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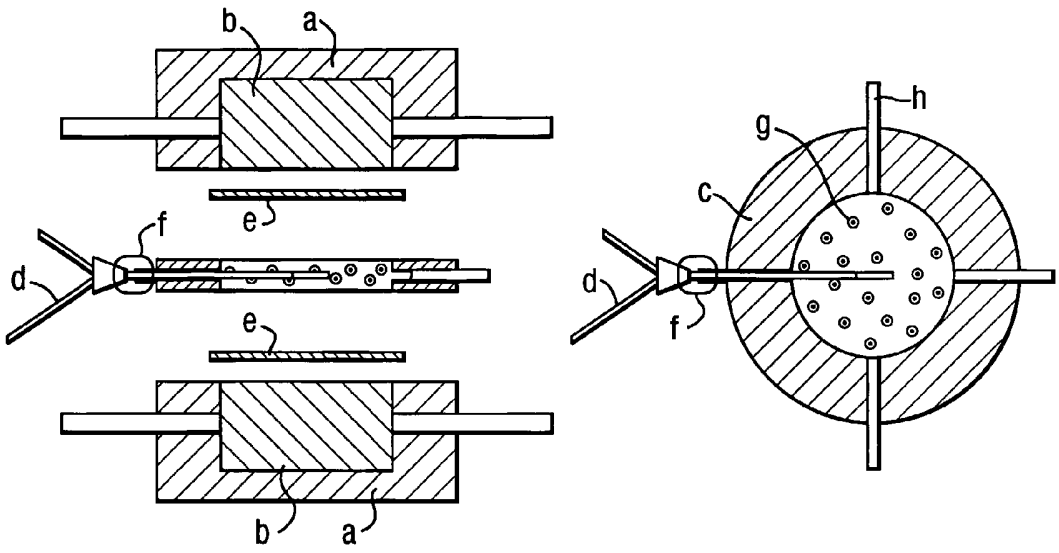
(54) Title: MONITORING METHOD USING MICROMEMBRANE PROBES



(57) Abstract: A method of monitoring the formation of a tissue in a tissue construct or the viability or function of cells in a tissue comprises contacting a micromembrane probe with the tissue construct; carrying out microdialysis and/or ultrafiltration to extract a marker into the dialysate and analysing the marker in the dialysate. The methods can be used to monitor formation, viability or function of tissues *in vitro* or to monitor viability or function *in vivo*.

WO 2004/111209 A1

MONITORING METHOD USING MICROMEMBRANE PROBES

Field of the Invention

The invention relates to methods of monitoring tissue formation and cell viability and function. The methods may be used for tissue engineering, or to monitor tissue function *in vivo*.

Background to the Invention

Every year, millions of surgical procedures are performed that require tissue or organ substitutes to repair or replace diseased or damaged tissues or organs. Tissue engineering is an emerging field in which tissues and organs are produced in the laboratory to replace or support the function of defective or injured body parts.

Cells have been cultured outside the body for many years. Cell cultures are grown in the laboratory either as a suspension or by adherence of cells to tissue culture plates, where they form two-dimensional monolayers. However, these colonies of cells do not become organised into three-dimensional arrangements. Cells need a complex range of external signals to develop into functional three-dimensional tissues or organs. *In vivo*, many of these signals originate from or relate to the extracellular matrix (ECM), which is produced by the cells themselves and also gives the tissues of the body their form and shape.

It has recently become possible to develop complex three-dimensional tissue constructs *in vitro* that will ideally mature into fully functional tissues and organs. Such constructs are based on the seeding of cells onto a scaffold, which form a base for the growth and development of the cells into the desired tissue. In addition, many growth factors and other signals must be present in the construct for the tissue to develop in the correct manner.

Summary of the Invention

The present inventors have shown that microdialysis and ultrafiltration using micromembrane probes can be used to monitor tissue viability and function of cells and to monitor the formation of tissues. The methods may be used *in vitro* for tissue engineering purposes or may be used to assess tissue function *in vivo*, for example

following transplantation. Microdialysis is based on the perfusion of a microdialysis probe containing a semipermeable membrane. Substances from the environment surrounding the probe diffuse though the membrane into the perfused solution. Perfusate can be analysed on-line or collected for *ex situ* analysis. The related technique of ultrafiltration involves applying a small negative pressure to the fluid in the probe so that the molecules in the surrounding fluid are drawn rapidly into the probe. These methods allow the analysis of markers present in the tissue both non-destructively and quantitatively.

In accordance with the present invention there is thus provided a method of monitoring the formation of a tissue in a tissue construct *in vitro* or the viability or function of cells of a tissue in a tissue construct being grown *in vitro*, which method comprises:

contacting a micromembrane probe with the tissue construct;

carrying out microdialysis and/or ultrafiltration to extract a marker into the dialysate; and

analysing the marker in the dialysate;

thereby to monitor the formation of the tissue or the viability or function of the cells.

The invention also provides the use of microdialysis and ultrafiltration to monitor the formation of a tissue in a tissue construct *in vitro* or the viability or function of cells of a tissue in a tissue construct being grown *in vitro*.

The invention also provides a method of monitoring the viability or function of cells of a tissue, which method comprises:

contacting a micromembrane probe with the tissue construct;

carrying out microdialysis and/or ultrafiltration to extract a marker into the dialysate; and

analysing the marker in the dialysate;

thereby to monitor the viability or function of the cells of the tissue.

Brief Description of the Drawings

Figure 1 is a schematic diagram of the flow cell used: a. flow cell cup (outside diameter (od) 30 mm); b. glass mesh (od 20mm); c. spacer (od 30mm; inside diameter (id) 20mm; width 4.5mm); d. microdialysis probe; e. isopore polycarbonate

membrane filters (pore size 0.22 μm); f. site of fixation of the microdialysis probe by dental cement; g. chondrocytes in alginate gel; h. sleeves for inserting the micromembrane probes

Figure 2 is a schematic diagram of the microdialysis set-up.

Figure 3a represents typical dependence of lactic acid concentration in dialysate on concentration of the lactic acid in the solution *in vitro*. Figure 3b shows the dependence of the recovery on lactic acid concentration.

Figure 4 shows recovery of the microdialysis probe before and after autoclaving.

Figure 5 is a typical curve of the monitored lactic acid level in alginate chondrocyte construct.

Figure 6 shows the concentration of (a) lactic acid, (b) 3-methyl-glucose and (c) 3 kDa dextran in the middle of the alginate gel with the time.

Figure 7 shows dependence of the recovery of one type of microdialysis probe under equilibrium conditions on MW of solutes.

Figure 8 shows the diffusion of lactic acid in an alginate gel formed inside of a bioreactor (data for four probes shown).

Figure 9 shows the distribution of lactic acid in a gel at different time points.

Figure 10 represents D_{app} of lactic acid and glucose in comparison with their diffusion coefficients in aqueous solution (D_{free}).

Figure 11 a and b show the diffusion of lactic acid and glucose respectively in alginate gel, monitored by microdialysis probe positioned in the centre of the gel.

Figure 12 shows the level of lactic acid in dialysate monitored over time during the bioconstruct culture using two probes, one at the centre and one at the edge of the gel.

Figure 13 shows the probe permeability over the course of time.

Figure 14 shows the cell count across the construct.

Figure 15 shows the amount of glycosaminoglycan across the construct.

Detailed Description of the Invention

The invention provides methods of monitoring the formation of a tissue in a tissue construct *in vitro* or the viability or function of cells of a tissue *in vitro* for example, in a tissue construct.

In another embodiment, the present invention provides a method of monitoring the viability or function of cells of a tissue, for example *in vivo*. The methods can be used to monitor the performance and viability of transplanted tissue or to assess viability or function across the tissue, for example in response to different stimuli. In one embodiment the tissue is transplanted tissue. In the methods of the invention, a micromembrane probe is contacted with the tissue. Typically, the micromembrane probe is inserted into the tissue.

The present invention relates in part to tissue engineering, and in particular to the growth of cells in three dimensions to produce functional tissues and organs. Three-dimensional cell growth occurs within a tissue construct. By tissue construct in accordance with the present invention is meant the cells or tissue growing *in vitro*. The term "tissue construct" may also include a scaffold, if present. By a scaffold is meant a suitable three-dimensional cell support. The scaffold can act as a temporary extracellular matrix (ECM) to direct the formation of the tissue in three dimensions. The scaffold is made of a natural or synthetic material and is preferably biodegradable. A typical scaffold is an alginate gel. The scaffold may be seeded with the cells to be grown and cell culture medium and/or any other necessary growth factors may be added to the tissue construct.

The present invention can also be used to monitor tissue function *in vivo* or *in vitro*. In particular, the invention may be used to monitor the growth and function of cells in two or three dimensions.

The present invention detects markers which can be used to monitor the formation of a tissue or the viability or function of cells of a tissue. Suitable markers include soluble markers. Typically, the molecular weight (MW) of the marker is in the range of up to 100 kD (kilo Dalton), for example 0.01 to 100 kD, 0.02 to 50 kD, 0.05 to 10 kD, 0.09 to 5kD or 0.1 to 3 kD, although larger proteins could be of interest. For example, markers with a molecular weight of up to 500 kD, for instance 200 kD, 300 kD or 400 kD, can be used. Markers for use in the invention include

metabolic products, cell nutrients, cell type markers, soluble proteins or other macromolecules synthesised by the cells and extracellular matrix turnover markers. By detecting the levels of such markers over time or in particular locations within the tissue construct, the viability of the cells can be assessed and the formation of the tissue can be analysed. Detection of markers can include detecting a portion of the marker.

In more detail, the methods of the invention can be used to monitor the levels of metabolic products produced by cells in the tissue. This can be used to give an indication of cell viability and function and the rate of turnover of cells. For instance, lactic acid can be analysed to monitor glycolysis. Glycerol can be monitored as a marker for lipolysis. This allows, for example, the calculation of the metabolic rate of the cells in the tissue to give an indication of the viability of the cells. For instance, a constant metabolic rate indicates that the population of cells is stable and a fall in metabolic rate for instance warns that the cells are dying or losing activity. Oxygen tension and pH can also be used as markers.

Methods of the invention can also be used to monitor the levels of cell nutrients. In this way, uptake of nutrients by the cells can be analysed and this can be used to assess cell viability and function. Markers such as glucose may be monitored.

Methods of the invention can also be used to monitor markers for a specific tissue. These types of markers are cell type specific markers. They may be markers of tissue formation or breakdown. For instance, for monitoring the development of cartilage, extracellular matrix (ECM) turnover can be analysed. The levels of procollagen can be monitored to track collagen synthesis. The level of proteoglycans can be monitored. The level of glycosaminoglycans (GAG) or collagen fragments can be monitored to track matrix breakdown. Other suitable markers may be used for different tissues. Such markers can be monitored over particular periods of time to analyse the development or breakdown of a tissue with time. The presence of the markers in specified spatial locations can also be monitored. This gives an indication of the development or breakdown of the tissue in a particular area and allows the assessment of three-dimensional tissue formation.

Differentiation of stem cells can also be monitored by the method of the invention. Markers of differentiation, such as osteocalcin for osteoblasts and albumin for hepatocytes can be monitored.

