of the polymers progressively increased as the MW increased from 6 kD to 170 kD (Table 1); the relationship between the half and the MW of PEGs is sigmoidal (Figure 3), which appears to be one of the characteristics of the kinetics of macromolecules.

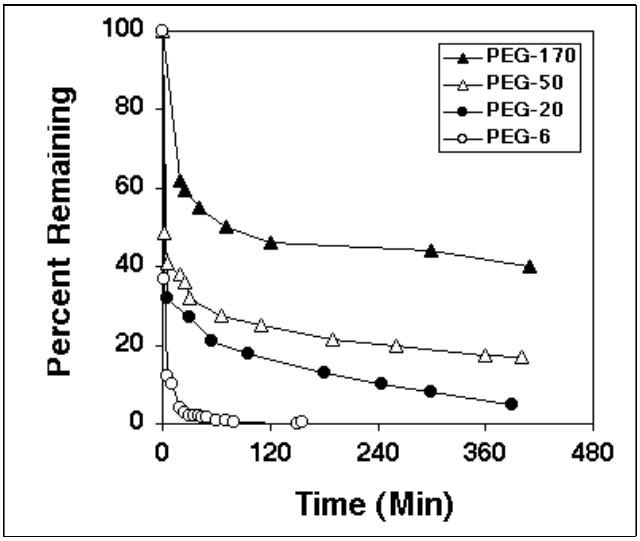

Figure 2. Blood radioactivity-time courses after iv administration of PEG with different molecular weights. Key: (▲) PEG-170; (Δ) PEG-50; (●) PEG-20; (○) PEG-6. From Ref. (2).

Table 1. Mean ± SD of AUC and terminal half life of PEGs with different MWs after iv administration to micea

Parameter	PEG-6	PEG-20	PEG-50	PEG-170
AUC,	6.17	110	600	1110
%dose.hr/mL	± 2.18	± 7.17	± 11.9	± 27.0
$t_{1/2}$, min	17.6	169	987	1390
	± 5.90	± 20.0	± 79.0	± 57.0

[a] *Source: Reference (2)*

With regard to the site of injection, PEG-50 is retained at the injection site longer than PEG-6 after im and sc injections (3), suggesting that the absorption of PEG from im and sc sites is MW dependent. However, after the ip administration, the injection site disappearance profiles of both MWs were very similar (3).

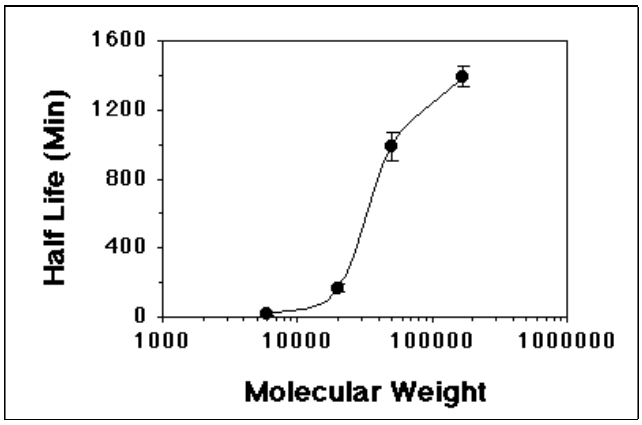

Figure 3. Relationship between the plasma half life of PEG and its molecular weight. From data presented in Table 1, Ref. (2).

The differences among the plasma concentration-time courses of PEGs with different MWs (Fig. 2) are mostly due to the size of these PEGs in relation to the pore sizes of the vascular beds in kidneys. Chang et al. (6) reported that, in rats, renal elimination of another linear polymer, neutral dextrans, with a MW of ~ 10 kD occurred without any molecular restriction. However, the renal clearance of dextrans of larger MWs progressively decreased and approached zero at a MW of ~40 kD. This is in agreement with a study (2) in mice using radiolabeled PEG, demonstrating a sigmoidal relationship between the renal clearance and the log MW of PEGs. This type of sigmoidal relationship (2) agrees well with the theoretical models of renal excretion of macromolecules based on the pore sizes of the glomerular capillary wall.

