Various “on column” PEGylation methods are discussed in Pfister (Reac React. Chem. Eng., 2016, 1,204), Ingold (2016) and Fee & Van Alstine (2006).

Methods of providing mono-PEGylated proteins may comprise performing a PEGylation reaction to provide a mixture comprising non-PEGylated protein (unreacted protein), mono-PEGylated protein, and oligo-PEGylated protein, and then performing a purification step to purify the mono-PEGylated protein.

WO 2009/010270 and WO 2012/035037 relate to methods for purifying mono-PEGylated EPO from a mixture comprising non-PEGylated protein, mono-PEGylated protein, and oligo-PEGylated protein. The purification methods involve subjecting the mixture that results from a PEGylation reaction to at least one cation exchange chromatography (CEC) step. The CEC step is performed in bind and elute mode and different elution factions comprise mostly non-PEGylated, mono-PEGylated or oligo-PEGylated EPO. Such CEC methods must be carried out below the isoelectric point of the protein, which in the case of EPO (isoelectric point in the range 4.0 to 5.5) involves CEC at a pH of around 3.0.

Methods for producing mono-PEGylated proteins by performing a PEGylation reaction and then purifying the mono-PEGylated protein from the resultant mixture may involve recycling unreacted (non-PEGylated) protein. These methods involve recovering unreacted protein and adding it to a subsequent PEGylation reaction. Such recycling improves the overall yield of mono-PEGylated protein.

Pfister (Biotech and Bioeng, 2016) describes a process in which a PEGylation reaction is performed followed by purification of mono-PEGylated protein from a mixture comprising unreacted (non-PEGylated), mono-PEGylated, and oligo-PEGylated protein. In the process unreacted protein is recovered and used in a subsequent PEGylation reaction. The purification process comprises cation exchange chromatography (CEC) in bind and elute mode, wherein unreacted protein, mono-PEGylated protein and oligo-PEGylated protein are separated in sequential elution. Unreacted protein is eluted last, using a high salt elution buffer. Recycling of unreacted protein therefore requires removal of salt by diafiltration before it can be subject to a further PEGylation reaction. The requirement for removal of salt reduces productivity and increases complexity of the process.

The present invention has been devised in light of the above considerations.

Summary ~~of the Invention~~

The present ~~invention~~disclosure relates to processes for providing PEGylated protein compositions comprising mono-PEGylated protein and oligo-PEGylated protein and processes for providing mono-PEGylated protein compositions. The processes of the ~~invention~~disclosure are particularly suitable for providing PEGylated EPO compositions comprising mono-PEGylated EPO and oligo-PEGylated EPO, as well as mono-PEGylated EPO compositions. Advantages of the process described herein include high yield and productivity.

In a first aspect, the present ~~invention~~disclosure provides a process for producing a PEGylated protein mixture~~,~~ the process comprising: (a) reacting a non-PEGylated protein with a PEGylation reagent to produce a mixture of reaction products comprising non-PEGylated protein and PEGylated protein, wherein the PEGylated protein comprises mono-PEGylated protein and oligo-PEGylated protein; (b) subjecting the mixture of reaction products to an ion exchange chromatography (IEC) step to provide an IEC flow-through solution in which the fraction of PEGylated protein is increased relative to the mixture of reaction products; the IEC step comprising applying the mixture of reaction products to an IEC material under conditions suitable for binding non-PEGylated protein; and (c) collecting the IEC flow-through solution from step b) to provide a PEGylated protein mixture comprising mono-PEGylated protein and oligo-PEGylated protein, wherein non-PEGylated protein is recovered in step b) by eluting an IEC eluate from the IEC material and the eluted non-PEGylated protein is used in a subsequent PEGylation reaction.

The non-PEGylated protein may be recovered in step b) by elution and used in a subsequent PEGylation reaction. That is, step b) may further comprise eluting non-PEGylated protein in an IEC eluate. The IEC eluate comprises non-PEGylated protein.

