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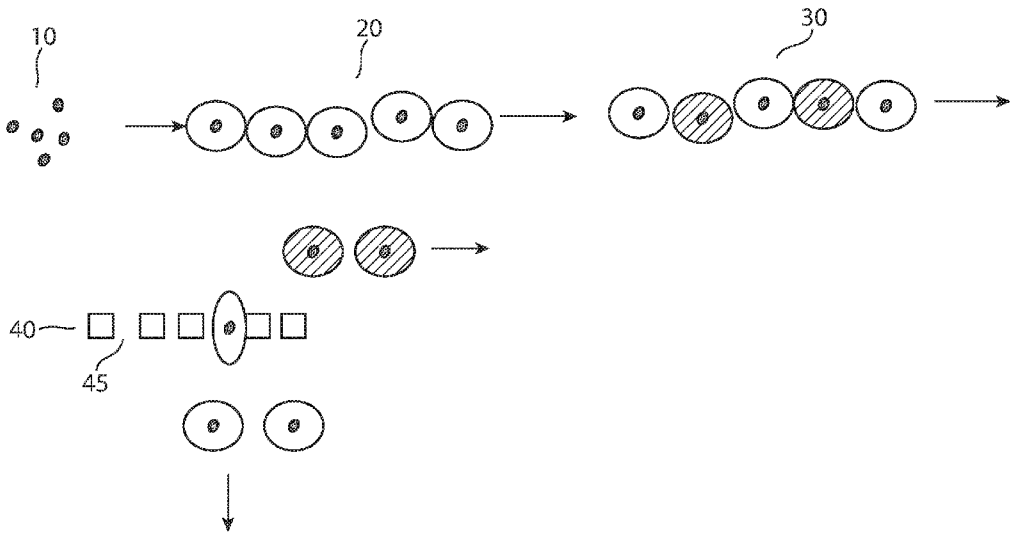

FIG. 1

(57) Abstract: The present invention generally relates to systems and methods for high throughput selection. In one aspect, cells are contained within gel droplets, and the cells may interact with the gel droplet in some manner, e.g., to form the gel and/or to degrade the gel. The interaction of cells with the gel droplet may result in some cells being contained within gel droplets and other cells not being contained within gel droplets, which may form the basis by which the cells are sorted. Such cells can be relatively rapidly sorted, e.g., on the basis of size, for example, using filtration, centrifugation, or other similar techniques. In some cases, unlike sorting techniques which can only sort one entity, such as droplets, at a time, such techniques can allow for more than one entity (and in some cases, relatively large numbers of entities) to be simultaneously sorted, dramatically increasing throughput.

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SYSTEMS AND METHODS FOR HIGH THROUGHPUT SELECTION

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application Serial No. 62/810,779, filed February 26, 2019, by Weitz, *et al.*, incorporated herein by reference in its entirety.

FIELD

The present invention generally relates to systems and methods for high throughput selection.

BACKGROUND

Currently, great amounts of labor and monetary resources are invested into directed evolution because this technique allows biomolecules to be adapted to the specific needs of a study or application. Directed evolution can increase the specificity or turnover of enzymes, adapt the temperature or pH optima, and even increase the expression yield of proteins. The general approach for directed evolution experiments is (a) generating variation in the gene of interest that is to be evolved, (b) expressing the different variants, and (c) selecting the best variants for the specific application. The best variants are used as a starting point for the next cycle to create further improved variants. However, as mentioned, as great amounts of labor and monetary resources are needed, improved selection for directed evolution are needed.

SUMMARY

The present invention generally relates to systems and methods for high throughput selection. The subject matter of the present invention involves, in some cases, interrelated products, alternative solutions to a particular problem, and/or a plurality of different uses of one or more systems and/or articles.

In one aspect, the present invention is generally directed to a method. In one set of embodiments, the method comprises encapsulating cells within liquid droplets, causing the liquid droplets containing the cells to form gel droplets, causing at least some of the cells within the gel droplets to degrade the gel, and separating the gel-degrading cells from the non-gel-degrading cells via a filter.

According to another set of embodiments, the method comprises encapsulating cells within liquid droplets, causing at least some of the cells to gel the liquid droplet to form a gel droplet, and separating the cells contained within gel droplets from cells contained within liquid droplets via a filter.

In another aspect, the present invention is generally directed to devices able to perform any of the methods described herein.

In another aspect, the present invention encompasses methods of making one or more of the embodiments described herein. In still another aspect, the present invention encompasses methods of using one or more of the embodiments described herein.

Other advantages and novel features of the present invention will become apparent from the following detailed description of various non-limiting embodiments of the invention when considered in conjunction with the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

Non-limiting embodiments of the present invention will be described by way of example with reference to the accompanying figures, which are schematic and are not intended to be drawn to scale. In the figures, each identical or nearly identical component illustrated is typically represented by a single numeral. For purposes of clarity, not every component is labeled in every figure, nor is every component of each embodiment of the invention shown where illustration is not necessary to allow those of ordinary skill in the art to understand the invention. In the figures:

Fig. 1 is a schematic diagram illustrating droplet filtration, in one embodiment of the invention;

Fig. 2 is a schematic diagram illustrating separation of cells using a filter, in another embodiment of the invention;

Fig. 3 is a schematic diagram of a microfluidic device in yet another embodiment of the invention; and

Fig. 4 is the DNA sequence of a plasmid containing the subtilisin gene (SEQ ID NO: 1).

DETAILED DESCRIPTION

The present invention generally relates to systems and methods for high throughput selection. In one aspect, cells are contained within gel droplets, and the cells may interact with the gel droplet in some manner, e.g., to form the gel and/or to degrade the gel. The interaction of cells with the gel droplet may result in some cells being contained within gel droplets and other cells not being contained within gel droplets, which may form the basis by which the cells are sorted. Such cells can be relatively rapidly sorted, e.g., on the basis of size, for example, using filtration, centrifugation, or other similar techniques. In some cases, unlike sorting techniques which can only sort one entity, such as droplets, at a time, such

techniques can allow for more than one entity (and in some cases, relatively large numbers of entities) to be simultaneously sorted, dramatically increasing throughput.

One aspect of the invention is thus generally directed to relatively high throughput selection of cells. For instance, enzymes such as proteases that are expressed by cells can be sorted relatively quickly. Thus, such techniques may be useful to simultaneously assay relatively large numbers of different variants of a protein, e.g., 10^7, 10^8, 10^9, or more different variants, without necessarily requiring separate assays for each possible genetic variant.

In some cases, microfluidic droplets are used, which can easily and cheaply be parallelized with microfluidic techniques to increase the throughput as necessary. Cells may be encapsulated within gelatin hydrogel droplets or microspheres, e.g., such that each droplet has, on average, 1 cell (or less). For example, for proteases, at least some of the encapsulated cells may secrete a protease that can degrade the gelatin hydrogel. If the gelatin hydrogel is sufficiently degraded, the encapsulated cell may be able to escape the gelatin droplet, and those cells can be separated from the cells that do not produce a protease (or produce inadequate amounts of proteases, or less active proteases, etc.), and which are accordingly still contained within hydrogels, for example, using filtration or other techniques. The selection stringency can be controlled, for instance, by altering the gelatin concentration or reducing the incubation time, etc.

Such techniques may also be used in a variety of other contexts, not just for proteases or directed evolution experiments. For example, enzymes that degrade other hydrogel-forming polymers may be may be screened, or cells that are able to form a hydrogel may be retained. One non-limiting example of a hydrogel-forming reaction is the cross-linking of tyrosine residues in proteins with horseradish peroxidase and hydrogen peroxide. Since many enzymes can produce hydrogen peroxide, and/or can be coupled to hydrogen peroxide-forming reactions, such enzymes can be screened by determining if cells containing such enzymes can produce a hydrogel when contained within a droplet.

An illustrative example is now provided with respect to Fig. 1. In this figure, a plurality of cells 10 are to be sorted. There may be at least a million cells, or more (e.g., at least 10^7, at least 10^8, at least 10^9, etc.) to be sorted, for example, on the basis of protease production, enzymes that produce hydrogen peroxide, acid secretion, or the like. In this example, the cells initially are encapsulated within droplets, such as microfluidic droplets, using techniques such as flow-focusing that are known to those of ordinary skill in the art. See, for example, U.S. Pat. Nos. 7,708,949, 8,337,778, 8,765,485, or Int. Pat. Apl. Pub. Nos. WO 2004/091763 and WO 2006/096571, each incorporated herein by reference in its

entirety. The cells may be encapsulated within droplets 20 within a carrier fluid, for example, such that they are encapsulated at an average of 1 cell/droplet, or less (for example, such that most or all of the droplets contain either no cell or 1 cell).

After encapsulating the cells, the droplets may be converted into gel droplets 30. In some cases, the droplets may be produced containing a gel precursor, although in some cases, the gel precursor may be added to the droplet after the droplet has been formed, e.g., using picoinjection, merging the droplet with another droplet, or other techniques known by those of ordinary skill in the art. See, for example, Int. Pat. Apl. Pub. No. WO 2010/151776 incorporated herein by reference in its entirety. As an example, in one set of embodiments, a liquid droplet containing cells and gelatin may be produced at an elevated temperature, and the droplets subsequently cooled to cause the gelatin to gel, causing the droplet to form a gel droplet.

In addition, in some embodiments, the cell may take part in the formation of a gel droplet. For example, in one set of embodiments, a cell may indirectly or directly produce hydrogen peroxide, which can react with a peroxidase and proteins (for example, with tyrosine residues) to cause the droplet to form a gel (e.g., shown as shaded in Fig. 1). Thus, if the cell produces sufficient hydrogen peroxide, or compounds which can be coupled through enzymatic or non-enzymatic reactions to hydrogen peroxide formation, the droplet containing the cell forms a gel; otherwise, the droplet stays liquid, as is shown here.

A variety of enzymes that produce hydrogen peroxide, or can be coupled to hydrogen peroxide (e.g., through enzymatic or non-enzymatic reactions), thus can be determined. One non-limiting example is glucose oxidase, which reacts glucose with oxygen to produce gluconic acid and hydrogen peroxide. Such reactions can also be coupled to other reactions in certain cases, such as with a dehydrogenase, in which glucose can be prepared from other sources. Accordingly, a variety of reactions, including enzymatic reactions, can be coupled to gel formation in various embodiments.

