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(71) Applicant: THE UNIVERSITY OF NOTTINGHAM
[GB/GB]; University Park, Nottingham, NG7 2RD (GB).

(72) Inventors: **SHAKESHEFF, Kevin**; The University of Nottingham, University Park, Nottingham, NG7 2RD (GB). **ALEXANDER, Cameron**; The University of Nottingham, University Park, Nottingham, NG7 2RD (GB). **SAEED, Aram Omer**; The University of Nottingham, University Park, Nottingham, NG7 2RD (GB). **SAUNDERS, Brian**; School of Materials, The University of Manchester, Manchester, M13 9PL (GB).

(74) Agent: **BARKER BRETTELL LLP**; 100 Hagley Road, Edgbaston, Birmingham, West Midlands, B16 8QQ (GB).

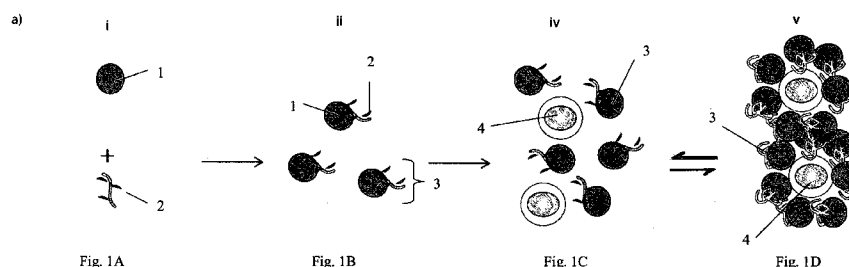
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(57) Abstract: A reversible scaffold for growing cells or bacteria, the reversible scaffold comprising magnetic particles, wherein the magnetic particles exist in a liquid phase under a first set of conditions and in a semi-solid or solid phase under a second set of conditions, thereby providing a scaffold for growing cells or bacteria, the reversible scaffold being capable of being repeatedly cycled from the liquid phase to the semi-solid or solid phase and back again by changing from the first set of conditions to the second set of conditions and back again.

SCAFFOLD

The present invention relates to scaffolds or supports for maintaining and/or growing cells or bacteria and/or for other biomedical applications, e.g. for controlled release applications. In particular, the invention relates to such supports or scaffolds comprising polymeric materials, e.g. stabilised polymer particles. The invention also relates to methods of manufacture of and/or the use of such supports or scaffolds.

Regenerative medicine, in particular cell therapy, has great potential for treating many illnesses, diseases and conditions. The research and development of new cell therapies requires cells to be grown or cultured.

Traditionally, cells have been cultured on planar surfaces. However, this does not reflect the natural environment in which cells exist. A three-dimensional cell culture environment may more accurately reflect the natural environment in which cells exist. Accordingly, the development of three-dimensional rather than two-dimensional (e.g. planar) culture environments has attracted considerable interest, especially with regard to potential cell therapies, e.g. using stem cells, where temporal and spatial environmental cues may be critical.

There is therefore a need for cell culture systems, in particular three-dimensional (3D) cell culture systems, that possess the features required for repeated cell culture. Ideally, for instance, materials or materials systems for 3D cell culture should possess the ability to assemble and disassemble rapidly and reversibly, in an easily tuneable manner and with a simple external input to allow multiple cycles of cell growth in 3D. With this goal in mind, a number of synthetic systems inspired by the intrinsic ability of responsive polymers to reversibly assemble and disassemble in temperature dependent manners have been investigated, e.g. as reported by S. Hopkins et al, *Soft Matter* 2009, 5, 4928.

N. Matsuda et al, *Adv. Mater.* 2007, 19, 3089 described a cell sheet technology, which is said to enable reversible cell adhesion to and detachment from the culture substrate by controlling the hydrophobicity of the surface of the substrate using temperature-responsive polymers, mainly poly (N-isopropylacrylamide) (PIPAAm) polymer. However, most of these systems adopted two-dimensional (2D) cell culture strategies.

Furthermore, the polymers employed have been reported to show toxicity above the phase transition (H. Vihola et al, Biomaterials 2005, 26, 3055).

5 After cell growth, it is necessary to sub-culture or harvest the cells, i.e. to separate or detach them from a substrate, support or scaffold, e.g. for further processing or passaging. Typically, this is achieved using an enzymatic treatment, e.g. using trypsin.

10 Cell growth may be carried out in a series of stages. For instance, repeated cell culture (growth) may routinely involve cell sub-culturing (detachment from the culture substrate) when cells reach high density. This step is often carried out through trypsin digestion for detaching the cells followed by reseeding them onto fresh culture substrates. In addition to this process being time consuming and tedious, repetitive exposure to trypsin may lead to poor cell quality owing to the proteolytic activity of
15 trypsin which can damage cell surface receptors or membrane proteins.

Thus, it may be desirable to reduce, minimise or even eliminate the use of enzymes such as trypsin when subculturing or harvesting cells.

20 WO 2010/043892 discloses a responsive particulate dispersion system comprising polymer particles that have a biodegradable core and thermoresponse biocompatible co-polymer corona. These dispersions can rapidly assemble at body temperature (37°C) into a 3D gel scaffold that can encapsulate and maintain cells, and revert to a free-flowing suspension when the temperature is reduced. An advantage of this
25 system is that no enzymatic treatment may be required, in order to detach the cells from the scaffold. However, rapid recovery of the cells from the suspension or scaffold may be difficult to achieve by physical separation techniques such as centrifugation. Also, there may be a risk of the cells being damaged if centrifugation were used to separate them from the suspension.

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In the context of growing cells for use in cell therapies or other biomedical applications, there remains a need for cell growth techniques that can produce a good yield of substantially undamaged cells and that can be scaled-up to produce relatively large quantities of cells in an efficient manner.

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A first aspect of the invention provides a reversible scaffold for growing cells or bacteria, the reversible scaffold comprising magnetic particles, e.g. magnetic polymer particles, wherein the magnetic particles exist in a liquid phase under a first set of conditions and in a semi-solid or solid phase under a second set of conditions, thereby
5 providing a scaffold for growing cells or bacteria, the reversible scaffold being capable of being repeatedly cycled from the liquid phase to the semi-solid or solid phase and back again by changing from the first set of conditions to the second set of conditions and back again.

10 A second aspect of the invention provides responsive particles, e.g. polymer particles, which are also magnetic. The particles may be thermoresponsive.

In an embodiment, the particles may each have a magnetic core or bulk phase and/or a responsive corona. The corona may comprise a thermoresponsive polymer or
15 copolymer.

In an embodiment, the responsive, e.g. thermoresponsive, corona may comprise a polymer that may be adsorbed on to a surface of the core or bulk phase.

20 In addition to or as an alternative to surface adsorption, the or a polymer may be physically attached and/or chemically bonded to the surface of the core or bulk phase by one or more of hydrophobic interaction, electrostatic or ionic bonding and covalent bonding, e.g. dative covalent bonding. In some embodiments, the or a polymer may be grown on and/or from the surface of the core or bulk phase.

25

The magnetic core or bulk phase may comprise a magnetic microparticle, e.g. a microsphere.

The magnetic core or bulk phase may comprise iron or an iron oxide such as
30 magnetite. The magnetic core may comprise nickel or cobalt.

The magnetic polymer particles may be ferromagnetic, paramagnetic or diamagnetic.

The thermoresponsive polymer may display a lower critical solution temperature, in
35 aqueous or non-aqueous media, that may be at least 10°C and/or may be up to 90°C.

A lower critical solution temperature may also be referred to as LCST or as an inverse-solubility temperature relationship. An LCST may be determined by heating at $1.0^{\circ}\text{C}\cdot\text{min}^{-1}$ in a Beckman DU-640 spectrophotometer, with the LCST being taken as the temperature where there is the onset of a sharp increase in absorbance at 550 nm.

The thermoresponsive polymer may be prepared by a method comprising: the use of one or more monomers selected from polymerisable alkylene glycol acrylate monomers and polymerisable alkylene glycol methacrylate monomers as monomers in a polymerisation reaction.

The method of preparation of the thermoresponsive polymer may involve the steps of: providing one or more monomers selected from polymerisable alkylene glycol acrylate monomers and polymerisable alkylene glycol methacrylate monomers; and carrying out polymerisation of the monomers to generate polymers with a lower critical solution temperature, in aqueous or non-aqueous media, of preferably from 10°C to 90°C .

The method of preparation may involve the use of polymerisable alkylene glycol acrylate and methacrylate monomers, either singly, or in various combinations, to generate polymers such that the lower critical solution temperatures in aqueous or non-aqueous media may be varied between 10°C and 90°C .

The polymerisable alkylene glycol acrylate monomers and polymerisable alkylene glycol methacrylate monomers may be selected from: di(ethylene glycol)-acrylate, oligo(ethylene glycol)-acrylate, poly(ethylene glycol)-acrylate, di(ethylene glycol)-methacrylate, oligo(ethylene glycol)-methacrylate, poly(ethylene glycol)-methacrylate, poly(propylene glycol)-acrylate, and poly(propylene glycol)-methacrylate, either singly, or in various combinations.

In one embodiment, two monomers may be used in combination, i.e. the polymer obtained may be a copolymer. It may be, for example, that the method involves the polymerisation of poly(ethylene glycol) methacrylate (PEGMA) and poly(propylene glycol) methacrylate (PPGMA). In one embodiment the PEGMA may have a number average molecular weight (M_n) of about 475 and the PPGMA may have a M_n of about 430. It may be that the PEGMA-PPGMA copolymer has a M_n of about 15,500.

Any polymerisation technique can be used in the method of the invention to generate the polymer materials. Typically, free-radical methods and controlled free-radical methods may be used, including, but not exclusively, atom transfer radical
5 polymerisation (ATRP).

The combinations of the monomer units in the generated thermoresponsive polymer can be random, or can be controlled, such that sequence specific block-co-polymers may be produced in any combination of monomers.

10

The choice of starting monomers (in particular their molar mass) and the polymerisation conditions can be controlled to vary the molar mass of the generated polymer. The molar masses of the generated polymers can be varied to be from 1 kDa up to over 1000 kDa. Preferably, for biomedical applications, the molar masses of the
15 polymers may be between 25-75 kDa with polydispersity (M_w/M_n) indices between 1 and 2.5.

The thermoresponsive polymers may hereafter be referred to as LCST polymers.

20 In some embodiments, the thermoresponsive polymers may be biocompatible amphiphilic copolymers that are chain-extended and exhibit high water solubility just below body temperature, but are insoluble at 37°C. Thus, these polymers can act to stabilise colloidal particles under the or a first set of conditions, but aggregate the particles under the or a second set of conditions.

25

The thermoresponsive polymers may be used, either singly or in combination, as surface-engineering surfactants during preparation of magnetic polymer particles by a method selected from emulsion methods, diffusion methods and evaporation methods.

30 Although the invention is mainly described in relation to the use of a temperature change to evoke a change in the support or scaffold, the support or scaffold may be responsive to changes in one or more conditions other than temperature and/or as well as temperature. For instance, the LCST of an LCST polymer can vary with other properties of the solution in which the particles reside. For example, LCST can be
35 affected by ionic strength, electric field, pH, solvent composition and any agents used

as co-solvents. Any factor that changes the lower critical solution temperature of the LCST polymers can be varied to cause a change in the steric stabilisation properties of the LCST polymers and therefore cause a change in stability of the magnetic polymer particles.

5

Thus, it can be envisaged, for example, that any change in solvent property, i.e. the ability of a liquid or liquid mixture to dissolve a solute, that affects lower critical solution temperature, could be used as a trigger to associate/disassociate the LCST polymers and their combinations with magnetic particles.

10

Thus, the invention may relate to magnetic particles, in particular magnetic polymer particles, that can reversibly associate from solution/suspension and their application in the biomedical field.

15 The magnetic particles may disperse with cells, or other biological materials such as bacteria, at temperatures below 37°C to form free-flowing suspensions, and form space-filling gels at body temperature that support cell growth.

Advantageously, the reversible scaffold of the first aspect of the invention and/or the
20 magnetic particles of the second aspect of the invention may be reusable. Thus, for instance, once cells or bacteria have been separated from the reversible scaffold or magnetic particles, the reversible scaffold or magnetic particles may be re-used, typically following sterilisation, to help grow a subsequent batch of cells or bacteria.

25 The composition and properties of the reversible scaffold or magnetic particles, e.g. of the magnetic bulk phase or core and the responsive corona, may be selected to suit the intended application.

In a third aspect, the invention provides a product which comprises the reversible
30 scaffold of the first aspect and/or the particles of the second aspect and biological material such as cells or bacteria.

Such a product may be a dispersion under the or a first set of conditions, e.g. below the lower critical solution temperature of the polymer particles. It may therefore be
35 initially provided as a free flowing suspension.

Such a product can be used to encapsulate the cells or other biological material under a second set of conditions, e.g. above the lower critical solution temperature, of the polymer particles. At such a temperature it may typically form a space filling gel or microgel that supports the growth of the cells or other biological material.

Preferably, the lower critical solution temperature of the thermoresponsive polymer may be just below body temperature such that the product is a dispersion at room temperature and a gel or microgel at body temperature.

10

Essentially, when cells are provided together with the particles, the particles can form a porous cocoon for the cells during cell growth; this will allow the generation of scaffold or matrix architectures that can promote cell-cell contact and may foster enhanced tissue generation.

15

The polymer particles of the invention can therefore be viewed as a colloidal cell delivery system.

The invention also provides, in a fourth aspect, the use of the polymer particles of the second aspect as a cell or bacteria support or scaffold.

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The invention also provides, in a fifth aspect, a cell or bacteria support or scaffold comprising the magnetic particles of the second aspect.

The invention also provides, in a sixth aspect, a free flowing suspension comprising (i) cells and (ii) the magnetic particles of the second aspect, at a temperature below 37°C.

25

The invention also provides, in a seventh aspect, a space filling gel or microgel that supports cell or bacteria growth comprising (i) cells or bacteria and (ii) the particles of the second aspect, at body temperature.

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An eighth aspect of the invention provides a method of production of cells or bacteria comprising:

providing a reversible scaffold for growing cells or bacteria, the reversible scaffold comprising magnetic particles, e.g. magnetic polymer particles, wherein the magnetic particles exist in a liquid phase under a first set of conditions and in a semi-solid or solid phase under a second set of conditions, thereby providing a scaffold for growing cells or bacteria, the reversible scaffold being capable of being repeatedly cycled from the liquid phase to the semi-solid or solid phase and back again by changing from the first set of conditions to the second set of conditions and back again;

providing seed cells or bacteria in the scaffold and promoting growth of further cells or bacteria within the scaffold.

