

PEG-proteins: Reaction engineering and separation issues

Conan J. Fee^{a,*}, James M. Van Alstine^b

^aDepartment of Materials and Process Engineering, University of Waikato, Private Bag 3105, Hamilton 2020, New Zealand

^bProtein Separations, GE Healthcare, Björkgatan 30, Uppsala 75184, Sweden

Received 25 January 2005; received in revised form 2 April 2005; accepted 2 April 2005

Available online 8 November 2005

Abstract

Poly(ethylene glycol)-conjugated (or PEGylated) proteins are an increasingly important class of therapeutic proteins that offer improved in vivo circulation half lives over their corresponding native forms. Their production involves covalent attachment of one or more poly(ethylene glycol) molecules to a native protein, followed by purification. Because of the extremely high costs involved in producing native therapeutic proteins it is important that subsequent PEGylation processes are as efficient as possible. In this paper, reaction engineering and purification issues for PEGylated proteins are reviewed. Paramount considerations for PEGylation reactions are specificity with respect to the conjugation site and overall yield. Batch PEGylation reaction methods are discussed, along with innovative methods using packed bed or “on-column” approaches to improve specificity and yield. Purification methods are currently dominated by ion exchange and size exclusion chromatography. Other methods in common use for protein separations, including hydrophobic interaction chromatography, affinity chromatography and membrane separations, are rarely used in PEGylated protein purification schemes. A better understanding of the effects of PEGylation on the physicochemical properties of proteins (isoelectric point, surface charge density and distribution, molecular size and relative hydrophobicity) and interactions between PEGylated proteins and surfaces is needed for the future development of optimal purification processes and media.

© 2005 Published by Elsevier Ltd.

Keywords: PEGylation; Proteins; Biochemical engineering; Separations; Reaction engineering; Chromatography

1. Introduction

Poly(ethylene glycol) or PEG is a neutral, hydrophilic polyether which exhibits little reactivity unless modified with functional groups. It has many uses and applications including those related to its use as a non-toxic, non-immunogenic lubricant or carrier in pharmaceutical formulations. The polymer can be prepared with relatively controlled average MW and modified with various functional groups. Abuchowski et al. (1977) observed that covalent attachment of PEG to proteins results in active conjugates that are non-immunogenic, non-antigenic and have greatly increased in vivo circulation half-lives. These changes

appear to be mainly due to significantly increased molecular size (hydrodynamic radii), plus surface alteration and protection (in effect masking) by the neutral, chemically inert, hydrophilic PEG polymers; whose coating effects were also noted by other researchers including Sehon et al., Merrill et al., Hoffman et al., Harris et al., Johansson et al., and Holmberg et al. (see in Harris, 1992a,b; Harris and Zalipsky, 1997; Veronese and Harris, 2002; Zalipsky, 1995). PEGylation of therapeutic proteins reduces renal clearance rates and protects from proteolytic and other degradation often resulting in enhanced medical efficacy. Other benefits of PEGylation may include improved physical and thermal stability, as well as solubility. The latter is particularly important with regard to biopharmaceutical formulation and delivery. Over the past three decades many proteins and other substances have been PEGylated for use as pharmaceuticals, reaction catalysts, drug delivery agents,

* Corresponding author. Tel.: +64 7 838 4206; fax: +64 7 838 4835.

E-mail addresses: c.fee@waikato.ac.nz (C.J. Fee), james.van.alstine@ge.com (J.M. Van Alstine).

and biopharmaceuticals (Harris, 1992b; Harris and Zalipsky, 1997). Successful protein biopharmaceuticals include PEGylated α -interferons, for use in the treatment of hepatitis C (PEGasys[®] from Hoffman-LaRoche and PEG Intron[®] from Schering-Plough/Enzon), PEGylated growth hormone receptor antagonist (PEG Somavert[®] from Pfizer), PEG-asparaginase (Oncospar[®] from Enzon), adenosine deaminase (ADAGEN[™] from Enzon), and granulocyte colony stimulating factor (Neulasta[®] from Amgen). Many other PEGylated proteins are currently under development.

Protein PEGylation has been reviewed extensively by many authors including a recent review by Veronese and Harris in a special issue of *Advanced Drug Delivery Reviews* dedicated to PEGylation (Veronese and Harris, 2002). Other notable reviews include books edited by Harris (1992b), plus Harris and Zalipsky (1997), as well as papers by Zalipsky (1995), Zalipsky and Lee (1992), Roberts et al. (2002), Kodera et al. (1998), Greenwald (2001) and Pasut et al. (2004). However apart from a brief 1998 review (Bailon and Berthold, 1998), little attention has been paid to production and separation aspects related to PEG-proteins.

This review focuses on some biochemical and biomolecular engineering aspects of protein PEGylation. Specific issues of clinical efficacy, biomedical effects, biological function, analysis and conjugation chemistry are not explored except in so far as they are relevant to engineering aspects, which mainly involve reaction engineering (physical control of reaction specificity and yield), separations and other aspects related to production. Some of the points discussed are relevant for PEGylation and processing of substances other than proteins including lower MW pharmaceuticals, or for substances modified by hydrophilic polymers other than PEG.

Processing challenges that arise in the development of PEGylated proteins, as well as other PEGylated substances, often concern controlling the position of conjugation (positional isomers or PEGamers) and the number of PEG adducts or extent of PEGylation (N). Therapeutic drugs should ideally be homogeneous products with well-defined activities, and acceptable side-effects. Variations in the position and number of PEG adducts gives rise to variations in characteristics relevant to clinical and other application related effects. These include enzymatic or other activities, circulation half-life, immunogenicity and clearance (Harris, 1992b; Harris and Zalipsky, 1997; Pasta et al., 1988; Zalipsky, 1995). Product homogeneity can be obtained by maximising reaction specificity and/or implementing effective product purification. Given the high cost of producing therapeutic proteins prior to their PEGylation, achieving high purity of PEGylated forms at the expense of yield (the usual trade-off) is not an economically wise option. Improving reaction specificity and developing effective and efficient purification processes are critical areas where the biochemical engineer can make valuable contributions. Several approaches are available. Related engineering can be organised in relation to (a) PEGylation site and chemistry engineering, (b) PEGylation

reaction engineering, and (c) purification process engineering. The first is affected by recent trends to (i) undertake controlled PEGylation at defined protein residues (sites) and (ii) use larger molecular PEG molecules in an effort to reduce the number of sites (N) which need to be modified to effect the desired results. In addition all three engineering aspects are affected by the physical properties of PEG polymers, including their neutral, hydrophilic nature and relatively large viscosity radii.

2. PEGylation site, chemistry and polymer engineering

The most common chemistry for PEGylation targets the ϵ -amino group of surface lysine residues. Lysines account for approximately 10% of amino acids in a typical protein so most proteins including antibodies have numerous surface-available lysine residues, which present both opportunities and challenges. Their availability makes conjugation straightforward but the large number of conjugation sites presents difficulty in obtaining a specific number of PEG adducts and gives rise to PEGamer mixtures. Control of PEGylation site(s) reduces random PEGylation. In one simple approach amino group modification can be controlled to some extent by reaction pH so that conjugation tends to occur at the α -amino of the N -terminal (Kinstler et al., 2002, 1996). Lysine or other amine groups are not the only protein (residue) groups open to covalent modification. For example, the sulfhydryl group of free cysteine groups or carboxyl groups may be targeted.

Specific chemistries can be used to target conjugation to a variety of sites on a protein e.g. (Zalipsky, 1995). Such an approach eliminates problems related to PEGylation altering a residue so as to negatively affect the activity of the protein or molecules being modified. If the native protein has a free cysteine residue not associated with the active site, then conjugation with a thiol-selective functionalised PEG (such as maleimide-PEG) may be an option. However, such cases, when they occur naturally, are fortuitous and by no means typical. Proteins can be engineered by site-directed mutation or otherwise chemically modified to fabricate a suitable conjugation site (e.g. see Chapman, 2002; Chapman et al., 1999; Roberts et al., 2002; Zalipsky, 1995). Such approaches have disadvantages in terms of time to market, cost of development and risk compared with developing a PEGylated form of an already-approved protein. The latter is significant given that the new mutated or chemically altered protein will require more certification to obtain regulatory approval (see Hoyle, 1991). Nevertheless approaches related to PEGylation of mutated or modified proteins may be justifiable in terms of ensuring the PEGylated product has adequate activity and function (see Chapman, 2002; Harris, 1992b; Harris and Zalipsky, 1997).

Yamamoto et al. (2003) produced a lysine-deficient tumour necrosis factor (TNF), which was PEGylated at the N -terminus to avoid PEGylation of lysine residues close

to the active site in the natural form. Remarkably, despite belief that TNF requires some lysine groups for activity, acceptable activity was obtained. Pettit et al. (1996) used site directed mutagenesis to control PEGylation and the resulting activity of interleukin protein. Yang et al. (2003) used site-directed mutagenesis to insert a free cysteine group into human Fv fragments followed by thiol coupling with maleimide-activated PEG. He et al. (1999) introduced a cysteine group into trichosanthin (the native form of which has no cysteines) for PEGylation. More exotic approaches have also been used. Sato (2002) used enzymatic catalysis with microbial transglutaminase for site-specific PEGylation of recombinant interleukin-2.

In many cases modification of a target substance by only one polymer molecule may achieve the desired pharmacological or other effects. One example is PEG-Intron which involves monoPEGylation of an interferon by a 12 kDa PEG (Wang et al., 2002). However in other cases more extensive PEGylation may be required, e.g. total addition of 20–60 kDa PEG (Fung et al., 1997; Heathcote et al., 1999; Yamaoka et al., 1994). Present availability of 20–40 kDa functionalised PEGs makes it possible to achieve this with mono- or di-PEGylation. Such low degrees of PEGylation reduces the PEG-protein fractions of varied N , and PEGamers of similar N , which must be separated from each other. Many researchers have related the effects of PEGylation to the large molecular size of PEGs (e.g. Clark et al., 1996). Recent work by the authors suggests that, as measured by (SEC) chromatography, the viscosity radius of a protein that is tetra-PEGylated with 5 kDa PEG is equivalent to that which results from mono-PEGylation with a 20 kDa PEG (Fee and Van Alstine, 2004). Another reason for reducing N is that negative effects of PEGylation on protein activity decrease with N and such factors are more dominant than the MW of the PEG coupled to the protein. In the case of antibodies and enzymes binding constants tend to directly decrease with N in a semilog manner (e.g. Chapman, 2002; Chapman et al., 1999; Clark et al., 1996; Karr et al., 1986; Veronese et al., 1992). The main argument for PEGylation involving more sites is that it allows use of smaller PEGs which may be more rapidly cleared from the body. Non-medical uses of PEG-proteins may also offer situations where multiple PEGylation sites are attractive.

