target tissue than a hemoglobin molecule with higher oxygen affinity. Therefore it would be desirable to control the oxygen affinity of the crosslinked tetrameric hemoglobin. Thus, there is a need in the art to control the type of cross-linking and cross-linking conditions to produce crosslinked tetrameric hemoglobin with precise levels of oxygen binding.

Summary of Invention

[0010] The present disclosure~~invention~~ provides a method for processing a non-polymeric, heat stable purified crosslinked tetrameric hemoglobin suitable for use in mammals without causing severe renal injury, vascular detrimental effects and severe adverse events including death. The present disclosure~~invention~~ removes the dimeric form of hemoglobin, non-crosslinked tetrameric hemoglobin, phospholipids and protein impurities. The present disclosure~~invention~~ also provides a technique for controlling the oxygen affinity of the resultant crosslinked tetramer by controlling the type of cross-linking in the tetramer (e.g., the amount of beta-beta cross-linking, alpha-alpha cross-linking, alpha-beta cross-linking in the tetramer), the quaternary structure of tetramer, and the cross-linking conditions. Lower oxygen affinity crosslinked hemoglobin is useful for applications requiring rapid tissue oxygenation (e.g. hemorrhagic shock) while higher oxygen affinity crosslinked hemoglobin is useful for applications requiring a slower rate of oxygenation (e.g. cancer adjunct therapy). Additionally, the present disclosure~~invention~~ uses (1) an instant cytolysis apparatus for precise and controlled hypotonic lysis, (2) a flowthrough column chromatography, (3) a high temperature short time (HTST) apparatus for heat processing the hemoglobin solution in the purification process to remove the undesirable non-stabilized dimers of hemoglobin and to remove the protein impurities, for example immunoglobin-G, so that renal injury, vascular detrimental effects and other toxicity reactions can be avoided, and (4) an air-tight infusion bag packaging to avoid oxygen intrusion into the final product.

treatment and cooling, N-acetyl cysteine is optionally added at a concentration of approximately 0.025% to 0.4% to further prevent formation of met-hemoglobin. The heat treatment is preferably a high temperature short time treatment conducted at approximately 70°C to 95°C for 30 seconds to 3 hours with subsequent cooling to 25°C. Any precipitates formed during the heat treatment are removed by centrifugation or filtration.

[0015] The dimer-free, phospholipid-free, protein impurities-free, heat stable, non-polymeric crosslinked tetrameric hemoglobin is then added to a pharmaceutically acceptable carrier.

[0016] Thereafter, the heat stable, crosslinked tetrameric hemoglobin is formulated and packaged in a custom-made and air-tight polyethylene, ethylene-vinyl-acetate, and ethylene-vinyl alcohol (PE, EVA, EVOH) infusion bag. The packaging prevents oxygen contamination which results in the formation of inactive met-hemoglobin.

[0016a] Accordingly, in one aspect, there is provided a method for the preparation of a highly purified and heat stable oxygen carrier-containing

pharmaceutical composition, the oxygen carrier-containing pharmaceutical composition including hemoglobin, the hemoglobin consisting essentially of non-polymeric crosslinked tetrameric hemoglobin having a beta-beta cross-linking of greater than 40%, the method comprising:

a) providing mammalian whole blood including at least red blood cells and plasma;

b) separating the red blood cells from the plasma in the mammalian whole blood;

c) filtering the red blood cells that were separated from the plasma to obtain a filtered red blood cell fraction;

d) washing the filtered red blood cell fraction to remove plasma protein impurities, resulting in washed red blood cells;

e) disrupting the washed red blood cells to create a solution comprising a lysate of disrupted red blood cells;

f) performing filtration to remove at least a portion of the waste retentate from the lysate;

g) extracting a first hemoglobin solution from the lysate;

h) performing at least one purification process to remove one or more of viruses, waste retentate, or protein impurities;