The relatively limited information on the metabolism of PEG in the body (7, 8) indicates that PEG undergoes cytochrome P-450 oxidation, resulting in the formation of ketone, ester, and aldehyde groups (8). Additionally, smaller MW PEGs are excreted into bile (7).

In terms of tissue distribution, it appears that PEGs with MWs between 6 kD to 170 kD distribute insignificantly to tissues such as heart, lung, liver, spleen, kidney, and thyroid gland (2). However, the distribution of PEGs to gastrointestinal tract and feces is relatively substantial (2). Additionally, no clear MW dependency is observed for the accumulation of PEG in tissues (2).

PEG-PROTEIN CONJUGATES

During the last three decades, PEG has been investigated extensively for delivery of various proteins via parenteral routes. Some examples are listed below.

Anticancer Agents

Generally, polymers have been most widely used for the delivery of both traditional (small molecule) drugs and proteins/enzymes in the treatment of cancer. However, PEGs have been specifically investigated for the delivery of anticancer proteins/enzymes as discussed below.

Antibodies: One of the major problems for the use of xenogenic monoclonal and polyclonal antibodies for the treatment of tumors is their immunogenicity which results in a rapid removal of the antibodies from the body and the possibility of allergic reactions after multiple administration. Kitamura et al. (9) conjugated the F(ab')2 fraction of the murine monoclonal antibody A7 to PEG 5 kD and studied the tumor accumulation and the kinetics of the conjugate in mice. The conjugate had a longer plasma half life and higher tumor accumulation, compared with the free F(ab')2 fraction. However, the tumor: blood ratio of the free F(ab')2 fraction was higher than that for the conjugate (9).

Takashina et al. (10) studied the pharmacokinetics and dynamics of conjugates of monoclonal antibody A7 to PEG 5 kD and dextran 70 kD. In vitro studies showed that the conjugates retained the antigen binding activity of the antibody. Additionally, after the iv administration of the conjugated and free antibody, the PEG conjugate had a plasma half life twice of that for the free antibody (10). On the other hand, the dextran conjugate showed higher clearance and shorter half life, compared with the free and PEG conjugated antibody. Additionally, the tumor accumulation of dextran-antibody conjugate was less than those for the free and PEG conjugated antibody. This study (10) suggests that the kinetics of polymer-

monoclonal antibody A7 are significantly dependent on the structure of the polymer.

Arginase: A PEG 5 kD conjugate of arginase retained 65% of the activity of the enzyme and prolonged its plasma half life in mice after multiple dose therapy (11); 30 days after the start of the treatment, the half life of the native enzyme was 1 hr, while the half life of the conjugate was 12 hr. In terms of effects, the conjugate increased the survival time in mice with Taper liver tumor. However, the free enzyme did not show any improvement in the survival time (12). With regard to the effects of the enzyme against L5178Y mouse leukemia cells, whereas the conjugate was more effective than the native enzyme in vitro, neither was able to stop the growth of tumor in vivo (12).

Asparaginase: Asparaginase, isolated from Escherichia Coli and Erwinia Carotovora, metabolizes asparagine, a necessary nutrient for sensitive tumors. However, after multiple injection of the enzyme, antibodies raised against the enzyme would quickly remove the enzyme from the circulation, and also significant immunogenicity may be observed. Several studies (13-19) have documented the usefulness of a conjugate of asparaginase with PEG for the treatment of various cancers in both humans and animals. Ho and his colleagues (15, 17) showed that the conjugate would alter the pharmacokinetics of the enzyme drastically in both humans and rabbits. In humans (15), conjugation resulted in an increase in the plasma half life from 20 hr (for native enzyme) to 357 hr (for the conjugate). In rabbits (17), the half life values of the free and conjugated asparaginase were 20 and 144 hr, respectively. The increase in the plasma half lives in both species was due to a significant decrease in the clearance of the enzyme (15, 17). The alterations in the kinetics of the enzyme by PEG conjugation also resulted in significant improvements in the toxicity and efficacy profile of the enzyme after in vivo administrations to animals (14, 16, 18) and humans (13, 19). A conjugate of asparaginase and PEG (pegaspargase) was marketed (Oncaspar®) in 1994 for the treatment of acute lymphoblastic leukemia (ALL) in patients who are hypersensitive to native forms of L-asparaginase. Oncaspar® is marketed by Rhône-Poulenc Rorer Pharmaceuticals, Inc. in the U.S. and Canada.