Thus, in some cases, some of the cells are contained within gel droplets, while others are not. Such cells may be contained within liquid droplets, and/or may be free-floating, e.g., in the carrier fluid. Such cells contained within gel droplets can then be sorted from cells not contained within gel droplets. In some cases, sorting can be applied to more than one cell or droplet at a time, which can allow for more rapid sorting than techniques that can only sort one droplet at a time. One example of such a technique is filtration, as is shown in Fig. 1. For instance, a filter 40 having pores 45 having a size that allows free cells to pass, but does not allow cells contained within gels to pass can be used to efficiently separate the cells.

Thus, the pore size of the filter may be selected based on the types of cells being studied. Different levels of stringency can also be applied, for example, by using filters with different pore sizes.

After separation, a variety of techniques can be used to determine the cells, and/or enzymes or other chemical reactions, as discussed above, and many of these will be known by those of ordinary skill in the art. For example, the cells may be removed from the droplets, nucleic acids (coding for proteins such as enzymes) may be sequenced, amplified, etc., and/or other techniques applied to the some or all of the sorted droplets. A variety of suitable techniques for analyzing cells will be known to those of ordinary skill in the art.

The above discussion is a non-limiting example of some embodiments of the present invention that can be used to sort cells. However, other embodiments are also possible. Accordingly, more generally, various aspects of the invention are directed to various systems and methods for the high throughput selection of cells.

For instance, certain aspects are generally directed to containing cells in droplets, such as microfluidic droplets. In some cases, a relatively large number of droplets may be created, e.g., at least about 10, at least about 30, at least about 50, at least about 100, at least about 300, at least about 500, at least about 1,000, at least about 3,000, at least about 5,000, at least about 10,000, at least about 30,000, at least about 50,000, at least about 100,000, at least 10^6, at least 10^7, at least 10^8, at least 10^9 droplets, etc. As discussed below, some or all of the droplets may contain cells, and in some cases, such that substantially each of the droplets contains 1 cell or no cell.

The droplets and the carrying fluid may be substantially immiscible, i.e., the droplets may be aqueous while the carrying fluid may be an oil, or vice versa. It should be understood that the term "oil" merely refers to a fluid that is generally more hydrophobic, or not miscible or soluble in water, as is known in the art. Thus, the oil may be a hydrocarbon in some embodiments, but in other embodiments, the oil may comprise other hydrophobic fluids.

Any suitable method may be chosen to create droplets, i.e., within a carrying fluid, and a wide variety of different techniques for forming droplets will be known to those of ordinary skill in the art. For example, a junction of channels may be used to create the droplets. The junction may be, for instance, a T-junction, a Y-junction, a channel-within-a-channel junction (e.g., in a coaxial arrangement, or comprising an inner channel and an outer channel surrounding at least a portion of the inner channel), a cross (or "X") junction, a flow-focusing junction, or any other suitable junction for creating droplets. See, for example, International Patent Application No. PCT/US2004/010903, filed April 9, 2004, entitled

"Formation and Control of Fluidic Species," by Link, *et al.*, published as WO 2004/091763 on October 28, 2004, or International Patent Application No. PCT/US2003/020542, filed June 30, 2003, entitled "Method and Apparatus for Fluid Dispersion," by Stone, *et al.*, published as WO 2004/002627 on January 8, 2004, each of which is incorporated herein by reference in its entirety. Other techniques for creating droplets include, but are not limited to, bulk emulsification, ink-jet printing, acoustophoretic printing, or the like.

In some embodiments, the junction may be configured and arranged to produce substantially monodisperse droplets. The droplets may also be created on the fluidic device, and/or the droplets may be created separately then brought to the device. For instance, the droplets may be of substantially the same shape and/or size (i.e., "monodisperse"), or of different shapes and/or sizes, depending on the particular application. In some cases, the droplets may have a homogenous distribution of cross-sectional diameters, i.e., the droplets may have a distribution of diameters such that no more than about 5%, no more than about 2%, or no more than about 1% of the droplets have a diameter less than about 90% (or less than about 95%, or less than about 99%) and/or greater than about 110% (or greater than about 105%, or greater than about 101%) of the overall average diameter of the plurality of droplets. Some techniques for producing homogenous distributions of cross-sectional diameters of droplets are disclosed in International Patent Application No. PCT/US2004/010903, filed April 9, 2004, entitled "Formation and Control of Fluidic Species," by Link et al., published as WO 2004/091763 on October 28, 2004, incorporated herein by reference. Those of ordinary skill in the art will be able to determine the average diameter of a population of droplets, for example, using laser light scattering or other known techniques. The droplets so formed can be spherical, or non-spherical in certain cases. The diameter of a droplet, in a non-spherical droplet, may be taken as the diameter of a perfect mathematical sphere having the same volume as the non-spherical droplet.

In certain cases, the average diameter of the droplets may be less than about 1 mm, less than about 500 micrometers, less than about 300 micrometers, less than about 200 micrometers, less than about 100 micrometers, less than about 75 micrometers, less than about 50 micrometers, less than about 30 micrometers, less than about 25 micrometers, less than about 20 micrometers, less than about 15 micrometers, less than about 10 micrometers, less than about 5 micrometers, less than about 3 micrometers, less than about 2 micrometers, less than about 1 micrometer, less than about 500 nm, less than about 300 nm, less than about 100 nm, or less than about 50 nm. The average diameter of the droplets may also be at least about 30 nm, at least about 50 nm, at least about 100 nm, at least about 300 nm, at least about

500 nm, at least about 1 micrometer, at least about 2 micrometers, at least about 3 micrometers, at least about 5 micrometers, at least about 10 micrometers, at least about 15 micrometers, or at least about 20 micrometers in certain cases. The "average diameter" of a population of droplets is the arithmetic average of the diameters of the droplets.

The cells contained within the droplets may be added during formation of the droplet, and/or added after formation of the droplet, for example, using techniques such as picoinjection, merging the droplet with another droplet, or the like. See, for example, Int. Pat. Apl. Pub. No. WO 2010/151776 incorporated herein by reference in its entirety. Those of ordinary skill in the art will be familiar with systems and methods for encapsulating a cell in a liquid droplet.

The cells may arise from any suitable source, and may include one, or more than one, cell type. The cells may be for example, from a specific population of cells, such as from a certain organ or tissue (e.g., cardiac cells, immune cells, muscle cells, cancer cells, etc.), cells from a specific individual or species (e.g., prokyaryotes, eukaryotes, human cells, mouse cells, bacteria, mammalian cells, etc.), cells from different organisms, cells from a naturally-occurring sample (e.g., pond water, soil, etc.), or the like. In some cases, the cells may be dissociated from tissue.

In certain embodiments, relatively large number of cells may be determined, sorted, etc., e.g., at least about 10, at least about 30, at least about 50, at least about 100, at least about 300, at least about 500, at least about 1,000, at least about 3,000, at least about 5,000, at least about 10,000, at least about 30,000, at least about 50,000, at least about 100,000, at least 10^6, at least 10^7, at least 10^8, or at least 10^9 cells, etc.

In one set of embodiments, the droplets are loaded such that, on the average, each droplet has 1 cell in it, or less. For example, the average loading rate may be less than about 1 cell/droplet, less than about 0.9 cells/droplet, less than about 0.8 cells/droplet, less than about 0.7 cells/droplet, less than about 0.6 cells/droplet, less than about 0.5 cells/droplet, less than about 0.4 cells/droplet, less than about 0.3 cells/droplet, less than about 0.2 cells/droplet, less than about 0.1 cells/droplet, less than about 0.05 cells/droplet, less than about 0.03 cells/droplet, less than about 0.02 cells/droplet, or less than about 0.01 cells/droplet. In some cases, lower cell loading rates may be chosen to minimize the probability that a droplet will be produced having two or more cells in it. Thus, for example, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 97%, at least about 98%, or at least about 99% of the droplets may contain either no cell or only one cell.

In one set of embodiments, after encapsulation of the cells within droplets, the droplets may be caused to form gels. In some cases, the gel may be formed initially, and the cells assayed by their ability to degrade the gel. In other cases, the cells may be sorted by their ability to form a gel.

In some cases, a gel may be formed after forming the droplet, and the cells assayed by their ability to degrade the gel. For example, in one set of embodiments, a gel may be formed using a protein, and a protease or other enzyme may be assayed for its ability to degrade the gel. The protease or other enzyme may be one that is excreted by the cell, and/or the cell may be lysed to release the protease into the gel. For instance, the cells may be lysed via exposure to a lysing chemical or a cell lysis reagent (e.g., a surfactant such as Triton-X or SDS, an enzyme such as lysozyme, lysostaphin, zymolyase, cellulase, mutanolysin, glucanases, proteases, mannase, proteinase K, etc.), or a physical condition (e.g., ultrasound, ultraviolet light, mechanical agitation, etc.). If a lysing chemical is used, the lysing chemical may be introduced into the droplet after formation of the droplet, e.g., through picoinjection, through fusion of the droplets with droplets containing the chemical or enzyme, etc.

Examples of gels that can be formed within droplets include, but are not limited to, hydrogels include, but are not limited to gelatin, collagen, agarose, or acrylamide-based gels, such as polyacrylamide, or poly-N-isopropylacrylamide. Other non-limiting examples of gels can be seen in Int. Pat. Apl. Pub. No. WO 2008/109176, incorporated herein by reference. For example, an aqueous solution of a monomer or other gel precursor may be dispersed in a droplet, and then polymerized or reacted, e.g., to form a gel. Another example is a hydrogel, such as alginic acid that can be gelled by the addition of calcium ions. As yet another example, a temperature change (e.g., cooling) may be used to cause a gel to form, e.g., with gelatin or agarose (e.g., low-melt agarose gel or ultra low-melt agarose gel). For instance, the gel may be chosen to be able to solidify upon exposure to relatively low temperatures, e.g., below about 60 °C, below about 50 °C, below about 40 °C, below about 35 °C, below 30 °C, below about 25 °C, below about 20 °C, below about 15 °C, below about 10 °C, below about 5 °C, etc. Such low-melt agarose gels may be readily obtained commercially.

