Freeze drying method

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ABSTRACT

The present invention relates to a method of freeze drying, in particular to the freeze drying of cell based biological material and subsequent reconstitution. The biological material may be from any source such as microbial cells, protozoal cells, animal cells or plant cells. In particular the invention relates to freeze drying mammalian cells, such as blood cells, nucleated cells and bacterial cells for therapeutic use and use in food products. The invention further provides a method of reconstitution of the freeze dried cells resulting in better viability and functionality of the cells after reconstitution.

DESCRIPTION

Freeze Drying Method

Field of the Invention

The present invention relates to a method of freeze drying, in particular to the freeze drying of biological material. The biological material may be cell based and can be from any source such as microbial cells, protozoal cells, animal cells or plant cells. In particular, the invention relates to freeze drying mammalian cells, such as blood cells and nucleated cells, as well as to bacterial cells for therapeutic use and use in food products. The present invention further provides a method of reconstitution.

Background to the Invention

There has long been a need for preservation and long term storage of biological materials, particularly bacterial cells for use in food products, red blood cells for use in transfusions, stem cells for regenerative medicines, bone marrow for transplants, cord blood for a future source of stem cells, as well as many other products.

In the past, cells such as red blood cells were preserved using sodium citrate but those would last only a few days after harvesting from the donor. Since then, nearly a hundred years ago, there have been improvements in blood preservation and processing arising from the development of modern aseptic processing techniques and reliable refrigeration systems, which enables organisations such as the UK's National Health Service Blood and Tissue (NHSBT) to assign treated donated blood a shelf life of 35 days at 2-8°C.

Nowadays, freeze drying or lyophilisation, is a commonly used technique for a variety of materials allowing preservation and long term storage of such materials. Freeze drying is being used more frequently for the preservation and storage of living materials such as bacteria and cells such as blood cells. However, viability of the freeze dried biological material after re-hydration or reconstitution provides further challenges.

Currently, the freeze drying of cells involves addition of protective agents to render the cells tolerant to freezing and desiccation, followed by cooling (freezing), sublimation and desorption steps. Since scientific studies were carried

CLAIMS (29)

Claims:

1. A method of freeze drying cell based biological material comprising the steps of incubating the material in a freeze-drying medium and a solvent, cooling the material with the medium and solvent until frozen, removing the frozen solvent and desorption of any unfrozen solvent wherein the rate of cooling is controlled to be at or greater than 0.5°C per minute.

2. A method according to claim 1 comprising an additional step of concentrating the biological material prior to freeze drying.

3. A method according to claim 1 or 2 wherein the biological material is incubated at a temperature of between 15 °C to 45°C for a period of between 1 hour to 24 hours prior to freeze drying.

4. A method according to claims 1, 2 or 3 wherein the biological material is selected from mammalian cells, microorganisms, protozoal cells, bacterial cells, viruses, fungal matter and components thereof.

5. A method according to any preceding claim wherein the freeze-drying medium comprises one or more of a buffer solution, a bulking agent, a pH stabiliser, a pH adjuster, a thermal stabiliser, a cryoprotectant, a lyoprotectant, an anti-oxidant, a biopolymer or an agent to enable active loading of the biological material with at least one of the aforementioned components.

6. A method according to claim 5 wherein the biopolymer is selected from PLP, PV-50, PL-50, PP-30, PP-50, PP-60 and PP-75.

7. A method according to claim 5 or 6 wherein the anti-oxidant is selected from Vitamin A (retinol), Vitamin C (ascorbic acid) and Vitamin E (comprising tocotrienol and tocopherol), Vitamin co-factors (such as co-enzyme Q10), minerals (such as manganese and iodine), hormones (such as melatonin), carotenoid terpenoids, flavonoid polyphenolics, phenolic acids and esters thereof, and non-flavonoid phenolics (such as curcumin, silymarin, xanthones and eugenol and biopolymers such as PP-50, PLP, PV- 50, PL-50, PP-30, PP-50, PP-60 and PP-75.

8. A method according to any one of claims 5 to 7 wherein the buffer

damage during freezing and/or drying. Investigations involving a range of potential protective agents that might be employed in the freeze-drying of cells and micro-organisms have been documented in recent decades, with the most promising agents purported to be dimethlysulfoxide (DMSO), small carbohydrates and starches (Morgan et al (2006); Wolkers et al (2001)). Studies to date with red blood cells have typically been limited to the use of trehalose as a cryo- lyo-protectant, primarily due to its historical use as a biological stabilising agent. Saccharides other than trehalose have also been found to be excellent cryo- or lyo- protectants for a number of proteins and whole organisms (Morgan et al, 2006).

Many biological materials are further dissolved or suspended in pH buffers such as PBS, prior to freeze drying in order to prevent damage by pH shifts in the liquid state. However, it has been established that some of the most widely used buffers, including PBS, can undergo pH shifts during the freezing process. In the case of PBS, an acidic pH shift has been

demonstrated by many workers, which is attributable to the crystallisation of the disodium salt, although it has also been shown that this can be moderated to some extent by adjusting the initial buffer concentration, its ratio with the protein in question, or freezing conditions (Murase and Franks (1989); Pikal et al (1991); Roy et al (1992)).

