ISSN : 2347 - 2243

Indo - American Journal of Life Sciences and Biotechnology




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Indo-American Journal of Life Sciences and Biotechnology

The PE gylation of key biotech molecules: delivery developments

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Abstract

Background: Although various injected peptide and protein therapeutics have been developed successfully over the past 25 years, several pharmacokinetic and immunological challenges are still encountered that can limit efficacy of both novel andestablished biotech molecules.
Objective and Method: PEGylation is a popular technique to address such properties**.** PEGylated drugs exhibit prolonged half-life, higher stability, water solubility, lower immunogenicity and antigenicity, as well as potential for specific cell targeting. AlthoughPEGylated drug conjugates have been on the market for many years, the technology has steadily developed in respect of site-specific chemistry, chain length, molecular weights, and purity of conjugate. These developments have occurred in parallel to improvements in physicochemical methods of characterization.
Result/Conclusion: This review will discuss recent achievements in PEGylation processes with emphasis on novel PEG-drugs constructs, the unrealized potential of PEGylation for non-injected routes of delivery, and also on PEGylated versions of polymeric nanoparticles including dendrimers and liposomes.

Key words: PEG, PEGylation, drug delivery, PAMAM dendrimers, PEGylatedliposomes

Introduction

Biotechnology produces important novel recombinant molecules, able to mimic endogenous hormones, cytokines and antibodies [1]. One of the major hurdles has been the difficulty in synthesizing sufficient quantities for subsequent testing. However, due to more efficient genetic engineering, recombinant polypeptides can now be produced in relatively large quantities at a much lower cost than before. Despite this, inadequate delivery is the barrier to the effective administration of many promising biotech molecules. Parenterally-administered proteins tend to be either cleared from circulation by the reticuloendothelial system (kidney, spleen, liver) or metabolized by serum peptidases leading to loss of biological activity. Oral delivery is more problematic than the parental route, as protein-based therapeutics are rapidly destroyed by the digestive system and are poorly absorbed across the small intestinal epithelium [2].

Many approaches have been examined to enhance parenteral polypeptide delivery. These include alteration of amino acid sequences to reduce degradation by serum and liver enzymes and to reduce antigenic side effects. Peptides can also be fused to immunoglobulin or albumin to increase half-life [3] and, in addition, they can be entrapped in insoluble matrices and immobilized onto polymer resins for use with extracorporeal shunts. By far the most successful approach is the covalent coupling of polyethylene glycol (PEG) chains to the molecule (PEGylation) [4-6]. Important pioneering work was performed by Davis and Abuchowski in the late 1970s [7, 8]. Site- specific PEGylation modifies many peptide features to reduce clearance, although biological functions of the peptide can be compromised by reducing access to receptors [9]. PEGylation has been shown to increase drug efficacy and this has been attributed to a combination of increased molecular size [10, 11], (which reduces glomerular filtration), and masking of the protein surface (which decreases immuno-recognition response and proteolytic degradation) [10, 12]. A typical example of this balanced benefit is PEGylated interferon α-2a (Pegasys®, Roche, USA) which retains only 7% of the antiviral activity of the native protein, but still shows a greatly improved pharmacokinetic performance following weekly injections *in vivo* in hepatitis C patients compared with the unmodified enzyme [13]. PEG also confers new physicochemical properties and can modify both biodistribution and solubility [14]. Due to these favorable properties, PEGylation plays an important role in parenteral drug delivery, enhancing the potential of peptides and proteins as therapeutic agents. Even though several attempts have been undertaken to develop new polymers which can also confer improved pharmacokinetics on conjugated peptides (e.g. polysialylation (Lipoxen, UK) [15], it remains to be seen if these approaches can compete with PEGylation in terms of better efficacy, reduced numbers of injections, adequate biocompatibility and versatility in polymer design. While progress in PEGylation for injectables has led to many marketed biotech products, its use in oral and pulmonary delivery formats is only beginning to be explored

