PEGylation - The Magic Wand ○ IRIS Biotech GmbH

Turning Proteins and other Biopharmaceuticals into Super Performing Block Busters.

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Introduction

The market of modern biopharmaceuticals in 2006 has reached a volume of over $45 billion and is projected to grow to over $90 billion per year within the next 5 years. The big advantages of proteins, antibodies, siRNA, and other natural products in their usage as drugs is their high specifity in combination with their low side effects. They normally interact with the dedicated target only, and thus do not have activities at any other place in the body. Ideal drugs in theory. A significant drawback, however, is their low stability under physiological conditions. Due to the fact that they are similar to biological components, they are also easily attacked by the immune system of the body, i.e. by antibodies and proteolytic degradation enzymes. Many efforts have been made by high sophisticated formulation techniques, special application methods (depots) and chemical modification to improve their pharmacokinetic properties.[1,2]

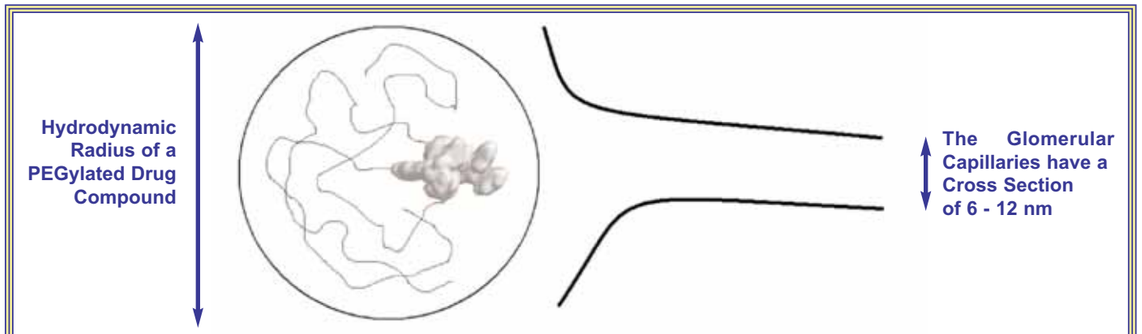


Fig. 1: Attaching polyethylene glycol chains to a biopharmaceutical increases the hydrodynamic radius to an extent larger than the renal glomerular capillary, which is normally between 6 and 12nm. The renal clearance is therefore drastically reduced.

One recent approach, which shows much better results than other methods tried in the past, is **PEG**ylation, i.e. attaching **P**oly**E**thylene **G**lycol chains (**PEG**) to the active component. The PEG shields the compound against attacks by the immune system, in particular by antibodies and degradation enzymes. The half life in the body is significantly improved.

One important mechanism to remove foreign substrates out of the body is the clearance through the kidneys. Attaching PEG compounds to proteins increases the hydrodynamic radius of the whole component to an order of magnitude, which is larger than the normal cross section of the glomerular capillaries.[3] The renal clearance is therefore reduced and the compound stays longer in the body.

Since the PEGylated drug stays much longer in the body, lower doses of application are required, which leads to a much higher acceptance, less toxic side reactions and an over all much more convenient pharmacokinetic behaviour. **Figure 2** illustrates how kinetic of a bioactive compound is changing. Small drug molecules or proteins suffer a rapid clearance, the concentration of the drug compound drops rapidly as it is removed from the body. Application has to be repeated, in order to keep the concentration over a certain threshold. Otherwise immunogenic reactions and forming of resistant derivatives start. PEGylated drugs show suppressed renal clearance and reduced immunogenic reaction, so the concentration is being reduced slowly over the time of treatment. In the ideal case only a single application is required over the time of treatment.