The markers that can be monitored in accordance with the present invention can be divided into small markers such as metabolic products and cell nutrients such as lactic acid or glucose (MW 0.1 to 1 kD), intermediate molecules such as growth factors, cytokines, hormones (MW 1 to 50 kD) and large molecules such as proteins or protein fragments (50 to 500 kD). Typically, the micromembrane probes for such markers will be provided with an appropriate pore size (molecular weight cut-off, MWC) such that the probes for small nutrients will have a MWC of 10 kD, while those for larger proteins will have a MWC of 500 kD. Alternatively, the probe has a MWC of 50 kD, 20 kD or 10 kD. Typically, small markers will be in the MW range of 0.01 to 50 kD, such as 0.03 to 20 kD, 0.05 to 5 kD, 0.07 to 10 kD, 0.09 to 5 kD or 0.1 to 3kD. The present invention can also be used to monitor larger molecule such as macromolecules such as proteins. The micromembrane probes are then selected to have a larger pore size, for example having a 300 kD cut off or a 100 kD cut off. Markers may typically be in the molecular weight range of 2 to 300 kD, preferably 5 to 200 kD, preferably 10 to 100 kD, preferably 20 to 50 kD.

Any suitable micromembrane probe can be used in the invention. The micromembrane probe can be, for example, a commercial microdialysis probe. A microdialysis probe generally consists of a thin capillary, typically of 200 microns in diameter, for example from 50 to 1000 microns in diameter, and has porous, semi-permeable walls. The pore size of the micromembrane is selected based on the particular marker to be monitored. For example, the pore size may be selected to have a cut off of between 10 to 20 kD, for monitoring smaller molecules such as glucose and lactic acid. For larger markers such as protein markers, a larger pore size is selected, for example having a cut off of 300 kD.

In a preferred embodiment, microdialysis is carried out by feeding a perfusion liquid into the microdialysis probe and removing the perfusion liquid as a dialysate from the probe. By dialysate is meant the perfusion liquid containing molecules from the environment surrounding the probe. When the perfusion liquid is

fed into the microdialysis probe, molecules diffuse across the semi permeable membrane of the probe and into the perfusion liquid.

The perfusion liquid is typically fed into the microdialysis probe by means of a pump. A negative pressure may be applied to the perfusion fluid when the perfusion liquid is withdrawn from the probe by the pump and if this is carried out the technique is called ultrafiltration. This has the effect of increasing the rate of diffusion of the molecules into the dialysate.

Any suitable perfusion liquid can be used in the microdialysis probe. The perfusion liquid may be a physiological salt solution. The perfusion liquid may be phosphate buffered saline (PBS) or another appropriate solution. Osmolytes such as serum albumin or polyethylene glycol can be added to provide iso-osmotic conditions between probe and interstitial fluid. Alternative perfusion liquids include physiological salt solutions such as Krebs-Ringers solution or tissue culture media such as Dulbecco's modified Eagles medium.

When microdialysis is carried out, the dialysate can be analysed on line, for instance using a flow through cell. Alternatively, the dialysate can be removed from the microdialysis probe. The dialysate is analysed to detect the presence, absence or level of a particular marker. The microdialysis probe can be left *in situ* while the dialysate is being removed and analysed.

Any suitable method can be used to assay the marker. Suitable methods include standard enzymatic procedures, enzyme-linked immunosorbent assay (ELISA), detection of radioactivity and detection using optical methods such as a fluorimeter or electrochemical methods. For instance, lactic acid concentration can be measured by a standard enzymatic procedure. Typically, a reagent containing lactate oxidase and colourless chromogen precursors is added to the sample obtained by collecting dialysate. The lactic acid is converted by oxidase to pyruvate and hydrogen peroxide, H_2O_2. The H_2O_2 formed catalyses oxidative condensation of the chromogen precursors to produce a coloured dye. Absorbance can then be read at 540 nm and is proportional to the original lactic acid concentration. Absorbance is calibrated against a standard curve, produced from known concentrations of lactic acid in PBS or blank medium with components presented in the sample.

In the method of the invention, the microdialysis probe is contacted with the tissue. The probe can be placed in the interstitial space within the scaffold in which the cells are embedded. This allows the sampling of markers which are produced or taken up by the cells. The microdialysis probe may be placed near the boundary of the construct or in the centre of the construct. Gradients in nutrients and metabolites, such as oxygen and lactic acid, which develop can be measured. These gradients depend, for instance, on how quickly the marker diffuses into, out of or away from the cell and how rapidly it is metabolised or produced.

The level of a particular marker can be monitored spatially. This is particularly useful in the analysis of tissues and three dimensional constructs. By detecting particular markers at specific locations within the tissue or construct, the development or viability of the tissue or construct in three dimensions can be followed. Typically, more than one microdialysis probe is placed at different locations within the tissue or tissue construct and each probe monitors the same or different marker(s) at each location. Alternatively, the same probe can be inserted sequentially at different locations to detect the concentration of the marker at each location. Preferably, three or more micromembrane probes are used in order to assess the three dimensional distribution of particular markers. The distribution of the markers can be used to give an indication of the status, viability and function of the tissue under analysis.

The methods of the invention can also be used to monitor more than one marker at a time. The dialysate removed from the microdialysis probe can be analysed for the presence, absence or level of a number of different markers. Alternatively, more than one type of microdialysis probe is inserted into the tissue construct at different locations to monitor different markers.

The relationship between the extracellular concentration of the solute and dialysate concentration can be characterised by the probe recovery. The probe recovery is expressed as a percentage of the extracellular concentration of the solute:

$$\text{Recovery} = \frac{[\text{Dialysate}]}{[\text{Extracellular}]} \times 100\%$$

The levels of particular markers can be monitored over time. Measurements of the level of the marker can be made by collecting dialysate at regular intervals, for instance every 5, 10, 15, 20, 30 or 45 minutes. Alternatively, dialysate may be collected every 1, 2, 3, 4, 12 or 24 hours. The microdialysis probe can be perfused continuously or for a set period of time. Typically, the time for a measurement to be taken is from 15 minutes to 3 hours, from 30 minutes to 2 hours or from 45 minutes to 1 hour. The duration of perfusion depends on the marker being analysed. The duration of the monitoring may be from 1, 2, 3, 4 or 5 days and preferably at least 7 days, up to 10, 12, 14, 21, 28 or 35 days.

The methods of the invention can be used to monitor particular markers over time. A microdialysis probe can be inserted into the scaffold or tissue and remain there for the entire culture period or for a selected period of time. Typically, the probe remains in the scaffold and new perfusion liquid is perfused and dialysate removed at desired intervals.

The present invention can be used to monitor the formation of a range of different tissues containing various cells. For instance, the cells can be chondrocytes and the tissue can be cartilage. Alternatively, the cells can be keratinocytes, stem cells, hepatocytes, osteoblasts, fibroblasts, muscle cells, nerve cells or thyroid cells. The tissue can be skin, liver, bone, blood vessel, muscle, nerve or thyroid.

In one embodiment, more than one micromembrane probe is used. The probes can be used to monitor for diffusion of substances through the tissue, or the effect on cell function to stimuli, such as in the presence of selected agents, such as compounds under investigation to determine their physiological effect on the tissue.

Comparative experiments can be carried out with healthy tissue with a view to identifying expected changes in marker levels in response to certain stimuli or expected diffusion rates. Differences between the control tissue and tissue under analysis can be used to assess cell function and viability in the tissue under test.

In a typical method of the invention, the microdialysis probe is used to monitor levels of markers for a construct of cells growing in a scaffold in a bioreactor. For example, lactic acid can be monitored in a chondrocyte cell culture growing in alginate gel in a bioreactor. By a bioreactor is meant any suitable vessel in which cells and tissues are grown *in vitro*. The cell can be grown, for example, in

a glass flow cell consisting of two identical flow cell cap-glass mesh assemblies. The apparatus is suitably set up to provide culture medium, such as Dulbecco's modified Eagle medium (DMEM), to the cultured cells. There can also be provided means to define the construct size and means to retain the construct. The spacer may have more than one means to introduce microdialysis probes, which can be fixed in place. A multichannel peristaltic pump can be used to provide the microdialysis flow and culture medium perfusion. Dialysate is then collected and analysed by any of the methods described above.

The following Examples illustrate the invention:

Example 1

Materials

Dulbecco's modified Eagle medium (DMEM) and foetal bovine serum was obtained from Gibco (Paisley, UK); antibiotic and antimycotic mixture, collagenase type II, diagnostic reagent for lactic acid assay and phosphate buffer solution (PBS) tablets were obtained from Sigma (Poole, UK); alginate was obtained from Fluka (UK); ascorbic acid was obtained from Wako (Japan); $CaCl_2$ was obtained from BDH Laboratory Supply (Poole, UK). Autoclavable Microbiotech microdialysis probes, PEP microdialysis tubing and adapters were supplied by Royem Scientific (UK). Peristaltic pump PVC tubing of the inside diameter (id) 0.25mm was supplied by Anachem (Luton, UK). Peristaltic pump silicon tubing (id 2.06 mm) was supplied by Alkey (Basingstoke, UK); Isopore polycarbonate membrane filters of pore size 0.2μm were obtained from Millipore (Watford, UK). The peristaltic pump used was a Gilson 8 channel pump.

Methods

Preparation of chondrocyte-alginate construct

Articular chondrocytes were isolated from the metacarpal-phalangeal joint of 18-24 month steers using enzymatic digestion procedure with collagenase following the procedure described by Hopewell and Urban (Biorheology. 2003; 40(1-3):73-7). The isolated cells were washed and resuspended in DMEM supplemented with antibiotic-antimycotic solution (1% v/v). 1.2 % w/v alginate solution was prepared in 0.9% w/v sodium chloride solution and sterilised by filtration through

polyethersulfone (PES) filters of pore size 0.22μm. The cell suspension and alginate solution were mixed together to yield a final concentration $16x10^6$ cells ml^{-1}.

Flow cell

A glass flow cell was developed to maintain chondrocyte cell culture in alginate gel (see figure 1). It consists of two identical flow cell cap-glass mesh assemblies (id 20mm) to provide DMEM supply to the cultured cells (a & b); a spacer (id 20mm, high 4.5 mm) to define the construct size (c); two isopore polycarbonate membranes to retain the gel-cell construct and separate alginate gel from the circulating solution (e). The spacer has four sleeves (h) to introduce microdialysis probes (d). In this experiment, one probe was fixed inside of the sleeve by dental cement (f). Other sleeves were blocked by silicon glue. The flow cell with fixed microdialysis probe was assembled and sterilised by autoclaving.

Microdialysis set-up

The experimental set-up to monitor lactic acid concentration in the alginate gel containing bovine cartilage chondrocytes is shown in figure 2. A multichannel (8 channel) peristaltic pump was used to provide the microdialysis flow and culture medium perfusion. The pump rate was 0.08 rpm. A PVC tubing of id 0.25 mm produced pulse flow of PBS for microdialysis probe at a flow rate 0.6±0.08μl/min while the silicon tubing of id 2.06 mm provided flow of DMEM above and below the spacer at 30.5±0.5 μl/min at the given pump rate. DMEM was not re-circulated. To connect FEP microdialysis tubing to the peristaltic pump PVC tubing (ID 0.25 mm) syringe stainless steel needles G 19 were used. Dialysate was collected every 20 min. The flow cell and reservoirs with DMEM were kept in the incubator under 37°C and 5% CO_2.