Therefore, buffers employed in freeze-drying should be examined on the basis of their freezing and drying behaviour in addition to their ability to maintain a stable pH range in the liquid state. Tris and citrate buffers have been shown not to undergo pH shifts during freezing with proteins (Carpenter et al, 2002).

Most cells and tissues are currently stored in liquid nitrogen after addition of cryoprotectants such as DMSO or glycerol. One disadvantage of this method of storage is that the level of liquid nitrogen needs regular checking and maintaining. Some tissues are stored at -80°C in freezers that are expensive to run and lead to sample loss due to, for example, ice crystal formation in long term storage.

A further disadvantage is that the cryoprotectant must be removed from the freeze-thawed sample prior to use. Several parameters in a freeze drying process affect the preservation of cells, for example, container type, fill volume, degree of container-shelf contact, sample cooling rate, freezing temperature, primary drying conditions (temperature, pressure, time), extent of desorption of unfrozen water in the 'secondary drying' phase of the lyophilisation cycle, rehydration media and methodology. Additionally, the formulation medium in which cells are suspended prior to the lyophilisation process is critical in preventing, or at least minimising, damage to cells that may be induced by the freezing or drying processes.

Freeze drying biological material such as live bacteria or cells such as red blood cells, so that they are still viable and retain cell integrity after reconstitution, presents some challenges. Maintaining the integrity of the cell membrane after reconstitution is also critical for a cell based therapy to be effective. Many regenerative medicine products rely on the delivery to patients of products that incorporate live cells. This presents a significant challenge in providing viable cells following reconstitution or re-hydration of the freeze dried cells.

Current reconstitution steps for lyophilised medical products typically involve addition of sterile water or saline solution in one or more steps and where the water or saline may be warmed prior to use in reconstitution. However, the problem associated with applying these steps to the rehydration of cells is that it creates concentration gradients in the sample during rehydration, leading to lysis of the cells.

Cryopreservation and lyophilisation have been applied to bacterial cultures (De Paz et al, 1 88), human platelets (Wolkers et al, 2001 ; WO 93/14191) and more recently to human haematopoietic stem and progenitor cells (Natan et al, 2009). EP0668013 discloses a freeze drying method involving granulation. This method

orthophosphate, disodium hydrogen orthophosphate, potassium dihydrogen orthophosphate, dipotassium hydrogen orthophosphate, citrate buffer, Tris buffer, amino acid-based buffers, TES, MOPS, PIPES, Cacodylate, SSC, MES and HEPES.

9. A method according to any one of claims 5 to 8 wherein the cryoprotectant is selected from disaccharides, lactose, trehalose, sucrose, maltose, mannose, sorbitol, amino acids, peptides, polymers and proteins.

10. A method according to any one of claims 5 to 9 wherein the lyoprotectant is selected from disaccharides, lactose, trehalose, sucrose, maltose, mannose, sorbitol, amino acids, peptides, polymers and proteins

1 1. A method according to any one of claims 5 to 10 wherein the thermal stabiliser is selected from mannitol, polymers, dextran, polyethylene glycol, polyvinyl pyrrolidone, saccharides and proteins.

12. A method according to any one of claims 5 to 11 wherein the bulking agent is selected from mannitol, polymers, dextran, polyethylene glycol, polyvinyl pyrrolidone, disaccharides, lactose, trehalose, sucrose, maltose, mannose, sorbitol and proteins.

13. A method of reconstituting freeze dried cell based biological material comprising the steps of adding a solution to the biological material that results in the final solute concentration in the solution being hypertonic to the material wherein said solution is hypertonic to the cells.

14. A method according to claim 13 wherein the reconstitution is conducted between 2°C and 40°C.

15. A method according to claim 13 or 14 wherein the solution comprises one or more of a buffer solution, an aqueous solution of a salt, phosphate buffer, hydroxy ethyl starch, anti-oxidant, glucose or dextran.

16. A method according to claim 15 wherein the salt in the salt solution is selected from sodium chloride, potassium chloride, sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate, sodium citrate or tris(hydroxymethyl)aminomethane.

17. A freeze dried composition comprising cell based biological material that has been freeze dried in a hypertonic solution said solution comprising one or more of a buffer solution, cryoprotectant, lyoprotectant, anti-oxidant, biopolymer, thermal stabiliser, antioxidant or bulking agent .

18. A freeze dried composition according to claim 17 wherein the biological material is red blood cells.

19. A freeze dried composition according to claim 17 wherein biological material is nucleated cells.

20. A freeze dried composition according to claim 17 wherein the biological material is bacterial cells.

21. A freeze dried composition according to claim 20 wherein the bacterial cells are selected from Lactobacillus crispatus, L. plantarum, Bifidobacteria, Bacillus coagulans and Saccharomyces boulardii.

22. A freeze dried composition prepared according to the method defined in any one of claims 1 to 12.

23. A freeze dried composition according to any one of claims 17 to 22 reconstituted according to the method defined in claims 13 to 16.

destabilisation of the cells and is therefore undesirable. Thus, the methods available, have not fully addressed the challenges faced in long term storage by freeze drying and rehydration or reconstitution to provide viable or intact cells.