Properties of PEG

PEGylation usually employs functionalized mono-methoxy PEG (mPEG) molecules to covalently attach to surface-available groups on the protein, most commonly through multi-site binding to the □-amino group of lysine residues or through site specific bindingto free cysteine residues or via protein engineering [16]. PEG is a linear polyether diol, with the chemical formula $HO–(CH_2CH_2O)_n–H$. It can be made in various molecular weights and functionalized architectures (e.g., amino-, carboxyl-, and sulfhydryl-
terminated). Low-molecular-weight (<500 Da) PEGs are viscous, colorless liquids, whereas higher-molecular-weight PEGs are waxy solids due to crystallization whose melting points increase towards an upper limit of 70 °C [17]. PEGs are hydrophilic and dissolve in most solvents including methanol, benzene, dichloromethane and water; they are insoluble in saturate hydrocarbons. PEGs are also eliminated by a combination of renal [18] and hepatic pathways [19, 20], their simple elimination pathways making them useful in pharmaceutical applications. PEG has the lowest level of protein or cellular adsorption of any known polymer; it is non-toxic, non-immunogenic, non-antigenic, and has been FDA-approved for many injected biotech products. The first examples of PEG-protein drugs were commercialized in the early 1990s (Table 1), and since then, a number of clinical trials involving polymer-drug conjugates have shown promising results.

The technique of PEGylation has expanded to include a wide range of chemical and enzymatic conjugation methods [21, 22]. This variety offers the possibility to address specific requirements of different proteins. The choice of appropriately end- functionalized PEG allows the modification of only the desired amino acids in the sequence. Amino groups were the first target of PEGylation, by acylation or alkylation reactions [23], but now conjugation of PEG to thiol or hydroxyl groups and to disulfide bridges has also been made possible by using several specific chemical or enzymatic methods. A number of authors have provided thorough reviews of novel PEGylation chemistry [16, 24].

Designing PEG conjugates

Polymeric conjugates must be carefully designed for their individual use, taking into account the nature of the individual drug payload and the location of each molecular pharmacological target. Typical PEG-conjugates contain the polymer, a linker and the bioactive agent. The following are important points to consider:

The molecular mass and physico-chemical properties of the polymer [25, 26], are frequently the most important drivers governing biodistribution, elimination and metabolism of the conjugate, therefore the choice of a suitable PEG is important.

The water-soluble biocompatible polymeric carrier must be suitable for repeated administration. PEG polymers have been typically limited to < 30 kDa in relation to its viscosity radius in order to ensure eventual glomerular elimination [10]. However, there is a debate regarding this issue. For example, comb-shaped PEGs can have a different viscosity radius compared to linear PEG of the same molecular weight [10].

In the case of non-biodegradable high molecular weight polymers, the linker to the molecule is an important design feature. The *in vivo* protein stability of most polypeptidesis limited and therefore need to be attached to a polymer of high molecular weight. However, PEGylation of high molecular weight non-biodegradable polymers can alter the structure of the drug and reduce bioactivity when compared to the original protein. Drug-polymer conjugates or complexes need to be stable in the blood prior to the drug being liberated at the site of action. In principle, the polymer-bound drug can be released by non-specific hydrolysis by enzymes, by reduction, or by pH-dependent metabolism. Tissue-specific expression of enzymes and altered local pH as well as cellular
endocytotic pathways offer several options for designing conjugates that can be preferentially cleaved at the preferred site of delivery. For example, the microenvironment of tumors can be acidic in both animal models and human patients [27]. Low pH in endosomes and lysosomes as well as the presence of lysosomal enzymespresent in tumor cells can therefore be exploited for releasing the drugs bound to polymers through acid-sensitive linkers [28]. For the purpose of this review, polymer- protein hybrids have been divided in three groups based on the macromolecular architecture of the conjugating polymers. (Fig. 1A-C).

