THREE-DIMENSIONAL BIOREACTOR FOR T-CELL ACTIVATION AND EXPANSION FOR IMMUNOTHERAPY

Field Of The Invention

The present disclosure relates to the application of a three-dimensional (3D) bioreactor for T- cell expansion for immunotherapy. The bioreactor described in the current invention can also be used for the expansion of other lymphocytes such as B cells and natural killer cells, or non-adherent cell in general.

Background

Cancer immunotherapy, representing the most recent phase of biotechnology revolution in medicine, is the use of a patient's own immune system to treat cancer. A recent successful case is the chimeric antigen receptor (CAR) T-cell based cancer treatment. A typical CAR T-cell therapy process is one in which T lymphocytes are collected from a patient's own blood are genetically engineered to produce a special receptor CAR on their surface so that the T cells are able to recognize and attack cancer cells. The engineered CAR T cells are grown in the laboratory and expanded to billions of numbers and then injected back to the patient to kill cancer cells.

With the successful of CAR T-cell therapy, the next question is how to make it safer and cost-efficient. The current T-cell engineering process is still generally based on the use of magnetic beads to incubate together with T-cells. The magnetic beads are coated with CD3 and CD28 on their surface to act as the antigens to activate the T-cells so they can proliferate. The micro-beads suspended in cell culture medium with T-cells provide a relatively large surface area for T-cells to contact and temporarily bind to CD3 and CD28 and then activate. After T-cells are grown to a certain density, the T-cells and magnetic beads are moved into a relatively large bioreactor such as the GE's WAVE bioreactor to continue the process of stimulation and expansion. The process is repeated multiple times to grow relatively large numbers of T-cells. At harvesting, the T cells have to be separated from the beads using a magnetic separator. Accordingly, the current T-cell expansion process generally relies upon magnetic beads, multi-stage processing, and manual interactions, which is not cost-effective. In addition, it is an open system, which can easily

introduce contaminations and make it relatively more expansive to meet good manufacturing process (GMP) requirements.

Accordingly, a need remains for methods and devices to improve cellular expansion, and in particular T-cell expansion, by offering improved bioreactor designs, cost-effective fabrication techniques, and improved bioreactor operating capability in order to achieve clinical application dose requirements.

Summary

A method for T-cell expansion comprises: (a) supplying a 3D bioreactor comprising a plurality of voids having a surface area for cell expansion, said plurality of voids having a diameter **D**, a plurality of pore openings between said voids having a diameter **d**, such that **D>d** and wherein: (a) 90% or more of said voids have a selected void volume (**V**) that does not vary by more than +/- 10.0%; and (b) 90% or more of said pore openings between said voids have a value of **d** that does not vary by more than +/- 10.0%; (b) providing a polydopamine coating on said bioreactor surface area for cell expansion; (c) providing a tetrameric protein, such as avidin or streptavidin, attached to said polydopamine coating; (d) providing one or more biotinylated antibodies immobilized on said tetrameric protein; (e) flowing T-cells through said 3D bioreactor having T-cell surface receptors where said T-cell receptors bind to said biotinylated antibody and are activated; (e) exposing the activated T-cells to a perfusion media containing a signaling molecule (such as cytokine) to promote T-cell expansion.

Brief Description Of The Drawings

FIG. 1 illustrates a section view of the 3D bioreactor fixed-bed.

FIG. 1a illustrates a unit negative model of the bioreactor that shows the overlapping of the neighborhood spheres.

FIG. 1b illustrates a unit negative model with each sphere surrounded by 12 identical neighborhood spheres.

FIG. 1c illustrates a 3D bioreactor fixed-bed geometry showing an interconnected void system.

FIG. 1d illustrates a 3D bioreactor fixed-bed geometry in cross-sectional view.

FIG. 1e illustrates in 2D view the identified spherical voids of a 3D bioreactor, and their overlapping areas to form interconnected pores between the spherical voids.

FIG. 2 illustrates a 3D bioreactor fixed-bed positioned in a housing with inlet and outlet for fluid perfusion.

FIG. 3 illustrates a typical 3D bioreactor perfusion system.

FIGS. 4a, 4b, 4c and **4d** show flow rate profiles through a 3D bioreactor.

FIG. 4e indicates the scale of flow rate through a 3D bioreactor.

FIGS. 5a and **5b** illustrate the distribution of surface shear stress in a 3D bioreactor.

FIG. 5c indicates a scale of shear stress in unit Pa.

FIG. 6a illustrates the pressure drop (gradient) along the flow direction in a cylindrical 3D bioreactor.

FIG. 6b indicates a scale of pressure.

FIG. 7a illustrates a 3D bioreactor fixed-bed generated by FDM 3D printing.

FIG. 7b illustrates the 3D bioreactor fixed-bed together with a bioreactor chamber.

FIG. 7c illustrates the inlet and outlet of a 3D bioreactor.

FIG. 7d illustrates an assembled 3D bioreactor.

FIG. 7e illustrates a 3D bioreactor fixed-bed generated by SLA 3D printing.

FIG. 7f illustrates a 3D bioreactor fixed bed generated by DLP 3D printing.

FIG. 8 illustrates two fluid distributors placed at the inlet and outlet of the 3D bioreactor to approach a laminar flow.

FIG. 9 illustrates a flow rate profile through the 3D bioreactor when using the fluid distributor.

FIGS. 10a and **10b** illustrate the formation of a 3D bioreactor by the alternative porogen-leaching method.

FIG. 11 illustrates immobilization of antibodies on the 3D bioreactor's spherical void volume for binding and activation of T-cells.

FIG. 12 illustrates a bead-free, closed loop perfusion based 3D bioreactor for T-cell activation and expansion.

FIG. 13 illustrates fluorescence intensity of avidin and streptavidin coatings at different concentrations. The fluorescent-labeled avidin and streptavidin were used to show the concentration of avidin and streptavidin coated on the bioreactor surface.

FIG. 14 illustrates fluorescence intensity of the biotin coating on avidin or streptavidin. The fluorescent-labeled biotin was used to show the concentration of biotin bound onto the avidin or streptavidin.

FIG. 15 illustrates T-cell growth (after beads activation) incubated (static) with antibody-coated magnetic beads and bioreactor matrix versus a bioreactor matrix without an antibody coating.

FIG. 16 illustrates T-Cell activation and expansion incubated (static) with beads, bioreactor matrix with antibody coating and bioreactor matrix without antibody coating, respectively.