The present invention relates to an improved method of freeze drying and reconstitution of cell based biological materials which address some of the challenges in this field. Summary of the Invention

According to a first aspect of the invention there is provided a method of freeze drying a cell based biological material comprising the steps of incubating the biological material in a suitable freeze-drying medium and solvent, cooling until frozen, removing the frozen solvent by sublimation, desorption of any unfrozen solvent and where the cooling rate is at or greater than 0.5°C per minute.

Preferably the cooling rate is between 0.5 °C to 1000 °C per minute. More preferably, the cooling rate is between 10 °C to 100 °C per minute. The cooling can be done by placing the material in a pre-cooled environment such as a freeze-dryer, a freezer or a cold liquid such as liquid nitrogen. Preferably, the material and medium is cooled down to between -20 °C to -196 °C. More preferably the material and the medium are cooled to between -40 °C and -70 °C.

24. A composition comprising reconstituted freeze dried cell based biological material prepared according to any one of the methods defined in claims 1 to 16.

25. A composition according to claim 24 wherein the cells are intact.

26. A composition according to claim 25 wherein the cells are red blood cells.

27. A kit comprising a freeze dried composition according to any one of claims 17 to 23 and a reconstituting medium wherein the medium is hypertonic to the cells in the composition.

28. A method according to claim 2 wherein the concentration step comprises suspending the cell based biological material in an aqueous medium, contacting the medium with an osmotic driver solution separated by a semi-permeable membrane.

29. A method according to claim 28 wherein the osmotic driver solution is composed of one or more of saccharides, proteins and salts.

In one embodiment, prior to freeze drying, the cell based biological material was incubated in the medium at a temperature of between 15 °C to 45°C for a period of between 1 hour to 24 hours. Preferably, the incubation temperature was 37 °C. More preferably, the incubation was carried out for 3 to 11 hours.

Preferably, the medium may contain one or more of a buffer solution, bulking agents, pH stabilisers, pH adjusters, thermal stabilisers, cryoprotectants , lyoprotectants, an antioxidant, a biopolymer or an agent that may enable the active 'loading' of the cell or organism with at least one of the aforementioned components. Preferably, the medium is isotonic (iso-osmotic) with or hypertonic (hyperosmotic) to the biological material (such as the intracellular compartment of a red blood cell). For the purposes of clarity, isotonic (iso-osmotic) is defined as the medium having the same concentration or osmotic pressure as the biological material or the medium contained within the biological material, while hypertonic (hyperosmotic) is defined as the medium having a greater concentration or osmotic pressure compared with the biological material or the medium contained within the biological material.

Preferably, the solvent may be selected from water, tertiary butanol, aqueous or organic solvent. The buffer solution may be selected from phosphate buffered saline, citrate buffer, Tris buffer, amino acid-based buffers (such as histidine buffer, glycine buffer) , sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate, potassium dihydrogen orthophosphate, dipotassium hydrogen orthophosphate, TES, MOPS, PIPES, Cacodylate, SSC, MES and HEPES.

The cryoprotectant or lyoprotectant may be selected from disaccharides (such as lactose, trehalose, sucrose, maltose, and mannose), sorbitol, amino acids, peptides, polymers and proteins such as albumins (bovine serum albumin, human serum albumin) or gelatins.

The biopolymer may be selected from PLP, PV-50, PL-50, PP-30, PP-50, PP-60 and PP-75.

The thermal stabiliser may be select from mannitol, polymers (such as dextran, polyethylene glycol, polyvinyl pyrrolidone) and proteins.

The bulking agent may be selected from mannitol, polymers (such as dextran, polyethylene glycol, and polyvinyl pyrrolidone), disaccharides (such as lactose, trehalose, sucrose, maltose, and mannose), sorbitol and proteins such as albumins and gelatins.

The cell based biological materials may include mammalian cells and tissue, bacteria, viruses, fungal matter or components thereof. Examples of cells include, but are not limited to stem cells, epithelial cells, endothelial cells, red blood cells. Also included are prokaryote microorganisms, gram positive bacteria, gram negative bacteria, eukaryotic organisms, fungal spores, Caudovirales, Herpesvirales, Mononegavirales, Nidovirales, Picornavirales, and protozoal cells.

Preferably the bacterial cells may be selected from Lactobacilli such as Lactobacillus crispatusa and L. plantarum, Bifidobacteria, Bacillus coagulans and Saccharomyces boulardii.

Preferably, the antioxidant may be selected from: Vitamin A (retinol), Vitamin C (ascorbic acid) and Vitamin E (comprising tocotrienol and tocopherol), Vitamin co-factors (such as coenzyme Q10), minerals (such as manganese and iodine), hormones (such as melatonin), carotenoid terpenoids, flavonoid polyphenolics, phenolic acids and esters thereof, and non-fiavonoid phenolics (such as curcumin, silymarin, xanthones and eugenol and biopolymers such as PP-50, PLP, PV-50, PL-50, PP-30, PP-50, PP-60 and PP-75

Preferably, the agent that may enable the active 'loading' of the cell or organism with a cryoprotectant, may be a biopolymer selected from PP-50, PLP, PV-50, PL-50, PP-30, PP- 50, PP-60 and PP-75. Some recent developments have been made in the cryoprotection of cells by the use of biopolymers that have been shown to render cell membranes permeable on a temporary basis, thus allowing 'loading' of cryoprotectant such as trehalose into the intracellular compartment. Polymer PP-50 has been shown to act as a membrane permeabilising agent to enable intracellular loading of trehalose prior to the freeze drying of red blood cells (Lynch et al (2010); GB 1000999.1).

