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(54) Title: FERROMAGNETIC CELL AND TISSUE CULTURE MICROCARRIERS BACKGROUND OF THE INVENTION

(57) Abstract: A porous, collagen coated, ferromagnetic cell culture microcarrier, which is suitable for in vitro cell and tissue culture and which facilitates 3D multicellular construct generation. Also provided is a method for creating batches of microcarriers which have inserted within them magnetite (Fe3O4) in the presence of collagen, thus creating a microcarrier which becomes magnetic in nature when placed in a the presence of a magnetic field and which facilitates cellular adherence (via the collagen coating) for 3D construct development.

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FERROMAGNETIC CELL AND TISSUE CULTURE MICROCARRIERS

BACKGROUND OF THE INVENTION

It has long been established that cells and tissue growing in microgravity behave differently than those on Earth. The ongoing challenge for the experimental study of cell behavior under these conditions has been simulating the environment of microgravity so that complete laboratory studies can be conducted on Earth. This provides the obvious advantages of cost-effectiveness and safety.

To address this issue, NASA developed the bioreactor in the 1980s. Essentially, the bioreactor is a cylindrical vessel equipped with a membrane for gas exchange and ports for media exchange and sampling. As the bioreactor turns, the cells continually fall through the medium yet never hit bottom. Under these conditions, the cells form clusters that sometimes grow and differentiate much as they would in the body. Unfortunately however, on Earth the clusters become too large to fall slowly. This requires the research to be continued in the true weightlessness of space.

It has been well established that a number of cell types grow in the bioreactor on Earth for extended periods in ways that resemble tissue-like behavior. For this reason, the bioreactor provides cell culture studies with a new tool for the study of 3-dimensional cell growth and differentiation.

Bioreactors have been used aboard the Mir space station to grow larger cultures than even terrestrial Bioreactors can support. Several cancer types, including breast and colon cancer cells, have been studied in this manner. Continued research using the NASA Bioreactor is planned aboard the International Space Station.

NASA-developed tissue engineering technology has greatly facilitated advancements in the design of three-dimensional cellular constructs that exhibit many tissue-like qualities. The NASA rotating wall vessel (RWV) is a low shear, optimized suspension culture which, like a clinostat, maintains growing cellular constructs in a state of free fall via randomization of the gravity vector. Multicellular constructs are cultured under spatially unrestricted conditions during constant rotation of the vessel about its horizontal axis, resulting in time-averaging of the g vector to near zero. Significant changes in gene expression, cellular physiology and morphology occurring during three-dimensional growth in the RWV have been attributed to a variety of factors particular to this culture paradigm, including significantly reduced shear stress, altered gravitational influence (sometimes referred to as modeled or simulated microgravity), adequate mass transfer of nutrients and waste removal, and the generation of three-dimensional architecture itself. Each of these parameters is readily addressed in cell culture studies

performed in the environment of space, where three-dimensional development occurs under conditions of true microgravity, fluid shear is absent and mass transfer may be controlled. In ground based studies, however, it has been difficult to separate these parameters from one another in order to examine the influence of each on three-dimensional cellular growth and function.

Therefore, what is needed is an efficacious method of simulating a microgravity environment thus allowing long-term three-dimensional (3D) development during in vitro cell and tissue culture. Commercially available magnetic beads are either too small for use as microcarriers in cell culture (diameters on the order of <10 um which are not feasible for use as cell culture supports), and/or they lack appropriate surface matrix coating to facilitate cell adherence, a requisite factor to maintain cell-cell interactions for 3D construct development during in vitro culture. Typical uses for commercially available magnetic beads are for cell and protein separation technology.

SUMMARY OF INVENTION

In one embodiment of the present invention a method is provided for developing a three-dimensional cell culture model. This is accomplished by providing a plurality of microcarriers, an upper and lower graphite plate, an adjustable platform (whereby the upper and lower plate are adjustably spaced) and an upper strong magnet. Next at least one cell is adhered to the magnetic microcarriers in a gas-permeable tissue culture bag. The tissue culture bag is then placed between the upper graphite plate and the lower graphite plate, situated below the upper magnet whereby the growing cells are levitated by adjustment of the platform to the appropriate height and are held in suspension via natural magnetic forces stabilized by the graphite plates, thereby allowing the study of three-dimensional cell growth in suspension in a constant and nonrandomized environment.In another embodiment of this method the microcarriers are ferromagnetic microcarriers. The method may also be employed wherein the upper and lower graphite plates are pyrolyric graphite plates. Continued growth of the cellular constructs is made possible by placing the adjustable platform in a standard incubator.

