A Review on PEG-ylation in Anti-Cancer Drug Delivery Systems

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ABSTRACT
PEGylation is a well known method used to improve the pharmaceutical properties of bioactive compounds. The main objective of this review is to provide a clear idea about pegylation and its use in drug delivery systems of cancer therapy. The conjugation of polyethylene glycol (PEGylation) is an effective method for overcoming the problem of rapid elimination of the drug from the body. Hence pegylation has paved the way for drug delivery by using proteins and peptides, liposomes, enzymes, and nanoparticles. This review explores the work directed towards cancer therapy, through more specific methods of drug delivery. This review also discusses about the various conjugation of protein compounds occurring in pegylation. The ability to treat cancer using pegylated liposomes and enzymes are discussed. Delivery methods through pegylated proteins and peptides and nanoparticles are also highlighted. This article explores the importance of pegylation in the field of cancer therapy and how this improves the properties of the drugs thereby providing safe treatment in patients.

Keywords: PEGylation, Conjugation, Nanoliposomes, Nanoparticles, Spherulite formulation.

INTRODUCTION
Approximately 11 million people are diagnosed with cancer every year, and cancer is responsible for about 7 million deaths/year (12.5% of deaths worldwide), making this disease a huge factor in worldwide mortality. The occurrence of cancer is expected to increase continuously as the world population ages, and it has been estimated that there will be around 16 million new cancer cases every year by 2020; and despite tremendous efforts to treat cancer, there has been very little improvement in cancer therapeutics over the past 50 years or so. To effectively improve cancer therapy, our knowledge of cancer pathophysiology, discovery of new anticancer drugs, and developing novel biomedical technologies should be thereby improved. Consequently, cancer therapy has become a multidisciplinary challenge requiring close collaboration among clinicians, biological and material scientists, and biomedical engineers.

Targeted cancer therapies may be more effective than current treatments and less harmful to normal cells. Targeted therapy blocks the growth of cancer cells by targeting specific molecules needed for carcinogenesis and tumor growth (NCI Dictionary of cancer Terms 2009) and this has paved the way for cancer therapy. The major drawbacks in targeted drug delivery systems include the following: Most of the anti-cancer drugs are insoluble in water; Nanoparticles which have been a promising aid in cancer therapy are removed by the Reticulo-endothelial system and thereby the circulating half-life of the drug is reduced. However, conjugation of therapeutic drugs with poly (ethylene glycol) has been successfully employed to increase their circulating half-life by enhanced permeation and retention and solubility as well as to reduce immunogenicity and toxicity (Markus Deckert et al. 2000). Agent-specific pegylation methods have been used in recent years to produce pegylated drugs that have biologic activity that is the same as, or greater than, that of the parent drug. These agents have distinct in vivo pharmacokinetic and pharmacodynamic properties, as exemplified by the self-regulated clearance of pegilgrastim, the prolonged absorption half-life of pegylated interferon alpha-2a, and the altered tolerability profile of pegylated liposomal doxorubicin. Pegylated agents have dosing schedules that are more convenient and more acceptable to patients, and this can have a beneficial effect on the quality of life of patients with cancer (Molineux 2002).

Properties of PEG
Polyethylene glycols are non-toxic, water soluble polymers that, owing to their hydrodynamic volume create a shield around the pegylated drug, thus protecting the drug from renal clearance, enzymatic degradation and recognition cells on the immune system. In its most common form poly (ethylene glycol), PEG, is a linear or branched polyether
terminated with hydroxyl groups and having the general structure:

\[
\text{HO-} \left(\text{CH}_2\text{CH}_2\text{O}\right)_n \text{-CH}_2\text{CH}_2 \text{-OH}
\]

PEG is synthesized by anionic ring opening polymerization of ethylene oxide initiated by nucleophilic attack of a hydroxide ion on the epoxide ring. Most useful for polypeptide modification is monomethoxy PEG, mPEG, having the general structure:

\[
\text{CH}_3\text{O-} \left(\text{CH}_2\text{CH}_2\text{O}\right)_n \text{-CH}_2\text{CH}_2 \text{-OH}
\]

Monomethoxy PEG synthesized by anionic ring opening polymerization is initiated by methyloxideions. Commercially available mPEG contains a small amount of diol PEG due to the presence of trace amounts of water during polymerization. This diol PEG is of relatively high molecular weight due to polymerization. The amount of diol PEG can exceed 15% of the composition of mPEG. A solution to the problem of diol contamination has been developed in laboratories (Roberts et al. 2002). This crude benzyl-PEG, containing diol impurity, is methylated and then hydrogenated to remove the benzyl group. Thus diol is converted to the inert dimethyl ether, which can be subsequently removed after activation and polypeptide attachment.