The processes may involve two or more cycles of steps (a), (b) and (c), in which non-PEGylated protein is recovered in step b) by eluting an IEC eluate from the IEC material and the eluted non-PEGylated protein is used in a subsequent PEGylation reaction.

The IEC step may be an anion exchange chromatography (AEC) step, or a cation exchange chromatography (CEC) step.

The present ~~invention~~disclosure provides a process for producing a PEGylated protein mixture, the process comprising: (a) reacting a non-PEGylated protein with a PEGylation reagent to produce a mixture of reaction products comprising non-PEGylated protein and PEGylated protein, wherein the PEGylated protein comprises mono-PEGylated protein and oligo-PEGylated protein; (b) subjecting the mixture of reaction products to an anion exchange chromatography (AEC) step to provide an AEC flow-through solution in which the fraction of PEGylated protein is increased relative to the mixture of reaction products; the AEC step comprising applying the mixture of reaction products to an AEC material under conditions suitable for binding non-PEGylated protein; and (c) collecting the AEC flow-through solution from step b) to provide a PEGylated protein mixture comprising mono-PEGylated protein and oligo-PEGylated protein, wherein non-PEGylated protein is recovered in step b) by eluting an AEC eluate from the AEC material and the eluted non-PEGylated protein is used in a subsequent PEGylation reaction.

The processes may involve two or more cycles of steps (a), (b) and (c), in which non-PEGylated protein is recovered in step b) by eluting an AEC eluate from the AEC material and the eluted non-PEGylated protein is used in a subsequent PEGylation reaction.

The mixture of reaction products may contain a relatively low proportion of oligo-PEGylated protein. For example, the mixture of reaction products may comprise less than 25%, 20%, 15%, 10% or 5% oligo-PEGylated protein.

The mixture of reaction products may comprise at least about 20%, 30%, 40%, 45%, 50%, 55%, 60% or 70% of non-PEGylated protein.

The IEC flow-through solution (AEC or CEC flow-through solution) may contain a relatively high proportion of PEGylated protein. For example, the IEC flow-through solution may comprise at least about 90%, 95%, 98%, 99% or at least about 99.9% PEGylated protein. The AEC flow-through solution may contain a relatively high proportion of PEGylated protein. For example, the AEC flow-through solution may comprise at least about 90%, 95%, 98%, 99% or at least about 99.9% PEGylated protein.

The protein may be erythropoietin (EPO). The protein may be a hormone. The protein may be a hormone, a cytokine, an enzyme or an antibody.

The processes of the ~~invention~~disclosure involve performing a PEGylation reaction and recycling non PEGylated protein. Such processes are advantageous because they enable relatively high yield.

The processes of the ~~invention~~disclosure may involve performing a PEGylation reaction at about pH 7.0 to 9.0, which may be performed, for example, using an N-Hydroxysuccinimide (NHS) activated PEG reagent. The PEGylation reaction may be performed at about pH 7.5 to 8.5, or at about pH 8.0, and may be performed using an NHS activated PEG reagent. This provides a relatively rapid PEGylation step, which contributes to the overall rapidity of and high productivity of the processes described herein. The protein to by PEGylated may be erythropoietin.

The processes involve performing a PEGylation reaction to provide the mixture of reaction products, and recycling the non-PEGylated protein (unreacted protein) from that PEGylation reaction. The non PEGylated protein is recovered from the mixture of reaction products as part of an IEC step. The IEC step involves contacting the mixture of reaction products with an ion exchange material. The IEC flow-through comprises a relatively high proportion of PEGylated protein (mono-PEGylated and oligo-PEGylated protein) because most of the non-PEGylated protein binds to the anion exchange material. Thus non-PEGylated protein may be recovered from the mixture of reaction products by eluting it from the IEC material. The recovered non-PEGylated protein is added to a subsequent PEGylation reaction, thereby recycling the non-PEGylated protein.