In some cases, gelation initiators (ammonium persulfate and TEMED for acrylamide, or Ca^{2+} or other divalent ions such as Zn^{2+}, Mg^{2+}, etc. for alginate and other gels) can be added to a droplet, for example, by co-flow with the aqueous phase, by co-flow through the oil phase, or by coalescence of two different drops, e.g., as discussed in U.S. Patent Application Serial No. 11/360,845, filed February 23, 2006, entitled "Electronic Control of Fluidic Species," by Link, et al., published as U.S. Patent Application Publication No.

2007/000342 on January 4, 2007; or in U.S. Patent Application Serial No. 11/698,298, filed January 24, 2007, entitled "Fluidic Droplet Coalescence," by Ahn, et al.; each incorporated herein by reference in their entireties. In addition, in some cases, light, such as ultraviolet light, may be used to initiate gel formation.

In some cases, the droplet may be converted to a gel within a droplet without fusing the droplet to another droplet, or by injecting or otherwise adding an external species to the droplet after formation of the droplet. This may be advantageous, for example, in reducing errors that might be created by fusing two sets of droplets together. In one example, a droplet may be formed containing a gel precursor, which may then be caused to form a gel (for example, by polymerization) upon an appropriate stimulus.

In some embodiments, additional materials may also be present in the gels, e.g., before or after formation of the gels. For instance, some materials may be added to the droplets, before they are formed into gel, or the gels after formation may be exposed to the materials. In some cases, such materials may be able to react with agents released by the cells, e.g., to facilitate or inhibit degradation of the gels.

After (or during) formation of the gel, the cells may release, or caused to be released, one or more agents that can degrade the gel. Thus, for example, a plurality of cells may be contained within a plurality of droplets, and after the droplets form gels, the cells may be assayed for their ability to secrete proteins (e.g., proteases) that are able to degrade the gels.

For example, the cells may secrete such agents, and/or the cells may be lysed to release the agents. The agents can interact with the gel, e.g., to degrade the gel, via a variety of mechanisms, depending on the application. As non-limiting examples, certain types of proteases may cleave protein-based gels (such as gelatin, collagen, silk, peroxidase, transglutaminase polymerized proteins, etc.), agarases may cleave agarose, cellulases may cleave cellulose-derived gels, or pH changes (e.g., caused by secretion of acid or acidic compounds) may disrupt gel formation. Thus, for instance, cells may be assayed for their ability to produced different types of protease, produce acid, or the like. In some cases, the cells may be incubated within the gels to allow such degradation to occur, e.g., at temperatures of between 35 °C and 40 °C, 30 °C and 40 °C, 30 °C, and 50 °C, etc., and/or at temperatures normal to the cells (for example, 37 °C for mammalian and some bacteria cells), and in some cases, for a suitable amount of time, e.g., at least 1 hour, at least 2 hours, at least 3 hours, at least 4 hours, at least 6 hours, at least 9 hours, at least 12 hours, at least 24 hours, etc.

In addition, in another set of embodiments, the cell may actively take part in gel formation, which can be used to assay the cells, e.g., based on their ability to form the gel. For example, the cell may secrete one or more agents that can take part in gel formation. As a non-limiting example, a gel may be formed through the cross-linking of tyrosine residues in proteins with horseradish peroxidase and hydrogen peroxide. Cells may be assayed based on their ability to secrete a protein or other enzyme that produces hydrogen peroxide.

In one example, a suitable substrate (e.g. protein or synthetic polymers containing phenols) may react with hydrogen peroxide to form a hydrogel. The reaction may, for example, be catalyzed using a peroxidase, such as horseradish peroxidase, which produces a peroxidase-mediated crosslinking reaction, which chemically crosslinks phenolic groups between different molecules to form a gel. For instance, cells may be assayed based on their ability to secrete proteins, enzymes, synthetic polymers, or the like, or the activity of such secreted compounds, based on how much gel forms within a droplet.

In some cases, glucose oxidase reacts glucose with oxygen to produce gluconic acid and hydrogen peroxide. As an example, sorbitol may be oxidized with NAD^+ or $NADP^+$ by an alcohol dehydrogenase (e.g., EC 1.1.1.2) to produce glucose, and the enzyme to be assayed may form NAD^+ or $NADP^+$. Thus, for example, an enzyme efficient at producing NAD^+ or $NADP^+$ may provide the NAD^+ or $NADP^+$ for alcohol dehydrogenase to oxidize sorbitol to glucose, and a more efficient enzyme may result in more production of NAD^+ or $NADP^+$, more production of glucose, more production of hydrogen peroxide, and consequently, better and/or faster gel formation.

As another example, gluconic acid delta-lactone may be reduced using NADH or NADPH (or another aldehyde) to produce glucose, for example, using a glucose dehydrogenase, such as glucose 1-dehydrogenase or EC 1.1.1.47. Thus, an enzyme efficient at forming NADH or NADPH (or another aldehyde) may provide the substrate for glucose dehydrogenase in the form of NADH or NADPH, and a more efficient enzyme may result in more reduction of gluconic acid, more production of glucose, more production of hydrogen peroxide, and consequently, better and/or faster gel formation.

Thus, in certain embodiments, reactions such as these may be useful for assaying NADH/NAD^+ or NADPH/$NADP^+$ dependent enzymes, e.g., by causing the droplets to gel. Non-limiting examples of NADH-dependent enzymes include dehydrogenases (e.g., which may be NAD^+/NADH or $NADP^+$/NADPH dependent, oxidoreductases acting on the CH-OH group of donors with NAD^+ or $NADP^+$ as an acceptor (EC 1.1) (i.e., alcohol oxidoreductases), acting on the aldehyde or oxo group of donors (EC 1.2), acting on the CH-

CH group of donors with NAD^+ or $NADP^+$ as an acceptor (EC 1.3) (i.e., CH-CH oxidoreductases), acting on the CH-NH_2 group of donors with NAD^+ or $NADP^+$ as an acceptor (EC 1.4) (e.g., amino acid oxidoreductases, monoamine oxidases, etc., acting on the CH-NH group of donors with NAD^+ or $NADP^+$ as an acceptor (EC 1.5), acting on NADH or NADPH with NAD^+ or $NADP^+$ as an acceptor (EC 1.6), acting on other nitrogenous compounds as donors, with oxidase NAD^+ or $NADP^+$ as an acceptor (EC 1.7), acting on a sulfur group of donors with NAD^+ or $NADP^+$ as an acceptor (EC 1.8), or the like. Those of ordinary skill in the art will be familiar with such dehydrogenases and oxidoreductases.

It should be understood that in any of the methods herein using proteins such as enzymes (e.g., proteases, dehydrogenases, etc.), such proteins may be endogenous within the cell, or transfected or transformed into the cell. For instance, in some embodiments, any of the enzymes described herein may be transfected into a suitable cell. Those of ordinary skill in the art will know of systems and methods for transfecting a gene, such as a protease, into a cell, e.g., using electroporation, calcium phosphate, cationic polymers, lipofection, sonoporation, cell squeezing, protoplast fusion, optical transfection, gene guns, viruses, or the like.

In one aspect, the cells and droplets may accordingly be sorted or separated based on their size, for example, using filtration or other techniques. For instance, some cells may be contained within fully-gelled droplets, while other cells may be contained within liquid droplets or free of any droplets. While in some cases, such droplets can be sorted using sorting techniques which can only sort one droplet at a time, such as FACS or microfluidic single-cell sorters, in other cases, relatively large numbers of droplets can be simultaneously sorted using techniques that operate on larger numbers of cells, such as filtration or centrifugation. These may operate on the basis of size, or other suitable parameters.

For example, in one set of embodiments, the cells (which may or may not be in gel droplets) may be passed through a filter. Larger or more rigid droplets (e.g., gel droplets) may be unable to pass through the filter and accordingly stay within the retentate, while smaller or more flexible droplets, or free cells, may be able to pass through the filter and can be collected within the permeate.

A wide variety of filters may be obtained commercially, made from a variety of wide variety of materials (e.g., polymers such as polytetrafluoroethylene (PTFE), polyvinylidene fluoride (PVDF), polyethersulfone (PES), ceramics, etc.), and may be selected to allow different levels of stringency or filtration, i.e., based on the percentage of cells that are desired in the filtrate versus the retentate. For example, the filter may have an average pore

size of less than about 1 mm, less than about 500 micrometers, less than about 300 micrometers, less than about 200 micrometers, less than about 100 micrometers, less than about 75 micrometers, less than about 50 micrometers, less than about 30 micrometers, less than about 25 micrometers, less than about 20 micrometers, less than about 15 micrometers, less than about 10 micrometers, less than about 5 micrometers, less than about 3 micrometers, less than about 2 micrometers, less than about 1 micrometer, less than about 500 nm, less than about 300 nm, less than about 100 nm, or less than about 50 nm, and/or at least about 30 nm, at least about 50 nm, at least about 100 nm, at least about 300 nm, at least about 500 nm, at least about 1 micrometer, at least about 2 micrometers, at least about 3 micrometers, at least about 5 micrometers, at least about 10 micrometers, at least about 15 micrometers, or at least about 20 micrometers. Of course, it should be understood that the filtration process may not necessarily be perfect, and some gel droplets may pass through into the filtrate and/or some non-gel droplets may stay within retentate, e.g., based on the pore sizes and droplets sizes used. In addition, more than one filter may be used in some cases.

As another example, of a technique that acts on relatively large numbers of droplets, centrifugation may be used to sort droplets, e.g., based on their size. For example, after sorting, cells free of gel, and/or incomplete or degraded gel droplets, may be smaller and lighter than cells contained within gel droplets. Accordingly, by centrifuging such mixtures, the cells may be sorted and selectively removed. For example, in one set of embodiments, a sample containing cells (which may or may not be contained within gels) is centrifuged to cause differential separation of the cells/gels to occur, e.g., at speeds of at least 100 g, at least 300 g, at least 500 g, at least 1,000 g, etc. In some cases, the speeds may be sufficient to cause differential separation to occur, e.g., without lysing large percentages of the cells. Those of ordinary skill in the art will be aware of techniques for differentially centrifuging cells.

