

DRY CRYOPRESERVATION OF CELLS ON NANOFIBER SCAFFOLD

Field of the Invention

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Background

For a long time, bio preservation technique that maintains ex-vivo cells, including hepatocytes/HepG2 and other cell lines, viability and functioning is one of the most
20 important research aspects of modern tissue engineering and pharmacokinetic in cell culture. The ability to preserve the integrity of cells (such as hepatocytes/HepG2) outside the native environment while maintaining a high biological activities for an extended period of time not only will save time and effort in primary culture, it may also be possible to establish a ready-to-use cells platform to support the fast pace
25 pharmacological and toxicological testing that is required when out-break arises. Such a ready-to-use cells platform would support the implementation of routine cryopreservation practices during preparation of even engineered cells and tissues for the clinical applications when needed.

30 Other limitation to cryopreservation is the use of Dimethyl Sulfoxide (DMSO). DMSO is frequently used in cell banking applications as a cryoprotectant. When added to a media, DMSO prevents intracellular and extracellular crystals from forming in the cells during the freezing process. Without a cryoprotectant, these crystals cause cell death, thus rendering the cells useless for transplant and research studies. However, the

downsides to DMSO are that DMSO can induce neuronal differentiation in stem cells and a cytotoxic effect on the cell viability at 30% v/v. This coupled with the cellular toxicity of DMSO at room temperature with high concentration has led to the search for a low cryopreservant that provides the same level of benefit as 10% DMSO but without the drawbacks.

It is therefore desirable to provide a nanofiber scaffold for use as a cryopreservation platform that seeks to address at least one of the problems described hereinabove, or at least to provide an alternative.

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Summary of Invention

In accordance with one aspect of this invention, a nanofiber scaffold for cryopreservation of cells in dry condition is provided. The nanofiber scaffold comprises electrospun nanofibers forming the scaffold, wherein the scaffold comprises a network of one or more nanofibers electrospun from a blend comprising polycaprolactone, dimethyl sulfoxide (DMSO) and a cryopreservation agent selected from galactose or collagen.

In accordance with one embodiment of this invention, the cryopreservation agent is galactose. In accordance with another embodiment of this invention, the cryopreservation agent is collagen.

In accordance with a second aspect of this invention, a method of producing a nanofiber scaffold for cryopreservation of cells in dry condition is provided. The method comprises providing an electrospinning blend comprising polycaprolactone, dimethyl sulfoxide (DMSO) and a cryopreservation agent selected from galactose or collagen; and electrospinning the blend to form a nanofiber scaffold comprises a network of one or more nanofibers on a surface of a substrate.

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In accordance with one embodiment of this invention, the method further comprises sterilizing the nanofiber scaffold; subjecting the nanofiber scaffold to ultra-violet radiation; and neutralizing the nanofiber scaffold with a growth medium.

In accordance with a third aspect of this invention, a method for cryopreserving cells in dry condition is provided. The method comprises introducing target cells onto an electrospun nanofiber scaffold, wherein the nanofiber scaffold comprises a network of one or more nanofibers electrospun from a blend comprising polycaprolactone, dimethyl sulfoxide (DMSO) and a cryopreservation agent selected from galactose or collagen; culturing the target cells in a growth medium on the nanofiber scaffold; removing the growth medium; and cryopreserving the target cells on the nanofiber scaffold.

- 10 In accordance with an embodiment of this invention, the step of cryopreserving the target cells comprises cooling the target cells at a rate of $1^{\circ}\text{C}/\text{min}$ to a temperature of -70°C and cryopreserving the target cells at the said temperature.

Brief Description of the Drawings

- 15 The above advantages and features of a method and apparatus in accordance with this invention are described in the following detailed description and are shown in the drawings:

- 20 Figure 1 is a schematic illustration of an electrospinning process.

Figure 2(a) shows a scanning electron microscope (SEM) image of a successfully spun random electrospun nanofibers. Figure 2(b) shows the electrospun nanofibers on coverslip backing.

- 25 Figure 3 is a graph showing the weight of membrane and the volume of polymers solution used.

- 30 Figure 4 is a bar chart showing post thaw cell viability after 24hr of 7-day cryopreservation period on different membranes using resazurin. Bar: standard deviation ($n = 6$). The results show that polycaprolactone/DMSO/Galactose blend has the best performance compared with other different blends tested.

