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. The elution of the unreacted protein in the final stage of CEC delays the recycling of unreacted protein, since it is processed by diafiltration before recycling, thereby reducing the overall productivity 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 and high purity of mono-PEGylated product.

The present ~~invention~~disclosure relates to a process in which a protein mixture comprising non-PEGylated, mono-PEGylated and oligo-PEGylated protein is subjected to a two-stage hydrophobic interaction chromatography (HIC) step. The two-stage HIC step involves applying the protein mixture to two HIC materials under specific conditions (“two-stage HIC conditions”): the first HIC material binds only oligo-PEGylated protein under those conditions, and the second HIC material binds mono-PEGylated protein under those same conditions. That is, the first HIC material binds oligo-PEGylated protein but not

non-PEGylated protein or mono-PEGylated protein under the two-stage HIC conditions. The second HIC material binds mono-PEGylated protein under the two-stage HIC conditions, and is capable of binding oligo-PEGylated protein under the two-stage HIC conditions but in the processes disclosed herein substantially all of the oligo-PEGylated protein may have already been removed by the first HIC material, so in carrying out the ~~invention~~disclosure the second HIC material does not necessarily bind oligo-PEGylated protein, or does not bind significant amounts of oligo-PEGylated protein.

In ~~particular~~one aspect, the present ~~invention~~disclosure provides a process for producing a mono-PEGylated protein composition comprising at least ~~about~~ 90% mono-PEGylated protein, the process comprising: a) providing a protein mixture comprising non-PEGylated protein, mono-PEGylated protein and oligo-PEGylated protein; b) subjecting the protein mixture to a two-stage hydrophobic interaction chromatography (HIC) step, comprising: applying the protein mixture to a first HIC material to provide a first HIC flow-through solution; and applying the first HIC flow-through solution to a second HIC material to provide a second HIC flow-through solution, wherein the second HIC material is different from the first HIC material; and wherein the two-stage HIC step is performed under two-stage HIC conditions, which two-stage HIC conditions are suitable for binding oligo-PEGylated protein to the first HIC material and binding mono-PEGylated protein to the second HIC material; and c) eluting the mono-PEGylated protein from the second HIC material to provide a second HIC eluate, wherein the second HIC eluate provides the mono-PEGylated protein composition.

The “second HIC eluate” may also be referred to as a “second HIC material eluate”, because it is the eluate from the second HIC material in the two-stage HIC step.

In the processes disclosed herein 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 protein may be erythropoietin (EPO). The protein may be a hormone. The protein may be a hormone, a cytokine, an enzyme or an antibody.

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 particularly desirable. Processes that provide good yields of mono-PEGylated EPO (Mircera®) are particularly desirable.

The processes disclosed herein are advantageous because they may enable rapid recovery of non-PEGylated protein (unreacted protein) for subsequent processing and recycling in a subsequent PEGylation reaction. The processes disclosed herein may enable relatively high yield because non-PEGylated EPO may be recovered and recycled, thus a greater proportion of the starting protein becomes mono-PEGylated.

The processes disclosed herein are advantageous relative to processes that recycle non-PEGylated protein in which the non-PEGylated protein is recovered after the mono-PEGylated protein. This is because the processes disclosed herein enable recovery of the non-PEGylated protein at an early stage of the process, before recovery of the mono-PEGylated protein, which means the non-PEGylated protein may be processed and recycled in parallel with later stages of the process. This increases the overall productivity of processes that involve a step of recycling non-PEGylated protein. The processes disclosed herein are advantageous because they involve HIC, which can be performed at a pH close to physiological pH. This may improve the stability of the protein. The processes of the ~~invention~~disclosure are particularly suitable for producing mono-PEGylated EPO compositions. This is advantageous over processes involving cation exchange chromatography (CEC) for producing mono-PEGylated EPO which are generally performed at a pH of 3.0 or lower. Acidic 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.

Purification of proteins by HIC is based on hydrophobicity of the protein rather than charge. The processes disclosed herein may be advantageous because they are relatively unaffected by acidic forms or by glycosylation variants that affect protein charge. Because PEGylation may greatly affect the hydrophobicity of a protein, the processes disclosed herein are particularly useful for providing mono-PEGylated protein compositions.