The processes involve performing a PEGylation reaction to provide the mixture of reaction products, and recycling the non-PEGylated protein (unreacted protein) from that PEGylation reaction. The non PEGylated protein is recovered from the mixture of reaction products as part of an AEC step. The AEC step involves contacting the mixture of reaction products with an anion exchange material. The AEC flow-through comprises a relatively high proportion of PEGylated protein (mono-PEGylated and oligo-PEGylated protein) because most of the non-PEGylated protein binds to the anion exchange material. Thus non-PEGylated protein may be recovered from the mixture of reaction products by eluting it from the AEC material. The recovered non-PEGylated protein is added to a subsequent PEGylation reaction, thereby recycling the non-PEGylated protein.

The recycling of non-PEGylated protein in the processes disclosed herein is achieved by eluting non-PEGylated protein in the IEC step. The recycling of non-PEGylated protein in the processes disclosed herein is achieved by eluting non-PEGylated protein in the AEC step. Elution of non-PEGylated protein from an anion exchange material is relatively straightforward and fast, thereby enabling simple and rapid recovery of unreacted protein for recycling. The rapidity of the elution for non-PEGylated protein recycling contributes to the overall rapidity and productivity of the processes disclosed herein.

The processes are performed using a mixture of reaction products that contains non-PEGylated, mono-PEGylated and oligo-PEGylated protein. The proportion of oligo-PEGylated protein in the mixture of reaction products may be relatively low. The processes involve an ion exchange chromatography (IEC) step that provides an IEC flow through solution in which the fraction of PEGylated protein is increased relative to the mixture of reaction products. The processes involve an ion exchange chromatography (IEC) step that provides an IEC flow through solution in which the fraction of non-PEGylated protein is decreased relative to the mixture of reaction products. The IEC flow-through solution may contain a high proportion of PEGylated protein, wherein the PEGylated protein consists of mono-PEGylated protein and oligo-PEGylated protein. That is, the processes of the ~~invention~~disclosure involve an IEC step that is performed in flow through mode. The IEC step in flow though mode is relatively rapid. The relatively fast IEC step of the ~~invention~~disclosure contributes to the relatively overall high productivity of the processes of the ~~invention~~disclosure. The IEC step may be an AEC step or a CEC step.

The IEC flow-through solution has an increased fraction of PEGylated protein relative to the mixture of reaction products. The AEC flow-through solution has a decreased fraction of non-PEGylated protein relative to the mixture of reaction products

The processes may involve an anion exchange chromatography (AEC) step that provides an AEC flow through solution in which the fraction of PEGylated protein is increased relative to the mixture of reaction products. The processes involve an anion exchange chromatography (AEC) step that provides an AEC flow through solution in which the fraction of non-PEGylated protein is decreased relative to the mixture of reaction products. The AEC flow-through solution may contain a high proportion of PEGylated protein, wherein the PEGylated protein consists of mono-PEGylated protein and oligo-PEGylated protein. That is, the processes of the ~~invention~~disclosure involve an AEC step that is performed in flow through mode. The AEC step in flow though mode is relatively rapid. The relatively fast AEC step of the ~~invention~~disclosure contributes to the relatively overall high productivity of the processes of the ~~invention~~disclosure.

An AEC flow-through solution has an increased fraction of PEGylated protein relative to the mixture of reaction products. The AEC flow-through solution has a decreased fraction of non-PEGylated protein relative to the mixture of reaction products.

An AEC step comprises applying the mixture of reaction products to an anion exchange material. The anion exchange material may be a strong anion exchange material. Suitable materials are well known in

exchange material. The non PEGylated protein may be recovered rapidly by step elution, which may provide an eluate comprising the non-PEGylated protein in a relatively concentrated form which may be suitable for direct addition to a PEGylation reaction. The non PEGylated protein may be eluted from the anion exchange material in an elution step performed at a relatively low conductivity, or low salt concentration, which facilitates recycling of non-PEGylated protein for example because removal of high salt concentrations (for example by diafiltration, reconcentration or buffer exchange) is not required before the eluate is added to a subsequent PEGylation reaction. The use of an ion exchange material with a low binding capacity for the PEGylated form of the protein is thus advantageous because it provides for rapid recovery of unreacted (non-PEGylated) protein in a form that is particularly suitable for recycling in a subsequent PEGylation reaction.

