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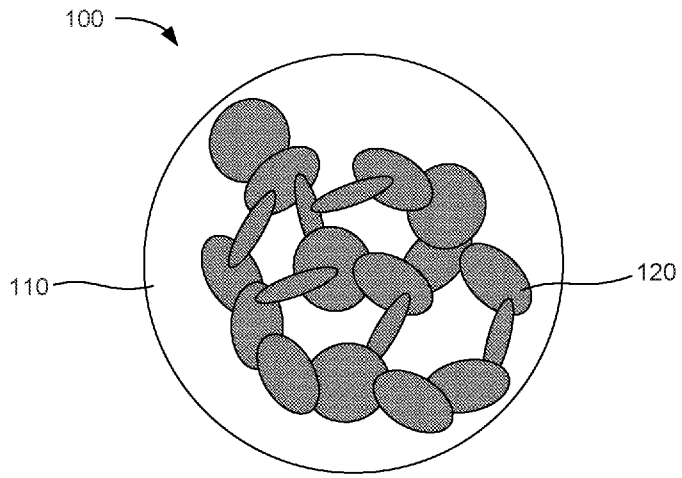


FIG. 1

(57) Abstract: An example bio-ink for three-dimensional printing can include a liquid vehicle including deionized water. Gelatin can be dissolved in the liquid vehicle. Nanoclay platelets can be dispersed in the liquid vehicle. The bio-ink can also include a gelling compound that includes a polysaccharide. An example bio-ink three-dimensional printer can include a bio-ink and an extrusion print head connected to or connectable to the bio-ink. The extrusion print head can include an extrusion nozzle and a driver to push the bio-ink through the extrusion nozzle.

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BIO-INKS FOR THREE-DIMENSIONAL PRINTING

BACKGROUND

[0001] Bio-printing is used for many purposes including performing various types of assay testing, toxicology testing, disease modelling, fundamental biology studies, in-vitro drug screening, and others. The ability to deposit living cells using bio-printers can be very useful in these applications. For example, automated fabrication of in-vitro tissue models using 3D bio-printing of living cells can be very useful for creating tissue models in a scalable and repeatable manner. Demand for bio-printing is expected to increase significantly in the future. The recent prevalence of COVID-19 cases and the increasing prevalence of chronic diseases are some of the many factors that may contribute to this growth in demand for in-vitro testing models.

BRIEF DESCRIPTION OF THE DRAWING

[0002] FIG. 1 is a schematic view of an example bio-ink for three-dimensional printing in accordance with the present disclosure.

[0003] FIG. 2 is a schematic cross-sectional view of an example bio-ink three-dimensional printer in accordance with the present disclosure.

[0004] FIG. 3 is a graph of Tan(delta) from a temperature sweep test of example bio-inks in accordance with the present disclosure.

[0005] FIG. 4 is a graph of compressive modulus in dry conditions for example bio-inks in accordance with the present disclosure.

[0006] FIG. 5 is a graph of compressive modulus in wet conditions for example bio-inks in accordance with the present disclosure.

[0007] FIG. 6 is a graph of fluorescence from a cell viability test of example bio-inks in accordance with the present disclosure.

DETAILED DESCRIPTION

[0008] The present disclosure describes bio-inks for three-dimensional printing. These bio-inks can be printed, for example, from an extrusion-type three-dimensional (3D) printer to form three-dimensional printed objects. The objects formed of the bio-ink can be used to incubate live cells for various bio-printing applications. A bio-ink for three-dimensional printing according to the present disclosure includes a liquid vehicle including deionized water. Gelatin is dissolved in the liquid vehicle. Nanoclay platelets are dispersed in the liquid vehicle. The bio-ink also includes a gelling compound that includes a polysaccharide. In certain examples, the bio-ink can also include an osmotic agent that includes a sugar, mannitol, sorbitol, glycerol, glycine, or a combination thereof, dissolved in the liquid vehicle. Sugars that can be used include sucrose, glucose, fructose, galactose, lactose, maltose, and combinations thereof. The osmotic agent can be included at a concentration from about 260 milliosmoles per liter of bio-ink to about 320 milliosmoles per liter of bio-ink. The gelling compound can be gellan gum, agar, agarose, guar gum, carrageenan, xanthan gum, or a combination thereof. In certain examples, the nanoclay platelets can include laponite nanoclay. The gelatin can be included in an amount from 6 grams/100 mL to 12 grams/100 mL, with respect to 100 mL of the bio-ink. The nanoclay platelets can be included in an amount from 1 gram/100 mL to 2 grams/100 mL, with respect to 100 mL of the bio-ink. The gelling compound can be included in an amount from 0.25 gram/100 mL to 1 gram/100 mL, with respect to 100 mL of the bio-ink. In some examples, the bio-ink has a melting point from 37 °C to 100 °C.

[0009] The present disclosure also describes three-dimensional printers that can print objects using the bio-inks. An example bio-ink three-dimensional printer includes a bio-ink and an extrusion print head connected to or connectable to the bio-ink. The extrusion print head includes an extrusion nozzle and a driver to push the bio-ink through the extrusion nozzle. The bio-ink includes a liquid vehicle including deionized water; gelatin dissolved in the liquid vehicle; nanoclay platelets dispersed in the liquid vehicle; and a gelling compound including a polysaccharide. The bio-ink can also include an osmotic agent including a sugar, mannitol, sorbitol, glycerol, glycine, or a combination thereof, dissolved in the liquid vehicle. The nanoclay platelets can include laponite nanoclay. The gelling compound can be

gellan gum, agar, agarose, guar gum, carrageenan, xanthan gum, or a combination thereof. The osmotic agent can be a sugar including sucrose, glucose, fructose, galactose, lactose, maltose, or a combination thereof. Gelatin can be included in an amount from 6 grams/100 mL to 12 grams/100 mL, with respect to 100 mL of the bio-ink. Nanoclay platelets can be included in an amount from 1 gram/100 mL to 2 grams/100 mL, with respect to 100 mL of the bio-ink. The gelling compound can be included in an amount from 0.25 gram/100 mL to 1 gram/100 mL, with respect to 100 mL of the bio-ink. The osmotic agent can be included at a concentration from about 260 milliosmoles per liter of bio-ink to about 320 milliosmoles per liter of bio-ink.

[0010] The present disclosure also describes methods of three-dimensional printing bio-inks. One example method includes extruding a bio-ink from an extrusion print head including an extrusion nozzle and a driver to push the bio-ink through the extrusion nozzle. The bio-ink includes a liquid vehicle including deionized water, gelatin dissolved in the liquid vehicle, nanoclay platelets dispersed in the liquid vehicle, and a gelling compound including a polysaccharide. The method can also include forming a three-dimensional printed structure from the bio-ink and introducing living cells onto the three-dimensional printed structure. In various examples, the nanoclay platelets can include laponite nanoclay. The gelling compound can be gellan gum, agar, agarose, guar gum, carrageenan, xanthan gum, or a combination thereof. The bio-ink can also include an osmotic agent including a sugar, mannitol, sorbitol, glycerol, glycine, or a combination thereof.

[0011] It is noted that when discussing bio-inks, bio-ink three-dimensional printers, and methods, these discussions can be considered applicable to other examples whether or not they are explicitly discussed in the context of that example unless expressly indicated otherwise. Thus, for example, when discussing a bio-ink composition, such disclosure is also relevant to and directly supported in context of bio-ink three-dimensional printers and methods of three-dimensional printing bio-ink, and *vice versa*. Furthermore, for simplicity and illustrative purposes, the present disclosure is described by referring mainly to certain examples. In the following description, numerous specific details are set forth in order to provide a thorough understanding of the present disclosure. It will be readily apparent however, that the present disclosure can be practiced without limitation to some of these specific details. In other instances, certain methods, compounds, compositions, and

structures have not been described in detail so as not to unnecessarily obscure the present disclosure.