Also provided is a microcarrier bead having a supporting surface for the attachment of cells, the microcarrier bead further comprising, at least one magnetically charged molecule and a cellular matrix material. In one embodiment the magnetically charged molecule is magnetite (Fe_3O_4) and the microcarrier cellular matrix material is Type I solubilized collagen. The support material may be constructed from porous gelatin.

Another embodiment includes a method of manufacturing a gelatin microcarrier bead having a supporting surface for the attachment of cells, comprising the steps of (a) swelling a porous gelatin microcarrier in culture media; (b) sterilizing the swelled microcarrier; (c) suspending the sterilized microcarriers in an acidic solution; (d) rotating the solution for a first predetermined time, at a first predetermined temperature; (e) rotating the solution for a second predetermined time, at a second predetermined temperature; and (f) stabilizing the loaded microcarriers prior to use. The gelatin microcarriers range in size from about 100-400μM. In a preferred embodiment the culture media is void of calcium and magnesium. The ratio of microcarriers to culture media is about 0.5g beads/ 25 ml media. After swelling the microcarriers are sterilized by conventional methods, as in an autoclave. The acidic solution further comprises 1 mg Type I solubilized collagen and 25 mg sterile fine granular magnetite (Fe_3O_4) and has a final volume of about 2 ml. In one embodiment the solution is then rotated at room temperature overnight, about 4 to 8 hours. Next the solution is rotated at about 37 degrees Celsius for about 24 to 48 hours.

The stabilizing step of the abovementioned embodiment further comprises the steps of (a) washing the microcarriers to remove any excess collagen and magnetite (Fe_3O_4) and (b) storing the microcarriers in a protein-containing media at a neutral pH under sterile conditions at 4 degrees Celsius.

Another embodiment of the present invention provides a method of manufacturing an alginate microcarrier bead having a supporting surface for the attachment of cells, comprising the steps of (a) providing a solution comprising alginate and culture media lacking calcium and magnesium, (b) adding sterile fine granular magnetite (Fe_3O_4) to the solution, (c) expressing the combined solution in droplet form into a solution of calcium chloride wherein the microcarrier bead is formed in about 1-2 hours, (d) washing the microcarrier beads with culture media; and (e) coating the microcarrier beads with a collagen solution. In this embodiment the solution comprising alginate and culture media lacking calcium and magnesium contains about 2% alginate (about 0.5g alginate per 25 ml). The amount of sterile fine granular magnetite (Fe_3O_4) used is about 150 mg. The solution of calcium chloride is about 25 mM calcium chloride and the collagen solution is about 0.3 mg solubilized Type I collagen.

BRIEF DESCRIPTION OF THE DRAWINGS

For a fuller understanding of the nature and objects of the invention, reference should be made to the following detailed description, taken in connection with the accompanying drawings, in which:

Fig. 1 is a side elevational view of the inventive method, wherein a gas-permeable tissue culture bag is placed on an adjustable platform;

Fig. 2 is a flowchart outlining the protocol for creating ferromagnetic gelatin microcarriers;

Fig. 3 is a diagrammatic representation of the protocol for creating ferromagnetic alginate microcarriers; and

Fig. 4 is a diagrammatic representation of an alternative protocol for creating ferromagnetic alginate microcarriers.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

In the following detailed description of the preferred embodiments, reference is made to the accompanying drawings, which form a part hereof, and within which are shown by way of illustration specific embodiments by which the invention may be practiced. It is to be understood that other embodiments may be utilized and structural changes may be made without departing from the scope of the invention.