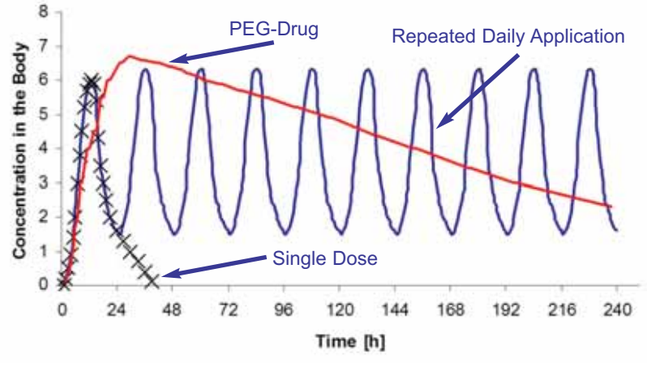


Figure 2: A single application leads to a rapid increase of the concentration followed by a rapid decrease caused by immunogenic reactions, proteolytic degradation and renal clearance. In order to keep the concentration above a certain threshold repeated applications are required. A PEGylated drug requires much less applications, because degradation and clearance are drastically reduced.

The clinical relevance of PEGylated drugs in the meantime is well established. PEG-adenosine deaminase (ADAGEN from Enzon) was the first PEG-protein conjugate to enter the market in 1990.[4] It is used to treat X-linked severe combined immunogenicity syndrome, as an alternative to bone marrow transplantation and enzyme replacement by gene therapy. Since then more and more PEG-Protein and PEG-Peptide conjugates have entered the market as pharmaceuticals **(Table 1)**.

Conjugate	Trade Name	Indication	Year to Market
PEG-adenosin deaminase	**Adagen®**		**1990**
PEG-L-asparaginase	Oncaspar®	Acute lymphoblastic leukaemia	1994
PEG-interferon α2b	**PEG-INTRON®**	**Hepatitis C (cancer, MS, HIV)**	**2000**
PEG-interferon α2a	PEGASYS®	Hepatitis C	2002
PEG-growth hormone receptor antagonist	**Pegvisomant®**	**Acromegaly**	**2002**
PEG-G-CSF	PEG-filgrastim®, Neulasta®	Treatment of neutropaenia during chemotherapy	2002
PEG-anti-VEGF aptamer	**Pegaptanib®, Macugen®**	**Macular degeneration**	**2004**
PEG-anti TNF Fab	CD870®	Rheumatoid arthritis	2005
PEG-aspargase	**Oncaspar®**	**Acute lymphoblastic leukaemia**	**2006**

Table 1: PEG conjugates in the pharmaceutical market.[1,5]

The increasing number of products in the market is encouraging due to the fact that it turns an active biopharmaceutical into a robust and clean drug with low side effects and ideal pharmacokinetic behaviour. However, it is not as simple as it seems to be, as there are also certainly several limitations which have to be considered.

Advantages and Limitations of PEGylation

Conjugation with a PEG shields the biopharmaceutical against attacks by the immune system. The much larger hydrodynamic radius prevents renal clearance. This leads to the following advantages and improvements of an active drug molecule, if conjugated with a PEG chain:

- Increased protein solubility

Polyethylene glycol solvates good in both polar AND non-polar solvents. Therefore the solubility of a PEG conjugate is improved in blood and in general in many different solvents. Higher concentrations can be achieved in formulation solutions for applications, as well as in physiological liquids.

- Enhanced proteolytic resistance and stability

The PEG “shield” prevents uptake by cells of the retinal endothelial system (macrophage system), degradation of proteolytic enzymes and attacks by antibodies. A more favourable biodistribution of drugs can be achieved, as the compound stays longer in the organism and the uptake by a tumor or any other target tissue has a higher probability to occur.

- Prevention of renal clearance

Whenever the increased hydrodynamic radius is increased to a larger value than the cross section of the globular capillaries, the renal clearance is strongly reduced, which leads in combination with the improved stability to a prolonged pharmacokinetic half-life.