Methioninase: It is known that all the tumor cells have elevated requirement for methionine. Therefore, methioninase may be used in cancer therapy. However, the recombinant enzyme, obtained from bacteria, has a short plasma half life and may be immunogenic upon multiple dose administration. Very recently, Tan et al. (20) demonstrated the potential of a conjugate of methioninase and PEG 5 kD in cancer therapy. In vitro tests demonstrated that the conjugate retained 70% activity of the enzyme. Additionally, in rats, the plasma half life of the enzyme was increased by a factor of 2 when it was conjugated to PEG 5 kD (20). Further, the effects of the conjugate lasted for 8 hr, as opposed to 2 hr for the free enzyme. In vitro studies in human lung and kidney cancer cells showed identical IC50 values for the conjugated and free methioninase, demonstrating the effectiveness of the enzyme in the conjugated from. Also, after the injection of the conjugate to tumor-bearing mice, the tumor: blood enzyme ratio was higher for the conjugate (1:6), compared with the free enzyme (1:10) administration (20). More studies are needed to confirm these promising findings.

Enzyme Replacement

Adenosine Deaminase: A deficiency of the enzyme adenosine deaminase (ADA) results in combined immunodeficiency disease (CID). For several years, conjugates of PEG and ADA have been used successfully for enzyme replacement in the treatment of CID in children (21-23). A conjugate of PEG and ADA, which is also named pegademase, was marketed (Adagen®) by Enzon, Inc. (Piscataway, NJ) in the US in 1990. The outcome of therapy with the conjugate appears to be better than red blood cell transfusion (23), which is another treatment for ADA deficiency. Studies (21-23) have shown that weekly intramuscular injections of the conjugate of PEG with bovine ADA would reverse the symptoms of ADA deficiency in most cases without substantial toxicity or hypersensitivity. The conjugate appears to have a very long half life of 48-72 hr in children (21). From a historic perspective, the PEG-ADA conjugate

served as one of the earliest examples of polymer conjugates marketed in the US and prompted more research interest in this area.

Uricase: Uricase is an enzyme which converts uric acid to allantoin and is lacking in humans. When the enzyme is administered to humans, it causes a significant reduction in the plasma and urine levels of uric acid. Therefore, it can be effective in the treatment of gout and other diseases related to high levels of urate. However, after multiple administration, the antibodies against this enzyme would deactivate it very rapidly. Several conjugates of uricase with PEGs (24-28) have been investigated to overcome this problem. Yasuda et al. (28) reported that conjugation of uricase with PEG resulted in a decrease in antibody production and reactivity towards uricase. When administered intravenously to rats, the enzymatic activity half life of the PEG conjugate (~ 7 hr) was almost 10 times of that for the parent enzyme (0.6 hr) (28).

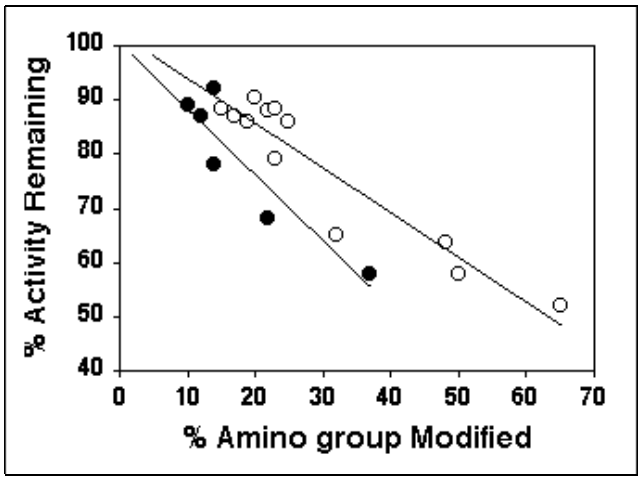

Figure 4. Relationship between the percentage of amino groups of uricase modified with dextran 10 kD (○) or PEG 10 kD (●) and the percentage of remaining enzymatic activity of uricase. From Ref. (28).

