

Claims:

1. A process for producing a mono-PEGylated protein composition comprising at least about 90% mono-PEGylated protein, the process comprising:
 - 5 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:
 - 10 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,
 - 15 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.
- 20 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.
- 25 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.
- 30 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.
- 35 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
 - 40 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.
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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.
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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.
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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 Na₂SO₄, 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.
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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.
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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.
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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.