In an effort to dissect the influence of fluid shear and randomized gravity from three-dimensional development, one embodiment of the present invention, shown in Fig. 1, applies the concept of diamagnetically stabilized levitation to develop a new type of three-dimensional cell culture model. Cells are adhered to ferromagnetic microcarriers within gas-permeable tissue culture bags **10**. The bags are placed between an upper **20** and lower **30** pyrolytic graphite plate on an adjustable lifter platform **40**, under the influence of a strong NdFeB magnet **50** located above the upper graphite plate. The growing constructs are levitated by adjustment of the platform to the appropriate height, and the apparatus is placed into a standard incubator. The unit requires no source of electrical power, since the growing cellular constructs are held in suspension via natural magnetic forces stabilized by diamagnetic (repelling) plates, thereby allowing the study of three-dimensional cell growth in suspension in a constant and nonrandomized 1g environment. Because the constructs remain fixed in position, this configuration also replicates the mass transfer conditions inherent in undisturbed cell culture in the true microgravity of space where mass transfer is limited to simple diffusion; a perfusion model is under development in order to impart control of mass transfer conditions to this culture

system. Controlled introduction of fluid shear may be accomplished in two ways: placement of the apparatus on an orbiting platform or by rotation of the upper magnet to induce spin of the cellular constructs within the culture bag.

The presence of a magnetic field during three-dimensional cell culture obviously introduces a variable with the potential to influence cell growth, the effects of which have yet to be fully investigated in this system. However, this technique affords a unique opportunity to examine mechanisms by which natural magnetic fields may influence multicellular construct growth and physiology, and associated changes in gene expression – both in the absence and presence of other physical parameters known to affect cell function, such as fluid shear and mass transfer. Studies performed thus far demonstrate several positive growth effects in this system, including rapid adherence to the microcarrier surface and multilayered outgrowth resulting in the generation of large-sized constructs on the order of 1 cm in diameter.

Another embodiment of the invention is a ferromagnetic microcarrier bead for in vitro cell and tissue. The invention describes methodology for creating the beads which have inserted within them magnetite (Fe_3O_4) in the presence of cellular matrix material such as collagen. Cells grown on these microcarriers readily form complex multicellular, tissue-like 3D constructs when cultured in a spatially unrestricted environment using the principle of diagmagnetic levitation, that is, the use of magnetic, paramagnetic, ferromagnetic and diamagnetic (repelling) fields to create a suspension culture capable of sustained, stable levitation. It is also envisioned that these ferromagnetic microcarriers may find application in standard in vitro tissue culture, wherein following enzymatic release of single cells and/or multicellular constructs from the microcarrier surface, the microcarriers are very easily removed from the cell suspension via a removal magnet. It is further envisioned that the magnetite could also be incorporated into the microcarriers in the presence of biodegradable encapsulated drug, for use in magnetically guided drug delivery to a specific site or target tissue.

The beads described above are porous, collagen coated, ferromagnetic cell culture microcarriers, which are suitable for in vitro cell and tissue culture and which facilitate 3D multicellular construct generation. The invention describes a method for creating batches of microcarriers which have inserted within them magnetite (Fe3O4) in the presence of collagen, thus creating a microcarrier which becomes magnetic in nature when placed in a the presence of a magnetic field and which facilitates cellular adherence (via the collagen coating) for 3D construct development.

The cells grown on these ferromagnetic microcarriers readily form complex, multicellular, tissue-like 3D constructs when cultured in a spatially unrestricted environment using the principle of diagmagnetic levitation, that is, the use of magnetic, paramagnetic, ferromagnetic and diagmagnetic (repelling) fields to create a suspension culture capable sustained, stable levitation. Although it is typically desirable for most microcarriers to have density close to 1.0 g/cm3 (e.g., near the density of standard culture media) in order to facilitate neutral buoyancy, the density of the microcarriers described herein is not of consequence for diagmagnetic levitation, since they are held in suspension via magnetic fields. The multicellular constructs generated may be useful for the creation of bioengineered tissue (for human and veterinary purposes), and for research purposes to understand mechanisms of cell growth and disease formation. These ferromagnetic microcarriers may also be of use in standard in vitro cell culture, wherein following enzymatic release of single cells and/or multicellular constructs from the microcarrier surface, the microcarriers are very easily removed from the cell suspension via a removal magnet. Moreover, it is also envisioned that the magnetite could be incorporated into the microcarriers in the presence of biodegradable nanospheres of encapsulated drug. Through the use of a guiding magnetic field, these magnetite/drug loaded microbeads could be directed to allow for magnetically directed drug delivery to a specific site or tissue. Finally, based upon their distinct striped appearance, these microcarriers have been named "Tiger Beads".