i) cross-linking the first hemoglobin solution by bis-3,5-dibromosalicyl fumarate to form crosslinked hemoglobin in an oxygenated environment wherein the crosslinked hemoglobin is non-polymeric crosslinked tetrameric hemoglobin having at least 40% beta-beta cross-linking;

j) removing any residual chemicals;

k) heat treating the crosslinked hemoglobin in a deoxygenated environment to denature and precipitate any residual non-stabilized/non-crosslinked hemoglobin, any dimeric hemoglobin and any other protein impurities such that the resulting heat stable crosslinked tetrameric hemoglobin has an undetectable concentration of dimer and consists essentially of non-polymeric crosslinked tetrameric hemoglobin with a beta-beta cross-linking of at least 40% and an oxygen affinity greater than the oxygen affinity of native hemoglobin of the same species measured under substantially similar conditions;

l) removing precipitate by a centrifugation or a filtration to form a clear solution; and

m) adding the purified and heat stable crosslinked tetrameric hemoglobin to a pharmaceutically acceptable carrier.

[0016b] In another aspect, there is provided a method for the preparation of a highly purified and heat stable oxygen carrier-containing pharmaceutical composition, the oxygen carrier-containing pharmaceutical composition including hemoglobin, the hemoglobin consisting essentially of non-polymeric crosslinked tetrameric hemoglobin having a beta-beta cross-linking of greater than 50%, the method comprising:

a) providing mammalian whole blood including at least red blood cells and plasma;

b) separating the red blood cells from the plasma in the mammalian whole blood;

c) filtering the red blood cells that were separated from the plasma to obtain a filtered red blood cell fraction;

d) washing the filtered red blood cell fraction to remove plasma protein impurities, resulting in washed red blood cells;

e) disrupting the washed red blood cells to create a solution comprising a lysate of disrupted red blood cells;

f) performing filtration to remove at least a portion of the waste retentate from the lysate;

g) extracting a first hemoglobin solution from the lysate;

h) performing at least one purification process to remove one or more of viruses, waste retentate, or protein impurities;

i) cross-linking the hemoglobin by bis-3,5-dibromosalicyl fumarate to form crosslinked hemoglobin in a deoxygenated environment wherein the crosslinked hemoglobin is non-polymeric crosslinked tetrameric hemoglobin having at least 50% beta-beta cross-linking;

j) removing any residual chemicals;

k) heat treating the crosslinked hemoglobin in a deoxygenated environment to denature and precipitate any residual non-stabilized/non-crosslinked hemoglobin, any dimeric hemoglobin and any other protein impurities such that the resulting heat stable crosslinked tetrameric hemoglobin has an undetectable concentration of dimer and consists essentially of non-polymeric crosslinked tetrameric hemoglobin with a beta-beta cross-linking of at least 50% having an oxygen affinity less than the oxygen affinity of native hemoglobin of the same species measured under substantially similar conditions;

l) removing precipitate by a centrifugation or a filtration to form a clear solution; and

m) adding the purified and heat stable crosslinked tetrameric hemoglobin to a pharmaceutically acceptable carrier.

Brief Description of the Drawings

[0017] FIG. 1 is a flow-chart depicting an overview of a process of the present invention.

[0018] FIG. 2 schematically depicts an instant cytolysis apparatus used in the process of the present invention.

[0019] FIG. 3 depicts high performance liquid chromatography analysis for (a) non-heat treated crosslinked tetrameric hemoglobin, and (b) heat stable crosslinked tetrameric hemoglobin which has undergone a heat treatment at 90°C for 45 seconds to 2 minutes or 80°C for 30 minutes.

[0020] FIG. 4 is an elution profile for flowthrough column chromatography; the hemoglobin solution is in the flowthrough fraction.

[0021] FIG. 5 schematically depicts a flowthrough CM column chromatography system with ultrafiltration for an industrial scale operation.

[0022] FIG. 6 is a schematic depiction of an apparatus used for HTST heat processing step.