PEGylation: An Overview and Recent Advances Reported in the Patent Literature

Marianela González[2], Santiago E. Vaillard[3] and Ricardo J. Grau[1,4,*]

[1]*INTEC, Instituto de Desarrollo Tecnológico para la Industria Química. Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Universidad Nacional del Litoral (U.N.L), CCT-CONICET Santa Fe, Colect. Ruta Nacional 168 Km 472 Paraje "El Pozo", (3000) Santa Fe. Argentina;* [2]*Research Assistant from CONICET;* [3]*Member of CONICET's Research Staff;* [4]*Professor at U.N.L. and Member of CONICET's Research Staff*

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Abstract: Pegylation technologies have improved since chemistry and purification processes have advanced over the years. Furthermore, a gradual better understanding of the clinical responses has spurred the need for more effective therapeutic agents. Nowadays, it is necessary to early identify therapy needs and technical capabilities to develop customized, innovative products that reach the market successfully. Consequently, the main technical efforts are directed to solve some intricate problems associated with the drug delivery where improved solubility, *in vivo* stability, targeted delivery, and enhanced efficacy and safety are the major challenges. Too many alternative approaches for too many needs open up the possibility for follow-on developers and competitors, and provide opportunities for innovation that provoke an intense worldwide competition in a complex intellectual property environment. This paper presents an overview of general trends and patents supporting PEGylation platforms. Since the golden rule is that PEGylation has to be tailored to each specific drug and application, the review format is structured by specific conjugated species alphabetically sorted within the following groups: PEG-protein conjugates, PEG-antibody and antibody fragment conjugates, PEG-oligonucleotide conjugates, PEG-small molecule drug conjugates, and PEGylated materials and miscellaneous applications.

Keywords: Drug delivery, patented conjugates, PEGylated drugs, PEGylated ODNs, PEGylation, poly(ethylene glycol), polymer therapeutics.

INTRODUCTION

The challenge in overcoming some limiting physicochemical and pharmacokinetic properties of the first generation of biopharmaceuticals is claimed to be the driving force towards a new class of biopharmaceuticals of commercial relevance. Many different methods are being explored, targeting at improved physicochemical qualities and biomedical efficacy. While some approaches are still in the discovery phase, others have already been successful in the marketplace [1]. Among the latter, the exciting technology of covalent attachment of poly(ethylene glycol) (PEG) chain(s) to a bioactive drug, so-called PEGylation, is now recognized as a useful procedure for the modification of proteins, peptides, enzymes, antibody fragments, oligonucleotides and also small synthetic drugs. After initial skepticism about the usefulness and profitability of PEGylation technology in the 1980s, FDA-approval of PEG-adenosine deaminase (Adagen®) and PEG-L-asparaginase (Oncaspar®) gave way to cautious optimism in the pharmaceutical industry in the 1990s. The subsequent therapeutic and commercial success has become powerful enough to produce a worldwide breakthrough in the generation of bio-better drugs at the beginning of the 2000s.

*Address correspondence to this autor at the Instituto de Desarrollo Tecnológico para la Industria Química. Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Universidad Nacional del Litoral (U.N.L), CCT-CONICET Santa Fe, Colect. Ruta Nacional 168 Km 472 Paraje "El Pozo", (3000) Santa Fe. Argentina; Tel: +54-342-4511546; Fax: +54-342-4511079; E-mail: cqfina@santafe-conicet.gov.ar

Fig. (**1**) shows the collected outcome data obtained by invoking the keyword "PEGylation" from three well-known scientific publication databases and the FDA approval year for the current marketed PEGylated products. The number of records displayed by publication year shows increasing interest and research activity in the last decade, *i.e*., once the proofs of concept and technology development were convincing enough to the academic and industrial communities. It is worth mentioning that only a relatively small number of products have currently received FDA approval, but several innovative PEG-conjugates of proteins or small organic drugs are undergoing clinical trials [1,2]. PEGylated drugs are expected to create a multi-billion dollar market in the next future. To mention only as a market size barometer, the leading genotype-1 hepatitis C treatments are currently based on PEGylated alpha-interferons (PEGasys® and PEGIntron®) which capture a large portion of an estimated US $ 5 billion worldwide market, and the PEGylated Granulocyte Colony-Stimulating Factor (Neulasta®) has sales over US $ 4 billion per year. Spending on these improved biopharmaceutics is steadily increasing and the prices per doses are 10-20 times higher than those of their parent unconjugated compounds.

Fig. (**2**) illustrates the technology evolution and the foundation core competencies. The PEGylation technology has evolved as the PEGylation chemistry and purification processes have advanced over the years, and a gradual better understanding of the clinical responses spurs the need for more effective therapeutic targets. Nowadays, specific clinical skills, including early identification of therapy needs

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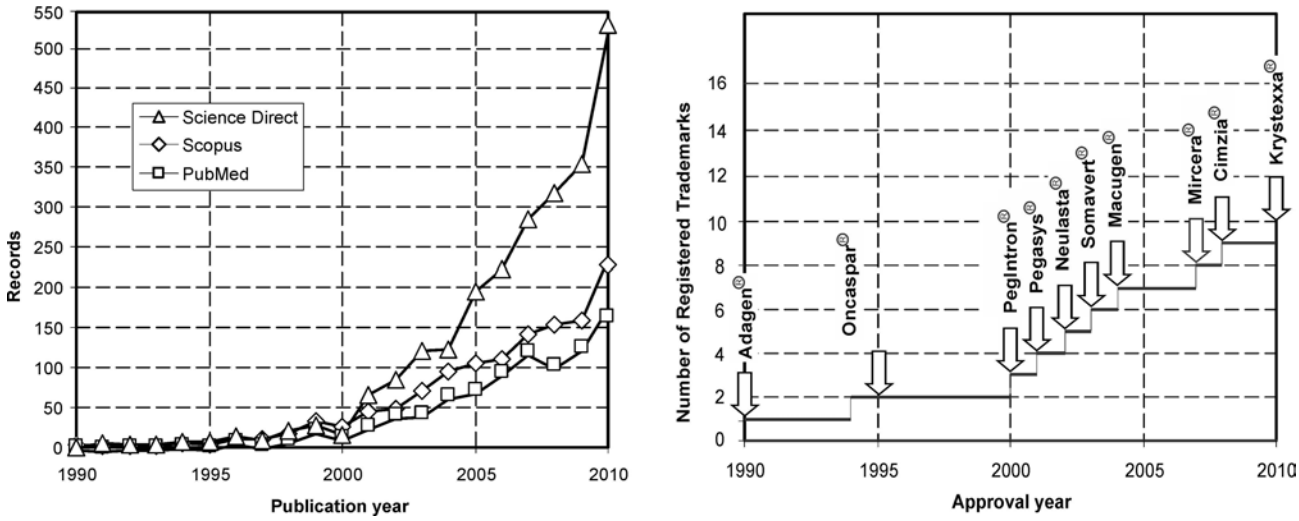

Fig. (1). Number of papers containing the keyword "PEGylation" and marketed PEGylated products in the last 20 years.

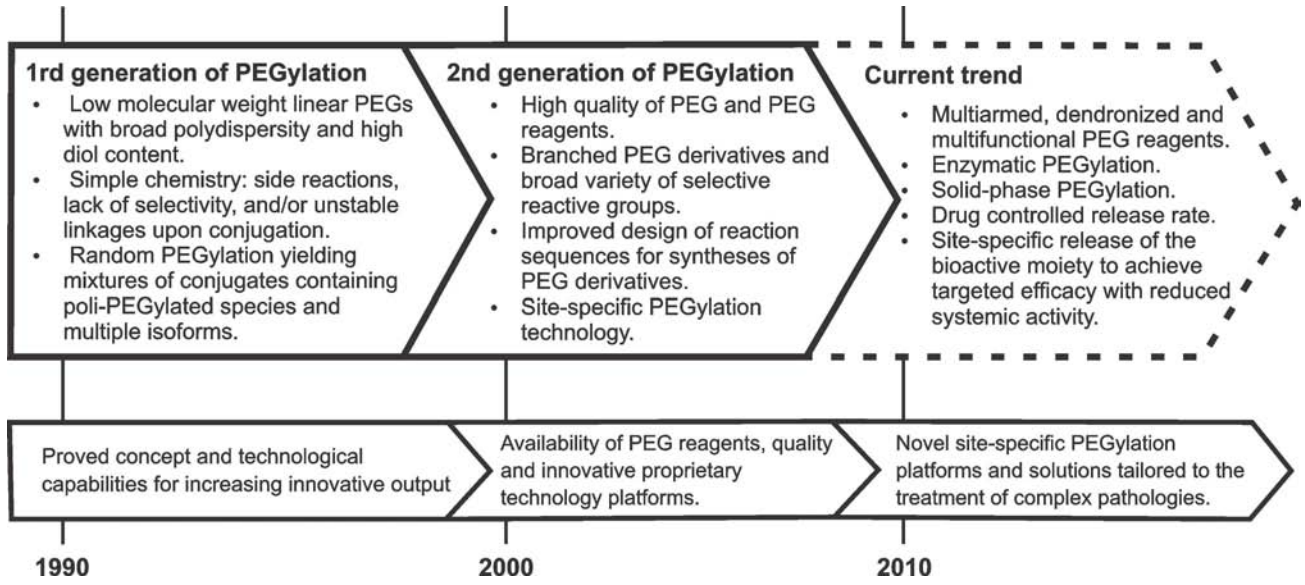

Fig. (2). The evolution of the PEGylation technology and core competencies.

and technical capabilities to develop sophisticated and innovative customized products, are increasingly required for future success. Consequently, the main technical efforts rely upon designing bio-better molecules, preparing high-quality customized PEG derivatives, and avoiding drawbacks associated with existing PEGylation methods.

