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BIOTECHNOLOGY BASED DRUG DELIVERY BY PEGYLATION METHOD

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ABSTRACT
PEGylation methods represent promising means for delivering many bioactive agents, including peptide and protein drugs. The importance of these methods grew with the advancement in the understanding of peptide and protein pharmacology as well as the ability to mass-produce these compounds. This polymer is non-toxic, non-immunogenic, non-antigenic, and highly soluble in water and FDA approved. These PEGylation systems have several advantages over conventional methods such as ease of manufacture, ease of administration, a prolonged residence in body, a decreased degradation by metabolic enzyme and a reduction or elimination of protein immunogenicity. In this article, we introduce the different strategies that have been developed and patented for use of poly ethylene glycol (PEG) in delivering peptide and protein drugs. The advantage, disadvantage, possibilities, and limitations of each of the PEGylation systems have been discussed.

KEYWORDS: Protein, peptide, conjugation, growth hormone, graft

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INTRODUCTION

The rapid growth in the field of biotechnology has led to the development of numerous recombinant protein drugs such as hormones and vaccines. These protein drugs are usually high molecular weight proteins and very sensitive to environmental conditions. Most proteins cannot be delivered orally due to problems related to degradation in the acidic environment of the gastrointestinal tract. Moreover the high molecular weight of these substances often results in poor absorption in to the blood stream when administered orally. Alternative routes, such as nasal, pulmonary, rectal, buccal, vaginal, and transdermal, have been investigated. It has been shown that protease activities in the homogenates of the nasal, buccal, rectal, and vaginal mucosa of rabbits are substantial and quite comparable to those in the intestinal mucosa.[1] In another study, degradation of luteinizing hormone releasing hormone (LHRH) was reported in buccal, liver, and nasal tissues.[2] Consequently most of the new protein based therapeutics are administered by frequent injections through the parenteral routes such as intravenous, intramuscular, and subcutaneous. With the exception of life-threatening conditions, this form of delivery has traditionally been poorly accepted by patients. Implantable drug delivery systems of biocompatible polymers, such as ethylene-vinyl acetate[3] cellulose acetate, and polymethyl methacrylale[4] have been studied. These implanted devices release drugs with zero order kinetics for an extended period which is desired. However these devices must be surgically implanted and, in some cases, explanted. Consequent disadvantages of using these implants include patient discomfort, possibility of infection and medical costs.[5] Chemical attachment of the hydrophilic polymer PEG, which is also known as poly (ethylene oxide) (PEO), to molecules and surfaces is of great utility in biotechnology. As an example of biotechnical applications of PEG, some active derivatives of PEG have been attached to proteins and enzymes with beneficial results. PEG is soluble in organic solvents. PEG attached to enzymes can result PEG-enzyme conjugates that are soluble and active in organic solvents. Attachment of PEG to protein can reduce the immunogenicity and rate of kidney clearance of the PEG-protein, which may result in dramatically increased blood circulation life times for the conjugate.[6] PEG attached to surfaces can reduced protein and cell adsorption to the surface and alter the electrical properties of the surface. Similarly, PEG attached to liposome's can result in a great increase in the blood circulation life time of these particles and there by possibly increase their utility for drug delivery.

Since then, the procedure of PEGylation as initially described was expanded and developed tremendously and now a wide range of chemical and enzymatic methods for conjugation are at hand.[7] This variety of modification procedures offers the possibility to address the requirements of different proteins. In this regard, this present article is an attempt to review several advances made in PEGylation technologies and discuss how these advances may be applied to resolve the challenges face the development of PEGylation systems for the delivery of protein drugs.

ADVANTAGES OF PEGYLATION

1. PEG conjugation can shield antigenic epitopes of the polypeptide, thus reducing reticuloendothelial (RES) clearance and recognition by the immune system and also reducing degradations by proteolytic enzymes.
2. PEG conjugation increases the apparent size of the polypeptide, thus reducing renal filtration and altering biodistribution.
3. PEGylation increases the plasma half life of protein and reduces the immunogenicity of proteins and in addition, the proteins remain biologically active.
4. Decreased dosing frequency due to prolonged residence in the body.
5. The advance PEGylation also offered new opportunities for creating viable peptides and protein drugs by site-specific PEGylation.