Figure 5 is a bar chart showing post thaw cell counting data after 24hr of different cryopreservation method (dry and wet) on PCL/DMSO/GAL membrane. The results show that PCL/DMSO/GAL supports dry cryopreservation up to 7 days.

- 5 Figure 6 is a bar chart illustrating resazurin results showing the metabolic activities of post thaw different cell lines tested in nanofibrous scaffolds. The results demonstrate the statistical differences between the control (2D coverslip) and the nanofiber membrane after 3, 5 and 7 days after 7 days of cryopreservation ($n = 6$; $p < 0.05$; $p < 0.01$).

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Figure 7 is a bar chart showing the average fluorescent intensity percentages of the different nanofibers scaffolds (Random and Aligned) after incubation for 1 day before cryopreservation.

- 15 Figure 8 is a bar chart showing the average number of cells counted that were attached to the respective nanofibers scaffolds (Random and Aligned) after incubation for 1 day before cryopreservation.

- Figure 9 is a bar chart showing the average number of cells counted that were attached to the respective nanofibers scaffolds (Random and Aligned) after 7 days of cryopreservation. Results were obtained for wet cryopreservation and dry cryopreservation.
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- Figure 10 is a bar chart showing the average number of cells counted that were attached to the respective nanofibers scaffolds (Random and Aligned) after 14 days of cryopreservation. Results were obtained for wet cryopreservation and dry cryopreservation.
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- Figure 11 is a bar chart showing the average fluorescent intensity percentages of the different nanofibers scaffolds (Random and Aligned) after 7 days of cryopreservation. Results were obtained for wet cryopreservation and dry cryopreservation.
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- Figure 12 is a bar chart showing the average fluorescent intensity percentages of the different nanofibers scaffolds (Random and Aligned) after 14 days of cryopreservation. Results were obtained for wet cryopreservation and dry cryopreservation.
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Figure 13 is a flow chart showing the timeline and steps for cryopreservation of target cells.

5 Detailed Description

This invention relates to a nanofiber scaffold for use as a cryopreservation platform to freeze/cryopreserve target cells in dry condition with cryopreservation agent.

- 10 According to one aspect of the invention, a 3-dimensional nanofiber scaffold for cryopreservation of target cells in dry condition is provided. The nanofiber scaffold comprising electrospun nanofibers forming the scaffold. The scaffold comprises a network of one or more nanofibers electrospun from a blend comprising polycaprolactone, dimethyl sulfoxide (DMSO) and a cryopreservation agent. The
15 cryopreservation agent is one selected from galactose or collagen.

In one embodiment, the nanofiber scaffold is electrospun from a blend comprising polycaprolactone, DMSO and galactose. The nanofiber scaffold prepared from this blend is suitable for use in cryopreserving HepG2 cells.

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In another embodiment, the nanofiber scaffold is electrospun from a blend comprising polycaprolactone, DMSO and collagen. The nanofiber scaffold prepared from this blend is suitable for use in cryopreserving Caco-2 cells.

- 25 The nanofiber scaffold of the present invention is defined by the network of one or more nanofibers having an average diameter of 100 to 500nm, preferably 100 to 350nm.

- The nanofiber scaffold is prepared by electrospinning method. Fabrication process of scaffolds has been reported to have an influence on the cell attachment and
30 proliferation. Electrospinning is an attractive fabrication technique because it can produce continuous fibers ranging in nanometer dimension. The nanofibers prepared from electrospinning are in the diameters ranging between 50 to 500nm. The fibrous structure facilitates cellular attachment, proliferation and 3D culture properties for successful development of tissue engineered scaffold construct as compared to porous
35 scaffold. Therefore, scaffold used in this invention is prepared by electrospinning

method, keeping in view the superiority of nanofiber architecture rather than microfibrinous scaffold.

5 In another aspect of the invention, a method of producing a nanofiber scaffold for cryopreservation of cells in dry condition is provided. The method comprises providing an electrospinning blend comprising polycaprolactone, dimethyl sulfoxide (DMSO) and a cryopreservation agent selected from galactose or collagen; and electrospinning the blend to form the nanofiber scaffold comprising a network of one or more nanofibers on a surface of a substrate. The nanofiber scaffold produced by the method of the present
10 invention is 3-dimensional.