The seed cells or bacteria may be provided whilst the magnetic particles are in liquid phase and/or they may be added to the scaffold once formed.

The method of the invention may further comprise the steps of:

changing from the second set of conditions to the first set of conditions, thereby providing a suspension or dispersion comprising the cells or bacteria and the magnetic particles; and

separating the cells or bacteria from the magnetic particles by applying an external magnetic field to the suspension or dispersion.

In an embodiment, the reversible scaffold may be thermoresponsive. The particles may exist in the semi-solid or solid phase at body temperature, i.e. approximately 37°C.

The first set of conditions may include a temperature below a lower critical solution temperature.

The second set of conditions may include a temperature above a lower critical solution temperature.

The lower critical solution temperature may be selected such that at body temperature, i.e. approximately 37°C, the particles exist in the semi-solid or solid phase.

In an embodiment, the lower critical solution temperature may be less than 37°C.

In an embodiment, the semi-solid or solid phase may comprise a space-filling gel or microgel.

5 In an embodiment where the particles are thermoresponsive, and cells or bacteria are introduced whilst the particles are in the liquid phase, the change to the semi-solid or solid phase may be achieved by placing the particles and cells or bacteria into a liquid medium which is at a temperature appropriate to caused scaffold formation. The particles and cells or bacteria may be dropped into a liquid medium at a temperature
10 appropriate to cause scaffold formation such that a scaffold in the form of a droplet is formed.

By starting with particles and cells or bacteria together in the liquid phase it is possible to produce patterned or layered structures which may have different particles,
15 and/or different cell types, and/or different bacteria in different places or layers in the structure.

There may be relative movement between the or a source of the external magnetic field and the suspension or dispersion. The source of the external magnetic field may
20 comprise an electromagnet.

In an embodiment, separating the cells or bacteria from the magnetic particles may be carried out by causing the suspension or dispersion to flow through the external magnetic field.

25 For instance, the suspension or dispersion may flow along a channel at least a portion of which is located close to the or a source of the external magnetic field. The channel may be configured such that the portion located close to the or a source of the external magnetic field is relatively large or long. Accordingly, a tortuous, e.g.
30 coiled, channel portion may be preferred.

The channel may comprise filtering means arranged to ensure that magnetic particles can be removed from the solution or dispersion as they flow through the external magnetic field.

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The external magnetic field may be controllable, e.g. the strength of the magnetic field may be variable, either manually and/or automatically. For instance, the external magnetic field may be controllable in accordance with a computer program and/or based on input data, which may include information on the magnetic content of the magnetic particles.

Advantageously, the method may be automated at least partially and/or may be substantially continuous.

For instance, the method may be controlled, e.g. in accordance with input data. The method may be controlled at least partially by a computer or programmable logic controller (PLC).

Advantageously, the method may give a better yield per surface area of support or scaffold in terms of cell quality and/or quantity than prior art methods.

A ninth aspect of the invention provides an apparatus for carrying out at least a portion of the invention according to the eighth aspect of the invention, wherein the apparatus includes a magnetic separation means configured to apply, in use, an external magnetic field to a suspension or dispersion comprising cells or bacteria and magnetic particles.

Advantageously, in some embodiments, the invention may provide magnetic polymer microparticles with surface-associated, e.g. adsorbed, thermo-responsive polymers, which assemble in the presence of cells at 37°C into cell-supporting or cell-encapsulating scaffolds that may support 2D or 3D cell growth, but which can be switched to a free-flowing particulate dispersion upon temperature modulation. For instance, the thermoresponsive polymers may be physically adsorbed on the surfaces of the magnetic polymer microparticles to form stabilised and free-flowing dispersions below the Lower Critical Solution Temperature (LCST) of the thermoresponsive polymer but transform reversibly within a short period of time to form a space-filling gel above the LCST. Upon cooling of the gel, the system may revert to a free-flowing dispersion and the stabilised polymer particles can be separated from the cell-polymer mixture via application of an external magnetic field, thus allowing the cells to be harvested and/or passaged. Advantageously, this process eliminates the need for

enzymatic treatments normally required for cell detachment. The person skilled in the art will appreciate that this combined thermoresponsive and magnetic, e.g. paramagnetic, polymer system may offer significant potential advantages for cell culture in 2D and 3D formats, for *in vitro* cell expansion and for larger-scale cell manufacturing. In particular, magnetic separation may be more reliable and/or quicker and/or more cost-effective and/or cleaner and/or simpler to operate and/or control and/or may cause considerably less, if any, damage to cells than other chemical and/or physical separation techniques.

Advantageously, the incorporation of a magnetic material such as magnetite within the thermoresponsive particles may serve to enhance the recovery of cells encapsulated/cultured within thermoresponsive particle gels. Typically, for instance, magnetic thermoresponsive particles of the invention may be separated away from the cells in a magnetic separator. In contrast, in conventional immunomagnetic separation (IMS), cells are typically separated with the magnetic particles.

The present invention may be particularly suited for potential applications in large scale cell population, culture and expansion. Typically, a thermoresponsive polymer may be physically adsorbed on to a surface of a polymer-based, e.g. polystyrene-based, magnetic microparticle, in order to yield temperature responsive dispersions that are also magnetic. For instance, polystyrene-based magnetic microparticles may be prepared or obtained commercially with various size and iron content specifications.

In some embodiments, one or more cytoadhesive moieties, e.g. peptides or sugars, may be provided on the magnetic particles and/or freely dispersed within a suspension or dispersion comprising the magnetic particles. For instance, cytoadhesive moieties may be provided on, e.g. attached or bonded to, the or a magnetic core or bulk phase and/or the or a responsive, e.g. thermoresponsive, polymer.

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The materials system described herein may be fully reversible, e.g. temperature modulation may trigger assembly into and disassembly of a 2D or 3D support or scaffold. A further advantage is the ability to separate the particles in a non-invasive and enzyme-free manner from cells or bacteria by applying an external magnetic field,

thereby allowing for cell or bacteria recovery on demand. Moreover, the cells or bacteria typically may not be damaged by the separation process.

5 In some embodiments, the invention may relate to the use of magnetic polymer particles that can reversibly assemble into 2D or 3D aggregates (viscoelastic materials) using simple modulation steps, e.g. by modulating temperature, and their application in the biomedical field. The invention may further comprise the preparation and application of new surface-functional polymeric colloidal particles with embedded inorganic components that bestow magnetic properties in addition to
10 the reversible association of the particles as a consequence of their surface-functional polymer layer. These materials may be especially suitable for application in 2D and 3D cell culture for routine cell processing (such as cycles of passaging and splitting) or as injectable cell support matrices to facilitate cell growth and proliferation.

15 Advantageously, the responsive, e.g. thermoresponsive, gels may be used for scale up and manufacturing of different cell or bacteria types in both 2D and 3D formats or for generation of 3D models of particular tissues or organs.

In some embodiments, the polymer particles which can have a bulk phase or core of
20 any organic-magnetic composition may have the form of a core-shell or core-corona architecture, or may have the form of homogeneous or heterogeneous mixtures, or of layer-by-layer or surface-coated particles.

The organic, typically polymeric, compositions may comprise biodegradable or non-
25 degradable polymers including, but not limited to: polystyrene, polystyrene sulfonate, polycaprolactone, polylactic-co-glycolic acid, polylactic acid and hydrophobic polyesters.

The magnetic polymer particles may be prepared by methods that include but are not
30 limited to: emulsion techniques, emulsion polymerisation techniques, suspension techniques, seeding polymerisation and spray drying.

The size, e.g. diameter, of these particles may be at least 100 nm, e.g. at least 500 nm and/or up to 10 μm , e.g. up to 5 μm . The magnetic content of the particles may be any
35 weight or molar percentage. For instance, the magnetic core or bulk phase, e.g.

magnetic microparticles, may have a magnetic content of up to 50 wt %, e.g. at least 5 wt% and/or up to 30 wt%, preferably at least 10 wt% and/or up to 20 wt%.

5 The thermoresponsive polymer or copolymers may be biocompatible and/or bioresorbable and/or biodegradable, if or as required by their application. The invention may involve the synthesis and application of biocompatible amphiphilic co-polymers that are chain-extended and exhibit high water solubility just below body temperature, but are insoluble at 37°C. As a consequence of these characteristics, these polymers can act to stabilise magnetic colloidal particles under a first set of
10 conditions, but aggregate the particles under a second set of conditions. The particles can form free-flowing suspensions below the LCST, but can reversibly form porous space-filling gels at or above the LCST as a result of chain collapse of the co-polymer corona. The LCST is typically below body temperature, i.e. below approximately 37°C. These particles can therefore be mixed with biological materials at less than the
15 LCST, and then can form into stable encapsulating gels at body temperature; these stable encapsulating gels can support cell growth within the gel. If the temperature is reduced to below the LCST, the gel liquefies and the polymer particles can easily be separated from the cells by a magnetic separation stage, without the necessity for enzymatic cell processes or any other chemical treatment that is normally utilized for
20 cell detachment from supporting substrates.

Advantageously, this materials system may be used to support cell attachment and proliferation without the use of specific cell adhesive motifs or ligands, but alternatively these ligands can be conjugated to the particle surfaces or side chains,
25 termini of the thermo-responsive polymer or physically mixed with main components to enhance cell attachment if required. The combination of the ease of preparation, potential for scale-up, and the wide variations possible in the co-polymer corona layer due to controlled synthesis indicates that these systems may be suitable for use as a new class of biological delivery agent and as a support for tissue, e.g. organ, growth.

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The present invention may provide, inter alia, dual-responsive (e.g. magnetic and thermoresponsive) smart particles, the process by which these materials can be produced and their use in biological applications, in particular a support or scaffold for growing cells or bacteria. The invention may also provide apparatus for use in at

least a portion of a method of growth of biological material, e.g. cells or bacteria, disclosed herein.

The non-limiting figures, which illustrate the invention, are as follows:

5

Figures 1A, 1B, 1C and 1D show a schematic illustration of the adsorption of a thermoresponsive polymer on to a magnetic microsphere;

Figures 2A, 2B, 2C and 2D schematically illustrate a method of cell growth in accordance with the invention;

10 Figure 2E is a schematic representation of cell expansion *in vitro* showing cell proliferation, trypsin-free passaging and cell recovery on a 3D thermoresponsive and magnetic colloidal gel;

Figures 3A, 3B, 3C and 3D relate to the preparation of particles according to the invention and characterisation of the properties of the particles;

15 Figures 4A, 4B and 4C are graphs showing the reversible behaviour of a thermoresponsive, magnetic scaffold according to the invention;

Figures 5A, 5B, 5C, 5D, 5E and 5F illustrate cell recovery in accordance with the invention;

20 Figures 6A and 6B include SEM micrographs showing *in vitro* cell processing of NIH3T3 cells seeded on a 2D thermoresponsive magnetic particle gel according to the invention;

Figures 7A and 7B demonstrate the proliferation and temperature induced subculture of cells on thermoresponsive magnetic microspheres;

25 Figure 8 is a schematic presentation of the formation of thermoreversible magnetic droplets for use as a 3D culture substrate;

Figure 9 illustrates cellular proliferation on thermoreversible magnetic droplets;

Figure 10 includes SEM micrographs showing the proliferation and morphology of cells on thermoreversible magnetic droplets;

30 Figure 11 is a schematic diagram of a magnetic separator for use in cell recovery in accordance with the invention; and

Figure 12 includes SEM micrographs of (a) SEM of polystyrene microspheres obtained by non-aqueous dispersion polymerisation, (b) SEM of magnetic polystyrene microspheres after impregnation with Fe₃O₄ particles in chloroform, (c) ESEM of colloidal gel particles of polystyrene (33%w/v and 3w/v DD-pMEO2MA) assembled at 37°C, (d) ESEM of gel disassembled by cooling to below LCST.

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Figures 1A, 1B, 1C and 1D schematically illustrate the concept underlying physical adsorption of the polymer on the surface of microparticles to provide steric stabilisation.

5

Referring to Figure 1A, there is shown a magnetic polystyrene microsphere 1 and a thermoresponsive polymer, pMEO₂MA, 2. Figure 1B shows a plurality of magnetic particles 3, only one of which is labelled for clarity. Each magnetic particle 3 comprises a core comprising a magnetic polystyrene microsphere 1 and a corona comprising the thermoresponsive polymer 2. Figure 1C shows a suspension of the magnetic particles 3 mixed with cells 4 at a temperature below the LCST of the thermoresponsive polymer. Figure 1D illustrates a microgel scaffold which forms above the LCST of the thermoresponsive polymer, in which the cells 4 are encapsulated by the assembled magnetic particles 3. Modulating the temperature with respect to the LCST causes the scaffold to change reversibly from the microgel shown in Figure 1D to the suspension shown in Figure 1C and vice versa.

Figure 2A shows a container 5 containing a suspension 6 of the cells 4 and the magnetic particles 3 below the LCST. Figure 2B shows the container 5 containing a microgel 7, in which the cells 4 are encapsulated by the assembled magnetic particles 3. Modulating the temperature with respect to the LCST can cause the scaffold to change reversibly from the microgel of Figure 2B to the suspension of Figure 2A and vice versa.

Further cells 4 may be grown, e.g. by exposing or immersing the scaffold within a nutrient medium (not shown). After a period, typically a predetermined period, of cell growth, the temperature is reduced to below the LCST, thereby providing a suspension containing the cells and the magnetic particles. After a further period, typically of around five minutes, as illustrated by Figure 2C, an external magnetic field 8 is applied to the suspension within the container 5 to separate the magnetic particles 3 from the cells 4. As illustrated in Figure 2D, the cells may then be harvested and split for passaging, e.g. into the container 5 and one or more further containers 5'.

Figure 2E shows an alternative schematic representation of cell expansion in vitro showing cell proliferation, trypsin-free passaging and cell recovery on 3D

thermoresponsive and magnetic colloidal gel. (1) Magnetic polystyrene microspheres (33%w/v) re-suspended in DMEM containing DD-5 pMEO2MA (3%w/v) at below LCST temperature ($< 37^{\circ}\text{C}$); (2) gel deposited by dispensing the colloidal suspension into tissue culture plate which quickly assemble to form gel at 37°C , (3) cells seeded in media suspension on the top of formed gel at 37°C and (4) incubated to allow cell proliferation, (5) colloidal gel disassembled rapidly by temperature reduction to obtain cell-particle suspension followed by subculture of the cell particles suspension on fresh gel, (6) cells separated from colloidal particles by using external magnetic separator.