\[
\text{BzO-PEG-OH+HO-PEG-OH} \rightarrow \text{HO-PEG-OCH}_3 \text{+CH}_3\text{O-PEG-OCH}_3
\]

The other common route for diol removal is to convert the PEGs to PEG-carboxylic acids that can then be purified by ion-exchange chromatography (Harris and Kozlowski 1997). PEG with various end groups can be prepared by use of suitable initiator and/or termination reagents. Numerous functionalities can be introduced as end groups on PEG in this manner, including heterobifunctional products. For instance, Kataoka et al. synthesized a heterobifunctional PEG derivative containing aldehyde and thiol end groups (Akiyama et al. 2000). Compared with other polymers, PEG has a relative narrow polydispersity \((M_w/M_n)\) in the range of 1.01 for low molecular weight PEGs (<5 kDa) to 1.1 for high molecular weight PEGs (>50 kDa). The unique ability of PEG to be soluble in both aqueous solutions and organic solvents makes it suitable for its application in cancer therapy. Studies of PEG in solution have shown that PEG typically binds 2–3 water molecules per ethylene oxide unit. Due to the high flexibility of the backbone chain and the binding of water molecules, the PEG molecule acts as if it were five to 10 times as large as a soluble protein of comparable molecular weight. These factors have been suggested as the reason that PEG exhibits the ability to precipitate proteins (Polson 1977), exclude proteins and cells from surfaces (Gombotz 1992), reduce immunogenicity and antigenicity (Working et al. 1997) and prevent degradation by mammalian cells and enzymes (Richter and Akerblom 1983). Low molecular weight oligomers of PEG (<400 Da) have been shown to be degraded in vivo by alcohol dehydrogenase to toxic metabolites, however the lack of toxicity of PEGs with a molecular weight above 1000 Da has been revealed over many years of use in foods, cosmetics and pharmaceuticals (Richter and Akerblom 1984).

PEG is rapidly cleared in vivo without structural change and clearance is dependent on molecular weight. Below a molecular weight of about 20 kDa the molecule is cleared in the urine, and higher molecular weight PEGs are cleared more slowly in the urine and feces. PEG is only weakly immunogenic even at high molecular weights. Antibodies to PEG have been generated when attached to a highly immunogenic molecule under an immunization protocol with Freund’s adjuvant (Richter and Akerblom 1984; Cheng et al. 1999; Goodson and Katre 1990). There are no known situations in which anti-PEG antibodies have been generated under ‘normal’ clinical administration of a PEG-modified protein.

PEGylation in protein and peptide drug delivery for treatment of cancer

Among the approaches in the field of drug delivery, PEGylation has so far been the best choice for protein delivery. Proteins, have played a major role in drug delivery, however formulation of such proteins have become difficult. Unfortunately, formulation alone cannot fulfill all the requirements to yield a safe and successful protein preparation for cancer therapy applications. Increasing the natural body defense mechanisms and balancing deregulated processes involved in tumorigenesis, such as regulation of cell cycle progression, angiogenesis, and apoptosis provide rational targets for novel therapies. In most cases, interfering with these mechanisms can be achieved by using biomolecules as active agents, most commonly hormones, proteins or nucleic acids, whose use has become economically advantageous after the advent of the recombinant DNA technology (Gianfranco Pasut et al. 2008). The importance of PEG reagents for peptide and protein modification has only been realized in the last several years as more and more PEG-conjugates make it to late phase clinical trials. Therefore, the results of the ongoing clinical trials to determine future product candidates.
are very much awaited. For the coupling of PEG to a molecule, it is necessary to activate the PEG by preparing a derivative of the PEG having a functional group at one or both the terminals. For proteins, the PEG is activated by the following conjugations.

**Amine conjugation**
The conjugation of electrophilic PEG’s to amino acid residues on proteins is highly dependent on the nucleophilicity of each amino acid residue. Nucleophilic attack will only take place when the pH of the protein solution is near or above the residue’s pK\(_a\). Therefore the reactivity of each residue also depends on neighboring amino acid residues. Coupling of aldehydes to primary amines proceeds through a Schiff base, this is reduced in situ to give a stable secondary amine linkage.

**Cysteine conjugation**
PEGylation of free cysteine residues in proteins is the major approach for site-specific modification because reagents that specifically react with cysteines that have been synthesized and the number of free cysteines on the surface of a protein is much less than that of lysine residues (Goodson and Katre 1990). The advantage of this approach is that it makes possible site-specific PEGylation at areas on the protein that will minimize a loss in biological activity but decrease immunogenicity.

