Purification of PEGylated Proteins

Mina Sepahi\textsuperscript{1}, Shahin Hadadian\textsuperscript{2}, Reza Ahangari Cohan\textsuperscript{3} and Dariush Norouzian\textsuperscript{3}

1 Introduction

Proteins are biomolecules produced by living cells. They can be of medicinal or industrial interest. Thereby, they need to be appropriately modified chemically in order to increase their stability against mostly proteolysis. This can be achieved by conjugating the targeted proteins to polyethylene glycol reagents. Conjugation of therapeutic proteins through polyethylene glycol technology causes increase in half life circulation of such drug in the body. But the adverse effect could be loss of pharmacokinetic properties of the drug under investigation.

Proteins can be either randomly or selectively PEGylated. However, the PEGylated forms of protein must be separated from the unpegylated one which can be achieved by size exclusion, ion exchange and reverse phase chromatography methods depending on the strategy employed.

Polyethylene glycol (PEG) is generally regarded as a non-biodegradable polymer that reacts and attaches covalently to particular functional groups present in proteins. Non-biodegradability is not hundred percent accepted and some reports show oxidative degradation by various agents and enzymes, such as alcohol and aldehyde dehydrogenases (Jevsevar et al., 2010; Roberts et al., 2002) furthermore, PEG chains with molecular weight less than 400 Da (Dalton) are in vivo metabolized by alcohol dehydrogenases to toxic metabolites.

\textsuperscript{1} Recombinant Biopharmaceuticals Production Department, Production and Research Complex Pasteur Institute of Iran, Iran
\textsuperscript{2} Research and Development Department, Production and Research Complex, Pasteur Institute of Iran, Iran
\textsuperscript{3} Pilot Nano Biotechnology Department, Pasteur Institute of Iran, Iran
The PEG larger than 400 Da which are used for PEGylation of proteins, are not susceptible to enzymatic dehydrogenation/oxidation to yield such toxic metabolite requiring different mechanisms to clear the toxic substances (Veronese & Pasut, 2005). Such removal mechanism varies with the molecular weight of employed polyethylene glycol. The detoxification/removal of toxic metabolites is brought about through renal filtration system for PEG molecules having a molecular weight of less than 20 kDa (Veronese & Pasut, 2005). There are other cleaning pathways like liver uptake and immune system (Veronese & Pasut, 2005).

2 Types of PEG

However, different lengths, shapes and chemistries of polyethylene glycol (PEG) reagents are available in international market. The most famous brands in PEG market are NOF Corporation (Japan); Creative PEG Works (USA); Chirotech Technology Limited (UK), Dr. Peddy’s Lab (India); and JenKem (China) (Jevsevar et al., 2010).

At the beginning of using PEG as a technology, researcher used linear PEG but nowadays new practices employ both linear and branched PEGs with the molecular weight up to 40 kDa with good results and improvement in pharmacokinetic properties (Jevsevar et al., 2010; Veronese & Pasut, 2005; Gaberc-Porekar et al., 2008). New PEG formats such as forked, multi-arm and comb-shaped PEGs show promises for the future. According to PEG reactivity with proteins or non proteinicous drugs, two distinct generations of PEG appeared viz, first and second and third generations.

2.1 First-Generation of PEG

This generation was begun with the alpha or epsilon amino groups of lysine reactivity. By introducing this generation, a wide range of proteins were coupled to commercially available PEG. Due to disadvantages such as small range of molecular weights, unstable linkages between proteins and PEG, importantly non selective modification and PEG impurities shifted the route to next generation (Jevsevar et al., 2010; Roberts, Bentley & Harris, 2002; Veronese & Pasut, 2005; Ryan, Mantovani, Wang et al., 2008).

2.2 Second–Generation of PEG

This generation created to cover some problem like lack of selectivity in modification and unstable linkages. This generation is also known as site-specific PEGylation. According to PEG site, there are different modified polyethylene glycols such as PEG-propionaldehyde, PEG-acetaldehyde and the well known one is free cysteine PEGylation reagent like malemidyl-PEG (Roberts et al., 2002; Shaunak, et al., 2006; Zhang et al., 2012).