Linear PEGylating Reagents

One or more linear PEG chains with a medium-to-high molecular weight between 1 and 40 kDa are bound to the surface of the protein (Fig. 1A). Suitable PEGylating agents are typically prepared by chemical modification of the PEG terminal hydroxyl moieties [23, 29]. With notable exceptions [30, 31], synthetic routes in the early years of the PEGylation era lacked the capacity to produce pure mono-functional PEG derivatives of high molecular weight. Since the diol content of high molecular weight PEGs could be as high as 15%, first-generation PEG chemistry tended to be inefficient for protein conjugation due to the inevitable presence of cross-linked conjugates. Another drawback of such early PEGylation agents was lack of site-specificity in the conjugation step, which typically led to heterogeneous mixture of regioisomers. Despite this, several first- generation candidates received regulatory approval due to their superior and reproducibleefficacy over the parent molecules. PEGylated granulocyte colony stimulating factor (PEG-G-CSF), (Neulasta®, Pegfilgastrim, Amgen, USA), was conjugated with a 20-kDa
linear PEG chain and was approved in 2002. This treatment is more convenient than injections of human recombinant G-CSF (Neupogen®, Filgastrim, Amgen, USA), since only one injection of Neulasta® is required every three weeks compared to daily injections of Neupogen® over two weeks [32]. Similarly, Pegasys®, using a 40-kDa PEG chain, is a competitor of the first-generation conjugate of interferon α2b, PEG-Intron® (Schering, USA). Both PEG-Intron® and Pegasys® have shown improved efficacy in the treatment of hepatitis C than native interferon, especially when combined with the antiviral agent ribavarin [33].

At present, PEGylation to free cysteine residues probably remains the most efficacious strategy for site-specific conjugation, mainly using maleimide- or 2-pyridyl disulfide- terminated conjugating polymers (Table 2). Free cysteine residues are rather rare in native (poly)peptides and often they need to be introduced via protein engineering.

Preferential targeting of *N*-terminus have been achieved by reductive amination using aldehyde-terminated PEGylating agents that are able to react with protein primary amine fragments to form Schiff bases that can be then reduced *in situ* to give hydrolytically stable secondary amines. It was found that at a relatively low pH (typically < 6); most of the □-amino group of lysine residues are protonated, while the *N*-terminus nitrogen atoms remain relatively non-protonated and are able to react with aldehyde-functional PEGylating agents. Recently, Brocchini and coworkers [34-37] introduced an elegant strategy in which disulfide bridges are first reduced, then reacted with methoxy PEGs bearing a bi-functional reacting agent at the chain end. Bis-alkylation of the resulting free thiols gave a three-carbon bridge to which PEG was covalently attached (Table 2).

Y-shaped PEGylating Reagents

Functional Y-shaped PEGs (Fig. 1B) have received growing interest [38-42] due to some reports indicating improved biological efficacy compared to the analogous therapeutics in which a linear conjugating PEG was used. The macromolecular structure of the conjugating polymer has proven to be crucial for the improved properties of the corresponding bio-conjugates. Compared to linear PEG, branched PEGs can further reduce resistance to proteolysis and reduce immunogenicity, and this is thought to be in part due to the "umbrella-like" shape [24].