The thermoresponsive, magnetic cell culture systems were prepared from a combination of PEG-based thermoresponsive polymers, copolymers and magnetic microspheres. Examples of monomers used to prepare thermoresponsive polymers are poly (ethyleneglycol) ethyl ether methacrylate (polyPEGMA₂₄₆-EE, PEGMA), 2-(2-Methoxyethoxy) ethyl methacrylate (pMEO₂MA) and poly (ethyleneglycol) methyl ether methacrylate (PEGMA₂₄₆-EE-co-PEGMA₄₇₅-ME), used at various feed ratios. Conventional free-radical polymerisation (FRP) and controlled living polymerisation routes (Reversible addition-fragmentation chain transfer (RAFT) and Atom Transfer Radical Polymerisation (ATRP)) were utilised to generate homopolymers and copolymers that exhibited Lower Critical Solution Temperatures (LCSTs) both in aqueous media (e.g. phosphate buffered saline (PBS) at pH 7.4) and in cell culture media. Preferably, the polymers may provide steric stabilisation when mixed with the microparticles below the LCST, assemble to form a stable gel with the particles above the LCST, but liquefy upon simple temperature modulation.

Examples of polymers and copolymers which exhibit LCST just below body temperature in distilled water (dH₂O) and Dulbecco Modified Eagles Medium (DMEM) and which can reversibly form a thermoresponsive gel or microgel are given in Table 1 below:

Composition of polymer and copolymer	Mn	PDI	LCST/°C (dH ₂ O)	LCST/°C (DMEM)
DDT-PEGMA-EE ₂₄₆ (100%)	23,170	1.8	21	18
DDT-PMEO ₂ MA (100%)	18,500	1.8	22	19
HOOC-t-PMEO ₂ MA (100%)	20,000	1.7	25	23
PEGMA-EE ₂₄₆ co-PEGMA-ME ₄₇₅ (93.55: 6.45%)	22,020	1.7	25	22

Table 1

With reference to Table 1, polymerisation of polyPEGMA246-EE and PEGMA246-EE-co-PEGMA475-ME was carried out via conventional free-radical polymerisation (FRP). For FRP, AIBN was used as initiator, AIBN/monomer (weight ration) = 0.5%, monomer/solvent (v/v) = 1:1. The solvent was butanone and the reaction conditions were 70°C for 1 to 24 hours.

The magnetic microspheres were prepared by conventional techniques such as single oil in water (o/w) emulsion and emulsion polymerisation technique. Alternatively or additionally, other magnetic microspheres may be purchased directly from commercial suppliers in various diameter sizes, preferably within the low-micron size ranges. Different magnetic contents of the microparticles can be obtained either via the above-mentioned techniques or as purchased from commercial sources. The particles were used as surface unmodified or modified. For instance, the particles may be surface modified with different polymers and stabilisers using surface adsorption techniques or plasma surface polymerisation process or surface engineering techniques. The core of magnetic microspheres can be chosen from a range of polymers, depending, for example, on whether end-use in cell culture or *in vivo* is required. Examples include hydrophilic, hydrophobic or amphiphilic polymers such as polystyrene sulfonate, poly lactic-co-glycolic acid, poly lactic acid, poly caprolactone, poly methylmethacrylate, polystyrene and other related polymers.

Figure 3A, 3B, 3C and 3D illustrate an example of the synthesis and properties of thermoresponsive magnetic microparticles according to the invention.

Referring to Figures 3A and 3B, PEG-based monomers are polymerised via free-radical polymerisation (FRP) techniques to form DDT-PEGMA polymer 2'.

Magnetic polymer particles 3' comprising magnetic polystyrene microspheres 1' and the DDT-PEGMA polymer 2' may be prepared as follows. A solution of 2-3 wt% DDT-PEGMA polymer 2' in water (or DMEM) is added to magnetic polystyrene microspheres 1' (33wt %) and vortexed to generate a stable suspension 6'. Figure 3B illustrates cycles of heating-cooling showing transformation of the particle-polymer suspension 6' in a tube 9 from liquid in the left-hand image, to a gel 7' in the middle image and back to a suspension 6' in the right-hand image. In the middle and right-hand images the tube 9 is inverted, as compared with the left-hand image.

Figure 3C is a graph showing % transmission at 550 nm on the y-axis plotted against temperature on the x-axis for 3 mg.ml⁻¹ of DDT-pMEO₂MA in DMEM. The graph illustrates the transformation from coils to globules, i.e. suspension to a gel, and it also indicates the LCST for the polymer.

Figure 3D is an SEM micrograph, which indicates spherical particles of low polydispersity.

Preparation of Thermoresponsive Magnetic Microparticle Gel

Method A:

The thermoresponsive magnetic microsphere suspension was prepared by mixing the two components of thermoresponsive polymer with magnetic microspheres in various weight ratios. Example of these ratios may range from 0.1 to 10 wt %, e.g. from 0.1 to 6 wt%, and from 15 to 50 wt%, e.g. from 19 to 45 wt%, for polymer and microparticles respectively. The polymer and microparticles solutions were prepared separately in predetermined concentrations and then mixed together or the thermoresponsive polymer can be prepared first and then added to the microparticles or vice versa.

Tube inversion assay, e.g. as shown in Figure 3B, confirmed the formation of space-filling particle gel from free-flowing particle suspension upon heating above the LCST and inverting back to its liquid state upon cooling to below the LCST. This thermogelation was observed for the various combinations of ratios of the polymer and microparticles.

Method B:

The thermoresponsive magnetic microsphere suspensions were prepared by re-suspending magnetic microspheres, surface engineered with thermoresponsive polymers, at predetermined concentrations, using emulsion techniques or emulsion
5 polymerisation techniques. The gel formation experiment (tube inversion assay) was carried out as described above in Method A with reference to Figure 3B.

Method C

The gel-forming particle dispersion was obtained by mixing pre-determined weight
10 ratios of magnetic polystyrene microspheres with DD-pMEO₂MA polymer dissolved in DMEM at temperatures below the LCST of DD-pMEO₂MA (~ 20 °C). Adsorption of the DD-pMEO₂MA polymer at the surface of the magnetic polystyrene microspheres at these temperatures produced a stable colloidal suspension. However when the suspension was heated to a temperature above the LCST (i.e. 37 °C), chain
15 collapse of the DD-pMEO₂MA polymer and subsequent loss of steric repulsion between the coronae of the coated microspheres caused a particle gel to form as colloidal stability was lost.

A tube inversion assay (identical results to Figure 3B.) was used to show how a
20 dispersion containing 33%w/v magnetic polystyrene microspheres and 3%w/v DD-pMEO₂MA polymer (dissolved in DMEM) transformed reversibly from a free-flowing suspension to a self-supporting gel within two minutes incubation at 37 °C. Environmental Scanning Electron Microscopy (ESEM), (Figure 12c) showed the morphology of the gel at 37 °C and also the formation of a continuous network of
25 flocculated polystyrene microspheres.

Temperature dependent rheological properties of the magnetic particle gels

The temperature dependent gelation of the components was further confirmed by rheological analysis where an oscillatory strain amplitude sweep experiment carried
30 out on a gel composed of 33% wt/v magnetic polystyrene microspheres particles and 3% w/v DD-pMEO₂MA showed that G' (the storage modulus) was higher than G'' (loss modulus) at 37°C, and on examination of the data in (Figure 4c) shows that $\tan \delta$ ($= G''/G'$) remains less zero at all of the strains measured, the gel remains gel-like at high strains at 37 °C, consequently, the particle gel showed remarkably good ductility.

Figures 4A and 4B are graphs showing an example of multiple heating-cooling cycles of a suspension containing 33wt% magnetic polystyrene microspheres and 3 wt% thermoresponsive polymer. Figure 4B shows the temperature profile of the heating-cooling cycles. In each heating-cooling cycle, the temperature was increased sharply from 10°C to 37°C, where it was held for a while before being reduced fairly sharply to 10°C.

The magnetic particle gels could be taken through multiple cycles of fluid-gel transitions and a full temperature-triggered switch between the two states was observed over very short time scales. As shown in 4A, which shows storage modulus G' and loss modulus G'' plotted as a function of time, at 10°C the material behaved as a fluid with $G' < G''$. However, G' and G'' increased rapidly when the temperature was raised to 37°C. At this temperature, the aggregated particles formed continuous elastic networks, giving a space-filling gel as indicated by G' becoming higher than G'' .

Lowering the temperature back to 10°C led to a significant drop in G' and G'' back to their initial values. G'' became eventually higher than G' , indicating full reversibility of gelation into a liquid state on cooling. The heating-cooling cycle was repeated over at least 3 cycles, and a similar dependence of the storage and loss moduli on temperature was observed in each case.

Recovery of Cells Mixed with Magnetic Particles

NIH3T3 cells were added to magnetic polystyrene microparticles suspended in nutrient media (DMEM). Cells were successfully recovered and were quantified by MTS assay. Optical microscope images of recovered cells showed that they maintained their characteristic morphology.

Cell Recovery via Magnetic Separation

NIH3T3 cells were encapsulated within thermoresponsive magnetic particle gels composed of magnetic polystyrene particles (33 wt %) and PEGMA polymers (2 wt %). Figure 5A shows such a gel within a vial. Following a brief (2 minutes) cell encapsulation at 37°C, the gels were liquefied and diluted. The cells were recovered from the suspension of magnetic particles in a MACS separator (not shown).

As shown in the right-hand graph in Figure 5B, as compared with the control (cells alone), a total of 84% of the encapsulated cells were successfully recovered after magnetic separation of the particles.

- 5 The left-hand graph in Figure 5B illustrates the situation when the cells are separated from the suspension without having been put through a heating-cooling cycle, i.e. without having been encapsulated in a gel. Under these conditions, as compared with the control, 99% of the cells were successfully recovered after magnetic separation of the particles.

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Moreover, in both cases, the recovered cells maintained their viability and normal characteristic morphology, as can be seen from the optical microscope images shown in Figure 5C – the left-hand micrograph relates to the left-hand graph in Figure 5B and the right-hand micrograph relates to the right-hand graph in Figure 5B.

15

- Further experimental data to demonstrate efficient cell recovery is provided in Figures 5D, 5E and 5F. In these experiments NIH3T3 cells were encapsulated within thermoresponsive magnetic particle gels composed of magnetic PS particles (33wt %) and PEGMA polymers (2wt %). Following 24 hours cell encapsulation at 37°C, the gels were liquefied and diluted. The cells were recovered from the suspension of magnetic bar. As it shown in Figure 5D, left, as compared to the control (0 hours), a total of 87% of the encapsulated cells were successfully recovered after magnetic separation of the particles. Figures 5E and 5F demonstrate further the process of magnetic extraction and recovery of cells in cell culture media using the method of the invention. More specifically, in Figure 5E the concept of gel liquefying and cell recovery via magnetic separation is illustrated, and in Figure 5F the concept illustrated in Figure 5E is shown to occur in an experimental environment in less than 1 minute.

30 Cell seeding on 2D thermoresponsive magnetic gel

Figures 6A and 6B include SEM micrographs showing *in vitro* cell processing of NIH3T3 cells seeded on a 2D thermoresponsive magnetic particle gel.

- In Figure 6A, cells are seeded on tissue culture treated plate (negative control) in the left-hand image, cells are seeded on 2D particle gel using carboxyl terminated

thermoresponsive polymer in the middle image and cells treated with triton (positive control) are seeded on 2D particle gel in the left-hand image. In Figure 6B, cells are seeded on tissue treated plate (negative control) in the left-hand image and cells are seeded on 2D particle gel using DDT-terminated thermoresponsive polymer in the middle and right-hand images. In all of the images in Figures 6A and 6B, the scale bar is 100µm.

Figure 7A demonstrates the proliferation and temperature induced subculture of 3T3 fibroblast cells on the particle gels of the invention compared to tissue culture plate surfaces versus tissue culture plate (normalised to initial cell seeding density). Figure 7B illustrates the re-proliferation of 3T3 fibroblasts on the surfaces of particle gels after single temperature induced subculture (that is to disassemble parent gel to liquid and re-assemble on the top of second gel to achieve subculture of proliferated cells), (normalised to initial cell seeding). The results demonstrate that cells will readily populate and proliferate on particles and gels of the invention.

Formulation of adroplet cell culture matrix for 3D ex vivo cell culture expansion

Cells were mixed with thermoreversible and magnetic particular gel in liquid form (below lower critical solution temperature of the polymer). The suspension was then dispensed into pre-warmed (37 °C) media in a dropwise dispensing manner to form spherical droplets instantly (Figure 8).

Preparation of thermoreversible and magnetic 3D droplet as cell culture substrate

Method A:

A thermoreversible and magnetic 3D droplet was prepared by dissolving a thermoreversible polymer in cell culture media (4%w/v) mixed with magnetic or non-magnetic microspheres in various ratios ranging (0.1-6 wt %) and (19- 45 wt %) respectively. The particulate dispersion was then dispensed in different amounts (ranging from 10ul – 500ul volume, but not limited to) into pre-warmed cell culture media to form different droplet sizes. The droplets were instantly formed.

Method B:

A thermoreversible and magnetic 3D droplet was prepared by dissolving a thermoreversible polymer in cell culture media (4%w/v) mixed with magnetic or non-magnetic microspheres in various ratios ranging (0.1-6 wt %) and (19- 45 wt %)

respectively. A known amount of live cells were pre-mixed with the particulate dispersion at or below the LCST of the polymer. The particulate dispersion was then dispensed in different amounts (ranging from 10ul – 500ul volume, but not limited to) into pre-warmed cell culture media to form different droplet sizes. The droplets were
5 instantly formed and incubated for different amounts of time.

The skilled man would readily appreciate that the method of A and B could be used to produce not only droplets but also other shapes, for example, a disk, a patterned surface or a multilayered structure.
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Cellular Proliferation of immortalised Human Mesenchymal Stem Cells (ihMSCs) on 3D thermoreversible magnetic droplets

Figure 9 demonstrates the efficient proliferation of ihMSCs on 3D droplets according to the invention. Several cell seeding densities were investigated for up to 5 days. Cell
15 proliferation was determined using PrestoBlue™ assay (cell viability assay) according to standard manufacture protocol.

Figure 10 shows microscopic images showing morphology and proliferation of GFP labelled immortalised Human Mesenchymal Stem Cells (ihMSCs) on 3D
20 thermoreversible magnetic droplets of the invention. Cells were seeded at 100×10^3 per 100ul gel and the cells morphology monitored up to day 6. Scale bar is 100um. Particle gel shown in light brown colour overlayed with GFP cells (left), GFP labelled cells (Right). Cells are proliferating throughout the gel and to have adopted an elongated morphology in day 6 compared to day 3, indicating cell migration and
25 interaction with the 3D droplet substrate.