Freezing is defined as the solidification of solvent and the rendering of most or all of the solute phase into a rigidified state by the removal of heat. Sublimation is defined as the direct conversion of solid solvent into the gaseous phase. Desorption is defined as the removal of unfrozen solvent from a material by the application of energy in line with the applicable sorption isotherm.

According to a second aspect of the invention there is provided a method for reconstitution of freeze-dried cell based biological material comprising the steps of adding a reconstituting solution to the material that results in the final extracellular concentration being hypertonic to the material and where the reconstituting solution is hypertonic to the cells

The hypertonic or hyperosmotic solution and can be added at a temperature between 2°C and 45°C. Preferably, the temperature is between 20 °C and 37 °C. The solution can be given in either a single shot or in several aliquots. The solution may comprise one or more of aqueous solutions of salt (sodium chloride), phosphate buffer or the polymer hydroxy ethyl starch (HES), glucose, or dextran.

In one embodiment, the solution may be isotonic or iso-osmotic to the cells.

Freeze dried cells are usually resuspended in water in order to maintain the same extracellular osmolality as the cell suspension possessed prior to freeze-drying. However, it was noted in studies carried out in the inventors' laboratories on red blood cells that the rehydration in this manner resulted in significant haemolysis, which was attributed to localised regions of hypotonicity during the rehydration step. Therefore, it was acknowledged that water does not represent a suitable rehydrating medium for lyophilised red blood cells but that, instead, the use of solutions that were not significantly hypotonic may lead to a decrease in lysis during the rehydration process. Further investigation of rehydration media comprising aqueous solutions of salt (sodium chloride), phosphate buffer or the polymer hydroxy ethyl starch (HES) demonstrated these solutions to be significantly more effective in maintaining red blood cell counts, which was attributed to the obviation of local regions of hypotonicity during the rehydration process; indeed, the resulting solutions were significantly hypertonic, which may have led to reversible crenulation (temporary shrinking of cells) but not lysis. A similar effect might be expected with standard rehydration media used in infusion drips in a medical environment such as glucose or dextran.

According to a third aspect of the present invention there is provided a method of concentrating a biological material in an aqueous medium by contacting the medium with an osmotic driver solution where the medium and the osmotic driver solution are separated by a semi-permeable membrane and an osmotic gradient is created across the membrane.

Conveniently, the semi-premeable membrane is a viral-excluding membrane.

Preferably, the membrane allows water and a range of solutes to pass, but the overall ratio of the buffer components remains isotonic with the biological material. This allows reduction of volume of biological sample thereby concentrating it. Preferably, the biological material is raw blood, red blood cell suspension or blood washed in phosphate buffered saline.

The osmotic drive solution can be selected from saccharides such as sucrose or proteins such as bovine serum albumin or metal salts such as sodium chloride. Concentrations may be greater than 278 mOsm (milliosmoles) and up to the saturation point of the solute in question. Preferably, the concentration may be 70% (w/v).

The concentration step may be used prior to the freeze drying method described, thereby reducing freeze drying time and making the process more efficient and cost effective. Concentration can mean the reduction in volume of a suspension to provide for increased cell concentration but with a proportionally lower level of increase in the concentration of solutes. According to another aspect of the invention there is provided a composition comprising freeze dried biological material and one or more of a freeze dried buffer solution, bulking agents, pH stabilisers, pH adjusters, thermal stabilisers, cryoprotectant, lyoprotectant, an antioxidant, a biopolymer or an agent that may enable the active 'loading' of the cell or organism with at least one of the aforementioned components.

The biological material may be cell based biological material and may have been freeze dried in a hypertonic solution.

The pH of the hypertonic solution may be acidic and may be pH 7.2 or below.

The osmolarity of the hypertonic solution may be greater than 300mOsmol.

Preferably the biological material is selected from bacterial cells or mammalian cells. More preferably, the mammalian cells are red blood cells or nucleated cells. Preferably, the bacteria] cells are selected from Lactobacilli such as Lactobacillus

plantarum, Bifidobacteria, Bacillus coagulans and Saccharomyces boulardii

According to a further aspect of the invention there is provided a composition comprising biological material that has been reconstituted according to the method of the present invention.

Preferably the biological material is cell based and may be selected from bacterial cells or mammalian cells. More preferably, the mammalian cells are red blood cells or nucleated cells. Preferably, the bacterial cells are selected from Lactobacilli such as Lactobacillus crispatus and L. plantarum, Bifidobacteria, Bacillus coagulans and Saccharomyces boulardii.

According to a further aspect of the invention there is provided a composition comprising intact, functional or viable biological material that has been freeze dried by the method of the present invention and reconstituted by the method of the present invention.