Nevertheless a heterogeneous product mixture results from batch-wise PEGylation that includes, depending on the conditions, un-reacted native protein, un-reacted functionalised PEG, and PEGylated species with a range of PEGylation sites and varying extents of conjugation. Further, functionalised PEGs such as succinimidyl-propionic-acid-PEG (PEG-SPA) are readily hydrolysed in water, yielding N -hydroxysuccinimide and un-reactive PEG residues, adding to the product complexity. Hydrolysis rates of amino-activated PEGs, though slower than aminolysis, are significant so molar excesses of PEG to protein as high as 50:1 may be used (for example, Brumeanu et al., 1995). Veronese et al. (1992) described other by-products of PEGy-

lation reaction such as cross-linked PEG-proteins, arising from impurities in commercial PEG reagents, which, although low level contaminants, may be significant in products intended for therapeutic use. Normally monomethoxy PEG reagents (mPEG-x) in which one terminal hydroxyl group on the PEG diol is replaced by a methyl group area used to prevent bifunctional activity such as cross-linking.

3. Reaction engineering

Reaction engineering involves controlling the conditions which influence the reaction. This often involves a trade-off between maximising the extent of the reaction and its specificity versus minimising cost. In biomolecular engineering, conditions must be gentle and the reactor and ancillary equipment must be able to be sterilised. Also, regulatory practices require traceability from end products back to source for all processes and in general it must be shown that all end products have been through identical processes. For these reasons, batch, unidirectional, single-pass processes are universally preferred, and these processes must be simple and robust to minimise variation between batches. In the case of protein PEGylation, the high cost of raw materials (particularly highly purified native protein) implies that maximising reaction extent and specificity are critical objectives.

In general only pure protein preparations are PEGylated. This means that PEGylated proteins, which require additional reaction, separation and analysis are inherently more expensive to produce. The main reasons for not PEGylating crude protein samples (e.g. crude or mildly purified fermentation samples) is to reduce validation (Hoyle, 1991) and separation challenges. These are of particular concern as regards production of PEGylated biopharmaceuticals and may be of less concern for other types of PEGylated-molecules (e.g. pesticides, catalysts, etc.). This is especially true if the PEGylation reaction can be confined to the target protein in a crude mixture, and such PEGylation helps render the desired PEG-protein sample readily separable from contaminants.

Modern trends are to carry out PEGylations using PEGs functionalised with groups which tend to readily hydrolyse. Such groups, such as NHS or SPA require specific care in regard to storage and often require use of excess molar ratio's of PEGylating agent to protein. This can be costly, especially if such reagents need to be GMP grade. Other reaction complications (and cost concerns) are related to rapid exposure of the PEG reagent to target protein, and controlled termination of the reaction typically via pH modulation. These requirements introduce the need for effective and costly process mixing and monitoring capabilities. Such reagent and process demands tend to reflect the high standards of biopharmaceutical production. If PEG-proteins and related substances are to be produced for less demanding applications it may be possible to work with nonGMP, slower reacting

reagents (e.g. PEG-aldehyde, PEG-epoxide) which have often been employed in other PEG-related coating applications (Harris, 1992b; Harris and Zalipsky, 1997).

Protein PEGylation may be carried out in a batch or fed-batch reactor or in a packed-bed reactor. A major advantage of the batch reactor is that it is simple to operate and easy to clean between batches. As such this is the main approach of choice used in the majority of papers cited in this review. Disadvantages are that the reactants, products and by-products remain in contact throughout the reaction process and must be separated after completion of the reaction. Batch reaction is particularly suitable when site-specific PEGylation chemistry is used. When a less specific chemistry is used, such as conjugation via ϵ -amino groups of lysine residues, both under- and over-PEGylated products are inevitable. In unpublished work in one of the author's laboratories (CJF), mainly mono-PEGylated protein will result from a fed-batch operation if the protein is kept in large excess and activated PEG slowly added as the limiting reagent but unless there is a partition or other way to constantly remove the PEGylated protein from the reactor, multiple PEGylation will result once appreciable amounts of PEGylated protein accumulate in the system. In other words, this method can control the extent of PEGylation but only at the cost of very low protein conversion. Targeting a specific PEGylation extent greater than $N = 1$ will invariably result in under- and over-PEGylated forms. (For experimental purposes, CJF has found that a broad range of N can be obtained from a single fed-batch reaction run by starting with a large excess of activated PEG and periodically adding native protein until the PEG reagent is exhausted.)

Packed-bed or "on-column" PEGylation has been used in attempts to influence both the site and the extent of conjugation. Solid-phase synthesis has been used for site-specific oligopeptide PEGylation (Felix, 1997). In this approach, the molecule is built up by tethering a peptide sequence to a solid phase and then adding peptides one at a time, with bi-functional PEG being attached at a specific step in the sequence. It may have some promise for smaller MW substances but its use for PEGylation of larger molecules such as most proteins appears questionable.

On-column PEGylation may be achieved by immobilising the protein to the solid phase during PEGylation, achieving at least partial separation by washing the column free of reactants and by-products in the mobile phase, and then eluting the product(s). Monkarsh et al. (1997) used this technique to PEGylate α -interferon before separating the positional isomers. They bound the native protein to an ion exchanger and then passed activated PEG through the column. Because PEGylation generally weakens (IEX) interactions, they were able to elute the PEGylated protein from the column at salt concentrations that did not elute the native form. They then replaced the equivalent amount of protein that was eluted and repeated the process. Unfortunately, this had virtually no effect on the distribution of PEGylated variants achieved in relation to batch PEGylation as the bound pro-

tein still presents multiple binding sites to the activated PEG in the mobile phase.

Others have attempted to use on-column PEGylation to orientate the protein so that the active site of the protein is held towards the solid-phase interface, thus hindering conjugation at sites that might interfere with activity. This may result in a higher activity of the PEGylated form over forms that are PEGylated at random in free solution. Lee and Lee (2004) reported that this technique gave site-specific mono-PEGylation of interferon at the N -terminus using reductive alkylation under acidic conditions with a yield of 50–60% of loaded native protein. However, the binding was on a cation exchange column, which would result in a largely random orientation of the protein on the exchanger surface and would thus be unlikely to significantly affect reaction specificity.

Kinstler et al. (2002) demonstrated site-specific PEGylation of recombinant human granulocyte colony stimulation factor (rhGCSF) at the N -terminus using reductive alkylation under mildly acidic free-solution conditions (pH 5.0). Wylie et al. (2001) showed that pH can significantly affect the PEGylation site, giving a preferential PEGylation of histidine with succinimidyl carbonyl-PEG under acidic free-solution conditions. Therefore, the on-column method of Lee et al., while achieving the advantage of reaction and isolation in a single unit operation, may not affect positional isomerism or PEGylation extent.

Baran et al. (2003) used an affinity media (Red Sepharose™ CL-4B, GE Healthcare) to protect the active site from access by the PEGylation reagent during on-column PEGylation of catalase and asparaginase. They reported higher retention of native activity for both proteins compared with literature values related to free solution PEGylation. However some of the latter studies used different chemistry, making it difficult to attribute higher retentions of activity to the solid phase technique rather than to reaction chemistry. As a result the evidence presented for protection of the active site by affinity immobilisation was only indirect.

A different approach to column PEGylation, size exclusion reaction chromatography (SERC), was used by Fee (2003), who exploited the differing linear velocities of species of differing sizes in SEC to control reaction extent. This method is analogous to membrane reactors, which, for example, may be used to control the extent of protein hydrolysis by removing products via a semi-permeable membrane once their molecular weights fall below a certain level. In SERC, the objective is to remove products whose size is larger than the reactants (as in PEGylation). Single pulses of the protein and the activated PEG are injected into the column sequentially, lowest molecular size first. The larger reactant has a higher linear velocity so catches up to the smaller one such that a moving reaction zone is formed as they migrate through the column. As the PEGylated protein formed is larger than either reactant, it moves ahead of the reaction zone. With a sufficient length of column, both reaction and separation of all species can be achieved in a

single unit operation. The approach is particularly useful with regard to PEGylation as many native proteins of therapeutic interest are less than 20 kDa, and smaller in colloidal size than even a 5 kDa PEG molecule.

4. Purification engineering

4.1. General considerations

Normally, in designing a bioseparation strategy, one seeks to exploit differences in physicochemical properties. These properties routinely include molecular size (often indirectly inferred from differences in molecular weight) for size exclusion chromatography and membrane separations, isoelectric point and surface charge distribution for ion exchange chromatography and relative hydrophobicity for hydrophobic interaction chromatography. Affinity chromatography, which targets highly specific sites on the protein, is a favoured method as it allows for significant target purification and concentration.

Purification of PEGylated proteins involves removing all molecular species that are not part of the target product. In some cases PEG-protein biopharmaceuticals may be comprised of a mixture of PEG-proteins of different grafting ratio (N). Such a mixture may offer optimal performance in terms of activity versus in vivo half life. Of course mixtures of PEGamers may be suitable as therapeutics only if they can be reproduced in type and relative amounts batch to batch.

If target proteins in a crude protein mixture (e.g. fermentation broth) are PEGylated in a specific manner then PEGylation may aid their preliminary purification. For example the log of the partition coefficient (K) of PEG-modified proteins between the two aqueous phases in a PEG-dextran two polymer, or PEG-salt two-phase system increases directly with degree of PEGylation of the protein (Delgado et al., 1997; Harris and Zalipsky, 1997; Karr et al., 1986). This is fortuitous as cell debris, endotoxin, some proteins and other contaminants often partition in favour of the non-PEG-rich phase. Other approaches may also be possible such as selective precipitation of PEG-proteins by calcium or other divalent salts (Vincentelli et al., 1999). In such cases subsequent purification will be performed in a manner similar to the normal situation where a pure protein sample is PEGylated.