The enzymatic activity of the polymer conjugated uricase is shown to be dependent on the degree of modification of the amino groups of the enzyme during the conjugation process (28). An increase in the modification would result in a decrease in the enzymatic activity of uricase for both dextran and PEG conjugates (Fig. 4). However, the decrease in the activity is more pronounced for the PEG conjugate, compared with dextran conjugation (Figure 4) (28).

Antioxidant Enzymes

Catalase: Similar to superoxide dismutase (SOD), catalse is an antioxidant enzyme, and several studies (29-32) have investigated the effects of PEG conjugates of SOD and catalse on the same animal model. The PEG-catalse conjugate was first prepared by Abuchowski et al. (33) using both PEG 1900 and 5 kD. These investigators (33) demonstrated that both conjugates retained significant (>90%) activity of the enzyme and were resistant to digestion by trypsin, chymotrypsin, and protease. Further, the half life of the conjugates was long even after their repeated administration to mice (33). A later study (34) using subcutaneous osmotic pumps delivering the conjugate showed the conjugate's beneficial effects in a rat model of lung injury due to asbestosis. Despite promising effects of the PEG-catalase conjugate, recent work in this area has concentrated more on a conjugate of SOD and PEG described below.

Superoxide Dismutase (SOD): Among the conjugates of PEG, SOD is the most widely studied. Superoxide dismutase is an antioxidant enzyme which eliminates superoxide anion, reducing tissue injury. After its iv administration in animals, the plasma half life of the enzyme is very short (5-10 min). Several investigators have reported the effects of conjugation of SOD with PEG on the pharmacokinetics and dynamics of the enzyme, some of which are summarized in Table 2 (29-32, 35-47). Although some of these studies have compared the effects of PEG-SOD with those of the free enzyme, most of the studies have concentrated on the effects of PEG-SOD without a comparison with the free SOD (Table 2). There is little doubt that conjugation of SOD with PEG increases its plasma half life (35) and reduces its immunogenicity (29, 35). However, conflicting reports (30-32, 36-40, 43, 45-47) exist with regard to the effects of PEG-SOD in various animal models of injury. Additionally, the results of clinical trials (42, 44) with PEG-SOD have not been unequivocal.

Table 2. Some of studies on the conjugates of PEG and SOD.

Type of Study	Comments	Reference
In vitro and in vivo kinetics and dynamics in rats	PEG 5 kD conjugate retained 51% enzyme activity; plasma half life of conjugate was longer than the native SOD after repeated dosing; anti-inflammatory effect of the conjugate was higher than SOD.	(35)
In vivo immunogenicity in mice	Decreased immunogenicity; antibody titer to the conjugate was 0.03%-0.07% of that observed with SOD.	(29)
In vivo effects in endotoxemia in pigs	No beneficial effects	(30)
In vivo effects in a dog model of ischemia/ reperfusion	Conflicting results: both no effect (37) or a reduction (36) in heart injury associated with reperfusion have been reported.	(36, 37)
In vivo effects in a rat model of brain ischemia	Administration of PEG-SOD before induction of focal cerebral ischemia resulted in a reduction in brain injury.	(31)
In vivo effects in a rabbit model of ischemia/ reperfusion	No effect in heart injury associated with reperfusion.	(38)
In vivo distribution into brain of piglets	IV injection of PEG-SOD did not increase the enzyme level in the brain in control piglets and in animals subjected to global ischemia/reperfusion.	(39)
In vivo effects in hemorrhagic shock in rats	Administration of a PEG-SOD conjugate to a rat model increased survival from 25% to 67%.	(40)
In vivo brain distribution in rats	The concentrations of PEG-SOD in the brain and CSF of normal rats were low; brain and CSF concentrations were higher after hypertensive brain injury	(41)
In vivo effects in piglets with hypoxic brain injury	Administration of PEG-SOD 5 min after reoxygenation did not have any positive effects.	(32)
Phase II clinical trial study in severe head injury	Improved outcome at 3 and 6 months after the treatment with PEG-SOD (10,000 U/kg), compared with placebo.	(42)
In vivo study in rats with oxygen toxicity	Insufflation of PEG-SOD increased survival time, in comparison with both placebo and free SOD.	(43)
Clinical trial in severe head injury	Percent of patients in a vegetative state or dead at 3 and 6 months postinjury was lower after the conjugate, compared with placebo.	(44)
In vivo effect in a rat model of ischemic renal failure	The PEG-SOD was more effective than an equivalent dose of free SOD.	(45)
In vivo effects in a rat model of warm renal ischemia	PEG-SOD conjugates were more protective, compared with free SOD.	(46)
In vivo effects in a rat model of ischemia/reperfusion	SOD conjugated to PEG showed a superior effect over that conjugated to polyacryloylmorpholine.	(47)

