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Pegylation-A Novel Approach in Protein Administration

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Protein administration is often associated with practical problems due to it biological degradation in a very short time. Pegylation modification with monopolyethylene glycol (mPEG) is one of the ways to enhance thermal stability and solubility in organic solvents. mPEG can be applied to the development of diagnostics, targeting or imaging agents, and can be used in kinetic analysis or receptor binding studies. The mechanism of the immune system can also be deduced using these modified compounds. Pegylation can also be applied to other system like liposomes to enhance effectiveness of the delivery system. Site directed attachment and noncovalent attachment of mPEG open up the entire new spectrum of possibilities. Molecular weight changes and charge of the mPEG attached also provide new possibilities, thus opportunities for producing longer acting and cost effective end products. Realization of new thrilling horizon in the field of drug delivery system with polyethylene glycol is in the offing.

Therapeutic proteins are those proteins with therapeutic potential. These are compounds derived or based upon native proteins, which often are endogenous substances with demonstrated autocrine or paracrine action *in vivo*. The majority of proteins are produced biotechnologically, in substantial quantity and purity to enable their clinical investigation. Potential therapeutic proteins are different in a number of important aspects from classical new drug entities, which are low molecular weight biologically active compounds. Therapeutic or pharmaceutical proteins have been defined by Blohm *et al.*[1] as proteins or polypeptides, which, by reason of their native functions in the human body, are suitable as therapeutic substances. Currently knowledge is lacking regarding complete range of biological behavior of the proposed therapeutic proteins. These therapeutic proteins are administered paranterally in concentration and circumstances, which differ markedly from their native counterparts, and this undoubtedly leads to untoward side effects. Therapeutic application of peptides, enzymes, hormones and other proteins are presented in Table 1. PEGylation is a procedure of growing interest for enhancing the therapeutic and biotechnological potential of peptides and proteins. When poly (ethylene glycol) (PEG) is properly linked to a polypeptide, it modifies many of its features while the main biological functions are retained. PEG conjugation masks the protein's surface and increases the molecular size of the polypeptide, thus reducing its renal ultrafiltration, preventing the approach of antibodies or antigen processing cells and reducing the degradation by proteolytic enzymes. PEGylation is therefore of interest in applied biotechnology because, upon modification, enzymes may become soluble and active in organic solvents[2]. This property opens new techniques in biocatalysis and in pharmaceutical technology where many insoluble drugs are solubilized by PEG conjugation and thus are, more easily administered[2,3].

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Problems associated with protein therapy:

There are a number of problems associated with administration of proteins. Owing to heterologous nature of most proteins, immune response may be elicited following repeated use and this can result in the development of neu-

TABLE1: CLINICAL USE OF THERAPEUTIC PROTEINS IN VARIOUS INDICATIONS.

Anamia	EPO
Cancer	G-CSF, GM-CSF, IL-1, IL-2. TNF-IFN
Cardiovascular	Hirudinn antithrombin III, Superoxide dismutase, artrial natriuretic factor, α antitrypsin
Diabetes	Insulin, Somatostatin, Proinsulin.
Growth Defects	hGH
Inflammation	IFN-Y
Respiratory disorders	α antitrysin, SOD
Thrombosis	Streptokinase, anisolyated-streptokinase activator complex, TPA, urokinase.

tralizing antibodies or hypersensitivity reactions. Pharmacokinetics is very poor, which limits the therapeutic application of these proteins. When proteins are administered parenterally, they are rapidly cleared from circulation by the reticuloendothelial system, kidney spleen or liver. Protein clearance depends on the charge, size and presence of cellular receptors. They are also metabolized by peptidases and thus rapidly lose their biological activity. Antigenic determinations on the surface of proteins may cause development of a humeral immune response. Antibodies can develop, which can inactivate the biological activity of the proteins and cause accelerated clearance. This immune response can also result in the development of hypersensitivity reactions and life threatening anaphylaxis. The native proteins are easily degraded by two distinct pathways (i) chemical instability i.e. deamidation, recemisation, hydrolysis, oxidation and (ii) physical instability i.e. denaturation, aggregation, precipitation and adsorption. Chemical instability is associated with specific amino acid or amino acid sequences. Asparagine and glutamine (Asn and Gin) residues are both susceptible to deamidation and the sequence Asparagine and glycine is susceptible to rearrangement via a cyclic imide intermediate to give a β-peptide structure[3]. Physical instability results due to association of unfolded proteins with surfaces (adsorption) or aggregation with other molecules (In aqueous solution protein exists in dynamic equilibrium with either folded or unfolded state).