PEGylation of a pure protein sample creates two basic types of purification challenges. The first is involves separation of PEG-proteins from other reaction products including, but not necessarily limited to, unreacted PEGs and proteins. The second is sub-fractionation of PEG-proteins on the basis of their degree of PEGylation and positional isomerism. One would think that effecting such separations should be straight-forward but it can be complicated by several factors related to the nature of PEG polymers.

As noted above, such polymers are neutral, hydrophilic and soluble in various aqueous solutions, as well as being somewhat hydrophobic and soluble in some organic solvents

such as acetone, or azeotropes (e.g. methanol/water 3:1 v/v). The amphipathic nature of PEG (Sheth and Leckband, 1997) appears related to the conformations it can have in solution (Karlström, 1985) and tend to make PEGs weak surfactants (detergents). As temperature affects these conformational states PEGs tend to be thermally responsive and their solubility in aqueous solution decreases inversely with temperature. PEGs are normally soluble in aqueous solution up to 100 °C but addition of salts can drastically reduce their solubility. Functionalisation also affects PEG solubility. PEGs are normally neutral but they can interact via their ether oxygens and terminal groups in various weak interactions such as hydrogen bonding or ion chelation. They are often used, together with different salts, to promote selective precipitation or crystallisation of proteins. PEGs represent fairly high MW polymers and they tend to increase the viscosities of aqueous solutions. They non-specifically adsorb to surfaces and in so doing affect the physical and other properties of such surfaces including charge group pKa's and protein adsorption (Harris, 1992b; Harris and Zalipsky, 1997).

Given the above it is perhaps not surprising that PEGylation reaction product mixtures containing PEGs and PEG-proteins can exhibit foaming, viscosity, and protein or polymer precipitation. These can lead to a need to reduce feed solution concentrations. So too column fouling associated with reduced column life may occur, and not be ameliorated by trying to clean the columns with concentrated salt or high temperature solutions. It is also understandable that proteins whose surfaces have been modified by coating with PEGs should show altered size, charge nature and expression, hydrophobicity, metal chelation, hydrogen bonding, and affinity interactions. Thus one can expect that chromatographic, filtration and other preparative and analytical separation methods related SEC, IEX, hydrophobic interaction chromatography (HIC), immobilised metal affinity chromatography (IMAC), specific affinity and other methods may all be affected. Analytical methods such as gel electrophoresis will also be affected.

Many FDA approved PEG-protein biopharmaceuticals have been developed from FDA-approved proteins. Given FDA approval of PEG, the ideal situation in terms of rapid process validation would be to purify PEG-proteins by the same process used for the native protein. This is possible in some cases. However the above process factors related to the nature of PEG, and the separation requirements related to fractionation of complex reaction product mixtures often make such approaches impractical.

Table 1 lists the proteins, PEGylation reagents and purification methods employed by many papers reviewed herein. The table is not intended to be exhaustive but gives a representative sample of published methods. (For further examples, the reader is referred to references Chapman, 2002; Harris, 1992b; Harris and Zalipsky, 1997; Nho et al., 1992). In many cases the methods listed in Table 1 reflect laboratory scale separations. Regardless, the separation methods are dominated by ion exchange and size exclusion

Table 1
PEGylated protein processing

Protein	PEG MW	Target product <i>N</i>	Conjugation method	Purification step	Purification step purpose	Reference
Anti-interleukin-8 F(ab') ₂	20,40 kDa	1–4	Succinimidyl propionate, <i>N</i> -hydroxy succinimide	Size exclusion cation exchange	Analytical separation removal of unreacted PEG and isolation of PEGylated forms	(Koumenis et al., 2000)
Anti-tumour necrosis factor- α scFv fragment	5,20,40 kDa		Maleimide	Size exclusion	Analytical separation	(Yang et al., 2003)
Asparaginase, catalase	5 kDa	69 (reported)	On-column affinity PEGylation with trichloro triazine	Affinity Procion Red diafiltration	Reaction with protection of active site desalting and buffer exchange	(Baran et al., 2003)
BSA, β -lactoglobulin, lysozyme, caricain	4.6 kDa	Various	Succinimide carbonate, dithiopyridyl	Hydrophobic interaction	Fractionation of PEGylated forms	(Vincentelli et al., 1999)
Calcitonin (salmon)	5 kDa	1	Succinimidyl carbonate	Size exclusion reverse phase	Isolation of mono-PEGylated forms fractionation of mono-PEGylated isomers	(Lee et al., 1999b)
Calcitonins (salmon)	12 kDa	1,2	Succinimidyl carbonate	Size exclusion	Fractionation of native and PEGylated forms	(Lee et al., 1999a)
Chymopapain	5 kDa	Various	Dithiopyridyl	Cation exchange	Removal of native protein	(Azarkan et al., 1996)
Chympapain	5 kDa	1,2	Dithiopyridyl	Cation exchange hydrophobic interaction	Fractionation of native and PEGylated forms removal of native protein	(Azarkan et al., 2003)
Epidermal growth factor	3.4 kDa	1	<i>N</i> -hydroxy succinamide	Size exclusion reverse phase	Isolation of mono-PEGylated form fractionation of mono-PEGylated isomers	(Lee and Park, 2002)
Epidermal growth factor	2,5 kDa	1–3	Succinimidyl propionate, succinimidyl succinamide	Dialysis and lyophilisation	Buffer exchange to stop reaction and recover product	(Kim et al., 2002)
Gelonin	2,5 kDa	Various	Succinimidyl succinate	size exclusion Ultrafiltration	analytical separation Removal of unreacted PEG	(Arpicco et al., 2002)
Granyulocyte colony-simulating factor (human)	6,12, 20,25,30 kDa	1	Aldehyde	Size exclusion	fractionation of PEGylated forms Analytical separation	(Kinstler et al., 2002)
Granulocyte hormone releasing factor	5 kDa	1,2	Succinimidyl ester	cation exchange Cation exchange	isolation of mono-PEGylated form Fractionation of PEGylated forms	(Esposito et al., 2003)
Growth hormone releasing factor 1-29 analogue	5 kDa	1,2	Norleucine succinimidyl ester	Cation exchange	Isolation of mono-PEGylated form	(Piquet et al., 2002)
Hemoglobin	5,10,20 kDa	2	Maleidophenyl	Unspecified ion exchange IDA/Cu+	Isolation of di-PEGylated forms	(Manjula et al., 2003)
Immunoadhesin	5 kDa	7.7–14.5	Aldehyde		Removal of unreacted PEG	(Chamow et al., 1994)
Immunoglobulin antigen binding domains (Fv fragments)	2,3,4 5,10,12,20 kDa	Various	Hydazide hydrochloride trichlorophenyl carbonate, <i>N</i> -hydroxy succinamide, succinimidyl	Size exclusion	Isolation of PEGylated forms	(Lee et al., 1999b)

Table 1 (continued)

Protein	PEG MW	Target product <i>N</i>	Conjugation method	Purification step	Purification step purpose	Reference
Immunoglobulins	5 kDa	Various	carbonate, thiazolidine-2-thione, bifunctional succinimidyl carbonate Trichloro-triazine	Ultrafiltration size exclusion anion exchange dialysis/ultrafiltration	Concentration removal of unreacted PEG isolation of mildly PEGylated forms buffer exchange/concentration	(Brumeanu et al., 1995)
Insulin	750 Da	1,2	Nitrophenyl chloroformate	Size exclusion	Fractionation of native and PEGylated forms	(Calceti et al., 2004)
Insulin	750 Da,	1	Succinimidyl propionate	Dialysis lyophilisation	Recovery of PEGylated form	(Hinds and Kim, 2002)
α -interferon 2b PEG Intron® (Schering–Plough)	12 kDa	1	Succinimidyl carbonate PEG	Cation exchange size exclusion cation exchange	Isolation of mono-PEG form analysis of species separation of positional isomers	(Wang et al., 2002)
α -interferon 2b, interleukin 10	12 kDa	1,2	Succinimidyl carbonate	Cation exchange size exclusion	Isolation of mono-PEGylated form analytical separation	(Wylie et al., 2001)
α -interferon 2a	20 kDa	1	On-column reductive alkylation	Cation exchange	Reaction and isolation of mono-PEG form	(Lee and Lee, 2004)
α -interferon 2a PEGasys® (Hoffmann–La Roche Inc.)	40 kDa	1	<i>N</i> -hydroxy succinimide PEG (branched)	Cation exchange	Isolation of mono-PEG form	(Reddy et al., 2002)
β -interferon (rat)	20 kDa	> 1	Propionaldehyde PEG	Cation exchange ultrafiltration size exclusion	Removal of reaction byproducts concentration of protein species separation of native and PEGylated forms	(Arduini et al., 2004)
Interleukin-2	3,10 12 kDa	1–3	Enzyme catalysed alkylamine PEG conjugation with transglutaminase, <i>N</i> -hydroxy succinimidyl PEG	Cation exchange	Isolation of PEGylated forms	(Sato, 2002)
α -lactalbumin, β -lactoglobulin, BSA	5 kDa	1,2,3	On-column, succinimidyl propionate, <i>N</i> -hydroxy succinimide	Size exclusion	Reaction and separation simultaneously	(Fee, 2003)
α -lactalbumin, β -lactoglobulin, BSA	2,5 10, 20,40 kDa	1–8	Succinimidyl propionate, <i>N</i> -hydroxy succinimide	Size exclusion	Analytical separation	(Fee and Van Alstine, 2004)
β -lactoglobulin	5 kDa		Succinimide carbonate, dithiolpyridyl	Dialysis chromatofocussing hydrophobic interaction	Removal of reaction byproducts separation of PEGylated forms separation of PEGylated forms	(Nijs et al., 1997)
Lysozyme	2,3 kDa	1–3	Biotinylated <i>N</i> -hydroxy succinamide	Dialysis size exclusion	Buffer exchange analytical separation	(Lee and Park, 2003)
Lysozyme, insulin	5 kDa	Various	Norleucine succinimidyl ester	Size exclusion reversed phase	Isolation of PEGylated forms isolation of PEGylated forms	(Veronese et al., 2001)

Table 1 (continued)