- Numerous functionalized PEG reagents are now commercially available from companies like NOF Corporation (Japan), Creative PEGWorks (USA), SunBio (South Korea), JenKem (China), and IRIS Biotech (Germany). The covalent attachment of PEG chains to bioactive drugs provides prolonged circulation half-lives *in vivo*, protecting the conjugates from proteolysis and shielding them from the immune system, but the effect of the PEG steric hindrance often leads to a drastic loss in the biological and the pharmacological potencies of the conjugates. Thus, an appropriate choice of the size and structure of the PEG reagent and the conjugation chemistry is crucial to balance the desired half-life *in vivo* while maintaining an acceptable biological activity. No simple technical approaches have been proposed to achieve these goals: i) the use of branched or multiarmed PEG derivatives instead of linear derivatives [3,4]; ii) the linkage of detachable PEG conjugates (reversible PEGylation) based upon chemical linkers sensitive to hydrolysis or capable of being cleaved enzymatically by serum proteases or estearases [5]; and, iii) the controlled and site-selective attachment of PEG derivatives to the bioactive moieties [6]; iv) and the PEGylation performed with enzymes that allows the attachment of PEG molecules in a site-specific manner [7].
- Random PEGylation leads to complex mixtures of conjugates, differing in both the number and the attachment site in the bioactive moiety. Even after extensive optimization, the coupling reactions commonly result in multiple PEGylated isoforms with non-uniform physicochemical and pharmaceutical properties due to the fact that each isomer might possess a different activity

and toxicity profile. Moreover the composition of the conjugate mixture can slightly vary from batch-to-batch. Although most of the marketed PEGylated biopharmaceutics (Adagen®, Oncaspar®, Pegasys®, PegIntron®, Somavert®, Mircera®) are mixtures of PEGylated conjugates the trend is shifting from the random to site-specific PEGylation. Proteins are generally PEGylated at nucleophilic sites such as amino groups or unprotonated thiols. Thus, there are successful chemical and enzymatic approaches to perform site-specific PEGylation: i) N-terminal specific PEGylation (Neulasta®) [8-10]; ii) thiol-selective PEGylation of free cysteine naturally present (or genetically introduced) in the protein (Cimzia®) [6, 11,12]; iii) disulfide site-specific PEGylation of native cysteine residues involved in disulfide bridges [13,14] (like TheraPEG™ technology) [15]; iv) PEGylation at a histidine tag that is expressed at one or both ends of a protein or peptide (HiPEG™) [16] (patent pending); v) incorporation of non-natural aminoacids by genetic manipulation [17] (like Amber technology [18]); vi) site-directed enzymatic PEGylation taking advantage of enzymes able to specifically modify some amino acid side chains, in particular glycosyltransferases and transglutaminases [19,20].

- Other approaches include: i) the use of heterobifunctional PEG derivatives to improve water solubility, biocompatibility, and flexibility, and capable of carrying two different biomolecules [21]; ii) sophisticated drug release systems based on PEGylated nano-particles like liposome and polymer micelles [22,23], and PEG linkers for antibody conjugated nano-particles [24]; iii) customized PEG derivatives for receptor-mediated targeting [25].

Fig. (**3**) shows some of the above mentioned alternatives to perform PEGylation. Too many alternative approaches for too many needs open up the possibility for follow-on developers and competitors, and provide opportunities for innovation that provoke an intense worldwide competition in a complex intellectual property environment. In this review, we present an update on patents supporting PEGylation platforms. Since the golden rule is that PEGylation has to be tailored to each specific drug, the review format is structured by specific conjugated compound alphabetically sorted within the following groups: PEG-protein conjugates, PEG-oligonucleotides, PEG-antibodies and antibody fragments, PEG-synthetic drug conjugates, and PEGylated materials and other molecules.

ADVANCES IN PEG-PROTEIN CONJUGATES

Proteins are the most modified biologically active molecules. Several state-of-the-art reactive amino acids are known which couple PEGylation reagents to proteins and peptides. A preferred route has been the acylation of the ε-amino groups of lysine residues. Because of the abundance and ready accessibility to these conjugation-sites, the conjugation reaction results in multiple PEGylated isoforms [3, 26,27]. Thus, selective methods of conjugating PEG to proteins are desirable for many reasons including retention of protein bioactivity, uniform chemical and pharmaceutical properties while simplifying downstream purification. The current approaches are subsequently moving from random to site-specific PEGylation. In order to address the issue of target site specificity, multiple methods and specific PEG reagents have been proposed in many innovative ways. Background, main underlying concepts and methods are well documented in the scientific literature. We refer the reader to [2] and [28] for a more comprehensive description; here we only mention strategies, embodiments, and related technologies claimed in some patents.

PEGylation at N-terminal residue is a well-known approach to site-specific PEGylation. Due to the multiple lysine residues in most proteins, N-terminal specific PEGylation has been a challenge. Taking into account differences in pKa values of α-amino groups of N-terminal lysine (7.6 – 8.0) and ε-amino group lysines of backbone polypeptide (10.0 – 10.2), it is possible to specifically attach a PEG chain to an N-terminal lysine. Reductive alkylation of these amines with PEG-aldehyde derivatives is carried out at slightly acid pH values and using sodium cyanoborohydride as reducing agent [29]. Conjugates of granulocyte-colony stimulating factor (G-CSF) and consensus interferon with PEG are used as examples. A similar approach to obtain N-terminally PEGylated conjugates has been carried out for granulocyte macrophage-colony stimulating factor (GM-CSF), and Interleukin-10 (IL-10) [30,31]. The most conspicuous example of this approach is Neulasta®, a PEGylated form of a G-CSF analog (filgrastim), which has been in the market since 2002 for the treatment of neutrophenia (see [32] for reviewed results). As previously mentioned, preceding studies have been also directed to GM-CSF PEGylation [33]. A single PEG chain has been attached to non-glycosylated human GM-CSF polypeptide increasing the ability to stimulate the replication of cells in cultures compared to the non-modified GM-CSF molecule [34]. PEGylated IL-10 has been prepared using different types of linkers and pHs to yield various forms of a PEGylated molecule [35,40]. Recently, the use of a buffer solution like an alcohol co-solvent has been demonstrated to be advantageous for reacting peptides with PEG-aldehyde at a free amino group, as exemplified for PEGylation of Calcitonin gene related peptides (CGRP) [41]. Similarly, an improved process has been proposed to increase PEGylation reaction yields of r-metHuG-CSF [42]. This approach consists in conjugating the free N-terminal-α-amino group of r-metHuG-CSF to a PEG aldehyde in the presence of a reducing agent in a buffer solution containing a polyol, or a carbohydrate. Another promising new PEGylation reagent for the selective attachment to the N-terminal α-amino group of proteins is PEG phenyl-isothiocyanate (PIT-PEG), which has been reported to be useful for modifying rhKGF-2 through PEGylation at N-terminal residue [43].

PEGylation at thiol groups of natural or engineered cysteines is a useful method if a protein contains a few or only one sulfhydryl group in the form of a cystein. The most common thiol-reactive PEGylating agents used for creating a stable thioether linkage are well known in the literature: PEG-maleimide (PEG-MAL) [44], PEG-iodoacetamide (PEG-IA) [45], PEG-vinylsulfonate (PEG-VS) [46] and PEG-orthopyridyl disulfide (PEG-OPSS) [47]. The facile and non-destructive cysteine-maleimide based chemistry is definitely the most preferred route of doing thiol-selective

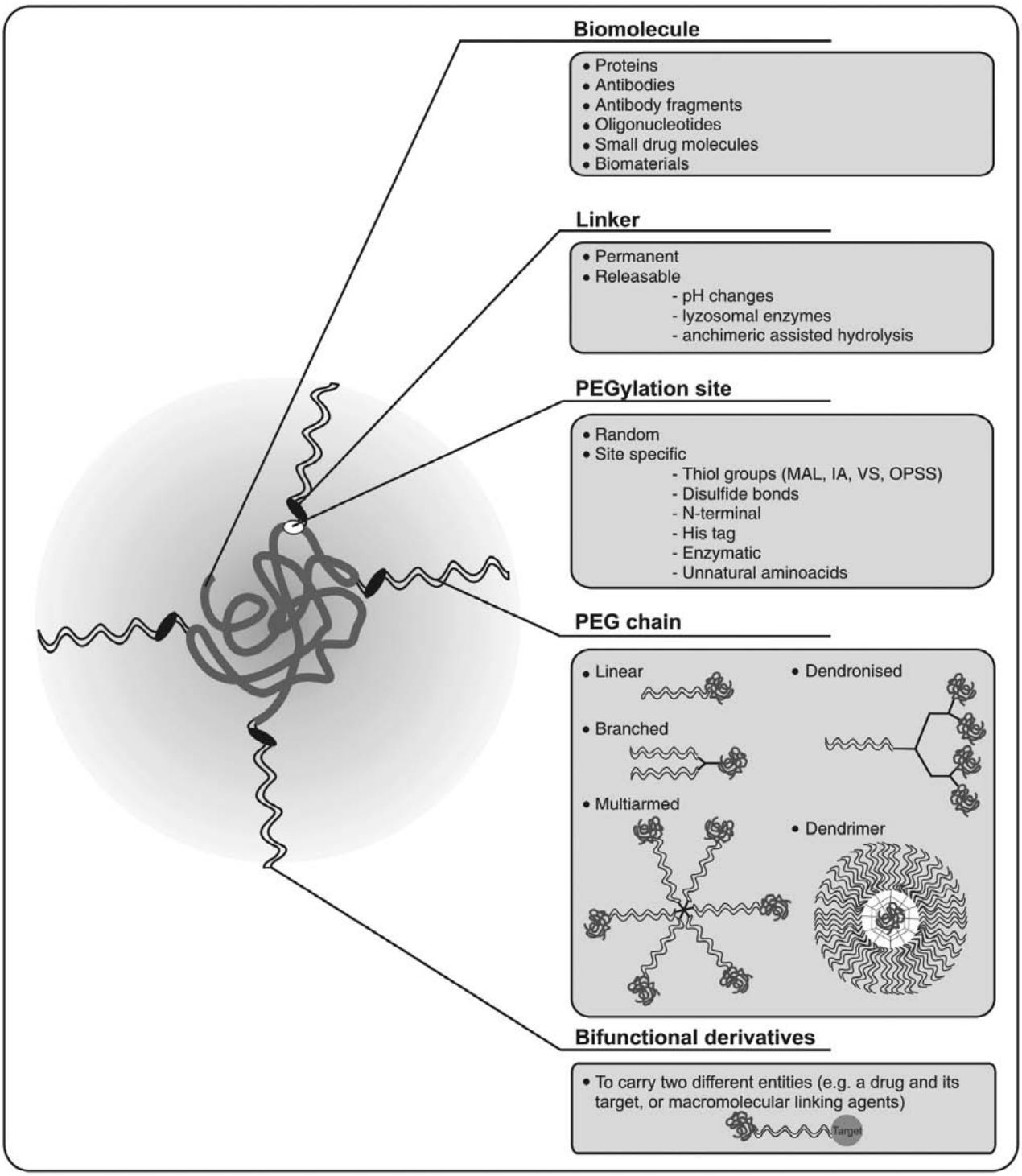

Fig. (3). Different alternatives to perform PEGylation.