Bio-Inks for Three-dimensional Printing

[0012] The bio-inks described herein are gelatin-based composites that can be used for three-dimensional bio-printing. In some cases, the bio-inks can be printed with an extrusion-based three-dimensional printer, which extrudes the bio-ink through an extrusion nozzle to form layers of bio-ink on a build platform, for example. The bio-inks can have good printability properties, viscosity recovery, thermal stability, and biocompatibility. As used herein, "viscosity recovery" refers to the ability of the bio-ink to exhibit a relatively high viscosity when at rest or under a small shear force, and then exhibit a lower viscosity when under a high shear force, and then return to the relatively high viscosity after the high shear force is removed. For example, a shear force can be applied to the bio-ink while the bio-ink is being extruded through an extrusion nozzle. After the bio-ink has been extruded, the bio-ink can recover its higher viscosity at rest so that the bio-ink can retain its three-dimensional shape.

[0013] The term "thermal stability" refers to the ability of the bio-ink to retain its shape and mechanical properties at elevated temperatures. In some cases, it can be useful to incubate living cells at a particular incubation temperature, which may vary depending on the type of cells. For example, human cells can be incubated at a temperature at or around 37 °C in some cases. Other cells may be incubated at higher or lower temperatures. In some examples, the bio-inks described herein can be stable at temperatures of 50 °C or higher, which can be sufficient for most types of cell incubation.

[0014] A composite of several materials is formed to yield the bio-inks described herein. The bio-inks include a liquid vehicle that includes deionized water. Gelatin is dissolved in the liquid vehicle. Nanoclay platelets are dispersed in the liquid vehicle. Additionally, a gelling compound is added to the bio-ink. The gelling compound includes a polysaccharide such as gellan gum, agar, agarose, guar gum, carrageenan, xanthan gum, or a combination thereof. In some examples, an osmotic agent can also be added to provide an appropriate osmolarity to make the bio-ink compatible with living cells. The osmotic agent can include a sugar, mannitol, sorbitol, glycerol, glycine, or a combination thereof. As a specific example, one

formulation can include deionized water, gelatin (type A), laponite nanoclay platelets, gellan gum as a gelling compound, and sucrose as an osmotic agent. When combined, these ingredients have surprisingly been found to provide excellent three-dimensional printing properties and thermal stability as explained above. Without being bound to a specific mechanism, it is believed that the nanoclay platelets form a loose "house of cards" configuration when dispersed in the deionized water. The nanoclay platelets can have opposite charges located at the edges of the platelets and the central portions of the platelets. The edges can therefore be attracted to the opposite charges in the central portions of neighboring platelets so that the platelets form a loose network of touching or nearly-touching platelets, referred to as the "house of cards" configuration. The charged portions of the nanoclay platelets can also interact with the gelatin and the gelling compound to form a reinforced, ionic-crosslinked gel. The osmotic agent can be added in an appropriate amount to tune the osmolarity to match living cells. The osmotic agent can also affect the viscosity and flowability of the bio-ink.

[0015] Many previously developed bio-inks for three-dimensional printing have utilized ultraviolet curing. For example, such bio-inks may include photo-initiators that can initiate a crosslinking process when exposed to ultraviolet radiation. These inks can often be used by extruding the ink and then exposing the extruded ink to ultraviolet radiation to crosslink the ink, which can increase the structural fidelity of the three-dimensional printed structure. However, any unreacted excess photo-initiator can be harmful to living cells that may be included in the bio-ink or introduced into the three-dimensional printed structure after printing. On the other hand, if an insufficient amount of photoinitiator is used in the bio-ink, then the structural integrity of the three-dimensional printed structure can be diminished. It can be difficult to find an amount of photoinitiator that can provide structural integrity without harming the cells encapsulated in the three-dimensional structure. In contrast with these previously developed bio-inks, the bio-inks described herein do not rely on ultraviolet curing. Sufficient structural integrity can be provided by the combination of gelatin, nanoclay platelets, gelling compound, and in some cases the osmotic agent, without any curing or photo-polymerization after printing. The bio-inks may be devoid of photo-initiators. Thus, the bio-inks can be safer for living cells.

[0016] Many previously developed bio-inks are also very temperature-sensitive. Some bio-inks are printed with an expensive temperature-controlled

printhead because the printing characteristics are poor outside a narrow temperature range. For example, unmodified gelatin has a melting point at about 28 °C. Such bio-inks often degrade over time, and swell or contract significantly after printing. The bio-inks described herein can have better performance in these areas. The bio-inks described herein are very thermally stable across a wide temperature range. The bio-inks described herein can be printable and stable across a temperature range from 25 °C to 50 °C, which can be a useful range for three-dimensional bio-printing. The bio-inks can also have a low degradation rate. It has been found that the bio-inks can retain more than 90% of their mass 14 days after being printed. The bio-inks also retain their printed dimensions very well instead of expanding or contracting after printing.

[0017] As mentioned above, the bio-inks described herein include a liquid vehicle with gelatin dissolved in the liquid vehicle, nanoclay platelets dispersed in the liquid vehicle, and a gelling compound also dissolved or dispersed in the liquid vehicle. FIG. 1 shows an example bio-ink 100 for three-dimensional printing in accordance with this disclosure. The bio-ink includes a liquid vehicle 110 and nanoclay platelets 120 dispersed in the liquid vehicle. The liquid vehicle includes deionized water. As explained above, the nanoclay platelets form a “house of cards” configuration in the deionized water due to the charge distribution in the nanoclay platelets. The bio-ink also includes gelatin and a gelling compound. These are dissolved in the liquid vehicle, and therefore these ingredients are not visible in FIG. 1.

[0018] The liquid vehicle includes deionized water. In certain examples, the liquid vehicle can be pure deionized water. The liquid vehicle can be mixed with the other ingredients of the bio-ink to form a stable gel. In some examples, the other ingredients of the bio-ink can be provided in a dry state, such as a powder. This powder can be mixed with the liquid vehicle to form the bio-ink. In certain examples, the powder can be mixed with deionized water to form the bio-ink. The amount of deionized water in the bio-ink can be from about 70 wt% to about 95 wt%, or from about 75 wt% to about 94 wt%, or from about 80 wt% to about 93 wt%, in various examples. In certain examples, deionized water can be the sole liquid ingredient in the bio-ink.

[0019] Gelatin can be included in the bio-ink. In some examples, the gelatin can be included in a greater amount than other solid ingredients of the ink. In various

examples, the amount of gelatin in the bio-ink can be from about 6 grams/100 mL to about 12 grams/100 mL, or from about 6 grams/100 mL to about 9 grams/100 mL, or from about 9 grams/100 mL to about 12 grams/100 mL. These amounts can be with respect to 100 mL of the bio-ink as a whole. The gelatin can be type A gelatin. Type A gelatin is a type of gelatin processed using acid. This type is different from type B gelatin, which is processed using alkali. However, in other examples, type B gelatin can be used in the bio-inks. The gelatin can have a gel strength expressed as a bloom value. The gelatin can have a bloom value from about 30 to about 325, or from about 200 to about 325, or from about 275 to about 325. The bloom value can be measured using the Bloom test, in which a 6.67 wt% gelatin solution is kept for 17-18 hours at 10 °C and then a plunger with a diameter of 0.5 inch is used to depress the surface of the gelatin by 4 mm without breaking the surface. The bloom value is the weight in grams that is applied to the plunger to depress the surface by 4 mm. The gelatin can be sourced from a variety of sources, such as porcine skin, bovine skin, fish skin, and others.

[0020] The bio-ink can also include nanoclay platelets. The platelets can have a flattened shape with a small thickness and a larger length and/or width. In some examples, the platelets can have an aspect ratio from about 1:10 to about 1:100, or from about 1:20 to about 1:100, or from about 1:20 to about 1:50. The aspect ratio can be defined as the thickness of the platelets divided by the longest dimensional of the platelets. The thickness of the platelets can be from about 0.5 nm to about 10 nm, or from about 0.5 nm to about 5 nm, or from about 1 nm to about 5 nm. The longest dimension of the platelets (i.e., length or width) can be from about 10 nm to about 100 nm, or from about 10 nm to about 50 nm, or from about 20 nm to about 50 nm. In certain examples, the nanoclay platelets can be shaped as discs.