The use of an anion exchange material with a low binding capacity for the PEGylated form of the protein is advantageous because it facilitates rapid separation of non-PEGylated protein from PEGylated protein. The AEC step can be performed in flow through mode to rapidly provide an AEC flow-through solution from which most, or almost all, of the unreacted (non-PEGylated) protein has been removed. Hence the flow through solution from the AEC step comprises a relatively high proportion of PEGylated protein. It may comprise at least about 80%, 85%, 90% or 95% PEGylated protein, or 80-95% PEGylated protein. It may comprise at least about 90% PEGylated protein. It may comprise at least about 95% PEGylated protein. It may comprise a low proportion, close to zero, or zero non-PEGylated protein. It may comprise less than about 20%, 15%, 10% or 5% non-PEGylated protein, or about 20-5% non-PEGylated protein. It may comprise less than 10% non-PEGylated protein. It may comprise less than 5% non-PEGylated protein.

A further advantage of using an anion exchange material with a low binding capacity for the PEGylated form of the protein is that it facilitates rapid recovery of unreacted (non-PEGylated) protein from the anion exchange material. The non PEGylated protein may be recovered rapidly by step elution, which may provide an eluate comprising the non-PEGylated protein in a relatively concentrated form which may be suitable for direct addition to a PEGylation reaction. The non PEGylated protein may be eluted from the anion exchange material in an elution step performed at a relatively low conductivity, or low salt concentration, which facilitates recycling of non-PEGylated protein for example because removal of high salt concentrations (for example by diafiltration, reconcentration or buffer exchange) is not required before the eluate is added to a subsequent PEGylation reaction. The use of an anion exchange material with a low binding capacity for the PEGylated form of the protein is thus advantageous because it provides for rapid recovery of unreacted (non-PEGylated) protein in a form that is particularly suitable for recycling in a subsequent PEGylation reaction.

The processes of the ~~invention~~disclosure involve providing a PEGylated protein mixture comprising mono-PEGylated protein and oligo-PEGylated protein, which comprises collecting the flow-through solution from the IEC step. Collecting the IEC flow-through solution may comprise collecting batches of IEC flow-

through solution from separate IEC cycles, in this way a PEGylated protein mixture is provided which is a pooled PEGylated protein mixture. The IEC step may be an AEC step or a CEC step.

The processes of the ~~invention~~disclosure involve providing a PEGylated protein mixture comprising mono-PEGylated protein and oligo-PEGylated protein, which comprises collecting the flow-through solution from the AEC step. Collecting the AEC flow-through solution may comprise collecting batches of AEC flow-through solution from separate AEC cycles, in this way a PEGylated protein mixture is provided which is a pooled PEGylated protein mixture. ~~In a second aspect, the~~The present ~~invention~~disclosure provides processes for producing a mono-PEGylated protein composition comprising at least about 90% mono-PEGylated protein, the process comprising: subjecting the PEGylated protein mixture comprising mono-PEGylated protein and oligo-PEGylated protein produced by a process according to the first aspect of the ~~invention~~disclosure to a purification process separating mono-PEGylated protein and oligo-PEGylated protein; and recovering a mono-PEGylated protein composition in which the fraction of mono-PEGylated protein is increased relative to the PEGylated protein mixture, wherein the mono-PEGylated protein composition comprises at least about 90% mono-PEGylated protein.

The mono-PEGylated protein composition may contain a relatively high proportion of mono-PEGylated protein. For example, the protein composition may comprise at least about 95%, 98%, 99%, or 99.9% mono-PEGylated protein.

PEGylation reactions tend to produce mixtures comprising non-PEGylated protein (unreacted protein), mono-PEGylated protein, and oligo-PEGylated protein. Mono-PEGylated proteins may be desirable because they provide a balance between increased half-life and comparable biological activity relative to non-PEGylated and oligo-PEGylated versions of the protein. Proteins, especially therapeutic proteins, are often expensive to manufacture and therefore processes that provide good yields of mono-PEGylated therapeutic protein are particularly desirable. Processes that provide good yields of mono-PEGylated EPO (Mircera®) are particularly desirable.