Preferably the biological material is cell based and may be selected from bacterial cells or mammalian cells. More preferably, the mammalian cells are red blood cells or nucleated cells. Preferably the bacterial cells are selected from Lactobacilli such as Lactobacillus crispatusa. and L. plantarum, Bifidobacteria, Bacillus coagulans and Saccharomyces boulardii

Preferably, the composition may additionally comprise an anti-oxidant. Preferably the antioxidant is a biopolymer such as PP-50. More preferably, the composition may further comprise a cryoprotectant selected from trehalose, lactose, sucrose, maltose or mannose.

According to another aspect of the invention there is provided the use of one or more antioxidants in the freeze drying of biological materials.

Preferably, the antioxidant may be selected from: Vitamin A (retinol), Vitamin C (ascorbic acid) and Vitamin E (comprising tocotrienol and tocopherol), Vitamin co-factors (such as coenzyme Q10), minerals (such as manganese and iodine), hormones (such as melatonin), carotenoid terpenoids, flavonoid polyphenolics, phenolic acids and esters thereof, and non-flavonoid phenolics (such as curcumin, silymarin, xanthones and eugenol), biopolymers such as PLP, PV-50, PL-50, PP-30, PP-50, PP-60 and PP-75. Preferably, the antioxidant is PP-50.

Preferably, the biological material is cell based and may comprise mammalian cells, such as stem cells, epithelial cells, endothelial cells, or red blood cells. Alternatively, the biological material may comprise bacterial cells such as Lactobacilli such as Lactobacillus crispatus and L. plantarum, Bifidobacteria, Bacillus coagulans and Saccharomyces boulardii.

Antioxidants are not widely considered in the development of formulations for freeze-drying. De Paz et al, (1988) used ascorbic acid and monosodium glutamate to reduce oxidation of Streptococcus lactis for the sole purpose reducing the bitterness that can occur due to the use of fast acid-producing strains of bacterial starter cultures in cheese production. The ability to prevent or reduce oxidation of haemoglobin by biopolymers such as PP-50 has not been identified previously. This surprising effect was first noted in the inventors' laboratory following the freeze-drying and reconstitution of red blood cell suspensions. The use of antioxidants to confer cell stability and viability in the freeze-drying process has not previously been contemplated. The use of anti-oxidants to prevent or reduce oxidation within a cell, or to enhance the antioxidant properties of other solutes by enabling intracellular loading, is a surprising new effect in the field of freeze drying and can be utilised to provide better cell viability and the ability of the cells to function normally even after being subjected to freeze drying and reconstitution. In the case of red blood cells the reduced oxidation of haemoglobin allowed the cells to carry oxygen to the same or similar extent as the normal unprocessed blood cells or blood cells in the natural state.

According to another aspect of the invention there is provided a method of providing intact, functional or viable reconstituted freeze dried biological material comprising the steps of adding an antioxidant to the material prior to freeze drying.

According to yet another aspect of the invention there is provided a kit comprising freeze dried cell based biological material prepared by the methods of the invention and a reconstituting medium. Preferably the rehydrating or reconstituting medium is hypertonic to the cells in the biological material.

The methods of the invention have allowed the development of formulations that will enable long-term storage and transport of a wide range of cell-based therapeutics without the need for ultralow temperature storage facilities or a refrigerated transport "cold chain". The methods of the present invention have also allowed better survivability, functionality or viability of the cells by ensuring they remain intact through the process. Thus, the cells have a greater ability to function normally, or as desired, after freeze drying and reconstitution.

Brief description of the drawings

The invention will now be described in the following examples by way of illustration only in which:-

Figure 1 is a graph showing "freeze-drying survival" for 30 RBC formulations incubated in the absence of the polymer PP-50, frozen by immersion in liquid nitrogen and subsequent primary drying carried out at a shelf temperature of 0°C.

Figure 2 is a graph showing "freeze-drying survival" for 30 RBC formulations incubated in the presence of the polymer PP-50, frozen by immersion in liquid nitrogen and subsequent primary drying carried out at a shelf temperature of 0°C. Figure 3 is a graph showing RBC "survival" and percentage haemoglobin oxidation for PBS- based formulations frozen by immersion in liquid nitrogen and subsequent primary drying at a shelf temperature of -40°C

Figure 4 is a graph showing RBC "survival" for incubated and freeze-dried PBS-based formulations following reconstitution with three different rehydration solutions.

Examples

All experiments were carried out on samples of approximately 40% haematocrit, in l.OmL volumes contained in standard 1.5mL Eppendorf tubes. Sample preparation: Red blood cell suspensions as received from the hospital were washed two or three times using PBS at physiological concentration and pH 7.05 by centriiugation and resuspension, before finally being resuspended in the appropriate freeze-drying medium and divided into 1.OmL replicate aliquots in 1.5mL Eppendorf tubes.

The study investigated a wide range of variables relating to the formulation and processing conditions, as shown in Table 1.