[0021] The nanoclay platelets can include laponite clay in some examples. Laponite is a synthetic clay, made up of a lithium sodium magnesium silicate. Specific examples include LAPONITE® XLG, LAPONITE® RD, LAPONITE® D, and LAPONITE® XL21 nanoclay platelets, available from BYK Additives and Instruments (Germany). The nanoclay platelets can be included in the bio-ink in an amount from about 1 gram/100 mL to about 2 grams/100 mL, with respect to 100 mL of the bio-ink. In specific examples, the nanoclay platelets can be included in an amount from about 1 gram/100 mL to about 1.5 grams/100 mL, or from about 1.5 grams/100 mL to about 2 grams/100 mL.

[0022] The bio-ink can also include a gelling compound. The gelling compound can include a polysaccharide. Examples of gelling compounds include gellan gum, agar, agarose, guar gum, carrageenan, xanthan gum, and others. These gelling compounds are made up of polysaccharides, such as exopolysaccharides, agarose, agaropectin, and polysaccharides made up of polymerized sugar monomers such as galactose, mannose, glucose, 3,6-anhydrogalactose, and other monomers. In various examples, the gelling compound can be included in an amount from about 0.25 gram/100 mL to about 1 gram/100 mL, or from about 0.25 gram/100 mL to about 0.5 gram/100 mL, or from about 0.5 gram/100 mL to about 1 gram/100 mL, with respect to 100 mL of the bio-ink.

[0023] Additionally, the bio-ink can include an osmotic agent. In various examples, the osmotic agent can include a sugar, mannitol, sorbitol, glycerol, glycine, or a combination thereof. In certain examples, the osmotic agent can be a sugar that includes sucrose, glucose, fructose, galactose, lactose, maltose, or a combination thereof. The osmotic agent can be included in an amount that provides an appropriate osmolarity for living cells. The concentration of the osmotic agent can be from about 260 milliosmoles per liter of bio-ink to about 320 milliosmoles per liter of bio-ink.

[0024] The bio-inks described herein can be thixotropic fluids, which can vary in viscosity depending on the shear force exerted on the bio-inks. In particular, the bio-inks can exhibit shear thinning behavior. Therefore, the bio-inks can have low viscosity at high shear rates and high viscosity at low shear rates. Additionally, the bio-inks can have good viscosity recovery, meaning that the viscosity returns to a high viscosity consistently when the shear is removed after having been exposed to high shear. The bio-inks can also return to a high viscosity quickly after the shear is removed, such as within about 1 second to several seconds. The viscosity of the bio-ink, when the shear is removed, can be sufficiently high to prevent the bio-ink from spreading so that three-dimensional self-supporting structures can be formed of the bio-ink.

[0025] In certain examples, the bio-ink can have a sufficiently low viscosity when the bio-ink is sheared by extruding the bio-ink through an extrusion nozzle. The shear rate of the bio-ink when being extruded through the extrusion nozzle can depend on several factors, including the rate of extrusion and the diameter of the nozzle. In many cases, the shear rate of the bio-ink can be from about 50 s^{-1} to

about 500 s^{-1} when the bio-ink is extruded from an extrusion nozzle of a three-dimensional printer. A useful range of viscosity for extruding the bio-ink can be from about 1 mPa·s to about 20 mPa·s. In certain examples, the bio-ink can have a viscosity from about 1 mPa·s to about 20 mPa·s at a shear rate of 100 s^{-1}. At a shear rate of 1 s^{-1}, the bio-ink can have a viscosity from about 40 mPa·s to about 1200 mPa·s. At a shear rate of 1000 s^{-1}, the bio-ink can have a viscosity from about 0.08 mPa·s to about 1 mPa·s. At very low shear rates, such as 0.1 s^{-1}, the bio-ink can have a viscosity from about 10 Pa·s to about 10,000 Pa·s. The viscosity can be measured using a rotational rheometer at a temperature of 25 °C, for example.

[0026] The bio-inks are viscoelastic materials that can be characterized by a storage modulus and a loss modulus. The storage modulus is a property that measures energy stored elastically when the bio-ink deforms. The loss modulus measures energy lost by dissipation as heat when the bio-ink deforms. These properties can also be measured using a rotational rheometer, such as a DISCOVERY® HR-2 rheometer from TA Instruments (Germany). The storage modulus and loss modulus can vary depending on the composition of the bio-ink. In particular, changing the amounts of gelatin, nanoclay platelets, and gelling compound can affect the storage modulus and loss modulus. These properties can also be temperature-dependent. By comparing the storage modulus and loss modulus at a particular temperature, it can be determined whether the bio-ink is acting as a solid or liquid. When the ratio of the storage modulus to the loss modulus is greater than 1, the bio-ink is a solid, and the temperature is above the melting point or sol/gel transition temperature. When the ratio is less than 1, the bio-ink is a liquid and the temperature is above the melting point or sol/gel transition temperature. The bio-inks described herein can remain in the solid state to elevated temperatures well above 50 °C, which can be sufficient for many bio-printing applications. In some examples, the melting point of the bio-ink can be from about 37 °C to about 100 °C, or from about 37 °C to about 100 °C, or from about 50°C to about 100 °C, or from about 50 °C to about 80 °C. In further examples, the melting point can be greater than 37°C, greater than 50 °C, greater than 60 °C, greater than 70 °C, or greater than 80 °C. The storage modulus of the bio-ink can vary from about 1,000 Pa to about 100,000 Pa in a temperature range of 25 °C to 50 °C, depending on the composition of the bio-ink and the temperature. The loss modulus can vary from about 100 Pa to about 10,000 Pa in the range of 25 °C to 50 °C, again

depending on the composition of the bio-ink and the temperature. For a given bio-ink composition, the storage modulus can remain above the loss modulus across a temperature range to well above 50 °C.

[0027] The stiffness of the bio-ink can be characterized by the compressive modulus. The compressive modulus can be different depending on whether the three-dimensional printed bio-ink is in dry or wet conditions. Dry conditions refers to bio-ink that has been three-dimensional printed in room-temperature air. Wet conditions refers to bio-ink that has been three-dimensional printed and then subjected to a tissue culture process, which includes submersion in cell culture medium in a cell incubator at 37 °C for 24 hours. In some examples, the bio-inks described herein can have a compressive modulus from about 1 kPa to about 50 kPa under dry conditions, and from about 1 kPa to about 6 kPa under wet conditions. The compressive modulus can also depend on the composition of the bio-ink. The compressive modulus can be measured, for example, using a compression tester such as an INSTRON® 3366 Universal Testing Machine from Instron (USA).

[0028] The bio-inks described herein can provide good cell viability for living cells that may be embedded within a three-dimensional printed bio-ink structure. For example, living cells can proliferate for 14 days or more when incubated in the three-dimensional printed bio-ink structure. In some cases, the composition of the bio-ink can affect the cell viability. Because the relative amounts of gelatin, nanoclay platelets, and gelling compound can affect the cell viability, compressive modulus, storage modulus, and loss modulus as explained above, different bio-ink formulations may be useful depending on the desired properties of the bio-ink.

[0029] In certain examples, the bio-ink can include: nanoclay platelets in an amount of about 1 gram/100 mL of bio-ink; gelatin in an amount of about 6 grams/100 mL of bio-ink; and a gelling compound in an amount of about 0.25 gram/100 mL of bio-ink.

[0030] In other examples, the bio-ink can include: nanoclay platelets in an amount of about 1 gram/100 mL of bio-ink; gelatin in an amount of about 9 grams/100 mL of bio-ink; and a gelling compound in an amount of about 0.25 gram/100 mL of bio-ink.

[0031] In other examples, the bio-ink can include: nanoclay platelets in an amount of about 1 gram/100 mL of bio-ink; gelatin in an amount of about 12

grams/100 mL of bio-ink; and a gelling compound in an amount of about 0.25 gram/100 mL of bio-ink.