Protein	PEG MW	Target product <i>N</i>	Conjugation method	Purification step	Purification step purpose	Reference
Methioninase	5 kDa	2–8	Succinimidyl propionate	ultrafiltration/dialysis Ultrafiltration size exclusion anion exchange	concentration and buffer exchange Removal of unreacted PEG removal of unreacted PEG removal of native protein	(Tan et al., 1998)
Myelopoietin	30 kDa	1	Aldehyde	Anion exchange cation exchange	Isolation of mono-PEGylated form	(Hall et al., 2004)
Parathyroid hormone	2,5 kDa	1	Succinimidyl propionate, aldehyde	Size exclusion	Isolation of mono-PEGylated forms	(Na and Lee, 2004)
Ribonuclease A, lysozyme	5 kDa	Various	<i>N</i> -succinimidyl succinate	Capillary electrophoresis	Analytical separation	(Li et al., 2001)
Staphylokinase	5 kDa	1	Maleimide	Size exclusion unspecified chromatography	Analytical separation isolation of mono-PEGylated form from native protein and free PEG	(Johnson et al., 2003)
Staphylkinase	5,10, 20 kDa	1	Site-directed mutagenesis insert of cysteine, maleimide PEG	Size exclusion cation exchange	Desalting concentration of protein species separation of PEGylated forms	(Moreadith and Collen, 2003)
Trichosanthin	5,20 kDa	1,2	Maleimide	size exclusion Cation exchange size exclusion	Fractionation of PEGylated forms analytical separation	(He et al., 1999)
Tumour necrosis factor α (lysine deficient mutant)	5 kDa	1	Site-specific <i>N</i> -terminal, succinimidyl propionate PEG	Size exclusion	Isolation of mono-PEG form	(Yamamoto et al., 2003)
Tumour necrosis factor receptor type 1	30 kDa	1	Aldehyde PEG sodium cyanoborohydride	Cation exchange ultrafiltration/dialysis	Removal of unreacted PEG and sodium cyanoborohydride then gradient NaCl to separate PEGylated forms concentration and buffer exchange	(Edwards et al., 2003)
Tumour necrosis factor-RI	30 kDa	—	—	Ultrafiltration/dialysis	Analytical separations for dialysis modelling	(Stoner et al., 2004)
Uricase	5,10 kDa	1,2	Norleucine succinimidyl ester, lysine succinimidyl ester	Size exclusion/unspecified ion exchange size exclusion	Removal of reaction byproducts and unreacted PEG analytical and preparative fractionation of PEGylated forms	(Schiavon et al., 2000)

chromatography. The most common IEX method is cation exchange which follows the preference for amine group PEGylation. Ultrafiltration and diafiltration are used less commonly and mainly for concentration or buffer exchange rather than separation of native and PEGylated forms of proteins. Hydrophobic interaction chromatography has not been fully investigated which is of interest given that (a) PEGylation should affect protein surface hydrophobicity—increasing or decreasing it depending on the native protein's hydrophobicity, and (b) reverse phase

chromatography (RPC) has been used in industry to analyse reaction mixtures with regard to PEGamers and proteins differing in *N* (private communication). Affinity chromatography has been used in only one case, for on-column PEGylation.

Given the above, plus the great variation in proteins which are PEGylated and their degree of PEGylation, it may not be possible to identify a generic separation process for PEG-proteins. However it is possible to list some general considerations. There will be at least three fractions to isolate

(PEGs, PEG-proteins and native proteins). The PEG-protein fraction will need to be subfractionated on the basis of N with perhaps some PEGamer fractionation. Given that PEGs are colloiddally active and can affect process operating conditions (foaming, viscosity, fouling) they should be removed as early as possible. One can take advantage of their properties (size, neutrality, hydrophilicity, amphipathic nature) and perhaps use filtration or other (e.g. HIC, SEC) approaches. Native proteins will tend to not be very “PEG-like” and may be isolated on the basis of their different properties (e.g. often smaller viscosity radii, greater surface charge, exposed affinity groups) using methods such as size exclusion, ion exchange, HIC, affinity chromatography, or even solubility. PEG-proteins will vary in properties between those which may be modified slightly (e.g. low N and small MW PEGs) to those more PEG-like in character. Surface property variation may be related to the weight fraction of PEG in the PEG-protein conjugate (Delgado et al., 1997) while size and other properties are related to volume fraction (keeping in mind the large viscosity radius contribution of the PEG) (Clark et al., 1996; Fee and Van Alstine, 2004). Thus approaches related to fractionation on the basis of size, charge, and hydrophobicity may be successful in terms of separating PEG-proteins from other reaction product mixture components. IEX media is generally eluted at higher salt concentration while HIC media is loaded under high salt; so normally HIC follows IEX. Thus it would thus appear that size exclusion followed by ion exchange approaches and then HIC may form the basis for a generic approach. As noted above SEC and IEX appear to be commonly used methods. PEGamer fractionation may require more exotic methods such as the above approaches with very long linear gradients, or HIC using complex and varied mobile phases.

There is presently no method of choice for PEGamer resolution on the preparative scale. Monkarsh et al. (1997) were able to separate 11 positional isomers of mono-PEGylated α -interferon using cation exchange chromatography but only in analytical scale. Differences between isomers in isoelectric point (pI), surface charge distribution and relative hydrophobicity are likely to be subtle and it is doubtful that these can be exploited at the preparative scale. Fortunately, to date at least, regulatory authorities appear to not have insisted on producing single-PEGylation site, molecularly defined PEGylated therapeutic proteins.

4.2. Size-based separations

The species to be separated after PEGylation may be classified according to molecular weight, as shown in Table 2.

Desalting with gel filtration will remove low molecular weight species and allow buffer exchange into a suitable buffer, such as a volatile buffer prior to freeze drying. Maeda et al. (1992) used hollow fibre ultrafiltration to remove < 40,000 MW species from the product mix after PEGylation of bilirubin oxidase. Bailon and Berthold (1998) included diafiltration in their generic purification scheme for PEGylated proteins. Tan et al. (1998) concentrated PEG-methioninase with 30,000 MW cut-off membranes. Edwards et al. (2003) used 5 kDa molecular weight cut-off membranes for concentrating PEGylated tumour necrosis factor receptor type I (TNF-RI), noting that unacceptable product losses occurred when using 10 kDa molecular weight cut-off membranes. This is surprising, as TNF-RI has a molecular weight of 55 kDa, and was mono-PEGylated with a 30 kDa PEG. The authors advised that they normally disregard the contribution of the PEG adduct when choosing a suitable pore size for ultrafiltration membranes (Edwards et al., 2003).

Low molecular weight hydrolysis and PEGylation reaction by products and un-conjugated PEG species are generally only weakly retained by ion exchange, so this could be used to remove these species. However, binding of PEGylated proteins becomes weaker as PEGylation extent increases. Therefore higher PEGylated species may well be contained in the flow through fraction. This is discussed in more detail below.

Size exclusion chromatography can be used to separate PEGylated species from other components but the effectiveness will depend greatly upon the molecular sizes of the species involved. The viscosity radius provides a consistent calibration curve between molecular size and elution volume for both proteins and polymers (specifically dextran) across all size exclusion chromatography media (Holdaas et al., 1998; Hagel et al., 1993, 1996), while other size or shape-related parameters such as molecular weight, the second virial coefficient, Stoke's radius and radius of gyration have been shown to be inadequate by comparison (Potschka, 1987). Although SEC has generally been thought to be unreliable for identifying PEGylated proteins from their elution profiles (Christakopoulos et al., 1998; Fortier and Laliberte, 1993; McGoff et al., 1988; Veronese, 2001) SEC columns calibrated in terms of molecular size rather than molecular weight give consistent results for protein and PEG standards (Benoit et al., 1966; Fee and Van Alstine, 2004). Using this technique, Fee and Van Alstine (2004) found that the size of PEGylated proteins can be accurately predicted from the radius of an hypothetical PEG molecule having the same molecular weight as the total conjugated PEG and the native protein radius, using the following equation:

$$R_{h,PEGprot} = \frac{1}{6} \left[108R_{h,prot}^3 + 8R_{h,PEG}^3 + 12(81R_{h,prot}^6 + 12R_{h,prot}^3 R_{h,PEG}^3)^{1/2} \right]^{1/3} + \frac{2}{3} \frac{R_{h,PEG}^2}{\left[108R_{h,prot}^3 + 8R_{h,PEG}^3 + 12(81R_{h,prot}^6 + 12R_{h,prot}^3 R_{h,PEG}^3)^{1/2} \right]^{1/3}} + \frac{1}{3} R_{h,PEG}, \quad (1)$$

Table 2
Classification of PEGylation products by molecular weight

Low molecular weight	High molecular weight
<ul style="list-style-type: none"> • By-products from hydrolysis of functionalised PEG • By-products of PEGylation • Buffer components 	<ul style="list-style-type: none"> • Unreacted functionalised PEG • Inactive PEG from hydrolysis of PEG-reagents • Native protein • Under-PEGylated protein • Over-PEGylated protein • Target PEGylated protein

where $R_{h,PEGprot}$ is the viscosity radius of the PEGylated protein; $R_{h,prot}$ is the viscosity radius of the native protein; and $R_{h,PEG}$ is the viscosity radius in free solution of a single PEG molecule of the same molecular weight as the total conjugated PEG.

For globular proteins, the viscosity radius (in Ångströms) of the molecule (assuming a spherical shape) is related to its molecular weight in Da, $M_{r,prot}$, by Eq. (2) (Hagel, 1998)

$$R_{h,prot} \approx (0.82 \pm 0.02)M_{r,prot}^{1/3} \quad (2)$$

Kuga (1981) collated data on the viscosity radius of PEG molecules from a range of sources and a subset of this data gave the following correlation ($r^2 = 0.9995$) with the PEG molecular weight in Da, $M_{r,PEG}$ (Fee and Van Alstine, 2004):

$$R_{h,PEG} = 0.1912M_{r,PEG}^{0.559} \quad (3)$$

As a general rule of thumb, preparative-scale size exclusion chromatography separates proteins well only if they differ in molecular weight by about 100%. Thus a protein of molecular weight x can be separated efficiently from one of $2x$. This corresponds to a size ratio between molecules, calculated from Eq. (2), of 1.26. In terms of size, a PEG molecule has a much larger hydrodynamic radius than a protein of corresponding molecular weight. In most aqueous environments PEGs exhibit large coils related to one in three chain atoms being oxygen. Various physical studies suggest that each ethoxy group affects approximately 16 water molecules (Harris, 1992b; Harris and Zalipsky, 1997). Proteins, on the other hand, generally contain chemical groups which can interact via covalent, hydrogen bond, van der Waals and other interactions even in pure water. Such interactions tend to determine and stabilise their solutions structures which are also significantly affected by a need to reduce water interaction with protein hydrophobic groups. Therefore, conjugating a single PEG of the same or higher molecular weight to protein adds much more to the molecular radius than simply doubling the protein molecular weight so these species should be easily separable by SEC. For low PEGylation extent then, SEC will be effective but the resolution between peaks will be expected to decrease as PEGylation extent increases.