The blend can be prepared by first blending polycaprolactone with the cryopreservation agent, followed by adding DMSO to the blend. The blend can also be prepared by first dissolving polycaprolactone in a solvent to obtain a solution, followed by mixing the
15 solution of polycaprolactone with the cryopreservation agent and DMSO to obtain the blend.

The blend is electrospun at a speed of 26 to 35kV to generate nanofibers at a flow rate of about 10ml/hr on the surface of the substrate. The substrate can be any suitable
20 substrate, such as a glass coverslip. The nanofiber scaffold on the glass coverslip is collected for further processing.

Any suitable apparatus for performing the electrospinning process may be employed. Figure 1 shows an exemplary electrospinning apparatus suitable for performing the
25 method according to the invention. The apparatus (10) comprises a syringe (12) for receiving an electrospinning solution (14), which in this case, is the blend. The syringe (12) is connected to a voltage source (16) to supply the desired voltage to the syringe (12) for generating nanofibers (18) at a desired flow rate onto the surface of a substrate (20). The substrate (20) is placed at the opposite of an outlet (22) of the syringe at a
30 distance of about 25 to 40cm. Any suitable substrate known in the art can be used including, but not limited to, glass coverslip, collector screen, etc. The collector screen can be rotating or stationary.

The nanofiber scaffold on the glass coverslip collected is subjected to further
35 processing including, sterilizing the nanofiber scaffold and subjecting the nanofiber

scaffold to ultra-violet (UV) radiation. The nanofiber scaffold is neutralized with a growth medium before seeding takes place.

5 In a further aspect of the invention, a method for cryopreserving cells in dry condition is provided. The method comprises introducing target cells onto an electrospun nanofiber scaffold, wherein the nanofiber scaffold comprises a network of one or more nanofibers electrospun from a blend comprising polycaprolactone, dimethyl sulfoxide (DMSO) and a cryopreservation agent selected from galactose or collagen; culturing the target cells in a growth medium on the nanofiber scaffold; removing the growth medium; and
10 cryopreserving the target cells on the nanofiber scaffold.

The step of culturing target cells in the growth medium involves seeding the target cells on the scaffold and allowing the target cells to incubate for a desired period of time, ranging from 1 to 5 days, before undergoing dry freezing/cryopreservation.

15 Before cryopreserving the target cells, the growth medium is removed from the scaffold. This is to provide a dry environment within the nanofiber scaffold for dry cryopreservation to take place.

20 The step of cryopreserving the target cells on the nanofiber scaffold comprises cooling the target cells at a rate of $1^{\circ}\text{C}/\text{min}$ to a temperature of -70°C and cryopreserving the target cells at the said temperature.

The blends used for producing the nanofiber scaffold are unique and novel. The unique
25 blends allow cryopreservation in 3-dimensional culture orientation. The blends also allow cryopreservation in dry condition without the addition of additional cryopreservation agent. The results obtained in the tests described in the examples below show that dry cryopreservation has relatively more cells counted together with a higher fluorescent intensity as compared to wet cryopreservation. Despite the lack of
30 media in the nanofiber scaffold, there were relatively more cells remaining in the nanofiber scaffold, suggesting that the cells had successfully adhered to the scaffolds and were protected within the scaffolds. The blends of the present invention can be applied to ready-to-use target cells slides or platforms for fast pharmacological and toxicological testing.

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The following examples illustrate various embodiments of this invention. One skilled in the art will recognize that the examples set out below are not an exhaustive list of the embodiments of this invention.

5 **EXAMPLES**

Example 1

Electrospinning condition and polymer solution

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4g of polycaprolactone (PCL) and 5mM of galactose were blended and the blend was made electrospinnable by dissolving the blend in a polymer mixture containing 100% acetone and 100% DMSO (final concentration at 5% v/v) in 95:5 volume/volume ratios at 60°C for 2 hours. The solution was electrospun at 30kv to generate nanofibers of an average diameter of 100 to 350nm, with a flow rate of about 10ml/hr, on a glass coverslip. A thick nanofiber scaffold on the glass coverslip at 8mg/cm² (or ranges from 5 to 15mg/cm², see Figure 3) was collected on a grounded roller collector with spinning distance of 30cm and with 21G needle at random fiber arrangements.