Table 1: Variables studied with regard to formulation and processing:

Variable:	Option 1	Option 2	Option 3
Buffer type	PBS	Citrate	Tris
Buffer concentration	High	Medium	Low
Protectant type	Trehalose	Sucrose	Dextran
Protectant concentration	High	Medium	Low
Cooling rate	High	Medium	Low
Sublimation shelf temperature	High	Medium	Low
Rehydration solution	0.9% NaCl	PBS	1% HES

Key for Table 1 :

PBS = phosphate buffered saline

Tris = tris (hydroxymethyl)aminomethane High protectant concentration was 0.7M for trehalose and sucrose, 5% w/v for dextran Medium protectant concentration was 0.36M for trehalose and sucrose, 3% w/v for dextran Low protectant concentration was 0.18M for trehalose and sucrose, 1 % w/v for dextran High cooling rate involved dipping samples into liquid nitrogen at -196°C

Medium cooling rate involved loading samples onto freeze-dryer shelf pre-cooled to -40°C Slow cooling rate involved loading samples onto freeze-dryer shelf at ambient temperature (15 °C to 25 °C) and cooling at 0.5°C per minute down to -40°C.

Sublimation (Primary drying) shelf temperatures: High = 0°C, Medium = -20°C, Low = -40°C. In all cases, the chamber pressure was controlled at 50 mTorr.

Additionally, the formulations were tested in the presence and absence of a bio-polymer, PP- 50, at 20(^g/ml, as reflected in the data shown in Figure 1 (absence of PP-50) and Figure 2 (presence of PP-50).

Selected formulations were then taken forward for comparison of other parameters:

• PBS only

• PBS + 0.7M Sucrose

• PBS + 0.7M Sucrose + 200µ /πι1 PP-50 (Incubated)

• PBS + 0.7M Trehalose

• PBS + 0.7M Trehalose + 200µg/ml PP-50 (incubated)

PBS tablets obtained from Sigma Chem. Co. Ltd., Poole, UK; Cat. No. P-4417;

Sucrose obtained from Sigma Chem. Co. Ltd., Poole, UK; Cat. No. S-9378;

Trehalose obtained from Sigma Chem. Co. Ltd., Poole, UK; Cat. No. T-9531;

Biopolymers PLP, PV-50, PL-50, PP-30, PP-50, PP-60 and PP-75 can be made by the process defined in Lynch et al (2010); Eccleston et al (2000); Chen et al (2009).

The pH of all the above solutions was adjusted to pH 7.05 prior to their addition to the red blood cell suspension, which had been washed using buffer three times using a standard centrifugation and resuspension method. Where employed, incubation was carried out at 37°C for between 3 hours and 11 hours. The biopolymer PP-50 was included in order to investigate the effects of freeze-drying red blood cells (RBC) with intracellular as well as extracellular buffer and protectant.

The inclusion of PP-50 was designed to increase RBC membrane permeability to buffer and protectant molecules, while also having the side-effect of reducing the extent of intracellular and extracellular solute concentration differential, which can lead to crenulations or lysis.

A series of experiments was carried out to investigate whether concentration of the blood solution prior to freeze drying would provide comparable levels of cell survival and oxidation while giving the additional benefit of reducing the volume of water needing to be removed during freeze drying, thus providing the potential for achieving shorter freeze-drying cycles and thereby a more efficient process.

These parameters were quantified by measuring absorbance of diluted samples by UV- visible spectrophotometry at wavelengths of 700nm, 630nm, 577nm and 560nm, from which the levels of haemoglobin, oxy-haemoglobin and met-haemoglobin could be calculated using a mathematical formula. All UV analyses were run in triplicate unless otherwise stated.

A graph showing the summary of % RBC survival and level of haemoglobin oxidation for different starting solutions (suspensions) frozen by immersion in liquid nitrogen and with primary drying carried out at a shelf temperature of -40°C, are provided in the Figure 3.

The sections below provide a brief description of each individual part of the study.

Example 1 - Cooling Rate

Two freeze-drying cycles were conducted in order to investigate the effect of cooling rate on red blood cell (RBC) survival and haemoglobin oxidation, one for non-incubated blood solutions in the absence of the PP-50 polymer and another which included the incubated solutions with PP-50 polymer included. Both cycles included the 'PBS only' solution as a control.

The following three cooling rates were used in each cycle:

1. Fast freeze by immersion in liquid nitrogen at -196°C

2. Medium freeze by loading samples onto freeze-dryer shelves pre-cooled to -40°C

3. Slow freeze by loading onto ambient freeze-dryer shelves followed by controlled cooling at 0.5°C per minute down to -40°C

The freeze-dried cakes produced were reconstituted using PBS solution and samples were taken from each blood solution for UV analysis, to quantify BC survival and resulting haemoglobin status (haemoglobin, oxy-haemoglobin and met-haemoglobin). The resulting data showed that the best average survival was obtained by using the fast freezing rate, with an average of 32.5% across all formulations, and an average haemoglobin oxidation of 18.5%. The medium freezing rate cycle produced the next best survival with an average of 23.8% and 19.0% haemoglobin oxidation; the slow freezing rate led to an average survival rate of 16.9%, with 3.7% haemoglobin oxidation. Across all the candidate blood solutions tested, the best survival was obtained by the incubated polymer-containing solutions, of which the sucrose candidate performed consistently the best with a maximum survival of 56% in the fast freezing rate cycle. The non-polymer sugar solutions performed the worst, with less than 10% survival on the medium and slow freezing rate cycles. The lowest haemoglobin oxidation was obtained by the polymer-containing solutions in which the % oxidation was below 10% for both solutions in all runs. A summary of results is provided in Figures 1 and 2.