Highly branched PEGylating Reagents

'Comb' shaped polymers with one specific site of attachment represent a new type of PEGylation agent. One such polymer is POLY PEG® where PEG 'teeth' are linked to a methacrylate backbone via an ester bond (Fig.1C). POLY PEG® has more degrees of freedom than linear PEG, enabling structure optimization. Conventional PEGylation agents are prepared by ring opening polymerization of ethylene oxide, usually initiated by an alkoxide derived from an appropriate alcohol. Depending on the synthetic route selected, subsequent chemical transformations result in the desired PEG for conjugation. This living ring opening polymerization is complicated by the inevitable presence of trace water/protic species which offer a competing initiating pathway resulting in a hydroxyl end group on the initiating end of the chain as well as the terminating end. In addition, multiple chemical transformations are often required to obtain the desired conjugating functionality. Since it is not possible to either have a) 100% chemical yield or b) to separate polymers which differ only in their end group functionality, the resulting PEG always is a mixture of desired conjugating agent and impurities. These complications escalate as the molecular weight of the polymer is increased, as is required for single site attachment, due to inherent problems with living ring opening polymerization of epoxides. The new generation of polymers are prepared by Transition Metal-Mediated Living Radical Polymerization (TMM-LRP, often called ATRP), a technique that allows tight control over the polymer molecular weight and over macromolecular architecture when compared to other polymerization chemistry [43,44]. The PEG is present in the form of commercially available (meth)acrylic monomers of different molecular weights. The polymer has an (meth)acrylic backbone with PEG teeth and one functional group per polymer. The quantity of PEG can be varied by altering the length of the PEG teeth using different PEG methacrylate monomers of varying molecular weight (typically 0.5-2kDa). Similarly, the length of the methacrylate backbone can be altered, allowing the molecular weight to be varied in 2-dimensions via changing the [monomer]/[initiator]. A toothless spacer segment can also be inserted to keep the "comb" further away from the protein. Thus, polymers can be tailored to incorporate active end-groups for specific amino acid targeting. For example, POLY PEG® allows conjugation through a number of active targeted end-groups such as aldehyde, succinimidyl ester and maleimide, which react with the *N*-terminus amine, lysine and cysteine units respectively [45 43, 46] .

Linear PEG normally has one hydroxyl end-group which can be chemically converted into a different functionality to enable conjugation. It is possible for some chains to have functionality at both ends which could lead to undesirable cross-linked protein-polymer biohybrid materials. In contrast to linear PEG, the living radical polymerization method used to synthesize comb polymers ensures that each polymer molecule is limited to a single terminal conjugating group [47]. Ongoing studies are examining whether these hybrid comb polymer conjugates have improved pharmacokinetics, reduced immunogenicity, reduced toxicity and a longer plasma half-life compared to parent protein and other PEGylated formats. However there is still the issue of the degradation of these 'comb' polymers and the release of the peptide attached. POLYPEG® has ester bonds connecting the PEG teeth to the methacrylate backbone which should break when they come into contact with protease enzymes, and this is the subject of ongoing studies. Importantly, incorporation of a labile linker between the peptide and other PEG polymers has been achieved [48] and this technology could be applied to comb polymers such as POLYPEG®.

Other comb polymers with acrylic backbones are also under investigation such as the pH- sensitive poly[2-(diethylamino)ethyl methacrylate] (PDMAEMA) backbone modified with poly(L-lysine) (PL) side chains, which has potential to be used as a DNA carrier.

Although not a PEGylating agent, this polymer has unique properties in that it is responsive to pH in solution [49]. A continuation of this work has seen the synthesis of comb-type copolymers consisting of a PL main chain, a DNA binding site, hyaluronic acid (HA) side chains and cell-specific ligands [47]. By using the PL-graft-HA comb- type copolymers, it may be possible to control the properties of the DNA complex such as complex size, solubility, and the degree of DNA compaction. Both of these comb-type copolymers might be used to increase the efficiency of cell targeting for siRNA cytosolic delivery. In another example, Srividhya *et al.*, [50] synthesized an amphiphilic comb polymer with PEG attached to poly(dimethyl siloxanes) (PDMS–PEG) via hydrosilylation with reactive epoxy groups for protein attachment. Thin membranes loaded with BSA were prepared and attached onto PDMS–PEG and the release profile was continuous and controlled over 72 hours. Thermo-responsive poly(N-isopropylacrylamide), (poly(NIPAM), has also been conjugated to streptavidin, allowing for thermal release at body temperature *in vivo* [51, 52]. Conjugates have also been used to produce self assembly into giant amphiphiles and to combine multiple properties along the backbone of the polymer when conjugated to BSA [53].