[0032] In other examples, the bio-ink can include: nanoclay platelets in an amount of about 1.5 gram/100 mL of bio-ink; gelatin in an amount of about 6 grams/100 mL of bio-ink; and a gelling compound in an amount of about 0.25 gram/100 mL of bio-ink.

[0033] In other examples, the bio-ink can include: nanoclay platelets in an amount of about 1.5 gram/100 mL of bio-ink; gelatin in an amount of about 9 grams/100 mL of bio-ink; and a gelling compound in an amount of about 0.25 gram/100 mL of bio-ink.

[0034] In other examples, the bio-ink can include: nanoclay platelets in an amount of about 1.5 gram/100 mL of bio-ink; gelatin in an amount of about 12 grams/100 mL of bio-ink; and a gelling compound in an amount of about 0.25 gram/100 mL of bio-ink.

[0035] In other examples, the bio-ink can include: nanoclay platelets in an amount of about 2 grams/100 mL of bio-ink; gelatin in an amount of about 6 grams/100 mL of bio-ink; and a gelling compound in an amount of about 0.25 gram/100 mL of bio-ink.

[0036] In other examples, the bio-ink can include: nanoclay platelets in an amount of about 2 grams/100 mL of bio-ink; gelatin in an amount of about 9 grams/100 mL of bio-ink; and a gelling compound in an amount of about 0.25 gram/100 mL of bio-ink.

[0037] In other examples, the bio-ink can include: nanoclay platelets in an amount of about 2 grams/100 mL of bio-ink; gelatin in an amount of about 12 grams/100 mL of bio-ink; and a gelling compound in an amount of about 0.25 gram/100 mL of bio-ink.

Bio-ink Three-dimensional Printers

[0038] The present disclosure also describes bio-ink three-dimensional printers that can be used to print bio-inks as described above. The bio-ink can be suitable for printing with an extrusion-type three-dimensional printer. Accordingly, the bio-ink three-dimensional printers can include an extrusion print head connected to or connectable to a supply of bio-ink as described above. The extrusion print head can include an extrusion nozzle and a driver to push the bio-ink through the

extrusion nozzle. Several types of drivers can be used to push the bio-ink through the extrusion nozzle. In some examples, the driver can include a syringe plunger that mechanically pushes on the bio-ink to force the bio-ink out of the extrusion nozzle. In other examples, the driver can include a rotary screw that can rotate to push bio-ink out of the extrusion nozzle. In further examples, pressurized air can be used to apply pressure to the bio-ink to push the bio-ink out of the extrusion nozzle.

[0039] As used herein, "connected or connectable" can refer to an extrusion print head that is either connected to a supply of bio-ink in a way that allows the bio-ink to flow from the supply to the print head, or which is designed to be connected to a supply of bio-ink in this way. In some cases, the bio-ink may be packaged separately from the printer. However, the print head can be designed to be fluidly connected to the bio-ink at some point so that the bio-ink can be extruded from the extrusion print head. In other examples, a bio-ink three-dimensional printer can include a reservoir of bio-ink that is already attached and connected to the print head. In certain examples, the bio-ink can be loaded inside the print head. This can also be referred to as having the print head "connected" to the bio-ink.

[0040] The bio-ink three-dimensional printers can also include a build platform and actuators to allow the print head to move in relation to the build platform. The print head can move in three dimensions (i.e., along an x, y, and z axis) in relation to the build platform so that three-dimensional objects can be formed. In various examples, the build platform can be physically stationary and the actuators can move the print head, or the print head can be stationary and the actuators can move the build platform, or a combination of actuators can be used on both the build platform and the print head. For example, the build platform can be configured to move along an x-axis, and the print head can move along the y-axis and z-axis.

[0041] FIG. 2 is a schematic diagram of an example bio-ink three-dimensional printer 200 in accordance with the present disclosure. The printer includes a bio-ink 100 connected to an extrusion print head 210. In this example, the bio-ink is loaded inside the extrusion print head. The extrusion print head includes an extrusion nozzle 220 and a driver 230 to push the bio-ink through the extrusion nozzle. In this example, the driver is a plunger similar to a syringe plunger. The plunger can move downward to push bio-ink out through the nozzle. This example printer also includes a build platform 240 below the extrusion print head. The bio-ink that is extruded out through the nozzle can form a three-dimensional printed structure 250. The bio-ink

that is printed with the three-dimensional printer can include any of the ingredients and formulations described above.

[0042] The extrusion nozzle can be sized to provide a good three-dimensional printing resolution. The diameter of the extrusion nozzle can be from about 50 micrometers to about 2,000 micrometers, or from about 100 micrometers to about 1,000 micrometers, or from about 200 micrometers to about 1,000 micrometers, in some examples.

[0043] Bio-ink three-dimensional printers can also include a variety of other components not illustrated in the figures herein. Additional components may include, a moveable print head carriage, an electronic controller to control the extrusion print head and the motion of print head and/or build platform, a power supply, communication or networking modules, and others. In some examples, an electronic controller can include a processor, memory components including volatile and/or non-volatile memory components, electronics for communicating with and controlling the extrusion print head, carriage, build platform, and so on. In certain examples, the electronic controller can receive data from a host system, such as a computer, and temporarily store data in memory. The data can include a representation of a three-dimensional structure to be printed, and/or instructions for moving the print head and/or build platform and for extruding bio-ink from the print head. The electronic controller can control the extrusion print head to form a three-dimensional printed structure.

Methods of Three-dimensional Printing Bio-ink

[0044] The present disclosure also describes methods of three-dimensional printing bio-ink. The bio-inks described herein can be printed using three-dimensional printing methods that involve extruding the bio-ink from an extrusion-based three-dimensional printer. As explained above, the extrusion-based three-dimensional printer can include an extrusion print head with an extrusion nozzle and a driver. Methods of three-dimensional printing bio-ink can include extruding the bio-ink from such an extrusion print head. The bio-ink can include a liquid vehicle including deionized water, gelatin dissolved in the liquid vehicle, nanoclay platelets dispersed in the liquid vehicle, and a gelling compound including a polysaccharide.

[0045] Methods can also include forming a three-dimensional printed structure of the bio-ink. Any desired shape can be formed. The bio-ink can have sufficient

stiffness and strength to allow relatively large structures to be formed. For example, structures that are several centimeters in height (i.e., the z-axis) can be formed without significant settling or spreading of the bio-ink. The horizontal dimensions (i.e., x-axis and y-axis) can be any desired size or shape, depending on the size of the three-dimensional printer. Living cells can also be introduced into the bio-ink by any suitable method. In some examples, cells can be mixed into the bio-ink before the bio-ink is printed. In other examples, a three-dimensional printed structure can be formed and then cells can be added to the three-dimensional printed structure after printing.

[0046] It is noted that, as used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the content clearly dictates otherwise.

[0047] As used herein, the term “about” is used to provide flexibility to a numerical range endpoint by providing that a given value may be “a little above” or “a little below” the endpoint. The degree of flexibility of this term can be dictated by the particular variable and can be determined based on experience and the associated description herein.

[0048] As used herein, a plurality of items, structural elements, compositional elements, and/or materials may be presented in a common list for convenience. However, these lists should be construed as though the individual members of the list are individually identified as separate and unique members. Thus, no individual member of such list should be construed as a *de facto* equivalent of any other member of the same list solely based on their presentation in a common group without indications to the contrary.

[0049] Concentrations, dimensions, amounts, and other numerical data may be presented herein in a range format. It is to be understood that such range format is used merely for convenience and brevity and should be interpreted flexibly to include the numerical values explicitly recited as the limits of the range, and also to include all the individual numerical values or sub-ranges encompassed within that range as if the numerical values and sub-ranges are explicitly recited. For example, a weight ratio range of 1 wt% to 20 wt% should be interpreted to include the explicitly recited limits of 1 wt% and 20 wt%, and also to include individual weights such as 2 wt%, 11 wt%, 14 wt%, and sub-ranges such as 10 wt% to 20 wt%, 5 wt% to 15 wt%, etc.