Advantageously, the magnetic particle gel may be simple to prepare in different ratios and compositions, may typically exhibit reversible, repeatable cycles of heating and cooling, and may allow for magnetic separation of particles from the residing medium.
30 This type of particle gel may offer many potential advantages for application in *in vitro* cell processing and cell manufacturing systems. The invention typically may not require the use of enzymes and chemical treatments normally used during cell manufacturing and processing which otherwise can decrease the quality of the cells produced for clinical application. Imparting magnetic properties combined with
35 reversible, e.g. thermoreversible, scaffold or support formation may allow for easy

cell processing and cell separation. The modular construction of the components may enable flexibility to adapt for different type of biological material, e.g. cells and/or bacteria, and different quantities (e.g. smaller quantities for basic research or larger quantities for an industrial manufacturing process), as well as growing biological material both in 2D and 3D format.

Materials

All solvents and reagents were of analytical or HPLC grade and purchased from Fisher Scientific unless otherwise stated. Deuterated solvents were purchased from Sigma. Polyethylene glycol ethyl ether methacrylate (PEGMA-EE 246, Mn 246), di (ethylene glycol) methyl ether methacrylate (pMEO2MA₁₈₈), 1-dodecanethiol (98%) styrene and divinylbenzene (DVB) was purchased from Sigma Aldrich and purified before use by passing through a column filled with neutral alumina. All these treated monomers were stored in a refrigerator prior to use. Polyvinylpyrrolidone (PVPK-30, Mw=40 000) and 4, 4'-Azobis 12 (4-cyanovaleric acid) (v501) were purchased from Sigma. Ferrous chloride tetrahydrate (FeCl₂.4H₂O, 98%), ferric chloride hexahydrate (FeCl₃.6H₂O, 99%), and ammonium hydroxide solution (30-33% in water) were purchased from Sigma Aldrich, and oleic acid (97%) from Tokyo Chemical Industry (TCI, Japan) were used for the preparation of magnetite nanoparticles. Magnetic microparticles (Fluka), of 5µm size, based on polystyrene (PS) and containing 20%w/w iron oxide were purchased from Sigma-Aldrich, UK. The purchased magnetic PS particle suspensions were diluted and taken through multiple centrifugation/re-dispersion washes with deionised water to remove any potential contaminants (e.g. surfactants). The particles were then lyophilised and characterised by SEM. Dulbecco Modified Eagles Medium (DMEM) was purchased from Invitrogen, UK.

Methods

Synthesis of Magnetic Polystyrene Microspheres

Synthesis of polystyrene Microspheres

Polystyrene microspheres were prepared by a non-aqueous dispersion polymerisation technique. In a 500 ml two neck round bottom flask equipped with a reflux condenser, ethanol (160g), water (18g) and PVP (3g) were added and stirred until the PVP completely dissolved. To this mixture styrene (10g, 96 mmol) and DVB (0.5g, 3.9 mmol) were added and stirred at 100 rpm for 0.5 h with nitrogen purging. The temperature was then elevated to 74 °C and (0.4 g, 2.4 mmol) of AIBN was added to

the reaction vessel and stirred for a further 24 hours at 120 rpm. The polystyrene microspheres were separated by centrifugation (3000 xG for 10 mins) and repeatedly washed by resuspension and centrifugation steps with distilled water. The resulting microspheres were dried overnight at 60 °C.

5

Synthesis of oleate coated magnetic nanoparticles

The magnetite nanoparticles were prepared by a coprecipitation method using iron chloride salts ($\text{Fe}^{2+}/\text{Fe}^{3+}$). $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (1.80 M) and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (1.20 M) were dissolved in degassed distilled water. Under a nitrogen atmosphere with vigorous mechanical stirring, 8 the mixture was heated to 80 °C. To this, 40 ml of ammonium hydroxide solution (25% in water) were rapidly added and reaction conditions were maintained for a further 30 minutes. To the magnetic precipitate (Fe_3O_4), oleic acid (15 mL) was added drop wise and the mixture heated up for a further 30 minutes at 70 °C degrees. At this time a deep black coagulate was observed indicating successful coating of the (Fe_3O_4) particles with oleic acid. The formed coated particles were separated by a magnetic decantation procedure before being washed extensively with a mixture of distilled water and acetone (1:1, v/v). The resultant particles were finally dried in vacuum desiccators for 48 h. The oleate coated magnetic nanoparticles was characterized by TEM imaging and used to analyse the diameter size using ImageJ software (1.44p).

20

Preparation of magnetic polystyrene-coated particles

Magnetic polystyrene microspheres were prepared by swelling/de-swelling steps in chloroform. Briefly, oleate coated magnetic nanoparticles (2 g) were re-suspended in chloroform (100ml) in a 500 ml round bottom flask. Polystyrene microspheres (10 g) were added to the above suspension, mixed, sonicated and stirred overnight at 165 rpm. The resulting magnetic polystyrene microspheres were filtered through a 1 μm pore glass mmicrofiber filter membrane (Whatman) and washed twice with 100 ml each of hexane and ethanol. These magnetic microspheres were left to dry in desiccators at room temperature for 48 hours.

30

Preparation of Thermoresponsive Polymer (example of)

Polymer synthesis was carried out by free radical routes, generally as previously reported by R. Cheikh et al Soft Matter 2010, 6, 5037. Briefly, PEGMA ($M_n = 246$, 40 mmoles), 1-dodecanethiol (0.25 mmoles) and AIBN (0.3 mmoles) were charged into a

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Schlenk flask containing butanone (15 ml) and the mixture was degassed with argon for 15 minutes. The polymerisation was conducted at 70°C for one hour and the polymer was recovered by precipitation in excess of hexane. The purified polymer was characterised by gel permeation chromatography (GPC) and nuclear magnetic resonance (NMR).

Determination of Lower Critical Solution Temperatures (LCST)

PEGMA based polymer solutions in PBS (pH 7.4, 3 mg.mL⁻¹) were heated at 1.0°C.min⁻¹ in a Beckman DU-640 spectrophotometer. The LCSTs of the copolymer systems were considered to be indicated by the onset of a sharp increase in absorbance at 550 nm.

Synthesis of Dodecane-pMEO₂MA polymer (DD-pMEO₂MA)

Polymer synthesis was carried out by free radical routes as previously reported. Monomer MEO₂MA (Mn= 188, 54 mmols), 1-dodecanethiol (0.40 mmols) and V501 (0.40 mmols) were charged into a 100 ml round bottom flask attached to a Schlenk line containing butanone (25 ml) and the mixture was degassed with argon for 15 minutes. The polymerisation was initiated by heating to 70 °C and maintained for two hours. The polymer was recovered by precipitation in excess hexane.

Gel Permeation Chromatography

Number average molecular weight (Mn), weight average molecular weight (Mw) and polydispersity (Mw/Mn) were obtained by Gel Permeation Chromatography (PL-120 and PL-50 Polymer Labs) with triple detection. The columns (30 cm PLgel Mixed-C, 2 in series) were eluted with THF and calibrated with polystyrene standards. All calibration and analysis were performed at 40°C and a flow rate of 1mL/min: under these conditions all of the products were fully dissolved in THF, and passed through 0.2 µm filter before injection with little or no backpressure observed.

Particle Size Measurements

Micelle and microparticle sizes and distributions were measured using Malvern Zetasizer Nano and Coulter LS 230 (Beckman Coulter) instruments. For dynamic light scattering experiments, PEGMA-co-PPGMA solutions (1mg.mL⁻¹) were prepared in distilled water and filtered prior to measurement using a 0.45µm disposable filter (PTPE Acrodisc CR) in a 12.5×12.5 mm polystyrene disposable cuvette. For

microparticle size measurements with the Coulter LS230, samples were resuspended in distilled water at an obscuration value between 8-12%.

Scanning Electron Microscopy

5 Scanning electron micrographs were recorded using a JEOL JSM-6060LV instrument. Polymer microparticles were placed on platinum stubs and sputter coated for 4 minutes, using a complex rotating planetary motion to allow irregular surfaces to be uniformly coated.

10 *Preparation of magnetic particle sol-gel system*

Particle suspensions with temperature dependent sol-gel properties were prepared by mixing ppHex-modified polystyrene magnetic microparticles with polyPEGMA246-EE in DMEM at fixed concentration of (33 wt %) and (3 wt %) respectively. Temperature induced gelation was confirmed by both simple tube inversion and rheological
15 methods.

Alternatively preparation of the particle dispersion was carried out as previously reported. A stock solution of DD-pMEO₂MA (3% w/v) was prepared in supplemented DMEM and refrigerated before use. Dried magnetic polystyrene microspheres (1g)
20 were dispersed in 15 ml of the prepared chilled polymer solution to give a concentration of particles in suspension of 33% (w/v). The above dispersion was vortexed for 5 minutes and stored in a refrigerator.

Mechanical Properties

25 Sample suspensions/gels containing 33wt% magnetic PS particles and 3wt% polyPEGMA246-EE were characterised by rheology. The tests were performed in an Anton Paar Physica MCR 301 rheometer, with a 25mm diameter serrated parallel plate at 0.6mm gap. To minimise solvent evaporation a solvent trap was used and the external chamber was filled with water. The gels were probed using the following two
30 oscillatory tests:

Strain amplitude sweeps were performed between 1 and 100% strain amplitude (at an angular frequency ω of 10 rad/sec) to determine the linear viscoelastic region of the gel at 37°C.

Heating-cooling cycles: an oscillatory rheology test was conducted on a sample of magnetic particle gel to determine the effects of temperature on the rheological properties of these materials. The gel was subjected to 3 cycles of consecutive heating to 37°C followed by cooling to 10°C, under an oscillatory strain of 0.01% applied at an angular frequency ω of 10 rad/sec, while the G' (storage) and G'' (loss) moduli were being recorded.

Cell Culture

NIH3T3 cells were cultured and maintained as monolayers in T-flasks (Nunc, Denmark), using Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin–streptomycin–amphotericin B solution (referred to as DMEM). Cell lines were maintained in a humidified incubator at 37°C and with 5% CO₂

Cell proliferation on magnetic polystyrene / DD-pMEO₂MA gel

Aliquots of particle suspensions (160 μ l) at 4 °C as prepared above were added into wells of non-tissue culture treated well plates which had been pre-chilled to 4 °C. The plates were then placed in the incubator at 37 °C for 10 minutes to form the gelled particle dispersion. To the above gel, aliquots containing 4×10^5 cells (200 μ l) in DMEM were seeded to the top of the gel. After 0.5 hours, 800 μ l of pre-warmed (37 °C) DMEM complete media was added to the top of the gel and the seeded gels were returned to the incubator. Cell proliferation was monitored via a PrestoBlue assay according to the standard manufacture's protocol. Cell viability was measured against a validated standard calibration curve constructed using cells seeded on to particle gels. Cell proliferation on the gel was also compared against cells seeded directly onto tissue culture plastic.

Cell subculture on magnetic polystyrene / DD-pMEO₂MA gel

Cell subculture and expansion were carried out on a particle gel via the temperature modulation procedure. Briefly, cells were maintained on the particle gel using the above protocol for three days, after which the time the gel was liquefied and centrifuged at 200xG for 5 minutes. The resulting particle and cell pellets were re-suspended in DMEM medium (150 μ l), split into three equal volumes (50 μ l) and added on the top of newly formed gels. These gels were then incubated at 37 °C for a further 48 hours. Cell proliferation was then monitored again via the PrestoBlue assay

as described above. It is stressed that this new methodology did not involve the use of Trypsin.

Cell recovery via magnetic separation

5 Over the series of gel formation, cell expansion and gel disassembly, cells were recovered from the polystyrene microspheres using magnetic separation. The gels were liquefied at different time intervals via temperature reduction, the suspensions containing cells were diluted with pre-chilled PBS (5 ml) and exposed to a magnetic separator to remove the magnetic particle fraction. The obtained supernatants
10 containing the cells alone were then centrifuged at 200 x G for 5 minutes. The cells recovered at this stage were counted via a haemocytometer.

Morphology and Cytotoxicity assay

The cells recovered from the magnetic particle gels were seeded on tissue culture
15 plates and left to attach for 3 hours. These were imaged using Nikon Eclipse TS100 microscope (Nikon, UK) to assess their morphology. MTS assay (Promega, UK) was performed on the recovered cell versus the control (cells 2X10⁵ seeded on tissue culture plastic without pre-manipulations). The efficiency of the cell recovery process was calculated from the ratio of MTS readings from the recovered and the control
20 cells.

It will be appreciated that the behaviour under different conditions of the materials systems of the invention may be characterised, predicted, controlled and tuned. Thus, for example, it may be relatively simple to determine under which set of conditions
25 the reversible scaffold exists in a liquid phase and under which set of conditions the reversible scaffold exists in a semi-solid or solid phase.

Moreover, the magnetic content of the particles may be tuned and/or the applied external magnetic field may be controlled and/or varied, in order to improve or
30 optimise the efficiency of the magnetic separation process.

Therefore, the methods of the invention may be well suited to being scaled up and/or at least partially automated.

Specific apparatus adapted to perform the methods of the invention or portions thereof may be designed. Figure 11 schematically illustrates an example of a magnetic separator that could be part of such an apparatus.

5 Referring to Figure 11, there is shown a magnetic separator 71. A suspension 72 containing cells 78 and magnetic particles 77 is passed to the magnetic separator 71 from a temperature variable cell growth apparatus or chamber (not shown). The magnetic separator 71 may be integral with, connected to or separate from and/or permanently, fixedly or releasably connectable to the apparatus, in which the cells are
10 grown.

The magnetic separator 71 comprises a flow channel, through which, in use, the cells and magnetic particles flow in the direction indicated by arrow 75. The channel comprises an inner tube 76, which is perforated and is arranged inside an outer tube
15 73, which is not perforated. A plurality of switchable magnets 74 are arranged inside the outer tube 73 and outside the inner tube 76, the magnets being configured to provide a magnetic field which is substantially perpendicular to the direction of flow indicated by arrow 75. The magnets 74 comprise electromagnets, which can be turned on and off and controlled to provide varying magnetic field strengths.

20

In use, and as shown in Figure 11, the suspension 72 flows into inner tube 76. The magnets 74 are operable to separate the magnetic particles 77 from the suspension. The separated magnetic particles 77 are attracted to the magnets and pass through the perforations in the inner tube and out of the main flow of the suspension 72. The
25 magnetic separator is configured and controlled such that, when the cells 78 have flowed past all of the magnets 74, the suspension 72 is substantially free of magnetic particles 77, while containing substantially all of the cells 78.