DISADVANTAGES OF PEGYLATION

1. As each PEG subunit is capable of binding 2-3 water molecules, a peptide drug conjugated with a PEG could be 5-10 folds larger in size compared to its unmodified counterpart. This increase in size, which might also be attained by multiple conjugations of PEG molecules, could decrease both the epithelial permeability and activity of the peptide drug.

2. PEG is obtained by chemical synthesis and, like all synthetic polymers, it is polydisperse, which means that the polymers batch is composed of molecules having different member of monomers, yielding a Gaussian distribution of the molecular weights. This leads to a population of drug conjugates, which might have different biological properties, mainly in body-residence time and immunogenicity.

3. PEGs are usually excreted in urine or feces but at high molecular weights they can accumulate in the liver, leading to macromolecular syndrome.

PHYSICO-CHEMICAL PROPERTIES OF PEG

Polyethylene glycol is a polymeric linear structure with repeating polyethylene oxide units. It has two ends, the α-end and the ω-end, which can carry the same or different functional groups. Then they are classified as homo-or-hetro biofunctional PEGs accordingly.

$$\alpha-O-CH_2-CH_2-(O\,CH_2\,CH_2)_n-O\omega$$

PEG can be produced as linear or branched chains, with functional groups at one or more termini to enable a variety of conjugation possibilities (**Fig.1**)

One characteristic parameter is the chain length n, which determines the molecular weight M of the compound. Depending, whether the polymer is consisting of one single molecular weight (only one n existing) or of a range of compounds with an average mass and a distribution of n around a mean value, PEG polymers are referred as monodisperse or polydisers. Polydispersivity value ranging approximately from 1.01 for low molecular weight oligomer (3-5kd_a), to 1.2 for high molecular weight (20 kd_a) may be expected. This polydispersivity is a negative property since it is reflected in polydispersivity of the conjugates.

A liner PEG has a α end and ω end. If one end is carrying a methoxy group it is unreactive at this end and cannot be used for any further chemical modification. The other end still carrying a reactive group can from a conjugate. The PEG is then called monofunctional and can be used to PEGylate a proteins or to form a thin PEG layer on a gold surface. If both ends are carrying a reactive group the PEG is called bifunctional and can be used as linker between two compounds. With a cleaver combination of reactive groups on both ends, involving orthogonal protecting group techniques a broad variety of applications is possible. Studies of PEG in solution have shown that PEG typically binds 2-3 water molecules per ethylene oxide unit. Due to both the high flexibility of the backbone chain and the binding of water molecules, the PEG molecules acts as if it were 5-10 times as large as a soluble protein of comparable molecular weight.

CONJUGATION OF POLYETHYLENE GLYCOL

Amino groups were the first target of PEGylation, by acylation or alkylation reactions, but now conjugation of PEG to thiol, hydroxyl or amide groups is also possible, by using several specific chemical or enzymatic methods. In the early days of PEGylation, researchers directed their attention towards the amino groups as suitable conjugation site because they are the most represented groups in proteins, generally exposed to the solvent and can be modified with a wide selection of chemical strategies. The PEG dichlorotriazine derivative can react with multiple nucleophilic functional groups such as lysine, serine, tyrosine, cysteine, and histidine, which results in displacement of one of the chlorides and produces a conjugate with retained charge in the form of a secondary amine linkage.[8]

$$\text{PEG—O—C}_3\text{N}_3\text{Cl}_2 \xrightarrow{\text{R-NH}_2} \text{PEG—O—C}_3\text{N}_3\text{Cl(NH-R)}$$

The remaining chloride is less susceptible to reactions with nucleophilic residues. Unfortunately, the reactivity is sufficient to allow cross linking of protein molecules containing additional nucleophilic residues. **Table 1** given below shows the various PEG derivatives that can react with amine to produce conjugated products.