Routes of Administration

Since biopharmaceuticals are now approaching 50% of all new drugs in development, there is considerable interest in non-injected delivery to improve patient acceptability [54]. Gene and siRNA delivery have additional barriers to negotiate over those of peptides, namely, intracellular escape from lysosomal decay, as well as intact delivery to either nuclear DNA or to cytoplasmic RNA. Incorporation of PEGylation concepts are being researched in order to overcome these different type of delivery barriers [55].

While oral delivery remains the preferred option for peptides, increasing interest in pulmonary delivery also indicates the potential of

that route for the delivery of biopharmaceuticals [56]. Inhalation is the fastest delivery system (apart from intravenous injections) for small peptides due to the large surface area of the lungs, good epithelial permeability and the highly dispersed nature of the aerosol. An inhaled dry powder
aerosol of insulin (Exubera®) was developed by Nektar and marketed by Pfizer in 2006.Although the delivery of inhaled insulin matched that of subcutaneous delivery, it was recently withdrawn due to poor sales, just a fraction of the total insulin market. While the reasons for failure are many, there was patient perception of long-term pulmonary safety issues in addition to acceptability issues with the device [57]. Recently, Novo Nordisk also halted development of Aradigm's AERx® inhaled insulin system which was in Phase III trials. However, there are a number of other fast-following inhaled insulin particle systems at late stages of development from Alkermes/Lilly (AIR® Insulin) and MannKind (Technosphere® Insulin System) [58-62], perhaps indicating that there is potential to improve upon the first inhaled insulin by using different types of particles, delivery devices and excipients. PEGylation can also promote pulmonary delivery to achieve longer-lasting peptide effects [63, 64].

BioAir™ (Biosante Pharma, USA) comprises calcium phosphate nanoparticulates (CaP) of insulin, and CaP-PEG particles significantly reduced the elimination of insulin in rats following pulmonary administration. Consequently, the amount of bioavailable insulin from this pulmonary formulation was also equivalent to or higher than that of insulin injected subcutaneously [65]. For potential treatment of lung cancer, transferrin- conjugated liposomes were PEGylated and tested for inhalation following nebulisation [66, 67]. These PEGylated targeted liposomes showed more stability and retained > 80% of their drug load over 48 hours. This was sufficient time for the drug carriers to be taken up by cancer cells over-expressing transferrin receptors in the lung.
POLY PEG® and oral delivery of salmon calcitonin

Injected and nasal salmon calcitonin (sCT) is used for the treatment of osteoporosis and Paget's disease, but upon oral delivery it is subject to proteolytic attack by intestinal peptidase enzymes and demonstrates poor intestinal epithelial permeability [68]. Nasally- administered sCT (Miacalcin®, Novartis, Switzerland) displays variable absorption and there is local cytotoxicity over time [69]. In addition, although the osteoporosis market is now dominated by long-acting oral bisphosphonates, these drugs have stringent administration requirements and are associated with patient perception of limited efficacyand side-effects [70]. There is substantial interest in developing an oral formulation of sCT and current approaches include conjugation to alkyl-PEG [10] and PEGylated chitosan nanoparticles [4]. Site-specific conjugation of linear PEG to Lys^{18}-amine (Lys^{18}-PEG_{2K}-sCT) has been achieved [71] and also of comb-shaped POLY PEG® to Cys^{1} in our own research.

A comparison of EC_{50} values was calculated from concentration-response curves comparing the bioactivity of sCT to sCT-POLY PEG®. The intracellular cyclic AMP values achieved in a breast cancer cell line over-expressing calcitonin receptors were 135
± 19 and 197 ± 31 pM for sCT and sCT-POLYPEG® respectively (Fig. 2). The conjugate also significantly improved the resistance of sCT to intestinal peptidases at relevant concentrations of enzymes (Fig. 3) and prolonged the half life of sCT *in vivo* over the sCT following intravenous injection (Fig. 4). POLY PEG® has also been successfully used to PEGylate lysozyme [45, 72] and BSA [46] to create conjugates that are also bioactive *in vivo*. Improvement of other delivery technologies by PEGylation