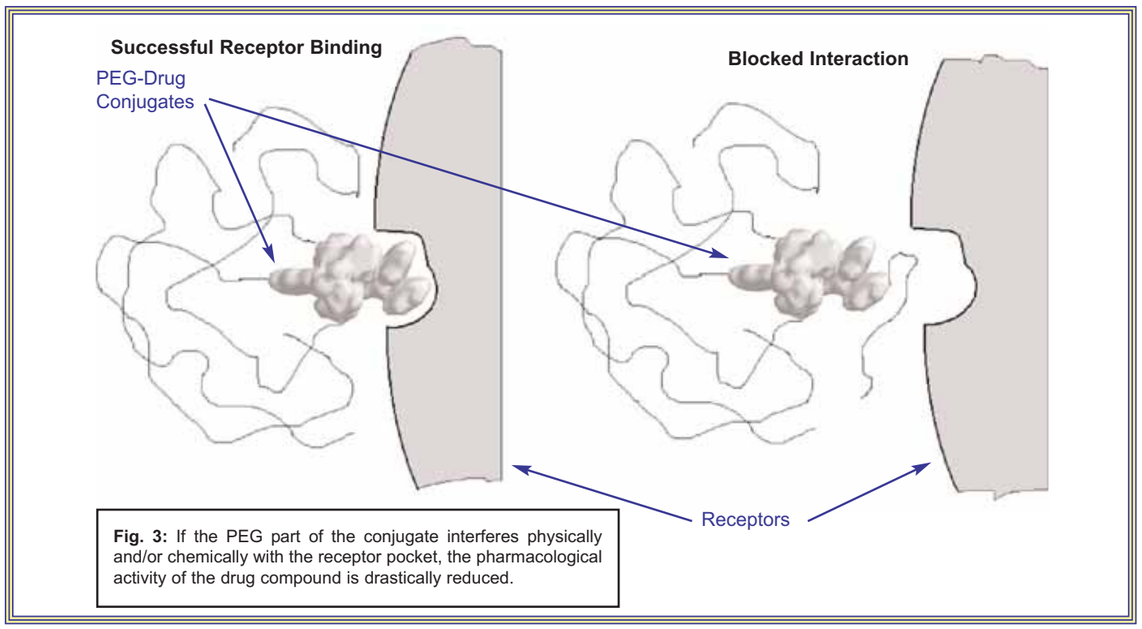


Fig. 3: If the PEG part of the conjugate interferes physically and/or chemically with the receptor pocket, the pharmacological activity of the drug compound is drastically reduced.

- Strong reduction of immunogenicity and improved drug properties

The PEG shield has characteristics rather like a solvent than a protein. Therefore recognition by the immune system is significantly reduced. The drug shows a better bioavailability, lower doses can be applied with lower administration frequency, which leads to an increased drug therapeutic index (benefit vs. toxicity). This is linked with lower toxicity, lower risks for an allergic response and side effects in general. In other words, the overall drug properties are simply improved for the welfare of the patient.

Besides all the convincing advantages, which are very reasonable in theory and also proved by an increasing number of drugs in the market, there is also an important drawback. The PEG shields the compound's backside, however, it should not “shield” the active side of the compound and then reduce or even block the drug receptor binding. It is obvious that the activity is then drastically reduced, if the PEG part interferes physically and/or chemically with the receptor. PEGylation of a protein done far away from the active part modifies it, nevertheless, in a certain manner. It is not surprising that PEGylation is often accompanied with a significant reduction or even loss of bioactivity. This is in many cases compensated by the prolonged bioavailability in the body. A typical example is PEG-interferon (Pegasys®), which retains only 7% of the antiviral activity of the native protein. Nevertheless, the in vivo performance is drastically improved compared with the unmodified enzyme due to the positive effects caused by the PEGylation.[6] Cytotoxicity, haematoxicity, carcinogenicity, tertatogenicity, as well as cellular and humoral immunogenicity of PEG derivatives, other hydrophilic substrates and hydrophobic polymers have been examined so far. PEI (polyethyleneimines) and other polycations show partially cytoxicity, while polyethylene glycol as conjugate fulfils all basic conditions of a drug component. However, one problem always related with polymers, is the excretion from the body. As renal clearance is reduced, compounds with higher molecular weight will accumulate in the liver, leading to macromolecular syndrome. Other factors certainly also play a role. Therefore, PEGylation has to be adapted to size, polarity, and pharmaceutical property of each lead compound.

Advanced Applications of PEGs in Drug Carriers

The simplest possibility of PEGylation is attaching a monofunctional PEG chain to a protein, antibody or small drug molecule. Using bifunctional PEGs with the same or different chemical functional groups enables the capabilities to bridge and link two compounds forming dimers or more complex conjugates. Many highly sophisticated compositions are under development and already published.