EXAMPLES

[0050] The following examples illustrate the technology of the present disclosure. However, it is to be understood that the following is merely illustrative of the application of the principles of the presented formulations and methods. Numerous modifications and alternative methods may be devised without departing from the scope of the present disclosure. The appended claims are intended to cover such modifications and arrangements. Thus, while the technology has been described above with particularity, the following examples provide further detail in connection with what are presently deemed to be certain acceptable examples.

Example 1 – *Bio-ink Formulations*

[0051] A series of example bio-inks were prepared having different proportions of ingredients. The ingredients included: deionized water; LAPONITE® XLG nanoclay platelets (available from BYK Additives and Instruments, Germany); GELZAN™ CM gellan gum as a gelling compound (available from Sigma Aldrich, USA); gelatin type A from porcine skin, bloom strength 300 (available from Sigma Aldrich, USA); and sucrose as an osmotic agent. The amounts of the nanoclay platelets, gelatin, and gellan gum in the example bio-inks are shown in Table 1. The names of the bio-inks indicate the amounts of the ingredients. In the bio-ink names, the letter "X" stands for the nanoclay platelets, the letter "G" stands for the gelatin, and the letter "Z" stands for the gellan gum. The bio-inks also included sucrose in an amount to provide an osmolarity compatible with living cells. The balance of the bio-inks was deionized water.

Table 1: Bio-ink Formulations

Bio-ink Name	Nanoclay Platelets (grams/100mL)	Gelatin (grams/100mL)	Gellan Gum (grams/100mL
0.5X6G0.25Z	0.5	6	0.25
1X6G0.25Z	1	6	0.25
1.5X6G0.25Z	1.5	6	0.25
2X6G0.25Z	2	6	0.25

0.5X9G0.25Z	0.5	9	0.25
1X9G0.25Z	1	9	0.25
1.5X9G0.25Z	1.5	9	0.25
2X9G0.25Z	2	9	0.25
0.5X12G0.25Z	0.5	12	0.25
1X12G0.25Z	1	12	0.25
1.5X12G0.25Z	1.5	12	0.25
2X12G0.25Z	2	12	0.25

Example 2 – *Viscosity of Bio-inks*

[0052] The viscosity of the example bio-inks was measured at shear rates of 1 s^{-1}, 100 s^{-1}, and 1,000 s^{-1}. The temperature was 25 °C. The viscosity measurements are shown in Table 2.

Table 2: Viscosity Measurements

Bio-ink Name	Viscosity at 1 s^{-1} (mPa·s)	Viscosity at 100 s^{-1} (mPa·s)	Viscosity at 1,000 s^{-1} (mPa·s)
0.5X6G0.25Z	50.52 ± 11.07	0.93 ± 0.09	0.14 ± 0.07
1X6G0.25Z	114.05 ± 11.79	1.73 ± 0.49	0.11 ± 0.07
1.5X6G0.25Z	84.01 ± 8.58	2.54 ± 0.38	0.19 ± 0.06
2X6G0.25Z	40.18 ± 3.11	1.02 ± 0.12	0.31 ± 0.01
0.5X9G0.25Z	365.61 ± 33.2	6.52 ± 1.78	0.24 ± 0.09
1X9G0.25Z	440.62 ± 65.17	8.16 ± 1.69	0.36 ± 0.24
1.5X9G0.25Z	386.39 ± 64.36	8.83 + 1.27	0.09 ± 0.07
2X9G0.25Z	338.38 ± 69.40	3.31 ± 0.97	0.12 ± 0.07
0.5X12G0.25Z	1174.02 ± 185.82	11.70 ± 4.95	0.74 ± 0.01
1X12G0.25Z	1027.61 ± 191.00	12.88 ± 2.36	0.17 ± 0.11
1.5X12G0.25Z	708.46 ± 136.28	14.66 ± 1.37	0.18 ± 0.10
2X12G0.25Z	850.90 ± 46.96	18.37 ± 0.40	0.62 ± 0.81

[0053] These results show that bio-inks exhibit shear-thinning behavior when the shear rate is increased from 1 s^{-1} to 1,000 s^{-1}. The amount of gelatin appears to

have a larger effect on the viscosity profile than the amount of nanoclay platelets. The viscosity of the bio-inks having 6%, 9%, and 12% gelatin, respectively, are different on the order of 10^1, 10^2, and 10^3 mPa·s, respectively. This may be because gelatin is a thermo-sensitive polymer that undergoes coil-to-helix transition at temperatures below 30 °C to form a triple helix structure, resulting in significantly higher viscosity with increasing gelatin concentration.

Example 3 – *Viscosity Recovery*

[0054] The viscosity recovery of the bio-inks was tested by measuring the viscosity while cycling the shear rate from a low shear rate of 0.1 s^{-1} to a high shear rate of 500 s^{-1} several times. The viscosity measurements in these examples were performed using a DISCOVERY® HR-2 rotational rheometer from TA Instruments (Germany). The results show that the bio-inks were able to recover to a high viscosity from about 100 Pa·s to about 1,000 Pa·s within about 1-2 seconds after switching from the high shear rate to the low shear rate. The viscosity also returned to nearly the same level consistently when the shear rate was cycled multiple times from high shear rate to low shear rate. This suggests that the bio-ink can be used for three-dimensional printing and can increase in viscosity quickly after being extruded from an extrusion nozzle, so that the printed bio-ink can hold its shape. This can allow for high printing resolution and lower printed layers of bio-ink can be sufficiently strong to hold up overlying layers.

Example 4 – *Temperature Sweep Profile*

[0055] A temperature sweep profile was measured for the example bio-inks. The storage modulus and loss modulus were measured across a temperature range from 25 °C to 50 °C. The storage modulus was above the loss modulus across the entire temperature range for all of the example bio-inks. This shows that the bio-inks remain in the solid state and are thermally stable up to 50 °C.

[0056] The tan(delta) was also measured across the temperature range. This measure refers to the tangent of the ratio of loss modulus to storage modulus. When the value of tan(delta) is low (less than 1) the bio-ink is in a solid state, and when the value is high (greater than 1) the bio-ink is in a liquid state. FIG. 3 shows the tan(delta) for the example bio-inks measured at 37 °C. This temperature is particularly useful for incubating cells.

Example 5 – *Compressive Modulus*

[0057] The compressive modulus of the example bio-inks was measured by casting the bio-inks in a mold to form a cylinder with a diameter of 14 mm and a height of 5 mm and testing the compressive modulus using an INSTRON® 3366 Universal Testing Machine from Instron (USA). The compressive modulus was measured both in dry conditions and wet conditions. Dry conditions means the bio-ink was casted in the cylinder shape and then tested. Wet conditions means the bio-ink was submerged in cell culture medium for 24 hours at 37 °C and then the compressive modulus was tested. The results for the dry conditions test are shown in FIG. 4. The results for the wet conditions test are shown in FIG. 5. The results show that in the dry state the compressive modulus tends to increase with higher gelatin concentration and decrease with higher nanoclay platelet concentration. In the wet state, the nanoclay platelets had a significant effect on compressive modulus. The bio-inks with high gelatin concentration (12%) have higher water absorption and caused swelling of the bio-ink when submerged in the cell culture medium. Overall, the compositions having a nanoclay platelet concentration of 1.5% or above could be picked up easily using a pair of forceps under wet conditions.

Example 6 – *Cell Biocompatibility*

[0058] The biocompatibility of the bio-inks was tested by encapsulating human fibroblast cells in the bio-ink. Live cells were stained green while dead cells were stained red. The fluorescence was measured on day 1, day 7, and day 14. The relative fluorescence measurements are shown in FIG. 6. The results show that lower nanoclay and gelatin concentrations tend to be better for cell biocompatibility. The bio-inks having a nanoclay concentration of 2% appear to be detrimental to the biocompatibility.

[0059] While the present technology has been described with reference to certain examples, various modifications, changes, omissions, and substitutions can be made without departing from the disclosure. It is intended, therefore, that the disclosure be limited by the scope of the following claims.