Polyamidoamine (PAMAM) dendrimers

PAMAM dendrimers consist of a central core, branching units and terminal functional groups and they were the first complete dendrimer family to be synthesized, characterized and commercialized [73, 74]. They have attracted great attention in for potential in biomedical applications [75-77] and are commercially available macromolecules that can be used as carriers for plasmid DNA, antisense oligonucleotides [78-80] and siRNA [81-83]. Although the PAMAM dendrimer has already been tested as a carrier for drug and gene delivery, there are potential problems with regard to payload solubility and cytotoxicity. Cytotoxicity of PAMAM dendrimers increases with generation (G), independent of surface charge, for both full generation cationic dendrimers (G2–G4) and the 'half-generation' anionic intermediates (G2.5, G3.5). A partial derivatization with four PEG chains on a G4-PAMAM, respectively, was sufficient to lower cytotoxicity [84, 85].
Many workers have examined the usefulness of PEGylated dendrimers to enhance drug solubility. Bhadra *et al*. devised PEGylated "nanocontainers" using G4 PAMAM dendrimers. The PEGylated PAMAM displayed improved entrapment efficiency of the poorly water-soluble anticancer agent 5- fluorouracil [86]. The effect of PEG chain length on dendrimer-mediated solubility was also studied [87]. Anticancer drugs were encapsulated using a PAMAM dendrimer having MPEG (polyethylene glycol monomethyl ether) grafts of different molecular weights. Two anticancer drugs- methotrexate (MTX) and doxorubicin (DOX), were encapsulated with high efficiency in the hydrophobic interiors of PEGylated G3 and G4 dendrimers. It was observed that mostof the ADR was complexed and solubilized on the surface of mPEG chains, while in caseof MTX, ,the number of encapsulated molecules increased due to electrostatic interactionresulting from an acid-base reaction between the dendrimer and MTX. With increasing dendrimer generation and PEG molecular weight, more drug molecules can be encapsulated [87]. One way in which dendrimers can assist PEGylation is by increasing the drug loading capacity of PEG polymers, which possesses only one or two hydroxyl terminal groups. To overcome this limitation, some research groups have constructed dendrimeric structures at the level of the polymer termini to produce PEG dendrons. The increase in the number of active groups to increase loading capacity is achieved by using branching molecules (e.g. bicarboxylic amino acid) [24, 88].

PEGylated Liposomes and Stealth® Technology

Liposomes are an important drug delivery technology with many approved injectable products [89-93]. Conventional liposomal formulations suffered from extensive uptake by the reticulo-endothelial system (RES), which is comprised largely of Kupffer cells inthe liver and, to a lesser extent, the bone marrow and lymphatic tissue [94-96].
Liposomes were improved by Johnson & Johnson's (USA) Stealth® technology [97], which is composed of lipid nanoparticles that incorporate a PEG coating on the liposome surface, which attracts a water shell to surround the liposome. This shell succeeds in reducing the adsorption of opsonins to the liposome surface that would otherwise enhance recognition and uptake by the RES. The 100 nm diameter size of Stealth® liposomes balances the drug-carrying capacity and circulation time, and also permits extravasation through endothelial gaps in the capillary bed of target tumours [98-101]. Kaposi's sarcoma tumours, in particular, have an inherently leaky capillary bed due to the open-ended or highly permeable vessels that occur during tumour angiogenesis [102].

Paclitaxel (Taxol®) is used for treatment for ovarian carcinoma, breast

cancer, head and neck cancers and non-small cell lung cancer [103, 104]. However, one of the biggest shortcomings of this drug is its low aqueous solubility. PEGylated liposomes significantly increased the biological half-life of paclitaxel after intravenous injection in rat, and showed high accumulation of the drug in tumor tissue, thereby more effectively inhibiting the tumor growth in mice. Therefore, this PEGylated liposomal formulation of paclitaxel was suggested to be a better alternative to passive targeting of breast tumors.
Similarly, Doxil® (Ortho Biotech L.P. Johnson & Johnson, USA) is a long-circulating Stealth® PEGylated liposomal formulation of doxorubicin.