FIG. 17 illustrates T-cell activation and expansion in a 3D bioreactor with continuous perfusion.

Detailed Description of Preferred Embodiments

The present disclosure relates to a perfusion-based scalable bioreactor design and with corresponding operating capability to achieve T-cell expansion for immunotherapy purposes. The activation and expansion of T-cells from a patient, after for example, gene-modification, provides a therapeutic T-cell product that can be infused back to the patient and uses patient's own immune system in a manner that selectively targets and kills the patient's tumor cells.

Reference to a bioreactor herein refers to the disclosed 3D reactor in which biological and/or biochemical processes can be implemented under selected environmental and operating conditions. This includes control of one or more of the following: geometry/size of the voids, interconnected pore size between the voids and total number of voids included (determining the overall dimension of the bioreactor). In addition, one may selective control surface coatings, flow characteristics through the voids within the bioreactor, pH, temperature, pressure, oxygen, nutrient supply, and/or waste removal. Clinical dosage requirements is reference to the ability to provide a dose of 10^9 cells or greater.

The 3D bioreactor's preferred fixed-bed **10** is generally illustrated in cut-away view in **FIG. 1,** which shows an example of a preferred packed and spherical void structure and their interconnected pores between the spherical voids. More specifically, the bioreactor includes a continuous interconnected 3D surface area **12** that provides for the ability for the cells to bind in the present of antibodies and also defines within the bioreactor a plurality of interconnected non-random voids **14** which as illustrated are preferably of spherical shape with internal concave surfaces to maximize the surface to volume ratio. A void is understood as an open space of some defined volume. By reference to non-random it should be understood that one can now identify a targeted or selected number of voids in the 3D bioreactor that results in an actual repeating void size and/or geometry of a desired tolerance.

By reference to a continuous surface, it is understood that the expanding cells can readily migrate from one surface area location into another within the 3D bioreactor, and the surface does not include any random interruptions, such as random breaks in the surface or random gaps of 0.1 mm or more. Preferably, 50% or more of the surface area within the 3D bioreactor for cell expansion is a continuous surface, more preferably, 60% or more, 70% or more, 80% or more, 90% or more, 95% or more or 99% or more of the surface area within the 3D bioreactor is continuous.

In addition, the bioreactor fixed-bed **10** includes non-random interconnecting pore openings **16** as between the voids. Again, reference to non-random should be understood that one can now identify a targeted or selected number of pores for the voids, of a selected pore diameter, that results in an actual number of pores having pore diameters of a desired tolerance. The bioreactor as illustrated in cut-away view also ultimately defines a layer of non-random voids (see

arrow "**L**") and it may be appreciated that the multiple layers of the bioreactor may then allow for identification of a plurality of such non-random voids within a column (see arrow "**C**").

The bioreactor may be made of biocompatible or bio-inert polymeric materials such as polystyrene, polycarbonate, acrylonitrile-butadiene-styrene (ABS), polylactic acid (PLA), polycaprolactone (PCL) used in FDM (fused deposition modeling) 3D printing technology. Reference to biocompatible or bio-inert should be understood as a material that is non-toxic to the culturing cells. In addition, the polymeric materials for the 3D bioreactor are preferably selected from those polymers that at not susceptible to hydrolysis during cell cultivation, such that the amount of hydrolysis does not exceed 5.0 % by weight of the polymeric material present, more preferably it does not exceed 2.5 % by weight, and most preferably does not exceed 1.0 % by weight. The bioreactor may also be made of biocompatible materials (e.g., poly(methyl methacrylate) or PMMA , etc.) used in SLA (stereolithography) and DLP (digital light processing) 3D printing technologies.

It is preferable that the material used to fabricate the bioreactor is not degradable in aqueous medium and can provide a mechanical stable structure to tolerate aqueous medium flow during cell expansion. It is preferable that the material and manufacturing process can result a solid and smooth interconnected surface area for monolayer cell expansion. By reference to a solid surface, it should be understood that the surface is such that it will reduce or prevent penetration or embedding by the culturing cells, which typically have a diameter of about 20 microns to 100 microns. Preferably, the 3D bioreactor herein is one that has a surface that has a surface roughness value (**Ra**), which is reference to the arithmetic average of the absolute values of the profile height deviations from the mean line, recorded within an evaluation length. Accordingly, it is contemplated herein that **Ra** of the 3D bioreactor surface will have a value of less than or equal to 20 μm, more preferably, less than or equal to 5 μm.

The 3D bioreactor herein is also preferably one that is formed from material that indicates a Shore D Hardness of at least 10, or in the range of 10-95, and more preferably in the range of 45-95. In such regard, it is also worth noting that the 3D bioreactor herein is one that does not make use of a hydrogel type structure, which may be understood as a hydrophilic type polymeric structure, that includes some amount of crosslinking, and which absorbs significant amounts of water (e.g., 10-40 % by weight). It is also worth noting that the 3D bioreactor herein is one that

preferably does not make use of collagen, alginate, fibrin and other polymers that cells can easily digest and undergo remodeling.

Furthermore, the 3D bioreactor herein is preferably one that is made from materials that have a Tensile Modulus of at least 0.01 GPa. More preferably, the Tensile Modulus has a value that is in the range of 0.01 GPa to 20.0 GPa, at 0.01 GPa increments. Even more preferably, the Tensile Modulus for the material for the 3D bioreactor is in the range of 0.01 GPa to 10.0 GPa or 1.0 GPa to 10 GPa. For example, with respect to the earlier referenced polymeric materials suitable for manufacture of the 3D bioreactor herein, polystyrene indicates a Tensile Modulus of about 3.0 GPa, polycarbonate at about 2.6 GPa, ABS at about 2.3 GPa, PLA at about 3.5 GPa, PCL at about 1.2 GPa, and PMMA at about 3.0 GPa.

The 3D bioreactor design herein with such preferred regular geometric characteristics and continuous surface area is preferably fabricated by additive manufacturing technologies, such as FDM, selective laser sintering (SLS), stereolithography (SLA), digital light processing (DLP) 3D printing technologies, etc., according to computer generated designs made available by, e.g., a SolidWorks™ computer-aided design (CAD) program.