It is important to notice that PEGylation methods described here mostly belong to second generation of PEGylation. It means that researchers exactly predict where the reaction can take place. Bioinformatics is an important tool in second generation PEGylation. Here we describe some methods that cover both generations (Ahangar Co-
han et al., 2011). There are different methods to categorize the PEGs, according to length, shape, etc. Each company, brand or book uses a method to explain different derivative of PEG. Here, we explain some example of this subject.

2.2.1 Amine PEGylation

This kind of PEG reacts with amino group present on protein or peptide surface, includes an activated N-hydroxysuccinimide (NHS) form of polyethylene glycol (NHS-PEG). Nowadays, there are different forms of NHS-PEG like, PEG succinimidyl ester, PEG succinimidyl carbonate with different molecular weights which are commercially available (Roberts et al., 2002).

2.2.2 Thiol PEGylation

A thiol group is a functional group having generally a sulfur atom bonded to hydrogen atom i.e. -SH. Site-specific PEGylation at native cysteine residues is seldom carried out because these residues are usually in the form of disulfide bonds or otherwise, are required for biological activity. On the other hand, site-directed engineering needed to be used to introduce a cysteine residue at the surface of engineered protein for thiol PEGylation. The cysteine mutated protein must be accessible to PEG and maintain its biological activity after modification by PEG (Roberts et al., 2002; Shaunak et al., 2006; Ahangar Cohan et al., 2011).

2.2.3 N-terminal PEGylation

N-terminal PEGylation, means using a reductive alkylation step with a PEG-aldehyde reagent and a reducing agent (Roberts, Bentley & Harris, 2002).

2.2.4 Oxidized Carbohydrates or N-terminus PEGylation

Oxidation of carbohydrate sited at N-terminal of serine or threonine is a new method for site-directed PEGylation of proteins. Oxidation of carbohydrate by enzyme or chemical reagent creates aldehyde groups which can react with PEG-hydrazide to build up a hydrazide linkage.

2.3 Third-Generation of PEG

In this generation, the polymers are available in Y, branched or combed shapes and have received growing attention because they show enhanced pharmacokinetic properties as compared to linear PEG conjugation (Jevsevar, Kunstelj & Porekar, 2010; Roberts, Bentley & Harris, 2002; Veronese & Pasut, 2005).

They demonstrate low viscosity and unable to be accumulated in the organs/tissues (Veronese & Pasut, 2005).
3 Type Techniques Used to Purify PEGylated Proteins

Lower immunogenicity, longer half life due to lower circulation clearance rate and higher resistance to proteolytic degradation has made PEGylated proteins as widely spreading technology in biopharmaceuticals. Biopharmaceuticals bear more complex structure in comparison to chemical therapeutics and amongst these, PEGylated biopharmaceuticals are more complex due to their structural heterogenicity with aspect to various PEGylation sites, extent of PEGylation (partially or fully PEGylation) and the length of PEG chains attached to the molecule. The most important parameters to be considered in protein-polymer conjugation are: a) protein structure, spatial distances between PEGylation and protein functional sites, the number of linked polymer chains, protein molecular weight and b) polymer composition, polymer molecular weight, shape and its conjugation chemistry (Caliceti, and Veronese, 2003). By keeping the above, the differences in bioactivity of PEGylated protein have been observed as compared to non-PEGylated protein. Such alteration in biological activity could be due to the i) spatial differences between protein functional and PEGylation sites in different isomers, ii) the steric hindrance on protein caused by PEG molecule (Grace et al., 2005).

Since PEG molecules are relatively inert and neutral hydrophilic polymer, differences in molecular size of protein-PEG conjugates and their native forms (unPEGylated forms) are not relatively small. Methods used to purify PEGylated form of proteins from unPEGylated form must show good resolution (Veronese, 2009). However, one of the most challenging tasks in conjugating proteins to PEG reagents is low yield of the conjugated forms of protein. Therefore, to purify the conjugated protein from non-conjugated counterpart, it is essential to implement the techniques which can separate the two forms of proteins with high resolution. Some of the techniques which can be used to attain the goals are as follows:

3.1 Separation Based on Size Differences

PEG molecules are more hydrophilic than proteins, so increase in polymer’s length increments the stoke radius of PEGylated protein significantly and apparent molecular weight anomalously. For instance, a 5 KDa PEG conjugated to interferon α-2b (23.1 KDa, 21.2 °A) results to a PEG-protein with 73.3 KDa apparent molecular weight (absolute molecular weight 28.1 KDa) and 33.6 °A.