Preparation of chondrocyte-alginate construct

The mixture of the cell suspension and alginate solution was injected inside of the spacer of the assembled flow cell by the syringe. The flow cell was then perfused by 102 mM $CaCl_2$ for 45 min at the rate 2 ml/min to crosslink the alginate. After given period of time $CaCl_2$ was replaced by DMEM supplemented with antibiotic-antimycotic solution (1% v/v), 6% v/v foetal bovine serum and 0.5% v/v ascorbic acid. The flow cell was perfused by DMEM in the course of the experiment at the rate 0.6±0.08 μl/min.

Microdialysis probe characteristics

For measurement of the extracellular solute concentration inside of the alginate-chondrocyte construct autoclavable microdialysis probes with an od of 0.5mm and 35 mm flexible polyurethane shaft were used. The membrane has an effective length 4 mm with 15 kDa cut-off.

The relationship between the extracellular concentration of the solute and dialysate concentration was characterised by the probe recovery. The probe recovery was expressed as a percentage of the extracellular concentration of the solute:

$$\text{Recovery} = \frac{[\text{Dialysate}]}{[\text{Extracellular}]} \times 100\%$$

For the experiments with the stirred solution *in vitro* (Figure 3a) the microdialysis probe was perfused by PBS. For calibration a perfusion rate of 1.3±0.07µl/min was used. Dialysate was collected every 5 min. Probe recovery was also determined before and after autoclaving.

In the *in vitro* experiments using the alginate-chondrocyte construct (Figure 5), the microdialysis probe was perfused by PBS. Perfusion rate was 0.6±0.08µl/min. Dialysate was collected every 20 min. Duration of the monitoring of the lactic acid was 11 days.

Lactic acid assay

Lactic acid concentration was measured by a standard enzymatic procedure (Sigma procedure no. 735). A reagent containing lactate oxidase and colourless chromogen precursors was added to the sample. The lactic acid was converted by oxidase to pyruvate and hydrogen peroxide, H_2O_2. The H_2O_2 formed catalysed oxidative condensation of the chromogen precursors to produce a coloured dye. Absorbance was read at 540 nm (Titertek Multiscan Plus plate reader, Diversified Equipment Co. Inc., Lorton, Virginia, USA), and was proportional to the original lactic acid concentration. Absorbance was calibrated against a standard curve, produced from known concentrations of lactic acid in PBS or blank medium with components presented in the sample.

Results

Figure 3a represents typical dependence of solute concentration in dialysate on concentration of the solute in stirred solution *in vitro*. Recovery of the probe was determined to be 79.7± 4.5% at given perfusion rate (Fig 3b).

Autoclaving slightly altered the probe recovery, but the difference was not statistically significant, as it is shown in figure 4.

Figure 5 represents a typical curve of the monitored lactic acid level in the middle of alginate-chondrocyte construct. Over the first 20 hours the concentration of the lactate changed from 0.73±0.05 mM up to 3.0±0.5 mM showing the cell metabolic activity increase following an initial adaptation. The concentration of the lactic acid in the construct remained on the constant level over the observed period of the monitoring. The average mean of the lactic acid concentration was 3.1±0.4 mM. The results showed a stable population of chondrocytes at relative steady metabolic rate, as it has been expected.

Example 2

Materials

Alginic sodium salt was obtained from Fluka (UK); $CaCl_2$ was obtained from BDH Laboratory Supply (Poole, UK). Autoclavable Microbiotech microdialysis probes with 15 kDa cut-off polyether sulphone (PES) membrane, PEP microdialysis tubing and adapters were supplied by Royem Scientific (UK). Peristaltic pump PVC tubing (id 0.25mm) was supplied by Anachem (Luton, UK). Peristaltic pump silicon tubing (id 2.06 inches) was supplied by Alkey (Basingstoke, UK); Isopore polycarbonate membrane filters of pore size 0.2μM were obtained from Millipore (Watford, UK). The peristaltic pump used was a Gilson 8 channel pump. Radioactive [^{14}C] 3-methyl-D-glucose was received from Amersham Pharmacia Biotech UK Ltd; 3 kDa fluorescent dextran was supplied by Molecular Probes Inc (Cambridge, UK). Other chemicals were supplied by Sigma (Poole, UK).

Methods

Glass flow cell was used to form the alginate gel discs (Fig 1). It consists of two identical flow cell cap-glass meshes (id 20mm) to provide supply the solutions to the gel (a & b); a spacer (id 20mm, height 4.5 mm) to define the construct size (c);

and two isopore polycarbonate membranes to retain the gel and separate alginate gel from the circulating solution (e). The spacer has four sleeves (h) to introduce microdialysis probes (d) in the middle of the gel. In this experiment, one probe was fixed inside of the sleeve by dental cement (f). Other sleeves were blocked by silicon glue.

The flow cell was assembled and 1.2 % alginate solution was injected into the spacer. In order to polymerise the alginate the solution contained 102 mM $CaCl_2$ was pumped at the rate 0.5 ml/min through the both cap-glass meshes for 0.5 hr. The alginate gel was formed as a disc with a diameter of 20 mm and a thickness of 5 mm. After 0.5 hr $CaCl_2$ solution was replaced by the buffer containing 25 mM HEPES, 150 mM NaCl, 1.8 mM $CaCl_2$, 0.1mM sodium azid, 15mg/ml phenol red, pH=7.4. The diffusion of the lactic acid as a main product of cell metabolism, 3-methyl-D-glucose as a model molecule of glucose and 3 kDa dextran as the model of bioactive molecules (growth factors, peptides etc) were tested. The concentrations of the lactic acid, 3-methyl-D-glucose and fluorescent dextran in the buffer were 10 mM, 1.20E-04 μCi/ml and 4.38E-05 mg/ml respectively.

The perfusion liquid for the microdialysis probe was a buffer consisting of 25 mM HEPES, 150 mM NaCl, 1.8 mM $CaCl_2$, 0.1mM sodium azid, pH=7.4 and was pumped at the rate of 2 μl/min.

Dialysate was collected every 15 min. The lactate assay kit (Sigma) was used to determine the lactic acid concentration in collected dialysate. 3-methyl-D-glucose concentration was determined by radioactive counting; 3 kDa dextran concentration was determined by fluorimeter.

Hydration of the gel was calculated from:

(wet weight – dry weight)/dry weight

Partition coefficients (K) were estimated as:

$$K = C_{A \text{ in tissue}}/C_{A \text{ in equilibrating solution}}$$

Recovery of the microdialysis probe was defined as the concentration of the analyte in the dialysate expressed as a percentage of the concentration of analyte present in the surrounding gel.

Results

Hydration of the alginate gel was found to be 32.1±4.2.

Partition coefficients of solutes tested are given in the table below:

Solute	MW	Partition coefficient (K)
Lactic acid	90.1	0.88±0.08
3-methyl-glucose	196	0.69±0.07
Dextran 3 kDa	30000	0.49±0.03

The distribution of the solutes tested in the middle of the alginate gel with the time is shown on figure 6 a,b,c.

The dependence of the recovery of the microdialysis probe on solute MW is shown on figure 7.

Example 3

The experiments described above were extended using multiple micromembrane probes.

Materials

See also Materials for Example 1 and 2. 2 x crystallized suspension of papain (23mg·ml^{-1}, 17 Units·mg^{-1}), chondroitine sulphate from bovine trachea, 1,9-dimethyl-metylene blue was obtained from Sigma-Aldrich (Pool, UK). 3-O-Viability/Cytotoxicity kit was purchased by Molecular Probes Inc. (Cambridge, UK).

Bioreactor used

Glass bioreactor was developed to form alginate gel discs and to maintain chondrocyte cell culture in alginate gel. It consists of two identical cap-glass mesh assemblies (id 20mm) to provide DMEM supply to the cultured cells; a spacer of id 20mm and different heights (4.5 mm, 15 mm and 16 mm) to define the construct size

for specific experiment, having 4 or 8 ports or sleeves allowing position of microdialysis probes according to the aim of the experiment. Unused ports were blocked by silicon glue; two Isopore polycarbonate membranes to retain the gel-cell construct and separate alginate gel from the circulating solution. An appropriate number of microdialysis probes were introduced into the ports by introducer and fixed by Epoxy glue. The middle of the microdialysis membrane was always situated along the geometrical axis (centre) of the cylindrical spacer. The bioreactor with fixed microdialysis probes was assembled and clamped by clamps. The bioreactor was sterilised by autoclaving for experiments conducted on chondrocytes-alginate gel constructs.

Microdialysis set-up

A multichannel (8 channel) peristaltic pump was used to provide the microdialysis flow and culture medium perfusion. The pump produced pulse flow for microdialysis probe at a flow rate 0.6 – 3 µl/min (PVC tubing of id 0.25mm) and for bioreactor 30 – 60 µl/min (silicon tubing of id 2.06 mm). Buffer/DMEM to supply bioreactor was not re-circulated. To connect FEP microdialysis tubing to the peristaltic pump PVC tubing (id 0.25 mm) syringe stainless steel needles G 19 were used. The bioreactor and reservoirs with buffer for probe and Buffer/DMEM for bioreactor were kept in the incubator under 37°C.

Preparation of chondrocyte-alginate construct

Articular chondrocytes were isolated from the metacarpal-phalangeal joint of 18-24 month steers using enzymatic digestion procedure with collagenase following the procedure described by Hopewell and Urban (2003). The isolated cells were washed and re-suspended in DMEM supplemented with antibiotic-antimycotic solution (1% v/v). 1.2 % w/v alginate solution was prepared in 0.9% w/v sodium chloride solution and sterilised by filtration through polyethersulfone (PES) filters pore size 0.22µm. The cell suspension and alginate solution were mixed together to yield a final concentration $13 \cdot 10^6$ - $16 \cdot 10^6$ cell ml^{-1}. The mixture was injected inside of the spacer of the assembled bioreactor by the syringe. The bioreactor was then

perfused by 102 mM $CaCl_2$ for 45 min at the rate 2 ml/min to crosslink the alginate. After given period of time $CaCl_2$ was replaced by DMEM supplemented with antibiotic-antimycotic solution (1% v/v), 6% v/v foetal bovine serum and 0.5% v/v ascorbic acid. The bioreactor was perfused by DMEM in the course of the experiment at the rate 0.6±0.08 μl/min.

Alginate gel polymerisation

Alginate solution (1.2% w/v in saline) or isolated chondrocytes suspension in alginate solution was injected in assembled bioreactor through the port by syringe. Probes were introduced and solution of 102 mM $CaCl_2$ was pumped through the glass mash for periods of time ranged from 0.5-5 hr in dependence of spacer high to form alginate gel.

Microdialysis probe characteristics

Probe used. For measurement of a solute concentration in alginate gel or alginate-chondrocyte construct autoclavable microdialysis probes with 15 kDa cut-off polyethersulfone (PES) dialysis membrane of an effective length 4 mm, od 0.6mm and 35 mm in length flexible polyurethane shaft were used.

In situ probe recovery and probe degradation assessment. The probe recovery was expressed as a percentage of the concentration of a solute in the probe surrounding.