CLAIMS

What Is Claimed Is:

1. A bio-ink for three-dimensional printing comprising:
a liquid vehicle comprising deionized water;
gelatin dissolved in the liquid vehicle;
nanoclay platelets dispersed in the liquid vehicle; and
a gelling compound comprising a polysaccharide.

2. The bio-ink of claim 1, further comprising an osmotic agent comprising a sugar, mannitol, sorbitol, glycerol, glycine, or a combination thereof, dissolved in the liquid vehicle.

3. The bio-ink of claim 2, wherein the osmotic agent is a sugar comprising sucrose, glucose, fructose, galactose, lactose, maltose, or a combination thereof.

4. The bio-ink of claim 2, wherein the osmotic agent is included at a concentration from about 260 milliosmoles per liter of bio-ink to about 320 milliosmoles per liter of bio-ink.

5. The bio-ink of claim 1, wherein the gelling compound is gellan gum, agar, agarose, guar gum, carrageenan, xanthan gum, or a combination thereof.

6. The bio-ink of claim 1, wherein the nanoclay platelets comprise laponite nanoclay.

7. The bio-ink of claim 1, wherein the gelatin is included in an amount from 6 grams/100 mL to 12 grams/100 mL, with respect to 100 mL of the bio-ink;

wherein the nanoclay platelets are included in an amount from 1 gram/100 mL to 2 grams/100 mL, with respect to 100 mL of the bio-ink; and

wherein the gelling compound is included in an amount from 0.25 gram/100 mL to 1 gram/100 mL, with respect to 100 mL of the bio-ink.

8. The bio-ink of claim 1, wherein the bio-ink has a melting point from 37 °C to 100 °C.

9. A bio-ink three-dimensional printer comprising:

a bio-ink comprising:

a liquid vehicle comprising deionized water,

gelatin dissolved in the liquid vehicle,

nanoclay platelets dispersed in the liquid vehicle, and

a gelling compound comprising a polysaccharide; and

an extrusion print head connected to or connectable to the bio-ink, wherein the extrusion print head comprises an extrusion nozzle and a driver to push the bio-ink through the extrusion nozzle.

10. The bio-ink three-dimensional printer of claim 9, wherein the bio-ink comprises an osmotic agent comprising a sugar, mannitol, sorbitol, glycerol, glycine, or a combination thereof, dissolved in the liquid vehicle

.

11. The bio-ink three-dimensional printer of claim 10, wherein the nanoclay platelets comprise laponite nanoclay;

wherein the gelling compound is gellan gum, agar, agarose, guar gum, carrageenan, xanthan gum, or a combination thereof; and

wherein the osmotic agent is a sugar comprising sucrose, glucose, fructose, galactose, lactose, maltose, or a combination thereof.

12. The bio-ink three-dimensional printer of claim 10, wherein the gelatin is included in an amount from 6 grams/100 mL to 12 grams/100 mL, with respect to 100 mL of the bio-ink;

wherein the nanoclay platelets are included in an amount from 1 gram/100 mL to 2 grams/100 mL, with respect to 100 mL of the bio-ink;

wherein the gelling compound is included in an amount from 0.25 gram/100 mL to 1 gram/100 mL, with respect to 100 mL of the bio-ink; and

wherein the osmotic agent is included at a concentration from about 260 milliosmoles per liter of bio-ink to about 320 milliosmoles per liter of bio-ink.

13. A method of three-dimensional printing bio-ink comprising:

extruding a bio-ink from an extrusion print head comprising an extrusion nozzle and a driver to push the bio-ink through the extrusion nozzle, wherein the bio-ink comprises:

a liquid vehicle comprising deionized water,

gelatin dissolved in the liquid vehicle,

nanoclay platelets dispersed in the liquid vehicle, and

a gelling compound comprising a polysaccharide.

14. The method of claim 13, further comprising forming a three-dimensional printed structure from the bio-ink and introducing living cells onto the three-dimensional printed structure.

15. The method of claim 13, wherein the nanoclay platelets comprise laponite nanoclay;

wherein the gelling compound is gellan gum, agar, agarose, guar gum, carrageenan, xanthan gum, or a combination thereof; and

wherein the bio-ink further comprises an osmotic agent comprising a sugar, mannitol, sorbitol, glycerol, glycine, or a combination thereof.