The processes disclosed herein are advantageous because they are relatively rapid, and therefore enable relatively high productivity. The processes disclosed herein also enable high yield, in that a high proportion of the starting protein becomes PEGylated, and in particular mono-PEGylated. The processes disclosed herein also provide mono-PEGylated protein compositions of relatively high purity.

The flow-through solution from the IEC step is used to provide a PEGylated protein mixture comprising mono-PEGylated protein and oligo-PEGylated protein For example the flow-through solution from an AEC step is used to provide a PEGylated protein mixture comprising mono-PEGylated protein and oligo-PEGylated protein. This mixture is subjected to a purification process to separate the mono-PEGylated protein from the oligo-PEGylated protein and thereby provide a composition comprising a relatively high proportion of mono-PEGylated protein. The purification process may comprise subjecting the PEGylated protein mixture to a hydrophobic interaction chromatography (HIC) step. Both the anion exchange step

and HIC step are relatively rapid, thereby providing a relatively rapid process for producing a mono-PEGylated protein composition. The HIC step may be performed in flow through mode, which is particularly rapid, to provide a rapid process for producing a mono-PEGylated protein composition.

The processes of the ~~invention~~disclosure involve collecting the flow-through solution from the IEC step (which may be an AEC or a CEC step). For example the processes of the ~~invention~~disclosure may involve collecting an IEC flow-through solution. Collecting the IEC (AEC or CEC) flow-through solution may comprise collecting batches or fractions of IEC (AEC or CEC) flow-through solution from separate IEC (AEC or CEC) cycles, in this way a PEGylated protein mixture is provided which is a pooled PEGylated protein mixture. Collecting the IEC (AEC or CEC) flow-through solution may comprise delivering the IEC (AEC or CEC) flow-through solution to a HIC material via an in-line connection, in an "in-line conditioning" operation. Collecting the IEC (AEC or CEC) flow-through solution may comprise delivering the IEC (AEC or CEC) flow-through solution to a vessel for conditioning the IEC (AEC or CEC) flow-through solution. Collecting the IEC (AEC or CEC) flow-through solution may comprise delivering the IEC (AEC or CEC) flow-through solution directly to the HIC material. When the IEC (AEC or CEC) flow-through solution is delivered directly to the HIC material the IEC (AEC or CEC) flow-through solution may be conditioned for example by in-line conditioning. There may be a continuous flow of IEC (AEC or CEC) flow-through solution (PEGylated protein mixture) from the IEC (AEC or CEC) material to the HIC material which is in-line conditioned to provide a continuous low of flow-through solution to the HIC material. The IEC (AEC or CEC) flow-through solution may comprise a low proportion of non-PEGylated protein and a high proportion of PEGylated protein (mono-PEGylated and oligo-PEGylated protein). The processes of the ~~invention~~disclosure may further involve separating the mono-PEGylated protein from the oligo-PEGylated protein in the PEGylated protein mixture by performing a hydrophobic interaction chromatography (HIC) step.

The processes of the ~~invention~~disclosure are particularly suitable for producing mono-PEGylated EPO compositions. In processes for producing mono-PEGylated EPO, the AEC step is performed at a pH of about 7.0 to 10.0, about 7.0 to 9.0, about 7.5 to 8.5, or about 8.0. This is advantageous over processes involving cation exchange chromatography (CEC) which is generally performed at a pH of 3.0 or lower. Acid conditions of about pH 3.0 or lower may have adverse effects on EPO quality, such adverse effects are reduced in the processes of the present ~~invention~~disclosure, which do not require CEC and thus do not require the low pH conditions necessary for CEC-based purification of EPO.