Example 2 - Sublimation or Primary Drying Shelf Temperature

Two cycles were conducted in order to investigate the effect of sublimation shelf temperature on RBC survival and haemoglobin oxidation. Both cycles were performed using both non- incubated blood solutions in the absence of the PP-50 polymer, and the incubated solutions with PP-50 polymer included. Both cycles included the PBS only solution as a control, and used the fast freezing rate as described above. The fast freezing cycle carried out in the cooling rate study above used a primary drying shelf temperature of 0°C, which is used as a high temperature primary drying cycle.

The following temperatures were used:

1. High temperature primary drying conducted at 0°C

3. Low temperature drying conducted at -40°C

The freeze-dried cakes produced were reconstituted using PBS solution and samples were taken from each blood solution for UV analysis. The resulting data showed that the best average survival was obtained by using the low temperature primary drying, with an average of 83.1% across all formulations, and an average haemoglobin oxidation of 23.1%. The medium temperature primary drying cycle produced the next best survival with an average of 81.3% and 29.7% haemoglobin oxidation; the high temperature primary drying led to an average survival rate of 32.5% and 18.5% haemoglobin oxidation. Across all the candidate blood solutions used, the best survival was obtained by the nonpolymer-containing trehalose solution, which achieved a maximum survival of 96.6% in the low temperature cycle, although haemoglobin oxidation was high (61.1 %).

Among the excipients traditionally added to the active material for freeze-drying are bulking agents, thermal stabilisers, pH stabilisers, pH adjusters, thermal stabilisers, cryoprotectants and/or lyoprotectants. While it has been well documented that non-reducing sugars can provide benefits over reducing sugars when included in formulations containing proteins, in order to avoid Maillard reactions, the addition of antioxidants per se is not something that is traditionally considered when designing formulations for freeze-drying. In the present study, it was noticed that while the polymer-containing trehalose solution led to a lower RBC survival (83.8%), more significantly, the haemoglobin oxidation was maintained below detectible levels. This is arguably a better overall result, since the benefits of higher RBC survival in the absence of polymer would be outweighed by the high levels of haemoglobin oxidation, which would render the RBCs far less efficient as oxygen carriers. A similar pattern was observed in the sucrose formulations in the low temperature cycle, in which there was a high RBC survival (90.2%) and high oxidation (47.4%) in the non-polymer solution, but with the addition of the polymer, although the RBC survival was only 57.1%, the haemoglobin oxidation level was below detectable limits. The 'PBS only' control (no sugar, no polymer) also gave excellent RBC survival at 87.7%, but oxidation levels were high at 66.9%.

Desorption or Secondary drying: All samples were subjected to identical secondary drying conditions, where the shelf temperature set point was 20°C and chamber pressure 50 mTorr.

Example 3 - Reconstitution Method

A single cycle was conducted in order to investigate the effect of the reconstitution method on RBC survival and haemoglobin oxidation. This was performed using both non-incubated blood solutions in the absence of the PP-50 polymer, and the incubated solutions with PP-50 polymer included. 'PBS only' solution was once again as a control. Samples were f ozen in liquid nitrogen (fast freezing) and primary drying was carried out with a shelf temperature of 0°C, as described above. The following three reconstitution solutions were used:

1. Reconstitution using PBS solution

2. Reconstitution using 0.9% (w/v) NaCl solution

3. Reconstitution using 1% (w/v) Hydroxyethyl Starch solution

The 0.9% NaCl solution is the same as would readily be available sterile in modern hospitals, so would be of benefit in the final product if proved to be acceptable. Hydroxyethyl Starch (HES) is commercially available as a blood plasma substitute and therefore would be beneficial to the final product if the RBCs could be resuspended in this solution prior to injection to maximise the benefit of the blood to the patient.

The freeze-dried cakes produced were reconstituted using one of the three solutions above and samples were taken from each blood solution for UY analysis. The resulting data

demonstrated the robustness of the incubation and freeze-drying process to subsequent reconstitution with each selected rehydration solutions, as shown in Figure 4. Reconstitution with PBS solution (source: Sigma Chemical Co. Ltd., Cat. No. P-4417, prepared by adding 1 tablet to 200mL of BDH NormapuR water, source: BDH, Poole, UK, Cat. No. 102927G) yielded an average survival of 70.0% across all formulations, and an average haemoglobin oxidation of 12.5%.

Reconstitution with saline (prepared by dissolution of sodium chloride powder [source: Sigma Chemical Co. Ltd., Poole, UK, Cat. No. S-7653] in analytical grade water) led to an average survival of 633% and 8.8% haemoglobin oxidation; reconstitution with HES (source: Sigma Chemical Co. Ltd., Poole, UK; Cat. No. H6382, dissolved in BDH NormapuR water to give a 1% w/v solution) led to an average survival rate of 71.4%, with 8.3% haemoglobin oxidation.