Novel approaches for protein delivery:

There are various approaches, which have been developed to circumvent above mentioned problems. The basic problems with the administration of native proteins are its elicited immunogenicity, poor pharmacokinetics and enzymatic degradation. The advent of recombinant DNA technology has flourished the protein therapy[4] which resolved the immunogenicity and antigenicity problems to greater extent[5] but the problems of circulating life and poor stability still existed[6]. Additionally it was again necessary to ensure therapeutic efficacy by improving the poor pharmacokinetics. Because the proteins are rapidly cleared from circulation by the reticuloendothelial system and metabolized by peptidases and thus rapidly lose their biological activity. To overcome the problems associated with the use of proteins for therapeutics, number of drug delivery systems are emerged which do not affect the natural property of protein. Delivery systems used for the protein administration have been presented in Table 2.

Nagai *et al.*[7] developed mucosal dosage form of insulin with a view to resolve the problem of administration by injection, using HPC-M and carbapol-934 and sodium glycolate. Transbuccal delivery systems for low molecular weight polysaccharides (heparin), peptides and proteins based on a hydrogen polyether urethane have been reported by Yang and Knutson[8], which remarkably improved the pharmacokinetics.

Miyachi *et al.*[9] attempted to prepare liposomes encapsulated superoxide dismutase for topical application and on the same ground Weiner *et al.*[10] reported reduction in lesion sores by interferon encapsulated in liposomes. In an effort to produce new forms of blood substitutes, human adult

TABLE 2: DELIVERY SYSTEMS FOR ADMINISTRATION OF PROTEINS.

Technology mode	Delivery system
Delivery technology	Controlled release (e.g. encapsulation, microadhesives and prodrugs
Materials	Synthetic polymers, (e.g. PEG) and natural Polymers (e.g. polysaccharides), hyaluronic acid, collagen.
Delivery vehicles	Soluble polymers, microspheres, hydrogels, red blood cells, monoclonal antibodies.

hemoglobin has been encapsulated in polymerized liposomes derived from conjugated diacetylene phospholipids[11] which prevent metabolic degradation.

Polyvinyl pyrrolidone-protein conjugate conveys to the conjugates many critical properties as resistance to denaturation, resistance to proteolysis, increased residence time, reduced immunogenicity similar to PEG, while it confers new properties of biodistribution[12] to conjugates. Erythrocytes based delivery of Gasparaginase[13] and albumin by Deloach *et al.*[14] and insulin by Fiddler *et al.*[15] have been reported. These systems helped to circumvent the proteins of short circulating life. Insulin has been successfully administered through both hydrogels and microspheres. Hydrogels fabricated using alginic acid[16] showed controlled release of insulin while nasal delivery of the same has been reported through starch microspheres and it offers feasibility for the nasal delivery of low molecular weight hydrogel proteins. Regulated delivery of insulin has also been reported from biodegradable dextran hydrogel containing polyethylene glycol[17]. Moriyama *et al.*[18] reported regulated insulin (peptide) release from polyethylene glycol modified polysaccharide in two phase system and they achieved controlled insulin release by forming a heterogeneous structured systems consisting of PEG as drug reservoirs and polysaccharide as biodegradable matrices. Similarly, immobilized enzymes are capable of prolonged functioning in the body without the loss of specific activity (due either to degradative inhibition or capture by the cells of the reticuloendothelial system). This technique requires certain carrier, which may either, be water soluble or insoluble, depending upon the property of enzymes or protein to be used.