**Oxidized carbohydrates or N-terminus**
Oxidation of carbohydrate residues is an alternative method for site-directed PEGylation of proteins. Carbohydrates are oxidized with enzymes, such as glucose oxidase, or chemically with sodium periodate. Oxidation of the carbohydrate residues generates multiple reactive aldehyde groups, which can be reacted with PEG-amine to produce a reversible Schiff base. Multiple attachment sites are generated using this method, but the modification site is specific to the carbohydrate. Another approach is to take advantage of an N-terminal serine or threonine which is converted to glyoxyl derivative by periodate oxidation.

**PE Gylated enzymes in the treatment of cancer**
Many enzymes, already well known or recently characterized, display antitumoral activity and can find application in cancer therapy, alone or in combination with classic therapies. To make these molecules more suitable for pharmaceutical purposes, PEGylation is one of the most commonly used methods. PEG-L-asparaginase is the only drug approved by FDA for the treatment of leukemia and most of them are under clinical trials. This enzymatic approach seems to be very promising since several enzymes have proven to be active against various types of cancer by acting through different mechanisms. The major advantage of using enzymes is because of their great specificity.

**PEG-asparaginase (PEG-ASNase)**
Tumour cells are auxotrophic for asparagine as they lack or have a very low level of asparagine synthetase, an enzyme normally expressed in healthy cells. Therefore, asparaginase catalyzes the hydrolysis of asparagine to aspartate and ammonia (Fig. 1), thereby selectively killing tumour cells that rely on asparagines supplied by the serum for survival. Currently, two forms are present for clinical use and they include Elspar and Erwinia L-asparaginase due to their high activity and stability and also the plasma residence is high. However, the limitations include clinical hypersensitivity and acute allergic reactions.

\[
\text{HOOC} - \text{NH}_2 - \text{O} + \text{H}_2\text{O} \rightarrow \text{HOOC} - \text{COOH} + \text{NH}_3
\]

Fig. 1: Asparagine deamidation catalyzed by asparaginase

**PEG-Methioninase (PEG-METase)**
Tumour cell lines are auxotrophic for methionine also, and its depletion can slow down or stop cell growth (Hoffman and Jacobsen 1980; Guo et al. 1993; Kokkinakis et al. 1997). The normal human cells are relatively resistant to exogenous L-methionine restriction (Guo et al. 1993; Cellarier et al. 2003) hence this amino acid is a good cancer metabolic target. Methioninase (methionine-a-deamino-\(\gamma\)-mercaptomethanelyase; METase) is a pyridoxal-L-phosphate-dependent enzyme that transforms L-methionine into \(\alpha\)-ketobutyrate, methanethiol and ammonia (Fig. 4) and is thus able to induce methionine depletion in the medium. The major practical disadvantage is that some antibodies were repeatedly formed against PEG-METase when tested in the primate model.
Some tumours are only auxotrophic for arginine because of their incomplete enzymatic pool. In this case, some cancer cells do not express ASS (Sugimura et al. 1992) and are therefore unable to synthesize arginine from its precursor. Hence, arginine-depleting enzymes are useful against certain tumours. Indeed, arginine deficiency inhibits tumourgrowth, angiogenesis and nitric oxide synthesis (Cheng et al. 2005). The literature specifies two types of arginine degrading enzymes, and both have been suggested as antitumour agents: Arginase (Savoca et al. 1979), an enzyme that converts arginine into ornithine and urea and ii. Arginine deiminase (ADI), which degrades arginine into citrulline and ammonia (Fig. 3).

PEG-arginase

PEG-arginasedeiminase and PEG-arginase are the two arginine-depleting enzymes used in arginine-dependantmalignancies. PEG-arginaseis an arginine-depleting enzyme in anticancer therapy has been reconsidered (Cheng et al., 2007). This enzyme has the advantage of being an endogenous protein, involved in the urea cycle, where it converts arginine to ornithine and urea (Fig. 4) (Morris 2002). It has demonstrated strong in vitro anti-tumoural activities.

PEG-uricase

Uricase enzyme, although not directly involved in cancer treatment, can be used to improve a cancer patient's condition by minimizing side effects of chemotherapy (urate oxidase, EC 1.7.3.3.) by catalyzing the oxidation of uric acid to yield the more soluble allantoin, readily excreted by the kidney (Fig. 5). The greatest disadvantage is that uricase is not produced by humans and all forms of this enzymes are antigenic and thereby resulting in allergic reactions, anaphylaxis and even death.