In situ *probe recovery in the experiments on assessment of the diffusion in aginate gel was calculated as:*

$R=C_{d\,eq}\cdot 100/C_{g\,eq}$

Where $C_{d\,eq}$ was a concentration of lactic acid or 3-methyl-D-glucose in dialysate after equilibration of a gel with a solute and $C_{g\,eq}$ was concentration of a solute in a gel.

In the experiments on cell-alginate gel tissue construct *in situ* probe recovery was assessed on the basis of the percentage of phenol red (PhR) in dialysate after equilibration of the construct with DMEM containing PhR.

Biochemical and histological analysis

Cell viability. At the end of experiment tissue engineered construct was removed from bioreactor, sectioned by blade and cell viability was assessed using Viability/Cytotoxicity kit for live and dead cells. Briefly, staining of living cells was based on detection of intracellular esterase activity. They convert non-fluorescent cell-permeating calcein AM to the intensely fluorescent calcein which is well retained within living cells producing bright green fluorescence at excitation/emission wavelengths of 495/515 nm. Dead cells were stained by ethidium homodimer-1 (EthD-1), which penetrates damaged cell membranes and undergoes the enhancement of fluorescence upon binging to nucleic acids, producing a bright red fluorescence at excitation/emission wavelengths 495/635 nm.

Papain digestion. In order to determine total sulphated glycosaminoglycan (GAG) content sections of the construct were dissolved in citrate buffer containing 55 mM sodium citrate, 50 mM EDTA and 0.15 M NaCl. A proportion of gel-cell construct:citrate buffer was 1:1.5 w/v. Papain suspension was add in the proportion of 1µl of papain suspension to 100µl of dissolved construct and samples were digested at 67° C for 18 hr.

Glycosaminoglycan content. Total sulphated glycosaminoglycan (GAG) content was determined colorimetrically using dimethylmethylene blue (DMB). Papain digest (20-100 µl) were mixed with DMB reagent (pH 4.6) in a proportion (1:10) and absorbance at 576 nm was immediately read using Perking-Elmer spectrophotometer (Perkin Elmer UK Ltd, Beaconsfield, Buckinghamshire, UK). Concentrations of GAG were determined against chondroitine sulphate standards containing corresponding amount of alginate.

Collagen content. Collagen content was calculated from hydroxyproline concentration, measured using a modification of the Stegemann method. Digests were hydrolysed in 6 N HCL for 18 hr at 110° C, neutralized with sodium hydrogen carbonate, mixed with oxidant containing chloramines T and a colour reagent

containing p-dimethylaminodenzaldehyde, incubated for 20 min at 67° C, left for 15 min at room temperature and the absorbance read at 550 nm on Perking-Elmer spectrophotometer. Collagen concentration was calculated from hydroxyproline concentration, using a factor of 7.14 grams collagen per gram of hydroxyproline.

Determination of phenol red concentration in dialysate

PhR is commonly used indicator for pH range 6.4- 8.2. Its dissociation in a solution can be described by:

$H_2PhR \rightarrow HPhR^- + H^+$

$HPhR^- \rightarrow PhR^{2-} + H^+$

$HPhR^-$ has a yellow colour and PhR^{2-} has a red colour. On pH > 8.2 molecules of PhR are converted predominantly to PhR^{2-} "red" form and absorbency read at 540 mm did not depend on pH. To determine total concentration of PhR, $HPhR^-$ form in dialysate was converted to PhR^{2-} by adding 2 µl of 5M NaOH to 40-50 µl of dialysate (final pH 12.5). Absorbance of the samples was read at 540 nm on Plate reader.

Determination of lactic acid in dialysate

Lactic acid concentration was measured by a standard enzymatic procedure (Sigma procedure no. 735). A reagent containing lactate oxidase and colourless chromogen precursors was added to the sample in a proportion sample:reagent 1:10. The lactic acid was converted by oxidase to pyruvate and hydrogen peroxide, H_2O_2. The H_2O_2 formed catalysed oxidative condensation of the chromogen precursors to produce a coloured dye. Absorbance was read at 620 nm and was proportional to the original lactic acid concentration. Absorbance was calibrated against a standard curve, produced from known concentrations of lactic acid in PBS or blank medium with components presented in the sample.

Measurement of apparent diffusion coefficient in alginate gel

To determine diffusion coefficients (D_{app}) of lactic acid and 3-methyle-glucose 8-port spacer of 14.8 mm high were used. Ports on spacer were positioned in a helix along the spacer with a distance of 1.64±0.17 mm between them. Probes were

introduced and gel was polymerised. After gel polymerisation $CaCl_2$ was replaced by buffer containing 25 mM HEPES, 150 mM NaCl, 1.8 mM $CaCl_2$, 15 mg/L phenol red and tracer molecules: 10 mM lactic acid or μCi/ml 3-methyle-glucose. The same buffer for probe perfusion contained 25 mM HEPES, 150 mM NaCl and 1.8 mM $CaCl_2$. The flow along the other side of the gel was ceased. The dialysate was collected during 27.5 hr and concentration of the solutes was in dialysate was determined by an appropriate assay.

To calculate the apparent diffusion coefficient of the solutes in a gel measured by microdialysis probes 8 time points ranged 2.4 hr to 5.1 hr were chosen. The solute concentration at the side of the gel, where flow was ceased was zero at time points given. D_{app} of lactic acid and 3-methyl-glucose were determined by the concentration-gradient technique according to the equation:

$$D_{app}=1/4 \cdot t \cdot \text{slope}$$

Where t is the time allowed for solute diffusion from the surface along the gel. The slope was obtained from a plot of $\ln(c_\chi/\Sigma\, c_\chi)$ vs χ^2, where c_χ was the concentration of solute measured in dialysate collected a probe, which was situated at a distance χ from the surface.

Experiments were conducted at 37° C.

Measurement of partition coefficient

The equilibration of alginate gel by lactic acid and/or 3-methyl-glucose was established as concentration of a solute in dialysate monitored by all 8 probes has reached its maximal and constant value. After equilibration of the gel by the solute, gel was removed from bioreactor and dissolved as described above. Concentration of the solute in the gel was determined by an appropriate methods. Partition coefficient (K) was estimated as

$$K= C_g/C_s$$

where C_g was the concentration of a solute in a gel on a wet weight basis obtained from the concentration of dissolved gel and C_s was the concentration of a solute in a buffer run through glass mesh of a bioreactor.

Comparison of experimental and fit curves

To monitor diffusion of lactic acid and 3-methyle-glucose in bioreactor by microdialysis probe a spacer of 4 mm high was used. The probe was positioned in the centre of the spacer and the distances from the surfaces of the gel to the probe were equal (2 mm each). A gel formed inside of the spacer was fed from top and bottom by a solution containing mixture of both solutes.

Concentration of the solutes in the gel equilibrated by solution supplied ($C_{g\ eq}$) was calculated as:

$C_{g\ eq} = C_s K$

where Cs was concentration of the solutes in supplied solution, K was a partition coefficient of corresponding solute.

Concentrations of a solute in a gel (C_g) monitored by probes were calculated as:

$C_g = 100 \cdot C_d / R$

where C_d was solute concentration in dialysate and R was a probe recovery.

A ratio $C_g / C_{g\ eq}$ was plotted versus t_i, where t_i was time interval a solute needed to rich a concentration C_g in the middle of the gel.

A fit curve was calculated according to the equation:

$$\frac{C-C_0}{C_1-C_0} = 1 - (4/\pi) \sum_{n=0}^{\infty} \frac{(-1)^n}{2n+1} e^{-D(2n+1)^2 \pi^2 t/4l^2} \cos \frac{(2n+1)\pi x}{2l} \qquad (1)$$

where C_0 was initial uniform concentration within a region $-l < x < l$ where probe was situated and l was a distance between probe and the surface of the gel, C was the concentration in a region x at time t; t was the time of diffusion of a solute from a surface of the gel to region x on a distance $\pm l$; C_1 was a constant concentration of a

solute on the surfaces of the gel; D was an apparent diffusion coefficient defined experimentally as described above.

If $C_0 = 0$, and for a probe situated in the middle of the gel $x = 0$, $t = t_i$, $C = C_i = 100 \cdot C_d/R$ at given Δt_i, equation (1) became:

$$\frac{C_i}{C_1} = 1 - (4/\pi) \sum_{n=0}^{\infty} \frac{(-1)^n}{2n+1} e^{-D(2n+1)^2 \pi^2 \Delta t_i / 4l^2} \qquad (2)$$

Calculated curve was plotted as C_i/C_1 vs t_i

Gel hydration

To determine gel hydration gel was dried at 67° C for 18 hr and the hydration was calculated as (wet weight – dry weight)/dry weight.

$HPhR^-$ has a yellow colour and PhR^{2-} has a red colour. On pH > 8.2 molecules of PhR are converted predominantly to PhR^{2-} "red" form and absorbance read at 540 mm exists in PhR^{2-} In a dialysate of usual pH range 6.8-7.4 both form are present in proportions depending on pH. To determine total concentration of PhR, $HPhR^-$ form was converted to PhR^{2-} by adding 2 µl of 5M NaOH to 40-50 µl of dialysate (final pH) . Absorbance of the samples was read at 540 nm on Plate reader

Results

The diffusion of glucose as main cell nutrient and lactic acid as main metabolic products were assessed.

Figure 8 represents monitoring of the diffusion of lactic acid in an alginate gel formed inside of a bioreactor by 8 microdialysis probes (data from probes 1, 3, 5 and 7 are not shown). In order to calculate a diffusion coefficient 8 time points ranged from 0.5-5 hr where chosen (Fig 8). Within this period of time the solute had not reached the edge of the gel and the construct is symmetrical we have considered the case of the diffusion occurred in a plane sheet.

A distribution of lactic acid in a gel at different time points is shown on Figure 9. The concentration of lactic acid in a gel of 14.8 mm high has reached the equilibrium within 27 hr.

Figure 10 represents D_{app} of lactic acid and glucose in comparison with their diffusion coefficients in aqueous solution (D_{free}). Diffusion coefficients decreased with increasing of solute molecular weight.

The diffusion of glucose and lactic acid in alginate gel as it takes place in bioreactor was monitored by microdialysis probe positioned in the centre of a gel (Figure 11). The theoretical curves for diffusion of lactic acid (Fig. 11, a) and glucose (Fig. 11, b) in the centre of a bioreactor were generated using D_{app} (solid lines). The experimental data were in a good agreement with the predicted values.

For monitoring of cell metabolic activity 2 probes were introduced inside of bioreactor. Edge probe was positioned on 2 mm distance from the edge of the gel and centre probe was positioned in the centre of the construct. On figure 12 the level of lactic acid in dialysate monitored by two probes is shown. After 7 hours the level of lactic acid production by the cell on the edge of the construct has reached its maximum and remained constant during the following 70 hr. The responses of cell lactic acid production to buffer changes were clearly shown. Level of lactic acid monitored in the centre of the bioreactor also reached its maximum after approximately 7 hr and after 13 hr a steep decline in lactic acid production was observed.

The probe permeability was assessed during the experiment. Concentration of phenol red in dialysate collected from the centre probe during the experiment is shown on figure 13. The decline of probe permeability to solutes was 2.5% for 24 hr.

