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 mono-PEGylated protein compositions. The processes of the ~~invention~~disclosure are particularly suitable for providing mono-PEGylated EPO compositions. Advantages of the process described herein include high yield and productivity.

~~The~~ In one aspect, the present ~~invention~~disclosure provides a process for producing a protein composition comprising at least ~~about~~ 90% mono-PEGylated protein, the process comprising: (a) providing a first mixture comprising non-PEGylated protein and PEGylated protein, wherein the PEGylated protein comprises mono-PEGylated protein and oligo-PEGylated protein (b) subjecting the first mixture to an ion exchange chromatography (IEC) step to provide an IEC flow-through solution; the IEC step comprising applying the first mixture to an IEC material under conditions suitable for binding non-PEGylated protein; (c) collecting the IEC flow-through solution from step b) to provide a second mixture comprising mono-PEGylated protein and oligo-PEGylated protein; and (d) subjecting the second mixture to a hydrophobic interaction chromatography (HIC) step to provide a

protein composition in which the fraction of mono-PEGylated protein is increased relative to the second mixture, wherein the protein composition comprises at least ~~about~~ 90% mono-PEGylated protein.

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 protein composition comprising at least about 90% mono-PEGylated protein, the process comprising: (a) providing a first mixture comprising non-PEGylated protein and PEGylated protein, wherein the PEGylated protein comprises mono-PEGylated protein and oligo-PEGylated protein (b) subjecting the first mixture to an anion exchange chromatography (AEC) step to provide an AEC flow-through solution; the AEC step comprising applying the first mixture to an AEC material under conditions suitable for binding non-PEGylated protein; (c) collecting the AEC flow-through solution from step b) to provide a second mixture comprising mono-PEGylated protein and oligo-PEGylated protein; and (d) subjecting the second mixture to a hydrophobic interaction chromatography (HIC) step to provide a protein composition in which the fraction of mono-PEGylated protein is increased relative to the second mixture, wherein the protein composition comprises at least about 90% mono-PEGylated protein.

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

The IEC material may have a binding capacity for PEGylated protein of less than about 1.5 g/L. The IEC material may have a binding capacity for PEGylated protein of less than about 1.0 g/L, 0.75 g/L, 0.5 g/L, 0.25 g/L, 0.10 g/L, or 0.05 g/L. The binding capacity may be the dynamic binding capacity. The AEC material may have a binding capacity for PEGylated protein of less than about 1.5 g/L. The AEC material may have a binding capacity for PEGylated protein of less than about 1.0 g/L, 0.75 g/L, 0.5 g/L, 0.25 g/L, 0.10 g/L, or 0.05 g/L. The binding capacity may be the dynamic binding capacity. The IEC material may be an AEC material and the PEGylated protein may be PEGylated erythropoietin.

The IEC material may have a relatively high binding capacity for non-PEGylated protein (e.g. non-PEGylated EPO) of 20 – 50 g/L, 30 – 40 g/L, or about 35 g/L. The IEC material may have a binding capacity for non-PEGylated protein (e.g. non-PEGylated EPO) of at least about 20 g/L, 25 g/L, 30 g/L or about 35 g/L. The binding capacity may be the dynamic binding capacity. The IEC material may be an AEC material and the non-PEGylated protein may be non-PEGylated erythropoietin.

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 process may comprise an anion exchange chromatography (AEC) step and the protein may be erythropoietin.

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. However PEGylation reactions tend to produce mixtures comprising non-PEGylated protein (unreacted protein), mono-PEGylated protein, and oligo-PEGylated 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 mono-PEGylated. The processes disclosed herein also provide mono-PEGylated protein compositions of relatively high purity.

The processes disclosed herein are performed using a first mixture that contains non-PEGylated, mono-PEGylated and oligo-PEGylated protein. The proportion of oligo-PEGylated protein in the first mixture may be relatively low. The processes 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 first mixture. 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 first mixture. 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 flow-through solution from the IEC step (either AEC or CEC) is used to provide a second mixture that is subjected to a hydrophobic interaction chromatography (HIC) step 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. Both the ion 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 relatively fast IEC (AEC or CEC) step of the ~~invention~~disclosure contributes to the relatively overall high productivity of the processes of the ~~invention~~disclosure.

The processes of the ~~invention~~disclosure involving anion exchange chromatography 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, acid forms (acidic variants) of EPO may have high 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 monoPEGylated proteins other than monoPEGylated EPO.

The processes of the ~~invention~~disclosure may involve performing a PEGylation reaction at about pH 7.0 to 9.0, which may be performed 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 process.

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 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 first mixture and applying the first mixture 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 may involve performing a PEGylation reaction and recycling non-PEGylated protein. Such processes are advantageous because they enable relatively high yield.