PEGylation. A method called CyPEG™ technology (patent pending) yields conjugates more stable than those PEGylated using the established non-proprietary PEG maleimide reagents. Improved preparation methods and novel structures of this type of PEGylation reagents are reported, like the synthesis of single and PEG2-MAL derivatives [48,49]. Some recent examples of thiol-selective PEGylation include the attachment of some of these PEG derivatives to a modified galectin-2 [50], salmon calcitonin analogues [51], glucagon-like peptide-1 [52], hemoglobins and albumins [53], and IFN β [54]. Site-directed mutagenesis can be used to incorporate cysteinyl PEGylation sites for thiol-specific linkers. As an illustration, the US 6,420,339 [55] patent describes an approach using leptin protein. A dual PEGylation with two

different PEG derivatives at two specific amino acid residues yields conjugates with pharmacokinetic advantages when compared with the native form.

PEGylation across one or more natural disulfide bonds may comprise reduction of protein accessible disulphide bonds to thiols under mild conditions, followed by a sequential bis-alkylation using PEG monosulfone reagents that yield a covalent linkage through a three-carbon bridge. As an example of this approach, the so-called TheraPEG^{TM} technology [56] has been successfully applied to a wide range of proteins and peptides, such as IFN α-2a, IFN α-2b, IFN β-1b, Anti-CD4 Fab, Anti-TNFα domain antibody, Asparaginase, Amylase, Leptin, EPO, Tumour imaging ligand, and coagulation factor. This technology can be considered as a site selective approach, if the protein being PEGylated contains only one disulfide bond, or two bridges but one of them is more susceptible to reduction. Moreover, it can be performed without the need of adding a free cysteine or a non-native amino acid by protein reengineering. Other advantages are the greater stability of the resulting conjugates owing to protection of sulphur atoms from disulphide cleavage or exchange reactions, and also the greater resistance to degradation and aggregation phenomena.

PEGylation at a histidine (His) residue of proteins such as IFN α-2b and IL-10 has been early reported [39]. Taking into account that genetically fused His-tags are widely used to facilitate expression and purification of proteins, an approach for PEGylation at a histidine tag has been proposed to take advantage of both PEGylation and purification technologies. In fact, the so-called HiPEG^{TM} technology enables PEG conjugation at two consecutive histidine residues without losing the ability to purify the protein [57]. This approach has been successfully applied to PEGylation of a wide range of proteins and peptides. Particularly, an improved version of PEGylated IFN α-2a by engineering a histidine-tag at the N-terminus of the protein is claimed to be eight-fold more active than Pegasys® (*in vitro* assays) with a comparable half-life (preclinical studies). A potentially attractive feature of this approach is the feasibility of optimizing the bioprocess, including both PEGylation and purification steps.

Increasingly broader and more complex approaches for PEGylation are being developed. For example, an attractive technique, known as dock-and-lock technology, has been proposed [58,59] to prepare conjugates with defined stoichiometry and structure. It consists in the attachment of the PEG moiety to an anchor domain sequence (AD), and the target to be PEGylated to a dimerization and docking domain (DDD) sequence. The monoPEGylated products are formed because dimmers of the DDD sequence bind with high affinity to monomers of AD sequence. The specificity of the DDD/AD interaction determines the stoichiometry of binding and location of the PEG residue. Other advances in PEGylation processes are clearly shown by a patent concerned with the use of a simulation technology that allows the optimization of location and molecular sizes of attached polymeric moieties in proteins, providing a computational method for designing polymer attachment sites and positions. In the invention, some simulation analysis and PEGylated proteins are exemplified [60].

Table **1** highlights the patents related to the preparation of PEG-protein conjugates. Only a few patents are cited with the aim to exemplify different strategies and different types of proteins and peptides that can be coupled to PEG derivatives.

ADVANCES ON PEG-ANTIBODY AND ANTIBODY FRAGMENTS CONJUGATES

The therapeutic and commercial success of the first-generation formulations of therapeutic antibodies, such as anti-VEGF antibody (Avastin^{TM}), anti-CD20 monoclonal antibody (Rituximab^{TM}) and IgG1 monoclonal antibody (Erbitux^{TM}), is forcing proprietary (innovative) companies to look into the next generation of antibody-based drugs as strategy to replace revenues lost from expiring patents. Among the different strategies are antibody engineering, changes in primary structure, fusions, and chemical modifications such as glycosylation and polymer conjugates, including PEGylation. Methods of attaching PEG molecules to monoclonal antibodies and antibody fragments (e.g., Fab', Fv and scFV) and some variants genetically engineered are proposed in the field [99-101], but these approaches have mainly been limited by the high cost of developing these conjugates and the long times required for new medicines and treatments to reach the market.

PEG-antibodies and antibody fragments conjugates raise an increasing interest for targeting cancerous tumors due to PEGylayed antibody fragments may have preferential accumulation in tumors (instead of normal tissues). Although antibody targeting oncology is the most active area, several products are also in developing stage for emerging drug targets to treat chronic diseases. The leading commercial product available today is Certolizumab pegol (Cimzia®), a PEGylated Fc-free antibody fragment that targets tumor necrosis factor alpha (anti-TNF-α), which was approved to treat Crohn's disease and rheumatoid arthritis [102-104]. A branched PEG (40kDa) was specifically attached to anti-TNF Fab' to facilitate less frequent therapeutic dosing regimes.

Unlike full antibodies, PEGylated antibody fragments do not contain the fragment crystallizable (Fc) region and, therefore, undesired side-effects caused by the Fc region can be avoided. Conjugation of PEG to antibody fragments has also shown to reduce their antigenicity and significantly increase the circulating half-life in mammals of full antibodies and fragments thereof. PEGylation advantages are better reached if the conjugation site is far from the antigen-binding region to retain the targeting specificity, and the covalent link of PEG chain(s) is achieved in a stable manner to prolong circulating half-life. Random PEGylation via lysine residues and thiolated derivatives often results in reduction or loss of binding affinity due to the fact that the conjugation is near the antigen binding domain. As a far better option than random attachment, specific sites in the constant and the hinge regions of antibodies can be engineered to allow site-specific linkage [105,106]. Critical lysine residues in antigen binding loops have been replaced with arginines to allow modification with less loss in immunoreactivity [107]. However, the above-mentioned aims can be best achieved by introducing a free cysteine to

Table 1. Highlighted Patents Covering the Technology of PEGylation of Proteins and Related Applications

Species	Comments	Conjugation Method and Polymer MWs	Patent [Reference]	Year
Arginine deiminase	rADI is produced, renatured and purified. PEG derivatives of different functionality and with a variety of molecular weight are tested to prepare rADI-PEG. Results of *in vivo* assays are reported.	Random 5-40 kDa	US 20050129706 [61]	2005
Antimicrobial agents	Lysostaphin, preprolysostaphin or prolysostaphin are attached to PEG by degradable and non-degradable linkages. The conjugates present reduced immunogenicity and decreased frequency of dosing. The products obtained are purified and characterized.	0,5-100 kDa (5 kDa)	WO2006110776 [62]	2006
Erythropoietin	EPO-PEG conjugates are prepared using butyric or propionic acid succinimidyl esters of PEG.	Random 20-100 kDa	US 6583272 [63]	2003
Erythropoietin	PEG-derivatives to use for conjugation to EPO are developed. Examples are given using several different derivatives (mPEG-MAL, mPEG2-butyraldehyde, mPEG2-NHS, mPEG-SPA, among others) with diverse molecular weight and chemical structure.	Random and site-specific 10-100 kDa	US 7714114 [64]	2010
Erythropoietin	EPO is attached not only to PEG but also to an organic molecule that increases even more the half-life of the conjugate. PEG-DSPE [disteroylphosphatidylethanolamine]-lysyl glycine is used for coupling.	Site specific 0.2-100 kDa	US 7074755 [65]	2006
Factor VII glycoforms	FVII or related polypeptides are bound to oligosaccharide chains and one or more of these groups are covalently linked to PEG. GlycoPEGylation of Factor VII or related polypeptides using sugar-nucleotides-PEG and glycosyl transferases enzymes is exemplified.	Glyco-PEGylation 0.3-100 kDa (10 kDa)	US 20050113565 [66]	2005
Factor VIII (FVIII)	A proteinaceous construct comprising a Factor VIII molecule, which is conjugated to a water-soluble polymer (PEG, polysialic acid or dextran), is claimed. The attachment of PEG-SS to lysine residues of FVIII, a branched form of PEG succinimidyl glutarate, or a PEGylation via carbohydrate moiety are exemplified among other procedures.	Random and Glyco-PEGylation Various MWs (3.3 kDa)	US 7645860 [67]	2010
Factor VIII and other blood coagulation factors	FVIII-PEG conjugates are obtained by first incubating the FVIII with FVIII specific antibody to form a complex, then, incubating the resulting complex with PEG, and finally releasing the PEG-FVIII construct. The process is also tried for other blood coagulation factors.	Random 2-150 kDa (20 kDa)	US 20100093934 [68]	2010
Galectin-2	An amino acid residue is modified with the purpose of providing a single cysteine residue in the protein structure to make the specific PEG attachment. The procedure using PEG-MAL derivative is exemplified.	Site specific 1-100 kDa (5,5 kDa)	WO 2009140722 [50]	2009
G-CSF	G-CSF-PEG conjugate having primarily 3 attached PEG chains per G-CSF molecule is prepared by first making a PEGylation reaction followed by a partial de-PEGylation by changing the pH of solutions. The composition of positional isomers and stability data are reported.	Random 1-20 kDa (5 kDa)	US 7381805 [69]	2008
G-CSF	G-CSF-PEG (with some different lysine residue compared with human protein) conjugates having 2-6 PEG chains are prepared. The obtained constructs have better *in vivo* behavior.	Random and site specific 5 kDa each PEG molecule	US 7696153 [70]	2010
Glucagon-like peptide-1	PEG-GLP-1 conjugate is made by reacting PEG2-MAL derivatives with cysteine residues of G-CSF. Improvements in half-life and clearance features are reported.	Site specific 3.4 and 40 kDa	US 7557183 [52]	2010

(Table 1) contd….