Number of enzymes has been immobilized using different types of carriers. Immobilization of asparaginase with albumin[19] was reported for oncology. Cholesterol oxidase immobilized with soluble polymer intended for atherosclerotic deposits were reported[20]. In the same way immobilization of collagenase with dextran[21], β-glucouronidase in red blood cells[22], uricase with soluble polymer[23], trypsin in the polyethylene glycol[24] have been reported. These technologies proved fruitful for the development of controlled delivery and extended circulating life of proteins, however they are rapidly sequestered in the liver, spleen, kidney and reticuloendothelial system (RES). Various protein modification technologies have been explored. These include acylation, succinylation, guanidation and deamination. Polymers such as albumin, dextran, polyvinyl pyrollidone, Q, D2-polyaminoacids, have also been conjugated to number of proteins in view to either increase blood circulating life, and/or reduction in immunogenicity. Activation of proteins employing acylation, succinylation and guanidation results in extremely well modified proteins with well preserved biological activities, however the resultant linkages has been reported to be spontaneously hydrolyzed at physiological pH and *in vivo* by nonspecific hydrolases[25]. Albumin, dextran and polyvinyl pyrollidone are undoubtedly potential immunogen while Q, D2-polyaminoacids didn't give any additional advantages over pegylation[26]. The advent of pegylation results in the elimination of problems associated with other methods of protein modification. Due to its structural simplicity and possession of only one derivatizable end group, the use of mPEG minimizes crosslinking possibilities and leads to improved homogeneity of the conjugates. The polyether backbone of PEG is inert in biological environments 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[27].

Modification with polyethylene glycol:

The recent discovery and most extensively studied drug delivery technology involves protein modification with mPEG, pegylation, which results in dramatic decrease in nonexistent immunogenicity, increased circulating life, increased stability opened a channel for the development of protein pharmaceuticals that would help to realize the promise held by protein therapy.

Polyethylene glycol is a linear, hydrophilic, uncharged flexible polymer, which is available in a variety of molecular weights. In addition to this, it has low order toxicity in oral, paranteral and epidermal applications[28,29] and is nonoimmunogenic. mPEG modified proteins exhibit increased stability, increased resistance to proteolytic in activation, increased circulating life, least to negligible immunogenicity and low toxicity. Monofunctionality of mPEG makes this polymer more advantageous over others, since it assures the absence of crosslinking during the conjugation reaction and moreover this allows the masking of epitopes as well as sites of proteolysis, while increasing of the macromolecular volume reduces the renal ulbrafiltration[30]. In addition, mPEG proteins are well solubilized in organic solvents, retaining their original activity[31]. mPEG-protein conjugation techniques were developed using linkage groups such as carbonyidiimidazole, succinic anhydride, trichloro-s triazine. All involve the preparation of activated PEG along with reactive functional group, which can successfully be coupled, to the lysine groups on proteins[32,33].

PEG is readily available in a variety of molecular weights. In general, the molecular weights of PEGs used for synthesis of biologically, active conjugates are in the range of 1000-2000 Da, which are homogeneous polymers. There are reports where half life of PEG is said to be directly proportional to the molecular weight. A study with SOD carried out by some workers[34] revealed that modifying SOD with PEG having molecular weight 72000 was having 24 times greater half life compared to SOD modified with PEG 1900. Different methods for PEG modification have been described in fig. 1.

In 1977, Abuchowski *et al.*[35] reported a method for the covalent attachment of mPEG to proteins. Most of the cases involve activation of mPEG molecule, which eventually leads to formation of reactive functional group, which is coupled to a specific site on the protein. An original strategy was devised by Zaiipsky to specifically pegylate carboxylic groups in proteins without cross-linking formation with amino group: it takes advantage of the linkage of PEG to carboxyl groups only, by the use of PEG-hydrazide[36].

However, the most common site to be modified is an amino group, and most commonly used reagents used for this activation are trichloro-s-triazine (cyanuric chloride)[35], carbonyidiimidazole[37], succinic anhydride[38] and succiniimidyl

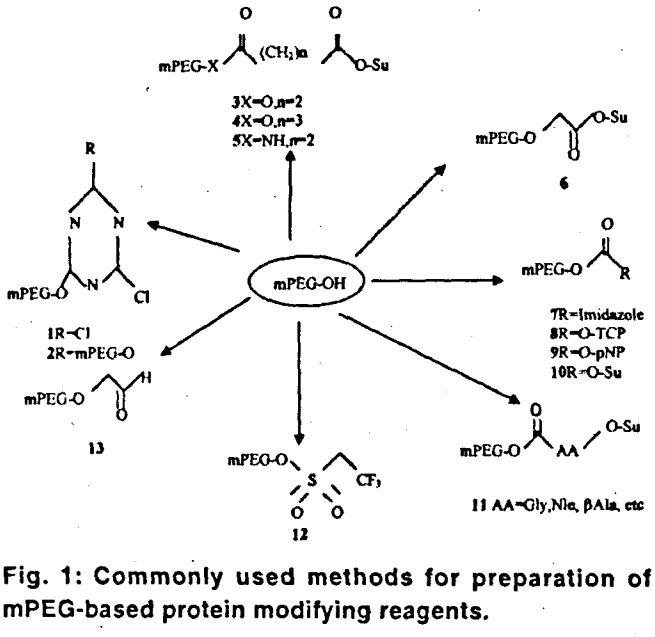

Fig. 1: Commonly used methods for preparation of mPEG-based protein modifying reagents.