Stokes radius and also linear PEG molecules can cause more increase in molecular weight of conjugated protein as compared to branched type of polyethylene glycol (Grace et al., 2005). The stock or hydrodynamic radius of PEGylated protein can be calculated by Equation 1 (Veronese, 2009):

\[
R_{h, \text{PEG-prot}} = \frac{A}{6} + \frac{2}{3A} R_{h, \text{PEG}}^2 + \frac{1}{3} R_{h, \text{PEG}},
\]

\[
A = [108 R_{h, \text{prot}}^3 + 8 R_{h, \text{PEG}}^3 + 12(81 R_{h, \text{prot}}^6 + 12 R_{h, \text{prot}}^3 R_{h, \text{PEG}}^3)]^{1/3}.
\]

\(R_{h, \text{PEG-prot}}\), \(R_{h, \text{prot}}\) and \(R_{h, \text{PEG}}\) are hydrodynamic radius of PEGylated protein, protein and
PEG molecule respectively. Two latter are calculated according Equations 2 (Fee & Alstine, 2004) and 3 (Hagel, 1998).

\[
R_{h, \text{PEG}} = 0.1912 M_{r, \text{PEG}}^{0.559},
\]

\[
R_{h, \text{PEG}} \approx (0.82 \pm 0.02) M_{r, \text{prot}}^{1/3},
\]

where \( M_r \) is the molecular weight.

One should consider that true molecular weight of commercial PEGylating reagents differs from their nominal molecular weight such as 5 KDa commercial PEG is

\[
5589 \pm 56 \text{ Da (Veronese, 2009)}.
\]

It is an essential to select an appropriate PEG size and a better shielding of the proteins which lead to delayed clearance from kidney ultra filtration and decrease immune system recognition (Caliceti & Veronese, 2003; Caserman, 2009).

The reactivity of PEG molecule and subsequently PEGylation reaction yield can be decreased by increasing PEG molecular weight, PEGylation reaction step will also increase the cost of production because more consumption of PEG (more excess molar ratio of PEG) is required (Zhai, Zhao, Lei et al., 2009). PEGylation of proteins starts with pure sample but there is no reaction with 100% conversion and yield, so separation of PEGylation reaction mixture which means separation of products from reaction feeds (unPEGylated protein and PEG molecules) is the first aim of purification process. High PEGylation may decrease bioactivity and low PEGylation may have no or low half life clearance (Daly, Przybycien & Tilton, 2005). Challenges start when the target protein has different PEGylation sites and the fractionation of positional isoforms is the real adversity. PEGylation extent (the number of PEG adducts on each molecule, \( N \)), and positional isomerism (the positions of the PEG adducts on each molecule) should be exactly defined.

Size exclusion chromatography makes the advantages of differences in molecular size which sometimes is related to molecular weight in normal practice during protein separations. But in PEGylated – conjugates separation, elution volume is correlated with molecular size / apparent molecular weight and the stock radius too. Good resolution between high resolution gel filtration fractionation is usually accessible when the two fractions molecular weight differences is more than two fold (Veronese, 2009; Skoog, Holler & Crouch, 2007). For example mono and di PEGylated chicken egg lysozyme (35.5, 56.2 KDa) showed two connected peak which need to lose peak overlapping section to avoid cross contamination of two fractions, but both as mentioned above have a good resolution with unPEGylated form (14.6 KDa) (Daly, Przybycien & Tilton, 2005). Hence size exclusion chromatography (SEC) is a useful technique for separation of PEGylated forms from PEGylation mixture. Therefore heavier PEG molecule will result better fraction resolution in high performance size exclusion chromatography (HPSEC) (Zhai, Zhao, Lei et al., 2009).