Example 4 - Volume Reduction (Concentration) of Blood Prior to Freeze-Drying

In conjunction with comparing a range of variables such as buffers, cryoprotectants and freezing rates, the feasibility of increasing the cell haematocrit as a method for reducing cell death rate during lyophilisation was also considered. Increasing the concentration of red blood cells per volume of suspension (haematocrit of the suspension), would reduce the mass of water per volume to be removed from the product, allowing less aggressive lyophilisation conditions to be used, for a shorter

centrifugation would impose upon the cells may cause additional cell death, and thus would outweigh the benefit gained from a shorter lyophilisation time.

Therefore we have investigated a novel method for blood concentration. This method employed purpose-built test chambers consisting of two compartments, one containing the substance to be concentrated, and the other containing a highly concentrated solution of an osmotic driver. The two compartments are separated by a novel viral-excluding membrane (obtained from HATS Ltd., Dorset). Water and a range of solutes are able to traverse the membrane, driven by the osmotic gradient generated by the driver, whilst maintaining the overall ratio of the buffer components largely isotonic with the red blood cells, which are themselves excluded by the membrane.

Initially, experiments were conducted to determine whether the solutes found in PBS buffer could traverse the membrane. Different solutions containing the same total osmolality of either sodium chloride, potassium chloride, sodium dihydrogen orthophosphate or disodium hydrogen orthophosphate were subjected to volume reduction using this method and conductivity measurements were taken before and at regular intervals during concentration, in order to determine whether each individual species was able to traverse the membrane.

As expected, the rates of concentration of the solutes were related to the concentration of the driver. However, there was only a slight reduction in uptake rates if its own concentration was reduced from twenty to fifteen percent. Consequently by starting experiments at 20% (w/v) driver concentration and ensuring there was an adequate mass of concentrating agent present, it was found that the rate of volume reduction in the sample compartment remained largely constant for an experiment lasting twenty four hours. Where washed blood was used as a comparison: after the raw RBC suspension had been subjected to the necessary washing steps, a haematocrit level of approximately 40% was attained by adjusting the volume of isotonic solution added.

Using the above method we were able to successfully concentrate the raw blood as well as a suspension of 3x PBS washed blood. Two sizes of test pot (chamber) were used, providing two different membrane surface areas. The results of the volume reduction step are shown in Table 2 and Table 3. Table 2: Volume Reduction of Raw RBC suspension

	Weight of Blood at Start (g)	Weight of Blood after 18 hours contact with membrane	pH change
Large pot	50.34	23.53	0.05
Small pot	48.11	17.18	0.14

Table 3: Volume Reduction of Washed RBC suspension

	Weight of Blood at Start (g)	Weight of Blood after 18 hours contact with membrane	pH change
Small pot	89	6.6	-
Large pot	222	120.6	0.02

Thus we were able to demonstrate that this method could be used to concentrate both the raw blood and red blood cells in suspension to provide concentrated suspensions of less than half their original weight. In the case of washed RBCs, a weight reduction of more than 90% was achieved, yielding a very concentrated 'paste' of RBCs. Moreover, it was shown that the pH of the blood did not significantly change during the concentration process, which may be a critical factor i ensuring RBC survival.

Freeze drying of the concentrated RBC suspensions was carried out, in order to determine whether the volume reduction process would have a significant impact on RBC death or haemoglobin oxidation. The concentrated PBS blood samples were included in the freezing rate study cycle so that a direct comparison could be made between concentrated and non-concentrated blood.

UV analysis of the freeze-dried and reconstituted samples produced results comparable with the non-concentrated PBS blood solution processed under same conditions. The non- concentrated PBS blood solution achieved a RBC survival of 30.0% and an oxidation level of 64.4%; the concentrated PBS blood achieved a RBC survival of 24.3% and an oxidation level of 65.6%. These data suggest that the volume reduction process does not appear to

significantly reduce the % RBC survival post-lyophilisation or significantly increase the haemoglobin oxidation level. Therefore, it is possible that when freeze-dried in the presence of saccharide, or after incubation with saccharide + polymer, levels of % RBC and

haemoglobin oxidation may be achieved that are in keeping with the data shown in sections 1- 3 above, while providing the additional benefit of shorter freeze-drying time due to the smaller volumes of water present.

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LEGAL EVENTS

Date	Code	Event	Description
Sep 12, 2012	121	Ep: the epo has been informed by wipo that ep was designated in this application	**Ref document number:** 12703564 **Country of ref document:** EP **Kind code of ref document:** A1
Jul 22, 2013	NENP	Non-entry into the national phase in:	**Ref country code:** DE

Date	Code	Event	Description
Aug 19, 2013	ENP	Entry into the national phase in:	**Ref document number:** 1314778 **Country of ref document:** GB **Kind code of ref document:** A **Free format text:** PCT FILING DATE = 20120122
Aug 19, 2013	WWE	Wipo information: entry into national phase	**Ref document number:** 1314778.0 **Country of ref document:** GB
Feb 12, 2014	122	Ep: pct app. not ent. europ. phase	**Ref document number:** 12703564 **Country of ref document:** EP **Kind code of ref document:** A1