Protocol for Creating Ferromagnetic Gelatin Microcarriers

Referring now to Fig. 2, porous cross linked gelatin microcarriers **10** of a size range of 100-400 μM are swelled in culture media lacking calcium and magnesium (0.5 g beads/25 ml media) after which they are sterilized by autoclaving **20**. In step 3, (represented by the numeral **30)** the sterile cross-linked gelatin microcarriers (20 mg) are placed into an acidic suspension containing 1 mg type I solubilized collagen and 25 mg sterile fine granular magnetite (Fe_3O_4); the final volume is approximately 2 ml. The suspension is rotated at room temperature **40** overnight then rotated at 37° C for an additional 24-48 hours **50** until visible evidence of magnetite incorporation is noted. The incorporation of magnetite into the microcarriers is readily observable using a dissecting or inverted phase contrast microscope at low magnification. Afterwards, the microcarrier suspension is washed to remove collagen and excess (unincorporated) magnetite, and stored under sterile conditions at 4° C in a high protein-containing media (such as standard cell culture media with 20% serum added) at neutral pH, to stabilize the magnetite-loaded microcarriers prior to use in cell culture.

Protocol for Creating Ferromagnetic Alginate Microcarriers

Referring now to Fig. 3, a 2% solution of alginate (0.5g alginate per 25 ml) **10** is prepared using culture media lacking calcium and magnesium to which is added 150 mg of sterile fine granular magnetite (Fe_3O_4). The solution is slowly expressed in droplet form from the tip of a tuberculin syringe **20** or via fine mechanized spray (**25**, Fig. 4) into a solution of 25 mM calcium chloride **30**. The beads **40** formed are allowed to gel for 1-2 hours and then washed with culture media after which they are coated overnight with a solution of 0.3 mg solubilized type I collagen.

It will be seen that the objects set forth above, and those made apparent from the foregoing description, are efficiently attained and since certain changes may be made in the above construction without departing from the scope of the invention, it is intended that all matters contained in the foregoing description or shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

It is also to be understood that the following claims are intended to cover all of the generic and specific features of the invention herein described, and all statements of the scope of the invention which, as a matter of language, might be the to fall therebetween. Now that the invention has been described,

What is claimed is

1. A method of developing a three-dimensional cell culture model, comprising the steps of;

providing a plurality of microcarriers;

providing an upper and lower graphite plate;

providing an adjustable platform, whereby the upper and lower plate are adjustably spaced;

adhering at least one cell to the microcarriers in a gas-permeable tissue culture bag;

placing the tissue culture bag between the upper graphite plate and the lower graphite plate; and

placing a magnet above the upper graphite plate whereby the growing cells are levitated by adjustment of the platform to the appropriate height and are held in suspension via natural magnetic forces stabilized by the graphite plates, thereby allowing the study of three-dimensional cell growth in suspension in a constant and nonrandomized environment.

2. The method of claim 1 wherein the microcarriers are ferromagnetic microcarriers.

3. The method of claim 1 wherein the upper and lower graphite plates are pyrolyric graphite plates.

4. The method of claim 1 wherein the adjustable platform is placed in a standard incubator.

5. A microcarrier bead having a supporting surface for the attachment of cells, the microcarrier bead further comprising;

at least one magnetically charged molecule; and

a cellular matrix material.

6. The microcarrier of claim 5 wherein the magnetically charged molecule is magnetite (Fe_3O_4).

7. The microcarrier of claim 5 wherein the cellular matrix material is Type I solubilized collagen.

8. The microcarrier of claim 5 wherein the support material is constructed from porous gelatin.

9. A method of manufacturing a gelatin microcarrier bead having a supporting surface for the attachment of cells, comprising the steps of;

swelling a porous gelatin microcarrier in culture media;

sterilizing the swelled microcarrier;

suspending the sterilized microcarriers in an acidic solution;

rotating the solution for a first predetermined time, at a first predetermined temperature;

rotating the solution for a second predetermined time, at a second predetermined temperature; and

stabilizing the loaded microcarriers prior to use.

10. The method of claim 9 wherein the gelatin microcarriers range in size from about 100-400μM.

11. The method of claim 9 wherein the culture media is void of calcium and magnesium.

12. The method of claim 9 wherein the ratio of microcarriers to culture media is about 0.5g beads/ 25 ml media.

13. The method of claim 9 wherein the microcarriers are sterilized in an autoclave.

14. The method of claim 9 wherein the acidic solution further comprises 1 mg Type I solubilized collagen and 25 mg sterile fine granular magnetite (Fe_3O_4).