Furthermore, acidic forms (acidic variants) of EPO may have higher therapeutic activity, making them particularly useful. However, recovery of mono-PEGylated acidic forms of EPO by CEC may be poor because their elution conditions in CEC are similar to those of di-PEGylated non-acidic forms of EPO. Acidic conditions result in the oxidation of the side chains of amino acids in a protein molecule. As CEC separates protein molecules according to their charge, basic variants of a species will elute later than acidic variants. For this reason, the CEC elution profile of di-pegylated non-acidic forms of EPO tends to overlap with mono-PEGylated acidic forms of EPO. The poor recovery of these useful mono-PEGylated

acidic EPO forms by CEC is reduced in the processes disclosed herein, which do not rely on CEC to separate mono-PEGylated from oligo-PEGylated EPO.

The advantages associated with the ~~invention~~disclosure to provide mono-PEGylated EPO may apply to other proteins, particularly other therapeutic proteins. That is, avoidance of low pH conditions and recovery of acidic variants may render the ~~invention~~disclosure useful for providing mono-PEGylated proteins other than mono-PEGylated EPO.The processes may comprise carrying out the IEC and HIC steps at substantially the same pH. The processes may comprise carrying out the PEGylation reaction, IEC and HIC steps at substantially the same pH. The processes may comprise carrying out the AEC step and purification process at substantially the same pH. The processes may comprise carrying out the AEC and HIC steps at substantially the same pH. The processes may comprise carrying out the PEGylation reaction, AEC and HIC steps at substantially the same pH. The pH may be about 7.0 to 9.0, or about 7.5 to 8.5, or about 8.0. Substantially the same pH may refer to ±0.5 pH units. Performing steps of the process at substantially the same pH is advantageous because it avoids the need for pH adjustments between steps, which pH adjustments may be time-consuming and therefore productivity-reducing. . The process may comprise carrying out a PEGylation reaction to provide a mixture of reaction products and applying the mixture of reaction products directly to the IEC material. That is, the process may comprise carrying out a PEGylation reaction and applying the resultant mixture to the IEC material without adjusting the pH of the mixture.

The processes of the ~~invention~~disclosure involve providing a PEGylated protein mixture comprising mono-PEGylated protein and oligo-PEGylated protein, which comprises collecting the flow-through solution from the IEC step. Collecting the IEC flow-through solution may comprise collecting batches of IEC flow-through solution from separate IEC cycles, in this way a PEGylated protein mixture is provided which is a pooled PEGylated protein mixture. The processes of the ~~invention~~disclosure involve providing a PEGylated protein mixture comprising mono-PEGylated protein and oligo-PEGylated protein, which comprises collecting the flow-through solution from the AEC step. Collecting the AEC flow-through solution may comprise collecting batches of AEC flow-through solution from separate AEC cycles, in this way a PEGylated protein mixture is provided which is a pooled PEGylated protein mixture.

The processes of the ~~invention~~disclosure involve subjecting a PEGylated protein mixture to a purification process. This may involve subjecting a pooled PEGylated protein mixture to a purification process. Collecting the IEC flow-through solution may comprise delivering the IEC flow-through solution to a HIC material via an in-line connection, in an “in-line conditioning operation”. Collecting the IEC flow-through solution may comprise delivering the IEC flow-through solution to a vessel for conditioning the IEC flow-through solution to provide a PEGylated protein mixture. The IEC flow-through solution may comprise a low proportion of non-PEGylated protein and a high proportion of PEGylated protein (mono-PEGylated and oligo-PEGylated protein). The purification process may comprise subjecting the PEGylated protein mixture to a hydrophobic interaction chromatography (HIC) step. The HIC step may be performed in flow

through mode. The HIC step in flow through mode may comprise contacting the PEGylated protein mixture with a hydrophobic interaction material, and collecting the flow-through. The flow-through comprises a relatively high proportion of mono-PEGylated protein. Performing the HIC step in flow through mode is advantageous because it can be performed relatively rapidly, thereby contributing to the overall rapidity and productivity of the process. Performing the HIC step in flow through mode, rather than bind and elute mode, is advantageous because it can be performed using a column of relatively small size.