Thrombolytic Agents

Streptokinase: Rajagopalan et al. (48) conjugated streptokinase to PEG 2 kD, 4 kD, and 5 kD, and investigated the thrombolytic activity and antigenicity of the conjugates. In vitro studies demonstrated comparable activity for the conjugates and the free enzyme. However, the binding of the conjugates to antibodies against streptokinase was reduced by 95% (48). In vivo studies in mice (48, 49) revealed low clearance of the conjugates attached to plasmin, resulting in a half life of 200 min for the conjugate, compared to a half life of 15 min for streptokinase itself. These studies (48, 49) demonstrate that PEG conjugation of streptokinase retains the activity of the enzyme, prolongs its plasma circulation by blocking plasmin degradation, and reduces the antigenicity of the enzyme.

Urokinase: In dogs, a conjugate of urokinase, a thrombolytic agent, with PEG 5 kD was shown (50) to have longer activity and more activation of fibrinolysis, compared with the native enzyme. Also, a polypropylene glycol-PEG conjugate of urokinase showed a decreased activity on plasminogen and had a longer plasma half life in rabbits, compared with the native enzyme (51). Later (52), it was shown that this conjugate blocked autolysis of the enzyme at 37^0C. Unfortunately, these early positive results have not been followed by more extensive in vivo studies.

Oxygen Carriers

Hemoglobin: Several studies have examined the feasibility of the conjugation of hemoglobin to PEG for use as a blood substitute. Hemoglobin binds to oxygen and can be used as an oxygen carrier. However, because of its rapid elimination, the plasma half life of the protein is very short. Additionally, the affinity of hemoglobin to oxygen is too high for release of oxygen in the tissues. A conjugate of PEG with pyridoxylated hemoglobin has been shown (53, 54) to have longer plasma half life and better therapeutic effects in rats, compared with the free hemoglobin. The benefits of PEG-hemoglobin conjugates as a blood substitute have been shown in several animal models, including a hemorrhagic hypotension pig model (55) and in partial exchange transfusion and top-loaded rat models (56). Additionally, a PEG-hemoglobin conjugate has been used (57) for an increase in the sensitivity of tumors to radiation by increasing oxygen delivery to the tumor. These studies point to the potential of hemoglobin conjugated to PEG for manipulation of the oxygen levels in normal and malignant tissues.

Cytokines and Hematopoietic Growth Factors

Interleukin-2 (IL-2): Both animal and clinical studies have been conducted using PEG conjugates of IL-2. Earlier studies in animals (58, 59) and humans (60) showed that PEG conjugation would increase stability, decrease clearance, and increase plasma half life (> 20 fold) of IL-2. Further, these studies (58-61) suggested promising effects for the PEG-IL-2 conjugate in the treatment of various cancers. However, more recent data (62-64), mostly in patients, have failed to clearly demonstrate an advantage for PEG-IL-2, compared with free IL-2, in terms of therapeutic or toxic end points for the treatment of cancer. On the other hand, it appears that recent interest in the PEG-IL-2 conjugate revolves around its potential beneficial effects in patients with human immunodeficiency virus (65-69). Recent studies (65-69) in patients with HIV show that low dose PEG-IL-2, alone or in combination with zidovudine, would increase the immune response by increasing the number of CD4 T cells without significant toxicity. Additional clinical studies, comparing free and PEG conjugated IL-2 will shed more light on these exciting results.