Species	Comments	Conjugation Method and Polymer MWs	Patent [Reference]	Year
Glutenase	Glutenase-PEG conjugates are obtained by attachment of PEG derivatives to this enzyme, so the protein is stabilized to resist digestion in acidic stomach conditions.	Random and site-specific 0,3-40 kDa (2-30 kDa)	US 20090304754 [71]	2009
Growth Hormones	GH is C-terminally PEGylated, being useful in therapeutic applications.	Site specific 10-40 kDa	US 20090105134 [72]	2009
Hemoglobin	Hgb is conjugated with PEG-SC maintaining the structural integrity of the heme oxygen binding site. In another approach, Hgb is modified first by bifunctional PEG and then further modified by monofunctional PEG.	Random 0,5-40 kDa	US 5386014 [73]	1995
Hemoglobins	Hgb-PEG conjugates are prepared by novel amidation, alkylation or thiolation-mediated maleimide chemistry-PEGylation.	Site specific 5 kDa	US 7169900 [74]	2007
Hemoglobins	Hgb-PEG conjugates are obtained using phenyl isothiocyanato PEG carbamate, isothiocyanato phenyl PEG carbamate, or isothiocyanato phenyl di-PEG carbamate.	Random 0,2-40 kDa (5 kDa)	US 7144989 [75]	2006
Human growth hormone (hGH)	Recombinant hGH is N-terminally monoPEGylated with branched mPEG-aldehyde (40kDa).	Site specific 20 and 40 kDa	EP 1591467 [76]	2005
Human-Growth Hormone-Releasing Factor	hGRF-PEG conjugates are prepared by attaching one, two or three PEG chains to different amino acid residues. Reactions are carried out covering a wide range of hGH:PEG ratios and using linear and branched PEG derivatives with different molecular weights.	Site specific 5-20 kDa	US 7317002 [77]	2008
Insulin	PEG is attached to extended insulin (with 1-4 extensions), in 1-4 amino acid residues in the mentioned extensions. The product is claimed to possess better bioavailability and longer time-action profile.	Site specific 2 kDa	US 20090306337 [78]	2009
Insulin analogues	An insuline analogue having an amino acid residue or a peptide C-terminally attached and a PEG moiety is prepared. The construction can be pulmonary administrated.	Site specific 2-5 kDa	WO 2009022005 [79]	2009
Insulin and proinsulin	PEG is attached to proinsulin polypeptides which can be cleaved to yield insulin. Different approaches are presented in the examples.	Site specific LowMW PEGs	US 7312192 [80]	2007
Insulin derivatives	Insulin is coupled to PEG by making functional group protection and deprotection reactions. Insulin-PEG derivatives are obtained where PEG is specifically attached to PheB1 residue of insulin.	Site specific 0.6-2 kDa	US 6323311 [81]	2001
Insulin-like growth factor binding protein 4 (IGFBP-4)	IDFBP-4-PEG conjugates are obtained. The resulting constructions are claimed to have better properties for therapeutic applications in tumor treatments and avoid undesired side effects *in vivo*. The procedure is exemplified by using random PEGylation (with branched mPEG2-NHS), N-terminally PEGylation (with mPEG-aldehyde), and specific cysteine PEGylation (with mPEG-MAL or branched mPEG2-MAL).	Random and site specific 20-40 kDa	US 20060100144 [82]	2006
Insulin-like growth factor-1	Lysine-PEGylated IGF-I (or variant) is prepared by obtaining the recombinant protein with a fusion protein, PEGylating the resulting construction, and then cleaving the fusion protein recovering the conjugate.	Site specific 20-100 kDa	US 7625996 [83]	2009

(Table 1) contd….

Species	Comments	Conjugation Method and Polymer MWs	Patent [Reference]	Year
Interferon α 1b	INF α 1b-PEG conjugate is prepared by using an N - maleimide derivative of a single chain mPEG, which can be attached to a specific cysteine residue. Pharmaceutical compositions are described.	Site specific 0.5-100 kDa (20 and 40 kDa)	US 20060029573 [84]	2006
Interferon γ	INF γ-PEGconjugates (which may contain at least one different amino acid residue) are prepared with one or two PEG chains.	Random and site specific 0.3-100 kDa	US 7230081 [85]	2007
Interferon α	IFN α-mPEG conjugate is prepared from IFN α and succinimidyl carbonate-activated mPEG derivative. The preparation method is exemplified with mPEG chains of 12 kDa. The conjugate showed activity retention and longer circulating half-life *in vivo*.	Random 2-20 kDa (12 kDa)	US 5981709 [86]	1999
Interferon β1a	IFN β1a-PEG is synthesized. A 20 kDa mPEG-aldehyde derivative is proposed to N-terminal PEGylation. The product obtained is usefully employed in therapeutic applications.	Site specific 5-40 kDa	US 6962978 [87]	2005
Interleukin-10	An IL-10 PEGylated version is presented. The conjugate is made by attaching mPEG in the aldehyde form to the amino terminus of IL-10, under reducing conditions and in a site-specific manner. Structurally intact mono-conjugates are obtained in high yields and as homogeneous products.	Site specific 10-36 kDa	US 7052686 [40]	2006
Linear Salmon Calcitonin	Site-specific PEGylated linear salmon calcitonin analogues, and the techniques to produce them are presented. The methodology is exemplified replacing one amino acid by cysteine and then coupling the hormone to PEG-MAL.	Site specific 2-100 kDa (5 kDa)	US 20100227815 [51]	2010
Megakaryocyte growth and development factors	MGDF derivatives attached to mPEG succinimidyl propionate (MW 20 kDa), poly-mPEG-MGDF conjugates (obtained by MGDF reductive alkylation with mPEG-aldehydes), and mPEG- MGDF conjugates with the site of attachment at the N-terminal α-amino residue are claimed.	Site specific 5-50 kDa	US 5795569 [88]	1998
Muteins of IL-2	Site-specific PEGylated Mutein is generated by the replacement of one amino acid naturally present in the native IL-2 sequence by a cysteine residue, which in turn is used as specific site for PEG attachment.	Site specific 3-100 kDa (10 and 40 kDa)	US 5206344 [89]	1993
Neurotrophic factors (BDNF and NT-3)	Brain-derived neurotrophic factor (BDNF) and Neurotrophin-3 (NT-3) are conjugated with different PEG derivatives. The PEGylated factors are achieved by alkylation, acylation and N-terminally conjugation. Mono and poly-PEGylated products and *in vitro* and *in vivo* tests of these conjugates are exemplified.	Random and site specific 2-100 kDa (6 and 20 kDa)	US 5770577 [90]	1998
Novel erythropoietin stimulating protein (NESP)	The conjugates are obtained by N-terminally attachment of PEG to NESP. The effect of different degrees of substitution and variations of the polymer size is analyzed using both mPEG-aldehyde and mPEG-NHS based chemistries.	Random and site specific 2-100 kDa (5, 20, 30 and 40 kDa)	US 6586398 [91]	2003
Obese (ob) protein compositions	Ob proteins can be used therapeutically for treating, preventing or controlling obesity and its related diseases. The invention is directed to obtain expression vectors, the host organism transformed by such vector, the DNA sequence of the human ob protein, and the PEGylated conjugate, which is exemplified by using a mPEG2-NHS (40 kDa) derivative.	Random 15-60 kDa (40 kDa)	US 6025325 [92]	2000
Prolactin Receptor Molecules	N-terminal PEGylated molecules that may bind prolactin receptor, improving its pharmacokinetic parameters without significant decrease in the binding to the receptor, are prepared. Conjugates using different PEG derivatives are exemplified.	Site specific 20 kDa	US 20100035814 [93]	2010

(Table 1) contd….

Species	Comments	Conjugation Method and Polymer MWs	Patent [Reference]	Year
Superoxide dismutase mimetic	SODm-PEG conjugates are obtained, which show lower toxicity levels than the unPEGylated counterparts. These constructions are reported to have better *in vivo* efficacy and increased plasma levels and half-life. The approach is exemplified by using different mPEG derivatives.	Random and site specific 0.2-44 kDa	US 20080318917 [94]	2008
T1249 polypeptide	PEGylated versions of T1249 (which is a polypeptide with antiretroviral activity against HIV) and related pharmaceutical compositions are prepared.	Random and site specific. A lot of derivatives are mentioned	US 7084261 [95]	2006
T20 polypeptide	T20 polypeptide (which is an antiviral agent against HIV) is PEGylated using different mPEG-adlehyde derivatives.	A broad diversity of derivatives.	US 7049415 [96]	2006
Transforming growth factor-α	TGF-α and related polypeptides are PEGylated. The approach is exemplified by using mPEG2-NHS with a lysine core.	Site specific 2-40 kDa	US 20030036509 [97]	2003
Uricase	Uricase-PEG conjugates are prepared. The preparation of conjugates is exemplified with mPEG-SS (5 and 20 kDa).	Random 20 kDa	US 6913915 [98]	2005

the residual hinge region of a Fab´ fragment by performing thiol-site specific PEGylation using maleimide-based chemistry, or by incorporating the hinge region on the C-terminus of a Fab and scFv. Reductive activation is required prior to PEGylation. Thus, before modification with a PEG-maleimide PEGylating reagent, a single cysteine must be activated through mild reduction with a thiol-based compound such as β-mercaptoethylamine (β-MA), β-mercaptoethanol (β-ME), or glutathione. A common problem in applying this procedure is the undesirable formation of thiol adducts which can reduce the efficiency of the conjugation reaction. Tris(butyl) phosphine (TBP) and tris(2-carboxyethyl) phosphine (TCEP) are reducing agents that cannot form thiol adducts. TCEP has been used for the activation of scFv thiols [108,109]. However, TBP and TCEP are not suitable for the activation of a single hinge because they are strong reducing agents.

The following are some remarkable published results. Site-specific attachment of PEG to divalent antibody fragments can be used to avoid the loss of immunoreactivity associated with random attachment processes. Fragments modified in this way have markedly improved binding and/or pharmacokinetic properties when compared to fragments which have been modified randomly with the same number and type of polymer molecules [110]. In practice, PEGylation of Fab has been usually performed by activating the hinge cysteine using mild reducing conditions in order to retain an intact interchain disulphide. Recently, evidence has been presented which proves that the final Fab-PEG product does not need to retain the interchain disulphide, therefore, strongly reducing conditions can be used [111].

Some promising applications and innovative approaches are worthy of notice. Currently, studies on cancer targeting agents using antibodies for cancer cells are in progress to obtain high efficacy and safety as antitumor agents [112]. PEGylated CEA-targeting Fab fragments have early shown the potential use of PEG-scFv in oncology to improve both the pharmacokinetics of small proteins and tumor specific binding [113]. Covalently linked rhuMabHER2 Fab´ and PEG-phosphatidyethanolamine to facilitate binding and internalization have been used in designing anti-HER2 immuno-liposomes [114]. The potential application of PEG-scFv as a platform technology for multi-valent tumor targeting has been reported for an anti-HER2 conjugate which is characterized by an improved tumor accumulation [115]. The utility of PEG-scFv in immuno-liposome development has been also described [116,117]. The Fab binds to platelet GPIIb/IIIa and blocks interactions with its ligands. Site-specific PEGylation of the Fab in the hinge region did hinder the overall extent of platelet binding, but the conjugates exhibited functional inhibition of platelet aggregation and the desired prolonged circulating times [118]. New attempts like the integration of site-specific PEGylation and refolding technologies have been applied to a scFv, allowing the production of therapeutically useful small antibodies from insoluble fractions [119]. Antibodies covalently linked to a signal-generating moiety through a heterobifunctional PEG linker moieties have been used in immunoassays for detecting specific target molecules in biological samples. The two different reactive groups are selected from a carbonyl-reactive group, an amine-reactive group, a thiol-reactive group and a photo-reactive group; more specifically, the thiol-reactive group includes a maleimide group, the amine-reactive group includes an active ester and the carbonyl-reactive group includes a hydrazine derivative [120]. Some examples of claimed conjugates, their preparation methods and related applications are indicated in Table **2**.