By way of preferred example, the process utilizing SolidWorks™ to create the 3D bioreactor design is described below. A computer model for the bioreactor negative is initially created. More specifically, what may therefore be described as a 3D bioreactor negative was created, e.g., using packed 6.0 mm diameter spheres that overlap to create 1.0 mm diameter connecting pores between spheres. Of course, other possible dimensions are contemplated within the broad context of this disclosure.

The spheres are preferably organized in a hexagonal close packed (HCP) lattice to create an efficiently (or tightly) packed geometry that results in each sphere surrounded by 12 neighborhood spheres. A unit cell of this exemplary geometry is shown in **FIG. 1a**. More specifically, in **FIG 1a** there is a unit cell of the HCP lattice where the top three spheres are displayed as translucent to show the 6 radial overlapping areas between the neighborhood spheres. The pores are formed at these overlapping areas. Preferably, the maximum number of pores is 12 to optimize packing. The minimum pore number is 2 in order to allow medium perfusion through the voids of the 3D bioreactor. Accordingly, at least 90.0 % to 100 % of the voids present in the

3D bioreactor have at least 2 pore openings per void. More preferably, at least 90.0 % to 100 % of the voids in the 3D bioreactor have 8-12 pore openings per void. In one particularly preferred embodiment, at least 90.0 % to 100 % of the voids in the 3D bioreactor have 12 pore openings per void.

In **FIG. 1b**, all spheres of the unit are illustrated. The bioreactor geometry is then preferably created by reversing the negative model to create the positive model comprising an interconnected spherical void system shown in **FIG. 1c**. Moreover, in **FIG. 1d** one can see the 3D bioreactor again in cross-sectional view providing another illustration of the interconnected voids shown in cut-away view at **14** with regular geometric characteristics (substantially the same control of void volume as described above) and the corresponding interconnected pore openings **16**.

In the preferred regular geometric 3D bioreactor described above, one can identify a relationship as between the void diameter and interconnected pore diameter. Attention is directed to **FIG. 1e**. For this preferred geometry, Spherical Void 1 is represented by a solid circle, diameter is D (indicated by the arrows). Diameter "D" may therefore be understood as the longest distance between any two points on the internal void surface. Spherical Void 2 is represented by a dash circle and would also have diameter D (not shown). Spherical Void 2 is one of the 12 of neighborhood voids of Spherical Void 1. Due to the overlap between the neighborhood voids, it forms interconnected pores between the spherical voids, with the diameter of "d" as also indicated by the generally horizontal arrow. Diameter "d" may therefore be understood as the longest distance between any two points at the pore opening. The total 3D spherical surface area of the void is $SA_{void} = 4\times\pi\times(D/2)^2$. The surface area between A and B, called $S_{cap} = \pi\times D\times h$, where $h = \frac{D-\sqrt{D^2-d^2}}{2}$. The useful void surface for a given void in the 3D bioreactor would be $SA_u = SA_{void} - [12\times S_{cap}]$.

The smaller the void diameter D, the larger the number of voids can be packed into a set 3D space (volume), and therefore results larger overall cell binding surface. However, to minimize or prevent cell aggregation to block the perfusion, the minimal diameter of the pores is preferred d = 0.2 mm for this geometry. The diameter of the pores d may fall in the range of 0.2 mm to 10

mm and more preferably 0.2 mm to 2.0 mm. Most preferably, $d \geq 0.5$ mm and falls in in the range of 0.5 mm to 2.0 mm.

If D = 0.40 mm or less, the computed SA_u is less than 0 when d = 0.2 mm, which leads to an impossible structure therefore, D has to be > 0.4 mm for this 3D bioreactor geometry. However, D can have a value between 0.4 mm to 100.0 mm, more preferably, 0.4 mm to 50.0 mm, and also in the range of 0.4 mm to 25.0 mm. One particularly preferred value of D falls in the range of 2.0 mm to 10.0 mm. Spherical voids with a relatively large value of D may reduce the objective of increasing cell culture surface area as much as possible within a same bioreactor volume. Accordingly, for the preferred geometry illustrated in **FIG. 1e**, D > 0.4 mm (the diameter of the void) and d > 0.20 mm (the diameter of the pores). It is also worth noting that with respect to any selected value of diameter D for the voids in the range of 0.4 mm to 100.0, and any selected value of diameter d for the pores in the range of 0.2 mm to 10.0 mm, the value of D is such that it is greater than the value of d (D>d).

It can now be appreciated that the 3D bioreactor herein can be characterized with respect to its non-random characteristics. Preferably, all of the voids within the 3D bioreactor are such that they have substantially the same volume to achieve the most efficient 3D space packing and offer the largest corresponding continuous surface area. With respect to the total number of interconnected voids present in any given 3D bioreactor, preferably, 90.0 % or more of such voids, or even 95.0 % or more of such voids, or even 99.0 % to 100 % of such voids have a void volume (V) whose tolerance is such that it does not vary by more than +/- 10.0%, or +/- 5.0%, or +/- 2.5% or +/- 1.0%, or +/- 0.5% or +/- 0.1%. It should be noted that while the voids in FIG. 1 are shown as generally spherical, other voids geometries are contemplated. The diameter of voids are chosen to minimize or avoid cell aggregation and to provide maximum useful surface area for cell culturing.

Another non-random characteristic of the 3D bioreactor herein are the pore openings between the voids, having a diameter **d** (see again **FIG. 1e**). Similar to the above, 90.0 % or more of the pore openings, or even 95.0 % or more of the pore openings, or even 99.0 % to 100 % of the pore openings between the voids, indicate a value of **d** whose tolerance does not vary more than +/- 10.%, or +/- 5.0%, or +/- 2.5% or +/- 1.0%, or +/- 0.5% or +/- 0.1%.

It can therefore now by appreciated that the 3D bioreactor herein for growth of non-adherent cells comprises a surface area for cell binding, a plurality of voids having a diameter **D** (the longest distance between any two points on the internal void surface), a plurality of pore openings between said voids having a diameter d (the longest distance between any two points at the pore opening), where **D>d**. In addition, 90% or more of the voids have a void volume (**V**) that does not vary by more than +/- 10.0%, and 90% or more of the pore openings have a value of **d** that does not vary by more than +/- 10.0%.