Few proteins possess thiols suitable for PEG binding. However this rare residue may be introduced at the desired position of the sequence by genetic engineering, a strategy that offers the conditions for site directed PEGylation. These sequence specific modification may take advantage of the few thiol reactive PEGs. Many mutant proteins have been described which were generated to PEGylate therapeutically important drug, such as human growth hormone or G-CSF.[14,15]

In the former case, the buried and less-accessible cysteine 17 was modified with a double step method. In the first step the cysteine was modified with a heterobifunctional low molecular weight PEG, bearing at one end a thiol reactive group and at the other an azide group. This polymer can reach the cysteine because of its low steric hindrance. In a second step, a high molecular weight PEG chain, with a group reactive towards azide, was specifically linked to the first PEG chain. Some selective thiol PEGylating agents are reported in **Table 2**.

Oxidation of carbohydrate residues or N-terminal serine or threonine is an alternative method for site directed PEgylation of proteins .Carbohydrates can be oxidized with enzymes, such as glucose oxidase, or chemically with sodium periodate. Oxidation of the carbohydrates residues generates multiple reactive aldehyde groups, which can be reacted with either PEG-hydrazide to produce a hydrazone linkage or with PEG-amine to produce a reversible Schiff base[20].

PEG-isocyanate is useful for hydroxyl group conjugation yielding a stable urethane linkage. PEG[21] –isocynate is infact highly reactive with amines also.

$$\text{PEG-O-CH2-CH2-N=C=O+R-OR} \rightarrow \text{PEG-O-CH}_2\text{-CH}_2\text{-NH-C(=O)-O-R}$$

$$\text{PEG-O-CH2-CH2-N=C=O+R-NH}_2 \rightarrow \text{PEG-O-CH}_2\text{-CH}_2\text{-NH-C(=O)-NH-R}$$

The earlier PEGylated conjugate aimed simply to use PEG to increase systemic exposure of the drug or reduce adverse reaction, without optimizing the effect on potency. More recent approaches however integrate the pharmacological properties of the drug and of PEG to minimize the loss of potency while maximizing exposure. **Table 3** lists the most important examples of PEG conjugates in the pharmaceutical market.

PROTEIN AND PEPTIDE DRUG DELIVERY BY PEGYLATION

Huang *et al*[22] evaluated PEGylated dendrigraft poly-L-lysines as novel gene delivery vectors. In his work, dendrigraft poly-L-lysines (DGELS) were evaluated as a novel gene vector for the first time. The results indicate that proper PEGylated DGLs could mediates efficient genes transfaction, showing their potential as an alternate biodegradable vector in the field of non viral gene deliver. In one study[23] PEGylation enhances penetration of fibroblast growth factor 2(FGF2) to injured spinal cord tissue from an intrathecal delivery system. Conjugation to PEG is known to improve penetration of proteins into tissue by reducing clearance and providing immunogenic shielding. Importantly, PEG conjugation nearly doubled the concentration of PEGF2 in the injured spinal cord when delivery locally. Qioa *et al*[24] synthesized PEGylated poly (2-dimethlyamino) ethyl methanoylate (PDMAEMA) by atom transfer radical polymerization .Interestingly, mice in vivo vaccination study clearly showed that PEGylated PDMAEMA used as DNA delivery vector significantly improved the prime effect of DNA vaccine through intranasal administration. Importantly PEGylated PDMAEMA was further proved its ability to induce cytokines production by murine macrophages. Overall, mPEG-b-PDMAEMA can be used as an efficient DNA vaccine vector which enhances adaptive immune responses by activating innate immunity. Higashi *et al*[25] prepared a insulin derivative substituted randomly with poly ethylene glycol (PEG,MW about 2200) and its polypseudorotaxanes with cyclodextrine (CyDs). The pegylated insulin /gamma CyD polypseudorotaxanes displayed a significantly higher resistance to proteolysis. The results indicated that the CyD polypseudorotaxanes could be formed with randomly pegylated insulin and work not only as a sustained release system ,but also as a stabilizing agent to enzymatic degradation of pegylated insulin. In one study, Wang *et al*[26] developed novel peptide targeted delivery systems for systemic and targeted delivery of therapeutic Si RNA based on a multifunctional carrier, (1-aminoethyl) iminobis[N-(oleicylcysteinyl histinyl-1-aminothhyl) propionamide](EHCO) ,which showed pH –sensitive amphiphilic cell membrane disruption.