ADVANCES ON PEG-OLIGONUCLEOTIDE CONJUGATES

Oligonucleotides (ODNs), especially antisense oligonucleotides, are promising as therapeutic agents, but the current ODN therapy is very expensive because they are rapidly degraded by nucleases before reaching the target cells.

Table 2. Some Patents Covering the Technology of PEGylation of Antibodies and Antibody Fragments, and Related Applications

Species	Comments	Conjugation Method and MW of PEGs	Patent [Reference]	Year
Amyloid beta (A.β.) peptide	A method to treat conditions associated with A.β. peptide activity both prophylactically and therapeutically is described. The method employs humanized antibody fragments that specifically bind human A.β. peptide between amino acid positions 13-28, wherein the antibody fragments are covalently attached to a PEG molecule.	Site-specific 0.5-30 kDa (20 kDa)	US 20100015155 [121]	2010
Antibody fragments	PEG is conjugated to various antibodies and antibody fragments like those directed against vascular endothelial growth factor, human p185 receptor-like tyrosine kinase, human CD20, human CD18, human CD11a, human IgE, human apoptosis receptor-2, human tumor necrosis factor-α, and human tissue factor. Conjugates of one or more molecules of antibody fragments with one or more molecules of polymer are also prepared.	Site-specific 5-300 kDa (5-100 kDa)	US 7507405 [122]	2009
Antibody fragments	Conjugates consisting essentially of one or more antibody fragments covalently attached to one or more nonproteinaceous polymer molecules (such as PEG) are described in this patent. At least one antibody fragment comprises an antigen binding site that binds to a polypeptide. This polypeptide may be selected from the group consisting of human vascular endothelial growth factor (VEGF), human p185 receptor-like tyrosine kinase (HER2), human CD20, human CD18, human CD11a, human IgE, human apoptosis receptor-2 (Apo-2), human tumor necrosis factor- α (TNF- α), human tissue factor, human α_4, β_7 integrin, human GPIIb-IIIa integrin, human epidermal growth factor receptor (EGFR), human CD3, and human interleukin-2 receptor α -chain (TAC).	Site-specific 20-40 kDa	US 7842789 [123]	2010
Antibody fragments of IL-8	Humanized anti-IL-8 monoclonal antibodies conjugates of at least 500 kDa are prepared with PEG. The *in vitro* and *in vivo* properties are evaluated.	Random 20-40 kDa	US 7005504 [124]	2006
Antibody/signal-generating moiety conjugates	Antibody/signal-generating moiety conjugates are disclosed that include an antibody covalently linked to a signal-generating moiety through a heterobifunctional PEG linker. The disclosed conjugates show exceptional signal-generation in immunohistochemical and *in situ* hybridization assays on tissue sections and cytology samples. Enzyme-metallographic detection of nucleic acid sequences with hapten-labeled probes can be accomplished using the disclosed conjugates as a primary antibody without amplification	Site specific- heterobifunctional PEG Final conjugate MW: 230-330 kDa	US 20090176253 [120]	2009
Divalent antibody fragments	Divalent antibody fragments are described, each of which has one or more interchain bridges containing a synthetic or naturally occurring polymer selected from a polyalkylene, polyalkenylene, polyoxyalkylene or polysaccharide. Fragments modified by site-specific PEGylation have improved binding and/or pharmacokinetic properties when compared to fragments which have been modified randomly with the same number and type of polymer molecules.	Site-specific 25-40 kDa (40 kDa)	EP1090037 B1 [110]	2004
E2 antibody fragments	A site specific PEGylated conjugate of monoclonal antibody fragment anti-E2 is prepared and its properties, like pharmacokinetics, pharmacodynamics, bioactivity and biocompatibility, are evaluated. This conjugate is equally broadly-neutralizing as the unmodified therapeutic monoclonal antibody fragments.	Site-specific 5-300 kDa	WO 2006028634 [125]	2006

(Table 2) contd….

Species	Comments	Conjugation Method and MW of PEGs	Patent [Reference]	Year
Monovalent antibody fragments	Antibodies and antibody fragments conjugates are prepared by site-specific and random PEGylation. Different PEG reagents, such as, maleimide, iodoacetamide and vinyl-sulphone, are used. Preparation, purification, and antigen binding measurements and pharmacokinetic procedures are exemplified for different antibodies and antibody fragments.	Site-specific 5 kDa	US 20040121415 [126]	2004
Tc-99m-radiolabeled antibody fragments	Radioimmunodetection of diseases involves the use of radiolabeled antibodies (like with technetium-99m isotope), which have a renal retention in kidneys leading to imaging difficulties in the area of this organ. This invention has the purpose of generating a method for preparing these radiolabeled antibody fragments that exhibit reduced renal uptake and retention by the conjugation to PEG.	Random 2-20 PEG moieties (5 kDa) per divalent fragment	US 5670132 [127]	1997
7E3, humanized 7E3, c7E3 antibody fragments	Modified antibodies with improved pharmacokinetic properties are disclosed. The antibodies bind to one or more human integrin receptors selected from the group consisting of GPIIb/IIIa, ccvP3 and Mac-1, and comprise from one to about six organic moieties which are covalently bonded to a carboxyl-terminus of the antibody and/or covalently bonded to the sulfur atom of a cysteinyl residue of the antibody. Preferably, the modified antibody is 7E3 or a humanized 7E3, more specifically c7E3.	Site-specific 0.8-120 kDa	WO/2000/026256 [128]	2000

Another disadvantage is that ODNs tend to form secondary and high-order structures in solution, which can produce non-specific side effects and insufficient amounts of active compound for binding. Furthermore, their negative charge on the phosphate group prevents easy penetration into the cells. Enhanced targeting and penetration of ODNs into tumor cells could potentially improve the utility of ODN based therapy. Thus, there is a need of methods for protecting antisense ODN compounds against degradation, preventing the formation of high-order structures and delivering effective amounts of active antisense ODN compounds to the target. Among the different methods of modifying ODNs to achieve these concerns, the most widely studied modifications have been made to the back-bone portion of the ODN molecules [129]. In fact, the addition of sulfur atoms to the back-bone slows down the enzymatic degradation rate, but increases toxicity and results in several diastereomers that may likely provoke undesirable side effects. Other efforts have been made for improving the transmembrane delivery by using protein or antibody carriers, liposomal delivery systems, electroporation, direct injection, cell fusion, viral vectors, and calcium phosphate-mediated transformations. However, many of these techniques are limited by the type of cells and by the conditions needed for achieving an efficient transport.

A particularly attractive alternative for improving ODN therapy consists in adding a linking moiety and PEG chain(s) [130,133]. Pegaptanib sodium (Macugen®) is a covalent conjugate of an ODN of twenty-eight nucleotides in length that terminates in a pentylamino linker to which two 20 kDa PEG chains are covalently attached via the two amino groups on a lysine residue. This PEGylated anti-VEGF aptamer binds with specificity to VEGF 165, a protein that plays a critical role in angiogenesis and increased permeability, which are two of the primary pathological processes responsible for the vision loss associated with neovascular AMD. Developers have usually failed in their efforts to stabilize ODNs against degradation and increase cell permeability by attaching permanent linking moieties. Releasable PEGylation (rPEGylation) employing customized linkers that reversibly attach the therapeutic moiety with PEGs appears to be a better option than PEGylation with permanent linkages [5]. The rPEGylation technology yielded attractive results for G3139 ODN, demonstrating differences between *in vitro* and *in vivo* models of oligonucleotide activity, as it occurred in the case of proteins [134]. Triplex-forming ODNs were PEGylated resulting in a product with improved biological properties (cellular uptake and nuclear penetration) [135]. Specific protocols for the PEGylation of oligonucleotides have widely varying yields and often unwanted reactions, such as precipitation, depending on the specific oligonucleotide that is being PEGylated. A method that optimizes the PEGylation for a given oligonucleotide, preferably an aptamer, has been claimed to be efficient, consistent, cost effective and directly scalable to large scale synthesis [136].

ODNs or specifically siRNA molecules can be conjugated to cell penetrating peptides with a hydrophilic polymer, preferably a PEG-based linker, to improve the efficiency of crossing biological membranes or physiological barriers to deliver the said nucleic acid into cells without compromising the ODN or siRNA molecule activity. A procedure for synthesizing this type of conjugates is known in

the literature [132]. However, the synthesis of ODN-PEG-peptide conjugates containing an ODN sequence longer than 10 nucleotides could be difficult to achieve due to the low yield of such a procedure. A detailed protocol for the preparation of a conjugate having the general formula P-L-N, wherein P is a cell penetrating peptide, N is a siRNA, and L is a PEG-based linker, has been presented and the anti-tumoral efficacy of siRNAVEGF when conjugated to DPV15b using a PEG4-based linker has also been demonstrated [137].