Based on Eq. (1), Fig. 1 shows the size ratio between species differing in PEGylation extent by a single PEG adduct for a protein of MW 15 kDa. The normal guideline for

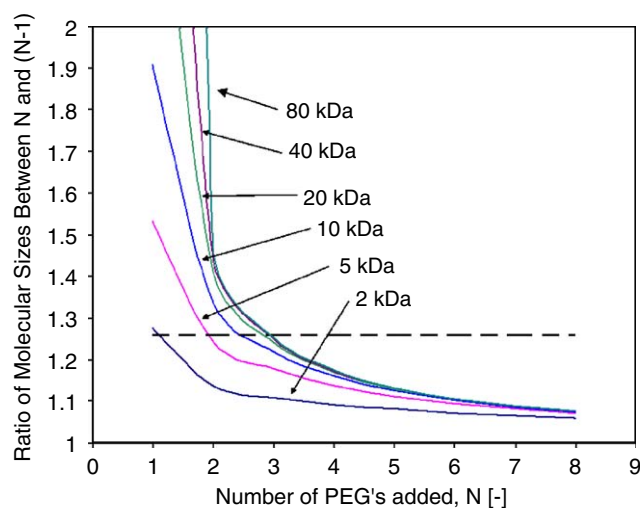


Fig. 1. Ratio of hydrodynamic radii of PEGylated species differing by a single PEG adduct. The dotted line shows the normal guideline for obtaining sufficient resolution for separation.

separating proteins with acceptable resolution (i.e., a size ratio of 1.26) is shown as a dotted line. All PEGylated species can be separated from the native protein in this case even with just a 2 kDa PEG adduct. Note that as the molecular weight of the PEG adduct increases, the ability to separate PEGylated species from one another increases. However, as expected, SEC becomes less effective in separating between species differing by a single PEG as the extent of PEGylation increases. The upper limit for good resolution appears to be between di- and tri-PEGylated species even for 40 kDa PEG adducts (the largest commercial PEG adduct readily available). Only mono- and di-PEGylated species can be efficiently separated by SEC when a 5 kDa PEG adduct is used. The curves are closer together for the larger molecular weight PEG adducts so larger PEG adducts will not allow separation of species with $N > 3$ from their $(N - 1)$ counterparts. Increasing the molecular weight of the protein reduces the proportional increase in size with each PEG added. Thus we conclude that the upper limit for effective SEC separation of PEGylated proteins differing by a single PEG adduct is between di- and tri-PEGylated proteins. This separation will become more difficult as the ratio of protein to PEG molecular weight increases.

Although the sizes of the target PEGylated species are equal, SEC can purify from its under- and over-PEGylated forms a protein that is mono-PEGylated with a 20 kDa PEG but not the same protein tetra-PEGylated with a 5 kDa PEG. It is worth repeating that the guideline for acceptable resolution between species is for obtaining acceptable yield and purity in preparative-scale purifications. For analytical purposes, peak elution volumes and therefore species can often be clearly identified at higher PEGylation extents.

Not shown in Table 2 are positional isomers of the same PEGylation extent, which will have identical molecular weights but different biological activities. Positional isomers are much more difficult to separate as they have closely similar physicochemical properties.

The SEC studies of Fee and Van Alstine suggest that existing SEC media is suitable for separation of PEG-proteins from native proteins and moderate fractionation of PEG-proteins of differing N . This work also suggests that grafting PEGs to proteins results in PEG-protein conjugates where, on the timescales and physical events of SEC, the PEG polymers appear to spread over the surface of the protein so as to maintain the surface to volume ratio they normally have in solution. This helps rationalise how modification of proteins with one PEG polymer can have a pronounced effect on both its size (e.g. SEC) and surface (e.g. partition or capture chromatography) properties (Delgado et al., 1997; Karr et al., 1986).

4.3. Charged-based separations

As noted above common problems with purification of PEG-proteins by IEX and other capture methods include a need to work with dilute feed solutions and fouling. In addition adsorption capacities in terms of mg protein per ml are often 10X lower than is normal for non-PEGylated proteins. However this may to some extent reflect the fact that much of volume of PEG-proteins is related to PEG not protein. Capacities of media in terms of mass per ml may be more comparable. IEX approaches offer the possibility to effect some separation of PEGs, native proteins, and PEGylated proteins in one step. Given the generally satisfactory performance of SEC media, IEX media is the most commonly used media which may be in need of improvement.

Most purification methods in the literature use ion exchange at some stage in the process. Invariably it is reported that interaction weakens with increased PEGylation extent. To the authors' knowledge, there has been no systematic study of the effects of PEGylation extent, the molecular weight of the PEG adducts or the binding site on ion exchange behaviour.

PEG is a neutral polymer but it may affect the charge properties of proteins in three ways. First, the presence of the PEG conjugate may shield the surface charges of a protein, thereby weakening the binding to ion exchange resins. Second, conjugation to amino acid residues that alter their

charge nature (e.g. convert amine groups to amides) or take on charge at certain pH values alter this potential charge and will affect the pI. Third, protein surface localised PEGs may hydrogen bond with acidic or other groups and raise their pKa (Delgado et al., 1997).

Azarkan et al. (1996) used thiol PEGylation as a strategy to purify chymopapain for X-ray crystallography and found that PEGylated species bound more weakly to a cation exchanger. Since the pI of the protein was unaltered by PEGylation, they concluded that charge-shielding was responsible.

Hecht et al. (1996) separated PEGylated from native brain-derived neurotrophic factor (BDNF) using a preparative electrofocussing device but were unable to separate BDNF of differing PEGylation extent. The authors suggested the reason for this was that the species with differing degrees of PEGylation "probably" varied only in the length of PEG chains attached rather than the number, and therefore the pIs of the PEGylated species would not differ. However, the origin of the PEGylated material was from authors (Chamow et al., 1994) who described the variation in the degree of PEGylation as resulting from differing PEGylation extent using a 5000 MW PEG. Nonetheless, it is possible that the pI of the various PEGylated species in Hecht et al.'s work did not vary significantly despite differing PEGylation extent. Fee and Mason (2004) have shown that changes in pI can be calculated from the amino acid sequence of the native protein and the reduction in charged residues by conjugation to PEG. Isoelectric focussing was able to resolve proteins with differing PEGylation extent when coupled via lysine residues in quantitative agreement with calculated pI changes. The change in calculated pI with PEGylation extent depended inversely on the slope of the net charge versus pH curve, with acidic and strongly basic proteins exhibiting little change in pI with PEGylation. Neutral and slightly basic proteins, on the other hand, often exhibit relatively large changes in calculated pI with increasing PEGylation extent. Fee and Mason were unable to separate these species by anion or cation exchange using running buffers at pH values between their pI values. This suggests that pI changes per se are not sufficient to ensure predictable separation on the basis of charge.

PEGylation dramatically affects molecular size and this may be reflected in lowered ion exchange media capacity due to associated decreases in diffusivity or through steric hindrance. PEGylated surfaces repel proteins (Harris, 1992b; Harris and Zalipsky, 1997), and bound PEG-proteins may hinder adsorption of other PEG-proteins.

Brumeanu et al. (1995) found that highly PEGylated species were contained in the flow through fraction during anion exchange, while mildly PEGylated species eluted at lower salt concentrations than native proteins. They also noted that prior removal of free PEG was extremely important in obtaining good resolution. This was also reported by McGoff et al. (1988) in charge-reversal capillary zone electrophoresis. Sherman et al. (1997) and Olson et al.