In addition, the 3D bioreactor herein for expansion of non-adherent cells like T-cells can include a first plurality of voids having a diameter D_1, a plurality of pore openings between said first plurality of voids having a diameter d_1, wherein $D_1 > d_1$, where 90% or more of the first plurality of voids have a void volume (V_1) with a tolerance that does not vary by more than +/- 10.0%. Such 3D bioreactor may also have a second plurality of voids having a diameter D_2, a plurality of pore openings between said second plurality of voids having a diameter d_2 wherein $D_2 > d_2$, wherein 90% of the second plurality of voids have a void volume (V_2) with a tolerance that does not vary by more than +/- 10.0%. The values of V_1 and V_2 are different and outside of their tolerance variations. Stated another way, the value of V_1, including its tolerance of +/- 10.0 % and the value of V_2, including its tolerance of +/- 10.0%, are different, or [V_1 +/- 10.0%] $\neq$ [V_2 +/- 10.0%].

The radius of curvature (Rc) of the surface within the voids is therefore preferably 1/0.5(D), or 1/0.2 mm = 5 mm^{-1} or lower. Preferably, Rc may have a value of 0.2 mm^{-1} to 1.0 mm^{-1}, which corresponds to a value of D of 10.0 mm to 2.0 mm. A high curvature (large Rc) surface provides a significantly different environment than the typical monolayer 2D culture, which may also induce cell phenotype changes.

Cells are preferably bound on the interconnected spherical void surfaces of the 3D bioreactor. Such 3D structure is preferably scalable and is able to provide a relatively high surface to volume ratio for relatively large cell expansion with a relatively small footprint cell expansion bioreactor. The surface area-to-volume ratio is also preferably determined by the diameter of the spherical voids. The smaller is the diameter, the higher is the surface area-to-volume ratio. Preferably, the voids provide a relatively "flat" surface (i.e., low radius of curvature $\leq$ 1.0 mm^{-1}) for cells having a size of 5 µm to 100 µm and also to reduce or avoid cell aggregation. In addition,

as alluded to above, cell aggregation is also reduced or avoided by controlling the diameter d of the interconnected pores, which diameter is preferably at least 500 μm, but as noted, any size greater than 200 μm.

The bioreactor fixed-bed **10** may therefore preferably serve as a single-use 3D bioreactor as further illustrated in **FIG. 2**. More specifically, the bioreactor **10** may be positioned in a housing **18** and then placed in the inlet and outlet compartment **20** for which inflow and outflow of fluid may be provided. Preferably, the bioreactor **10**, housing **18**, and the inlet and outlet compartment **20** can be fabricated as a single component using Additive Manufacturing technology. As shown in **FIG. 3**, the bioreactor **10** in housing **18** and inlet and outlet compartment **20** may become part of an overall 3D bioreactor system for MSCs expansion. More specifically, the 3D bioreactor is preferably positioned within a perfusion system which delivers a cell culture medium and oxygen through the 3D bioreactor for promoting cell growth. Multiple passage cell expansion methods used in 2D T-flask can also be directly applied to the 3D bioreactor except a 3D bioreactor has the cell culture area equivalent to 10s, 100s, or 1000s of T-flasks.

As may now be appreciated, the 3D bioreactor herein offers a relatively large surface-to-volume ratio depending upon the diameter of the interconnected voids. By way of example, a conventional roller bottle defining a cylinder of 5 cm diameter and 15 cm height, provides a cell growth surface area of 236 cm^2. If the same volume is used to enclose the 3D bioreactor herein with 2.0 mm diameter interconnected voids, a total of 44,968 spherical voids can be packed into the space, which can provide a matrix with about 5,648 cm^2 surface area, an almost 24-fold larger than the roller bottle surface area.

At least one unique feature of the 3D bioreactor herein in comparison with hollow-fiber or microcarrier-based bioreactors is the ability to provide a large interconnected continuous surface instead of fragmented surfaces. Continuous surfaces within the 3D bioreactor herein are therefore contemplated to enable cells to more freely migrate from one area to another. Using the perfusion system shown in **FIG. 3**, it is contemplated that one can readily create a gradient of nutrition or cell signals inside the bioreactor to induce cell migration.

In conjunction with the preferred 3D printing technology noted herein for preparation of the 3D bioreactor, computational fluid dynamics (CFD) can now be used to simulate the medium

flow inside the bioreactor and estimate the flow rate and shear stress at any location inside the 3D interconnected surface, and allow for optimization to improve the cell culture environment. More specifically, CFD was employed to simulate the flow characteristics through the 3D interconnected voids of the bioreactor herein and to estimate the distribution of: (1) flow velocity; (2) pressure drop; and (3) wall shear stress. It may be appreciated that the latter parameter, shear stress, is important for cell expansion. A reduction in shear stress can reduce or prevent shear induced cell differentiation.

A small-scale (to increase computer simulation speed) cylindrical 3D bioreactor with a diameter of 17.5 mm, height of 5.83 mm, void diameter of 2 mm, and pore diameter of 0.5 mm was used in the simulations reported below. In this case, the diameter (Φ=17.5 mm) to height (H=5.83 mm) ratio of the bioreactor is 3:1 (**FIG 1d**), which is a preferable ratio to reduce the gradient of nutrition and oxygen between the inlet and outlet of the bioreactor. Based on the cell density available on the fixed-bed spherical surface the oxygen and nutrition consumption rates were estimated, and how often the cell culture media needed to be replaced (i.e., the volume flow rate) was determined. An overall linear flow rate of 38.5 μm/sec was assumed in this simulation. Using 38.5 μm/sec rate laminar flow as the input to the 3D bioreactor, the CFD results are shown in **FIGS. 4-6**.

FIGS. 4a, **4b**, **4c** and **4d** show the flow velocity profile throughout the small-scale cylindrical 3D bioreactor. **FIG. 4e** indicates the scale of flow rate. More specifically, **FIG. 4a** indicates the flow rate distribution viewed from the side of the bioreactor. The flow passes each spherical voids through the pores along the flow direction. The white/gray areas in the figures are the solid regions between the spherical voids with no fluid flow. By comparing with the colored velocity scale bar in **FIG. 4e**, **FIG. 4a** indicates that the flow rate at the pores along the flow direction achieve the maximum flow rate of 200 μm/s to 240 μm/s. In contrast, the flow rates near the spherical surface reduce to a minimum of 0.06 μm/s to 19.0 μm/s, which will significantly reduce the flow caused shear stress to cells reside on the spherical surface.