The construct has been cultured for 12 days and cell viability, gel hydration, collagen and glycosaminoglycan content were determined.

Cells mainly survived in the region of up to 5 mm from the edges of the construct (Fig. 14).

Glycosaminoglycans were located around the areas containing live cells (Figure 15). Collagen was spread evenly through the construct. Hydration of the gel did not vary with the distance from the edge.

CLAIMS

1. A method of monitoring the formation of a tissue in a tissue construct *in vitro* or the viability or function of cells of a tissue in a tissue construct being grown *in vitro*, which method comprises:

contacting a micromembrane probe with the tissue construct;

carrying out microdialysis and/or ultrafiltration to extract a marker into the dialysate; and

analysing the marker in the dialysate;

thereby to monitor the formation of the tissue or the viability or function of the cells.

2. A method according to claim 1 wherein the microdialysis is carried out by feeding a perfusion liquid into the micromembrane probe and removing the perfusion liquid as a dialysate from the probe.

3. A method according to claim 2 wherein a negative pressure is applied to the perfusion liquid when it is fed into the micromembrane probe.

4. A method according to claim 2 or 3 wherein the perfusion liquid is phosphate buffered saline (PBS) or a cell culture medium.

5. A method according to any one of the preceding claims wherein the marker is a metabolic product, a cell nutrient, a cell type marker, a soluble macromolecule produced by the cells or a marker of extracellular matrix turnover, a macromolecule marker or a protein.

6. A method according to claim 5 wherein the marker is lactic acid, glycerol, glucose, oxygen, procollagen, a proteoglycan, a glycosaminoglycan (GAG) or collagen.

7. A method according to claim 5 or 6 wherein the marker has a molecular weight of 0.05 to 5 kD or 10 to 200 kD.

8. A method according to claim 7 wherein the marker has a molecular weight of 0.1 to 3 kD.

9. A method according to claim 7 wherein the marker has a molecular weight of 20 to 100 kD.

10. A method according to any one of the preceding claims wherein the analysis comprises the detection of the presence, absence or level of a marker.

11. A method according to claim 10 wherein the levels of the marker are monitored over time.

12. A method according to any one of the preceding claims wherein the probe or more than one probe is contacted within the tissue construct at more than one location.

13. Use of microdialysis and ultrafiltration to monitor the formation of a tissue in a tissue construct *in vitro* or the viability or function of cells of a tissue in a tissue construct being grown *in vitro*.

14. A method of monitoring the viability or function of cells of a tissue, which method comprises:

contacting a micromembrane probe with the tissue construct;

carrying out microdialysis and/or ultrafiltration to extract a marker into the dialysate; and

analysing the marker in the dialysate;

thereby to monitor the viability or function of the cells of the tissue.

15. A method according to claim 14 wherein the probe or more than one probe is contacted with the tissue at more than one location.

16. A method according to claim 15 wherein three or more probes are contacted with the tissue at three separate locations.

17. A method according to any one of claims 14 to 16 wherein the marker is selected from a metabolic product, a cell nutrient, a cell type marker, a marker of extra cellular matrix turnover, a macromolecule marker or a protein.

18. A method according to claim 17 wherein the marker has a molecular weight between 0.05 to 5 kD, or between 10 to 200 kD.

19. A method according to claim 18 wherein the marker has a molecular weight of 20 to 100 kD.

20. A method according to any one of claims 14 to 19 wherein the levels of the marker are monitored over time.

21. A method according to any one of claims 14 to 20 wherein the tissue is transplanted tissue.

Fig.1.

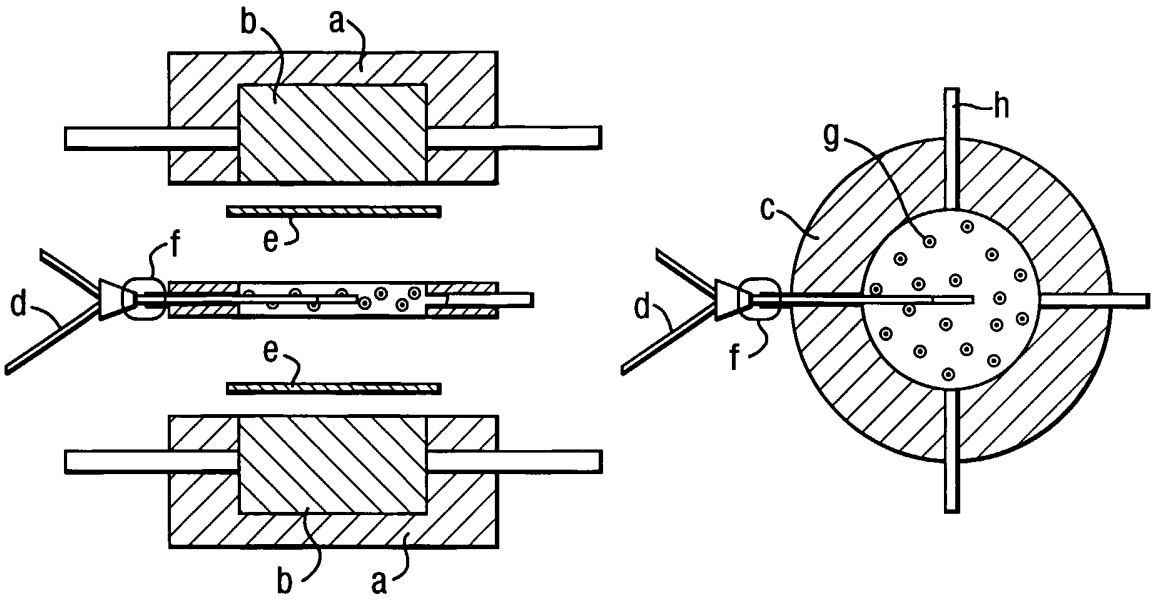
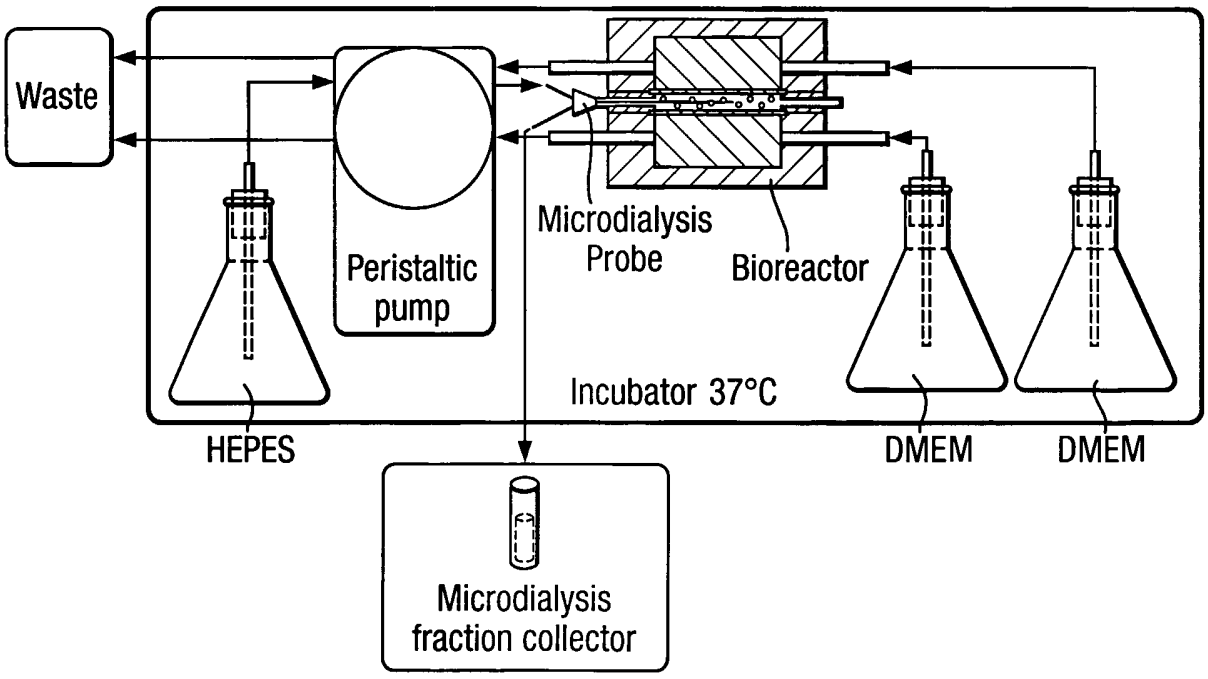

b
a
e
f
d
e
b
a
h
g
c
d
f

Fig.2.


Waste
Peristaltic pump
Microdialysis Probe
Bioreactor
Incubator 37°C
HEPES
DMEM
DMEM
Microdialysis fraction collector

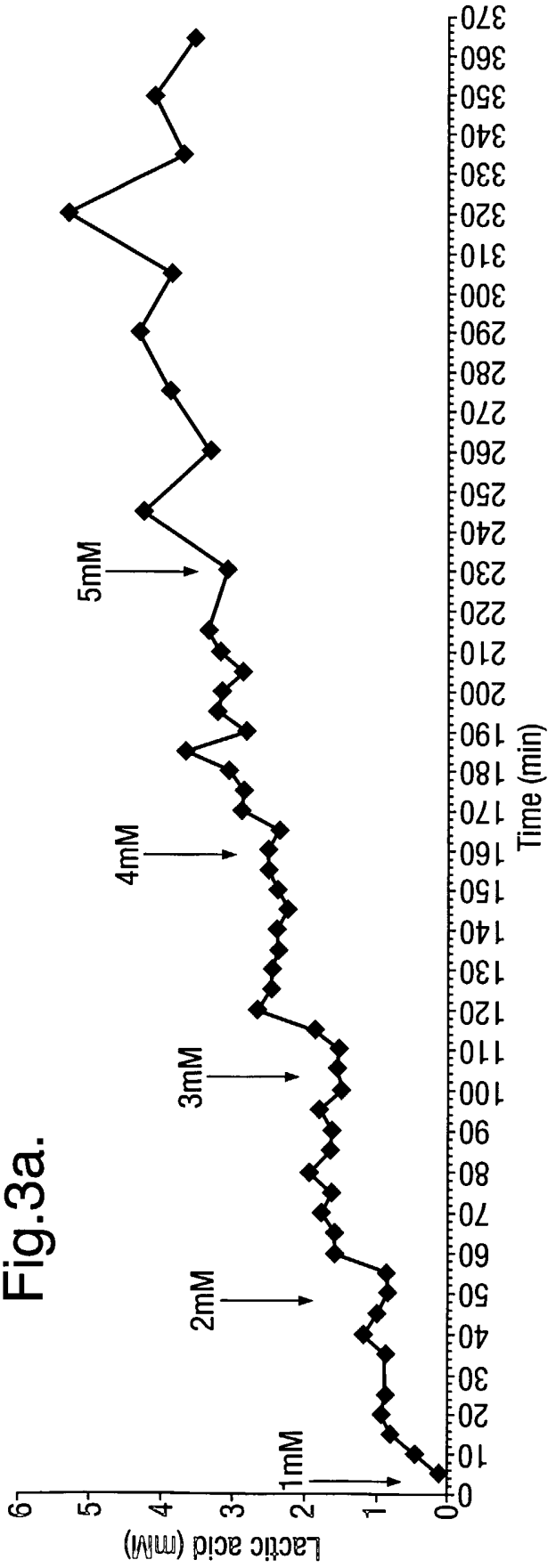

Lactic acid (mM)
Time (min)
1mM
2mM
3mM
4mM
5mM

Fig.3a.