RNA delivery and PEG

The potential of siRNA as a therapeutic agent in the treatment of chronic diseases and genetic disorders is set for major breakthroughs [105]. However, its delivery, especially *via* systemic routes, remains a challenge. Cells do not readily take up siRNA and therefore clinical applications of siRNA largely depend on the development of delivery systems that can bring intact siRNA into the cytoplasm of the target cells. Gene delivery systems should be designed to protect the genetic materials from premature degradation in systemic blood stream and to efficiently transfer the therapeutic genes to target cells. The PEGylation of these delivery systems protects the siRNA but other problems can occur such as reduction in gene transfer, due to a reduced cellular uptake or limited endosomal release [106].

Polycationic materials can protect nucleic acids from degradation and facilitate entry into the target cells however, unspecific interactions of the cationic complex can occur with negatively charged serum proteins and other blood components. By PEGylating the polycation, these undesirable effects can be greatly reduced. While PEGylation is a necessity to improve extracellular stability and circulation half-life, it often decreases the transfection efficiency due to reduced specificity and inhibiting cell association and uptake. Furthermore, PEGylation can hinder endosomal release and hence lessen the efficiency at a later phase of the entry pathway. To circumvent this, a pH-cleavable PEG shielding was specifically designed to strip the protective shielding during endosomal uptake [107].

Similarly Vandenbroucke *et al.*,[108], PEGylated siRNA–liposome complexes to prevent aggregation with serum proteins and to prolong circulation time. Ultrasound combined with microbubbles has been used to deliver siRNA, but the gene silencing efficiency is rather low and very high amounts of siRNA are required. To overcome the negative effects of PEGylation and to enhance the efficiency of ultrasound-assisted siRNA
delivery, PEGylated siRNA–liposome complexes were attached to the microbubbles. Ultrasound radiation of these microbubbles resulted in an improved intracellular release of unaltered PEG-siRNA–liposome complex. The PEG-siRNA–liposome loaded on microbubbles was able to enter cells after exposure to ultrasound and they induced muchhigher gene silencing than free PEG-siRNA–liposome. Additionally, the microbubble complex silenced the expression of genes only in the presence of ultrasound, which may enable space- and time-controlled gene silencing.

Conclusion

PEGylation has developed substantially over the past decade from an injectable biocompatible technology that was quite generic to one in which many key parameters can be tightly controlled and tailored for each molecule. Current research is exploring ways to use PEG advantageously for oral and inhaled peptide delivery, either in co-polymer or nanoparticle constructs. Combining PEG with other polymeric formats is also showing tremendous promise as it takes advantage of the inherent properties of PEG in providing protection against metabolism and the immune system.

Expert Opinion

PEGylation is already a mainstay of nanoparticulate formulation and there is a considerable research into combining PEG with chitosan, poly(lactide) co-glycolide (PLG), liposome and dendrimers. Apart from the obvious pharmacokinetic advantages,researchers are likely to achieve better targeting to tissue for diagnostic and treatments,
for example, by conjugating ligands to PEG-coated particles. In relation to pulmonary delivery, it is likely that PEGylation will continue to be used to improve the pharmacokinetics of peptides, delivered as non-adherent aerosol particles to the lung. However, the potential for local toxicity from chronic administration cannot be underestimated. Likewise, while PEG is biocompatible, it is not biodegradable (in the same way as PLG) and careful toxicology studies from the newer comb-shaped and co- polymer contructs will be required, with a focus on the potential for build up in the kidneys. Finally, we see great potential for targeted PEGylated polymers as an alternative to live vectors in the delivery of siRNA to the cytosol of cancer cells. To achieve these aims will require ever increased collaboration between chemists, formulators and biologists.