For low PEGylation extent, SEC will be effective but the resolution between peaks is expected to be decreased as PEGylation extent is increased (Fee & Alstine, 2006). Considering the PEGylation of protein with a 20 KDa PEG, if the number of PEG adducts on each molecule \( N \) is more than three, mono PEGylated form could not be separated
from the others efficiently (Veronese, 2009). Also, the low flow rates and small amount of sample loading onto the column are obstacles for scaling up (Yu & Ghosh, 2010).

The PEGylated protein peaks overlap at positions where total molecular weight of PEG adducts is equal, regardless of the number of PEG chains used to reach the total molecular weight (Fee & Alstine, 2006) so this method does not work properly for site specific PEGylation isomers with the same extent of PEGylations (Grace et al., 2005; Knudson, Farkas & McGinley, 2006). Superdex 75 (Yu & Ghosh, 2010), Superdex 200 (Grace et al., 2005; Zhai, Zhao, Lei, Su et al., 2009; Yu & Ghosh, 2010) and Sephacryl S-300 (Daly, Przybyszien & Tilton, 2005) are the most high performance size exclusion chromatography media, which have been used for this purpose.

3.2 Separation Based on Electrostatic Charges Differences Using Ion Exchange Chromatography

PEGylation changes the isoelectric point (pl) of a protein because it neutralizes a charged residue such as lysine on the protein surface depending on the size of protein, number of charged residues on the protein and the shape of protein titration curve near its isoelectric point (Veronese, 2009). It is applicable to divide the PEG molecule to two groups from the aspect of protein charge affection. In the first group, PEGylation on N terminal residue does not significantly change the net charge of protein, so PEGylated protein has the same charge of un-PEGylated form. But even in this condition, ion exchange chromatography could resolve PEGylated protein from reaction mixture. Thus shielding of steric effect and weakening the interaction of PEGylated species with the resin, could be the reason (Zhai, Zhao, Lei et al., 2009; Kusterle, Jevševar, and Porekar, 2008). The extent of shielding increases with the degree of PEGylation (Yu & Ghosh, 2010), usually higher PEGylated species including higher number of PEGylation or higher molecular weight of PEG elute at lower ionic strengths (Pabst, Buckley, Rama-subramanayn et al., 2007) and somehow in flow through fraction without binding to the column(Fee & Alstine, 2006). The first mentioned group consists of PEG-aldehyde (labels only the α- amino group), PEG- teryl or tosyl (not much used because the chemistry leads to a mixture of products), PEG-dichlorotriazine or chlorotriazine (abandoned for therapeutic application because of toxicity) and PEG- epoxide which is slowly reactive and rarely used (Veronese & Pasut, 2005). The second group consists of PEG-carboxilate and PEG-carbonate derivatives which decrease the positive charge of the protein conjugate after bonding to amino group. PEG- carbonates derivatives include PEG- nitrophenyl carbonate, PEG- benzotriazolyl carbonate, PEG-2, 3, 5, thrichlorophenyl carbonate and PEG- succinimidyl carbonate yield a urethane linkage with amine and all of them are slowly reactive. The PEG- carboxilate derivatives include derivatives with one or more CH2 groups between PEG and carboxylic group which the kinetic rate of conjugation depends on the numbers and eventual ramification of CH2 group linked to the carboxyl group. PEG- succinimidyl succinate, in which the ester bond between succinate acid and PEG is easily hydrolyzed, is another example of PEG- carboxilates group. The other members are PEG- amino acid- succinimidyl ester and PEG- peptide-succinimidyl ester. Easy quantification of the number of linked PEG chains is possible
by NLE or βAla amino acid analysis in PEG- amino acid- succinimidylester family and easily localization of PEGylation site is possible by CNBr treatment of Met- Nle or Met-βAla in the PEG- peptide- succinimidyl ester family (Veronese & Pasut, 2005). Ion exchange chromatography is the most commonly used technique for Purification of PEGylated proteins using different cationic and anionic resin are cited in literatures (Grace et al., 2005; Zhai, Zhao, Lei, Su et al., 2009; Yu & Ghosh, 2010; Abzalimov, Frimpong & Kaltashov, 2012). It is a diffusion limited process and diffusivity decreases adversely by increasing of PEGylation extent (hydrodynamic radius increasing). So restricted flow rate are allowed, a range of 90 cm/hr up to 136 cm/h are reported (Yu & Ghosh, 2010). The other disadvantage is small differences of the strengths of electrostatic interactions between positional isomers which cannot be exploited effectively at the preparative scale (Veronese, 2009). Lower dynamic binding because of the lower diffusivity and access to internal pores in chromatography media is another problem of this technique (Pabst, Buckley, Ramasubramanyan et al., 2007), sometimes adsorption capacity is 10 times lower than is normal for non-PEGylated proteins. Removal of free PEG before loading the sample to the column is a very important parameter in obtaining good resolution (Fee & Alstine, 2006). PEG molecule is not a UV visible substance so does not show a peak in chromatogram and should be detected by SDS- page stained with barium iodine (Zhai, Zhao, Lei et al., 2009) and the UV absorbance of PEGylated isoforms is due to the protein part of them. Its UV absorption but could be detected by Evaporative Light Scattering Detector (ELSD) (Yu et al., 2010). It is possible to use membrane for ion exchange chromatography. In membrane chromatography, porous chromatography media are substituted by the stacks of micro porous membranes. Low back pressure and rapid separation make this method as a effective technique for separation of PEG molecules at large scales. At a operational investigation research, Only a 1 bar backpressure at a 240 cm/h flow rate was seen and by comparing flow rates of 24, 96, 144 and 240 cm/h, the best resolution was reported at the highest one which is in contrary to observed behavior in packed bed column chromatography where both resolution and peak sharpness decrease at high superficial velocities. This can be explained by the enhancement of mass transfer phenomena. In membrane chromatography the convective transport is dominant to diffusion and diffusion only takes place within the stagnant thin film layer very closed to the pores walls and the thickness of this layer reduces by increasing the velocity which causes better mass transport and resolution consequently (Yu & Ghosh, 2010).