15. The method of claim 14 wherein the acidic solution has a final volume of about 2 ml.

16. The method of claim 9 wherein the first predetermined time is about 4 to 8 hours.

17. The method of claim 9 wherein the first predetermined temperature is about room temperature.

18. The method of claim 9 wherein the second predetermined time is about 24-48 hours.

19. The method of claim 9 wherein the second predetermined temperature is about 37 degrees Celsius.

20. The method of claim 9 wherein the step of stabilizing the loaded microcarriers further comprises the steps of:

washing the microcarriers to remove any excess collagen and magnetite (Fe_3O_4); and

storing the microcarriers in a protein-containing media at a neutral pH under sterile conditions at 4 degrees Celsius.

21. A method of manufacturing an alginate microcarrier bead having a supporting surface for the attachment of cells, comprising the steps of;

providing a solution comprising alginate and culture media lacking

calcium and magnesium;

adding sterile fine granular magnetite (Fe_3O_4) to the solution;

expressing the combined solution in droplet form into a solution of calcium chloride wherein the microcarrier bead is formed in about 1-2 hours;

washing the microcarrier beads with culture media; and

coating the microcarrier beads with a collagen solution.

22. The method of claim 21 wherein the solution comprising alginate and culture media lacking calcium and magnesium contains about 2% alginate (about 0.5g alginate per 25 ml).