1, 2-chloro-s-striazine, 3,4,5,6-Hydroxy succiniimidyl ester, 7,8,9,10 Carbonyloxy imidazolyl derivatives, 11 Carbonyl aminoacids 12, Tresylate (2,2,2-trifluroethane sulfonate), 13 Acetaldehyde.

carbonate[39], Depending on the final product desired, each coupling method has its own drawbacks. Coupling with cyanuric chloride may cause substantial decrease in biological activity of enzymes[40,41], coupled with hike in toxicity[42]. Modification with carbonyldiimidazole activator, reaction time is long-drawn-out. Activation of mPEG with succinic anhydride preserves the biological activity[38,43] but ester linkage thus produced are, prone to spontaneous hydrolysis, though at slow rates at physiological pH *in vivo* by nonspecific hydrolases in the blood[24,44]. The activation of mPEG with urethane linker results in an activated polymer with limited reactivity, thus necessitating long reaction times, though preservation of activity of modified enzymes is appreciable[37,45]. In some cases, the problem was reduced by carrying out the conjugation in the presence of an active-site protecting agent with specific affinity for the macromolecule[46]. The selection of coupling agent[47], molecular weight of mPEG[40,48], the percentage of groups modified[49], and the specific properties of the protein being modified[50] can alter the characteristics of the final mPEG adduct. It is quite possible to change any of these variables in order to 'custom design' the final product, though inherent characteristics of the protein dominate the final biological activity of the adduce[51]. A successful example of separation between mono and di-pegylated products was reported in Salmon calcitonin conjugation by PEG 12kDa. In this case, it could be demonstrated that the conjugated species were equally bioactive as the native peptide[52,53]. More sophisticated strategy to reach specific pegylation is based on a preliminary chemical reversible site directed protection of the peptide to leave some groups free for PEG conjugation as in the case of insulin[54-56].

Outcome of protein modification with polyethylene glycol:

It has been reported that mPEG molecules modify protein by forming shell around the protein which sterically hinder and prevents the recognition by the immune system[57] and this hindrance also prevents recognition by cell associated receptors, thus increasing circulating life from minutes or hours to days, as depicted in Table 3.

Apart from this mPEG modification effects stability, solubility, activity, immunogenicity and antigenicity. The PEG adducts results in remarkable increase in *in vivo* stability due to well-known predisposition of PEG to exclude proteins, other macromolecules and particulates from its surroundings. These properties of the polymer have been explained by its chain's high mobility associated with conformational flexibility and water binding ability[58]. As mPEG is extremely hydrophilic, the formation of water shell around protein mol-

TABLE 3: EFFECT OF PEG MODIFICATIONS ON VARIOUS PROTEINS.

Enzyme/protein	Comments	Ref.
Adenosine Deaminase	$T_{1/2}$ of PEG conjugated was increased from 30 min to 28 h.	41
Asparaginase	$T_{1/2}$ of PEG conjugated was increased from 2.9 h to 56 h	63
Arginase	$T_{1/2}$ of PEG conjugated was increased from 1 h to 12 h	64
Catalase	$T_{1/2}$ of PEG conjugated was increased from 10 min to 4 h	65
Superoxide Dismutase	$T_{1/2}$ of PEG conjugated was increased from 5 min to 4.2 h	66
Uricase	$T_{1/2}$ of PEG conjugated was increased from 30 min to 28 h	67
Gulonolactone oxidase	Conjugate most stable at 37° than native protein, $T_{1/2}$ was not extended.	68
Alkaline phosphatase	Conjugates were with well preserved activities	69
Ribonuclease	Improved dissociation constant as found for conjugated protein	70
Elastate	Completely lost anti-elastate binding Ability	71
Tissue Plasminogen activator	Clearance and inactivation were reduced after PEGylation	72
Trypsin	PEG-trypsin showed complete lack of autodigestion and slow rate of catalase cleavage	73
Chymotrypsin	Able to digest azoalbumin while failed to cleave bovine serum albumin	74
Bilirubin Oxidase	For detoxification of bilirubin	130