Liposomal and micellar formulations having a hydrophobic core and hydrophilic shell are currently successfully used as pharmaceutical carriers for water-insoluble drugs and demonstrate a series of attractive properties. However, a limited number of these systems comprising nucleic acids are known. Methods for encapsulating high molecular weight nucleic acids in liposomes have been early claimed [138-141]. Nano-particulate polyelectrolyte complexes formed by interaction of negatively charged ODN with cationic carriers, such as cationic liposomes, cationic lipids, and cationic polymers, have also been proposed to improve the poor cellular uptake. Despite their satisfactory performance in intracellular gene transfer *in vitro,* the use of polyelectrolyte complexes nano-aggregates appears to be limited due to the fast rate of clearance and undesirable pharmacokinetic profiles [142]. Poly(L-lysine)-PEG block copolymer [143-147] and a PEG-grafted PEI copolymer [148,149] were reported to form water-soluble micellar polyelectrolyte complexes when interacting with ODNs. The entrapment of nucleic acids in the core of these polyion complex micelles prevented serum protein binding of ODN and also significantly improved the nuclease resistance. Antisense *c-raf* ODN was conjugated to PEG via a noncleavable amide linkage to form polyelectrolyte complex micelles with polyethylenimine. The antisense *c-raf* ODN showed remarkable antiproliferative effects on several cancer cells, including lung, breast, and ovarian carcinoma cells *in vitro* and *in vivo* [150]. Polyelectrolyte complex micelle-based antisense oligodeoxynucleotide delivery system was designed to overcome intrinsic limitations of cationic lipid-mediated gene transfer. Cationic lipid and PEG conjugated ODN were ionically complexed to form self-assembled spherical polyelectrolite complex micelles. The micelles exhibited enhanced cellular uptake followed by rapid nuclear localization of ODN in human epithelial carcinoma cells [151]. Enzon Pharmaceuticals Inc. has developed a novel lipid formulation with PEG that enhanced cellular penetration of oligonuleotides *in vitro* and *in vivo* in animal models. Customized lipid-based PEG nanoparticles provide a promising approach for more efficient *in vivo* delivery of ODNs, including two types of RNA antagonists: Locked Nucleic Acid-based antisense molecules and siRNAs (Enzon, 2008).

Oligonucleotides can be incorporated to PEG shielded micelles facilitating its delivery across cellular membranes. This system presents advantages over other micelles since multifunctional PEGs provide greater shielding effectiveness. Enhanced uptake into cancer cells, stability against nucleases, high solubility, and non-binding to serum proteins are some characteristics that this shielded micelles demonstrated in *in vivo* assays. Moreover, the inventors present a novel gene carrier which is shown to be substantially non-toxic and is suitable for parenteral, oral, pulmonary, and transmucosal delivery of polynucleotides [152]. Another interesting approach to improve ODN performance is using oligonucleotides bounded to the surface of the controlled release polymer nanoparticles that selectively bind to a target cells or tissues. Polymers biologically degradable and/or chemically degradable, such as PEGylated poly(lactic acid), PEGylated poly(lactic-co-glycolic acid), PEGylated poly(ortho esters), and PEGylated poly(caprolactone), have been used to prepare the controlled release polymer nanoparticles [131]. Table **3** summarizes some examples of patents covering the PEGylation technology of ODNs.

ADVANCES IN PEG-DRUG CONJUGATES

While the most common application of PEGylation is the modification of relatively large proteins, PEGylation has also been used, yet still in a limited degree, to improve the bioavailability and ease of formulation of small molecule drugs having poor aqueous solubilities, untargeted biodistribution and rapid excretion. The field of small drug-polymer conjugates has been reviewed from a number of perspectives [157,158]. Although the number of low molecular weight drugs is relatively large when compared to protein pharmaceuticals and many studies have looked for PEG-small drug conjugates as effective therapeutics, few PEG conjugates are so far under clinical evaluation and none has still reached the market. For instance, a range of PEG-conjugated oncolytic drugs are under development or undergoing clinical trials with very promising results. A phase II study to determine the dose, safety and efficacy of PEG-irinotecan (NKTR 102) in combination with Cetuximab for the treatment of second-line colorectal cancer and multiple solid tumors, was completed. Clinical development programs are currently carried out to evaluate the safety and efficacy of NKTR-102 in patients with metastatic or locally advanced platinum-resistant ovarian cancer. PEG-docetaxel (NKTR-105) is in Phase I clinical trials for the treatment of multiple solid tumors. PEG-SN38 (EZN-2208) is in Phase II for the treatment of patients with metastatic breast cancer, and with and without Cetuximab in patients with metastatic colorectal carciroma. A Phase I development program to evaluate the safety and efficacy of PEG-paclitaxel in patients with advanced solid tumors and lymphomas was terminated. Poly-(L)-glutamic acid (PGA)-paclitaxel (Opaxio) will be the first of this class of therapeutics to reach the market since, in combination with carboplatin, it is currently under phase III clinical trials for the treatment of non-small cell lung cancer and ovarian cancer. At this time, as mentioned above, there is no FDA-approved PEG-small drug conjugates.

The first simple PEG-small drug conjugates did not fulfill the high expectations placed on PEG as polymeric carrier and several projects were stopped. There are numerous chemical and biological factors affecting the therapeutic effectiveness, which in turn have a major impact on the commercial feasibility of PEG-small drug conjugates. As briefly mentioned here, some factors are common to all small drug-polymer conjugates while others are specific of PEG conjugates. Small drugs enter cells primarily by diffusion, while high molecular weight drugs are mainly internalized by endocytosis. Thus, high molecular weight pro-drugs resulting from conjugation of low molecular weight drugs

Table 3. Some Examples of Patents Covering the Technology of PEGylation of Oligonicleotides and Related Applications

Species	Comments	Conjugation Method and MW of PEGs	Patent [Reference]	Year
Nucleic acid ligand	A controlled release polymer nanoparticle system bounded to a nucleic acid ligand is claimed. The ligand is capable of binding selectively to a target, such as a cell surface antigen, and therefore can be delivered to a diseased tissue for further releasing.	Site specific 2-3,5 kDa	US 7727969 [153]	2010
ODNs	With the aim of increasing stability and cell permeability, while decreasing toxicity, a variety of polymeric pro-drug platforms with releasable PEGs attached to ODNs are presented.	Site specific 3-100 kDa	US 7595304 [154]	2009
ODNs	A method based in the encapsulation of antisense ODNs in neutral biodegradable polymersome vesicles is described. This polymer may be used to transport selected active agents and deliver them to a determined cell or tissue target.	Polymersome	WO2008060557 [155]	2008
ODNs	Some possibilities of making conjugation reactions between ODNs and PEG in aqueous solution are proposed. In the procedures, pH 8.5 or greater and DMSO are used to avoid PEG side reactions and degradation.	Site specific 2-30 kDa	WO2009073820 [136]	2009
ODNs	A pure ODN is obtained by an ultrafiltration procedure, and further conjugation with PEG is performed. The resulting product may be purified by additional ultrafiltration under denaturing conditions. In the examples branched PEG-NH (40kDa) is used.	Site specific 0,2-60 kDa (40 kDa)	US 20100029924 [156]	2010
siRNAVEGF	ODNs (or specifically siRNA molecules) are conjugated to a PEG-based linker, which in turn is coupled to cell penetrating peptides. As result, biomolecule activity is not compromised in therapeutic applications while its properties are improved. Heterobifunctional PEGs are used, having one NHS group and one maleimide group in each end.	Site specific, low molecular weight PEG	US 20090186802 [137]	2009

with high molecular weight polymers may exhibit changes in the cellular drug entrance mechanisms relative to those of the primary drug. Moreover, the stable attachment of large polymers usually prevents the activity of small drugs. Alternatively, it is very important to achieve a suitable controlled drug release rate when using hydrolytically unstable bonds or enzymatically labile linkers because either the advantages of polymer conjugation can be annulled by a very fast release rate or drug activity can be reduced by a too slow release rate. Particularly, PEGylation itself confers a passive targeting to solid tumors, by effects of enhanced permeability and retention and/or by slowing the drug clearance from the body [159]. An attractive alternative consists of conjugates containing ligands that might promote receptor-mediated targeting, but so far only N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-doxorubicin-galactosamine conjugate has progressed into Phase I clinical trial for treatment of hepatocellular carcinoma or secondary liver disease. PEG molecules also present only one (in methoxy molecules) or two (in diol forms) possible attachment sites that directly conduct to a low loading. Significant efforts have been made to overcome this limitation, branched and multi-arm PEGs (now commercially available) or dendrimer PEG [160-162] are very attractive forms. In fact, dendrimers, dendrons, and hyperbranched polymers present the advantages of well-defined molecular weight, multivalent surfaces, and high drug carrying capacity. Such architectures can also display unique endocytic properties. Nevertheless, the costs of synthesizing too complex PEG-based architectures become too high in comparison with other multifunctional polymers, such as PGA and HPMA. Some examples of multi-arm PEG structures are remarkable: PEG-SN38 (EZN-2208) has coupled a four-arm star PEG [163], i.e., this conjugate presents a high drug loading (4 molecules of SN38 per PEG molecule). In a similar manner, melittin (powerful stimulator of phospholipase A2) has also been coupled to a four-arm PEG structure [164]. In another approach, dendrimers of aspartic acid were used to obtain branched PEG acid, and then coupled to Ara-C [165]. Clinical data collected over the last decade projected renewed expectations for multi-arm PEGs conjugates as polymeric carriers for designing better conjugates.

Table **4** cites some of the patents about PEG-drug conjugates. Often, pharmaceutical companies prefer to patent the technology of generating these products, than the specific conjugate *per se*, leading to a limited number of patents of this kind. Nevertheless, there are some scientific publications, exemplifying PEG conjugation to small molecule drugs like camptothecin [166], paclitaxel [167], doxorubicin [168,169], and Ara-C [170], among others.