Recombinant human granulocyte colony-stimulating factor (rhG-CSF): This is a 156 amino acid glycoprotein which is produced by Escherichia Coli and increases production and phagocytic and cytotoxic activities of neutrophils (70). The plasma half life of rhG-CSF is short (3.5 hr) (70), requiring daily injections to sustain the neutrophil levels in situations like cancer chemotherapy. In 1991, Tanaka et al. (71) reported that a conjugate of rhG-CSF with PEG increased the plasma half life of the growth factor from 1.8 hr (native factor) to 7 hr (conjugated factor) in mice. The increase in half life was associated with an increase in both the intensity and duration of the effect of the drug on the neutrophil count (71). These results were later (72) confirmed in mice made neutropenic by the administration of anticancer agents cyclophosphamide and fluorouracil. Recent studies (73-75) demonstrated that the in vivo activity of the conjugate is dependent on both the MW of PEG (73, 74) and the total number of PEG units attached to rhG-CSF (73, 75); there was a positive relationship between the total mass of the conjugate and the intensity and duration of the effect of rhG-CSF. Future studies should be conducted to determine whether these positive results in animals can be extended to humans.

Recombinant human granulocyte/macrophage colony-stimulating factor (rhGM-CSF): This is a 127 amino acid glycoprotein produced in yeast which acts similar to rhG-CSF to increase neutrophils, with

a broader action on monocytes, macrophages, and eosinophils (70). Similar to rhG-CSF, the plasma half life of rhGM-CSF is short (2-3 hr) (70), requiring daily injections to sustain the neutrophil levels in patients undergoing bone marrow transplantation or intensive chemotherapy. Compared with rhG-CSF, the studies on the conjugates of PEG with rhGM-CSF are scarce (76, 77). The limited information indicates that similar to rhG-CSF, PEG conjugation increases the plasma half life (76) and some biological activities of rhGM-CSF (77).

Other Proteins

Table 3 lists the use of PEGs for delivery of some other therapeutic agents (78-86) which are not discussed in detail in this review. These studies (Table 3) show that polymer conjugation could result in altered pharmacokinetics, decreased affinity of the conjugate to bind to the protein receptor, and/or a decrease in antigenicity of proteins.

CONCLUDING REMARKS

The examples provided in this review clearly point to the potential advantages of polyethylene glycols for parenteral delivery of proteins. Despite significant promise of protein therapeutics in cell culture and other in vitro studies, optimal delivery of these agents in humans is very challenging. This is mostly because of relatively high clearance and short plasma half life of these agents, especially after multiple administration which results in activation of the immune system and faster elimination of the proteins. The available studies on the use of PEG for delivery of proteins indicate that these polymers will continue to have a significant role in the delivery of proteins in the future.

Table 3. Additional studies on the conjugates of PEGs with proteins

Drug/Protein	Description
Antigen E	A preliminary study in man showed that a 5 kD conjugate may be useful for the immunotherapy of ragweed hay fever (78).
Batroxobin	A 10 kD conjugate retained the activity of the enzyme while losing its ability to bind to anti-batroxobin antibodies in dogs (79).
Bilirubin oxidase	In a rat model of jaundice, the conjugate reduced the blood and liver levels of bilirubin, but, did not improve the liver function tests (80).
Honeybee Venom	In a clinical study, a 5.7 kD conjugate showed lower systemic reactions during immunotherapy and less efficacy against honeybee sting (81).
Interferon-alpha	In humans, the half life of the conjugate was twice as long as that of free protein; however, this did not result in a substantial reduction in the frequency of the protein administration (82).
Interferon-gamma	A 5 kD conjugate had activity similar to that of free protein but with a reduced binding affinity; the plasma half life of the conjugate was significantly longer than that of free protein in rats (83).
Interleukin-6	A 12 kD conjugate showed significantly higher thrombopoietic effects (increase in the platelet counts), compared with free IL-6 in mice (84)
Tissue Plasminogen Activator	In mice, the half life of radioactivity after the injection of the radiolabeled conjugate with 5 kD and 20 kD was long; however, the effect disappeared much faster (85).
Trypsin	A 5 kD conjugate was resistant to anti-trypsin antibody precipitation and retained some of the activities of trypsin to varying degrees (86).

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