23. The method of claim 21 wherein the amount of sterile fine granular magnetite (Fe_3O_4) is about 150 mg.

24. The method of claim 21 wherein the solution of calcium chloride is about 25 mM calcium chloride.

25. The method of claim 21 wherein the collagen solution is about 0.3 mg solubilized Type I collagen.

Fig. 1

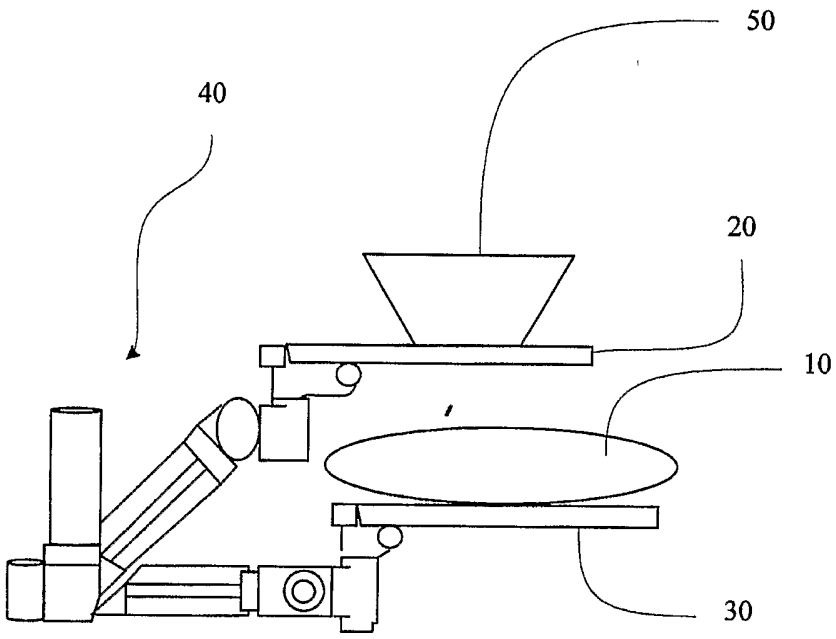

Fig. 2

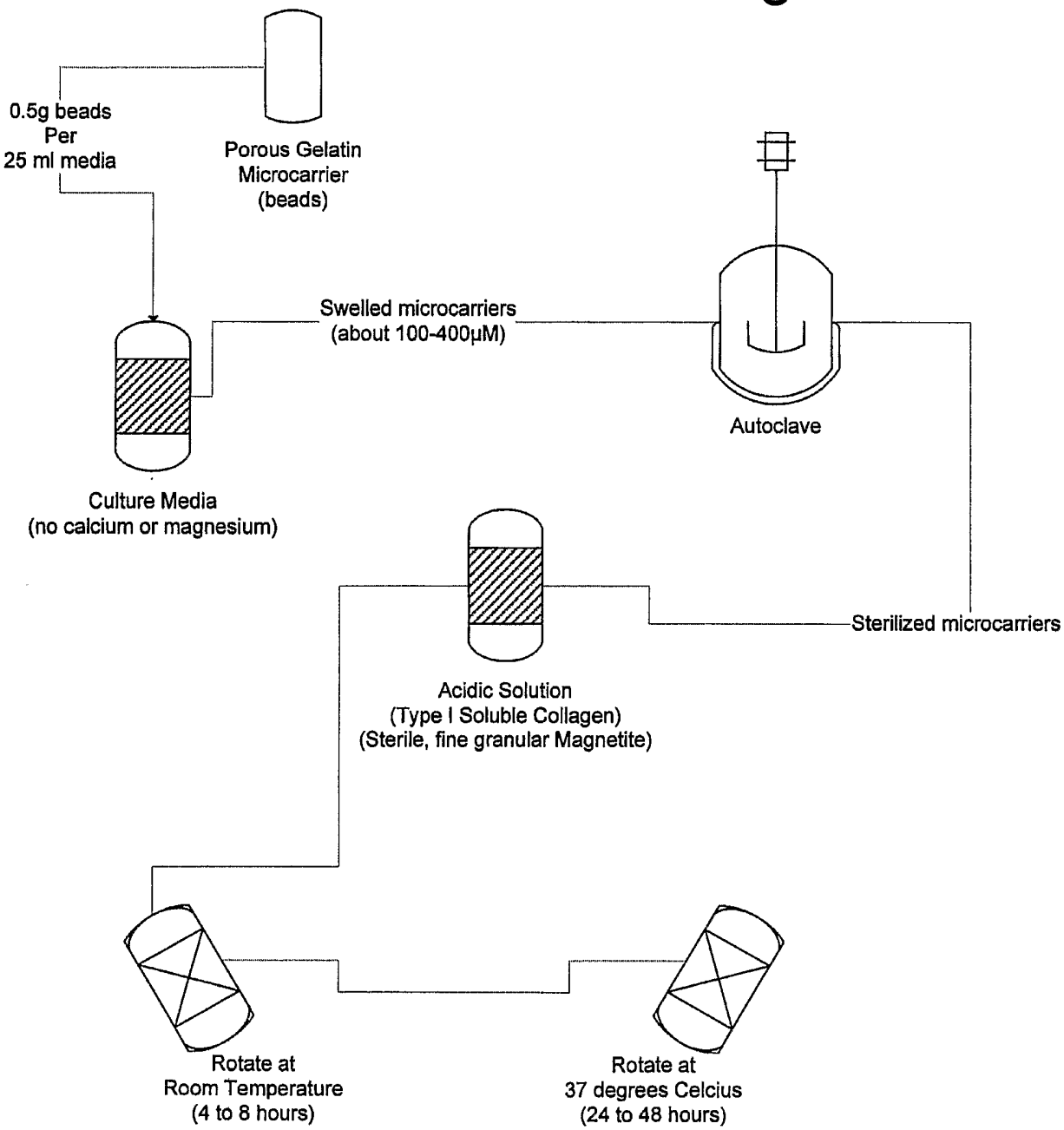
0.5g beads
Per
25 ml media
Porous Gelatin
Microcarrier
(beads)
Swelled microcarriers
(about 100-400µM)
Autoclave
Culture Media
(no calcium or magnesium)
Sterilized microcarriers
Acidic Solution
(Type I Soluble Collagen)
(Sterile, fine granular Magnetite)
Rotate at
Room Temperature
(4 to 8 hours)
Rotate at
37 degrees Celcius
(24 to 48 hours)