A variety of materials and methods, according to certain aspects of the invention, can be used to form articles or components such as those described herein, e.g., channels such as microfluidic channels, chambers, etc. For example, various articles or components can be formed from solid materials, in which the channels can be formed via micromachining, film deposition processes such as spin coating and chemical vapor deposition, laser fabrication, photolithographic techniques, etching methods including wet chemical or plasma processes, and the like. See, for example, *Scientific American*, 248:44-55, 1983 (Angell, *et al*).

In one set of embodiments, various structures or components of the articles described herein can be formed of a polymer, for example, an elastomeric polymer such as

polydimethylsiloxane ("PDMS"), polytetrafluoroethylene ("PTFE" or Teflon®), or the like. For instance, according to one embodiment, a microfluidic channel may be implemented by fabricating the fluidic system separately using PDMS or other soft lithography techniques (details of soft lithography techniques suitable for this embodiment are discussed in the references entitled "Soft Lithography," by Younan Xia and George M. Whitesides, published in the *Annual Review of Material Science*, 1998, Vol. 28, pages 153-184, and "Soft Lithography in Biology and Biochemistry," by George M. Whitesides, Emanuele Ostuni, Shuichi Takayama, Xingyu Jiang and Donald E. Ingber, published in the *Annual Review of Biomedical Engineering*, 2001, Vol. 3, pages 335-373; each of these references is incorporated herein by reference).

Other examples of potentially suitable polymers include, but are not limited to, polyethylene terephthalate (PET), polyacrylate, polymethacrylate, polycarbonate, polystyrene, polyethylene, polypropylene, polyvinylchloride, cyclic olefin copolymer (COC), polytetrafluoroethylene, a fluorinated polymer, a silicone such as polydimethylsiloxane, polyvinylidene chloride, bis-benzocyclobutene ("BCB"), a polyimide, a fluorinated derivative of a polyimide, or the like. Combinations, copolymers, or blends involving polymers including those described above are also envisioned. The device may also be formed from composite materials, for example, a composite of a polymer and a semiconductor material.

In some embodiments, various structures or components of the article are fabricated from polymeric and/or flexible and/or elastomeric materials, and can be conveniently formed of a hardenable fluid, facilitating fabrication via molding (e.g. replica molding, injection molding, cast molding, etc.). The hardenable fluid can be essentially any fluid that can be induced to solidify, or that spontaneously solidifies, into a solid capable of containing and/or transporting fluids contemplated for use in and with the fluidic network. In one embodiment, the hardenable fluid comprises a polymeric liquid or a liquid polymeric precursor (i.e. a "prepolymer"). Suitable polymeric liquids can include, for example, thermoplastic polymers, thermoset polymers, waxes, metals, or mixtures or composites thereof heated above their melting point. As another example, a suitable polymeric liquid may include a solution of one or more polymers in a suitable solvent, which solution forms a solid polymeric material upon removal of the solvent, for example, by evaporation. Such polymeric materials, which can be solidified from, for example, a melt state or by solvent evaporation, are well known to those of ordinary skill in the art. A variety of polymeric materials, many of which are elastomeric, are suitable, and are also suitable for forming molds or mold masters, for embodiments where

one or both of the mold masters is composed of an elastomeric material. A non-limiting list of examples of such polymers includes polymers of the general classes of silicone polymers, epoxy polymers, and acrylate polymers. Epoxy polymers are characterized by the presence of a three-membered cyclic ether group commonly referred to as an epoxy group, 1,2-epoxide, or oxirane. For example, diglycidyl ethers of bisphenol A can be used, in addition to compounds based on aromatic amine, triazine, and cycloaliphatic backbones. Another example includes the well-known Novolac polymers. Non-limiting examples of silicone elastomers suitable for use according to the invention include those formed from precursors including the chlorosilanes such as methylchlorosilanes, ethylchlorosilanes, phenylchlorosilanes, dodecyltrichlorosilanes, etc.

Silicone polymers are used in certain embodiments, for example, the silicone elastomer polydimethylsiloxane. Non-limiting examples of PDMS polymers include those sold under the trademark Sylgard by Dow Chemical Co., Midland, MI, and particularly Sylgard 182, Sylgard 184, and Sylgard 186. Silicone polymers including PDMS have several beneficial properties simplifying fabrication of various structures of the invention. For instance, such materials are inexpensive, readily available, and can be solidified from a prepolymeric liquid via curing with heat. For example, PDMSs are typically curable by exposure of the prepolymeric liquid to temperatures of about, for example, about 65 °C to about 75 °C for exposure times of, for example, about an hour. Also, silicone polymers, such as PDMS, can be elastomeric and thus may be useful for forming very small features with relatively high aspect ratios, necessary in certain embodiments of the invention. Flexible (e.g., elastomeric) molds or masters can be advantageous in this regard.

One advantage of forming structures such as microfluidic structures or channels from silicone polymers, such as PDMS, is the ability of such polymers to be oxidized, for example by exposure to an oxygen-containing plasma such as an air plasma, so that the oxidized structures contain, at their surface, chemical groups capable of cross-linking to other oxidized silicone polymer surfaces or to the oxidized surfaces of a variety of other polymeric and non-polymeric materials. Thus, structures can be fabricated and then oxidized and essentially irreversibly sealed to other silicone polymer surfaces, or to the surfaces of other substrates reactive with the oxidized silicone polymer surfaces, without the need for separate adhesives or other sealing means. In most cases, sealing can be completed simply by contacting an oxidized silicone surface to another surface without the need to apply auxiliary pressure to form the seal. That is, the pre-oxidized silicone surface acts as a contact adhesive against suitable mating surfaces. Specifically, in addition to being irreversibly sealable to itself,

oxidized silicone such as oxidized PDMS can also be sealed irreversibly to a range of oxidized materials other than itself including, for example, glass, silicon, silicon oxide, quartz, silicon nitride, polyethylene, polystyrene, glassy carbon, and epoxy polymers, which have been oxidized in a similar fashion to the PDMS surface (for example, via exposure to an oxygen-containing plasma). Oxidation and sealing methods useful in the context of the present invention, as well as overall molding techniques, are described in the art, for example, in an article entitled "Rapid Prototyping of Microfluidic Systems and Polydimethylsiloxane," *Anal. Chem.*, 70:474-480, 1998 (Duffy *et al.*), incorporated herein by reference.

Thus, in certain embodiments, the design and/or fabrication of the article may be relatively simple, e.g., by using relatively well-known soft lithography and other techniques such as those described herein. In addition, in some embodiments, rapid and/or customized design of the article is possible, for example, in terms of geometry. In one set of embodiments, the article may be produced to be disposable, for example, in embodiments where the article is used with substances that are radioactive, toxic, poisonous, reactive, biohazardous, etc., and/or where the profile of the substance (e.g., the toxicology profile, the radioactivity profile, etc.) is unknown. Another advantage to forming channels or other structures (or interior, fluid-contacting surfaces) from oxidized silicone polymers is that these surfaces can be much more hydrophilic than the surfaces of typical elastomeric polymers (where a hydrophilic interior surface is desired). Such hydrophilic channel surfaces can thus be more easily filled and wetted with aqueous solutions than can structures comprised of typical, unoxidized elastomeric polymers or other hydrophobic materials.

The following documents are incorporated herein by reference: Int. Pat. Apl. Pub. No. WO 2004/091763, entitled "Formation and Control of Fluidic Species," by Link *et al.*; Int. Pat. Apl. Pub. No. WO 2004/002627, entitled "Method and Apparatus for Fluid Dispersion," by Stone *et al.*; Int. Pat. Apl. Pub. No. WO 2006/096571, entitled "Method and Apparatus for Forming Multiple Emulsions," by Weitz *et al.*; Int. Pat. Apl. Pub. No. WO 2005/021151, entitled "Electronic Control of Fluidic Species," by Link *et al.*; Int. Pat. Apl. Pub. No. WO 2011/056546, entitled "Droplet Creation Techniques," by Weitz, *et al.*; Int. Pat. Apl. Pub. No. WO 2010/033200, entitled "Creation of Libraries of Droplets and Related Species," by Weitz, *et al.*; U.S. Pat. Apl. Pub. No. 2012-0132288, entitled "Fluid Injection," by Weitz, *et al.*; Int. Pat. Apl. Pub. No. WO 2008/109176, entitled "Assay And Other Reactions Involving Droplets," by Agresti, *et al.*; Int. Pat. Apl. Pub. No. WO 2010/151776, entitled "Fluid Injection," by Weitz, *et al.*; and U.S. Pat. Apl. Ser. No. 62/810,779, filed

February 26, 2019, entitled "Systems and Methods for High Throughput Selection," by Weitz, *et al.*

The following examples are intended to illustrate certain embodiments of the present invention, but do not exemplify the full scope of the invention.

EXAMPLE 1

Fig. 2 illustrates schematically an experiment demonstrating a selection assay. In this example, two bacterial populations, one of which produced a lot of protease and one of which produced less protease, were mixed at a ratio of 1:1. After encapsulation into liquid droplets containing gelatin, incubation at 37 °C, and cooling the droplets to 4 °C to gel the gelatin as shown in Fig. 2, the gelatin droplets were filtered and plated onto a suitable growth medium. The bacterial population producing increased protease was found to be highly enriched in the filtrate.

EXAMPLE 2

This example illustrates various material and methods useful in certain embodiments of the invention.