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The nanofiber scaffold on the glass coverslip was sterilized with 70% ethanol and subjected to UV radiation overnight (12hr). The sterilized scaffold was washed thoroughly with phosphate buffer saline (PBS) and neutralized with a growth medium comprising DMEM (Dulbecco's Modified Eagle's medium) with 5% FBS (Fetal bovine serum) at 37°C with 5% carbon dioxide.

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1 x10⁶ cells/ml of HepG2 cells were seeded on the scaffold and cultured in DMEM with 5% FBS and 1% antibiotics, for 3 days before freezing/cryopreservation.

Cryopreservation

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Cryopreservation was performed in -70°C freezer. The HepG2 samples on the nanofiber scaffold were cooled at a rate of 1°C/min to a temperature of about -70°C after the growth medium was removed. After 7 days of cryopreservation, the HepG2 cells were removed from the freezer and were thawed by shaking at 37°C. Cultured

HepG2 cells without nanofiber scaffold on coverslip was also prepared and used as a control.

The viability of cells of the cryopreserved HepG2 cells was assessed by regular
5 resazurin assay. The results are shown in Figures 4 and 6.

The results in Figure 4 show that the blend comprising polycaprolactone/DMSO/Galactose has the best performance compared to the other blends tested.

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Figure 6 shows the resazurin results obtained on the metabolic activities of post thaw different cell lines tested in the nanofiber scaffold. The results demonstrate the statistical differences between the control (2D coverslip) and the nanofiber scaffold after 3, 5 and 7 days after 7 days of cryopreservation. HepG2 cells achieved the best
15 results among the different cell lines.

The direct cell counting was carried out by regular trypsinization procedure and the results are shown in Figure. 5. The results show the post thaw cell counting data after 24hr using dry cryopreservation and wet cryopreservation methods on nanofiber
20 scaffold electrospun from the blend comprising PCL/DMSO/Galactose. The results show that PCL/DMSO/GAL supports dry cryopreservation up to 7 days.

Example 2

Electrospinning condition and polymer solution

4g of polycaprolactone (PCL) in 50mL of high-grade acetone, at a mixture concentration of 8% (w/v), was left on a hot plate stirrer at 60°C for 2 to 2.5 hours. 8ml of 8% of PCL was then mixed with 1.5ml collagen (Type I bovine skin collagen at
30 6mg/ml aqueous solution in 0.01 M HCl, at a pH of 2.0) and 0.5ml DMSO to form a spinning polymer solution. The solution was electrospun at 27kV to generate nanofibers of an average diameter of 100 to 500nm, at a polymer liquid flow rate of about 10 ml/hr, on a glass coverslip. Thickness of the nanofiber at 8mL was spun onto the coverslip. Random fibres scaffold was collected with a stationary coverslip; whereas

aligned fibres scaffold was collected while the coverslip is attached on a moving collector roll at 100 rpm/min rotation speed.

5 A normal glass coverslip without nanofibers scaffold disposed on the coverslip was used as a control.

10 The random and aligned nanofiber scaffolds on the glass coverslips were sterilized with 70% ethanol and subjected to UV radiation overnight (12hr). The sterilized scaffolds were washed thoroughly with phosphate buffer saline (PBS) and neutralized with a growth medium comprising DMEM with 10% FBS at 37°C with 5% carbon dioxide.

1 $\times 10^6$ cells/ml of Caco-2 cells were seeded on the random and aligned scaffolds, and cultured in DMEM with 10% FBS, for 1 day before freezing/cryopreservation.

15 Average fluorescent intensity of the control, the random fibres scaffold on coverslip and the aligned fibres scaffold on coverslip were measured after they were incubated on the coverslip for 1 day, before cryopreservation. The results obtained are shown in Figure 7.

20 The results show that both scaffolds with random fibres and aligned fibres express a greater amount of fluorescent intensity as compared to the control with normal glass coverslip (without the fibres). This could mean that there are either more Caco-2 cells on both types of scaffolds or those activities of the cells on the scaffolds are increased. Furthermore, random fibres scaffold also has 77.37% more fluorescent intensity than the aligned fibres scaffold which suggests that Caco-2 cells could grow better on
25 random fibres scaffold or that or that the bioactivities of the cells are most active on random fibres scaffold.

30 Readings on the average number of cells that were attached to the different scaffolds that had been incubated for 1 day before cryopreservation were taken and the results are shown in Figure 8. From the figure, we can see that the number of cells attached to the random fibres scaffold and the aligned fibres scaffold are about the same, while more cells are attached to the 2D non-scaffold coverslip.