Fig. 2: Conversion of methionine to a-ketobutyrate, methanethiol and ammonia, catalyzed by methioninase

PEG-arginase deiminase (PEG-ADI)

Fig. 3: Arginine converted to citrulline by arginine deiminase

Fig. 4: Conversion of arginine to ornithine by arginase

Fig. 5: Conversion of uric acid to allantoin by uricase
Several PEGylated proteins are on the market and more are coming, thanks to the improvement in knowledge of polymer conjugation to proteins and reduction in production costs. Thus more enzymes with antiproliferative properties will be available for cancer treatment in their PEGylated forms in the near future (Gianfranco Pasut et al. 2008).

**Nano-medicine in the treatment of cancer**

The polymer-based nanomedicine, includes the use of polymer–DNA complexes (polyplexes), polymer–drug conjugates, and polymer micelles bearing hydrophobic drugs, had increasing attention for its ability to improve the efficacy of cancer therapeutics. Due to their small size and excellent biocompatibility, nanosized polymer therapeutic agents can circulate in the bloodstream for long periods of time, allowing them to reach the target site. In addition, chemical modification of polymer therapeutic agents with ligands increases the therapeutic efficiency (Jae Hyung Park et al. 2008). Therefore, polymeric nano-medicine will continue to grow for the next few decades. This provides the researchers to overcome the limitations of chemotherapy and severe systemic side effects.

**Polymer based nano-medicine used in the treatment of cancer**

**PEG-poly (amino-acid)**

PEG has been widely used as the hydrophilic segment of polymeric micelles. Due to their biocompatibility and hydrophilic nature, PEG based polymeric micelles have shown no significant cytotoxicity and are rarely recognized by the RES system, allowing prolonged circulation in the bloodstream (Kwon et al. 1994). The PEG chains of polymeric micelles possess high chain mobility in an aqueous environment and have a large excluded volume, potentially decreasing the interactions of the polymeric micelles with constituents of biological fluids (Wang et al. 2000; Otsuka et al. 2003). The PEG molecules in the outer layer of the polymeric micelles can inhibit hydrophobic interactions between the inner cores of different micelles, thus blocking inter-particle aggregation.

When amphiphilic block copolymers are prepared with heterobifunctional PEG having different functional groups, the polymeric micelles can be modified with targeting moieties for drug delivery to specific cells and/or tissues (Akiyama et al. 2000). Micelles formed of poly (ethylene glycol)–poly (b-benzyl L-aspartate) (PEG–PBLA) have been physically loaded with various anticancer drugs, including DOX, KRN 5500 (KRN), and camptothecin (Yokoyama et al. 1998; Kataoka et al. 2006; Opanasopit et al 2004; Watanabe et al. 2006).

**PEG-polymers**

Polyesters have been widely used for drug delivery, as they gradually degrade in the body and thus do not require an additional removal procedure after implantation. They are also useful for preparation of amphiphilic block copolymers capable of forming micelles in aqueous solution. The representative polymers that can be used as the hydrophobic segments of the copolymers include poly (glycolic acid), poly (D-lactic acid), poly (e-caprolactone), and poly (D, L-lactic acid), as well copolymers of lactide/glycolide (Forrest et al. 2006; Lin et al. 2006). Aptamers-conjugated biodegradable PEG-PLGA and PEG-poly(lactic acid) (PLA) micelles were developed for encapsulating docetaxel to target the prostate-specific membrane antigen (PSMA) that was expressed on surface of prostate tumor cells (Farokhzad et al. 2004; Farokhzad et al. 2006).

**PEG–lipid**

Lipid is extensively studied as drug carriers. Liposomes, which were initially introduced (Bangham and Horne 1964), are a representative example of a lipid-based entity that has been successfully developed as drug delivery carriers (Wissing et al. 2004; Harashima and Kiwada 1996). However, the early lipidosome formulations were limited by difficulties in controlling the release rate of the drug, which rapidly diffused to the surrounding medium. By adjusting the liposome structure and incorporating excipients, researchers were able to subsequently achieve suitable release rate (Harashima and Kiwada 1996), while surface modification of liposomes with
PEG increased their residence times in the bloodstream and decreased their immune system recognition (Papahadjopoulos et al. 1991; Adams et al.2003).