Overnight cultures grown in Lauria-Bertani medium containing 6 micrograms/ml chloramphenicol of *Bacillus subtilis* 168 containing plasmid pSEVA3b61 and *Bacillus Subtilis* KO7-S (ΔnprE, ΔaprE, Δepr, Δmpr, ΔnprB, Δvpr, Δbpr, ΔsigF, Bacillus Genetic Stock Center ID: 1S145, The Bacillus Genetic Stock Center Columbus, OH, USA) containing a modified version of pSEVA3b61, with a gene for the overexpression of subtilisin (see Fig. 4), were mixed at a 1:1 cell ratio. The mixture of cells was diluted in mineral medium (22 mM KH_2PO_4, 20 mM Na_2HPO_4, 18.7 mM NH_4Cl, 11 mM NaCl,1 mM $MgSO_4$, 0.1 mM $CaSO_4$, Trace elements (per liter): 50 mg EDTA, 8.3 mg $FeCl_3 \cdot 6H2O$, 0.84 mg $ZnCl_2$, 130 micrograms $CuCl_2 \cdot 2H_2O$, 100 micrograms $CoCl_2 \cdot 6H_2O$, 100 micrograms H_3BO_3, 16 micrograms $MnCl_2 \cdot 6\ H_2O$) containing 15% OptiPrep™ (Cat. No.: D1556, Sigma Aldrich, Darmstadt, Germany) to an appropriate optical density (OD_{600}), for 40 micrometer drops an OD_{600} of 0.4 is optimal. This cell suspension was denoted as dispersed phase #1. Disperse phase #2 used 4% (w/w) gelatin from porcine skin (Cat. No. G2500, Sigma Aldrich, Darmstadt, Germany) in mineral medium at 37 °C.

The continuous phase was 3M™ Novec™ 7500 Engineered Fluid containing 2% (v/v) 008-FluoroSurfactant (RAN Biotechnologies, Beverly, MA 01915, USA).

With a polydimethylsiloxane (PDMS) microfluidics drop-maker of about 35 micrometers thick (see Fig. 3), cells were encapsulated by co-flowing dispersed phase #1 at a flowrate of 50-100 microliters/h and dispersed phase #2 at a flowrate of 150-300

microliters/h with the continuous phase at a flowrate of 1000-1500 microliters/h. Disperse phase #1 was kept cold with ice during the encapsulation and disperse phase #2 was kept at around 37 °C to keep the gelatin liquid. The encapsulated cells with gelatin were collected into a 1.5 ml Eppendorf™ tube which was kept on ice during the collection.

After collecting about 0.5 ml of total volume the droplets were incubated at 37 °C for 2-4 h, followed by a transfer back on ice, and maintained for 0.5 to 1 hour to ensure the complete gelling of gelatin in the droplets.

After cooling and complete gelling of the gelling agent, gelatin, the emulsion was disrupted with 1H,1H,2H,2H-perfluoro-1-octanol (Cat. No.: 370533, Sigma Aldrich, Darmstadt, Germany) and the gelatin hydrogel microspheres were passed through a pluriStrainer Mini 10 micrometer cell strainer (pluriSelect Life Science, Leipzig, Germany). The best protease-secreting cells pass through the filter because the gelatin which they were encapsulated in was digested while worse secreting cells are retained on the filter because they were entrapped in the gelatin hydrogel matrix.

The filtrate was plated on Luria-Bertani agar plates containing 6 microgram/ml chloramphenicol incubated overnight at 37 °C. After overnight growth, the best protease-secreting cells could be used for further analysis and validation.

While several embodiments of the present invention have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the functions and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be within the scope of the present invention. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the teachings of the present invention is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, the invention may be practiced otherwise than as specifically described and claimed. The present invention is directed to each individual feature, system, article, material, kit, and/or method described herein. In addition, any combination of two or more such features, systems, articles,

materials, kits, and/or methods, if such features, systems, articles, materials, kits, and/or methods are not mutually inconsistent, is included within the scope of the present invention.

In cases where the present specification and a document incorporated by reference include conflicting and/or inconsistent disclosure, the present specification shall control. If two or more documents incorporated by reference include conflicting and/or inconsistent disclosure with respect to each other, then the document having the later effective date shall control.

All definitions, as defined and used herein, should be understood to control over dictionary definitions, definitions in documents incorporated by reference, and/or ordinary meanings of the defined terms.

The indefinite articles "a" and "an," as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean "at least one."

The phrase "and/or," as used herein in the specification and in the claims, should be understood to mean "either or both" of the elements so conjoined, i.e., elements that are conjunctively present in some cases and disjunctively present in other cases. Multiple elements listed with "and/or" should be construed in the same fashion, i.e., "one or more" of the elements so conjoined. Other elements may optionally be present other than the elements specifically identified by the "and/or" clause, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, a reference to "A and/or B", when used in conjunction with open-ended language such as "comprising" can refer, in one embodiment, to A only (optionally including elements other than B); in another embodiment, to B only (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); etc.

As used herein in the specification and in the claims, "or" should be understood to have the same meaning as "and/or" as defined above. For example, when separating items in a list, "or" or "and/or" shall be interpreted as being inclusive, i.e., the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as "only one of" or "exactly one of," or, when used in the claims, "consisting of," will refer to the inclusion of exactly one element of a number or list of elements. In general, the term "or" as used herein shall only be interpreted as indicating exclusive alternatives (i.e. "one or the other but not both") when preceded by terms of exclusivity, such as "either," "one of," "only one of," or "exactly one of."

As used herein in the specification and in the claims, the phrase "at least one," in reference to a list of one or more elements, should be understood to mean at least one element selected from any one or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. This definition also allows that elements may optionally be present other than the elements specifically identified within the list of elements to which the phrase "at least one" refers, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, "at least one of A and B" (or, equivalently, "at least one of A or B," or, equivalently "at least one of A and/or B") can refer, in one embodiment, to at least one, optionally including more than one, A, with no B present (and optionally including elements other than B); in another embodiment, to at least one, optionally including more than one, B, with no A present (and optionally including elements other than A); in yet another embodiment, to at least one, optionally including more than one, A, and at least one, optionally including more than one, B (and optionally including other elements); etc.

When the word "about" is used herein in reference to a number, it should be understood that still another embodiment of the invention includes that number not modified by the presence of the word "about."

It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

In the claims, as well as in the specification above, all transitional phrases such as "comprising," "including," "carrying," "having," "containing," "involving," "holding," "composed of," and the like are to be understood to be open-ended, i.e., to mean including but not limited to. Only the transitional phrases "consisting of" and "consisting essentially of" shall be closed or semi-closed transitional phrases, respectively, as set forth in the United States Patent Office Manual of Patent Examining Procedures, Section 2111.03.

What is claimed is:

CLAIMS

1. A method, comprising:
encapsulating cells within liquid droplets;
causing the liquid droplets containing the cells to form gel droplets;
causing at least some of the cells within the gel droplets to degrade the gel; and
separating the gel-degrading cells from the non-gel-degrading cells via a filter.

2. The method of claim 1, wherein the gel droplets comprise gelatin.

3. The method of any one of claims 1 or 2, wherein the gel droplets comprise agarose.

4. The method of any one of claims 1-3, wherein the gel droplets comprise acrylamide.

5. The method of any one of claims 1-4, wherein the gel droplets comprise alginate.

6. The method of any one of claims 1-5, wherein the gel droplets comprise collagen.

7. The method of any one of claims 1-6, wherein the liquid droplets comprises a gel precursor.

8. The method of claim 7, wherein at least some of the gel precursor is added after encapsulating the cells.

9. The method of any one of claims 7 or 8, wherein at least some of the gel precursor is added at formation of the liquid droplets.

10. The method of any one of claims 1-9, wherein at least some of the cells secrete an agent able to degrade the gel.

11. The method of any one of claims 1-10, wherein the agent comprises an enzyme.

12. The method of any one of claims 1-11, wherein the agent comprises protease.

13. The method of any one of claims 1-12, wherein the agent comprises acid.

14. The method of any one of claims 1-13, wherein causing at least some of the cells within the gel droplets to degrade the gel comprises incubating the cells.

15. The method of claim 14, comprising incubating the cells at a temperature of between 35 °C and 50 °C.

16. The method of any one of claims 14 or 15, comprising incubating the cells at about 37 °C.

17. The method of any one of claims 1-16, wherein causing at least some of the cells within the gel droplets to degrade the gel comprises waiting for a period of time.

18. The method of claim 17, comprising waiting for at least 1 hour.

19. The method of any one of claims 17 or 18, comprising waiting for at least 2 hours.

20. The method of any one of claims 17-19, comprising waiting for at least 6 hours.

21. The method of any one of claims 17-20, comprising waiting for at least 12 hours.

22. The method of any one of claims 1-21, wherein causing the liquid droplets containing the cells to form gel droplets comprises cooling the liquid droplets to cause the liquid droplets to gel.

23. The method of claim 22, wherein the liquid droplets are cooled to below 20 °C.

24. The method of any one of claims 22 or 23, wherein the liquid droplets are cooled to below 5 °C.

25. The method of any one of claims 1-24, wherein causing the liquid droplets containing the cells to form gel droplets comprises exposing the liquid droplets to ultraviolet light.

26. The method of any one of claims 1-25, wherein causing the liquid droplets containing the cells to form gel droplets comprises exposing the gel droplets to an initiator.

27. The method of any one of claims 1-26, wherein causing the liquid droplets containing the cells to form gel droplets comprises exposing the gel droplets to a divalent ion.

28. The method of any one of claims 1-27, wherein causing the liquid droplets containing the cells to form gel droplets comprises exposing the gel droplets to Ca^{2+}.

29. The method of any one of claims 1-28, wherein the filter selectively passes gel droplets on the basis of size.

30. The method of any one of claims 1-29, wherein the filter selectively passes gel-degrading cells with respect to non-degrading cells.

31. The method of any one of claims 1-30, wherein the filter has a pore size of less than 20 micrometers.

32. The method of any one of claims 1-31, wherein the filter has a pore size of less than 10 micrometers.

33. The method of any one of claims 1-32, wherein at least some of the cells are prokaryotes.

34. The method of any one of claims 1-33, wherein at least some of the cells are eukaryotes.

35. The method of any one of claims 1-34, wherein at least some of the cells are bacteria.

36. The method of any one of claims 1-35, wherein at least some of the cells are mammalian cells.

37. The method of any one of claims 1-36, wherein at least some of the cells are transfected with a protease gene.

38. The method of any one of claims 1-37, wherein at least some of the cells are transfected with a dehydrogenase gene.

39. The method of any one of claims 1-38, wherein the liquid droplets are microfluidic.

40. The method of any one of claims 1-39, wherein the liquid droplets have an average diameter of less than about 1 mm.

41. The method of any one of claims 1-40, comprising encapsulating the cells within the liquid droplets within a microfluidic device.