The step of providing the PEGylated protein mixture may comprise pooling the flow-through solution from two IEC steps, or three IEC steps, or more than three IEC steps, or four IEC steps, or five IEC steps. Such pooling of IEC flow-through solution enables the HIC step to produce a mono-PEGylated protein composition from the products of multiple PEGylation reactions, which is relatively efficient. The IEC steps may be a series of AEC steps, or a series of CEC steps.The processes of the ~~invention~~disclosure involve subjecting a PEGylated protein mixture to a purification process. This may involve subjecting a pooled PEGylated protein mixture to a purification process. Collecting the AEC flow-through solution may comprise delivering the AEC flow-through solution to a HIC material via an in-line connection, in an “in-line conditioning operation”. Collecting the AEC flow-through solution may comprise delivering the AEC flow-through solution to a vessel for conditioning the AEC flow-through solution to provide a PEGylated protein mixture. Collecting the AEC flow-through solution may comprise delivering the AEC flow-through solution directly to the HIC material. When the AEC flow-through solution is delivered directly to the HIC material the AEC flow-through solution may be conditioned for example by in-line conditioning. There may be a continuous flow of AEC flow-through solution from the AEC material to the HIC material which is in-line conditioned to provide a continuous flow of conditioned flow-through solution to the HIC material. The AEC flow-through solution may comprise a low proportion of non-PEGylated protein and a high proportion of PEGylated protein (mono-PEGylated and oligo-PEGylated protein). The purification process may comprise subjecting the PEGylated protein mixture to a hydrophobic interaction chromatography (HIC) step.

Conditioning the IEC flow-through solution may be referred to as providing a conditioned second mixture. Conditioned second mixtures are described in more detail below. Conditioning an IEC flow-through solution (or providing a conditioned second mixture) may comprise the addition of salt. Conditioning an IEC flow-through solution the addition of bicine. Conditioning an IEC flow-through solution (or providing a conditioned second mixture) may comprise the addition of salt and bicine. Conditioning an IEC flow-through solution (or providing a conditioned second mixture) may comprise the addition of salt and/or bicine to provide a conditioned second mixture as described below.

The HIC step may be performed in flow through mode. The HIC step in flow through mode may comprise contacting the PEGylated protein mixture with a hydrophobic interaction material, and collecting the

Claims:

1. A process for producing a PEGylated protein mixture the process comprising:

a) reacting a non-PEGylated protein with a PEGylation reagent to produce a mixture of reaction products comprising non-PEGylated protein and PEGylated protein, wherein the PEGylated protein comprises mono-PEGylated protein and oligo-PEGylated protein;

b) subjecting the mixture of reaction products to an ion exchange chromatography (IEC) step to provide an IEC flow-through solution in which the fraction of PEGylated protein is increased relative to the mixture of reaction products;

the IEC step comprising applying the mixture of reaction products to an IEC material under conditions suitable for binding non-PEGylated protein; and

c) collecting the IEC flow-through solution from step b) to provide a PEGylated protein mixture comprising mono-PEGylated protein and oligo-PEGylated protein,

wherein non-PEGylated protein is recovered in step b) by eluting an IEC eluate from the IEC material and the eluted non-PEGylated protein is used in a subsequent PEGylation reaction.

2. The process according to claim 1, wherein the protein is a hormone, a cytokine, an enzyme or an antibody.

3. The process according to claim 1 or claim 2, wherein the protein is erythropoietin.

4. The process according to any one of the preceding claims, wherein the IEC step is an anion exchange (AEC) step.

5. The process according to any one of the preceding claims, wherein two or more cycles of steps a), b) and c) are performed, in which non-PEGylated protein is recovered in step b) by eluting an IEC eluate from the IEC material and the eluted non-PEGylated protein is used in a subsequent PEGylation reaction.

6. The process according to claim 5 wherein the process comprises three, four or five cycles, in which in step b) of each cycle non-PEGylated protein is recovered and used in a subsequent PEGylation reaction.

7. The process according to claims 5 or claim 6, wherein step c) further comprises pooling the flow-through solution collected from each IEC step to provide a PEGylated protein mixture which is a pooled PEGylated protein mixture.

8. The process according to any one of the preceding claims, wherein eluting non-PEGylated protein from the IEC material uses an elution buffer comprising less than or equal to ~~about~~ 45 mM salt.