The processes may involve performing a PEGylation reaction to provide the first mixture, and recycling the non-PEGylated protein (unreacted protein) from that PEGylation reaction. The non-PEGylated protein may be recovered from the first mixture as part of the IEC step. The IEC step involves contacting the first mixture with an ion exchange material. The IEC flow-through comprises a relatively high proportion of PEGylated

exchange) is not required before the eluate is added to a subsequent PEGylation reaction. The use of an ion exchange material, such as 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 second mixture, which comprises 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 providing a second mixture, which comprises collecting the flow-through solution from the AEC step. 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 second mixture is provided which is a pooled second 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 to provide a second mixture. 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 to provide a second mixture to the HIC material. There may be a continuous flow of IEC (AEC or CEC) flow-through solution from the IEC (AEC or CEC) material to the HIC material which is in-line conditioned to provide a continuous low of second mixture 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 further involve separating the mono-PEGylated protein from the oligo-PEGylated protein in the second mixture by performing a hydrophobic interaction chromatography (HIC) step.

For example, collecting the AEC flow-through solution may comprise collecting batches or fractions of AEC flow-through solution from separate AEC cycles, in this way a second mixture is provided which is a pooled second mixture. 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 second 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 to provide a second mixture to the HIC material. 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 second mixture 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 processes of the ~~invention~~disclosure further involve separating the mono-PEGylated protein from the oligo-PEGylated protein in the second mixture by performing a hydrophobic interaction chromatography (HIC) step.

Claims:

1. A process for producing a mono-PEGylated protein composition comprising at least ~~about~~ 90% mono-PEGylated protein, the process comprising:

    a) providing a first mixture comprising non-PEGylated protein and PEGylated protein, wherein the PEGylated protein comprises mono-PEGylated protein and oligo-PEGylated protein

    b) subjecting the first mixture 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 first mixture; the IEC step comprising applying the first mixture to an IEC material under conditions suitable for binding non-PEGylated protein;

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

    d) subjecting the second mixture to a hydrophobic interaction chromatography (HIC) step to provide a mono-PEGylated protein composition in which the fraction of mono-PEGylated protein is increased relative to the second mixture, wherein the mono-PEGylated protein composition comprises at least ~~about~~ 90% mono-PEGylated protein.

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 ion exchange chromatography (IEC) step is an anion exchange chromatography (AEC) step.

5. 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.

6. The process according to any one of the preceding claims wherein:
    i. the first mixture comprises less than 25% oligo-PEGylated protein; and/or
    ii. the IEC flow-through solution comprises at least 90% PEGylated protein; and/or
    iii. the mono-PEGylated protein composition comprises at least ~~about~~ 95%, 98%, 99%, or 99.9% mono-PEGylated protein.

7. The process according to any one of the preceding claims, wherein step a) further comprises performing a PEGylation reaction comprising reacting the non-PEGylated protein with a PEGylation reagent.

8. The process according to claim 7, 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.

9. The process according to claim 7 or claim 8, comprising

performing a first cycle comprising steps a), b) and c), wherein step b) further comprises eluting non-PEGylated protein from the IEC material to provide an IEC eluate, and

performing a second cycle of steps a), b) and c), in which the non-PEGylated protein eluted in step b) of the first cycle is added to the PEGylation reaction of step a).

10. The process according to claim 9, wherein eluting non-PEGylated protein from the IEC material uses an elution buffer comprising less than or equal to ~~about~~ 45 mM salt.

11. The process according to claim 9 or claim 10, wherein the process comprises three, four or five cycles, and wherein step b) of each cycle comprises eluting non-PEGylated protein from the IEC material to provide an IEC eluate, and wherein the non-PEGylated protein eluted in step b) is added to the PEGylation reaction of step a) in the next cycle.

12. The process according to any one of claims 9 to 11, wherein the IEC eluate from step b) of a cycle is added directly to the PEGylation reaction of step a) of the next cycle.

13. The process according to any one of claims 9 to 12, in which the non-PEGylated protein eluted in step b) of a cycle is added to the PEGylation reaction of step a) of the next cycle, and wherein fresh non-PEGylated protein is also added to step a) in order to maintain substantially constant PEGylation reaction conditions in step a) of each cycle.

14. The process according to any one of the preceding claims, comprising performing two or more cycles of steps a), b) and c), wherein

step c) further comprises pooling the flow-through solution collected from each IEC step to provide a second mixture which is a pooled second mixture, and wherein

step d) comprises subjecting the second mixture to an HIC step.

15. 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 first mixture 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.

16. The process according to any one of the preceding claims, wherein step d) comprises subjecting the second mixture to a 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 second mixture,

the HIC step comprising applying the second 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 according to any one of claims 1 to 15, wherein step d) comprises subjecting the second mixture to a 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 second 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 claim 16, wherein
    i. the HIC material is Toyopearl Phenyl 650M; and/or
    ii. the HIC step is performed at a pH of ~~about~~ 7.0 to 9.0; and/or
    iii. the HIC step is performed at a conductivity of ~~about~~ 30-40 mS/cm; and/or
    iv. the second mixture is applied to the HIC material as a HIC load solution comprising ~~about~~ 25 mM bicine and ~~about~~ 390 mM Na_2SO_4.

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.