A current focus is the study of modern drug carrier systems where polyethylene glycol linkers are connecting a recognition part with a drug-active part. Such conjugations can reach the size of a nanoparticle. The recognition part can be a peptide or hormone, which binds specifically to the surface of a certain cell. After internalization of the whole nanoparticle the active part (DNA or siRNA, for example) is released. Inhibition or activation of certain enzymes or the nucleus follows with the consequence to repair the sick cell or shut it down by initiating apoptosis.[7,8] In conjugation with hydrophobic compounds forming amphiphilc and biodegradable block-copolymers like PEG-PLA (polylactic acid) and PEG-PLGA (co-polylactic acid-glycolic acid) micelles are formed, where drug molecules can be masked and protected against attacks of the immune system.[9]

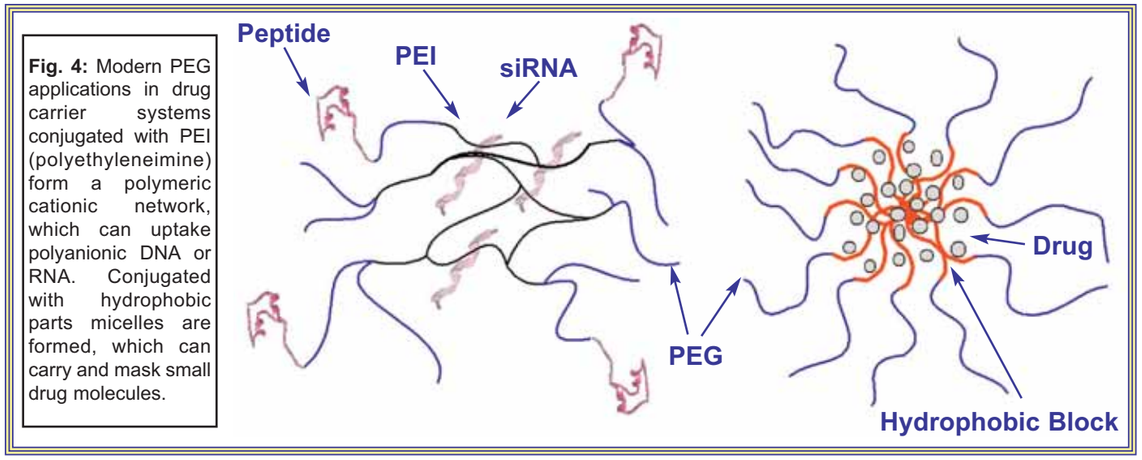


Fig. 4: Modern PEG applications in drug carrier systems conjugated with PEI (polyethyleneimine) form a polymeric cationic network, which can uptake polyanionic DNA or RNA. Conjugated with hydrophobic parts micelles are formed, which can carry and mask small drug molecules.

Physical and chemical properties of polyethylene glycols.[10]

Basic structure and polydispersity

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

One characteristic parameter is the chain length n, which determines the molecular weight M of the compound. Depending, whether the polymer is consisting of one single molecular weight (only one n existing) or of a range of compounds with an average mass and a distribution of n around a mean value, PEG polymers are referred as monodisperse or polydisperse. If n has a value of approximately 20, the molecular weight is then approximately 1000 Da. Typically “long” PEGs have n higher than 45 and a molecular weight of over 20 kDa. Some applications are reported with PEGs of 40kDa.

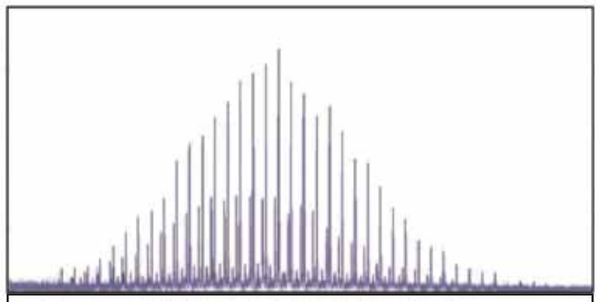
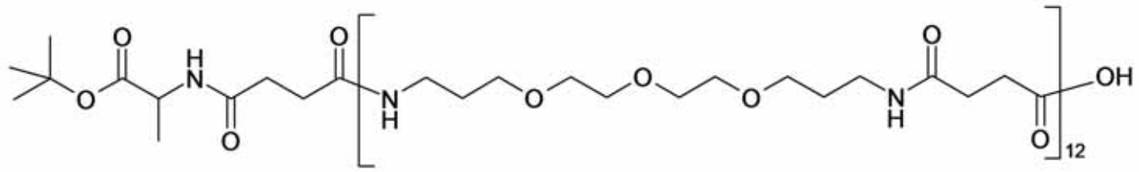