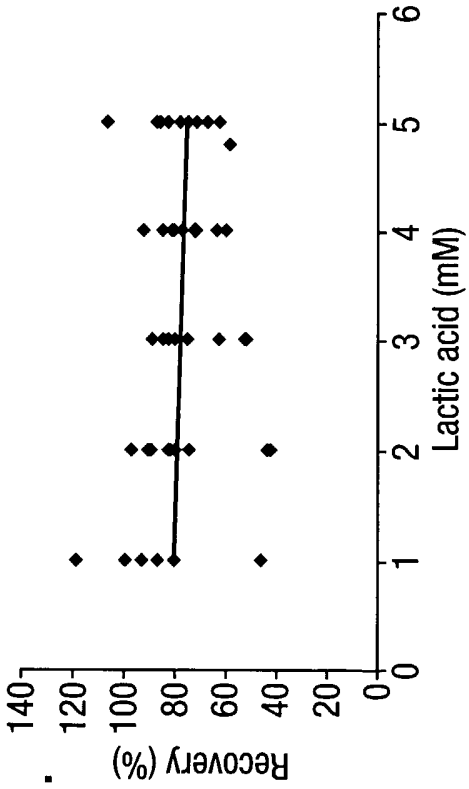

Recovery (%)
Lactic acid (mM)

Fig.3b.

Fig.4.

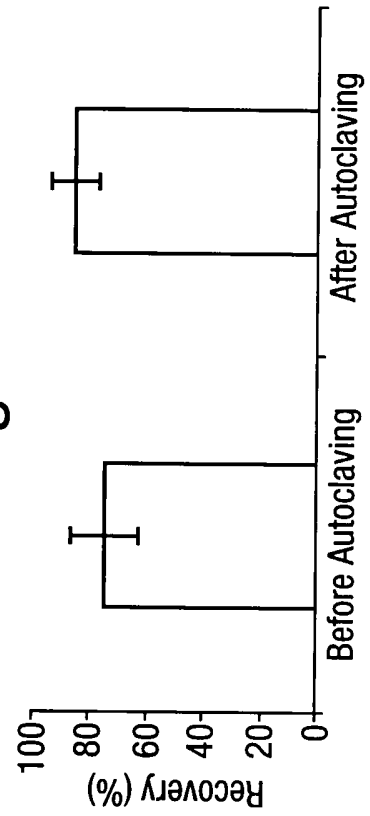

Recovery (%)
100
80
60
40
20
0
Before Autoclaving
After Autoclaving

Fig.5.

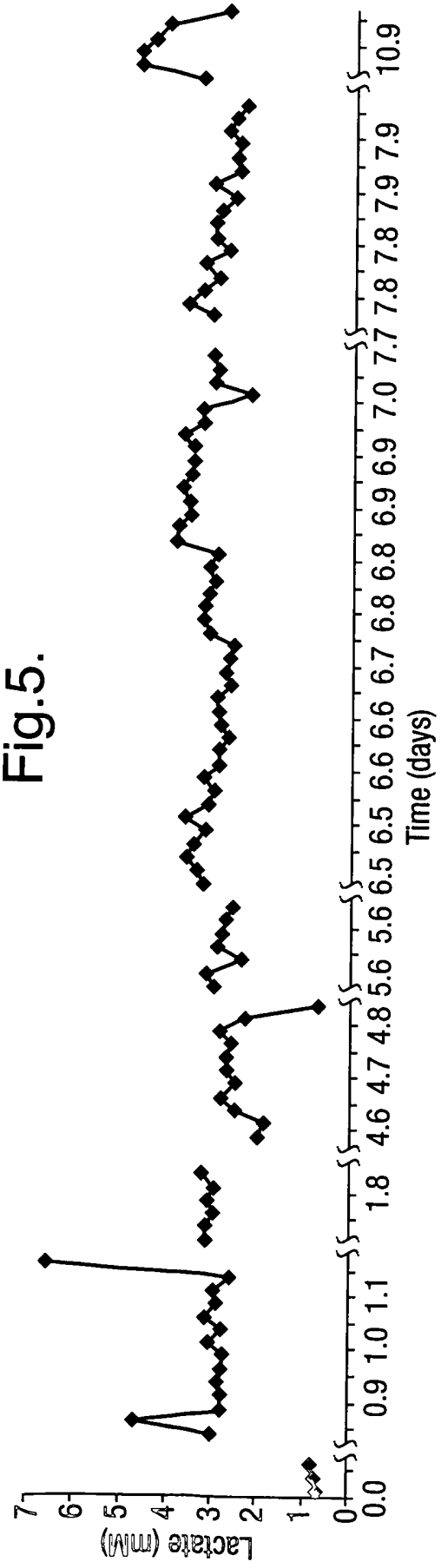

Lactate (mM)
7
6
5
4
3
2
1
0
0.0
0.9
1.0
1.1
1.8
4.6
4.7
4.8
5.6
5.6
6.5
6.5
6.6
6.6
6.7
6.8
6.8
6.9
6.9
7.0
7.7
7.8
7.8
7.9
7.9
10.9
Time (days)

Fig.6a.

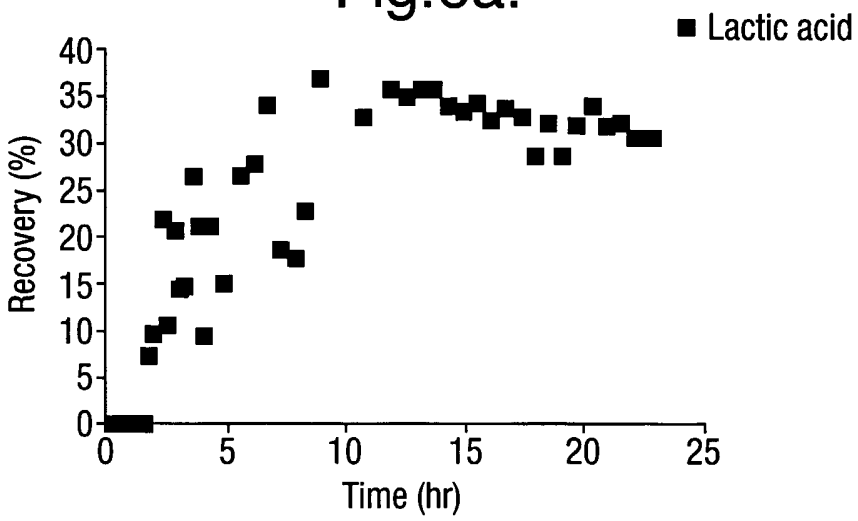


Fig.6b.

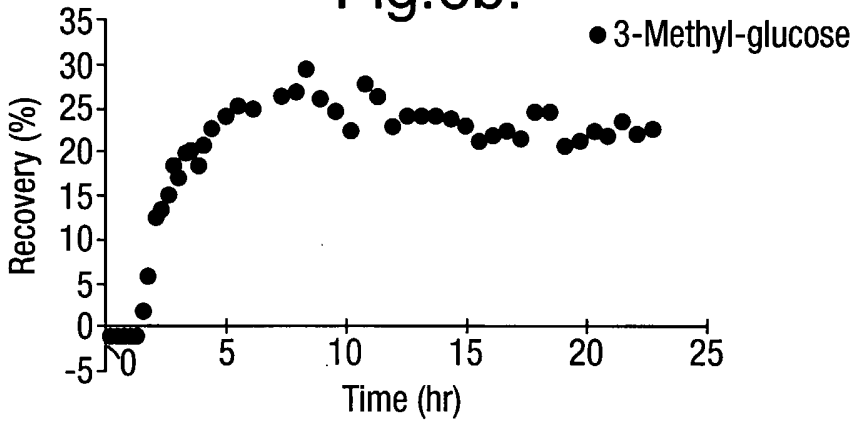


Fig.6c.

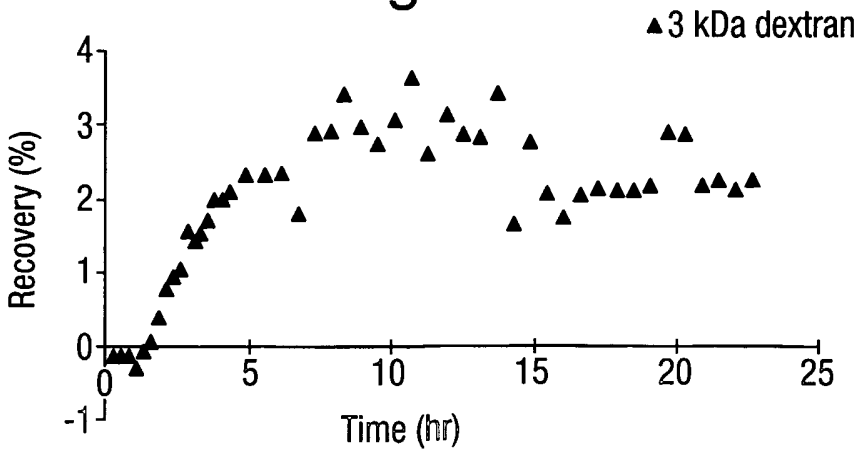


Fig.7.

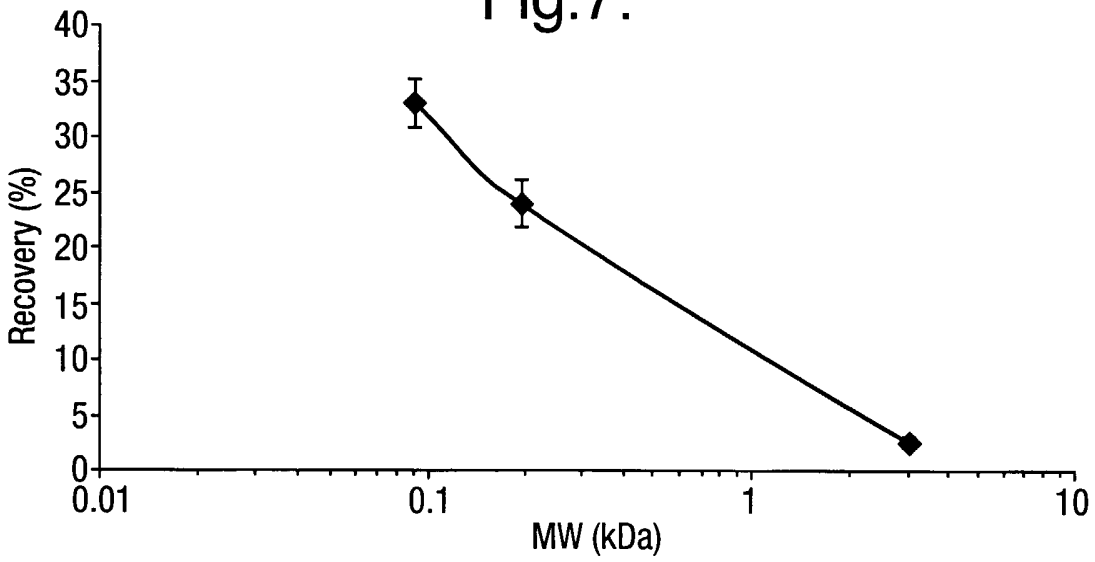

Recovery (%)
40
35
30
25
20
15
10
5
0
0.01
0.1
1
10
MW (kDa)

Fig.8.