ecule[16] results in remarkable increase in the solubility of the resultant conjugate in the organic solvent and retention of activity. The formation of mPEG shell protects adduct from the proteolytic inactivation[41] and decreases the activity of the protein, though this decrease in activity[40] is offset by increase in circulating life. The increase in circulating half-life can be correlated to increase in molecular size due to masking protein's surface, which reduces the renal ultrafiltration, preventing the approach of antibodies or antigen processing cells and reducing the degradation by proteolytic enzymes[59]. That is why the ability of protein to induce immune response or reacting to antibodies is altered following modification. The coating of mPEG shell around the protein results in enhanced stability (chemical, physical and thermal) of the native protein. The umbrella structure of branched proteins is also very effective in protecting proteins from proteolysis, in the approach of antibodies, and in reducing immunogenicity[60,61]. This is well demonstrated in alpha-interferon conjugation that, once modified with the linear polymer, gave rise to eleven positional isomers, whereas, with the branched polymer, only one or two lysine residues were sites for PEGylation[62]. However, PEG, being a synthetic polymer, is polydispersed and even in the best of cases, a polydispersivity value (Mw/Mn) ranging approximately from 1.01 for low molecular weight oligomer (3-5 kDa), to 1.2 for high molecular weight (20 kDa). Polydispersivity is a negative property since it is reflected in polydispersivity of the conjugates. The monomethoxylated form of PEG is generally used in protein conjugation, since its monofunctionality yields cleaner chemistry. However, a certain amount of PEG diol is always present, in the range of 1-10%, depending upon the molecular weight. High diol concentration generally yields unwanted cross-linked conjugates.

In certain cases, mPEG modified protein circulates similarly in virgin and immunized animals, indicating that the mPEG adduct is nonimmunogenic[41]. The degree of reduction in immunogenicity and antigenicity varies with each protein surface[40]. Substantial alterations following mPEG modification are significant increase in solubility and circulating life, reduced antigenicity, immunogenicity, enhanced stability and negligible loss of biological activity. This sort of modification makes a ground for the protein (both human and nonhuman) to use as a pharmaceutical.

THERAPEUTIC USES OF MONO POLY (ETHYLENE GLYCOL) MODIFIED PROTEINS

Enzyme replacement therapy:

This therapy is based on the fact that in certain cases loss of specific metabolite enzyme is associated with a dis-

eased state, adenosine deaminase (ADA) modified with mPEG (ADAGEN) was reported for the use of severe combined immuno deficiency disease (SCID) associated with adenosine deaminase deficiency[75]. ADA deficiency causes partial or total dysfunction of the immune system that is fatal, if left untreated[76]. Weekly injection of ADAGEN maintained therapeutic levels continuously for up to 3 years in 10 of 11 patients. Patients receiving ADAGEN demonstrated the development of immune function via antigen specific humoral and cellular immune responses[77,78]. Clinically, patients gain weight, thrive and show an improvement in neurologic and/or psychomotor development[79,80].

Cancer therapy:

The peculiar alteration in cancer cells from their counterparts, in that the synthesis of building blocks such as aminoacid is limited which translate these cells, to be dependent on the host to supply the necessary requirements. This compelled the search in the field of metabolite depletion for cancer therapy in which enzymes were used to deplete the body of the required nutrient, thus selectivity starving the cancer cells and not affecting normal cells and tissues. The enzyme therapy for cancer was restricted by the problem of short blood circulating life and immunogenicity. Asparaginase is the only enzyme, which is extensively studied for the treatment of acute lymphoblastic leukemia (ALL). In clinical studies, it has been reported that it introduces immunological side effects, ranging from mild allergic reaction to anaphylactic shock in one third of the patients[81,82]. Hypersensitivity reaction also varies from species to species. It has been noted that hypersensitivity reaction is higher in patients receiving *Erwinia* asparaginase after treatment with *E coli* asparaginase than those not previously treated[83]. It is possible to switch over from one source of enzyme to another, it is being reported that *in viro*, there is no cross reactivity between the two forms. In addition, this does not solve the problem of potential immunogenicity but merely prolongs the time the enzyme can be used. When firstly asparaginase was used clinically for the treatment of lymphoblastic leukemia[84,85] and canine lymphosarcoma[86], a promising treatment was achieved without provoking immunogenicity, which is currently in clinical trials. In man, mPEG-asparaginase showed increase in half-life from 20 h to 375 h with enzyme levels detectable 15 days post dosing[87]. Several other enzymes with potential as anticancer therapies for metabolic depletion have also been modified with mPEG, including[88,89], glutamine-asparaginase[19] and phenylalanine lyase[41]. In addition, uricase[90] has also been modified with mPEG for use as a treatment for hyperuricemia associated with chemotherapy. In one study of the comparison of efficacy and toxicity of mPEG asparaginase to native *E coli* asparaginase in children with acute lymphoblastic leukemia in second bone marrow relapse combined with a standard 4 w vincristine and prednisone induction, mPEG-asparaginase was administered on day I and 15 while *E. coli* asparaginase was given 3 times a week for a total of 12 doses, of these patients 88 % tolerated mPEG-asparaginase without hypersensitivity or other significant reactions. Half of the patients achieved a complete or partial response[91]. Monopolyethylene glycol-asparaginase conjugate has been reported to be a less toxic drug than asparaginase while retaining activity[92] and can be safely administered to patients with known hypersensitivity to other forms of asparaginase[91].

Red blood cell substitutes:

Two major routes are followed for the development of artificial blood. The first involves the formation of an inert group of organic chemicals called perflourocarbon[93,94] referred to as "white blood" which has no similarity to natural blood. "Red blood" is based on modification of the naked hemoglobin from red blood cells so that oxygen carrying capacity can be maintained even devoid of red cell membrane[95,96]. Red cell membrane provides a protective covering, so that free hemoglobin could not provoke toxicity after breaking down into toxic products[97]. In addition when Hb (hemoglobin) is removed from the red blood cell membrane, the loss of 2,3 diphosphoglycerate results in an increase in oxygen affinity[98]. Number of methodologies have been developed in view to address these problems, which include cross linking of hemoglobin subunits to improve tetrameric stability for polymerization of the hemoglobin molecules attachment of artificial polymers of high molecular weight or some combination thereof[99]. Modification of hemoglobin with polyethylene glycol plays an important role. Based on the molecular weight of mPEG and the number of mPEG molecules attached a wide variety of characteristics were note[98,100]. Iwashita *et al.*[101] have shown that the oxygen carrying capacity of PEG hemoglobin, using mPEG of molecular weight 3400 and outdated human blood is lower than that of the whole blood. Rats exchanged transfused upto two weeks[102], upon lyophilization, this material could be stored for over a year with methamoglobin increase of only 15%[103]. Pyridoxylated mPEG hemoglobin was an effective oxygen carrier demonstrating oxygen delivery to the tissues for at least 6 h as compared to RBCs hemoglobin as reported[102]. Labrude *et al.*[104] showed that after conjugation hemoglobin in the urine was less than 6% after 20 h. When

comparing crosslinked mPEG hemoglobin to noncrosslinked mPEG hemoglobin, it was found that the noncrosslinked adduct had a half-life of 9.6-13 h in rats while the mPEG-hemoglobin prepared from crosslinked hemoglobin had a half-life upto 18.8 h in rats. These studies establish that hemoglobin modified with mPEG is of extreme value.

Oxygen toxicity associated diseases:

In pathological conditions, the oxygen free radicals damage the tissues, when body's free radical scavengers are overwhelmed to remove these metabolites. Free radicals have been associated with tissue damage in inflammation[105], various lung diseases[106,107], thermal injury[108] and ischemia/reperfusion diseases[109,110]. The superoxide dismutase (SOD) and catalase are antioxidant enzymes, which are administered as therapeutic agents to bolster the body's defense against oxidative damage. SOD has been used in human for the treatment of rheumatoid arthritis[111], radiation cristics[112], broncho pulmonary dysplasia[113], and degenerative joint disease[114]. Catalase has been reported for the effective application in thermal injury[115] and arthritis and combination of these two enzymes are used in ischemia and reperfusion[116] and in vascular injury[117]. The problems with these enzyme therapy are, induction of allergic responses and very short circulating life. SOD has plasma half-life of 6 to 10 min[118] while the catalase has 23 min. When modified with mPEG the half-life of both enzymes increased upto 40 h[119]. Another dramatic effect noted was enhancement in cell binding to endothelial tissues and that modification resulted in the uptake of these normally membrane impermeable enzymes[120]. Michelson *et al.*[121] reported that antiinflammatory activity of SOD is not due to its molecular weight or its circulating half-life but rather due to its high binding affinity to membranes at the site of local inflammation. mPEG-SOD increased the survival of skin flaps to 80% as compared to 52% when administered native SOD. mPEG-SOD has been reported to provide protection in canine's undergoing regional myocardial ischemia[116]. Simultaneous administration of mPEG-SOD and mPEG-catalase at birth prevents lung damage secondary to oxygen treatment and mechanical ventilation in premature lambs with respiratory distress. MPEG catalase was shown to ameliorate the asbestos induced lung damage[122]. In addition, it has been reported that mPEG alone may have some antioxidant property in that it can inactivate hydroxyl radicals *in vitro*[123]. The protective action of either enzyme is related with specific oxidant causing damage in the tissue (superoxide radical or hydrogen peroxide) and selection of mPEG-enzyme must match the damaging agent present in the tissue.

Detoxification of bilirubin by PEG conjugated bilirubin oxidase:

Bilirubin, the end product of heme catabolism, is generally regarded as toxic and fatal in newborn infants and fulminant hepatitis. Bilirubin encephalopathy (kernicterus) is usually considered to be caused by the entry of circulating, free (albumin-unbound), unconjugated bilirubin into the cerebral tissue[124] Bilirubin conjugation with glucuronic acid takes place in the liver and the process is impaired in liver diseases. The tactic for treatment of jaundice is to decompose toxic bilirubin by employing its polymer conjugate to improve pharmacological properties.

Previously, various methods were used to remove bilirubin, such as plasma exchange, steroid therapy and phototherapy, but none has proven to have therapeutic value as a first choice regimen. Bilirubin oxidase is a specific enzyme and is reported to be useful for the treatment of neonatal jaundice with an immobilized bilirubin oxidase column system. However, the column system has many inconveniences, such as physical confinement, clotting problems, and high incidence of infections. PEG conjugated bilirubin has been synthesized by Maeda *et al.*[125] and reported to have increased plasma half life, reduced immunogenicity and more effective than native counterpart. They conjugated protein carboxyl group with diaminobutane as a spacer side chain to provide reactive amino group and finally reacted with PEG derivatized p-nitrophenyl chloroformate. It was found that PEG conjugated bilirubin oxidase oxidized free bilirubin 100-200 times more than native and completely safer when studied against C1300 neuroblastoma cells.

CONCLUDING REMARKS

By and large, pegylation has been applied to protein, with enzymatic activity. Nevertheless, mPEG can also be attached to peptides and hormones and as such has been applied successfully to modified calcitonin, insulin, bovine growth hormones, NAD[126] D-alpha tocopherol[127] and interleukin-2[128]. Monopolyethylene glycol has also been attached to antibodies and allergens with success[129]. Interestingly mPEG modification is not limited only to improvement of protein or pharmaceuticals, but can also be applied to other areas depending upon the property of the molecules. Of these various approaches, glycosylation and site directed mutagenesis are of much importance to enhance the therapeutic efficacy of proteins. Site directed mutagenesis offer the possibility of designing a new generation of proteins with greater cell specificity and fewer side effects than those available today. Furthermore, the upcoming of new pegylation

tools such as dendrimer PEG will further develop the biological and therapeutic applications that, began in the 190s with pioneering albumin and catalase.

Pegylation can be used to perk up thermal stability, solubility in organic solvents. mPEG can be used to stabilize or solubilize compounds. mPEG can be applied to the development of diagnostics, targeting or imaging agents, and can be used in kinetic analysis or receptor binding studies. These compounds can also be used to figure out the mechanism of the immune system. Pegylation can also applied to liposomes to enhance effectiveness. Site directed attachment and noncovalent attachment of mPEG open up the entire new spectrum of possibilities. Molecular weight changes and charge of the mPEG attached also provide new possibilities, thus opportunities for producing end product precisely having long acting life and cost effective. Realization of a new thrilling horizon in the field of drug delivery system with polyethylene glycol is in the offing.

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