Fig. 5: Mass spectrum of a polyethylene glycol showing the typical signals with a difference of 44 m/z

If the polymer is polydisperse it shows a mass spectrum as shown in **Figure 5**. In order to quantify the distribution of the molecular weight the *Polydispersity* D is defined as the ratio between the *weight average* molecular weight M_w° and the *number average* molecular weight M_n°. As this leads many times to confusion smart explanations are given in literature,[11] in order to make this ratio understandable.

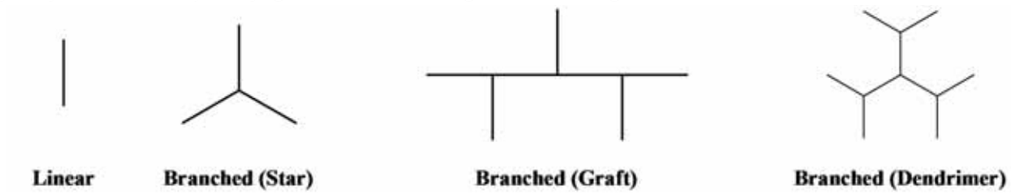
$$D = \frac{M_w^\circ}{M_n^\circ} \geq 1 \qquad \text{with} \quad M_w^\circ = \frac{\Sigma\, N_x M_x^2}{\Sigma\, N_x M_x} \qquad \text{and} \quad M_n^\circ = \frac{\Sigma\, N_x M_x}{\Sigma\, N_x}$$

Let's take an elephant and 4 mosquitoes flying around his tail as an example for a system composed of 5 units with different masses. The weight average molecular weight will be very close to the weight of the elephant, while the number average molecular weight will be closer to the weight of the mosquitoes. The weight average molecular weight does not “count” species just by their number, but takes into account the total weight of each species and is therefore a much more realistic indicator of the gross mechanical property. For a homogeneous sample, where the polymer chains have all the same length, M_w° is equal to M_n°, the polydispersity D is then equal to 1 and the sample is referred to be *monodisperse*. Whenever there is a distribution of molecular weights, the weight average M_w° is always greater than the number average M_n° and the polydispersity is greater than 1. The compound is then referred to be *polydisperse*. The polydispersity D of PEGs typically used in PEGylation is between 1.05 and 1.3. However, whenever a PEGylated new drug compound needs to be approved by the FDA and other authorities, it is easier and faster, if this compound shows only one signal in the mass spectrum and not a distribution pattern. Therefore the need for large, but monodisperse compounds is increasing. The longest monodisperse PEG reagent currently existing is the following one:



α-Alanine-t-butyl ester, ω-carboxylic acid PEG-dodecamer, $C_{179}H_{331}N_{25}O_{65}$, 3873,76 g/mole

Polymeric Architecture: In principle a simple polyethylene glycol is linear. However, by substituting multifunctional compounds like Lysine [12], Glycerine and Pentaerythritol branched structures can be created, which can have a much better shielding effect than a linear structure. Umbrella like structures have been developed and even dendrimeric structures formed by PEG-PEI (polyethyleneimmine) conjugates are investigated as drug carriers.[13]