FIG. 4b indicates the velocity profile viewed from the top of the bioreactor through a center cross-section of the 3D structure. Again, the image shows that the maximum rates are at each center of the pores of the spherical voids along the flow direction. This maximum rate is again in

the range of 200 μm/s to 240 μm/s. The flow rate near spherical surface is again low and has a value of 0.06 μm/s to 19.0 μm/s.

FIG. 4c indicates the velocity profile of an individual sphere void showing flow passing through the radial interconnected pores. **FIG 4c** therefore provides a useful illustration of the flow distribution inside a spherical void. The high flow rate is at the central empty space of a void where there are no cells and is at a level of 200 μm/s to 240 μm/s. The cells reside on the concaved void surface where the flow rate is reduced and where the flow rate is again at a level of 0.06 μm/s to 19.0 μm/s. This unique structure can therefore shield cells from exposure to relatively high flow stress. This is another distinct advantage of the 3D bioreactor described herein over, e.g., micro-carrier based reactors, where cells are grown on the outside surface surfaces of 300 μm to 400 μm diameter microbeads with convex spherical surfaces that are suspended in a cell culture medium and stirred in a bioreactor to deliver nutrition and oxygen to the cells. Cells residing on such convex spherical surfaces can be exposed to relatively large shear stress to 0.1 Pa, which is known to affect cellular morphology, permeability, and gene expression. **FIG. 4d** indicates the flow trajectory through the side pores along the flow direction, indicating that the 3D bioreactor herein provides a relatively uniform flow pattern to provide nutrients and oxygen throughout.

Accordingly, the maximum linear flow rate computed inside the preferred 3D bioreactor is 200 μm/s to 240 μm/s which occurs at the 0.5 mm diameter interconnected pores between 2.0 mm diameter voids along the flow direction. As shown in **FIGS. 4a-4e** while the flow is preferentially in the central direction along the flow, there is still flow (~19.0 μm/sec) near the spherical surface to allow nutritional supply to the cells residing on the spherical surface. Therefore, it is contemplated that the cells are able to reside anywhere throughout the structure and thrive in any location because nutrients can be supplied both through flow convection and diffusion throughout the 3D bioreactor structure.

FIGS. 5a and **5b** show the distribution of surface shear stress throughout the cylindrical 3D bioreactor described above as well as on a single spherical void surface. **FIG. 5c** indicates the scale of shear stress in units of Pa. The highest shear stress was observed on the edges of the interconnected pores. This is due to the higher flow rates at these locations. However, the majority of the useful spherical surface area within the bioreactor indicates a shear stress of less than $3x10^{-4}$ Pa, which may be understood as 90% or more of the surface area of the bioreactor. This provides

for cell proliferation, without shear induced differentiation. In addition, even the maximum shear stress of 4.0 x 10^{-3} Pa, is believed to be lower than the average shear stress that cells experience when cultured in hollow fiber based bioreactors, wave bioreactors, and micro-carrier based bioreactors. Therefore, the 3D bioreactor herein is contemplated to provide a relatively lower shear stress environment for cell growth in comparison to existing cell expansion bioreactors. See, e.g., *Large-Scale Industrialized Cell Expansion: Producing The Critical Raw Material For Biofabrication Processes*, A. Kumar and B. Starly, Biofabrication 7(4):044103 (2015).

FIG. 6a illustrates the pressure drop along the flow direction from bottom to the top of the cylindrical 3D bioreactor described above. **FIG. 6b** provides the applicable scale of pressure. The figure indicates that the overall pressure drop between the inlet and outlet of the bioreactor is less than or equal to 1.0 Pa. The pressure drop may therefore fall in the range of 0.1 Pa up to 1.0 Pa. In other words, cells near the inlet and outlet of the bioreactor will not experience significant differences in pressure. The low gradient of pressure suggests that such design will also produce a small gradient (or difference) in nutrition/metabolites concentrations between the inlet and outlet of the bioreactor. The low gradient is due to the design of the bioreactor such that the diameter **Φ** is larger than the height **H** while the total bioreactor volume remains the same. This is superior to the hollow fiber bioreactor. It is difficult to fabricate a hollow fiber bioreactor with **Φ>H** ratio to reduce the gradient of nutrition/metabolites between the inlet and outlet of the bioreactor.

A comparison was also made for the same total volume cylindrical 3D bioreactor with different aspect ratios (i.e. **Φ:H** ratio, **Φ**: overall diameter of the bioreactor fixed-bed, **H**: overall height of the bioreactor fixed-bed). See **FIG. 1d**. As shown in **Table 1**, for the same volume flow rate (volume flow rate = cross area of flow × linear velocity), the linear velocity increases significantly for a bioreactor with a low **Φ:H** ratio. The increase of linear velocity also increases the surface shear stress, pressure drop, as well as the gradient of nutrition/metabolites concentrations between the inlet and outlet, which would have an unfavorable effect for cell expansion. The disclosed fixed-bed 3D bioreactor is therefore preferably designed into a **Φ:H** ratio structure, e.g., a **Φ:H** ratio in the range of greater than 1:1 and up to 100:1. Preferably, the **Φ:H** ratio is greater than 1:1 and up to 10:1.

Table 1
Flow Rate Comparison For 3D Bioreactor
With Different Aspect Ratios

#	Ratio (Φ:H)	Diameter (Φ) (cm)	Height (H) (cm)	Flow Rate (μm/sec)
1	3:1	10.5	3.5	38.5
2	1:1	7.5	7.5	75.4
3	1:3	5	15	169.8

FIG. 7a illustrates a 3D bioreactor fixed-bed part generated by FDM 3D printing with interconnected 6 mm diameter voids and 1 mm interconnected pores. This 3D bioreactor was printed with ABS filament. The diameter (Φ) and height (H) of this particular 3D bioreactor is 4.28 cm and 1.43 cm respectively. Accordingly the Φ:H ratio is 3:1. There are about 134 interconnected open-voids included in the fixed-bed. The total interconnected continuous spherical surface area SA_u for cell culturing is about 152 cm^2. The inlet and outlet wall and fluid distributor **22** at the inlet and outlet (**FIG. 8**) provides an additional 88 cm^2 surface area for cell culturing. In other words, there is about 240 cm^2 total useful surface area in the 3D bioreactor for cell attachment. The fluid distributor can improve the laminar flow through the bioreactor. The fluid distributor is optional if the Reynolds number is <2100 or in the range of greater than 0 up to and not including 2100.

