production phase. Thus, there is a need for controlling cell growth once an optimal VCD has been obtained and thus minimizing cell bleed to increase the product recovered per perfusion run and generating a more efficient method for operating perfusion processes.

[013] In the present disclosure a perfusion culture medium comprising a retinoid and Fe ions and methods of culturing mammalian cells using said medium are provided. It is shown that vitamin A induced cell growth arrest in the presence of Fe ions of perfusion Chinese hamster ovary (CHO) cell cultures. This medium can inhibit proliferation without negatively affecting viability and/or cell specific productivity (qP). By controlling cell growth and thus minimizing cell bleed, the product recovered per perfusion run is greatly increased, generating a more efficient method for operating perfusion processes.

[014] The present disclosure provides for a method of culturing mammalian cells expressing a heterologous protein in a perfusion cell culture is provided comprising: (a) culturing mammalian cells expressing a heterologous protein in a serum-free culture medium; (b) culturing the mammalian cells during growth phase by perfusion with a serum-free perfusion medium; and (c) maintaining the mammalian cells during production phase by perfusion with a serum-free perfusion medium comprising Fe ions at a concentration of 230 µM to 3.5 mM and a retinoid at a concentration of 10 µM to 400 µM, wherein step (b) is optional. In one aspect, the present disclosure provides for a method of culturing Chinese hamster ovary (CHO) cells expressing a heterologous protein in a perfusion cell culture is provided comprising: (a) culturing CHO cells expressing a heterologous protein in a serum-free culture medium; (b) culturing the CHO cells during growth phase by perfusion with a chemically defined serum-free perfusion medium; and (c) maintaining the CHO cells during production phase by perfusion with a chemically defined serum-free perfusion medium comprising Fe ions at a concentration of 230 µM to 3.5 mM and a retinoid at a concentration of 10 µM to 400 µM, wherein the retinoid is retinol, retinal, retinoic acid or a retinyl ester; wherein the osmolarity of the chemically defined serum-free perfusion medium is in the range between 300 and 1400 mOsmol/kg; and wherein step (b) is optional. In one example of the method prior to step (c) the retinoid concentration in the mammalian cell culture is adjusted to the retinoid concentration in the serum-free perfusion medium of step (c), preferably by bolus addition.

[015] The present disclosure provides for a method of reducing cell bleeding in a perfusion cell culture expressing a heterologous protein is provided comprising: (a) culturing mammalian cells expressing a heterologous protein in a serum-free culture medium; (b) culturing the mammalian cells during growth phase by perfusion with a serum-free perfusion medium; and (c) maintaining the mammalian cells during production phase by perfusion with a serum-free perfusion medium comprising Fe ions at a concentration of 230 µM to 3.5 mM and a retinoid at a concentration of 10 µM to 400 µM, wherein step (b) is optional. In another aspect, the present disclosure provides for a method of reducing cell bleeding in a perfusion cell culture expressing a heterologous protein comprising: (a) culturing CHO cells expressing a heterologous protein in a serum-free culture medium; (b) culturing the CHO cells during growth phase by perfusion with a chemically defined serum-free perfusion medium; and (c) maintaining the CHO cells during production phase by perfusion with a chemically defined serum-free perfusion medium comprising Fe ions at a concentration of 230 µM to 3.5 mM and a retinoid at a concentration of 10 µM to 400 µM, wherein the retinoid is retinol, retinal, retinoic acid or a retinyl ester; wherein the osmolarity of the chemically defined serum-free perfusion medium is in the range between 300 and 1400 mOsmol/kg; and wherein step (b) is optional. In one example of the method prior to step (c) the retinoid concentration in the mammalian cell culture is adjusted to the retinoid concentration in the serum-free perfusion medium of step (c), preferably by bolus addition.

[016] The present disclosure provides for a method of increasing protein production in a perfusion cell culture expressing a heterologous protein is provided comprising: (a) culturing mammalian cells expressing a heterologous protein in a serum-free culture medium; (b) culturing the mammalian cells during growth phase by perfusion with a serum-free perfusion medium; and (c) maintaining the mammalian cells during production phase by perfusion with a serum-free perfusion medium comprising Fe ions at a concentration of 230 µM to 3.5 mM and a retinoid at a concentration of 10 µM to 400 µM, wherein step (b) is optional. In another aspect, the present disclosure provides for a method of increasing protein production in a perfusion cell culture expressing a heterologous protein comprising: (a) culturing CHO cells expressing a heterologous protein in a serum-free culture medium; (b) culturing the CHO cells during growth phase by perfusion with a chemically defined serum-free perfusion medium; and (c) maintaining the CHO cells during production phase by perfusion with a chemically defined serum-free perfusion medium comprising Fe ions at a concentration of 230 µM to 3.5 mM and a retinoid at a concentration of 10 µM to 400 µM, wherein the retinoid is retinol, retinal, retinoic acid or a retinyl ester; wherein the osmolarity of the chemically defined serum-free perfusion medium is in the range between 300 and 1400 mOsmol/kg; and wherein step (b) is optional. In one example of the method prior to step (c) the retinoid concentration in the mammalian cell culture is adjusted to the retinoid concentration in the serum-free perfusion medium of step (c), preferably by bolus addition.