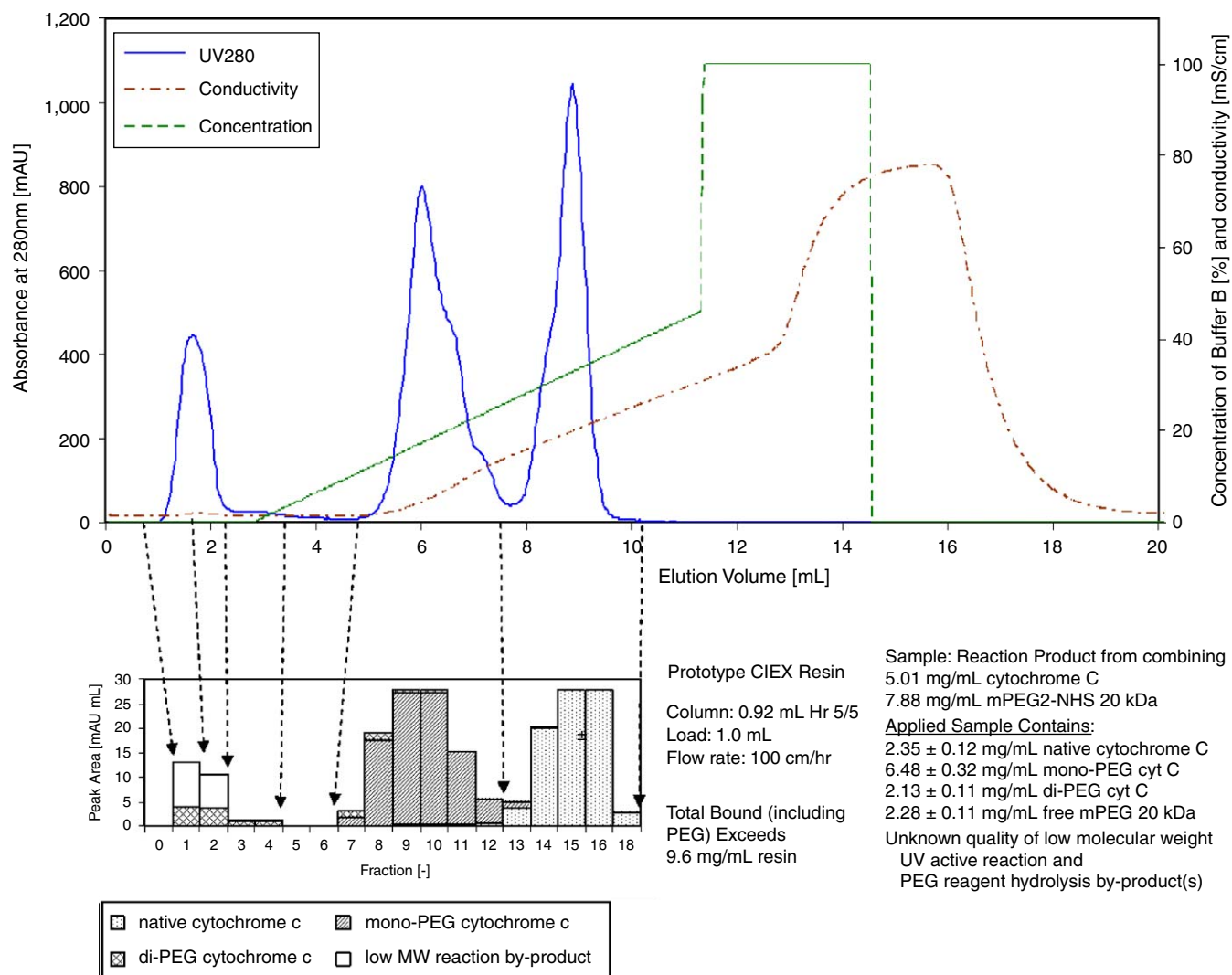


Fig. 2. Separation of native and PEGylated forms of cytochrome C using a prototype cation exchange resin designed for PEG-protein fractionation (GE Healthcare).

(1997) showed that increased PEGylation decreased the conductivity required for elution.

Fig. 2 shows the separation of native and PEG-cytochrome C proteins using a prototype sulphopropyl (SP) cation exchange resin from GE Healthcare (Uppsala, Sweden). This media was designed to fractionate PEG-protein reaction mixtures. The example shown involved PEGylation with 20 kDa PEG-SPA under conditions which resulted in primarily mono- and di-PEGylated protein. The figure clearly shows that reaction and hydrolysis by-products are contained in the flow through fraction, as well as the bulk of the di-PEGylated form. Mono-PEGylated cytochrome C eluted at a lower conductivity than the native protein, with good resolution between the two. However, under the conditions studied which featured a fairly steep elution gradient the di-PEGylated form was not sharply separated from the mono-PEGylated form. In addition a tail on the flow through peak extended to the mono-PEGylated peak.

Similar results were observed with SP Sepharose™ Fast Flow media and other commercial cation exchange media (data not shown), though with lower capacities, broader peaks and poorer resolution. In a variety of situations the prototype media appears to not only offer good selectivity but also much reduced fouling and the ability to work with 2x more concentrated feed solutions at linear flows of ≥ 100 cm/h. Note that faster flow rates may not be desired due to the large viscosity radii and slow diffusing nature of the PEGylated proteins.

Piquet et al. (2002) described large laboratory-scale chromatographic separation of PEGylated growth hormone release factor (GRF) using a gram-scale column 20 cm \times 5.5 cm with stepwise elution of monoPEG-GRF. Process yield (including PEGylation) was 41% and purity of monoPEG-GRF was approximately 97%. Hall et al. (2004) compared conventional anion and cation exchange media with monolithic columns and claimed much faster

processing in the latter while analytical separations were faster in monolithic columns but with poorer resolution. Chapman et al. (1999) fractionated PEGylated antibody fragments using SP Sepharose High Performance media. The fragments were modified using PEG-maleimide reagents (25 kDa or branched 40 kDa from Nektar Therapeutics, Huntsville, AL, USA) and PEGylated in a controlled fashion at low N which favoured their purification.

4.4. Hydrophobicity based separation

Very little work has been done on utilising hydrophobicity for separation. Clark et al. (1996) fractionated growth hormone modified with 5 kDa PEG-NHS ($N = 0-6$) using a process which began with HIC fractionation of the reaction product mixture on a phenyl TSK 5PW™ HIC column loaded at 2.75 mg of protein per ml media followed by elution with salt gradient 0.35 M NaCitrate, 0.05 M Tris pH 7.5 to 0.05 M Tris at 60 cm/h for 7 column volumes. This was followed by ultrafiltration, buffer exchange on a G-25 Sephadex™ column and then cation exchange on SP Sepharose High Performance media loaded at 2.1 mg protein per ml media and eluted with 7 column volumes and a gradient from 0 to 300 mM NaCl in 25 mM NaAcetate of N amers on SP Sepharose High Performance media run at pH 4 and loaded at 2.1 mg protein per ml gel with elution in 7 column volume gradient from 0 to 300 mM NaCl in 25 mM.

PEG forms a biphasic system at high salt concentrations. Vincentelli et al. (1999) examined the salting out effects of potassium fluoride and ammonium sulphate on PEG and related these to the effectiveness of hydrophobic interaction chromatography. They showed that PEG 4600 and native bovine β -lactoglobulin co-eluted on Fractogel TSK-Butyl 650 but that the conjugate was more tightly bound to the matrix, concluding that conformational changes to the protein may contribute to the surface hydrophobicity. Another explanation is that the PEG may surround the surface of the protein in such a way as to shield hydrophilic regions while leaving hydrophobic regions exposed. The authors and colleagues have noted interesting results with HIC (not shown) and this is an approach that requires more investigation.

4.5. Immunoaffinity and other specific affinity interactions

PEGylation can affect affinity interactions in two basic ways depending on whether the target or the binding protein is PEGylated. A general observation is that binding constant logarithms increase directly with N . This holds for enzymes, antibodies and other affinity proteins. However PEGylation may also eliminate binding depending on the site of modification. Karr et al. noted that significantly PEGylated polyclonal antibody preparations were capable of recognising and binding antigen and could be used for immunoaffinity separations (Karr et al., 1986). They also noted that PEG-modified protein A was also capable of binding antibody (Karr et al., 1988). Many studies (Chapman, 2002) suggest that even low level PEGylation of antibod-

ies can significantly affect Fc receptor binding. Veronese et al. (1992) investigated antibody interactions with PEGylated proteins and noted similar results. Thus K_d for antibody binding of PEG-ribonuclease decreased from 7.6×10^{-8} M to 1.3×10^{-7} M to 1.2×10^{-6} M as N went from 0 to 9. The above suggest that affinity interactions may be used to fractionate PEG-protein reaction mixtures and possibly even to sub-fractionate PEG-proteins. However care should be taken to undertake such separations under conditions where expensive affinity columns will not become fouled.

5. Conclusions

PEG-proteins as well as other polymer-conjugates present new challenges with regard to engineering both their preparation and purification. In many cases such challenges are related to such basic factors as polymer properties, and conjugation chemistry, as well as how these combine to alter the modified substance. Basic research related to elucidating these properties and effects should pay large dividends in terms of helping engineers design processes to deliver effective new products at reasonable costs. Research areas of particular interest also include improving control over PEGylation site, and separation of both PEG-reaction product mixtures as well as PEGamers. Some applications may benefit from the use of non-classical approaches such as two-phase partitioning, or on the column methods such as size exclusion reaction chromatography. While classic separation methods such as SEC, IEX and HIC are useful there is some concern that present media, which has typically been designed for macromolecules of smaller viscosity radius than many PEG-proteins, do not offer optimal performance. Though much work needs to be done it is promising to note that in many cases PEGylation may render proteins more similar and amenable to related process engineering solutions.

References

- Abuchowski, A., McCoy, J.R., Palczuk, N.C., Van Es, T., Davis, F.F., 1977. Effect of covalent attachment of poly(ethylene glycol) on immunogenicity and circulating life of bovine liver catalase. *Journal of Biological Chemistry* 252 (11), 3582–3586.
- Arduini, R.M., Li, Z., Rapoza, A., Gronke, R., Hess, D.M., Wen, D., Miatkowski, K., Coots, C., Kaffashan, A., Viseux, N., 2004. Expression, purification, and characterization of rat interferon- β , and preparation of an N-terminally PEGylated form with improved pharmacokinetic parameters. *Protein Expression and Purification* 34 (2), 229–242.
- Arpicco, S., Dosio, F., Bolognesi, A., Lubelli, C., Brusa, P., Stella, B., Ceruti, M., Cattel, L., 2002. Novel poly(ethylene glycol) derivatives for preparation of ribosome-inactivating protein conjugates. *Bioconjugate Chemistry* 13 (4), 757–765.
- Azarkan, M., Maes, D., Bouckaert, J., Thi, M.-H.D., Wyns, L., Looze, Y., 1996. Thiol PEGylation facilitates purification of chymopapain leading to diffraction studies at 1.4 Å resolution. *Journal of Chromatography A* 749 (1–2), 69–72.