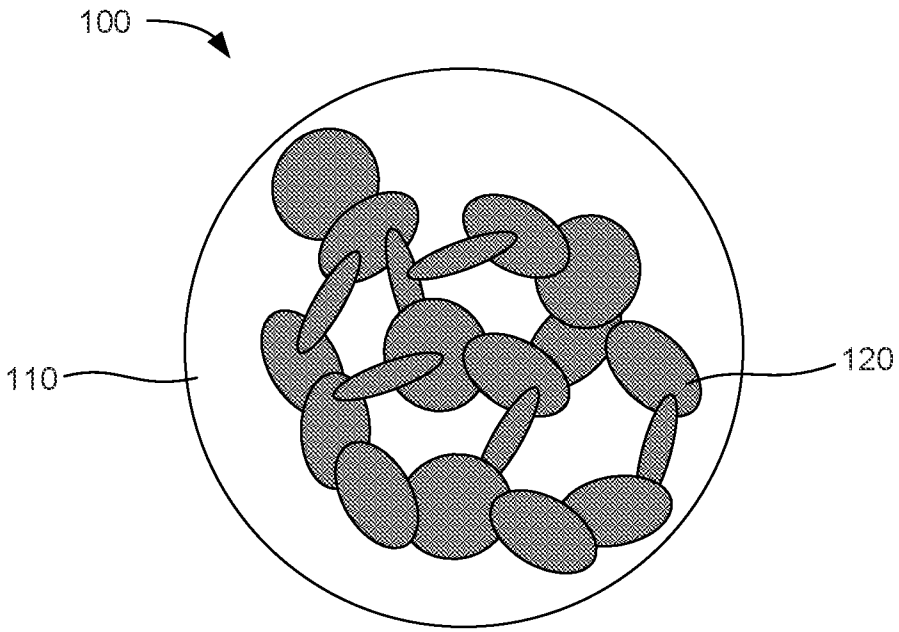


FIG. 1

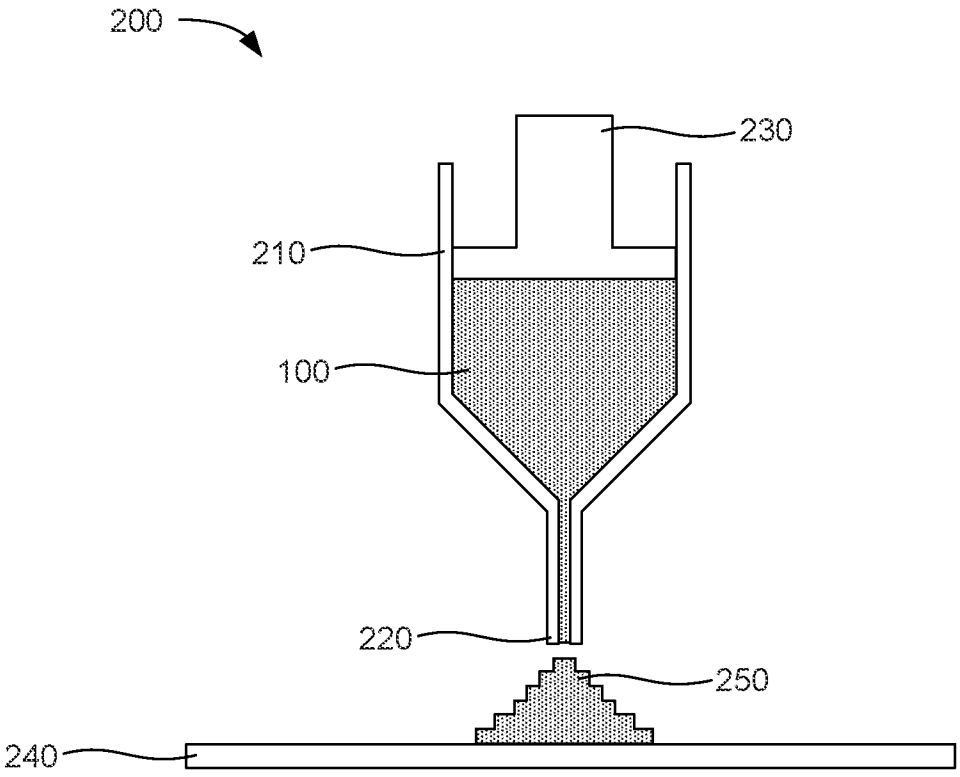


FIG. 2

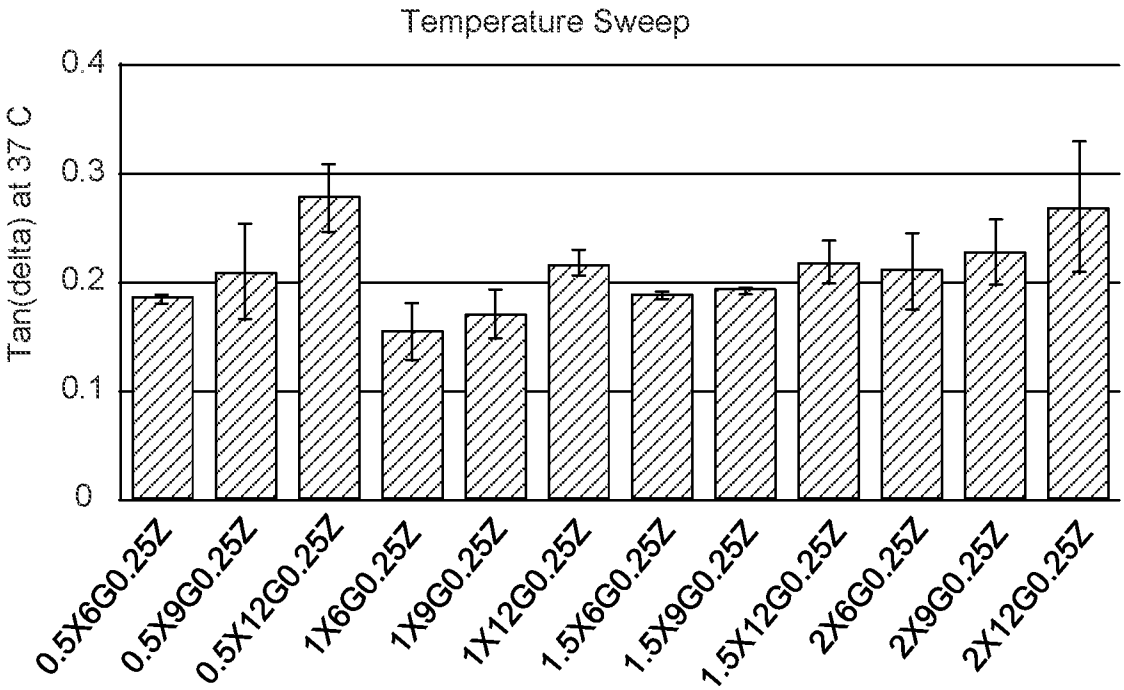

Temperature Sweep
Tan(delta) at 37 C
0.4
0.3
0.2
0.1
0
0.5X6G0.25Z
0.5X9G0.25Z
0.5X12G0.25Z
1X6G0.25Z
1X9G0.25Z
1X12G0.25Z
1.5X6G0.25Z
1.5X9G0.25Z
1.5X12G0.25Z
2X6G0.25Z
2X9G0.25Z
2X12G0.25Z

FIG. 3

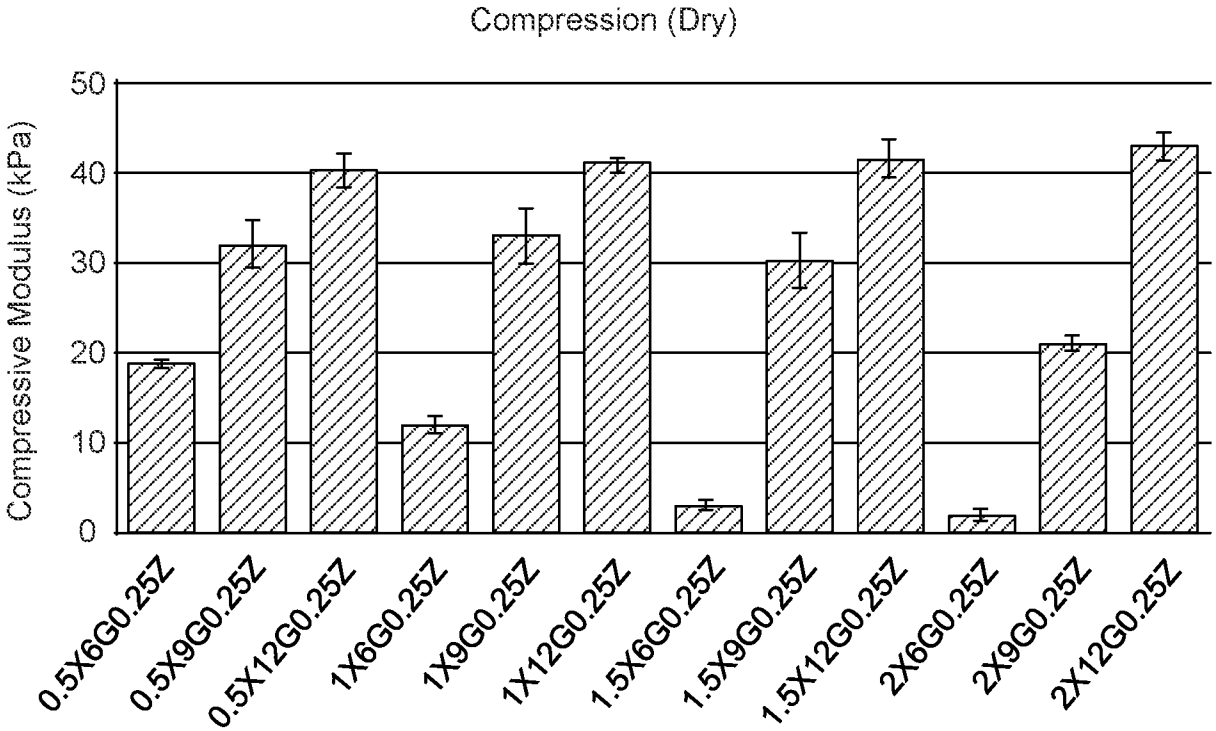

Compression (Dry)
Compressive Modulus (kPa)
50
40
30
20
10
0
0.5X6G0.25Z
0.5X9G0.25Z
0.5X12G0.25Z
1X6G0.25Z
1X9G0.25Z
1X12G0.25Z
1.5X6G0.25Z
1.5X9G0.25Z
1.5X12G0.25Z
2X6G0.25Z
2X9G0.25Z
2X12G0.25Z