[024] A method of producing a therapeutic protein using the methods of the disclosure is provided optionally comprising a further step of purifying and formulating the therapeutic protein into a pharmaceutically acceptable formulation. In another aspect, the present disclosure provides for a method of producing a heterologous protein using the method of culturing CHO cells as described herein. In another aspect, the present disclosure provides for a method of producing a heterologous protein using the method of reducing cell bleeding as described herein. In another aspect, the present disclosure provides for a method of producing a heterologous protein using the method of increasing protein production as described herein.

[025] The present disclosure provides for a serum-free perfusion medium is provided comprising Fe ions at a concentration ranging from 230 µM to 3.5 mM and a retinoid in a concentration ranging from 10 µM to 400 µM. In another aspect, the present disclosure provides for a chemically defined serum-free perfusion medium comprising Fe ions at a concentration ranging from 230 µM to 3.5 mM and a retinoid in a concentration ranging from 10 µM to 400 µM, wherein the retinoid is retinol, retinal, retinoic acid or a retinyl ester; wherein the osmolarity of the chemically defined serum-free perfusion medium is in the range between 300 and 1400 mOsmol/kg. In some examples the retinoid concentration is about 60 µM to 400 µM, preferably about 100 µM to about 200 µM and more preferably about 150 µM to about 200 µM. In some examples the Fe ion concentration is about 350 µM to 3.5 mM, preferably about 1 mM to 3.5 mM, more preferably about 1.4 mM to 3.5 mM and even more preferably about 1.6 mM to 3.5 mM or the Fe ion concentration is about 350 µM to about 3.1 mM, preferably about 1 mM to about 3.1 mM, more preferably about 1.4 mM to about 3.1 mM and even more preferably about 1.6 mM to about 3.1 mM.

[026] The retinoid may be retinol, retinal, retinoic acid or a retinyl ester and is preferably retinol or a retinyl ester. The retinyl ester may be retinyl acetate or retinyl palmitate, preferably retinyl acetate. The Fe ion may be provided as $FeSO_4$, Fe-citrate, Fe-transferrin, iron choline citrate, Fe-EDTA, $Fe(NO_3)_3$, $FeCl_2$, $FeCl_3$, or combinations thereof, preferably as $FeSO_4$, Fe-citrate, $Fe(NO_3)_3$, Fe-transferrin, or combinations thereof, more preferably as Fe-citrate.

[027] The osmolarity of the serum-free perfusion medium should be in the range of between 300 and 1400 mOsmol/kg, preferably between 300 and 500 mOsmol/kg, more preferably between 330 and 450 mOsmol/kg and even more preferably between 360 and 390 mOsmol/kg.

[028] The serum-free perfusion medium may be chemically defined and/or hydrolysate free. Preferably the serum-free perfusion medium is protein-free or protein-free except for recombinant insulin and/or insulin-like growth factor, more preferably the serum-free perfusion medium is chemically defined and protein-free or protein-free except for recombinant insulin and/or insulin-like growth factor.

[029] A use of the serum-free perfusion medium of the disclosure for culturing mammalian cells in a perfusion culture during production phase or for reducing the total cell bleed volume in a perfusion culture is provided. Alternatively a use of the serum-free perfusion medium of the disclosure for increasing protein production in a perfusion cell culture is provided. In another aspect, the present disclosure provides for a use of the chemically defined serum-free perfusion medium as described herein for culturing CHO cells in a perfusion culture during production phase. In another aspect, the present disclosure provides for a use of the chemically defined serum-free perfusion medium as described herein for reducing the total cell bleed volume in a perfusion culture. In another aspect, the present disclosure provides for a use of the chemically defined serum-free perfusion medium as described herein for increasing protein production in a perfusion cell culture.

BRIEF DESCRIPTION OF THE DRAWINGS

[030] Figure 1: CHO cells in small scale culture were exposed to control (diamonds), ethanol control (squares), 5 µM retinyl acetate (triangles) and 20 µM retinyl acetate (X) using a basal medium containing 3.1 mM ferric citrate. Shown in panel (A) is the viable cell density (e5 cells/mL, which is the technical spelling for "10^5 cells/mL") and in panel (B) the viability of CHO cells in the culture. Data represent average of triplicates; error bars represent standard deviation.