PEGylation of the SiRNA/EHCO nanoparticles significantly reduce non-specific cell uptake .The incorporation of a bombesin peptide or RGD peptide via a PEG spacer resulted in receptor-mediated cellular uptake and high gene silencing efficiency in 487 cells. Fluorescence confocal microscopic studies demonstrated that EHCO/SiRNA nanoparticles and PEG modified EHCO/SiRNA nanoparticles were able to facilitate endosomal escape of the SiRNA delivery systems. Tuesca *et al*[27] synthesized and purified mono substituted PEG-insulin conjugate. The site of conjugation was determined by MALDI-TOF-MS. Polyethylene glycol conjugate specifically to the amino terminus of the B-chain of insulin maintained the bioactivity of the protein and significantly extended the duration of the hypoglycemic effect. Brain–derived neurotrophic factor (BDNF) was covalently attached to PEG in order to enhance delivery to the spinal cord via the cerebrospinal fluid (intrathecal administration)[28]. A high degree of in vitro biological activity was maintained in mixtures enriched in primary and secondary conjugate products, while a substancial reduction in biological activity was observed in mixtures with tertiary and higher order conjugates. When a biologically active mixture of PEG–BDNF was administered intrathecally, it displayed a significantly improved half-life in the cerebrospinal fluid and an enhanced penetration into spinal cord tissue relative to native BDNF.

The conjugation of PEG to proteins serves as a mechanism of prolonging the residence time by decreasing degradation and prolonging half life in the lungs while reducing side effects by limiting systemic absorption[29]. It appears to be a safe carrier[30] and prolong the effect of rhG-CSF upon delivery to the airways of male rats. PEGylated superoxide dismutase (SOD) delivered to the lungs has been found to be effective in protecting rats from oxygen toxicity compared with SOD alone, which offered no protection[31]. Once the protein/peptide has reached the lungs, its site of action may be on the cells surface, intracellularly in airway cells, or in the systemic circulation. A carrier /drug can be modified in several ways to improve distribution into the desired target cell/tissue. For many proteins and peptides, rapid proteolytic degradation by circulating enzymes represents one of the principal challenges in producing viable therapeutics. PEG provides protection from proteases and peptidases by impairing access for proteolytic enzymes. Despite this, the continual mobility of the PEG domain provides sufficient flexibility to enable high affinity interactions between the target receptor and the drug moiety and thus yield a biological effect. Consequently, the PEGylated drug retains efficacy while acquiring greater stability in plasma. The most likely model to explain the protection from proteolysis involves a dynamic process in which the highly hydrated but mobile PEG moiety creates stearic obstruction over the domain of the protein that serves as an enzyme substrate, thus reducing the frequency of favorable collisions.

Growth Hormone (GH) is a 22 KD protein, secreted by somatotrophs of the anterior pituitary gland, which promotes growth and affects metabolism. GH contains two distinct binding sites for the receptor, termed BS1 and BS2, which display high and low affinity binding respectively. In vitro binding studies suggest that high affinity binding to a single receptor via the BS1 sites enables interaction of the lower affinity BS2 site with a second receptor in the performed dimmer, leading to activation of the intracellular signaling cascade[32]. Site directed mutagenesis experiments demonstrated that a single point glycine to lysine mutation in the BS2 site (G120k) generates an antagonist able to bind the GH receptor with high affinity but unable to activate signal transduction[33]. This G120k-GH antagonist displayed a very short plasma half life of approximately 30 minutes, similar to the 15 minutes half life reported for the parent GH[33]. To prolong the plasma circulating time, 5 kDa PEG was conjugated to the antagonist using a random conjugation approach that attached PEG at free amine groups on lysine residues .This yielded a protein with a dramatically longer half life at more than 100 h ,but which lost 186 –fold potency in receptor binding studies compared with GH.