Cryopreservation

In this experiment, wet and dry cryopreservation methods were carried out at -70°C for 7-14 days on cells attached to the random fibres scaffold and the aligned fibres scaffold. Wet cryopreservation involved the addition of a 5% dimethyl sulfoxide (DMSO) based DMEM culture media. This media replaced the normal culture media. The cell attached scaffolds were left in DMSO before the scaffolds were placed in -70°C freezer. Dry cryopreservation involved a complete removal of the growth media from the scaffolds and the cells were left attached to the scaffolds before the scaffolds were placed in the -70°C freezer.

Figures 9 and 11 show the results obtained after 7 days of cryopreservation, while Figures 10 and 12 show the results obtained after 14 days of cryopreservation.

After 7-14 days of cryopreservation, the cell attached scaffolds were allowed one day of growth/wake-up phase before biological assays.

The results obtained demonstrate that both the random fibres scaffold and the aligned fibres scaffold retained a good cell counting number against the control (without fibres scaffold) (see Figures 9 and 10).

In addition, the 3D orientation was retained on both the random and aligned fibres scaffolds after cryopreservation. These findings were supported by the good biological activities (resazurin) shown in Figures 11 and 12. When comparing the different cryopreservation conditions on aligned fibres scaffold, dry cryopreservation had more cells counted together with a higher fluorescent intensity as compared to wet cryopreservation. This could be due to the fact that ice crystals could still form in wet cryopreservation. In dry cryopreservation, crystals will not be formed as there is no media. Despite the lack of media, there were still more cells, suggesting that the cells on the scaffolds were protected.

The above is a description of the subject matter the inventors regard as the invention and is believed that those skilled in the art can and will design alternative embodiments that include of this invention as set forth in the following claims.

Claims:

1. A nanofiber scaffold for cryopreservation of cells in dry condition, comprising:
electrospun nanofibers forming the scaffold, wherein the scaffold comprises a
5 network of one or more nanofibers electrospun from a blend comprising
polycaprolactone, dimethyl sulfoxide (DMSO) and a cryopreservation agent selected
from galactose or collagen.
2. The nanofiber scaffold according to claim 1, wherein the cryopreservation agent
10 is galactose.
3. The nanofiber scaffold according to claim 1, wherein the cryopreservation agent
is collagen.
4. The nanofiber scaffold according to claim 2, wherein the nanofiber scaffold is to
15 be used for cryopreserving HepG2 cells.
5. The nanofiber scaffold according to claim 3, wherein the nanofiber scaffold is to
be used for cryopreserving Caco-2 cells.
6. The nanofiber scaffold according to claim 1, wherein the nanofiber scaffold is
20 defined by the network of one or more nanofibers having an average diameter of 100 to
500 nm.
7. A method of producing a nanofiber scaffold for cryopreservation of cells in dry
25 condition, the method comprising:
providing an electrospinning blend comprising polycaprolactone, dimethyl
sulfoxide (DMSO) and a cryopreservation agent selected from galactose or collagen;
and
30 electrospinning the blend to form a nanofiber scaffold comprises a network of
one or more nanofibers on a surface of a substrate.
8. The method according to claim 7, wherein the substrate is a glass coverslip.
9. The method according to claim 7, further comprising:
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sterilizing the nanofiber scaffold;
subjecting the nanofiber scaffold to ultra-violet radiation; and
neutralizing the nanofiber scaffold with a growth medium.

- 5 10. A method for cryopreserving cells in dry condition, comprising:
 introducing target cells onto an electrospun nanofiber scaffold, wherein the
nanofiber scaffold comprises a network of one or more nanofibers electrospun from a
blend comprising polycaprolactone, dimethyl sulfoxide (DMSO) and a cryopreservation
agent selected from galactose or collagen;
- 10 culturing the target cells in a growth medium on the nanofiber scaffold;
 removing the growth medium; and
 cryopreserving the target cells on the nanofiber scaffold.
11. The method according to claim 10, wherein the step of cryopreserving
- 15 comprises cooling the target cells at a rate of 1°C/min to a temperature of -70°C and
cryopreserving the target cells at the said temperature.
12. The method according to claim 10, wherein the targets cells are cultured in the
growth medium for 1 to 5 days before cryopreserving the target cells on the nanofiber
- 20 scaffold.