Fig. 3

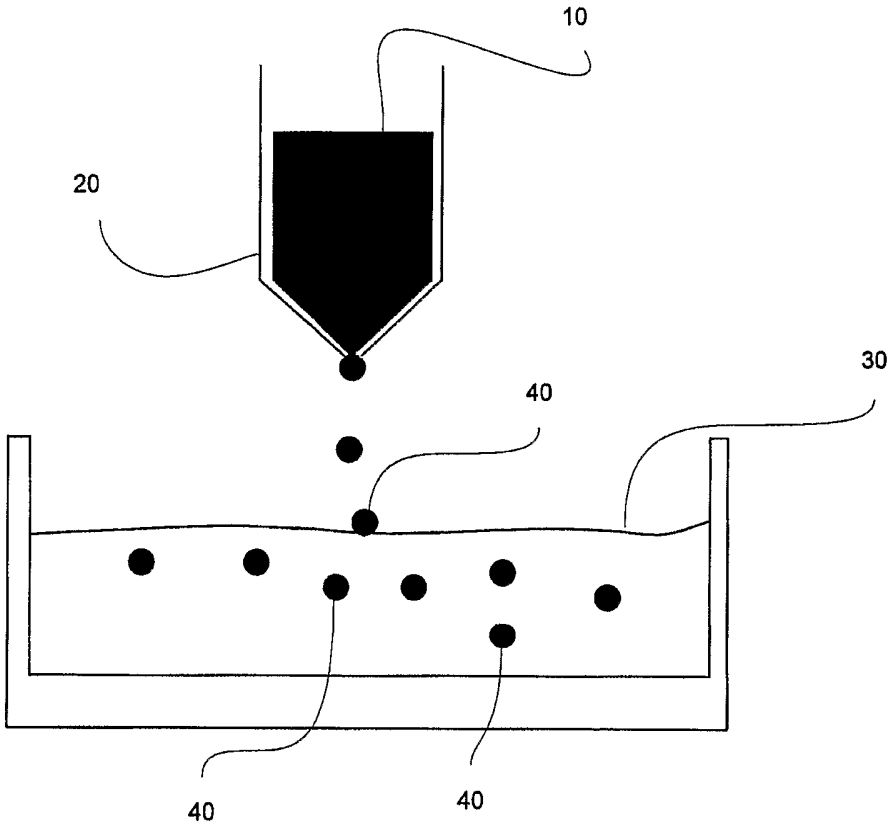
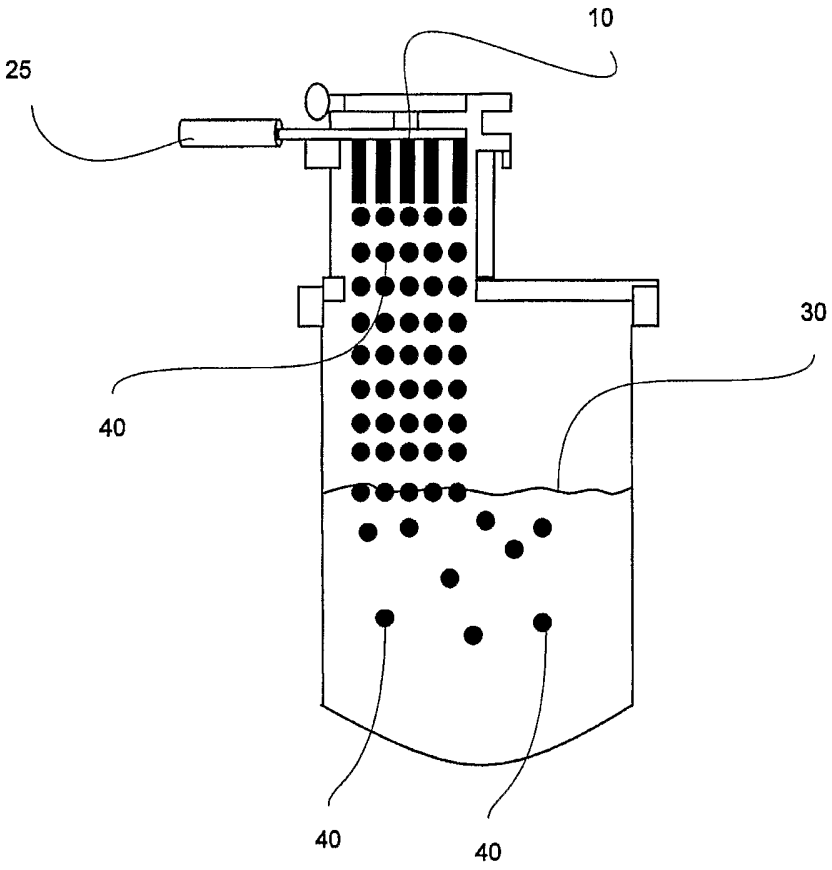
Fig. 4