42. The method of any one of claims 1-41, comprising encapsulating the cells within the liquid droplets at an average of 1 cell/droplet or less.

43. The method of any one of claims 1-42, comprising encapsulating the cells within the liquid droplets at an average of 0.1 cells/droplet or less.

44. The method of any one of claims 1-43, wherein separating the cells comprises filtering at least 10^3 cells.

45. The method of any one of claims 1-44, wherein separating the cells comprises filtering at least 10^4 cells.

46. The method of any one of claims 1-45, wherein separating the cells comprises filtering at least 10^5 cells.

47. The method of any one of claims 1-46, wherein separating the cells comprises filtering at least 10^6 cells.

48. The method of any one of claims 1-47, wherein separating the cells comprises filtering at least 10^7 cells.

49. The method of any one of claims 1-48, wherein separating the cells comprises filtering at least 10^8 cells.

50. The method of any one of claims 1-49, wherein separating the cells comprises filtering at least 10^9 cells.

51. A method, comprising:

encapsulating cells within liquid droplets;

causing at least some of the cells to gel the liquid droplet to form a gel droplet; and

separating the cells contained within gel droplets from cells contained within liquid droplets via a filter.

52. The method of claim 51, wherein causing at least some of the cells to gel the liquid droplet to form a gel droplet comprises causing the cells to produce hydrogen peroxide.

53. The method of claim 52, wherein the hydrogen peroxide reacts with a peroxidase and a protein within the droplet to form the gel.

54. The method of claim 53, wherein the peroxidase comprises horseradish peroxidase.

55. The method of any one of claims 52-54, wherein the cells produce hydrogen peroxide via reaction of glucose with oxygen to produce gluconic acid and hydrogen peroxide, catalyzed by an glucose oxidase.

56. The method of claim 55, wherein the glucose is produced via reaction of a sugar with NAD^+ and/or $NADP^+$, catalyzed via an alcohol dehydrogenase.

57. The method of claim 56, wherein the alcohol dehydrogenase comprises EC 1.1.1.2.

58. The method of any one of claims 55-57, wherein the glucose is produced via reaction of gluconic acid delta-lactone with NADH and/or NADPH, catalyzed via a glucose dehydrogenase.

59. The method of claim 58, wherein the glucose dehydrogenase comprises a glucose 1-dehydrogenase.

60. The method of any one of claims 58 or 59, wherein the glucose dehydrogenase comprises EC 1.1.1.47.

61. The method of any one of claims 51-60, wherein the filter has a pore size of less than 20 micrometers.

62. The method of any one of claims 51-61, wherein the filter has a pore size of less than 10 micrometers.

63. The method of any one of claims 51-62, wherein at least some of the cells are prokaryotes.

64. The method of any one of claims 51-63, wherein at least some of the cells are eukaryotes.

65. The method of any one of claims 51-64, wherein at least some of the cells are bacteria.

66. The method of any one of claims 51-65, wherein at least some of the cells are mammalian cells.

67. The method of any one of claims 51-66, wherein at least some of the cells are transfected with a protease gene.

68. The method of any one of claims 51-67, wherein at least some of the cells are transfected with a dehydrogenase gene.

69. The method of any one of claims 51-68, wherein at least some of the cells are transfected with a dehydrogenase gene which is NAD^+/NADH or $NADP^+$/NADPH dependent.

70. The method of any one of claims 51-69, wherein the liquid droplets are microfluidic.

71. The method of any one of claims 51-70, wherein the liquid droplets have an average diameter of less than about 1 mm.

72. The method of any one of claims 51-71, comprising encapsulating the cells within the liquid droplets within a microfluidic device.

73. The method of any one of claims 51-72, comprising encapsulating the cells within the liquid droplets at an average of 1 cell/droplet or less.

74. The method of any one of claims 51-73, comprising encapsulating the cells within the liquid droplets at an average of 0.1 cells/droplet or less.

75. The method of any one of claims 51-74, wherein separating the cells comprises filtering at least 10^3 cells.

76. The method of any one of claims 51-75, wherein separating the cells comprises filtering at least 10^4 cells.

77. The method of any one of claims 51-76, wherein separating the cells comprises filtering at least 10^5 cells.

78. The method of any one of claims 51-77, wherein separating the cells comprises filtering at least 10^6 cells.

79. The method of any one of claims 51-78, wherein separating the cells comprises filtering at least 10^7 cells.

80. The method of any one of claims 51-79, wherein separating the cells comprises filtering at least 10^8 cells.

81. The method of any one of claims 51-80, wherein separating the cells comprises filtering at least 10^9 cells.

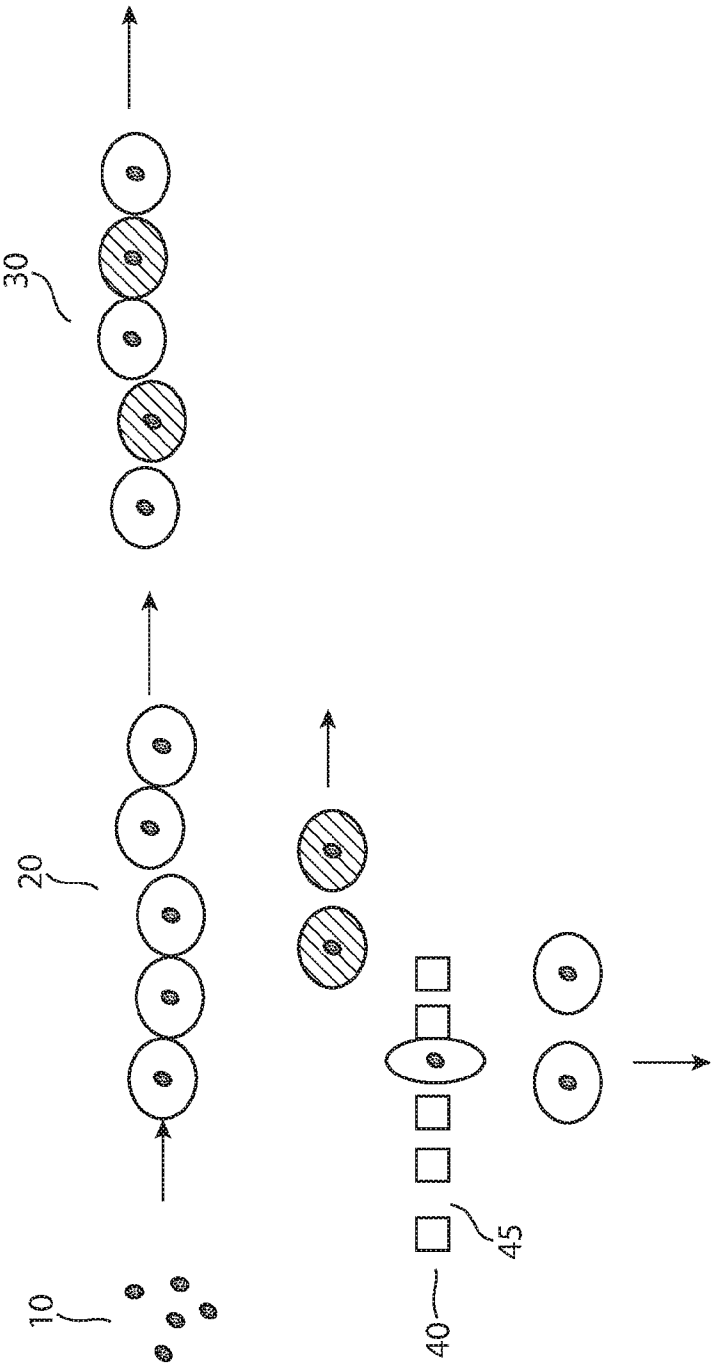

FIG. 1

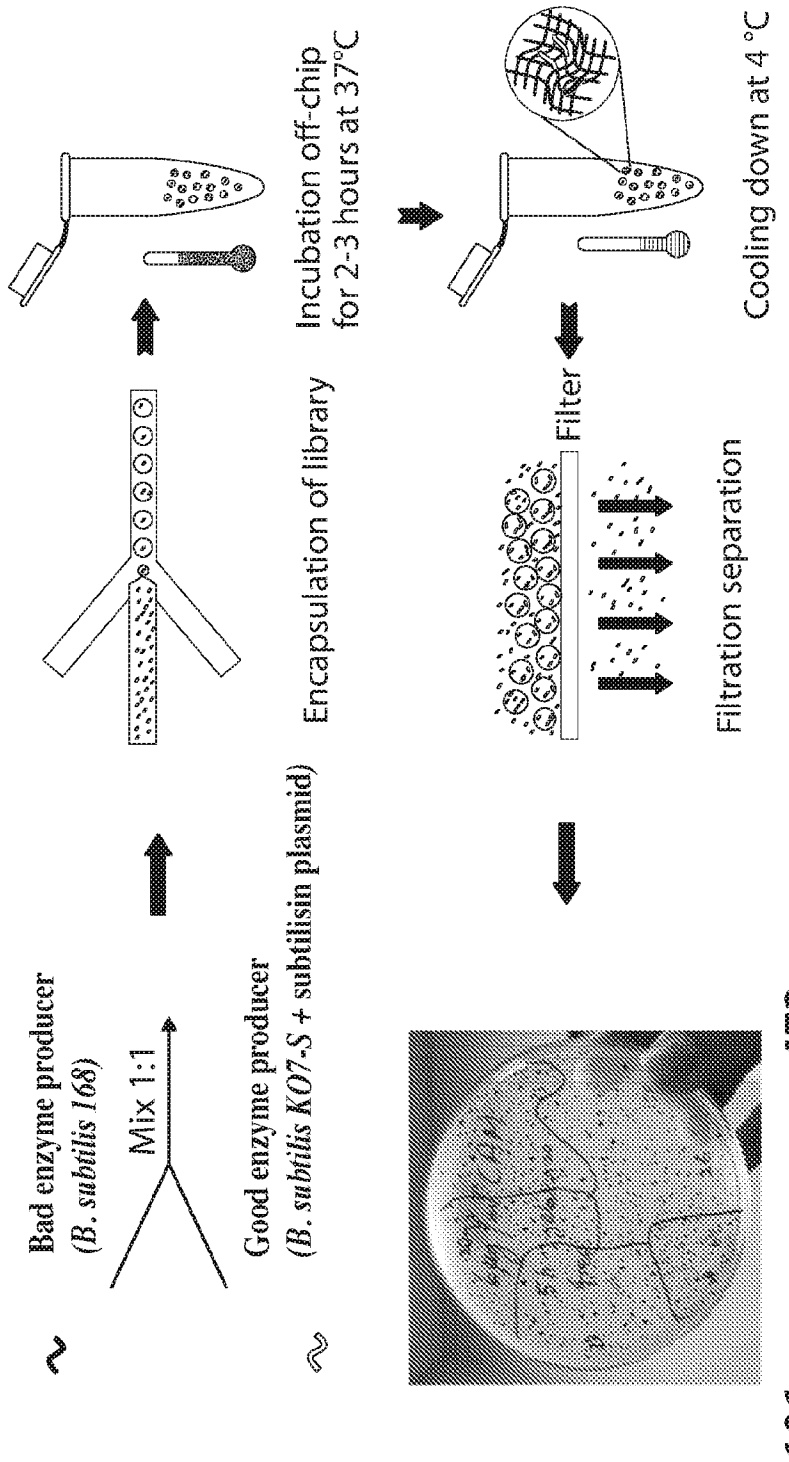
Bad enzyme producer
(B. subtilis 168)
Mix 1:1
Good enzyme producer
(B. subtilis KO7-S + subtilisin plasmid)
Encapsulation of library
Incubation off-chip
for 2-3 hours at 37°C
Filter
Filtration separation
Cooling down at 4 °C
106
473
B.Subtillis 168 with RFP
KO7-S with subtillisin