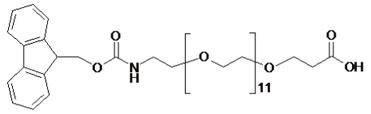
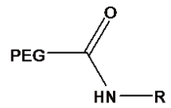
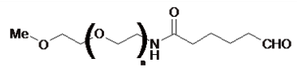
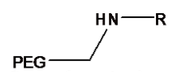
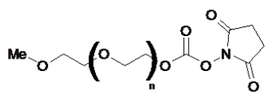
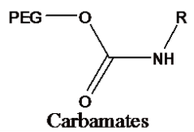
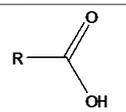
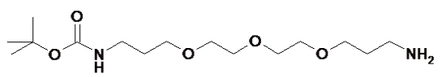
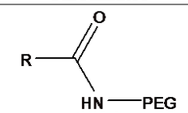
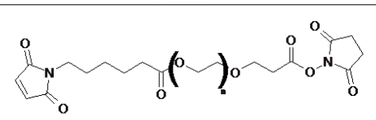
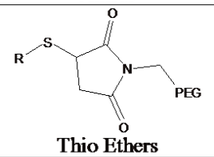
Linear **Branched (Star)** **Branched (Graft)** **Branched (Dendrimer)**

The synthesis of branched structures is more complex and therefore dedicated to specific projects, where a tailor made PEG with defined branches and individual chain lengths is developed, in order to match the unique and specific requirements of a certain lead compound.

Reactivity of the terminal groups: A linear PEG has an α-end and an ω-end. If one end is carrying a methoxy group it is unreactive at this end and cannot be used for any further chemical modification. The other end still carrying a reactive group can form a conjugate. The PEG is then called monofunctional and can be used to PEGylate a protein or to form a thin PEG layer on a gold surface. If both ends are carrying a reactive group, the PEG is called bifunctional and can be used as linker between two compounds. With a clever combination of reactive groups on both ends, involving orthogonal protecting group techniques a broad variety of applications is possible.

Attaching PEG to an amino group is widely used in protein conjugates. Lysine, ornithine or N-terminus are the anchoring possibilities. Less common is attaching to carboxylic acid side chains of aspartic acid, glutamic acid or to the C-terminus. The free thiol groups of any cysteine, which is not involved in disulfide bridges, can be used to couple with maleinimmides, vinyl-sulfones or pyridine disulfides. These conjugates form stable, covalent bounded conjugates. In case the bond to the PEG is pH sensitive and unstable towards hydrolysis, compounds will be released at certain pH (4 to 6.5) enabling for example intravesicular triggering drug liberation after internalization. This is realized by forming carbamates, which hydrolyze under certain conditions. In **table 2** the most popular methods for forming a conjugate are listed with examples of monodisperse and polydisperse PEG reagents. In recent review articles more details and variations can be found.[1,2,10]

Table 2: The most common targets for conjugation are amines, carboxylic acids and thiols, however, many more reactive groups can be used to attach PEGs to a target compound.

Target	PEG-Reagent	Conjugation
$R—NH_2$		
Amines	Carboxylic Acid, Activated Esters (NHS)	Amids
$R—NH_2$		
Amines	Aldehydes	Secondary Amines
$R—NH_2$		
Amines	Carbonates	Carbamates
		
Carboxylic Acids	Amines	Amids
$R—SH$		
Thiols	Maleinimides	Thio Ethers

Perspective

PEGylation is an efficient tool that can be used in order to improve the pharmacokinetic properties and the drug performance of biopharmaceuticals in general. It has shown promising results as drug delivery technology and seems to have a bright future, as products are stabilized, which tend to be degradated under physiological conditions rather quickly. Looking at lead compounds, which have failed in the past in toxicology or clinical studies, PEGylation might help to diminish the toxicologic property. Many more applications are expected in the future, which require tailor made PEG reagents protecting the drug compound properly against the immune system, but not against interactions with the target receptor. A renaissance of failed drugs substances is expected, as PEGylation technology can help to formulate these drugs into safe, stable and efficacious delivery systems.

Biography

Dr. Thomas Bruckdorfer studied chemistry in Erlangen, Germany and holds a PhD and an MBA. In previous positions he developed the analytical instruments business for Bio-Rad Laboratories GmbH and the business of Advanced ChemTech Europe N.V. in Central Europe in the areas of peptide synthesis, combinatorial chemistry and chemical libraries. He has been more than 15 years in the chemical and pharmaceutical business and is heading the marketing department of IRIS Biotech GmbH since 2002 .

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