to suppress cell growth in the perfusion state and avoid the need for cell bleeding would significantly help advance the art.

[011] Osmolality has been a known lever to impact cell growth.. Prior art using osmolality to affect cell growth is known in the literature (Zhu, et al (2005) Biotechnology Progress 21, 70-77; Han, Koo and Lee (2009) Biotechnology Progress 25, 1440-1447; Hu and Aunins (1997) Current Opinion in Biotechnology, 148-153). However, the ability to control a cell culture process to a target osmolality, particularly in perfusion culture has never been established. Also, chemical additives affect media composition and/or need to be cleared in subsequent purification steps, thereby increasing process complexity. Chemical additives including salts may also affect product quality.

[012] In view of the challenges with perfusion cell culture such as prepared media consumption and the desire to further increased productivity, media that improve logistic problems and methods that are effective in suppressing cell growth in the perfusion state and avoid the need for cell bleeding without further additives would significantly help advance the art.

SUMMARY

[013] The present disclosure relates in part to the discovery that feed media can be developed in a more concentrated form by compartmentalization to reduce the cell specific perfusion rate and the volume of prepared media consumed. These concentrated feeds are diluted in the bioreactor vessel. It is advantageously used with sterilized, de-ionized water as a diluent which does not require preparation other than filtering. Additionally, the unique combination of the 3 media concentrates (acidic, basic, and near neutral) designed in this disclosure allows for the use of higher-fold concentrates, thereby reducing the total media volume further. By reducing the prepared volume of media, a perfusion process has been developed that takes away media volume as a bottleneck, therefore can justify scale up of the perfusion cell culture process to 1000 L scale and potentially above.

[014] The use of separate concentrated feeds and a diluent also allows to control cell growth by culture osmolality. Using the concept of mass balance, an osmo balance was derived with known osmolality of each of the concentrated feeds, feed rates, calculated daily cell specific osmolality consumption rate, to predict the culture residual osmolality as an

output. Using this method, the growth of the cell culture can be controlled by increasing the residual osmolality to physiologically stressful levels (about 350-400 mOsm or higher) while remaining below the cytotoxic level (about 400 mOsm or higher). The physiologically stressful levels as well as the cytotoxic level may be cell line specific. However, this can be easily determined by measuring the viable cell concentrations and viability at different osmolality levels during cultivation, which may be performed in a small scale such as 3 ml working volume. In the current disclosure, the culture osmolality is controlled via an osmo balance which includes changes in feed rates of the media concentrates from a day-to-day basis at a fixed vessel volume per day (VVD) or changes in feed rates of the VVD from a day-to day basis at fixed feed rates of the media concentrates. The osmo balance is capable of targeting a higher or lower residual osmolality by adjusting concentrates and diluent rate, whereas the chemical additives approach that others have used can only adjust osmolality in the increasing direction. It was found that the osmo balance as described herein was effective in suppressing cell growth, and that this growth suppression led to an increase in cell specific productivity and helped in maintaining high viability in a cell culture.

[015] The cell growth suppression by the osmo balance described herein not only leads to an increase in cell specific productivity and sustained high cell viability, in perfusion cell culture it also reduces or eliminates the need to employ cell bleeding techniques during the perfusion state to otherwise maintain the cells in a steady state of growth. This reduces or eliminates product loss due to wasteful and undesirable cell bleeding techniques.

[016] The tripartite highly concentrated feed media provided herein can theoretically be used in connection with any type of cell culture system, but are particularly advantageous in continuous perfusion cell culture systems. Thus the serum-free cell culture perfusion medium according to the disclosure is particularly suitable for use in a continuous perfusion cell culture system. Also the osmo balance may theoretically be used in connection with any type of cell culture system. However, it is particularly advantageous when the cell culture system is a continuous perfusion cell culture system.