3.3 Separation Based on Hydrophobicity Differences Using Reverse Phase Chromatography

As mention PEGylation increase the hydrophilic properties of the protein, so cause a reduction in retention time of PEGylated forms in high performance reverse phase chromatography. In this case more PEGylated species elute sooner. Also this phenomena is detectable between different PEG molecule, the more heavy one has less retention time (Zhai, Zhao, Lei et al., 2009) although there are some literature reporting reverse (Lee, Kwon, Kim et al., 2007; Lakshmi & Palaniswamy, 2013; Na & Lee, 2004; Yu et al.,
2007). Stationary phase chemistry, temperature, mobile phase gradient and pH are important factors that need to be considered and optimized for good resolution in site specific isomers with similar PEGylation extent (Knudson, Farkas & McGinley, 2006). Fee and Alstine have prepared a detailed list of different chromatography methods for different PEGylated proteins in details (Fee & Alstine, 2006). A research shows that slightly improved resolution between different PEGylated forms can be achieved by increasing temperature in reverse phase chromatography also a C4 column shows a slightly better resolution than C18 but not significant improvement (Knudson, Farkas and McGinley, 2006).

4 Conclusion

Engineering purification of conjugated proteins is often of prime importance in designing the bioseparation strategy. In this chapter, the strategy is built on physicochemical properties of the biomolecule under study. These properties include molecular size, surface charge distribution and relative hydrophobicity for size exclusion, ion exchange and hydrophobic interaction chromatography respectively. Purification of PEGylated proteins involves the separation of unconjugated protein and unreacted PEG from the conjugated proteins. Therefore, it would be an essential to consider the physicochemical properties of the target biomolecule. Furthermore, PEGylation of already purified proteins imposes purification challenges. The challenges involve the separation of PEGylated protein from other reaction products like unreacted PEG and proteins and achievement of high resolution of the PEGylated protein on the basis of their extent of PEGylation. Thus, the purification strategy for separation of PEGylated proteins, extent of PEGylation from unreacted protein and PEG are discussed which seems to be state of arts in purification of PEGylated proteins of medicinal and industrial importance.

References