Treatment of hepatitis C virus (HCV) infection with unmodified interferons produces a sustained response only in a minority of patients however, as a result of host factors such as the viral load, viral factors such as the viral genotype, and most significantly pharmacokinetic properties of the interferon themselves[34]. The plasma half life of 4-6 h results in undetectable levels of IFN α 2a within 24h of a subcutaneous dose, and thus requires 3 times per week dosing by subcutaneous injection[35]. This produces continual fluctuations in plasma concentrations of IFN –α 2a, in which intervals of negligible IFN –α 2a creates periods of exposure to the virus, and opportunities for the virus to replicate and develop resistance mutations. Initial attempts at PEGylation employed a 5kDa PEG conjugated to the 19 kDa IFN –α 2a molecule, but generated an insufficient improvement over the unmodified IFN –α 2a, when tested in clinical trials[36]. A branched 40 kDa PEG was then used to conjugate to IFN-α 2a, and produce a substantially different serum concentration time profile. PEGlyation prolonged the serum half life from 3.8 to

65h, showed the clearance by more than 100 fold and reduced the volume of distribution 5 fold[37].
Therapeutic enzymes represent a growing class of biopharmaceuticals and PEGylation has played a central role in improving or enabling several of these products[38]. For most enzymes examined, reduction in immunogenicity represents the principal mechanism by which PEG prolongs the circulating half life. Early work on adenosine deaminase (ADA) demonstrated the ability of PEG to reduce the generation of neutralizing antibodies, extending the plasma half life from several minutes to approximate 24h and led to the development of the first PEGylated enzyme therapeutic, pegedemese[39]. Veiseh *et al*[40] has been developed a non viral nanovector by PEGylation of DNA complexing polyethylenimine (PEI) in nanoparticles functionalized with an Alexa Fluor 647 near infra red flurophore, and the chlorotoxin (CTX) peptide which binds specifically to many forms of cancer. With this nanovector, the potential toxicity to healthy cells is minimized by both the reduction of the toxicity of PEI with the biocompatible copolymer and the targeted delivery of the nanovector to cancer cells as evaluated by viability studies. The nano vector demonstrated high levels of targeting specificity and gene translation efficiency with both C6 glioma and DAOX medulloblastoma tumor cells.
Sonoke *et al*[41] designed PEGylated lipid carriers for achieving increased plasma concentration RNA and hence improved accumulation of RNA in tumor by the enhanced permeability retention effect .They compared the pharmacokinetics of SiRNA complexed with liposomes incorporating pegylated lipids with larger (C-17or C-18), shorter (C-12toC-16),or unsaturated (C-18:1) acyl chain. When longer acyl chains were used, the plasma concentrations of SiRNA obtained were dramatically higher than when shorter or unsaturated chains were used. Three fold higher accumulation of RNA in the tumors was achieved when pegylated liposome incorporating a C-18 lipid rather than nonpegylated liposomes was used, and sequence specific anti tumor activity was observed.
In one study, Jiang *et al*[42] modify pegylated recombinant human tumor necrosis factor alpha (PEG-TNF-alpha) with transferrin (Tf) to form Tf – PEG –TNF –alpha conjugates, which could maintain the advantages of pegylation and also achieve the function of active targeting to tumor cells. These result suggested that Tf–PEG –TNF –alpha was a useful long circulating conjugate with the capabilities of specific receptor binding resulting in enhanced antitumor activity of TNF –alpha. In another study, Vyas *et al*[43] investigated the effects of PEG modification and there, subsequent capsulation in multivesicular liposome (MVLs), on the release properties of IFN alpha. In this study interferon – alpha was conjugated with methoxy polyethylene glycol (mPEG, MW5000). Prepared IFN alpha-mPEG5000 conjugate (IFN alpha-mPEG5000) was purified with size exclusion chromatography. The relative in vitro antiviral activity of pegylated interferon alpha -2a was found to 87.9% of the unmodified IFN alpha.
Kushibixi *et al*[44] prepared cationized gelatins grafted with PEG (PEG- cationized gelatin) and evaluate the in vivo efficiency as a non viral gene carrier. Cationized gelatin was prepared by chemical introduction of ethylenediamine to the carboxyl group of gelatin. When the PEG –cationized gelatin-plasmed DNA complex was intramuscularly injected, the level of gene expression was significantly increased compared with the injection of plasmid DNA solution.