[017] In one aspect the disclosure relates to a compartmentalized serum-free cell culture perfusion medium comprising the medium components subgrouped into at least three separate aqueous concentrated feeds and a diluent, wherein the first concentrated feed is an alkaline concentrated feed, the second concentrated feed is an acidic concentrated feed and the third concentrated feed is a near neutral concentrated feed; wherein the

compartmentalized serum-free cell culture perfusion medium is pH-adjusting to neutral pH upon mixing of the at least three separate aqueous concentrated feeds and the diluent in the resulting serum-free cell culture perfusion medium. In a preferred example the at least three separate aqueous concentrated feeds are not premixed prior to addition to the cell culture and/or the reaction vessel of the bioreactor. The diluent is preferably sterile water. In one example the resulting serum-free cell culture perfusion medium has a pH of between 6.7 and 7.5, between 6.9 and 7.4, preferably between 6.9 and 7.2, upon mixing of the at least three separate aqueous concentrated feeds and the diluent. The compartmentalized serum-free cell culture perfusion medium according to the disclosure is suitable for separate addition of the alkaline concentrated feed, the acidic concentrated feed and the near neutral concentrated feed to a cell culture and/or a reaction vessel of a bioreactor; direct addition of the alkaline concentrated feed, the acidic concentrated feed and the near neutral concentrated feed to a cell culture and/or a reaction vessel of a bioreactor without prior pre-mixing; and/or direct mixing of the at least three separate aqueous concentrated feeds in a cell culture and/or a reaction vessel of a bioreactor.

[018] In certain examples, the alkaline concentrated feed is a 2x to 80x concentrated feed, the acidic concentrated feed is a 2x to 40x concentrated feed and the near neutral concentrated feed is a 2x to 50x concentrated feed. The near neutral concentrated feed has a pH of about 6.5 to about 8.5. Preferably, the alkaline concentrated feed has a pH of about 9 or higher, the acidic concentrated feed has a pH of about 5 or lower and the near neutral concentrated feed has a pH of about 7 to about 8.5. Also, the ratio (v/v/v) of the alkaline concentrated feed to the acidic concentrated feed to the near neutral concentrated feed is a fixed ratio to provide the resulting serum-free cell culture perfusion medium that is pH-adjusting to a neutral pH; and the ratio (v/v) of the diluent to the cumulative volume of the at least three separate aqueous concentrated feeds in the resulting serum-free cell culture perfusion medium that is pH-adjusting to a neutral pH determines the osmolality of the serum-free cell culture perfusion medium.

[019] The acidic concentrated feed may comprise trace elements, trace metals, inorganic salts, chelators, polyamines, and regulatory hormones. The acidic concentrated feed and/or the near neutral concentrated feed may comprise surfactants, anti-oxidants, and carbon sources. Further, the alkaline concentrated feed comprises amino acids with maximum solubility at alkaline pH of 9 or higher, preferably comprising at least aspartic acid, histidine

and tyrosine, and optionally cysteine and/or cystine and/or folic acid. The remaining amino acids are in the acidic and/or near neutral concentrated feed, preferably in the acidic concentrated feed. Preferably the vitamins and the metals are in separate feeds, preferably vitamins are in the near neutral feed and metals are in the acidic feed. Vitamins poorly soluble in aqueous solutions, such as choline chloride, are present in the neutral feed and the acidic feed.

[020] In another aspect, the disclosure also relates to an alkaline aqueous concentrated feed for combination with an acidic aqueous concentrated feed, a near neutral aqueous concentrated feed and a diluent to form a serum-free cell culture perfusion medium, wherein the pH of the resulting serum-free cell culture perfusion medium is automatically adjusted to a neutral pH. In another aspect, the disclosure relates to an acidic aqueous concentrated feed for combination with an alkaline aqueous concentrated feed, a near neutral aqueous concentrated feed and a diluent to form a serum-free cell culture perfusion medium, wherein the pH of the resulting serum-free cell culture perfusion medium is automatically adjusted to a neutral pH. In yet another aspect the disclosure relates to a near neutral aqueous concentrated feed for combination with an alkaline aqueous concentrated feed, an acidic aqueous concentrated feed and a diluent to form a serum-free cell culture perfusion medium, wherein the pH of the resulting serum-free cell culture perfusion medium is automatically adjusted to a neutral pH.