FIG. 7b shows that the fixed-bed of the 3D bioreactor was solvent bound into a bioreactor chamber. This will seal the gaps between the fixed-bed and the chamber wall, which will force the perfusion cell culture medium to pass through the interconnected pores instead of through those gaps. Preferably, the fixed-bed and chamber is printed together as an integrated part to increase the manufacturing efficiency. **FIG. 7c** illustrates the inlet and outlet of the bioreactor. They are designed geometrically to promote a laminar flow through the fixed-bed. The inlet of the bioreactor optionally contains a built-in rotation gear, which may be coupled to a stepper motor to control the

rotation of the bioreactor for uniform cell seeding (see below). The integrated bioreactor is shown in **FIG. 7d** and is able to connect to 1/8 inch tubing to conduct the fluid flow. Alternatively, the inlet and outlet can be made for repeated usage, where only the inside bioreactor fixed bed is disposable. Also shown in **FIG. 7e** is a relatively smaller-size 3D bioreactor fixed bed produced by DLA 3D printing having a 3.0 mm void and a 0.5 mm pore. **FIG. 7f** is a relatively larger-size 3D bioreactor fixed bed using DLP 3D printing having the same-diameter3.0 mm void and a 0.5 mm pore. When scaling up the bioreactor, the internal structure of the bioreactor is maintained. Therefore, the perfusion volume flow rate can be scaled up proportionally while keeping the local linear flow rate the same. In this way, minimum process change is required during the scale-up process which can reduce the process development costs.

It should next be noted that the fluid distributor **22** (**FIG. 8**) is preferably such that it will improve the flow uniformity through the 3D bioreactor. The design of the inlet, outlet, and fluid distributor also preferably takes into consideration the following: (1) improve the flow uniformity through the 3D bioreactor; (2) minimization of the dead-volume **24** at inlet and outlet to reduce the overall priming volume of the bioreactor; and (3) preventing bubble collection inside the bioreactor. **FIG. 9** shows the flow velocity profile throughout the 3D bioreactor based on CFD simulation by using the fluid distributor. The use of the fluid distributor (**FIG. 8**) improved the uniformity of the flow. The maximum flow rate (around 30 μm/s) and the minimum flow rate (around 10 μm/s) are relatively close to each other and serve to promote uniform laminar flow (i.e. flow of fluid in relatively parallel layers). A relatively uniform flow rate everywhere in the bioreactor will also provide smaller differences of shear stress to cells residing at different locations in the bioreactor.

The 3D bioreactor can be fabricated by other additive manufacturing technologies such as selective laser sintering (SLS), stereolithography (SLA), Digital Light Processing (DLP), and etc. **FIGS. 7e, 7f**.

In addition to preparing the 3D bioreactor herein via additive manufacturing or 3D printing, it is contemplated that the 3D bioreactor may be prepared by the traditional porogen-leaching method to provide an interconnected cell culture surface. **FIGS. 10a** and **10b** shows a 3D bioreactor utilizing a porogen-leaching methodology. This is reference to combining porogen and polymer in a mold, followed by leaching out of the porogen to generate pores. The 3D bioreactor

in **FIG. 10a** starts with the step of tightly packing 4.0 mm water-soluble spherical sugar beads (as porogen) in a cylindrical stainless mold by shaking, tapping, and pressing the beads, so that the beads are in contact. The gaps between the beads are filled with acetone containing 5.0% by weight deionized water. This is followed by evaporation the acetone and water under vacuum chamber overnight. The gaps between the beads are then filled with a low viscous polymerizable vinyl monomer such as styrene together with polymerization initiators such as benzoyl or tert-butylperoxybenzoate. The styrene monomer will then polymerize to form polystyrene. The sample remained at 90 °C for 8-12 hours, and then was heated to 115 °C for an additional 3 hours and removed from the oven to provide what is illustrated in **FIG. 10a**. The sugar beads were then leached out while submerged in an ultrasound water bath to leave the polystyrene 3D bioreactor fixed-bed with interconnected voids. See **FIG. 10b**. The 3D bioreactor is then extracted with methanol for three days to remove any residual styrene monomer. However, the porogen leaching method not only has a complex manufacturing process, but also is difficult to achieve exact reproducible structures since the packing of porogen beads is a random process.

For the 3D printed bioreactor (**FIG. 7d**) using ABS or PMMA, the hydrophobic internal surfaces of the bioreactor is preferably modified to allow for cell adherence. Polydopamine was used as a primer coating to the bioreactor surfaces so that other proteins can be easily adhere to the bioreactor surface via the polydopamine coating. Incubation of the bioreactor surface in a 0.25 mg/mL dopamine dissolved in 10 mM Tris buffer (pH = 8.5 at 25 °C) for a period of about 18 hours, resulted in an effective polydopamine layer for the subsequent protein coating. After polydopamine is deposited on the bioreactor surface, other proteins can then bind with functional ligands via Michael addition and/or Schiff base reactions. The ligand molecules therefore include nucleophilic functional groups, such as amine and thiol functional groups.

It should now be appreciated from all of the above that one of the additional features of the 3D bioreactor disclosed herein is that one may now design a 3D bioreactor, with particular geometric and void volume requirements, and corresponding available surface area requirements, and be able to achieve (i.e., during fabrication or manufacturing) such targets with relatively minimal variation. For example, one may now identify a design requirement for a 3D bioreactor wherein the one or more internal voids are to have a targeted void volume "V_t", and the 3D bioreactor itself is to have a targeted overall surface area for cell culturing "SA_t". Accordingly,

one may now form such 3D bioreactor wherein the one or more internal voids have an actual void volume "V_a" that is within +/- 10.0% of V_t, or more preferably, +/- 5.0% of V_t. Similarly, the actual surface area for cell culturing SA_a is within +/- 10.0% of SA_t, or more preferably +/- 5.0% of SA_t. Moreover, one may also identify for the internal surface within the targeted voids a targeted geometry for fabrication such as a targeted radius of curvature "Rc_t" and then in fabrication the actual radius of curvature "Rc_a" of the void internal surface can now be achieved that is within +/- 5% of Rc_t.