FIG. 4

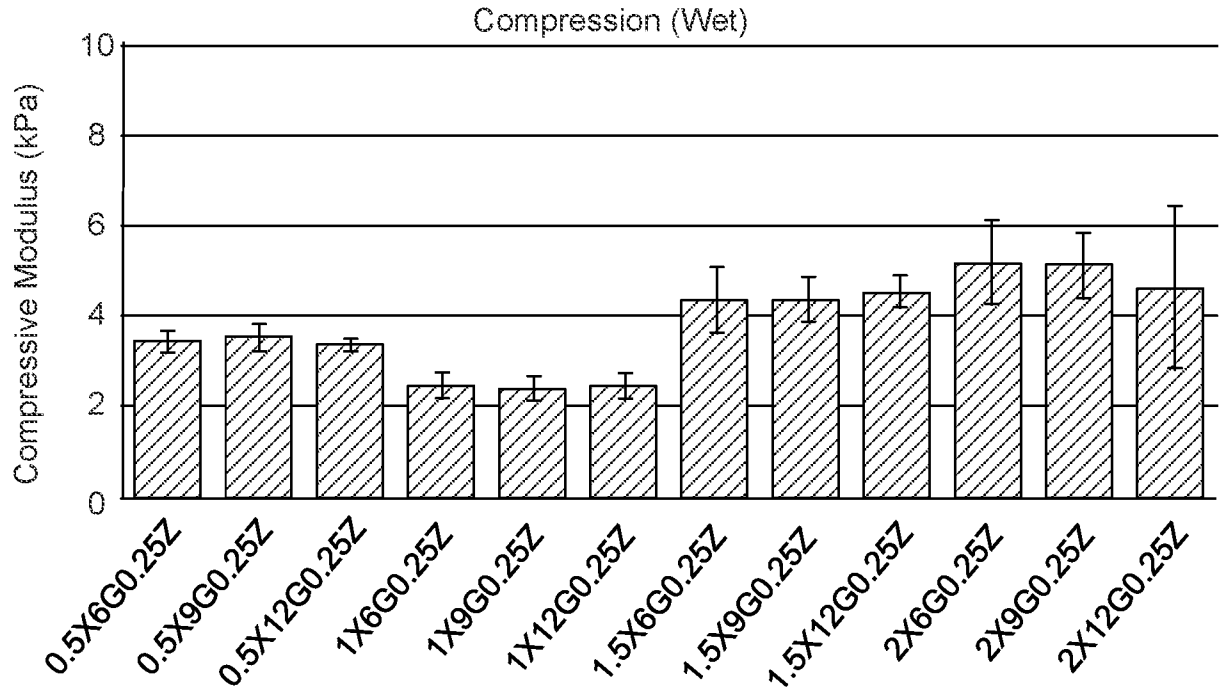

Compression (Wet)
Compressive Modulus (kPa)
10
8
6
4
2
0
0.5X6G0.25Z
0.5X9G0.25Z
0.5X12G0.25Z
1X6G0.25Z
1X9G0.25Z
1X12G0.25Z
1.5X6G0.25Z
1.5X9G0.25Z
1.5X12G0.25Z
2X6G0.25Z
2X9G0.25Z
2X12G0.25Z

FIG. 5

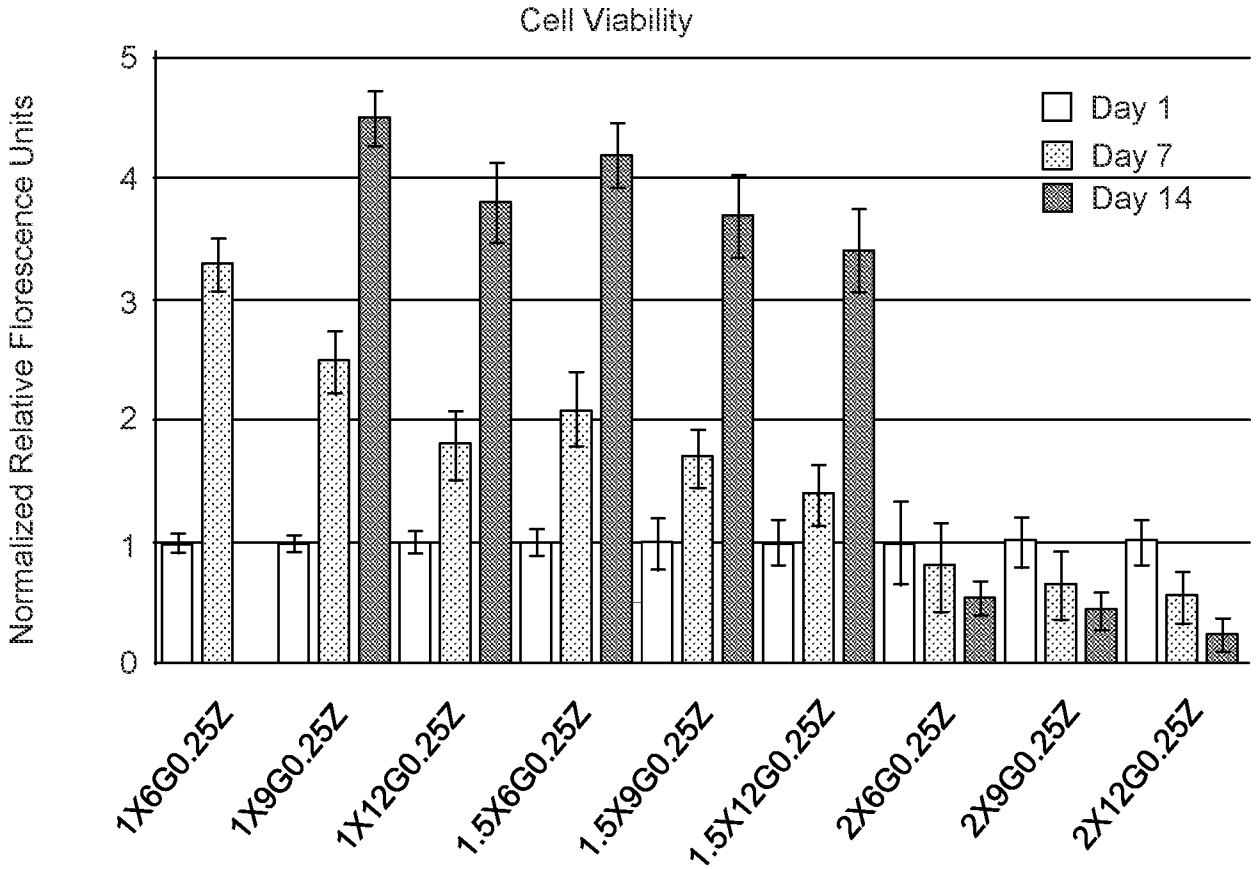

Cell Viability
Normalized Relative Florescence Units
5
4
3
2
1
0
Day 1
Day 7
Day 14
1X6G0.25Z
1X9G0.25Z
1X12G0.25Z
1.5X6G0.25Z
1.5X9G0.25Z
1.5X12G0.25Z
2X6G0.25Z
2X9G0.25Z
2X12G0.25Z

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2021/044261

A. CLASSIFICATION OF SUBJECT MATTER

B29C 64/106 *(2017.01)*
B33Y 70/10 *(2020.01)*
B33Y 30/00 *(2015.01)*

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)

B33Y 70/10, 30/00, B29C 64/40, 64/106

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)

PatSearch (RUPTO internal), USPTO, PAJ, K-PION, Esp@cenet, Information Retrieval System of FIPS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GIANLUCA CIDONIO et al «Printing bone in a gel: using nanocomposite bioink to print functionalised bone scaffolds» September 2019, Materials Today 4:100028, DOI:10.1016/j.mtbio.2019.100028 [он-лайн]. Найдено в Интернет <URL: https://www.researchgate.net/publication/335851475_Printing_bone_in_a_gel_using_nanocomposite_bioink_to_print_functionalised_bone_scaffolds>, p. 2 chapters 2.1.1.-2.1.4., p. 3 chapter 2.8.	1-15
Y	WO 2018/053404 A1 (THE UNIVERSITY OF FLORIDA RESEARCH FOUNDATION, INC.) 22.03.2018	1-15
Y	LIQUN NING et al «Biomechanical factors in three-dimensional tissue bioprinting» Cite as: Appl. Phys. Rev. 7, 041319 (2020); https://doi.org/10.1063/5.0023206. Published Online: 30 December 2020, p. 041319-3 right col.	2-4, 10-12, 15

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

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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	Date of mailing of the international search report
13 December 2021 (13.12.2021)	10 February 2022 (10.02.2022)
Name and mailing address of the ISA/RU: Federal Institute of Industrial Property, Berezhkovskaya nab., 30-1, Moscow, G-59, GSP-3, Russia, 125993 Facsimile No: (8-495) 531-63-18, (8-499) 243-33-37	Authorized officer E. Golubeva Telephone No. 499-240-60-15

Form PCT/ISA/210 (second sheet) (July 2019)