Table 4. Some Patents Covering the Technology of PEGylation of Small-Drugs

Species	Comments	MWs of PEG	Patent [Reference]	Year
Anticancer agents	A novel structure with a determinate formula which contains an aromatic or multi-substituted heterocyclic group is presented. This structure contains a polymeric moiety coupled to an anticancer drug. In the examples drugs like paclitaxel and Ara-C are conjugated.	0,2-100 kDa (20 kDa)	US 6936597 [171]	2005
Anti-inflammatory solution	A methodology to solubilize hydrophobic pharmaceutical active ingredients using PEGs is presented. This approach is exemplified using Indomethacin.	300-700 Da	US 4794117 [172]	1988
Ara-C	Different conjugates are obtained by the attachment of bis-thiazolidine-2-thione-activated PEG, SC-PEG or PEG-NH_2 to Ara-C, obtaining PEG-amide-Ara-C, PEG-carbamate-Ara-C and PEG-urea-Ara-C, respectively. Gemcitabine is also attached to PEG, and *in vivo* and *in vitro* experiments are carried out comparing the results with the uncoupled counterparts.	20-100 kDa (40 kDa)	US 6376470 [173]	2002
Calcitonin-drug	This drug is frequently used to treat bone marrow disorders. A conjugate of calcitonin drug and an oligomer (ethylene glycol moieties with subunits) is presented; and pharmaceutical compositions thereof. A broad diversity of PEG derivatives is exemplified.	Low MW PEGs	US 7713932 [174]	2010
Camptothecin	A conjugate is obtained by the functionalization of the drug to allow the attachment of PEG. *In vivo* efficacy of the conjugate is evaluated against human mammary carcinoma in nude mice.	20-100 kDa (40 kDa)	US 6608076 [175]	2003
Coumarin	Different conjugates are prepared attaching PEG (dithiazolidine thione) to coumarin. Conjugation reactions with daunorubicin hydrochloride and (L)-Asparaginase are also carried out.	2-100 kDa (40 kDa)	US 6214330 [176]	2001
Etoposide	This molecule is an inhibitor of topoisomerase II enzyme, frequently used in chemotherapy of various cancer types, including cancer of the central nervous system. The etoposide prodrug presented by the inventors is capable of crossing the blood-brain barrier, and consists of PEG molecules joined to etoposide by ester or carbonate bonds, which are hydrolizable *in vivo*.	Low MW PEG	US 6713454 [177]	2004
Posaconazole	It is a triazole antifungal drug applied in patients having invasive infections, immunocompromised, or cancer and metastatic diseases. The invention is exemplified by the attachment of posaconazole to bis-PEG-amine (20 kDa) and *in vitro* and *in vivo* tests were also conducted.	1-100 kDa (20 kDa)	US 7625898 [178]	2009
Rapamycin, tacrolimus and temsirolimus (CCI-779)	These macrolides are structurally similar and are potent immunosuppressants effective in animal models of autoimmune diseases. A conjugation process using mPEGSH, and making the attachment by a simple derivatization of the macrolides is presented.	5 kDa	US 7605257 [179]	2009
Taxanes	Taxanes are used to produce various chemotherapy drugs because they are mitotic inhibitors. One to three PEG oligomers can be attached to paclitaxel or docetaxel. The use of a salt forming PEG, like ammonium or carboxylate, is preferred	PEG oligomers having from 1 to 25 PEG units	US 6380405 [180]	2002
Taxanes	Conjugates of PEG to paclitaxel, docetaxel, and more potent taxanes are synthesized with the aim of conferring enhanced aqueous solubility and diminishing their cytotoxicity. Taxanes linked to a cell binding agent through a PEG-containing linking group, and its pharmaceutical preparation, are presented.	Not specified	US 6596757 [181]	2003

(Table 4) contd….

Species	Comments	MWs of PEG	Patent [Reference]	Year
Vancomycin	It is a glycopeptide antibiotic used in the treatment of infections caused by Gram-positive bacteria. Conjugates of vancomycin and polymers that are hydrolytically resistant *in vitro* are synthesized. The invention is exemplified using different PEG derivatives selectively attached to the sugar amino and /or N-methyl amino groups of vancomycin. Another approach is presented by the same group of inventors in US 7,273,845.	5-100 kDa (20-40 kDa)	US 7462687 [182]	2008
Various drugs	Polymeric-based prodrugs having reversible linkages involving aromatic moieties and biologically-active materials are presented. A PEG derivative is synthesized, and the attachment using camptothecin and paclitaxel derivatives is exemplified. Gemcitabine, triazole-based antifungal agents, such as fluconazole and other drugs, can be used in this approach.	At least 20 kDa	US 6936597 [171]	2005

ADVANCES IN PEGYLATED MATERIALS AND THEIR APPLICATIONS

Novel systems with special nanoscale functions are considered one of the key technologies for future progress in biochemistry, biotechnology and medicine. Specially, new nanotechnology-based delivery systems offer promise in drug delivery. In this regard, PEGylation is a valuable tool that allows advance in solving some intricate problems associated with the drug delivery where targeted delivery, formulation, *in vivo* stability, enhanced efficacy and safety are the major challenges. Advances in this field can directly be noted by the diversity of PEGylated materials and systems.

PEG coated micro and nano-particulate systems are suitable applicants for preparing long-circulating carriers since they have modified physicochemical properties that might provide improved clinical effectiveness of the encapsulated drug for oral, parenteral or ophthalmic administration [183-185]. PEGylation of such systems can be made by covalent attachment of PEG derivatives or by simple adsorption [186]. However, the drawback of simple adsorption is the quick loss of the coating due to the instability of the interaction. Thus, covalent binding is preferable. PEGylation of conventional nanoparticles for oral administration allows protecting them against enzymatic attack in digestive fluids and minimizing their interaction with mucin and other proteins present in the lumen. For ocular use, poly(alkyl-cyanoacrylate) (PACA) nanospheres coated with PEG have shown increased ocular absorption due to a greater interaction with the corneal epithelium [187]. Nanoparticles coated with PEG administered intravenously have demonstrated prolonged biological half-life due to PEG chains on the nanoparticle surface provide steric stabilization and reduced interaction with blood proteins [188]. There are thousands of interesting examples referring nanoparticles that are long-circulating and can label-specific locations or biomarkers with high selectivity. Some examples of interesting applications are given below.

PEG-PVM/MA nanoparticles demonstrated very high affinity to the intestinal mucosa rather than to the stomach wall [189]. PEG-PHDCA and PEG-PLGA nanoparticles seem to be promising as drug carriers across the blood-brain barrier [190, 191]. PEG-PHM-PEG nanoparticles did not possess any cytotoxic activity against K-562 cells and were able to escape from phagocytosis depending on the surface PEGylation degree [192]. PEG-PLGA nanoparticles encapsulating doxorubicin maximized the efficacy of doxorubicin and minimized dose-limiting cardiotoxicity [193]. Lactoferrin-PEG-PLA nanoparticles are promising as brain drug delivery system with low toxicity [194]. Heterobifunctional PEG has been used to prepare several varieties of nanoparticles conjugated to peptides, folic acid, or antibodies [195-198]. Di- and polythiol PEGs have been used to functionalize gold nanoparticles, improving monolayer stability [199,200]. PEGylated core-shell PMMA nanospheres have been found safe and represent promising vectors for oligonucleotide delivery [201]. PEGylated gold nanoparticles conjugated to monoclonal F19 antibodies as targeted labeling agents for human pancreatic carcinoma tissue have been developed [202]. In the biomedical imaging field, PEGylated ultrasmall rare earth based nanoparticles have been used as a contrast agent in magnetic resonance imaging for investigation of molecular and cellular events [203]. PEGylated core-shell microparticles have been synthesized by a one-pot procedure [204]. Adenovirus (Ad) has been PEGylated and then microencapsulated within PLGA microspheres showing improvements in gene transfection efficiency and stability [205]. Moreover, rosin derivatives have been PEGylated and then studied as microencapsulating materials for sustained drug delivery [206]. Vezzù and coworkers have functionalized bioactive molecules (like proteins) with PEG, and then studied lipid/PEG particles incorporating this active principle to obtain solid micro- and nanoparticles [207].

Polymeric micelles self-assembled from amphiphilic block copolymers have emerged as one of the most promising nanocarrier systems for drug and gene targeting, mainly for clinical administration of anticancer drugs. Novel approaches for the preparation of functionalized PEG layers as hydrophilic outer shell were proposed to attain receptor-mediated drug and gene delivery through PEG-conjugated ligands with a minimal non-specific interaction with other proteins. Furthermore, the utility of multimolecular assembly

of heterobifunctional PEGs and block copolymers were studied to systematically modify the properties of metal and semiconductor nanostructures by controlling their structure and surface properties, making them attractive for use in biological and biomedical applications, and, more importantly, for biorecognition [195, 208]. Cationic albumin-conjugated PEGylated nanoparticles allow gene delivery into brain tumors [209]. PEGylated liposomal doxorubicin in the treatment of AIDS-related Kaposi's sarcoma has shown greater tumor localization and consequent improved efficacy of doxorubicin, as well as reduced toxicity [210]. PR_b-targeted PEGylated liposomes have the potential to deliver a therapeutic payload to prostate cancer cells in an efficient and specific manner [211]. Cationic lipid-nucleic acid nanoparticles were made target-specific by insertion of an antibody-lipopolymer (anti-HER2 scFv (F5)-PEG-DSPE) conjugate into the particles, which are delivery vehicles capable of achieving a high degree of specific transfection activity [212]. Microscopic lipospheres for ceftriaxone sodium have been PEGylated improving stability in oral formulation [213]. Doxil® is a representative commercialized PEGylated liposomal formulation of doxorubicin [214]. In this formulation, a PEGylated liposome, *i.e.* STEALTH® liposome, enhances therapeutic effect and decreases serious side effects, such as cardiotoxicity of doxorubicin, by improving its biodistribution [215].

PEGylated dendrimers, which are characterized by a well-defined size, shape and controlled exterior functionality, are a newer class of drug carriers that are currently being evaluated for many *in vivo* biological applications, including drug and gene delivery, solubilization, tissue growth scaffolding, imaging (magnetic resonance, near-infrared, positron emission), and boron-neutron capture therapy [216-220]. Polyamidoamine (PAMAM) dendrimer, which is a typical dendrimer, has attracted great attention in terms of the above mentioned biomedical applications, but it has been reported to be cytotoxic [221]. Lysine dendrimers showed no significant cytotoxicity in cultured cells compared with PAMAM dendrimer [222]. A carboxylic acid functionalized dendrimer was elaborated to carry doxorubicin bound via a hydrazone bond. This drug-loaded carrier showed more accumulation in tumors and less in healthy organs than the clinically used PEGylated liposomal formulation Doxil [223]. 125I-labeled PEGylated dendrimers were found to be effective for single photon emission computed tomography (SPECT) imaging of tumor angiogenesis [224]. PEGylated dendrimers loaded with gold nanoparticles have been prepared and resulted novel theragnostic agents for photothermal therapy as well as CT imaging [225].

Miscellaneous applications of PEGylation are mentioned below. Surface PEGylated silk fibroin films could be useful antiadhesion and antithrombotic materials for biomedical applications [226,227]. Surface PEGylation is among the strategies to prevent molecular and cellular biofouling which is essential to the success of medical implants, biochips, sensors, and many other applications [228,229]. However, the immobilization of PEG to surfaces has remained largely unexplored. PEG derivatives for PEGylation of silica and gold surfaces with high level of specificity are commercially available (SunBio, Creative PEGWorks). A facile strategy of PEGylation on titanium oxide surfaces using NH_2-Cys-PEGs has been recently reported [230]. Chemical conjugation of Ad with PEG is one of the most promising strategies to overcome the problems arising from the systemic administration of Ad vectors, such as virus accumulation and undesirable transgene expression in the liver, with subsequent inefficient systemic cancer-targeted therapy and pronounced hepatotoxicity [231,232]. Some similar examples have been presented as patents and are shown in Table **5**.

CURRENT & FUTURE DEVELOPMENTS

Protein PEGylation is now a clinically proven strategy for accomplishing the primary goal of increasing protein half-life in vascular circulation while maintaining therapeutic activity. It therefore seems reasonable to speculate that PEGylation should become an early integral part of building up a successful protein-based medicine. On the other hand, even though there are many challenging scientific and policy questions about follow-on protein products, the increasing interest in the development of novel versions of approved PEGylated proteins cannot be ignored. In fact, the sales of PEGylated proteins are currently concentrated in few branded products, which may be attractive targets for innovative development since leading conjugates are losing patent protection over the next few years.