The present invention advances further on the use of the above referenced 3D bioreactor herein, to provide for T-cell expansion as applied for immunotherapy purposes. Reference is made to **FIG. 11,** which illustrates immobilization of antibodies on the bioreactor's spherical void volume surface for binding and activation of T-cells. More specifically, for the 3D bioreactor, one can now immobilize antibodies (biotin conjugated sphere-shaped proteins) such as anti-CD3 and anti-CD28 antibodies on the bioreactor surface via the biotin-avidin/streptavidin binding mechanism. Avidin or streptavidin are pre-coated on the bioreactor surface through the prime polydopamine coating as discussed above. More specifically, after a polydopamine coating is applied to the surface of the 3D bioreactor herein, one may attach a tetrameric protein (protein with quaternary structure of four subunits) such as avidin or streptavidin, which have affinity for biotin. Accordingly, proteins with quaternary structure (avidin or streptavidin) may be relied upon to immobilize biotinylated antibodies, for example, anti-CD3 antibody onto the bioreactor surface. A biotinylated antibody is reference to covalent attachment of biotin to the antibody. When T-cells flow through the bioreactor, they will bind and activate via the T-cell surface receptor, for example CD3, via the CD3, anti CD3 antibody binding. This is then followed by exposing the activated T-cells to a perfusion media containing a signaling molecule to provide T-cell expansion. The signaling molecule may preferably include a cytokine signaling molecule such as interleukin-2 (IL-2). It should be noted, however, that in the broad context of the present invention, it is contemplated that one may exclude the need for the use of a polydopamine coating and immobilize the antibody (e.g. the anti-CD3 antibody) directly on the surface of the 3D reactor, avidin/streptavidin directly on the bioreactor surface to bind biotinylated antibodies.

Using the 3D bioreactor herein, a closed-loop perfusion-based system for T-cell expansion is now possible as illustrated in **FIG. 12**. More specifically, **FIG. 12** illustrates a bead-free, closed-

loop perfusion based 3D bioreactor for T-cell activation and expansion. The perfusion system preferably contains a cell-safe peristaltic pump and a medium/cell reservoir, which is able to add media to dilute cell density for more effective cell expansion or exchange media to maintain the nutrient concentration during the expansion process. The medium reservoir may also serve as an oxygenator with gas infusion and stirring or shaking mechanism to mix the nutrients and oxygen. The suspended T-cells in the medium perfused through the 3D bioreactor have many chances of contacting CD3 and CD28 antibodies on the spherical surfaces and thus to be activated. The immobilized CD3 and CD 28 on the large surface is expected to provide equivalent stimulation to T-cells as the magnetic beads used in the current T-cell expansion system. Without the beads, the processes will be simplified significantly. In addition, this closed-loop system facilitates automation and cost-effective cGMP cell manufacturing.

Table 1 lists the dimensions, culture surface area, number of magnetic beads with equivalent total surface area, expected medium volume, etc. of three different sized bioreactors. PMMA, an FDA approved implantable biocompatible material, was used to fabricate the bioreactor using a DLP 3D printer. **Table 1** also lists the approximate material cost to construct the identified 3D bioreactors.

Table 1: Dimension and Cost of Fabricating Different Sized 3D Bioreactors Using SLA/DLP 3D Printing

PBMC Seeding Capacity	**2.5×10^7** [α]	**2.2×10^8**	**2.2×10^9**
Bioreactor diameter (cm)	2.1	4.2	9.0
Bioreactor height (cm)	0.7	1.4	3.0
Surface area (cm^2)	29	250	2500
Area equivalent to # of beads	7.5×10^7	6.5×10^8	6.5×10^9
Medium volume (mL)	2	14	136
Build material volume (mL)	0.78	5.4	53.6
Material cost[β]	$0.20	$1.34	$13.4

[α] based on 3:1 beads:cells ratio

[β] matrix only, does not include bioreactor's inlet and outlet; also assume 3 mm diameter hollow sphere and 0.5 mm pore size

Coating of Avidin (or Streptavidin) on the Bioreactor Surface

To mimic the Miltenyi MACSiBead system, avidin (streptavidin) and biotin binding mechanism was employed to immobilize anti-CD2, CD3 and CD28 antibodies on the bioreactor surface. First, different concentrations of fluorescence-labeled avidin and streptavidin were tested (**FIG. 13**) and 100 μg/mL of avidin or 30 μg/mL of streptavidin was found to achieve a relatively high avidin or streptavidin coating density on the bioreactor surface. **FIG. 13** shows florescence intensity of avidin and streptavidin coating at difference concentrations. The fluorescent-labeled avidin/streptavidin were used to test the concentration of avidin or streptavidin that can be bound on to the polydopamine prime coating. A preferred procedure of avidin/streptavidin coating is described as follows. The coating concentration of avidin or streptavidin can be further optimized:

1) The bioreactor surfaces are first coated by polydopamine herein.

2) Dissolve avidin or streptavidin in TRIS buffer (pH 8.5) to prepare the coating solution, with the avidin or streptavidin concentration of 100 μg/mL or 30 μg/mL, respectively. Immerse the bioreactor into the coating solution for 12 hours, in gentle shaking, and protected from exposure to light.

3) Wash the scaffolds thoroughly with phosphate-buffered-saline (PBS). Then leave the scaffolds in PBS ready for coating with biotinylated antibodies.

A comparison was then run with respect to different concentrations of fluorescence-labeled biotin as a second layer coating to bind to the avidin or streptavidin base layer (**FIG. 14**), and 1.0 μg/mL of biotin was identified as successfully binding to the immobilized avidin or streptavidin. See **FIG. 14**, which identifies fluorescence intensity (proportional to biotin concentrations) of the biotin coating on avidin or streptavidin. After confirming that biotin can successfully bind to the bioreactor surfaces coated with avidin or streptavidin, biotinylated anti-CD2, CD3, and CD 28 antibodies were applied onto the bioreactor for T cell activation and expansion.

To preferably apply the antibody coating on to the 2.1 cm diameter scaffolds (i.e., equivalent to about 7.5×10^7 beads total surfaces):

1) Take the bioreactor scaffolds out of the BPS

2) Remove as much PBS from the scaffolds as possible, but do not leave the scaffold dry.

3) Combine the 200 µL of 100µg/mL CD2-Biotin, 200 µL of 100µg/mL CD2-Biotin, and 200 µL of 100µg/mL CD28-Biotin in a 15-mL tube, add 1.4 mL of antibody labeling buffer (PBS without Ca2+ and Mg2+, pH = 7.2 plus 0.5% heat-inactivated fetal bovine serum and 2 mM EDTA), and mix well to make a total 2 mL biotinylated antibodies, with each the concentration of each antibody being 10 µg/mL.