Thus, substantially pure cells can be collected in cell culture media from the outlets of
30 the inner tube 76 and outer tube 73 (if any cells have passed through the perforations in the inner tube). After cell collection has finished, the magnetic field can be turned off and the magnetic particles attached to the magnets can then be washed off. The magnetic separator 71 may then be prepared for further use. The collected magnetic particles 78 may be sterilised and then re-used.

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The magnetic separator 71 will typically be housed in a cold chamber (not shown) or supplied with a cooling unit (not shown), in order to keep the suspension 72 at a low temperature to facilitate flow of the suspension 72. The temperature may be adjustable.

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Of course, various modifications of the magnetic separator shown in Figure 7 may be readily apparent to the person skilled in the art. For instance, the size, dimensions and distribution of the perforations or pores in the inner tube 76 may be varied. The perforated or porous inner tube may be sized and dimensioned such that magnetic particles may pass through them, but biological material, e.g. cells or bacteria, may not. Other filtering or screening means intended to facilitate removal of the magnetic particles from the flow of the suspension may be provided. For instance, such filtering means may or may not comprise a porous or perforated inner tube. Such filtering means may not be required, depending, for example, on the design of the flow channel.

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The arrangement of the channel and the magnets may be varied. Operation of the magnetic separator and/or an apparatus with which the magnetic separator is associated or to which it is connected may be at least partially automated. There may be any number of magnets. The or each magnet may or may not be an electromagnet.

20

Alternatively or additionally, a suspension may be passed through the magnetic field more than once, if necessary to remove substantially all of the magnetic particles from the suspension.

25

Similarly, the suspension may be passed through more than one magnetic field. Accordingly, the magnetic separator may be provided with an arrangement of magnets that is operable to provide two or more discrete magnetic fields.

The orientation of the magnetic field relative to the direction of flow of the suspension may vary and may be controlled and/or variable, in use. For instance, the magnets may be movable relative to the flow channel.

30

The or an apparatus in which biological material, e.g. cells or bacteria, is grown, and the magnetic separator may be comprised within a single device, instrument or machine.

- 5 Methods and uses of the invention may be relatively efficient, cost-effective, reproducible, safe and/or hygienic. For instance, there may be a reduced requirement for chemicals, e.g. enzymes, and therefore safety, operational and cost benefits. The reusability of the reversible scaffold or support and/or the reliability and/or controllability of magnetic separation may also provide significant benefits in terms of
10 cost, reproducibility, efficiency and/or safety.

While the invention may be used in growing cells, e.g. stem cells, it may also be suitable for use in growing bacteria or other biological materials.

- 15 The main applications envisaged for the invention may include: manufacture of stem cells or other types of cell culture; and pharmacological screening/disease diagnosis.

The invention may have application in controlled release, including flavour/fragrance release and especially in the biomedical field. For instance, the magnetic bulk or core
20 of the particles may incorporate actives, which for biomedical applications may typically be small molecule drugs and biopharmaceuticals for conventional drug delivery, or slow release growth factors and other soluble molecules for tissue growth, organ repair and regenerative medicine.

- 25 The particles of the invention may therefore comprise active ingredients selected from: flavours, fragrances, small molecule drugs and biopharmaceuticals, and slow release growth factors and other soluble molecules for tissue growth, organ repair and regenerative medicine. These active ingredients are suitably incorporated in the bulk or core of the particles.

30

Further applications of the invention, may, for example, be selected from flavour release applications, fragrance release applications and biomedical applications, such as conventional drug delivery applications. In particular, the applications may involve release of small molecule drugs or biopharmaceuticals for conventional drug delivery,

or release of slow release growth factors and other soluble molecules for tissue growth, organ repair and regenerative medicine.

CLAIMS

1. A reversible scaffold for growing cells or bacteria, the reversible scaffold comprising magnetic particles, wherein the magnetic particles exist in a liquid phase under a first set of conditions and in a semi-solid or solid phase under a second set of conditions, thereby providing a scaffold for growing cells or bacteria, the reversible scaffold being capable of being repeatedly cycled from the liquid phase to the semi-solid or solid phase and back again by changing from the first set of conditions to the second set of conditions and back again.
2. A scaffold according to claim 1, wherein the magnetic particles are thermoresponsive.
3. A scaffold according to claim 1 or claim 2, wherein the magnetic particles comprise iron, an iron oxide such as magnetite, nickel or cobalt.
4. A scaffold according to claim 1, claim 2 or claim 3, wherein the magnetic particles each have a magnetic core or bulk phase and/or a corona comprising a thermoresponsive polymer.
5. A scaffold according to claim 4, wherein the thermoresponsive polymer displays a lower critical solution temperature, in aqueous or non-aqueous media, that is at least 10°C and/or no more than 90°C.
6. A scaffold according to claim 4 or claim 5, wherein the thermoresponsive polymer may be prepared by a method comprising: the use of one or more monomers selected from polymerisable alkylene glycol acrylate monomers and polymerisable alkylene glycol methacrylate monomers as monomers in a polymerisation reaction.
7. A scaffold according to claim 6, wherein the polymerisable alkylene glycol acrylate monomers and polymerisable alkylene glycol methacrylate monomers may be selected from: di(ethylene glycol)-acrylate, oligo(ethylene glycol)-acrylate, poly(ethylene glycol)-acrylate, di(ethylene glycol)-methacrylate, oligo(ethylene glycol)-methacrylate, poly(ethylene glycol)-methacrylate, poly(propylene glycol)-acrylate, and poly(propylene glycol)-methacrylate, either singly, or in various combinations.

8. A scaffold according to any one of claims 1 to 7, which is reusable.
9. Thermoresponsive polymer particles, which are also magnetic.
- 5 10. Thermoresponsive polymer particles according to claim 9, which comprise iron, an iron oxide such as magnetite, nickel or cobalt.
- 10 11. Thermoresponsive polymer particles according to claim 9 or claim 10, wherein the polymer particles each have a magnetic core or bulk phase and/or a thermoresponsive corona.
12. Thermoresponsive polymer particles according to claim 11, wherein the thermoresponsive corona comprises a polymer that is adsorbed on to or chemically
- 15 bonded to or grown on and/or from a surface of the magnetic core or bulk phase.
13. Thermoresponsive polymer particles according to any one of claims 9 to 12, which display a lower critical solution temperature, in aqueous or non-aqueous media, that is at least 10°C and/or no more than 90°C.
- 20 14. Thermoresponsive polymer particles according to any one of claims 9 to 13, which were prepared by a method comprising: the use of one or more monomers selected from polymerisable alkylene glycol acrylate monomers and polymerisable alkylene glycol methacrylate monomers as monomers in a polymerisation reaction.
- 25 15. Thermoresponsive polymer particles according to claim 14, wherein the polymerisable alkylene glycol acrylate monomers and polymerisable alkylene glycol methacrylate monomers may be selected from: di(ethylene glycol)-acrylate, oligo(ethylene glycol)-acrylate, poly(ethylene glycol)-acrylate, di(ethylene glycol)-methacrylate, oligo(ethylene glycol)-methacrylate, poly(ethylene glycol)-methacrylate,
- 30 poly(propylene glycol)-acrylate, and poly(propylene glycol)-methacrylate, either singly, or in various combinations.
16. Thermoresponsive polymer particles according to any one of claims 9 to 15,
- 35 which are reusable.

17. A product which comprises the reversible scaffold of any one of claims 1 to 8 and/or the thermoresponsive polymer particles of any one of claims 9 to 16 and biological material such as cells or bacteria.

5

18. A product according to claim 17 further comprising one or more cytoadhesive moieties.

19. The use of the thermoresponsive polymer particles of any one of claims 9 to 16 as a support or scaffold for growth of biological material such as cells and/or bacteria.

10

20. A cell or bacteria support or scaffold comprising the thermoresponsive polymer particles of any one of claims 9 to 16.

15 21. A method of production of cells or bacteria comprising:

providing a reversible scaffold for growing cells or bacteria, the reversible scaffold comprising magnetic particles, wherein the magnetic particles exist in a liquid phase under a first set of conditions and in a semi-solid or solid phase under a second set of conditions, thereby providing a scaffold for growing cells or bacteria, the reversible scaffold being capable of being repeatedly cycled from the liquid phase to the semi-solid or solid phase and back again by changing from the first set of conditions to the second set of conditions and back again;

20

providing seed cells or bacteria in the scaffold and promoting growth of further cells or bacteria within the scaffold;

changing from the second set of conditions to the first set of conditions, thereby providing a suspension or dispersion comprising the cells or bacteria and the magnetic particles; and

25

separating the cells or bacteria from the magnetic particles by applying an external magnetic field to the suspension or dispersion.

30

22. A method according to claim 21, wherein the reversible scaffold is thermoresponsive.

23. A method according to claim 22, wherein the magnetic particles comprise a thermoresponsive polymer and the first set of conditions includes a temperature below

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a lower critical solution temperature of the thermoresponsive polymer and/or the second set of conditions includes a temperature above the or a lower critical solution temperature of the thermoresponsive polymer.

5 24. A method according to claim 21, claim 22 or claim 23, wherein the semi-solid or solid phase comprises a space-filling gel or microgel.

25. A method according to any one of claims 21 to 24, wherein there is relative movement between the or a source of the external magnetic field and the suspension
10 or dispersion.

26. A method according to any one of claims 21 to 25, wherein the external magnetic field is generated, in use, by an electromagnet.

15 27. A method according to any one of claims 21 to 26, wherein separating the cells or bacteria from the magnetic polymer particles is carried out by causing the suspension or dispersion to flow through the external magnetic field.

28. A method according to any one of claims 21 to 27, wherein the method is at
20 least partially automated.

29. An apparatus adapted to carry out at least a portion of the method of any one of claims 21 to 28, wherein the apparatus includes a magnetic separation means configured to apply, in use, an external magnetic field to a suspension or dispersion
25 comprising biological material such as cells and/or bacteria and magnetic particles.

30. A reversible scaffold substantially as described herein with reference to the accompanying drawings.

30 31. Responsive particles substantially as described herein with reference to the accompanying drawings.

32. A method of manufacture of biological material such as cells and/or bacteria substantially as described herein.

33. An apparatus comprising a magnetic separator substantially as described herein with reference to the accompanying drawings.

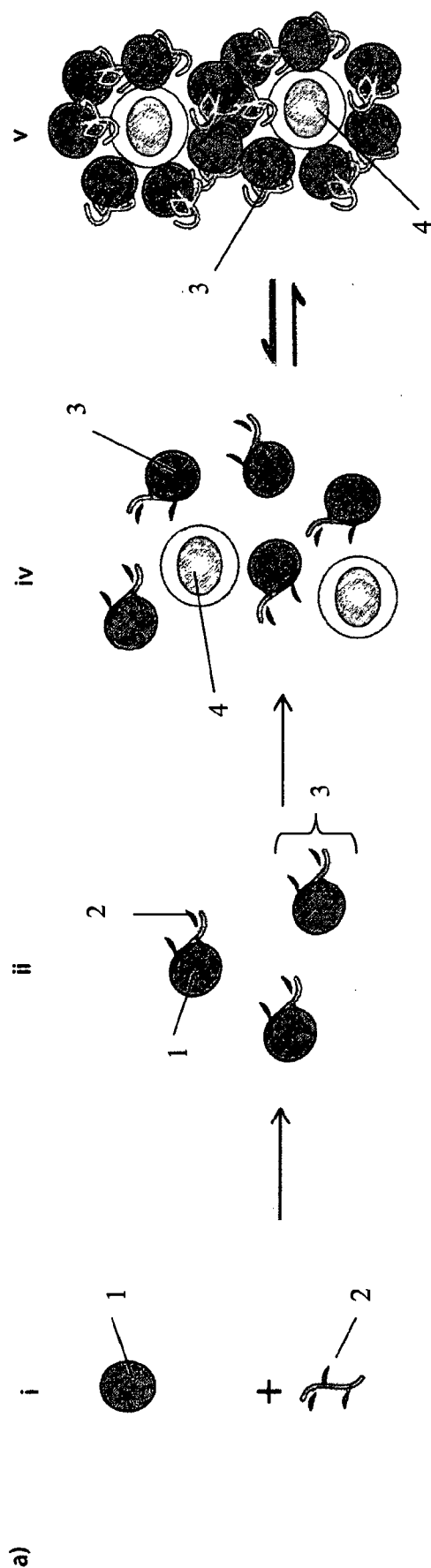
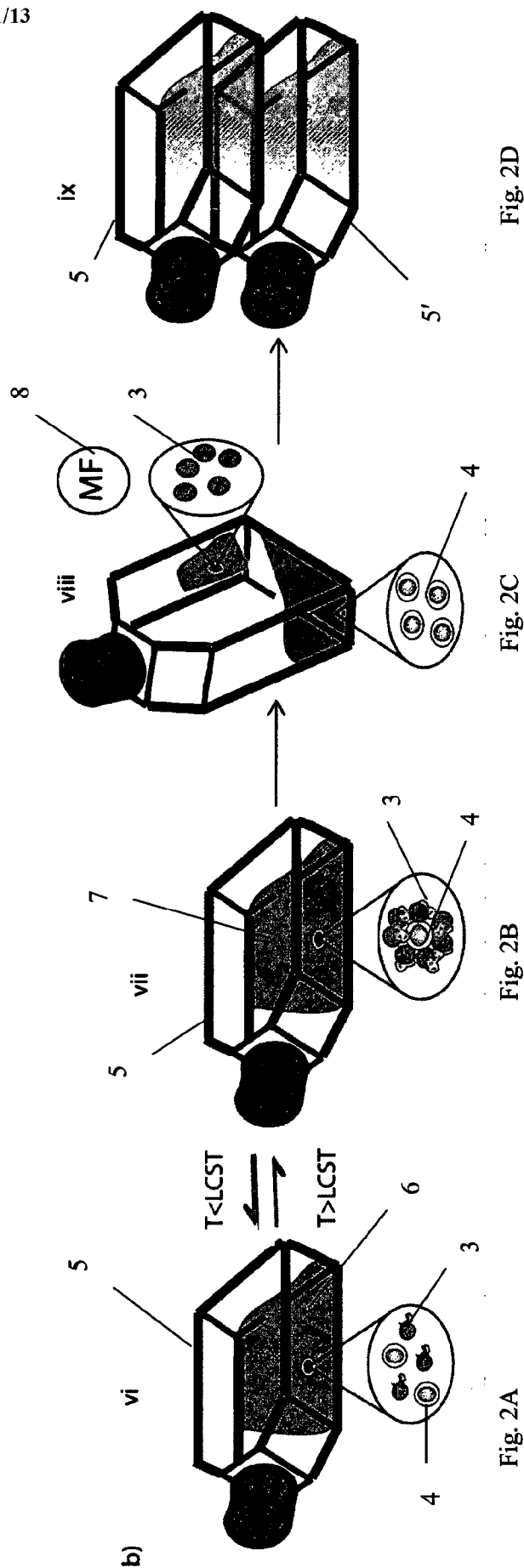