CLAIMS

1. A method of culturing Chinese hamster ovary (CHO) cells expressing a heterologous protein in a perfusion cell culture comprising:

   (a) culturing CHO cells expressing a heterologous protein in a serum-free culture medium;

   (b) culturing the CHO cells during growth phase by perfusion with a chemically defined serum-free perfusion medium; and

   (c) maintaining the CHO cells during production phase by perfusion with a chemically defined serum-free perfusion medium comprising Fe ions at a concentration of 230 µM to 3.5 mM and a retinoid at a concentration of 10 µM to 400 µM,

   wherein the retinoid is retinol, retinal, retinoic acid or a retinyl ester; wherein the osmolarity of the chemically defined serum-free perfusion medium is in the range between 300 and 1400 mOsmol/kg; and wherein step (b) is optional.

2. A method of reducing cell bleeding in a perfusion cell culture expressing a heterologous protein comprising:

   (a) culturing CHO cells expressing a heterologous protein in a serum-free culture medium;

   (b) culturing the CHO cells during growth phase by perfusion with a chemically defined serum-free perfusion medium; and

   (c) maintaining the CHO cells during production phase by perfusion with a chemically defined serum-free perfusion medium comprising Fe ions at a concentration of 230 µM to 3.5 mM and a retinoid at a concentration of 10 µM to 400 µM,

   wherein the retinoid is retinol, retinal, retinoic acid or a retinyl ester; wherein the osmolarity of the chemically defined serum-free perfusion medium is in the range between 300 and 1400 mOsmol/kg; and wherein step (b) is optional.

3. A method of increasing protein production in a perfusion cell culture expressing a heterologous protein comprising:

   (a) culturing CHO cells expressing a heterologous protein in a serum-free culture medium;

(b) culturing the CHO cells during growth phase by perfusion with a chemically defined serum-free perfusion medium; and

(c) maintaining the CHO cells during production phase by perfusion with a chemically defined serum-free perfusion medium comprising Fe ions at a concentration of 230 µM to 3.5 mM and a retinoid at a concentration of 10 µM to 400 µM,

wherein the retinoid is retinol, retinal, retinoic acid or a retinyl ester; wherein the osmolarity of the chemically defined serum-free perfusion medium is in the range between 300 and 1400 mOsmol/kg; and wherein step (b) is optional.

4. The method of any one of claims 1 to 3, wherein

(i) step (c) further comprises maintaining a cell density by cell bleeding, and/or

(ii) prior to step (c) the retinoid concentration in the CHO cell culture is adjusted to the retinoid concentration in the chemically defined serum-free perfusion medium of step (c), preferably by bolus addition.

5. The method of any one of the preceding claims, wherein the retinoid is retinol or a retinyl ester, preferably retinyl acetate or retinyl palmitate, more preferred retinyl acetate.

6. The method of any one of the preceding claims, wherein the Fe ion is provided as $FeSO_4$, Fe-citrate, Fe-transferrin, iron choline citrate, Fe-EDTA, $Fe(NO_3)_3$, $FeCl_2$, $FeCl_3$, or combinations thereof, preferably $FeSO_4$, Fe-citrate, $Fe(NO_3)_3$, Fe-transferrin, or combinations thereof, more preferred Fe-citrate.

7. The method of any one of the preceding claims, wherein the CHO cell is a CHO-DG44 cell, a CHO-K1 cell, a CHO DXB11 cell, a CHO-S cell, or a CHO GS deficient cell.

8. The method of any one of the preceding claims, further comprising harvesting the heterologous protein from the perfusion cell culture.

9. The method of any one of the preceding claims, wherein the heterologous protein is selected from the group consisting of an antibody, a fusion protein, a cytokine and a growth factor.

10. A method of producing a heterologous protein using the method of culturing CHO cells of any one of claims 1 and 4 to 9.

11. A method of producing a heterologous protein using the method of reducing cell bleeding of any one of claims 2 and 4 to 9.

12. A method of producing a heterologous protein using the method of increasing protein production of any one of claims 3 to 9.

13. A chemically defined serum-free perfusion medium comprising Fe ions at a concentration ranging from 230 µM to 3.5 mM and a retinoid in a concentration ranging from 10 µM to 400 µM, wherein the retinoid is retinol, retinal, retinoic acid or a retinyl ester; wherein the osmolarity of the chemically defined serum-free perfusion medium is in the range between 300 and 1400 mOsmol/kg.

14. Use of the chemically defined serum-free perfusion medium of claim 13 for culturing CHO cells in a perfusion culture during production phase.

15. Use of the chemically defined serum-free perfusion medium of claim 13 for reducing the total cell bleed volume in a perfusion culture.

16. Use of the chemically defined serum-free perfusion medium of claim 13 for increasing protein production in a perfusion cell culture.