[021] In yet another aspect the disclosure relates to a method of preparing a serum-free cell culture perfusion medium, the method comprising (a) providing the components of a cell culture media in at least three subgroups of components based on solubility at alkaline, acidic and neutral pH, (b) dissolving (i) the subgroup of components soluble at alkaline pH in an alkaline aqueous solution to form an alkaline concentrated feed; (ii) the subgroup of components soluble at acidic pH in an acidic aqueous solution to form an acidic concentrated feed; and (iii) the subgroup of components soluble at neutral pH in a neutral aqueous solution to form a near neutral concentrated feed; (c) optionally storing the prepared alkaline concentrated feed, acidic concentrated feed and near neutral concentrated feed in separate containers; and (d) adding the prepared alkaline concentrated feed, acidic concentrated feed and near neutral concentrated feed and the diluent to the cell culture and/or the reaction vessel of the bioreactor, wherein (i) the alkaline concentrated feed, the acidic concentrated feed and the near neutral concentrated feed are added separately to the cell culture and/or the reaction vessel of the bioreactor; and (ii) the diluent is added separately to the cell culture

and/or the reaction vessel of the bioreactor or the diluent is premixed with one of the at least three separate aqueous concentrated feeds immediately before addition to the cell culture and/or the reaction vessel of the bioreactor; wherein the pH of the resulting serum-free cell culture perfusion medium is automatically pH adjusted to a neutral pH upon mixing of the at least three separate aqueous concentrated feeds and the diluent. The diluent is preferably sterile water. In one example the resulting serum-free cell culture perfusion medium prepared by the method has a pH of between 6.7 and 7.5, between 6.9 and 7.4, preferably between 6.9 and 7.2, upon mixing of the at least three separate aqueous concentrated feeds and the diluent.

[022] In certain examples the at least three concentrated feeds are added drop-wise through separate ports to the cell culture and/or the reaction vessel of the bioreactor. The in-vessel mixing and dilution of the at least three separate aqueous concentrated feeds allows 50-90%, preferably 60-90% lower prepared medium consumption over a culture period of 14 days compared to a serum-free cell culture perfusion medium mixed and diluted prior to addition to the bioreactor. Typically the cell culture and/or the reaction vessel of the bioreactor comprise(s) mammalian cells. Also the method further comprises a step of sterilizing the concentrated feeds prior to storage and/or addition to the cell culture and/or the reaction vessel of the bioreactor.

[023] In certain examples the alkaline concentrated feed is a 2x to 80x concentrated feed, wherein the acidic concentrated feed is a 2x to 40x concentrated feed and the near neutral concentrated feed is a 2x to 50x concentrated feed. The near neutral concentrated feed has a pH of 6.5-8.5. Preferably, the alkaline concentrated feed has a pH of 9 or higher, the acidic concentrated feed has a pH of 5 or lower and the near neutral concentrated feed has a pH of 7 to 8.5. Also, the ratio (v/v/v) of the alkaline concentrated feed to the acidic concentrated feed to the near neutral concentrated feed is a fixed ratio to provide the resulting serum-free cell culture perfusion medium that is pH-adjusting to a neutral pH in the cell culture and/or the reaction vessel of the bioreactor; and the ratio (v/v) of the diluent to the cumulative volume of the at least three separate aqueous concentrated feeds added to the cell culture and/or the reaction vessel of the bioreactor to provide the resulting serum-free cell culture perfusion medium that is pH-adjusting to a near neutral pH determines the osmolality of the serum-free cell culture perfusion medium in the cell culture and/or the reaction vessel of the bioreactor. In certain example, the cell culture and/or the reaction vessel of the bioreactor

comprise at least about 100 L serum-free cell culture perfusion medium, preferably at least about 1000 L serum-free cell culture perfusion medium. Preferably the cell culture has a volume of at least about 100 L and/or the bioreactor has a volume of at least about 100 L. More preferably the cell culture has a volume of at least about 1000 L and/or the bioreactor has a volume of at least about 1000 L.

[024] In another aspect the disclosure relates to a serum-free cell culture perfusion medium obtainable by the method according to the disclosure.