4) Add 2 mL of the mixed antibodies to cover a bioreactor matrix (without inlet and outlet, **FIG. 7e**) in a 12 well plate, pipette up and down to mix.

5) Incubate in matrix in dark in the fridge for 2 hour under gentle shaking.

6) Wash away the unbounded antibodies from the scaffold thoroughly with PBS.

The coating procedures of polydopamine, avidin/streptavidin, and biotinylated antibodies can be extended to coat the internal surface of an intact bioreactor (that is, the bioreactor matrix plus the inlet and outlet).

Comparison of T Cells Incubated With Antibody-Coated Bioreactor Matrix and Magnetic Beads For T-cell Expansion

An antibody-coated matrix (without inlet and outlet) was compared with the Miltenyi MACSiBeadTM system. The magnetic beads (already attached with streptavidin) were coated with biotinylated anti-CD2, CD3, and CD28 antibodies according to the manufacturer's protocol, which is similar to the coating procedure. The relatively small 2.1 cm diameter by 0.7 cm height bioreactor (**FIG. 7e**), listed in **Table 1**, was used. The bioreactor matrix fits in a well of a 12-well plate so that one can easily compare the performance of beads and matrix. Three bioreactor matrices were coated with avidin or streptavidin first, and then the biotinylated CD2, CD3, CD28 antibodies using the preferred procedures described above. Same concentrations of antibodies were to coat the bioreactor matrix and the MACSiBeads. One bioreactor matrix with streptavidin coating was not further coated with antibodies, which was used as the negative control.

Human peripheral blood CD3+ Pan T cells (ReachBio Research Labs) were first activated and expanded (7-day) using the MACSiBeads according to the manufacturer's protocol and then de-beaded from the magnetic particles. Then 4.5×10^6 T cells were added to four wells of a 12-well plate, respectively. Four wells, each filled with 3 mL of culture medium (RPMI 1640 supplemented

with 10% fetal bovine serum and 20 IU/mL of human IL2), contains 1) 7.5×10^6 antibody-coated magnetic beads, 2) antibody-coated streptavidin-matrix, 3) antibody-coated avidin-matrix, and 4) streptavidin-matrix without antibody coating, respectively. Additional medium was added to the well on Day 3. On Day 5, the cells were divided into two wells with additional beads and matrices. The number of T cells in each well, after dissociated from the magnetic beads or the matrix, were counted on Days 3, 5 and 7.

FIG. 15 shows T-cell growth (after activation) incubated with antibody-coated magnetic beads and matrices versus a matrix without an antibody coating. The trend of T cell growth in antibody coated-matrices is similar to that of antibody-coated beads. However, T cells incubated with the matrix without antibodies did not proliferate, probably due to the lack of continued stimulation. In this experiment, the matrix was placed in a well under static condition. Therefore, the results are expected to be different from the perfusion based bioreactor. This experiment clearly indicate that the antibodies were successfully coated on the bioreactor's matrix surfaces, and they affected the T cell growth.

Comparison of Peripheral Blood Mononuclear Cells (PBMCs) Incubated with Antibody-Coated Bioreactor Matrix and Magnetic Beads for Both T-cell Activation and Expansion

A similar study to the above was performed with PBMCs instead of isolated T-cells. Typically, PBMCs, which include T cells and other mononuclear such as B cells, NK cells, monocytes, were collected from a patient and directly used for cell expansion without T-cell isolation. This is because non-T cells will not be activated by CD2, CD3, and CD28, and they will be naturally eliminated after several days without activation. Another difference of this experiment from the experiment in **FIG. 15** is the incorporation of the activation step when using the antibody-coated matrix. Briefly, 2 mL of culture medium containing PBMCs at density of $2.5\text{x}10^6$ cells/mL were added to three wells of a 12-well plate. The three wells contain 1) 7.5×10^6 antibody-coated magnetic beads, 2) antibody-coated streptavidin-matrix, and 3) streptavidin-matrix without antibody coating, respectively. Avidin coated matrix was not used in this experiment as streptavidin has shown superior to avidin in the last two experiments. PBMCs were incubated in each well for two days for activation. Then additional media containing IL2 cytokine were added to the well so that the media has 20 IU/mL of human IL2 to start the expansion phase. The change of the total cell number during the 5-day culture is illustrated in **FIG. 16**. This confirms that the

T-cells expanded with antibody-coated matrix resulted more T cells than that with antibody coated magnetic beads. This also confirms that the antibody-coated matrix can provide equivalent or better T-cell activation and expansion.

Dynamic Perfusion Based T-Cell Expansion

A perfusion-based 3D bioreactor was fabricated as described herein. The 3D bioreactor's internal surface was prepared by coating with polydopamine for 12 hours. The bioreactor internal surface was then coated with streptavidin for 12 hours. The bioreactor was then incubated with 70% ethanol for sterilization. After sterilization, the internal surface was washed PBS and coated with an equal mixture of CD2, CD3, and CD28-Biotin conjugates (10 µg/mL concentration for each antibody) to immobilize antibodies on the bioreactor's internal surfaces using the procedure described above.

A perfusion circuit was set up as illustrated in **FIG. 12.** A Masterflex L/S with Cytoflow pump head (Cole-Parmer), which is designed for pumping live cells and shear-sensitive fluids, was used for the perfusion system. As this application will use a relatively small bioreactor and the perfusion rate is expected to be relatively low, a custom-built oxygenation unit via gas diffusion to the medium and medium droplets was employed instead of an oxygenator.

About $20x10^6$ PBMCs were seeded into the primed bioreactor perfusion system. The cells were distributed evenly by circulating for 15 minutes at 2 mL/min. During the activation phase, that is the perfusion medium containing no cytokine IL2, the T-cells were perfused at the rate of 0.1 mL/min on Day 1 and 0.14 mL/min on Day 2. After two days of activation, cell density was determined and media with human IL-2 was added to the system so that the total IL-2 concentration is 20 IU/mL. T-cell expansion phase was carried out for 3 days at a perfusion rate of 0.2 mL/min. The cell density was determined on day 5 and the results are shown in **FIG. 17**. Similar to the static experiment shown in **FIGS. 15** and **16**, T cells were achieved at a three-time of expansion after activation.