**PEGylated liposomes in cancer therapy**

Liposomes represent the mainstream drug delivery technology. Liposomes are spherical vesicles with a membrane composed of a phospholipid and cholesterol bilayer and are used for drug delivery due to their unique properties. In cancer therapy, liposomes are used because of their natural ability to target cancer. The endothelial walls of all healthy human blood vessels are encapsulated by endothelial cells that are bound together by tight junctions, thereby prevents leaking of blood from the blood vessels whereas in the case of tumour cells the level of seals between cells are diagnostically leaky. Anti-cancer drugs such as doxorubicin and daunorubicin are in use today. Lorusso et al. studied pegylated liposomal doxorubicin as an emerging option for patients with recurrent ovarian carcinoma (Lorusso et al.2004). PEGylated liposomes are also used in the treatment of breast cancer (Keller et al. 2004), metastatic colorectal cancer (Hamaguchi et al.2004) and gastric cancer (Su et al. 2005). One of the major drawbacks of liposomes remains their relatively low entrapment efficiency (Hong and Mayhew 1989). An example inudes Galactosyldoxorubicilposomes containing PEGylated matrix metalloproteinase cleavable peptide-conjugated iodoeylphosphatidylethanolamine were developed for hepatocellular carcinoma-selective targeting (Terada et al. 2006).

**Nanopegylated liposomes in cancer therapy**

In contrast, nanopegylatedliposomes evades the reticuloendothelial system (RES) and remain in the circulation for prolonged periods, resulting in sufficient tumor targeting and efficacy in animal models (Torchilin2005). Nanopegylated liposomes are provided with passive targeting because of nanoliposome accumulation in tumor by means of enhanced permeability and retention (EPR) effect through leaky tumor vasculature (Torchilin2005; Huang et al. 1992; Huang et al.1993). Preclinical studies have shown that cytotoxic agents entrapped in pegylated liposomes tend to accumulate in tumors (Newman et al. 1999; Colbern et al. 1999). Nowadays, pegylated liposomal doxorubicin has been applied in patients with AIDS related Kaposi’s sarcoma, ovarian, breast and prostate carcinomas (Gabizon 2003).

**Nanoparticles in the treatment of cancer**

Nanoparticles are extremely promising delivery systems and constitute an extraordinary field of research at the interface between chemistry, biophysics, biochemistry, molecular biology, pharmacy and medicine. Nanoparticles conjugated with polymers enhance the solubility of the drug and thereby their circulating half-life is increased. However, their characterization is complicated because of their multicomponent formulation, their macromolecular structures and the rapid exchanges that occur with their changing microenvironment when they are injected in vivo. The work on nanoparticles which is currently underway is astonishing, and they will abundantly and rapidly enter the routine formulation of many drugs in the next few years. Several passive, stealth nanoparticles have already been successfully used in the clinic for the improved formulation of highly toxic drugs. Active targeting of nanoparticles is an issue, and this technology is not ready yet. The generation of highly sophisticated particles with coordinated and multifunctional properties should be obtained. The challenge today is to define a simple, robust, safe and reproducible method to produce complicated nanovectors (Liang-Cheng Chen et al.2007). An example is mAb-attached PLGA nanoparticles were reported to (immuno-nanoparticles) have the ability to recognize and target specific antigens to invasive breast tumors (Kocbek et al. 2007).

**PEGylatedspheruliteformulations in cancer therapy**

Spherulites are multilamellar vesicles obtained by shearing a lamellar phase of lipids and surfactants. They consist of concentric bilayers of amphiphiles alternating with layers of aqueous medium in which hydrophilic drugs can be sequestered with high yield. To be useful for drug targeting applications, spherulites should be small and long circulating. Spherulite technology to the encapsulation of an anticancer drug presented the first pharmacokinetic study conducted with such PEGylated vesicles. Furthermore spherulites are easier to prepare when compared to liposomes and their entrapment efficiency is high. When conjugated with polymers such as polyethylene glycol they exhibit long circulating half-life (Mignet et al.2000).

**CONCLUSION**

This review article, therefore, gives a clear idea about the happenings taking place in the treatment of cancer using pegylatedenzymes,spherulites,proteins and peptides, nano-medicine and nanoliposomes. This review also provides a clear gist of the various mechanisms and conjugation that takes place in cancer therapy. Clearly, it is seen that PEGylation can be a very useful and promising technique to improve the pharmacokinetic and
pharmacodynamic properties of the drug used and thus allow safe treatment in cancer therapy. PEGylated nanoparticles have exhibited prolonged circulation time, providing an increased opportunity for the drug to reach its site of action. Spherulites, owing to their simple preparation process and high entrapment efficiency, these vesicles appear as attractive alternative drug delivery system to conventional liposomes. In addition, like liposomes, they can be coated with PEG-lipid derivatives to acquire long circulation times. Future work should now be aimed at minimizing content leakage and downsizing the vesicles. Though this technology was failing for the past 40 years a great versatility has been achieved today and hence this proves to play a key role in the future of cancer therapy.

REFERENCES