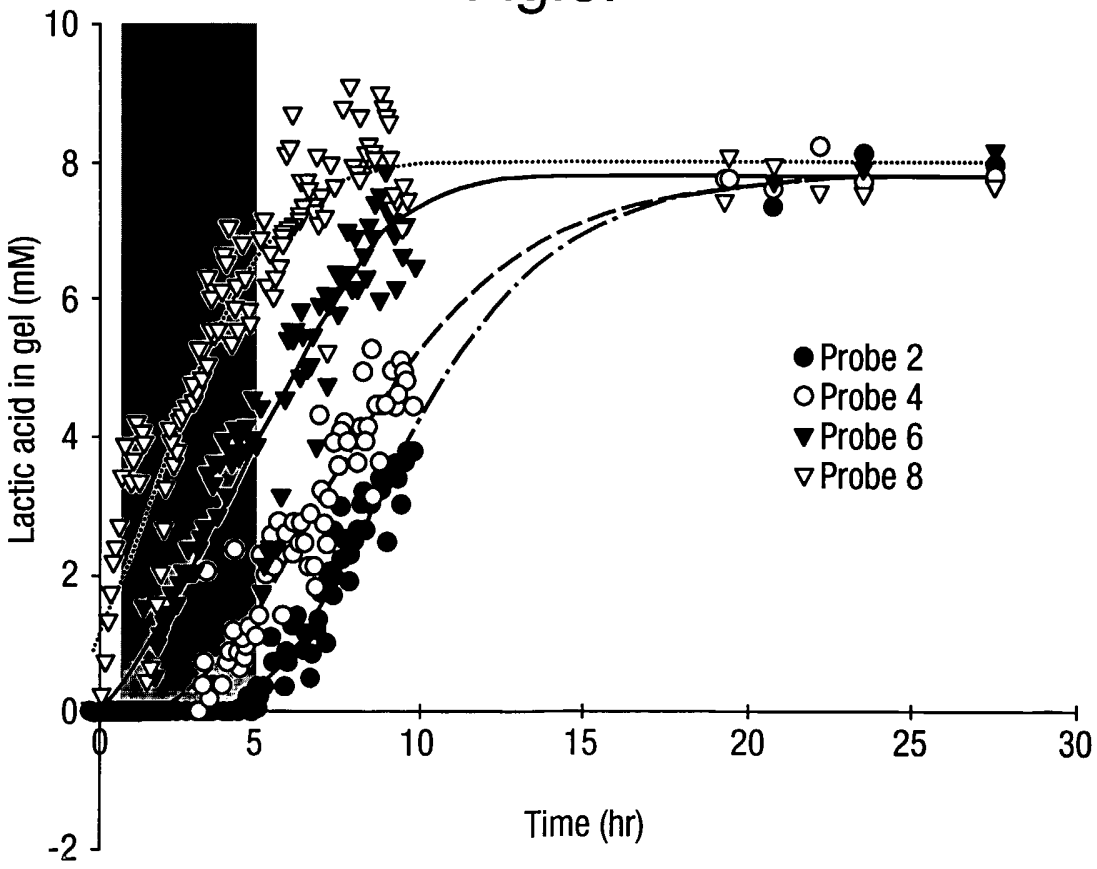

Lactic acid in gel (mM)
10
8
6
4
2
0
-2
0
5
10
15
20
25
30
Time (hr)
Probe 2
Probe 4
Probe 6
Probe 8

Fig.9.

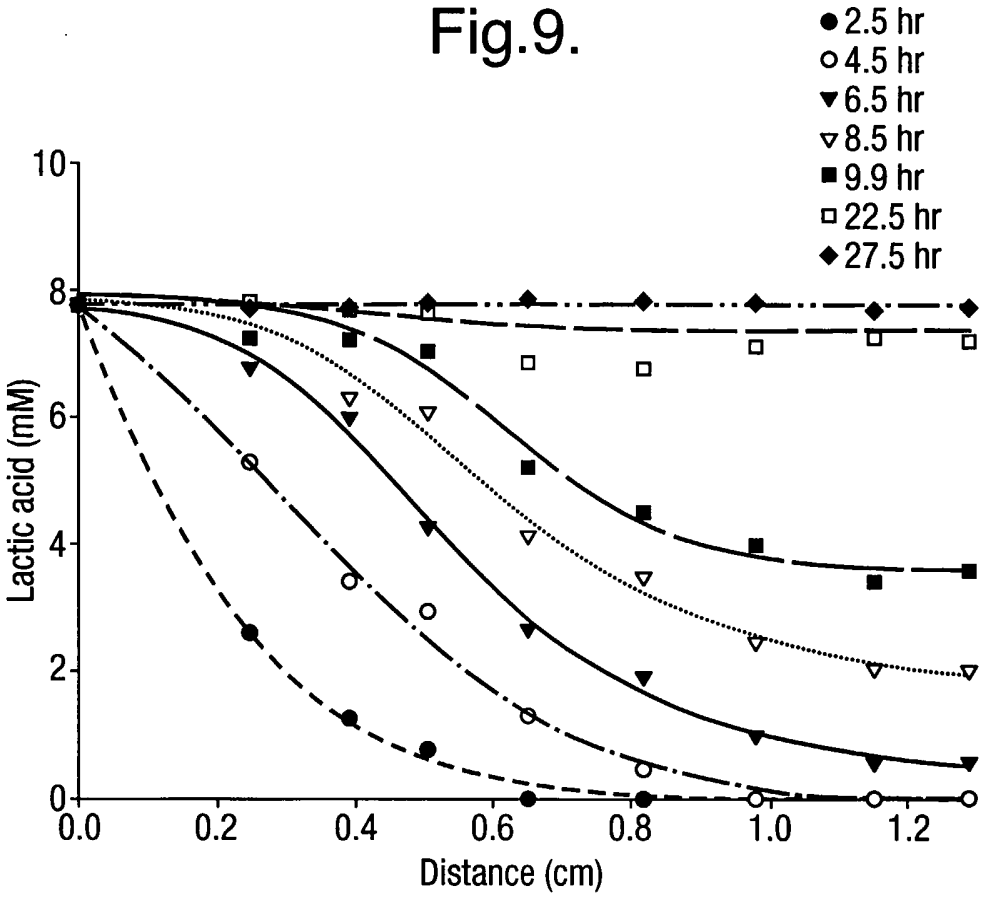

2.5 hr
4.5 hr
6.5 hr
8.5 hr
9.9 hr
22.5 hr
27.5 hr
Lactic acid (mM)
Distance (cm)
0
2
4
6
8
10
0.0
0.2
0.4
0.6
0.8
1.0
1.2

Fig.10.

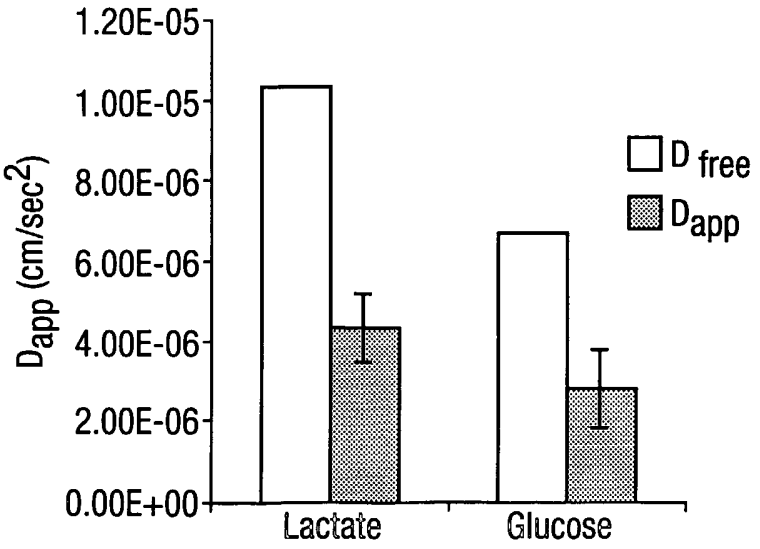

1.20E-05
1.00E-05
8.00E-06
6.00E-06
4.00E-06
2.00E-06
0.00E+00
Dapp (cm/sec2)
D free
Dapp
Lactate
Glucose

Fig.11a.

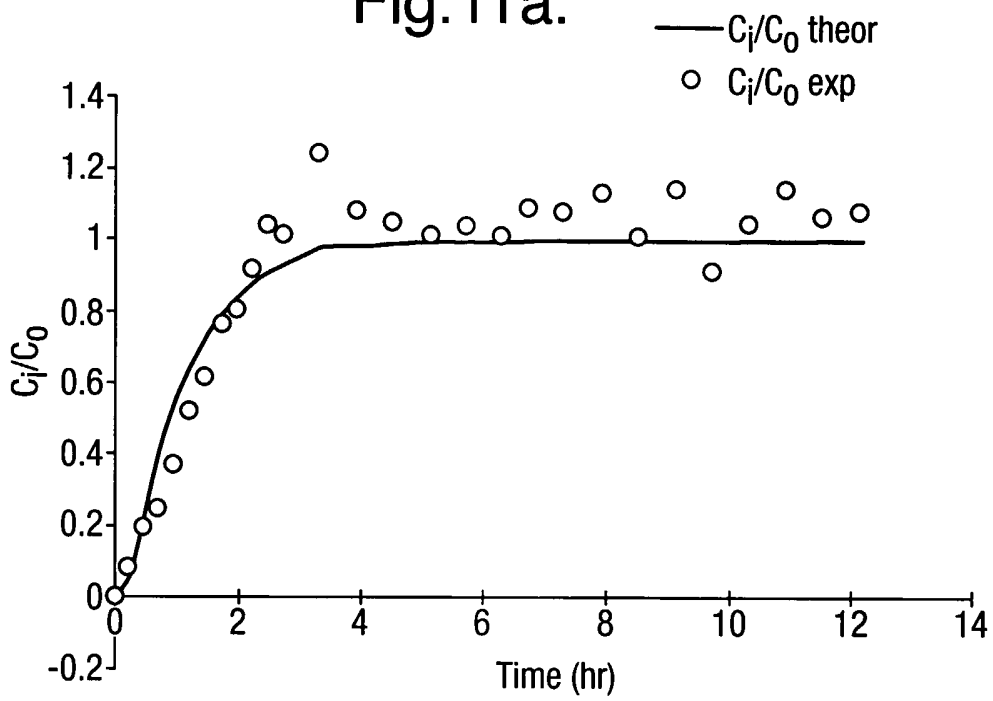


Fig.11b.