Fig. 1D

Fig. 1C

Fig. 1B

Fig. 1A



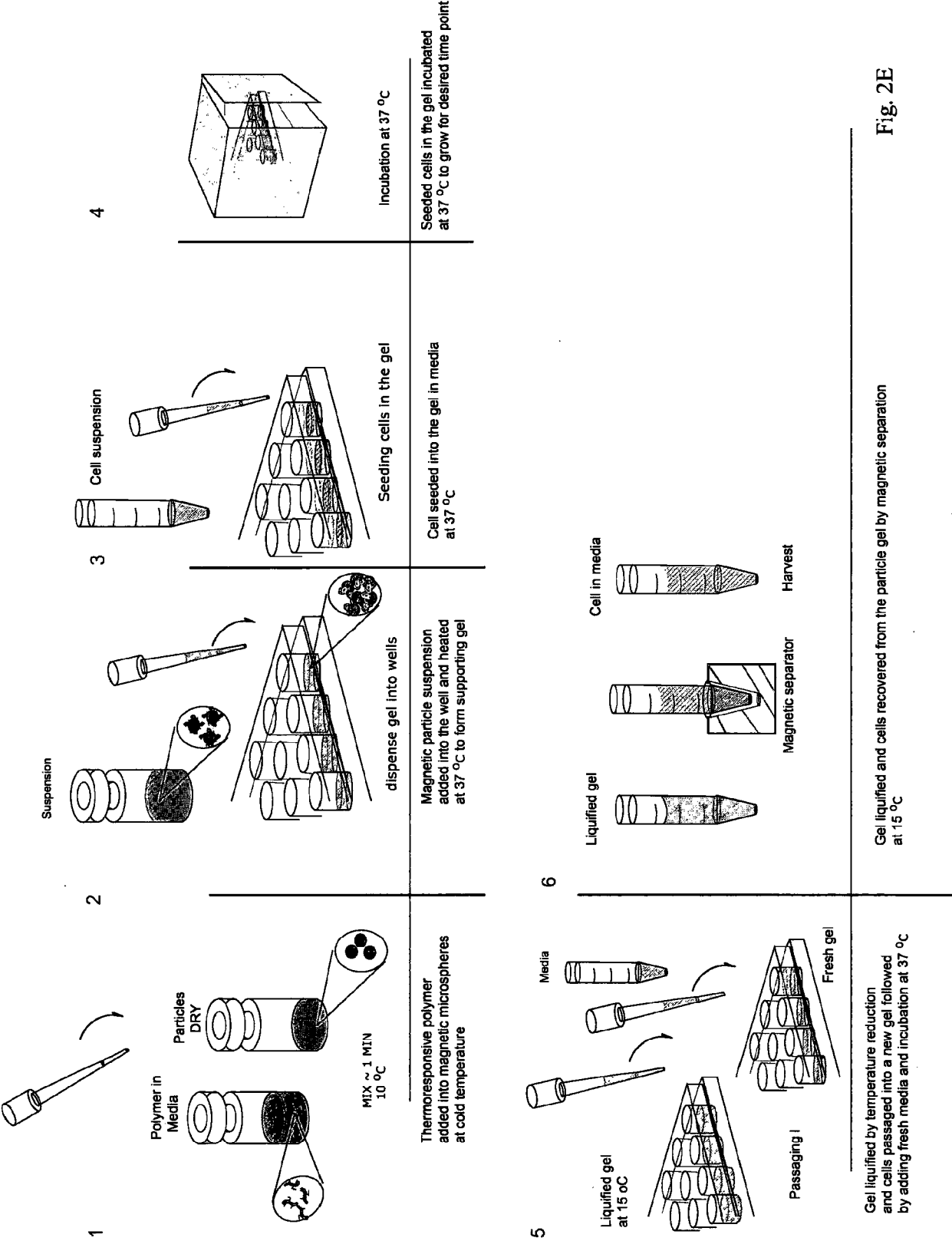


Fig. 2E

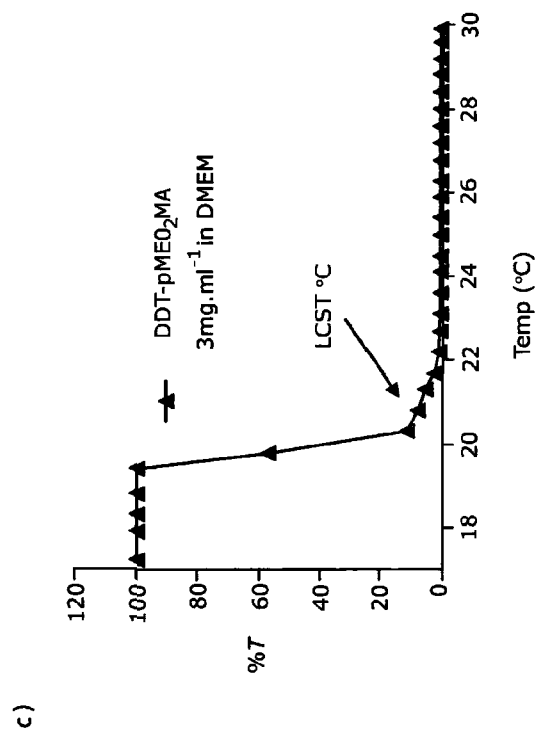


Fig. 3C

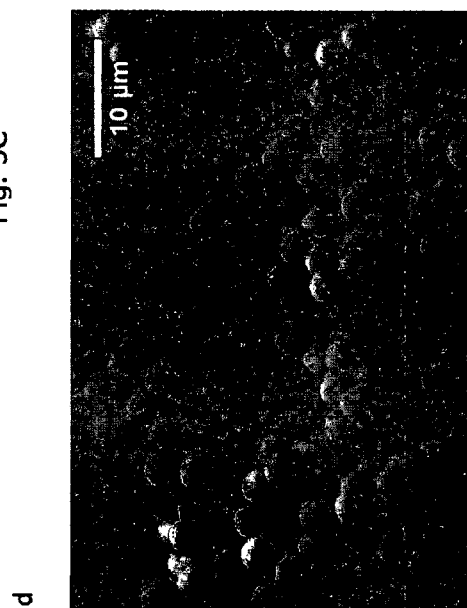


Fig. 3D

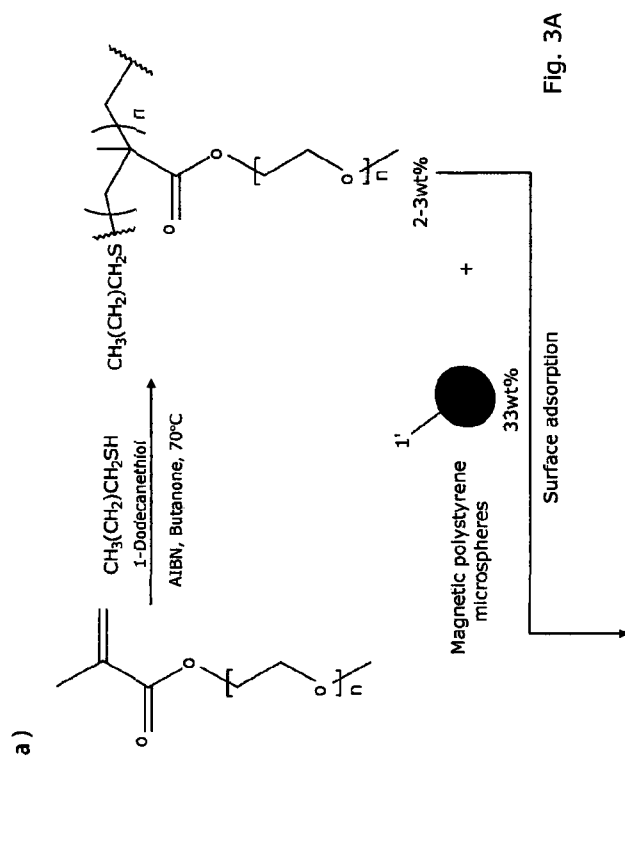


Fig. 3A

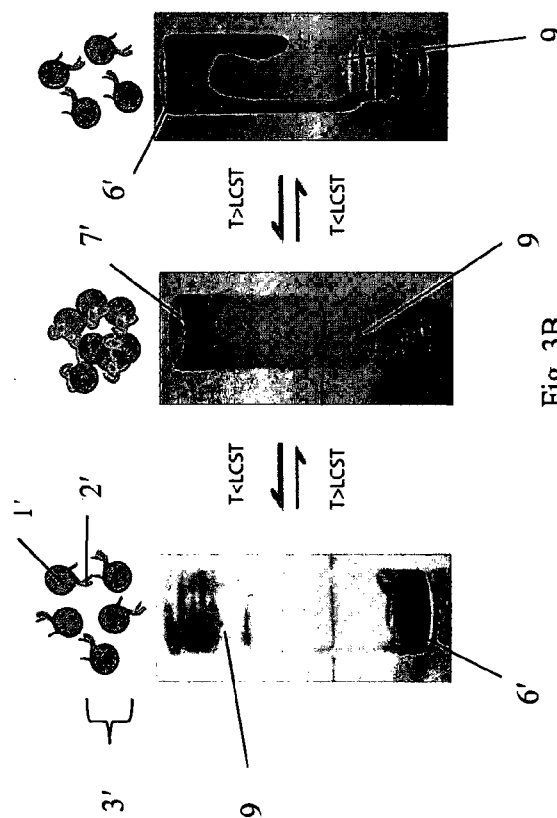


Fig. 3B

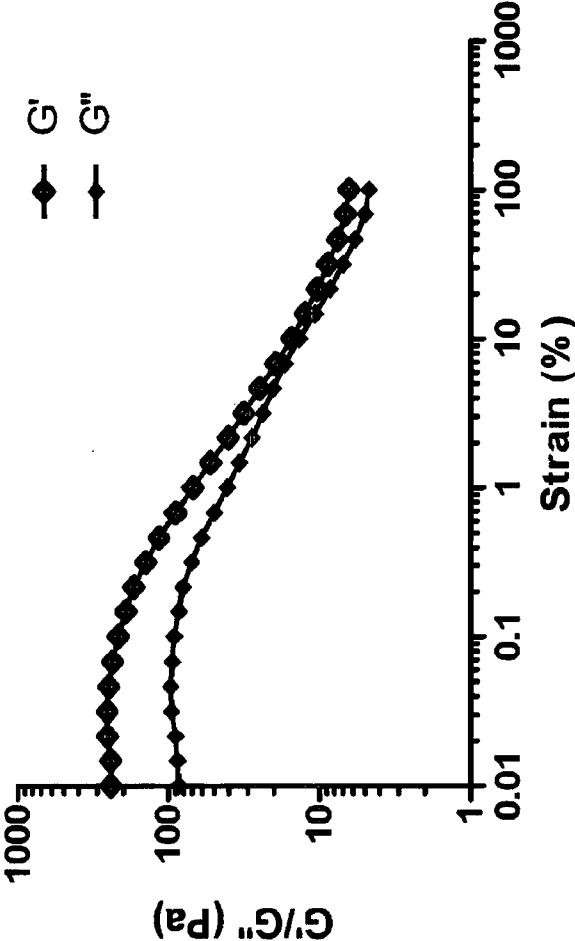


Fig. 4C

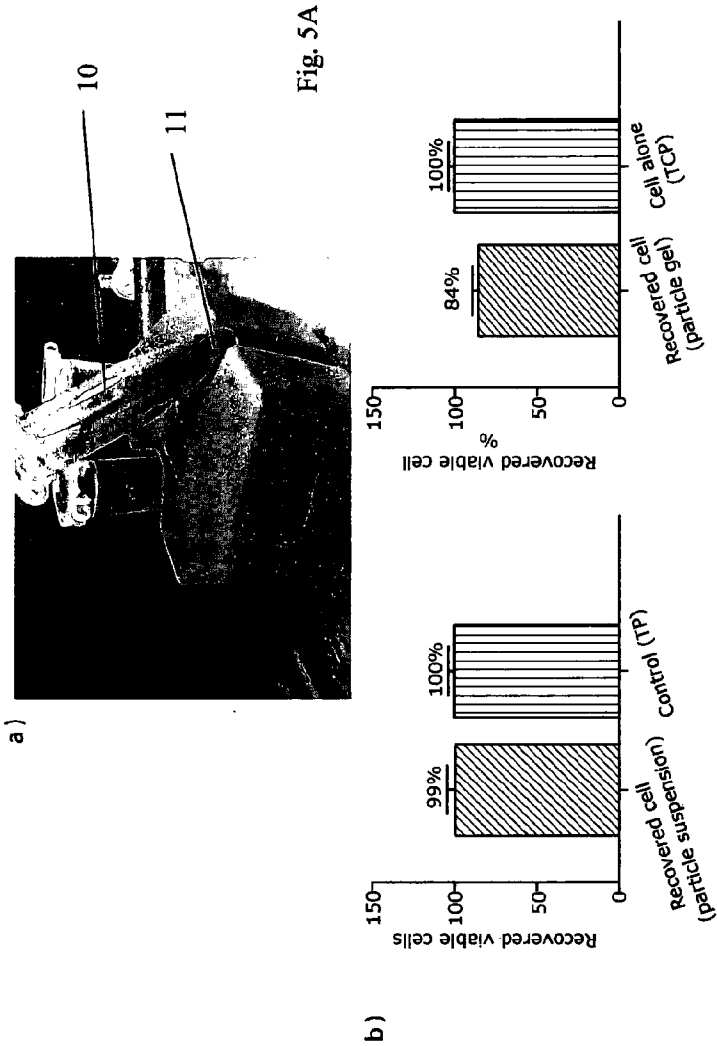


Fig. 5B

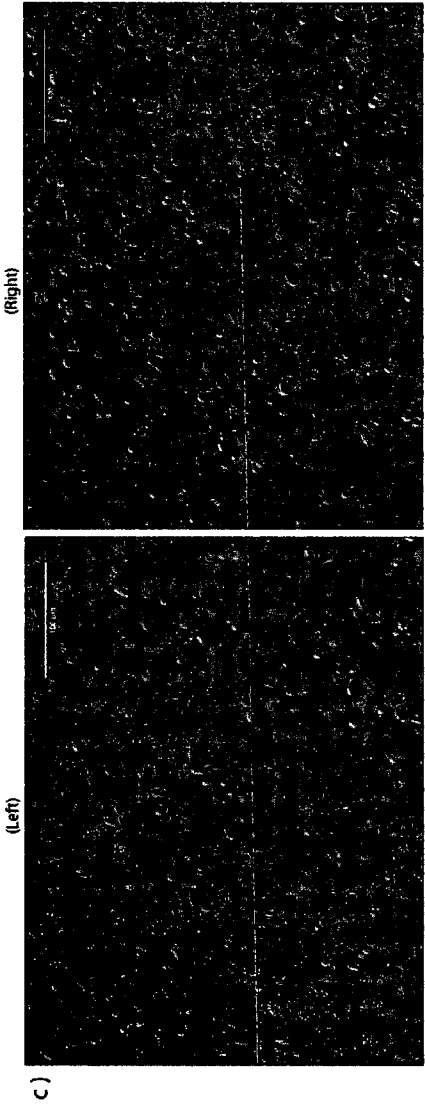


Fig. 5C

Fig 5E

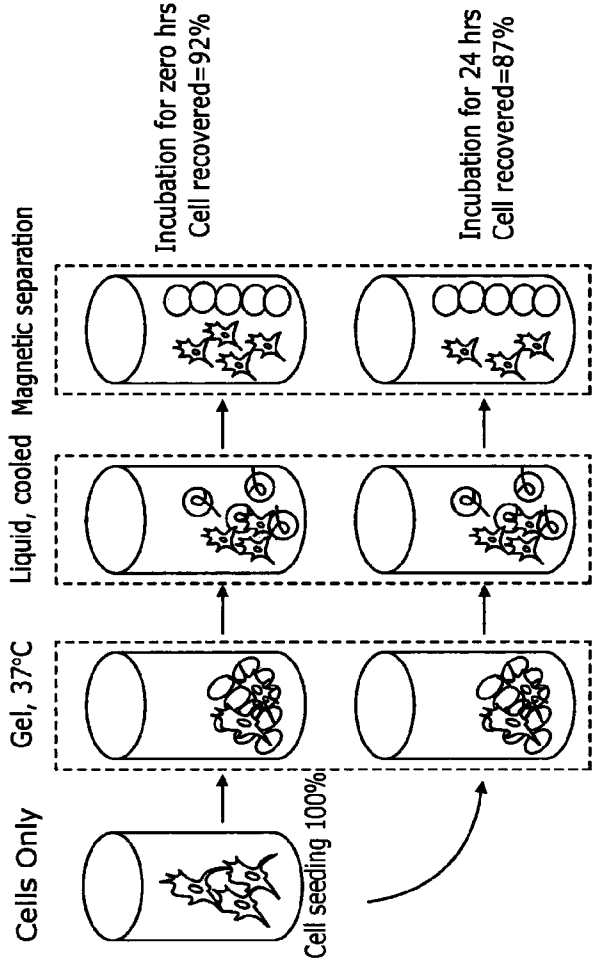
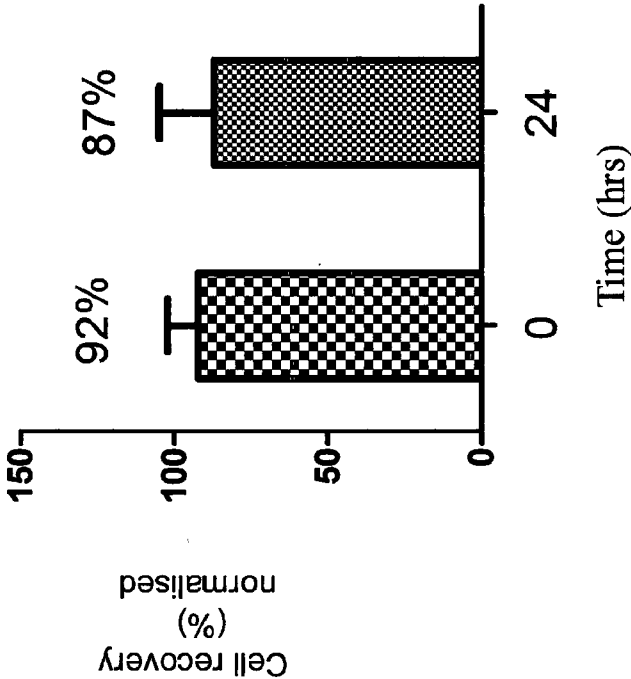