FIG. 2

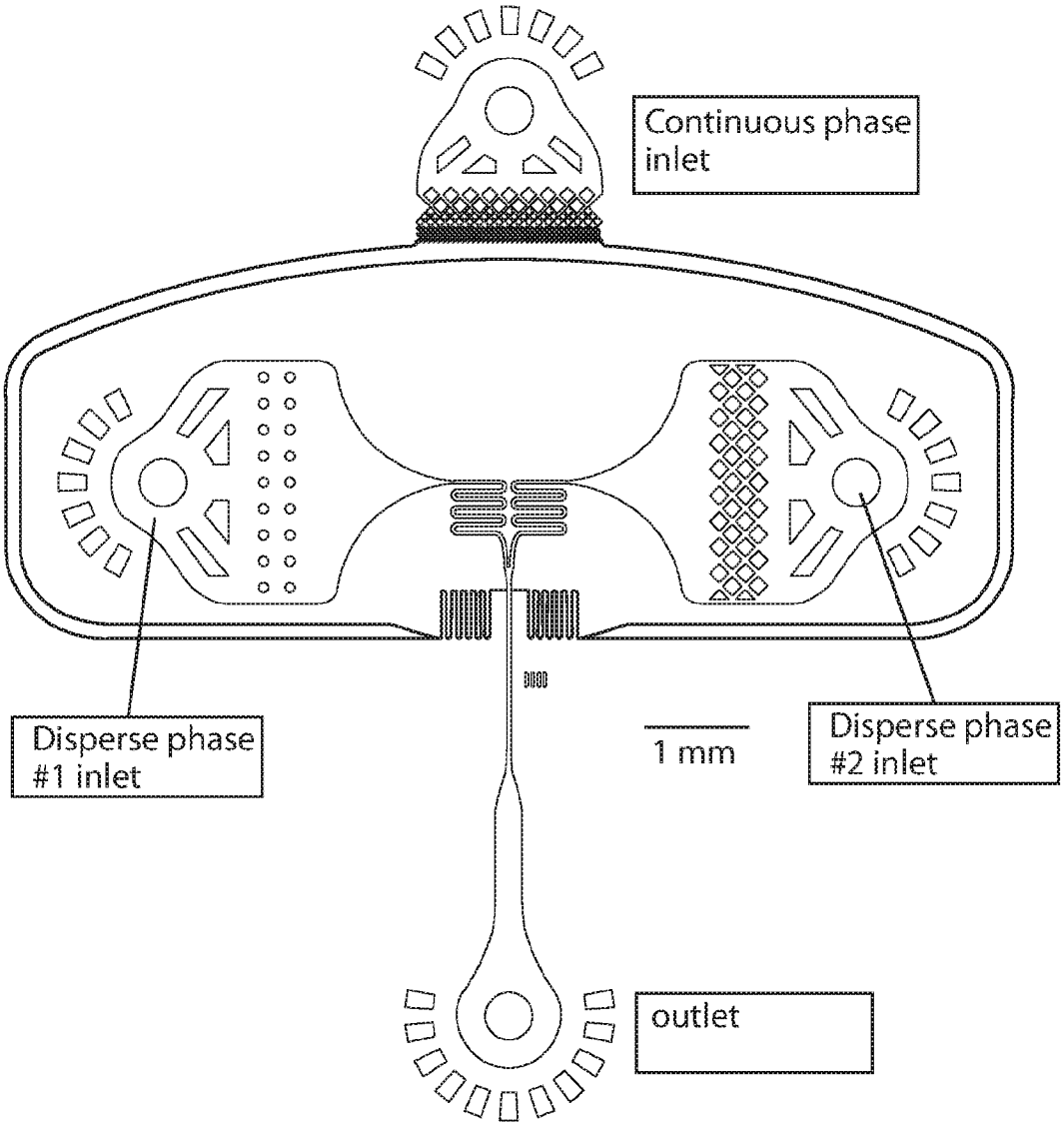
Continuous phase inlet
Disperse phase #1 inlet
1 mm
Disperse phase #2 inlet
outlet

FIG. 3

GCTGCGGGTGCGCTGCCACATGCGCGGTCCCGAGATCATGTCTGATGGCGGTCTC
ATGTTCCGGTTCTGAACATGCCGGTATGAAGAGGACGGATAAGATCAGCAGCATA
CTGAAAATCGGTTTCATCAGTTCCCCCTCTTTCGTTTTTCCGCAATTATATCATT
GACAATATCAACATCAATGATATTCATTATCATTATTTTTATAAAATGGTTTCAC
AGCTTTTCTCGGTCAAGAAAGCCAAAGACTGATTTCGCTTACGTTTCCATCAGTC
TTCTGTATTCAACAAAAGATGACATTTATCCTGTTTTTGGAACAACCCCCAAAAA
TGGAAACAAACCGTTCGACCCAGGAAACAAGCGAGTGATTGCTCCTGTGTACATT
TACTCATGTCCATCCATCGGTTTTTTCCATTAAAATTTAAATATTTCGAGTTCCT
ACGAAACGAAAGAGAGATGATATACCTAAATAGAAATAAAACAATCTGAAAAAAA
TTGGGTCTACTAAAATATTATTCCATACTATACAATTAATCAACAGAATAATCTG
TCTATTGGTTATTCTGCAAATGAAAAAAAGGAGAGGATAAAGAGTGAGAGGCAAA
AAAGTATGGATCAGTTTGCTGTTTGCTTTAGCGTTAATCTTTACGATGGCGTTCG
GCAGCACATCCTCTGCCCAGGCGGCAGGGAAATCAAACGGGGAAAAGAAATATAT
TGTCGGGTTTAAACAGACAATGAGCACGATGAGCGCCGCTAAGAAGAAAGATGTC
ATTTCTGAAAAAGGCGGGAAAGTGCAAAAGCAATTCAAATATGTAGACGCAGCTT
CAGCTACATTAAACGAAAAAGCTGTAAAAGAATTGAAAAAAGACCCGAGCGTCGC
TTACGTTGAAGAAGATCACGTAGCACATGCGTACGCGCAGTCCGTGCCTTACGGC
GTATCACAAATTAAAGCCCCTGCTCTGCACTCTCAAGGCTACACTGGATCAAATG
TTAAAGTAGCGGTTATCGACAGCGGTATCGATTCTTCTCATCCTGATTTAAAGGT
AGCAGGCGGAGCCAGCATGGTTCCTTCTGAAACAAATCCTTTCCAAGACAACAAC
TCTCACGGAACTCACGTTGCCGGCACAGTTGCGGCTCTTAATAACTCAATCGGTG
TATTAGGCGTTGCGCCAAGCGCATCACTTTACGCTGTAAAAGTTCTCGGTGCTGA
CGGTTCCGGCCAATACAGCTGGATCATTAACGGAATCGAGTGGGCGATCGCAAAC
AATATGGACGTTATTAACATGAGCCTCGGCGGACCTTCTGGTTCTGCTGCTTTAA
AAGCGGCAGTTGATAAAGCCGTTGCATCCGGCGTCGTAGTCGTTGCGGCAGCCGG
TAACGAAGGCACTTCCGGCAGCTCAAGCACAGTGGGCTACCCTGGTAAATACCCT
TCTGTCATTGCAGTAGGCGCTGTTGACAGCAGCAACCAAAGAGCATCTTTCTCAA
GCGTAGGACCTGAGCTTGATGTCATGGCACCTGGCGTATCTATCCAAAGCACGCT
TCCTGGAAACAAATACGGGGCGTACAACGGTACGTCAATGGCATCTCCGCACGTT
GCCGGAGCGGCTGCTTTGATTCTTTCTAAGCACCCGAACTGGACAAACACTCAAG
TCCGCAGCAGTTTAGAAAACACCACTACAAAACTTGGTGATTCTTTCTACTATGG
AAAAGGGCTGATCAACGTACAGGCGGCAGCTCAGTAAAACATAAAAAACCGGCGT
CGGCCTTGGCCCCGCCGGTTTTTTATTATTTTTCTTCCTCCGCATGTTCAATCCG
CTCCATAATCGACGGATGGCTCCCTCTGAAAATTTTAACGAGAAACGGCGGGTTG
ACCCGGCTCAGTCCCGTAACGGCCAAGTCCTGAAACGTCTCAATCGCCGCTTCCC
GGTTTCCGGTCAGCTCAATGCCGTAACGGTCGGCGGCGTTTTCCTGATACCGGGA
GACGGCATTCGTAATCGGATCAGAAGCAAAACTGAGCACGGATATAAGCAGCAAT
AACAGCGGGAGAGCGGCCAGATCTTCAGGCCCCTTTATATGAAGCATTCGGCCAA
AGGATTTGGACATCCGCCTGTAAAGCTTGTCAATGATATAGAACCCAACCAATGA
CAGCAGCAGATACCCGGCCAGTCCGATGTACACGTGCTTCATGACATAGTGCCCC
ATCTCATGCCCCATAATGAACAAAATTTCCTGATCCTTCAGTTTGTTAAGCGTCG
TATCCCACAAAACGATGCGCTTATTCGAGCCGATGCCCGTCACATACGCATTCAG
CGCCGCGCGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGC

FIG. 4

ATGCAAGCTTGCGGCCGCGTCGTGACTGGGAAAACCCTGGCGACTAGTCTTGGAC
TCCTGTTGATAGATCCAGTAATGACCTCAGAACTCCATCTGGATTTGTTCAGAAC
GCTCGGTTGCCGCCGGGCGTTTTTTATTGGTGAGAATCCAGGGGTCCCCAATAAT
TACGATTTAAATCCTTCAAACTTCCCAAAGGCGAGCCCTAGTGACATTAGAAAAC
CGACTGTAAAAAGTACAGTCGGCATTATCTCATATTATAAAAGCCAGTCATTAGG
CCTATCTGACAATTCCTGAATAGAGTTCATAAACAATCCTGCATGATAACCATCA
CAAACAGAATGATGTACCTGTAAAGATAGCGGTAAATATATTGAATTACCTTTAT
TAATGAATTTTCCTGCTGTAATAATGGGTAGAAGGTAATTACTATTATTATTGAT
ATTTAAGTTAAACCCAGTAAATGAAGTCCATGGAATAATAGAAAGAGAAAAAGCA
TTTTCAGGTATAGGTGTTTTGGGAAACAATTTCCCCGAACCATTATATTTCTCTA
CATCAGAAAGGTATAAATCATAAAACTCTTTGAAGTCATTCTTTACAGGAGTCCA
AATACCAGAGAATGTTTTAGATACACCATCAAAAATTGTATAAAGTGGCTCTAAC
TTATCCCAATAACCTAACTCTCCGTCGCTATTGTAACCAGTTCTAAAAGCTGTAT
TTGAGTTTATCACCCTTGTCACTAAGAAAATAAATGCAGGGTAAAATTTATATCC
TTCTTGTTTTATGTTTCGGTATAAAACACTAATATCAATTTCTGTGGTTATACTA
AAAGTCGTTTGTTGGTTCAAATAATGATTAAATATCTCTTTTCTCTTCCAATTGT
CTAAATCAATTTTATTAAAGTTCATTTGATATGCCTCCTAAATTTTTATCTAAAG
TGAATTTAGGAGGCTTACTTGTCTGCTTTCTTCATTAGAATCAATCCTTTTTTAA
AAGTCAATCCCGTTTGTTGAAGACTTTTGTCCTTTTCCGCTGCATAACCCTGCTT
CGGGGTCATTATAGCGATTTTTTCGGTATATCCATCCTTTTTCGCACGATATACA
GGATTTTGCCAAAGGGTTCGTGTAGACTTTCCTTGGTGTATCCAACGGCGTCAGC
CGGGCAGGATAGGTGAAGTAGGCCCACCCGCGAGCGGGTGTTCCTTCTTCACTGT
CCCTTATTCGCACCTGGCGGTGCTCAACGGGAATCCTGCTCTGCGAGGCTGGCCG
TAGGCCGGCCTAGAATCAATCCTTTTTTAAAAGTCAATCCCGTTTGTTGAACTAC
TCTTTAATAAAATAATTTTTCCGTTCCCAATTCCACATTGCAATAATAGAAAATC
CATCTTCATCGGCTTTTTCGTCATCATCTGTATGAATCAAATCGCCTTCTTCTGT
GTCATCAAGGTTTAATTTTTTATGTATTTCTTTTAACAAACCACCATAGGAGATT
AACCTTTTACGGTGTAAACCTTCCTCCAAATCAGACAAACGTTTCAAATTCTTTT
CTTCATCATCGGTCATAAAATCCGTATCCTTTACAGGATATTTTGCAGTTTCGTC
AATTGCCGATTGTATATCCGATTTATATTTATTTTTCGGTCGAATCATTTGAACT
TTTACATTTGGATCATAGTCTAATTTCATTGCCTTTTTCCAAAATTGAATCCATT
GTTTTTGATTCACGTAGTTTTCTGTATTCTTAAAATAAGTTGGTTCCACACATAC
CAATACATGCATGTGCTGATTATAAGAATTATCTTTATTATTTATTGTCACTTCC
GTTGCACGCATAAAACCAACAAGATTTTTATTAATTTTTTTATATTGCATCATTC
GGCGAAATCCTTGAGCCATATCTGACAAACTCTTATTTAATTCTTCGCCATCATA
AACATTTTTAACTGTTAATGTGAGAAACAACCAACGAACTGTTGGCTTTTGTTTA
ATAACTTCAGCAACAACCTTTTGTGACTGAATGCCATGTTTCATTGCTCTCCTCC
AGTTGCACATTGGACAAAGCCTGGATTTACAAAACCACACTCGATACAACTTTCT
TTCGCCTGTTTCACGATTTTGTTTATACTCTAATATTTCAGCACAATCTTTTACT
CTTTCAGCCTTTTTTAAATTCAAGAATATGCAGAAGTTCAAAGTAATCAACATTAG
CGATTTTCTTTTCTCTCCATGGTCTCACTTTTCCACTTTTTGTCTTGTCCACTAA
AACCCTTGATTTTTCATCTGAATAAATGCTACTATTAGGACACATAATATTAAAA
GAAACCCCCATCTATTTAGTTATTTGTTTGGTCACTTATAACTTTAACAGATGGG
GTTTTTTCTGTGCAACCAATTTTAAGGGTTTTCAATACTTTAAAACACATACATAC

FIG. 4 continued

CAACACTTCAACGCACCTTTCAGCAACTAAAATAAAAATGACGTTATTTCTATAT
GTATCAAGAATAGAAAGAACTCGTTTTTCGCTACGCTCAAAACGCAAAAAAAGCA
CTCATTCGAGTGCTTTTTCTTATCGCTCCAAATCATGCGATTTTTTCCTCTTTGC
TTTTCTTTGCTCACGAAGTTCTCGATCACGCTGCAAAACATCTTGAAGCGAAAAA
GTATTCTTCTTTTCTTCCGATCGCTCATGCTGACGCACGAAAAGCCCTCTAGGCG
CATAGGAACAACTCCTAAATGCATGTGAGGGGTTTTCTCGTCCATGTGAACAGTC
GCATACGCAATATTTTGTTTCCCATACTGCATTAATGAATCGGCCAACGCGCGGG
GAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTG
CGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATAC
GGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCA
GCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTC
CGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACC
CGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTC
TCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGA
AGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCG
TTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGC
CTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCA
CTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTA
CAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGG
TATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGA
TCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGA
TTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGGC
GCGCCCAGCTGTCTAGGGCGGCGGATTTGTCCTACTCAGGAGAGCGTTCACCGAC
AAACAACAGATAAAACGAAAGGCCCAGTCTTTCGACTGAGCCTTTCGTTTTATTT
GATGCCTTTAAT

FIG. 4 continued

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2020/019607

A. CLASSIFICATION OF SUBJECT MATTER
INV. B01J13/02 A01N1/02 A61K9/16 B01F13/00 B01J19/00
C12M1/12 C12N5/00 C40B30/00
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)
B01J C12M C12N A01N B01F G01N A61K C40B

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	WO 2010/004018 A2 (ETH ZUERICH [CH]; SWISSGENETICS [CH] ET AL.) 14 January 2010 (2010-01-14) page 9, lines 15-18; figure 13; page 23, line 32 - page 24, line 7. -----	1-50
A	FLURI D A ET AL: "A novel system for trigger-controlled drug release from polymer capsules", JOURNAL OF CONTROLLED RELEASE, ELSEVIER, AMSTERDAM, NL, vol. 131, no. 3, 12 November 2008 (2008-11-12), pages 211-219, XP025583899, ISSN: 0168-3659, DOI: 10.1016/J.JCONREL.2008.07.036 [retrieved on 2008-08-05] page 214-215. ----- -/--	1-50

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

[X] 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

"E" earlier application or patent 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
28 April 2020	02/07/2020

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, Fax: (+31-70) 340-3016	Mc Donnell, Shane

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2020/019607

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SAKAI S ET AL: "Enzymatically fabricated and degradable microcapsules for production of multicellular spheroids with well-defined diameters of less than 150@mm", BIOMATERIALS, ELSEVIER, AMSTERDAM, NL, vol. 30, no. 30, 4 August 2009 (2009-08-04), pages 5937-5942, XP026524658, ISSN: 0142-9612, DOI: 10.1016/J.BIOMATERIALS.2009.07.031 [retrieved on 2009-08-04] point 2.3	1-50

A	WO 2018/067792 A1 (HARVARD COLLEGE [US]; GEORGIA TECH RES INST [US]) 12 April 2018 (2018-04-12) the whole document	1-50

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2020/019607

Box No. 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. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

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 No. 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:

see additional sheet

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 fees, this Authority did not invite payment of additional fees.

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.:

1-50

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-50

A method, comprising:
encapsulating cells within liquid droplets;
causing the liquid droplets containing the cells to form gel droplets;
causing at least some of the cells within the gel droplets to degrade the gel; and
separating the gel-degrading cells from the non-gel-degrading cells via a filter.

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2. claims: 51-81

A method, comprising:
encapsulating cells within liquid droplets;
causing at least some of the cells to gel the liquid droplet to form a gel droplet; and
separating the cells contained within gel droplets from cells contained within liquid droplets via a filter.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2020/019607

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010004018 A2	14-01-2010	CA 2730292 A1	14-01-2010
		EP 2303245 A2	06-04-2011
		US 2011177162 A1	21-07-2011
		US 2013045190 A1	21-02-2013
		WO 2010004018 A2	14-01-2010
WO 2018067792 A1	12-04-2018	NONE	