- Azarkan, M., El Moussaoui, A., van Wuytswinkel, D., Dehon, G., Looze, Y., 2003. Fractionation and purification of the enzymes stored in the latex of carica papaya. *Journal of Chromatography B* 790 (1–2), 229–238.
- Bailon, P., Berthold, W., 1998. Polyethylene glycol-conjugated pharmaceutical proteins. *Pharmaceutical Science & Technology Today* 1 (8), 352–356.
- Baran, E.T., Ozer, N., Hasirci, V., 2003. Solid-phase enzyme modification via affinity chromatography. *Journal of Chromatography B* 794 (2), 311–322.
- Benoit, H., Gallot-Grubisic, Z., Rempp, P., Decker, D., Zilliox, J.G., 1966. Liquid-phase chromatographic study of branched and linear polystyrenes of known structure. *Journal de Chimie Physique* 63 (11–12), 1507–1514.
- Brumeanu, T.-D., Zaghouani, H., Bona, C., 1995. Purification of antigenized immunoglobulins derivatized with monomethoxypolyethylene glycol. *Journal of Chromatography A* 696 (2), 219–225.
- Calzeti, P., Salmaso, S., Walker, G., Bernkop-Schnurch, A., 2004. Development and in vivo evaluation of an oral insulin-PEG delivery system. *European Journal of Pharmaceutical Sciences* 22 (4), 315–323.
- Chamow, S.C., Kogan, T.P., Venuti, M., Gadek, T., Harris, R.J., Peers, D.H., Mordenti, J., Shak, S., Ashkenazi, A., 1994. Modification of cd4 immunoadhesin with monomethoxypoly(ethylene glycol) aldehyde via reductive alkylation. *Bioconjugate Chemistry* 5, 133–140.
- Chapman, A.P., 2002. Pegylated antibodies and antibody fragments for improved therapy: a review. *Advanced Drug Delivery Reviews* 54, 531–545.
- Chapman, A.P., Antoniw, P., Spitali, M., West, S., Stephens, S., King, D.J., 1999. Therapeutic antibody fragments with prolonged in vivo half-lives. *Nature Biotechnology* 17, 780–783.
- Christakopoulos, P., Kourentzi, E., Hatzinikolaou, D.G., Claeysens, M., Kekos, D., Macris, B.J., 1998. Enhancement of ph-stability of a low molecular mass endoglucanase from *fusarium oxysporum* by protein PEGylation. *Carbohydrate Research* 314 (1–2), 95–99.
- Clark, R., Olson, K., Fuh, G., Marian, M., Mortensen, D., Teshima, G., Chang, S., Chu, H., Mukku, V., Canova-Davis, E., Somers, T., Cronin, M., Winkler, M., Wells, J.A., 1996. Long-acting growth hormones produced by conjugation with poly(ethylene glycol). *Journal of Biological Chemistry* 271 (21), 969–977.
- Delgado, C., Malmsten, M., Van Alstine, J.M., 1997. Analytical partitioning of poly(ethylene glycol)-modified proteins. *Journal of Chromatography B* 692 (2), 263–272.
- Edwards, C.K., Martin, S.W., Seely, J., Kinstler, O., Buckel, S., Bendele, A.M., Cosenza, M.E., Feige, U., Kohno, T., 2003. Design of PEGylated soluble tumour necrosis factor receptor type i (PEG STNF-RI) for chronic inflammatory diseases. *Advanced Drug Delivery Reviews* 55, 1315–1336.
- Esposito, P., Barbero, L., Caccia, P., Caliceti, P., D'Antonio, M., Piquet, G., Veronese, F.M., 2003. Pegylation of growth hormone-releasing hormone GRF analogues. *Advanced Drug Delivery Reviews* 55 (10), 1279–1291.
- Fee, C.J., 2003. Size-exclusion reaction chromatography (SERC): a new technique for protein PEGylation. *Biotechnology & Bioengineering* 82 (2), 200–206.
- Fee, C.J., Mason, S., 2004. Charge-ph behaviour of PEGylated proteins: consequences for downstream processing. *Bioseparation 2004*, Uminonakamichi, Japan, October 15–17.
- Fee, C.J., Van Alstine, J.A., 2004. Prediction of viscosity radius and size exclusion chromatography behavior of PEGylated proteins. *Bioconjugate Chemistry* 15 (6), 1304–1313.
- Felix, A.M., 1997. Site-specific poly(ethylene glycol)ylation of peptides. In: Harris, J.M., Zalipsky, S. (Eds.), *Poly(ethylene glycol): Chemistry and Biological Applications*. American Chemical Society, Washington DC, pp. 218–238.
- Fortier, G., Laliberte, M., 1993. Surface modification of horseradish peroxidase with poly(ethylene glycol)s of various molecular masses. *Biotechnology and Applied Biochemistry* 17 (1), 115–130.
- Fung, W.-J., Porter, J.E., Bailon, P., 1997. Strategies for the preparation and characterization of poly(ethylene glycol) vinyl sulfone. *Polymer Preprints* 38, 565–566.
- Greenwald, R.B., 2001. PEG drugs: an overview. *Journal of Controlled Release* 74, 159–171.
- Hagel, L., 1998. Gel filtration. In: Janson, J.-C., Rydén, L. (Eds.), *Protein Purification*. Wiley, New York.
- Hagel, L., Hartmann, A., Lund, K., 1993. Analytical gel filtration of dextran for study of the glomerular barrier function. *Journal of Chromatography* 641, 63–70.
- Hagel, L., Ostberg, M., Andersson, T., 1996. Apparent pore size distributions of chromatography media. *Journal of Chromatography A* 743, 33–42.
- Hall, T., Wood, D.C., Smith, C.E., 2004. Preparative and analytical chromatography of PEGylated myelopoietin using monolithic media. *Journal of Chromatography A* 1041 (1–2), 87–93.
- Harris, J.M., 1992a. Introduction to biotechnical and biomedical applications of poly(ethylene glycol). In: Harris, J.M. (Ed.), *Poly(ethylene glycol) Chemistry: Biotechnical and Biomedical Applications*. Plenum Press, New York, pp. 1–14.
- Harris, J.M., Ed., 1992b. *Poly(ethylene glycol) chemistry: biotechnical and biomedical applications*. Topics in Applied Chemistry. Plenum Press, New York.
- Harris, J.M., Zalipsky, S. (Eds.), 1997. *Poly(ethylene glycol): chemistry and biological applications*. ACS Symposium Series 680, American Chemical Society, Washington DC.
- He, X.-H., Shaw, P.-C., Tam, S.-C., 1999. Reducing the immunogenicity and improving the in vivo activity of trichosanthin by site-directed PEGylation. *Life Sciences* 65 (4), 355–368.
- Heathcote, E.J., Pockros, P.J., Fried, M.W., Bain, M.A., DePamphilis, J., Modi, M., 1999. The pharmacokinetics of PEGylated-40k interferon alfa-2a (PEG-IFN) in chronic hepatitis c (CHC) patients with cirrhosis. *Gastroenterology* 116–135.
- Hecht, R., Tressel, T., Goldshteyn, V., Winters, D., Wilson, J., Boone, T., Daniels, M., 1996. Preparative electrofocusing for industrial applications. *Preparative Biochemistry & Biotechnology* 26 (3–4), 201–216.
- Hinds, K.D., Kim, S.W., 2002. Effects of PEG conjugation on insulin properties. *Advanced Drug Delivery Reviews* 54 (4), 505–530.
- Holdaas, H., Hartmann, A., Berg, K.J., Lund, K., Fauchald, P., 1998. Renal effects of losartan and amlodipine in hypertensive patients with non-diabetic nephropathy. *Nephrology Dialysis Transplantation* 13, 3096–3102.
- Hoyle, P.C., 1991. Polymer-modified proteins: preclinical development and regulatory strategies. *Advanced Drug Delivery Reviews* 6 (2), 219–233.
- Johnson, C., Royal, M., Moreadith, R., Bedu-Addo, F., Advant, S., Wan, M., Conn, G., 2003. Monitoring manufacturing process yields, purity and stability of structural variants of PEGylated staphylokinase mutant sy161 by quantitative reverse-phase chromatography. *Biomedical Chromatography* 17 (5), 335–344.
- Karlström, G., 1985. A new model for upper and lower critical solution temperatures in poly (ethylene oxide) solutions. *Journal of Physical Chemistry* 89, 4962–4964.
- Karr, L.J., Shafer, S.G., Harris, J.M., Van Alstine, J.A., Snyder, R.S., 1986. Immuno-affinity partition of cells in aqueous polymer two-phase systems. *Journal of Chromatography* 354, 269–282.
- Karr, L.J., Van Alstine, J.A., Snyder, R.S., Shafer, S.G., Harris, J.M., 1988. Cell separation by immunoaffinity phase partitioning with polyethylene glycol-modified protein a in aqueous polymer two-phase systems. *Journal of Chromatography* 442, 219–227.
- Kim, T.H., Lee, H., Park, T.G., 2002. PEGylated recombinant human epidermal growth factor (rhEGF) for sustained release from biodegradable PLGA microspheres. *Biomaterials* 23 (11), 2311–2317.
- Kinstler, O.B., Brems, D.N., Lauren, S.L., Paige, A.G., Hamburger, J.B., Treuheit, M.J., 1996. Characterization and stability of N-terminally PEGylated RHG-CSF. *Pharmaceutical Research* 13, 996–1002.