Fig 5D
Cells recovered from gel after 0hrs
Cell recovered from gel after 24hrs



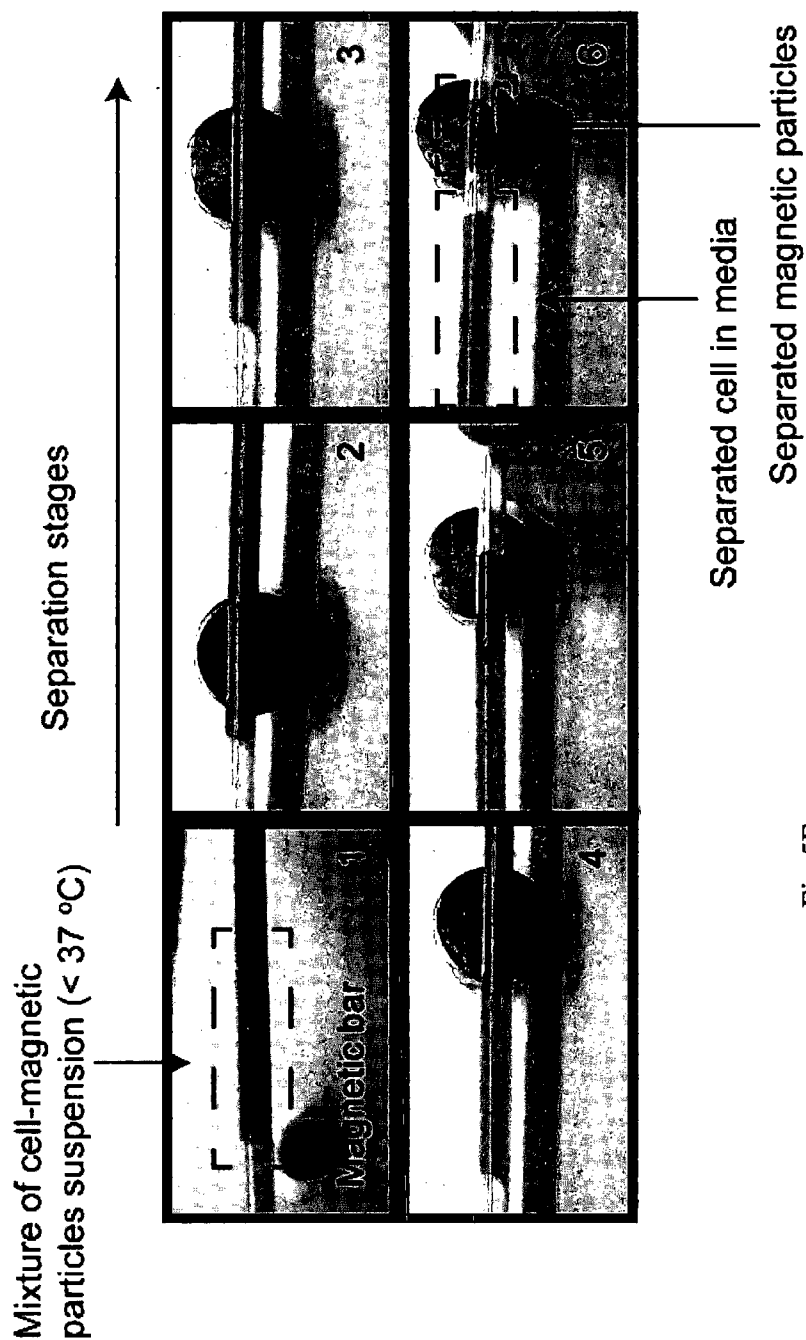


Fig 5F

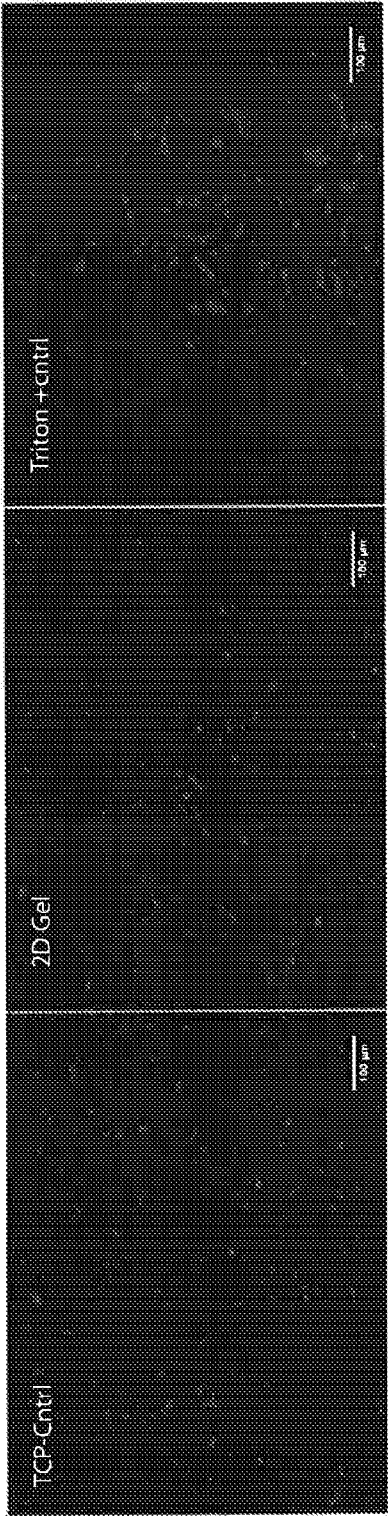


Fig. 6A

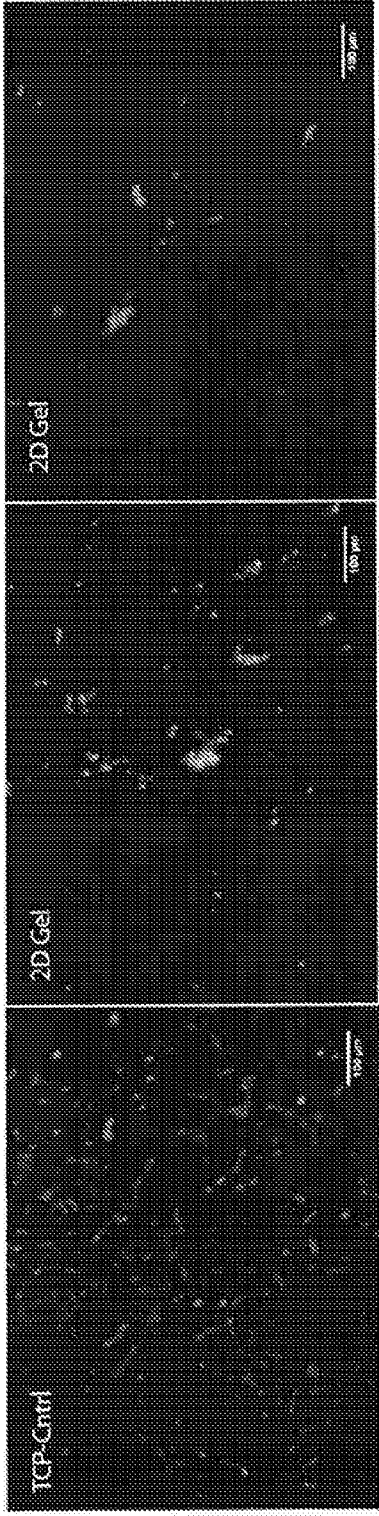


Fig. 6B

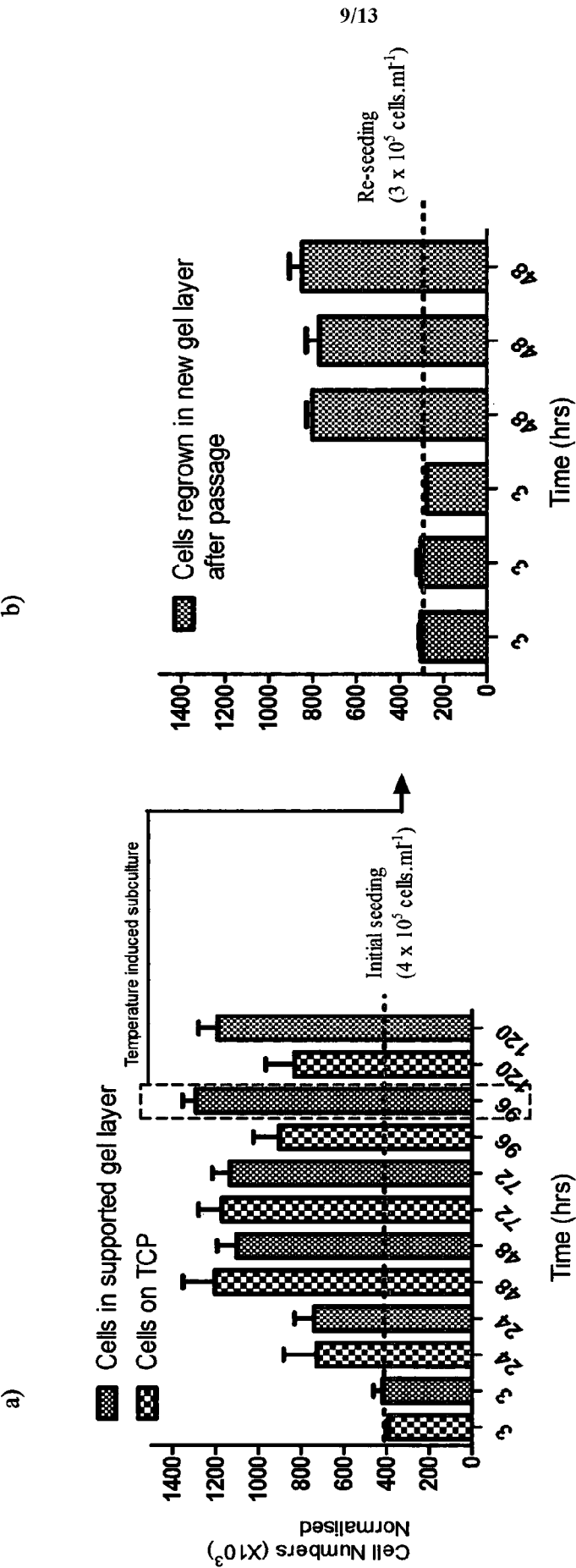


Fig. 7

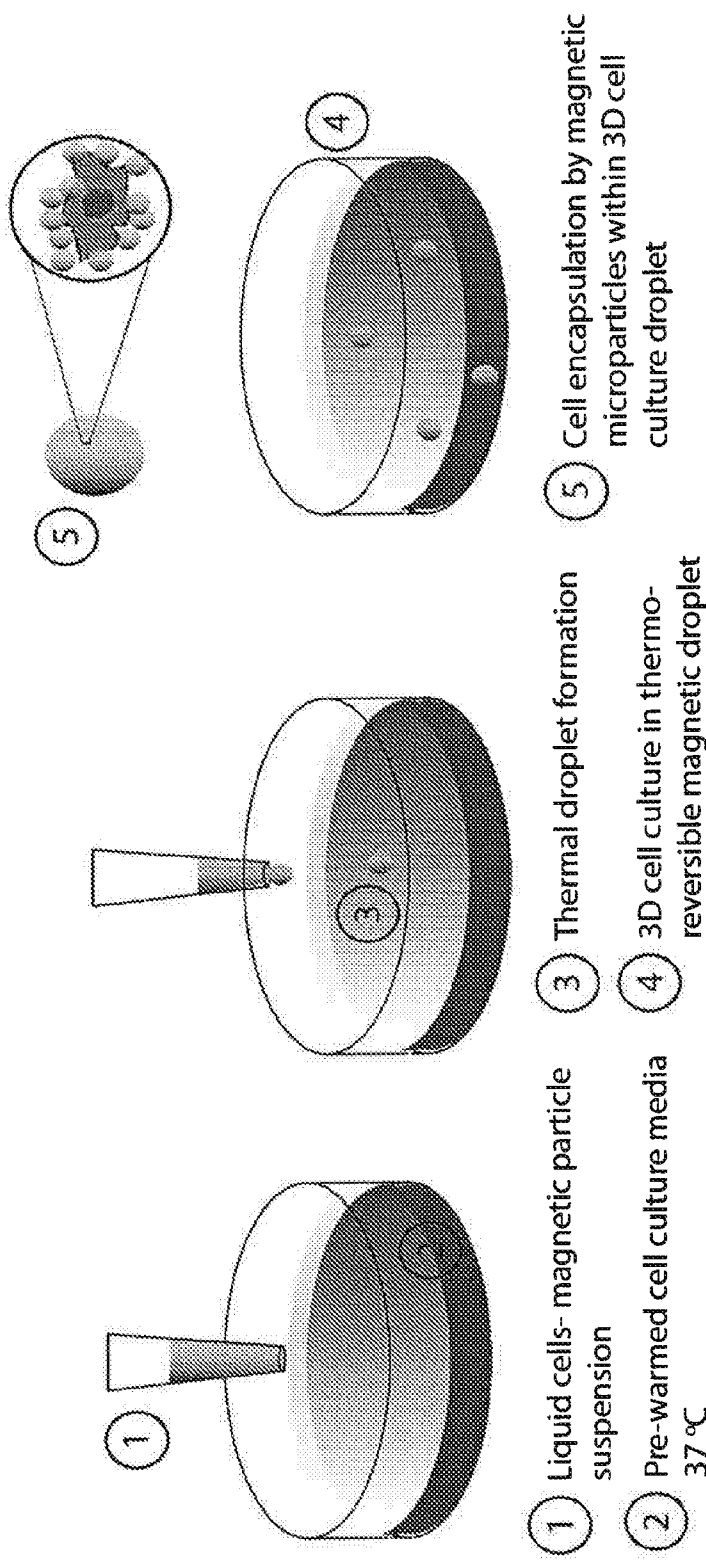


Fig. 8

ihMSCs grown inside droplet gels
(e.g. 200k cells/50ul Particle gel)

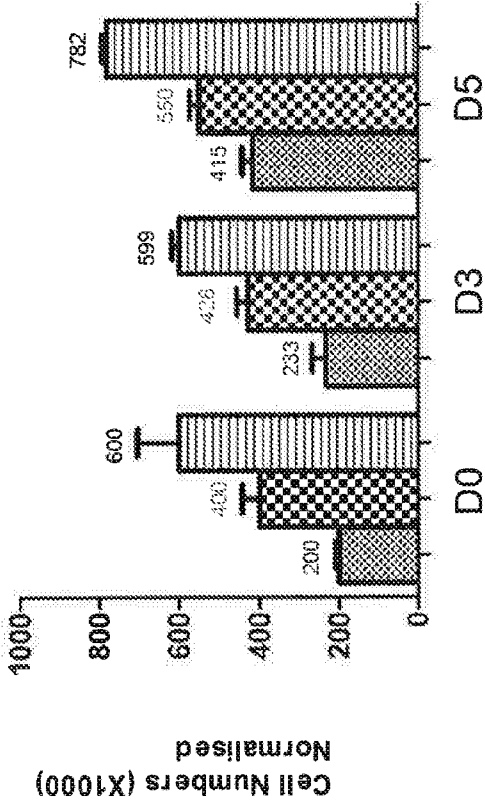
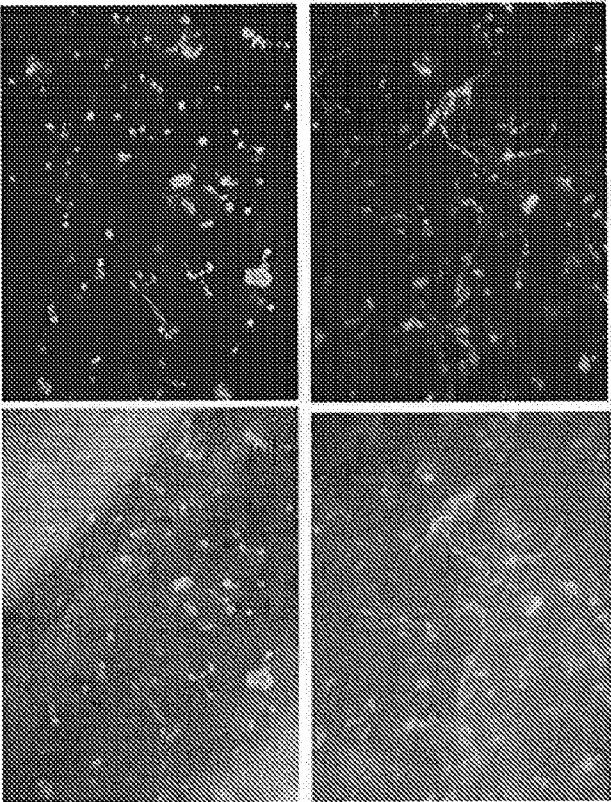


Fig. 9

Fig. 10



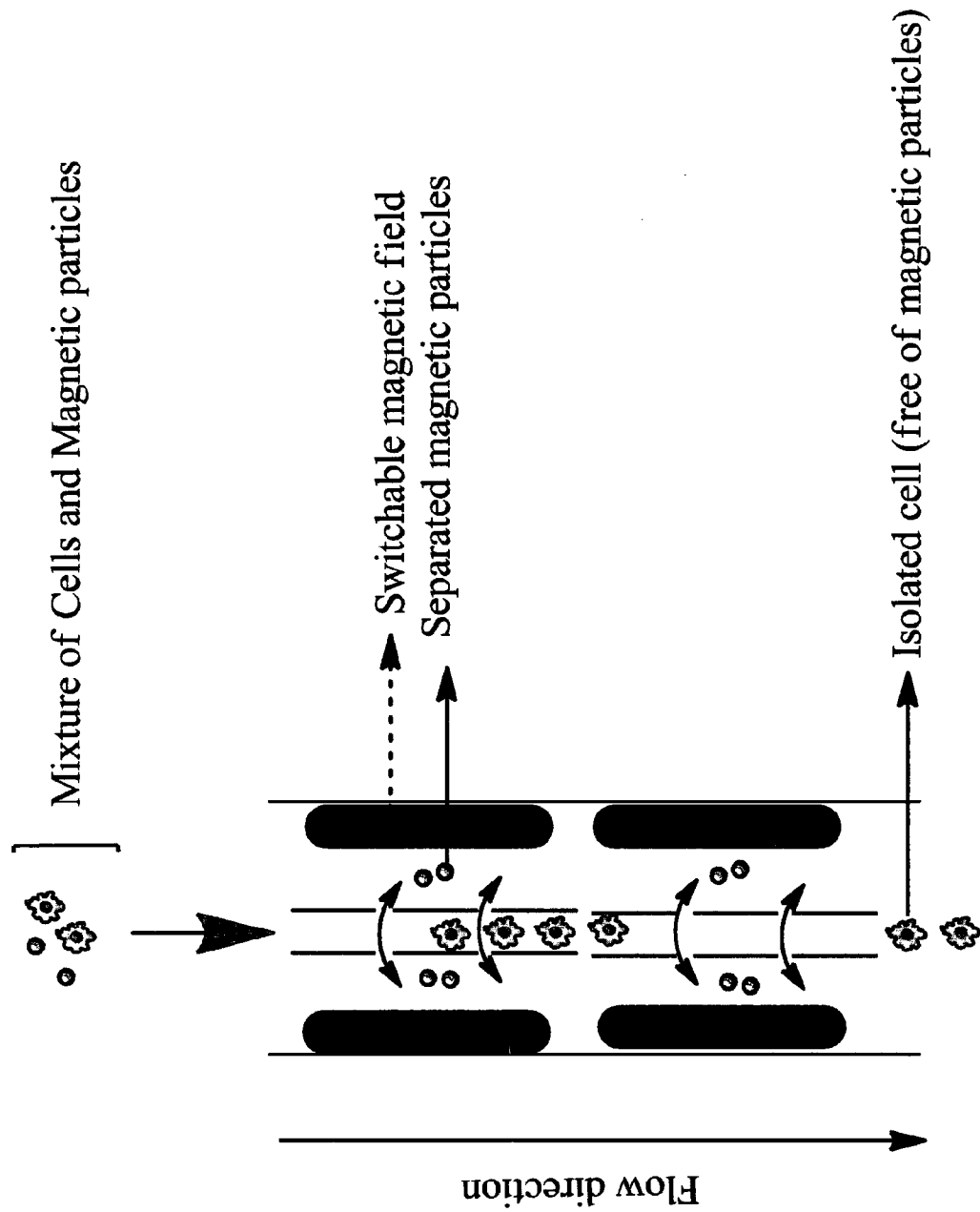


Fig. 11

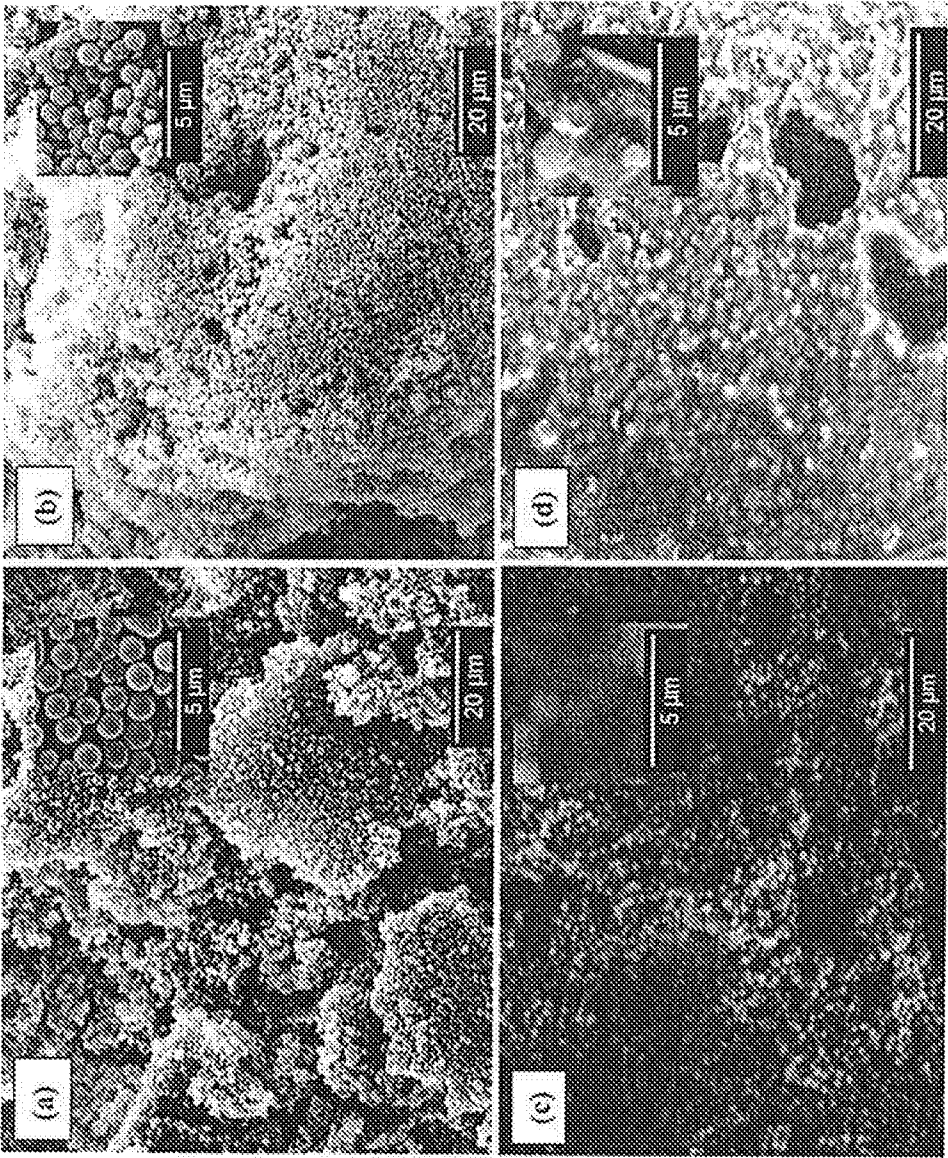


Fig. 12

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2013/051894

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61L27/04 A61L27/14 A61L27/38
ADD.

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)
A61L

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 practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	C Ghanami: "Novel Thermoresponsive Particle Gels For Tissue Engineering Applications", Nottingham eThesis, 13 July 2012 (2012-07-13), pages 1-256, XP55079038, Internet Retrieved from the Internet: URL: http://etheses.nottingham.ac.uk/2318/1/Thesis-5.pdf [retrieved on 2013-09-12] page 182 - page 208 ----- -/-	1-33



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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

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"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

12 September 2013

Date of mailing of the international search report

20/09/2013

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Dudás, Eszter

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2013/051894

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	C Ghanami: "Novel thermoresponsive particle gels for tissue engineering applications", Nottingham eThesis, 13 July 2012 (2012-07-13), pages 1-1, XP55079034, Internet Retrieved from the Internet: URL:http://etheses.nottingham.ac.uk/2318/ [retrieved on 2013-09-12] the whole document -----	1-33