[025] In another aspect the disclosure relates to a method of culturing mammalian cells expressing a heterologous protein in perfusion culture, comprising: (a) inoculating a bioreactor with mammalian cells expressing a heterologous protein in a serum-free cell culture medium; (b) culturing the mammalian cells in a perfusion culture by continuously feeding the mammalian cells with a serum-free cell culture perfusion medium feed and removing spent media while keeping the cells in culture, wherein the serum-free cell culture perfusion medium feed is (i) a compartmentalized serum-free cell culture perfusion medium comprising the medium components subgrouped into at least three separate aqueous concentrated feeds and a diluent, wherein the first concentrated feed is an alkaline concentrated feed, the second concentrated feed is an acidic concentrated feed and the third concentrated feed is a near neutral concentrated feed; and wherein the compartmentalized serum-free cell culture perfusion medium is pH-adjusting to neutral pH upon mixing of the at least three separate aqueous concentrated feeds and the diluent in the resulting serum-free cell culture perfusion medium; and/or (ii) the serum-free cell culture perfusion medium obtainable by the method according to the disclosure, and wherein the alkaline concentrated feed, the acidic concentrated feed and the near neutral concentrated feed of the compartmentalized serum-free cell culture perfusion medium feed are added separately to the cell culture and/or the reaction vessel of the bioreactor and wherein the diluent is added separately to the cell culture and/or the reaction vessel of the bioreactor or the diluent is premixed with one of the at least three separate aqueous concentrated feeds immediately before addition to the cell culture and/or the reaction vessel of the bioreactor. The method typically further comprises harvesting the heterologous protein from the cell culture.

[026] The mammalian cells may initially be cultured as a batch culture before perfusion culture is started and/or perfusion culture starts from days 0 to day 3 of the culture, i.e., post-inoculation. Typically the perfusion rate increases after perfusion has started until a target

viable cell density has been reached. In certain examples the perfusion rate increases from less or equal to 0.5 vessel volumes per day to about 5 vessel volumes per day, or from less or equal to 0.5 vessel volumes per day to about 2 vessel volumes per day.

[027] In certain examples the osmolality of the serum-free cell culture perfusion medium is increased above the optimal osmolality level for growth, resulting in growth suppression at a target viable cell density, preferably wherein the osmolality level of the serum-free cell culture perfusion medium is increased gradually or stepwise starting at about half the target viable cell density. The target viable cell density is about 30 x 10^6 cells/ml or higher, about 60 x 10^6 cells/ml or higher, about 80 x 10^6 cells/ml, preferably about 100 x 10^6 cells/ml or higher. Osmolality may be controlled using (a) a constant concentrated feed perfusion rate and a varying diluent perfusion rate, resulting in a varying overall perfusion rate; or (b) a constant overall perfusion rate and a varying concentrated feed perfusion rate; wherein the at least three concentrated feeds are added at a fixed ratio (v/v/v) to each other depending on their fold-concentration to maintain the relative proportion of the medium components in the 1x serum-free cell culture perfusion medium. Thus, the osmolality may be increased using (a) a constant concentrated feed perfusion rate and a decreased diluent perfusion rate, resulting in a decreased overall perfusion rate; or (b) a constant overall perfusion rate and an increased concentrated feed perfusion rate with a decreased diluent perfusion rate; wherein the at least three concentrated feeds are added at a fixed ratio (v/v/v) to each other depending on their fold-concentration to maintain the relative proportion of the medium components in the 1x serum-free cell culture perfusion medium. Preferably no further additive is added to the culture for increasing the osmolality.

[028] The person skilled in the art will know how to determine the optimal osmolality level for growth of the mammalian cell. In one example the optimal osmolality level for growth of the mammalian cell is about 280 to less than 350 mOsm. The osmolality is maintained at a level optimal for growth until about half the target viable cell density is reached. Preferably the osmolality is increased gradually or stepwise starting at about half the target viable cell density, preferably to about 10-50% of the optimal osmolality level for growth. The osmolality is increased to and maintained at an osmolality level that suppresses cell growth at about the target viable cell density, wherein the osmolality level that suppresses cell growth of the mammalian cell in one example is about 350 mOsm or higher, preferably about 380 mOsm

or higher. Increasing the osmolality reduces or eliminates the need for cell bleeding during production phase.

[029] When increasing osmolality, cell growth is suppressed to maintain a sustainable viable cell density without cell bleeding. The yield of the heterologous protein produced in the cell culture is increased by at least 5-50% relative to the yield in a control cell culture, wherein the osmolality is not increased.

[030] Generally using the method of the present disclosure the cell specific perfusion rate (pl/cell/day) is reduced by at least 50% relative to the cell specific perfusion rate of a 1 x serum-free cell culture medium.