Antibody PEGylation and a number of techniques for producing humanized and human monoclonal antibodies, show promise toward the goal of avoiding obstacles encountered in early attempts to make use of these biomolecules as therapeutic agents. Most of the antibody drugs on the market and in clinical development are humanized antibodies and among about 30 FDA approved antibody therapeutics there is only one PEG-antibody conjugate. PEGylation is now an attractive tool to facilitate the optimization of humanized antibodies, and this combination is expected to drive new innovations in the field of antibodies for the treatment of cancer, immune-related and infectious diseases.

ODN PEGylation offers a viable approach for researchers looking for stabilizing ODNs against degradation and increasing cell permeability. Specially, rPEGylation employing customized linkers for reversible attachment appears to be a better option than PEGylation with permanent linkages. rPEGylation of ODNs is also most attractive when compared to other delivery strategies, such as nano carriers formulated through the self-assembly of PEG-block-polycation/ODN or PEG-block-ODN/polycation. Due to the great potential of ODNs as therapeutic agents in several human diseases, such as viral diseases, malignancies and dominant hereditary diseases, and the mandatory needs for modifying their structures to improve stability and potency, an increasing research activity in this exciting field would be expected.

Small molecule drug PEGylation is still a clinical challenge and so far no successful commercial conjugate exists as a "good new" result for renewing the level of expectation initially placed on PEG as polymeric carrier for preparing pro-drugs. The challenge for future work will be to address a number of crucial issues, such as solving the limitation of low drug loading capacity and overcoming the disadvantages related to cost-expensive not-easy scalable synthesis of multi-arm PEG structures when compared with the commercially available poly-functional polymers like PGA and HPMA.

Table 5. Some Patents Covering the Technology of PEGylation of Materials or Other Molecules

Material	Comments	Conjugation Method and MW of PEGs	Patent	Year
Ceramide lipids	Ceramide lipids are modified with PEG molecules, being less susceptible to hydrolysis. When these lipids are used to form liposomes, the resulting liposomes show an increased longevity in circulation, allowing their use in drug delivery.	Random 2-5 kDa	US 5820873 [233]	1998
Drugs containing an amino group	An approach of combining the technology of derivatization of drugs with Fmoc or FMS (2-sulfo-9-fluorenyl-methoxycarbonyl) with PEGylation, though modifying PEG with Fmoc or FMS and then conjugating its moiety to drugs or proteins containing amino groups, is presented. Several examples are shown using different biologically active molecules.	Site specific 5-40 kDa	US 20100041867 [234]	2010
Fibrinogen based biomatrix	This interesting invention presents the use of fibrin-based biomatrix to deliver stem cells or progenitor cells to damaged heart tissue. The fibrin biomatrix may be prepared using a PEGylated fibrinogen solution.	Random, not specified	US 20090142305 [235]	2009
Fullerenes	Fullerenes may be used as solvent-free or solid electrolytes in Li+ batteries. The invention supplies a PEGylated fullerene being applicable to the mentioned field.	Random, not specified	US 20070202413 [236]	2007
Hyaluronic acid	A composition comprising a PEGylated hyaluronic acid (HA) and a PEGylated non-HA polymer is presented. The composition may be used to form coatings on implantable medical devices or to form the implantable medical devices themselves, and also may include a bioactive agent.	Random, 7-320 kDa	US 7713637 [237]	2009
Hydrogel biochip	A method to produce with low cost and efficiently a sensitive hydrogel biochip is claimed. The process uses a star-like PEG derivative having an epoxy terminal group, a hydrophilic polymeric cross-linking agent and a probe, in aqueous solution.	Random, 60-100 kDa	US 7695910 [238]	2010
Hydrogels	An approach to prepare PEG hydrogels by reaction of active derivatives of PEG with biologically active molecules and the crosslink of these units is described. The weak linkages suffer hydrolytic cleavage and release the bioactive substance.	Random, 0,3-200 kDa	US 6258351 [239]	2001
Immuno-conjugates	The approach consists in modifying with PEG a linker molecule that attaches the toxin moiety to the targeting moiety of an immunotoxin. A mutant of an immunotoxin, in which one or more cysteines are introduced in a peptide connector that attaches the antibody to the toxin, is prepared and then PEG-maleimide is attached to this cysteine. The construct presents improved characteristics: stability, plasma half-life, antitumor activity, immunogenicity and non-specific toxicity.	Site specific, 1-100 kDa (5 and 20 kDa)	US 20040018203 [240]	2004
Ion channel modulating compounds	A variety of cardiac pathological conditions may be treated and/or prevented by the use of ion channel modulating compounds. PEG moieties are attached to these compounds and a pharmaceutical composition that can be useful in the treatment of arrhythmia is exemplified.	Random Not specified	US 20080021005 [241]	2008
Lipids	Various PEG-lipids conjugates and cationic-based lipid encapsulation systems that can comprise a therapeutic agent are presented. The systems can be used to deliver different therapeutic agents (like DNa, RNA, rRNA, antigens etc).	Random, 0,5-10 kDa	US 20100099738 [242]	2010
Lipid (nanoparticules)	PEG chains are attached to a lipid and adsorbed to an insoluble organic drug, forming nanoparticules. The conjugate PEG-lipid can be PEG-phospholipid, PEG-cholesterol and PEG-vitamin A or E, among others. The conjugates shows improved characteristics (prolonged blood residence times, dose-independent pharmacokinetic, ability to cross biological barriers, etc.)	Random 0,75-5 kDa	US 6270806 [243]	2001

(Table 5) contd…

Material	Comments	Conjugation Method and MW of PEGs	Patent	Year
Lipids (in gene delivery systems)	PEG is conjugated to stable nucleic-acid–lipid particle (SNALP), stabilized plasmid-lipid particles (SPLP), or liposomes, being stabilized in aqueous media. By controlling the length of the alkyl or acyl chains of the lipid portion of the PEG-lipid conjugate, one can preferentially target the liposomal, SNALP or SPLP drug delivery system to a tumor or other target tissue of interest.	PEG-lipids 0,55-10 kDa	WO 2006007712 [244]	2006
Liposome-entrapped gemcitabine	A composition for better drug delivery consisting of a liposome-entrapped gemcitabine is presented. The system involves negatively charged phospholipids linked to polyethylene glycol derivatives.	Not specified	US 20050249795 [245]	2005
Liposomes of cardiolipin analogues	PEGylated cardiolipin analogues and variants that can be used in the preparation of liposomes containing therapeutic agents used in drug delivery for the treatment of mammalian diseases are presented. Stability of circulating liposomes is improved.	Site specific, 0,2-50 kDa	US 20080286351 [246]	2008
Nanoparticles	Nanoparticles are used as drug administration systems. PEGylated nanoparticles present stability in oral administration, have good bioadhesive characteristics for interacting with mucosae, of carrying a wide range of active molecules, release the active molecule in a controlled manner and prevent its elimination from the blood system. The nanoparticles comprise a biodegradable polymer (preferably the vinyl methyl ether and maleic anhydride (PVM/MA) copolymer) and are PEGylated with a PEG derivative.	Random 0,4-35 kDa	US 20080248125 [247]	2008
Phospholipids in liposomes	PEGylated liposomes containing a hydrophobic photosensitizer within the lipid bilayer membrane are presented for use in photodynamic therapy. The formulation shows improved pharmacokinetic properties since PEGylated liposomes help to maintain the drug level within the therapeutic window and longer circulating half-life *in vivo*.	Not specified	US 20060127471 [248]	2006
Red blood cells	There is a great interest in the production of a system that may mask cell antigens, such as red cell antigens, to generate red cells compatible with all recipients. This aim has been achieved by PEGylation, allowing the production of cells that may serve as universal donor for transfusion. A thiolated amino group on a membrane protein of red blood cell is conjugated to PEG. The masking of A, B and Rh antigens is achieved by the claimed methodology.	Site specific 5-20 kDa	US 7521174 [249]	2009
Red blood cells	A second generation technique, in which the surface of red blood cells is coated with multiple layers of PEG-albumin copolymers, is presented. The improved procedure generates less damage of red cells and is more effective in blocking the antigens.	4 arm PEG albumin copolymer	US 6129912 [250]	2000
Silk fibroin matrices	The surface of silk fibroin matrices with PEG is modified to altere protein adsorption and cell adhesion and/or proliferation on the surface of the matrix. The control of the degree of PEGylation on surface of silk fibroin matrix regulates both the degradation rate of the matrix and the differentiated adhesion of cells or adsorption of proteins on the surface of the matrix.	Random 5 kDa	WO 2010057142 [226]	2010
Styrenic block copolymer matrix	PEGylated styrenic block copolymer matrix is presented as a system for treating heart disease and other vascular conditions. The aim is to provide methods of manufacturing drug-polymer coated stents, and to overcome the deficiencies and limitations in this type of devices.	Not mentioned	US 6918929 [251]	2005

Micelles, liposomes, and nanoparticles capable of site-specific and intracellular delivery combined with optimal RNA-design are needed to maximize the therapeutic efficacy and to reduce dosage and non-specific effects. Several studies suggest that these systems as drug carriers could make a significant impact in treating various malignancies. PEGylation is an attractive alternative in the search of minimizing cytotoxicity and immunogenicity. Exciting fields of applications are delivery and imaging in the treatment of cancer, and also the use as drug carriers across the blood-brain barrier. The challenge for future work will be to address a number of crucial issues such as release response, cell surface interactions, biodegradability, environmental nanotoxicity, safety, scale-up, and discovering attractive combinations of novel drugs.

PEGylation is no doubt a diverse and hot field, which is embedded within emerging technologies to the development of bio-better and novel therapeutics, where positive information demonstrating an appropriate safety profile in preclinical testing is not necessarily indicative of clinical efficacy and does not ensure that later stage or larger scale clinical trials will be successful. PEGylation takes place at the interface of chemistry and biomedical sciences. Thus, it is synonym of cross-disciplinarity, a key success factor not new but scarce in a scenario where finding a common language is sometimes difficult. The combination of these and other factors, such as the ever growing demand and increasing complexity emerging from the need for more effective therapeutic targets, will indubitably require a far greater investment of capital and risk management, as well as more and more talent and creativity. Scientific and clinical communities and partner (pharma and biotech) companies increasingly need to collaborate to share risks on early-stage projects due to the high-risk profile that is perceived at present.

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