Acknowledgments

We are grateful to Science Foundation Ireland (Investigator Grant 04 IN3 B575 to DB) for funding for the PEGylated sCT work and to Warwick Effect Polymers Ltd. for providing polymer conjugates.
Figure Legends

Figure 1: Polymer-protein hybrid therapeutics divided into three groups based on their macromolecular architecture of the conjugating polymers. 1A: Linear PEGylating reagents), 1B: Y-shaped PEGylating reagents and 1C: Comb shaped PEGylating reagents.

Figure 2: Concentration response curves of sCT and sCT-POLY PEG® (Mw of conjugate is 9.5 kDa) for the stimulation of cAMP secretion in the breast cancer cell line, T47D. A comparison of EC_{50} values for sCT and sCT-POLY PEG® were 135 ± 19 and 197 ± 31 pM respectively. Conjugation of POLY PEG® onto sCT therefore does not inhibit bioactivity.

Figure 3: Stability of sCT-POLY PEG® (Mw of conjugate is 9.5 kDa) and sCT when incubated with the intestinal enzyme trypsin at the gastro-intestinal concentration (50µM). sCT-POLY PEG® has significantly increased resistance to the peptidase enzyme trypsin,

compared to free sCT which is degraded at 30 mins. Samples were analysed by SEC-HPLC.

Figure 4: sCT in rat serum after i.v.-administration of sCT and sCT-POLY PEG® (Mw of conjugate is 40 kDa) in rats (n=3-5). Serum samples were obtained before sCT/sCT- POLY PEG® were injected and after at time intervals. sCT was measured by ELISA.

PEG conjugates	**Name (Company)**	**PEGylation**	**Status**	**Disease**
1-asparaginase[109]	Oncaspar® (Enzon)	Multiple linear PEGs 5kDa	Market, 1994	Acute lymphoblastic leukemia
Adenosine deaminase [110]	Adagen® (Enzon)	Multiple, linear PEGs 5kDa	Market, 1990	Severe combined immunodeficiency disease (SCID)
Growth hormone receptor antagonist [111]	Somavert® (Pfizer Pharmacia)	4-6 linear 5 kDa PEG chains per protein molecule	Market, 2002	Acromegaly
Zn- protoporphyrin (ZnPP) [112]	Patent	Random, linear PEG 5kDa	Pre-clinical	Supression ofbilirubin
Alsterpaullone[113]	Alsterpaullone (A.G. Scientific Inc.)	40 kDa urea linked	Pre-clinical	Treatment of Alzheimer's disease
PEG–anti-TNF Fab [114]	CDP870, Cimzia® UCB (formly Celltech)	Not available	Market, 2008	Rheumatoid arthritis and Crohn's disease
interferon α2a[114]	Pegasys® (Roche Pharmaceuticals)	Random, branched withtwo 20 kDa PEG chains	Market, 2002	Hepatitis C
interferon α2b[115]	PEG–Intron® (Schering-Plough)	Random, linear PEG 12kDa	Market, 2000	Hepatitis C, cancer, multiple sclerosis, HIV/AIDS
GCSF(PEG-filgrastim) [116]	Neulasta™(Amgen)	20 kD PEG is bound to N-terminal methionyl residue of Filgrastim	Market 2002	Prevent severe cancer chemotherapy- induced neutropenia
Insulin (CaP-polyethylene glycol (PEG)- insulin nanoparticles) [65]	BioAir™ (BioSante Pharmaceuticals)	Not available	Pre-clinical	Diabetes

Branched PEG–anti-VEGF	Pegaptanib, Macugen™(OSI	Selective, 40 kDa branched PEG, each	Market, 2004	Macular degeneration (age-

PEG conjugates	**Name (Company)**	**PEGylation**	**Status**	**Disease**

Table 1

aptamer (Pegaptanib, Macugen™)[117]	Pharmaceutical)/PfizerUCB)	branch 20 kDa		related)

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●● Very accessible review for those entering the field

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