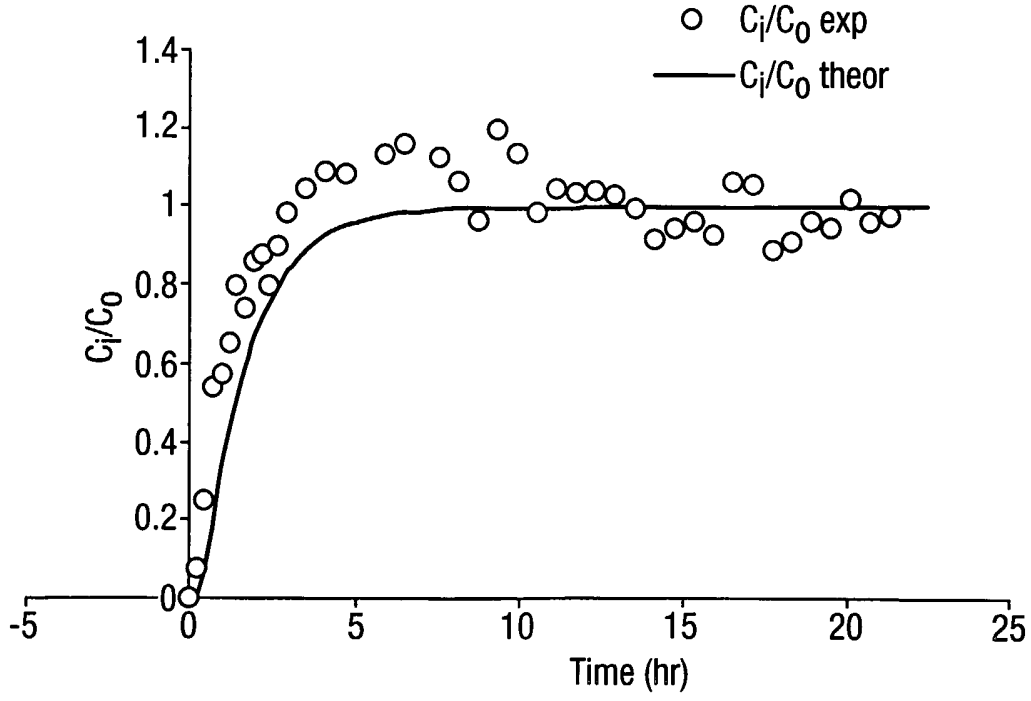


Fig.12.

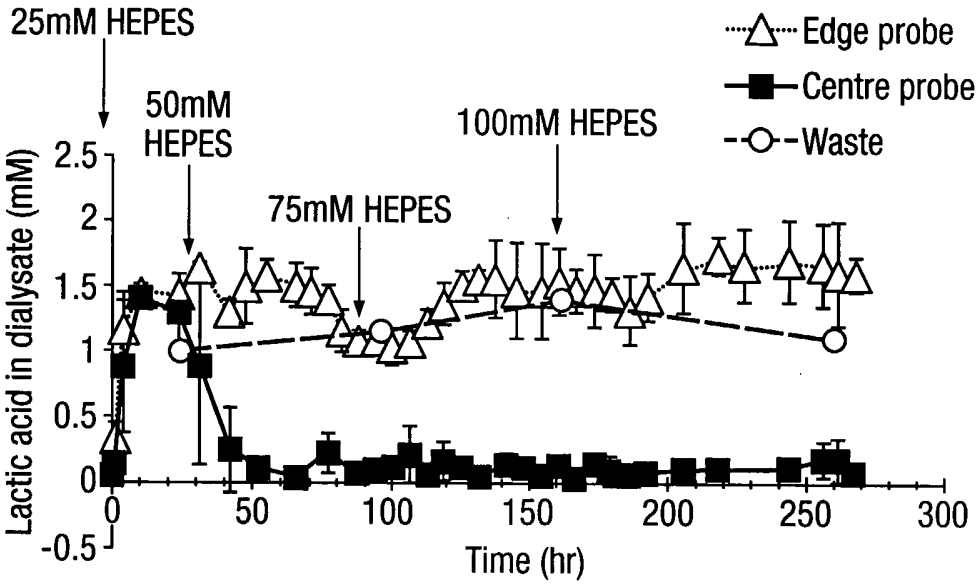

25mM HEPES
50mM HEPES
75mM HEPES
100mM HEPES
Edge probe
Centre probe
Waste
Lactic acid in dialysate (mM)
2.5
2
1.5
1
0.5
0
-0.5
0
50
100
150
200
250
300
Time (hr)

Fig.13.

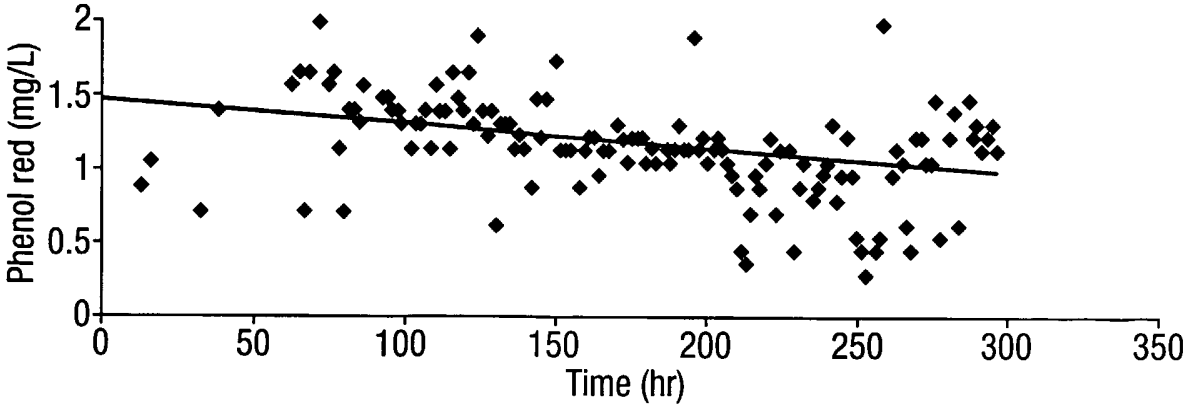

Phenol red (mg/L)
2
1.5
1
0.5
0
0
50
100
150
200
250
300
350
Time (hr)

Fig.14.

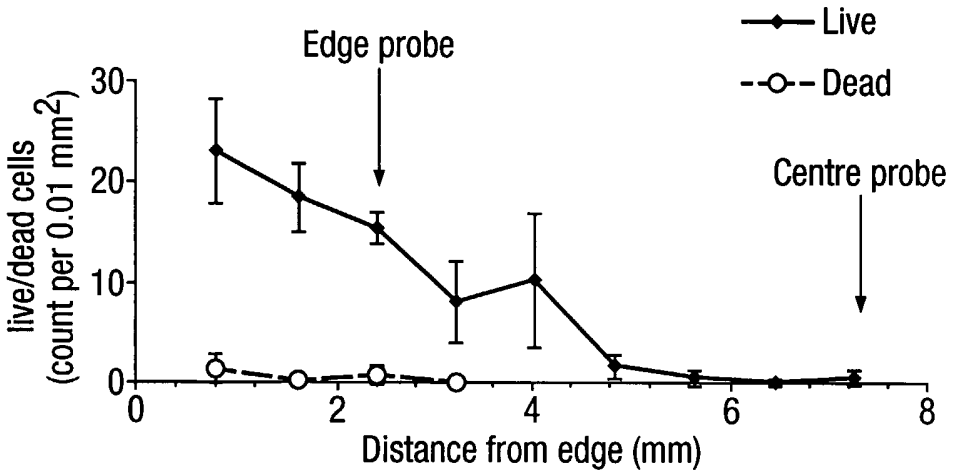
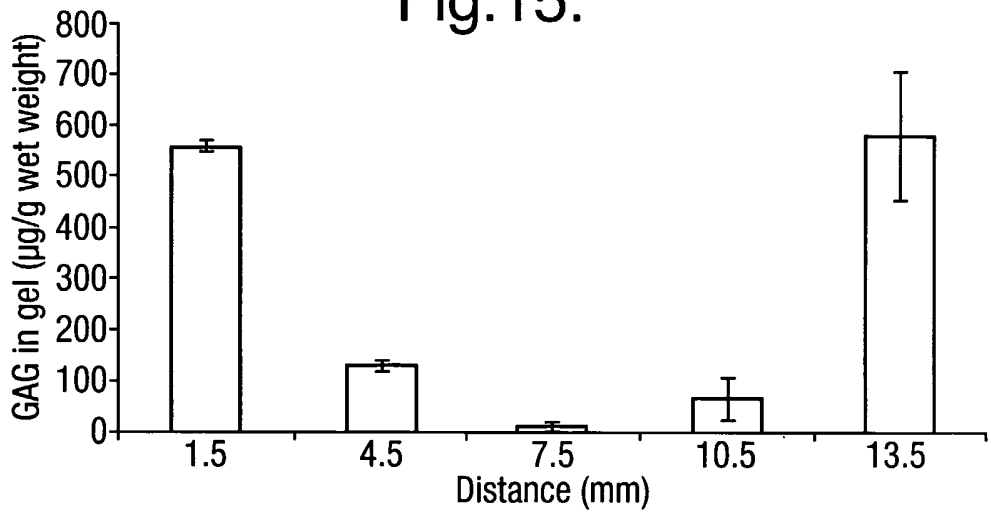

Live
Dead
Edge probe
Centre probe
live/dead cells
(count per 0.01 mm2)
30
20
10
0
0
2
4
6
8
Distance from edge (mm)

Fig.15.


GAG in gel (µg/g wet weight)
800
700
600
500
400
300
200
100
0
1.5
4.5
7.5
10.5
13.5
Distance (mm)

INTERNATIONAL SEARCH REPORT

In ional Application No
PCT/GB2004/002510

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 2002/082490 A1 (HOERAUF CHRISTIAN ET AL) 27 June 2002 (2002-06-27) abstract figure 1	14-21 1-13
X	WO 01/03763 A (JOHANSSON ROGER ; CMA MICRODIALYSIS AB (SE)) 18 January 2001 (2001-01-18) abstract page 1, line 16 - page 2, line 26; figure 1	14-21
Y	WO 01/00783 A (ADVANCED TISSUE SCIENCES INC) 4 January 2001 (2001-01-04) page 2, paragraph 3 - page 3, paragraph 1 page 4, paragraphs 2,3	1-13

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 September 2004	12/10/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Weiland, S

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INTERNATIONAL SEARCH REPORT

In ional Application No
PCT/GB2004/002510

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PARK JONG-CHUL ET AL: "Viability evaluation of engineered tissues" YONSEI MEDICAL JOURNAL, vol. 41, no. 6, December 2000 (2000-12), pages 836-844, XP002297029 ISSN: 0513-5796 page 839, right-hand column, paragraph 3 - page 841, left-hand column, paragraph 1	1-21

INTERNATIONAL SEARCH REPORT

ırnational application No.
PCT/GB2004/002510

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 14-21
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 14-21 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the product.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB2004/002510

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2002082490 A1	27-06-2002	DE 10038835 A1	28-02-2002
		EP 1177759 A1	06-02-2002
		JP 2002126074 A	08-05-2002
WO 0103763 A	18-01-2001	SE 514552 C2	12-03-2001
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		WO 0103763 A1	18-01-2001
		SE 9902694 A	15-01-2001
WO 0100783 A	04-01-2001	AU 5767900 A	31-01-2001
		WO 0100783 A2	04-01-2001