CONCLUTION

In general, protein delivery methods have continued to evolve over the past year; however, a great deal of research and development is still required to make most of these methods feasible for commercialization. PEGlyation of protein and peptides has advanced considerably over the last two decades, and the appearance of PEGlyated drugs promises to continue to accelerate as the field of biotherapeutics expands. The products already approved by the FDA are a clear demonstration of the usefulness of PEGylation in the improvement of the therapeutic value of drugs .The most relevant advantages are the prolonged body-residue time, which allows less frequent administration, the increase in stability towards proteases or nucleases and the reduction of immunogenicity. We expect that the approval of PEG-Intron for Hepatitis C and the FDA filing of Pegasys (PEG-IFN-alpha 2a), PEG-Neupogen® (PEG-G-CSF) and PEGVisomant (PEG-hGHra) will bring new life to a seasoned technology.

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Table 1: PEG derivative with conjugate product

PEG derivative	Reacting amine	Conjugated product	References
PEG—O H O PEG aldehyde	R-NH_2	PEG—O NHR	9
PEG—O O—so$_2$ cf$_3$ Tresylated - PEG	R-NH_2	PEG—O NHR	10
PEG—O O —NO_2 O PEG-P-nireophynyl carbonate	R-NH_2	PEG—O NHR O	11
Cl PEG—O O —Cl O Cl PEG-trichlorophynyl carbonate	R-NH_2	PEG—O NHR O	11
PEG—O C —N^+ N O PEG oxycarbonylimidazole	R-NH_2	PEG—O NHR O	12
PEG—O O—N N N O PEG-benzotrizole carbonate	R-NH_2	PEG—O NHR O	13

Table 2: Thiol reactive PEG derivative

PEG derivative	Reacting group	Conjugated product	References
PEG—S—S— N PEG-ortopyridyl - disulphide	R—SH	PEG—S—S—R	16
O PEG —N O PEG-maleimide	R—SH	O S —R PEG —N O	17
O PEG—S—CH=CH_2 O PEG-vinylsulphone	R—SH	O PEG—S—CH_2–CH_2—S—R O	18

O II PEG-NH-C-CH$_2$I PEG-iodoacetamide	R—SH	O II PEG-NH-C-CH$_2$-S-R	19

Table 3: Partial list of PEGylated marketed product

Trade name	Parent drug	use	Year to market
Somavert	Growth harmone receptor antagonist	Acromegaly	2002
Pegasys	Interferon α 2A	Hepatitis C	2002
PEG-intron	Interferon α 2B	Hepatitis C and clinical trials for cancer, multiple sclerosis, HIV/AIDS	2000
Neulasta	Granulocyte-colony stimulating factor	Treating of neutropania during chemotherapy	2002
Macugen™	Anti-vascular endothelial growth factor	Macular degeneration	2004
Oncaspar	asparginase	Acute lymphoblastic leukemia.	1994
Adagen	Adenosine deaminase	Severe combined immuno deficiency diseases	1990
Pegvisomant	Growth hormone	Acromegaly	2002
CD870	Anti TNF Fab	Rheumatoid arthritis	2005

$$H—O—CH_2—CH_2—(O\,CH_2\,CH_2)_n—OH \quad \text{------- (A)}$$

$$H_3C—O—CH_2—CH_2—(O\,CH_2\,CH_2)_n—OH \quad \text{------- (B)}$$

mPEG —O—C(=O)—C—NH—C(—C(=O)OH)—(CH$_2$)$_4$—NH—C(=O)—O— mPEG -(C)

Figure1: structural formula of-PEG molecules. (A) Linear PEG (B) Linear mPEG (C) Branched mPEG$_2$