9. The process according to any one of the preceding claims , wherein the step b) IEC eluate is added directly to the subsequent PEGylation reaction.

10. The process according to any one of the preceding claims, in which the non-PEGylated protein is recovered and used in a subsequent PEGylation reaction, and wherein fresh non-PEGylated protein is also added to the subsequent PEGylation reaction in order to maintain substantially constant PEGylation reaction conditions in each PEGylation reaction.

11. The process according to any one of the preceding claims wherein the IEC material has a binding capacity for the PEGylated protein of less than ~~about~~ 1.5 g/L.

12. The process according to any one of the preceding claims, wherein:

i. the mixture of reaction products comprises less than 25% oligo-PEGylated protein; and/or

ii. the IEC flow-through solution comprises at least 90% PEGylated protein.

13. The process according to any one of the preceding claims, wherein the IEC step is an AEC step and wherein,

i. the AEC material is Toyopearl Super Q 650 M; and/or

ii. the AEC step is performed at pH of ~~about~~ 7.0 to 9.0; and/or

iii. the AEC step is performed at a conductivity of ~~about~~ 1.0 to 3.0 mS/cm; and/or

iv. the mixture of reaction products is applied to the AEC material as a AEC load solution comprising ~~about~~ 10 – 30 mM bicine and ~~about~~ 1 – 10 mM Na_2SO_4.

14. The process according to any one of the preceding claims, wherein the PEGylation reaction is performed at a pH of ~~about~~ 7.0 to 9.0, and wherein the PEG/protein molar ratio is ~~about~~ 0.6 – 1.0.

15. ~~A process for producing a mono-PEGylated protein composition comprising at least about 90% mono-PEGylated protein, the process~~The process according to any one of the preceding claims, further comprising:

(d) subjecting the PEGylated protein mixture produced in step (c) of claim 1 and comprising mono-PEGylated protein and oligo-PEGylated protein ~~produced by the process of any one of claims 1 to 14~~ to a purification process separating mono-PEGylated protein and oligo-PEGylated protein; and

(e) recovering a mono-PEGylated protein composition in which the fraction of mono-PEGylated protein is increased relative to the PEGylated protein mixture~~,~~ and wherein the mono-PEGylated protein composition comprises at least ~~about~~ 90% mono-PEGylated protein.

16. The process of claim 15, wherein the purification process comprises subjecting the PEGylated protein mixture to a hydrophobic interaction chromatography (HIC) step in flow-through mode to provide a HIC flow-through solution in which the fraction of mono-PEGylated protein is increased relative to the PEGylated protein mixture,

the HIC step comprising applying the PEGylated protein mixture to a HIC material under conditions suitable for binding oligo-PEGylated protein, wherein the HIC flow-through provides the mono-PEGylated protein composition.

17. The process of claim 15, wherein the purification process comprises subjecting the PEGylated protein mixture to a hydrophobic interaction chromatography (HIC) step in bind and elute mode to provide a HIC eluate in which the fraction of mono-PEGylated protein is increased relative to the PEGylated protein mixture, the HIC step comprising

applying the second mixture to a HIC material under conditions suitable for binding mono-PEGylated protein and oligo-PEGylated protein,

eluting the mono-PEGylated protein from the HIC material to provide a HIC eluate, wherein the HIC eluate provides the mono-PEGylated protein.

18. The process according to any one of claims 15 to 17 wherein the mono-PEGylated protein composition comprises at least ~~about~~ 95%, 98%, 99%, or 99.9% mono-PEGylated protein.

19. The process according to any one of the preceding claims, wherein

i. the IEC step and HIC step are performed at substantially the same pH; or

ii. the PEGylation reaction, IEC step and HIC step are performed at substantially the same pH.

20. The process according to any one of the preceding claims, wherein mono-PEGylated protein comprises a PEG residue having a molecular weight of at least ~~about~~ 20kDa.

21. The process according to any one of the preceding claims, further comprising formulating the protein composition with a pharmaceutically acceptable carrier to provide a pharmaceutical composition.