[031] In certain example, the cell culture and/or the reaction vessel of the bioreactor comprise at least about 100 L serum-free cell culture perfusion medium, preferably at least about 1000 L serum-free cell culture perfusion medium. Preferably the cell culture has a volume of at least about 100 L and/or the bioreactor has a volume of at least about 100 L. More preferably the cell culture has a volume of at least about 1000 L and/or the bioreactor has a volume of at least about 1000 L.

[032] The heterologous protein may be a therapeutic protein, an antibody, or a therapeutically effective fragment thereof. The mammalian cell may be any cell line, such as selected from the group consisting of Chinese Hamster Ovary (CHO) cells, Jurkat cells, 293 cells, HeLa cells, CV-1 cells, or 3T3 cells, or a derivative of any of these cells. The CHO cell can be further selected from the group consisting of a CHO-DG44 cell, a CHO-K1 cell, a CHO DXB11 cell, a CHO-S cell, and a CHO GS deficient cell or a mutant thereof.

[033] According to the method according to the disclosure further one or more supplements selected from the list of anti-foaming agents, base, glutamine and glucose may be added separately (i.e., additionally) to the cell culture.

[034] In another aspect the disclosure relates to a method of producing a therapeutic protein using the method according to the disclosure.

[035] Also provided is a use of the compartmentalized serum-free cell culture perfusion medium according to the disclosure or the serum-free cell culture perfusion medium obtainable by the method according to the disclosure for culturing mammalian cells, particularly for culturing mammalian cells in a perfusion culture. In another aspect the disclosure relates to use of the compartmentalized serum-free cell culture perfusion medium according to the disclosure or the serum-free cell culture perfusion medium according to the disclosure for culturing mammalian cells. In another aspect the disclosure relates to use of the compartmentalized serum-free cell culture perfusion medium according to the disclosure or the serum-free cell culture perfusion medium according to the disclosure for culturing mammalian cells in a perfusion culture. In certain examples, the cell specific

perfusion rate (pl/cell/day) is reduced by at least 30% relative to the cell specific perfusion rate of a 1 x serum-free cell culture medium. In another aspect the disclosure relates to use of the compartmentalized serum-free cell culture perfusion medium according to the disclosure for separate addition of the at least three separate aqueous concentrated feeds to a cell culture and/or a reaction vessel of a bioreactor.

[036] Furthermore the disclosure provides using the compartmentalized serum-free cell culture perfusion medium according to the disclosure or the serum-free cell culture perfusion medium obtainable by the method according to the disclosure for controlling osmolality in a perfusion cell culture. In another aspect the disclosure relates to use of the compartmentalized serum-free cell culture perfusion medium according to the disclosure or the serum-free cell culture perfusion medium according to the disclosure for controlling osmolality in a perfusion cell culture. Increasing the osmolality in the cell culture suppresses cell growth and increases heterologous protein production. The yield of the heterologous protein produced in the cell culture is increased by at least 5-50% relative to the yield in a control cell culture, wherein the osmolality is not increased. In one example growth suppression is sufficient to maintain a sustainable viable cell density without cell bleeding.

BRIEF DESCRIPTION OF THE DRAWINGS

[037] **Figure 1.** Bioreactor and feed set-up illustrating separate inlet additions of: acidic, basic, and neutral feeds, and diluent.

[038] **Figure 2.** Reactor volume exchanges or Perfusion rate over time, given in liter of the media (L_{media}) per liter of the bioreactor (L_{br}) and day, for an example of a typical operating perfusion rate for perfusion cultures with a feeding strategy using the combination of three media concentrates: the combination of the three media concentrates (MCs; lower dashed line), MCs combined with diluent (solid line), and a potential maximum perfusion rate of the combine feeds (upper dashed line; VVD means “vessel volumes per day”).

[039] **Figure 3.** Viable cell density (+/- 3 standard deviations; solid lines) and viability (+/- 3 SD; dashed lines) for three 100L bioreactor runs using the concentrated media feed + diluent feeding scheme. Inherent peak VCD (that is, without high osmo inhibition of growth) for this cell line is >200e6 c/mL. By increasing osmolality pre-peak, the culture growth is inhibited and peak VCD suppressed.