- Kinstler, O., Molineux, G., Treuheit, M., Ladd, D., Gegg, C., 2002. Mono-N-terminal poly (ethylene glycol)-protein conjugates. *Advanced Drug Delivery Reviews* 54 (4), 477–485.
- Kodera, Y., Matsushima, A., Hiroto, M., Nishimura, H., Ishii, A., Ueno, T., Inada, Y., 1998. Pegylation of proteins and bioactive substances for medical and technical applications. *Progress in Polymer Science* 23 (7), 1233–1271.
- Koumenis, I.L., Shahrokh, Z., Leong, S., Hsei, V., Deforge, L., Zapata, G., 2000. Modulating pharmacokinetics of an anti-interleukin-8 f(ab')₂ by amine-specific PEGylation with preserved bioactivity. *International Journal of Pharmaceutics* 198 (1), 83–95.
- Kuga, S., 1981. Pore size distribution analysis of gel substances by size exclusion chromatography. *Journal of Chromatography* 206 (3), 449–461.
- Lee, E.K., Lee, J.D., 2004. Solid-phase, N-terminus-specific, mono-PEGylation of recombinant interferon- α -2a: purification, characterization, and bioactivity. *International Symposium on the Separation of Proteins, Peptides and Polynucleotides*, Aachen, Federal Republic of Germany, October 19–22.
- Lee, H., Park, T.G., 2002. Preparation and characterization of mono-PEGylated epidermal growth factor: evaluation of in vitro biologic activity. *Pharmaceutical Research* 19 (6), 845–851.
- Lee, H., Park, T.G., 2003. A novel method for identifying PEGylation sites of protein using biotinylated PEG derivatives. *Journal of Pharmaceutical Sciences* 92 (1), 97–103.
- Lee, K.C., Moon, S.C., Park, M.O., Lee, J.T., Na, D.H., Yoo, S.D., Lee, H.S., DeLuca, P.P., 1999a. Isolation, characterization, and stability of positional isomers of mono-PEGylated salmon calcitonins. *Pharmaceutical Research* 16 (6), 813–818.
- Lee, L.S., Conover, C., Shi, C., Whitlow, M., Filpula, D., 1999b. Prolonged circulating lives of single-chain Fv proteins conjugated with polyethylene glycol: a comparison of conjugation chemistries and compounds. *Bioconjugate Chemistry* 10 (6), 973–981.
- Li, W., Zhong, Y., Lin, B., Su, Z., 2001. Characterization of polyethylene glycol-modified proteins by semi-aqueous capillary electrophoresis. *Journal of Chromatography A* 905 (1–2), 299–307.
- Maeda, H., Kimura, M., Sasaki, I., Hirose, Y., Konno, T., 1992. Toxicity of bilirubin and detoxification by PEG-bilirubin oxidase conjugate. In: Harris, J.M. (Ed.), *Poly (ethylene glycol) Chemistry: Biotechnical and Biomedical Applications*. Plenum Press, New York, pp. 153–169.
- Manjula, B.N., Tsai, A., Upadhyay, R., Perumalsamy, K., Smith, P.K., Malavalli, A., Vandegriff, K., Winslow, R.M., Intaglietta, M., Prabhakaran, M., Friedman, J.M., Acharya, A.S., 2003. Site-specific PEGylation of hemoglobin at cys-93(b): correlation between the colligative properties of the PEGylated protein and the length of the conjugated PEG chain. *Bioconjugate Chemistry* 14 (2), 464–472.
- McGoff, P., Baziotis, A.C., Maszkiewicz, R., 1988. Analysis of polyethylene glycol modified superoxide dismutase by chromatographic, electrophoretic, light scattering, chemical and enzymatic methods. *Chemical and Pharmaceutical Bulletin* 36 (8), 3079–3091.
- Monkarsh, S.P., Spence, C., Porter, J.E., Palleroni, A., Nalin, C., Rosen, P., Bailon, P., 1997. Isolation of positional isomers of mono-poly(ethylene glycol)ylated interferon/ α -2a and the determination of their biochemical and biological characteristics. In: Harris, J.M., Zalipsky, S. (Eds.), *Poly(ethylene glycol) ACS Symposium Series 680*. American Chemical Society, Washington DC, pp. 207–216.
- Moreadith, R., Collen, D., 2003. Clinical development of PEGylated recombinant staphylokinase (PEG-SAK) for bolus thrombolytic treatment of patients with acute myocardial infarction. *Advanced Drug Delivery Reviews* 55, 1337–1345.
- Na, D.H., Lee, K.C., 2004. Capillary electrophoretic characterization of PEGylated human parathyroid hormone with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Analytical Biochemistry* 331 (2), 322–328.
- Nho, K., Zalipsky, S., Abuchowski, A., Davis, F.F., 1992. Peg-modified haemoglobin as an oxygen carrier. In: Harris, J.M. (Ed.), *Poly(ethylene glycol) Chemistry: Biotechnical and Biomedical Application*. Plenum Press, New York, pp. 171–182.
- Nijs, M., Azarkan, M., Smolders, N., Brygier, J., Vincentelli, J., Vries, G.M.P., Duchateau, J., Looze, Y., 1997. Preliminary characterization of bovine beta-lactoglobulin after its conjugation to polyethylene glycol. *Biotechnology & Bioengineering* 54 (1), 40–49.
- Olson, K., Gehant, R., Mukku, V., O'Connell, K., Tomlinson, B., Totpal, K., Winkler, M., 1997. Preparation and characterization of poly(ethylene glycol)ylated human growth hormone antagonist. In: Harris, J.M., Zalipsky, S. (Eds.), *Poly(ethylene glycol): Chemistry and Biological Applications*. American Chemical Society, Washington DC, pp. 170–181.
- Pasta, P., Riva, S., Carrea, G., 1988. Circular dichroism and fluorescence of polyethylene glycol-subtilisin in organic solvents. *FEBS Letters* 236, 329–332.
- Pasut, G., Guiotto, A., Veronese, F.M., 2004. Protein, peptide and non-peptide drug PEGylation for therapeutic application. *Expert Opinion on Therapeutic Patents* 14 (6), 859–894.
- Pettit, D.K., Bonnert, T.P., Eisenman, J., Srinivasan, S., Paxton, R., Beers, C., Lynch, D., Miller, B., Yost, J., Grabstein, K.H., Gombotz, W.R., 1996. Structure-function studies of interleukin 15 using site-specific mutagenesis, polyethylene glycol conjugation and homology modelling. *Journal of Biological Chemistry* 272, 2312–2318.
- Piquet, G., Gatti, M., Barbero, L., Traversa, S., Caccia, P., Esposito, P., 2002. Set-up of large laboratory-scale chromatographic separations of poly(ethylene glycol) derivatives of the growth hormone-releasing factor 1–29 analogue. *Journal of Chromatography A* 944 (1–2), 141–148.
- Potschka, M., 1987. Universal calibration of gel permeation chromatography and determination of molecular shape in solution. *Analytical Biochemistry* 162 (1), 47–64.
- Reddy, K.R., Modi, M., Pedder, S., 2002. Use of PEGinterferon alfa-2a (40kd) (Pegasys) for the treatment of hepatitis c. *Advanced Drug Delivery Reviews* 54, 571–586.
- Roberts, M.J., Bentley, M.D., Harris, J.M., 2002. Chemistry for peptide and protein PEGylation. *Advanced Drug Delivery Reviews* 54 (4), 459–476.
- Sato, H., 2002. Enzymatic procedure for site-specific PEGylation of proteins. *Advanced Drug Delivery Reviews* 54 (4), 487–504.
- Schiavon, O., Caliceti, P., Ferruti, P., Veronese, F.M., 2000. Therapeutic proteins: a comparison of chemical and biological properties of uricase conjugated to linear or branched poly(ethylene glycol) and poly(*n*-acryloylmorpholine). *Il Farmaco* 55 (4), 264–269.
- Sherman, M.R., Williams, L.D., Saifer, M.G.P., French, J.A., Kwak, L.W., Oppenheim, J.J., 1997. Conjugation of high molecular weight poly(ethylene glycol) to cytokines: granulocyte macrophage colony-stimulating factors as model substrates. In: Harris, J.M., Zalipsky, S. (Eds.), *Poly(ethylene glycol): Chemistry and Biological Applications*. American Chemical Society, Washington DC, pp. 155–169.
- Sheth, S.R., Leckband, D., 1997. Measurements of attractive forces between proteins and end-grafted poly(ethylene glycol) chains. *Proceedings of the National Academy of Science USA*. 94, 8399–8404.
- Stoner, M.R., Fischer, N., Nixon, L., Buckel, S., Benke, M., Austin, F., Randolph, T.W., Kendrick, B.S., 2004. Protein-solute interactions affect the outcome of ultrafiltration/ diafiltration operations. *Journal of Pharmaceutical Sciences* 93 (9), 2332–2342.
- Tan, Y., Sun, X., Xu, M., An, Z., Tan, X., Tan, X., Han, Q., Miljkovic, D.A., Yang, M., Hoffman, R.M., 1998. Polyethylene glycol conjugation of recombinant methioninase for cancer therapy. *Protein Expression and Purification* 12 (1), 45–52.
- Veronese, F.M., 2001. Peptide and protein PEGylation: a review of problems and solutions. *Biomaterials* 22, 405–417.
- Veronese, F., Harris, J.M., 2002. Introduction and overview of peptide and protein PEGylation. *Advanced Drug Delivery Reviews* 54 (4), 453–456.
- Veronese, F., Caliceti, P., Schiavon, O., Sartore, L., 1992. Preparation and properties of monomethoxypoly(ethylene glycol)-modified enzymes for

- therapeutic applications. In: Harris, J.M. (Ed.), *Poly(ethylene glycol) Chemistry: Biotechnical and Biomedical Applications*. Plenum Press, New York, pp. 127–137.
- Veronese, F.M., Saccà, B., Laureto, P.P.d., Sergi, M., Caliceti, P., Schiavon, O., Orsolini, P., 2001. New PEGs for peptide and protein modification, suitable for identification of the PEGylation site. *Bioconjugate Chemistry* 12 (1), 62–70.
- Vincentelli, J., Paul, C., Azarkan, M., Guermant, C., El Moussaoui, A., Looze, Y., 1999. Evaluation of the polyethylene glycol-kf-water system in the context of purifying PEG-protein adducts. *International Journal of Pharmaceutics* 176 (2), 241–249.
- Wang, Y.-S., Youngster, S., Grace, M., Bausch, J., Bordens, R., Wyss, D.F., 2002. Structural and biological characterization of PEGylated recombinant interferon alpha-2b and its therapeutic implications. *Advanced Drug Delivery Reviews* 54 (4), 547–570.
- Wylie, D.C., Voloch, M., Lee, S., Liu, Y.-H., Cannon-Carlson, S., Cutler, C., Pramanik, B., 2001. Carboxyalkylated histidine is a pH-dependent product of PEGylation with SC-PEG. *Pharmaceutical Research* 18 (9), 1354–1360.
- Yamamoto, Y., Tsutsumi, Y., Yoshioka, Y., Nishibata, T., Kobayashi, K., Okamoto, T., Mukai, Y., Shimizu, T., Nakagawa, S., Nagata, S., Mayumi, T., 2003. Site-specific PEGylation of a lysine-deficient TNF-alpha with full bioactivity. *Nature Biotechnology* 21 (5), 546–552.
- Yamaoka, T., Tabata, Y., Ikada, Y., 1994. Distribution and tissue uptake of poly(ethylene glycol) with different molecular weights after intravenous administration to mice. *Journal of Pharmaceutical Sciences* 83, 601–606.
- Yang, K., Basu, A., Wang, M., Chintala, R., Hsieh, M.-C., Liu, S., Hua, J., Zhang, Z., Zhou, J., Li, M., Phyu, H., Petti, G., Mendez, M., Janjua, H., Peng, P., Longley, C., Borowski, V., Mehlig, M., Filpula, D., 2003. Tailoring structure-function and pharmacokinetic properties of single-chain fv proteins by site-specific PEGylation. *Protein Engineering* 16 (10), 761–770.
- Zalipsky, S., 1995. Chemistry of polyethylene glycol conjugates with biologically active molecules. *Advanced Drug Delivery Reviews* 16 (2–3), 157–182.
- Zalipsky, S., Lee, C., 1992. Use of functionalized poly(ethylene glycol)s for modification of polypeptides. In: Harris, J.M. (Ed.), *Poly(ethylene glycol) Chemistry: Biotechnical and Biomedical Applications*. Plenum Press, New York, pp. 347–370.