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 disclosed herein are for producing a mono-PEGylated protein composition using a protein mixture that comprises non-PEGylated, mono-PEGylated and oligo-PEGylated protein. In the processes disclosed herein two HIC materials are used in series in a two-stage HIC step. The two HIC materials are different from each other and are selected such that, under the conditions used for the two-stage HIC step, the first HIC material binds oligo-PEGylated protein (but not mono-PEGylated protein), the second

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 protein mixture comprising non-PEGylated protein, mono-PEGylated protein and oligo-PEGylated protein;
    b) subjecting the protein mixture to a two-stage hydrophobic interaction chromatography (HIC) step, comprising:
        applying the protein mixture to a first HIC material to provide a first HIC flow-through solution; and
        applying the first HIC flow-through solution to a second HIC material to provide a second HIC flow-through solution,
        wherein the second HIC material is different from the first HIC material; and
        wherein the two-stage HIC step is performed under two-stage HIC conditions, which two-stage HIC conditions are suitable for binding oligo-PEGylated protein to the first HIC material and binding mono-PEGylated protein to the second HIC material; and
    c) eluting the mono-PEGylated protein from the second HIC material to provide a second HIC eluate, wherein the second HIC eluate provides the mono-PEGylated protein composition.

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 any one of the preceding claims, wherein the protein is erythropoietin.

4. The process according to any one of the preceding claims wherein the first HIC material and the second HIC material are directly connected in series.

5. The process according to any one of the preceding claims, wherein under the two-stage HIC conditions the first HIC material does not bind non-PEGylated protein or mono-PEGylated protein, and wherein the second HIC material does not bind non-PEGylated protein.

6. The process according to any one of the preceding claims, wherein in comparative HIC elution chromatograms the first HIC material and the second HIC material have well-resolved peaks for mono-PEGylated protein.

7. The process according to any one of the preceding claims, wherein
    a) the first HIC material has substantially the same selectivity as Phenyl Sepharose HP under the two-stage HIC conditions; and

b) the second HIC material has substantially the same selectivity as Toyopearl Phenyl 650M under the two-stage HIC conditions.

8. The process according to any one of the preceding claims, wherein
   a) the first HIC material is Phenyl Sepharose HP; and/or
   b) the second HIC material is Toyopearl Phenyl 650M.

9. The process according to any one of the preceding claims, comprising the further step of:
   d) eluting the oligo-PEGylated protein from the first HIC material to provide a first HIC eluate.

10. The process according to claim 9, wherein eluting the oligo-PEGylated protein from the first HIC material comprises a linear gradient elution.

11. The process according to any one of the preceding claims, wherein
   i. the two-stage HIC conditions are at a conductivity of ~~about~~ 54-55 mS/cm; and/or
   ii. eluting the mono-PEGylated protein from the second HIC material is at a conductivity of ~~about~~ 40-41 mS/cm; and/or
   iii. eluting the oligo-PEGylated protein from the first HIC material is carried out by linear gradient from ~~about~~ 54-55 mS/cm to ~~about~~ 1-5 mS.cm
   and wherein optionally the protein is EPO.

12. The process according to any one of the preceding claims, wherein the process is carried out using a mixture of buffer A and buffer B, wherein buffer A comprises 25mM HEPES, pH 7.5, 500 mM Na_2SO_4, and wherein buffer B comprises 25mM HEPES, pH 7.5, and wherein
   i. the two stage HIC conditions are at ~~about~~ 13.5% buffer B; and/or
   ii. eluting the mono-PEGylated protein from the second HIC material is at ~~about~~ 40% buffer B; and/or
   iii. eluting the oligo-PEGylated protein from the first HIC material is carried out by linear gradient from ~~about~~ 13.5% to ~~about~~ 80% buffer B
   and wherein optionally the protein is EPO.

13. The process according to any one of the preceding claims, wherein elution of the mono-PEGylated protein from the second HIC material comprises a step elution, optionally followed by a gradient elution.

14. The process according to any one of the preceding claims, wherein the mono-PEGylated protein composition comprises at least ~~about~~ 99% mono-PEGylated protein.

15. The process according to any one of the preceding claims, wherein the protein mixture comprises at least 10% oligo-PEGylated protein.

16. 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.

17. The process according to any one of the preceding claims wherein the mono-PEGylated protein composition is a pharmaceutical composition, the process further comprising formulating the second HIC eluate with a pharmaceutical excipient.