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(54) Title: METHOD AND APPARATUS FOR ACOUSTICALLY ASSISTED DROPLET GENERATION

(57) Abstract: Methods and apparatus for acoustically assisted droplet generation with or without cell encapsulation.

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Method and apparatus for acoustically assisted droplet generation

Cross-reference to Related Applications

[001] The following application claims benefit of U.S. Provisional Application No. 62747721, filed 10/19/2018, which is hereby incorporated by reference in its entirety.

Background

[001] Alginate matrices have been shown to be a robust and exceptional environment for 3D culture of mammalian cells¹ which is important in the development of therapeutics for cancer as well as in the fabrication of synthetic tissue via bioprinting.^{2,3} Alginate matrices provide a model biocompatible matrix for cell culture because their composition can be engineered to imitate that of natural polymers which promote cell adhesion and proliferation.⁴ Alginate matrices have been integrated into droplet microfluidics and have been shown to create droplets for 3D cell culturing.⁵ Methods of synthesizing aqueous droplets via microfluidics are useful but limited in that the drops are stabilized using oil-water emulsion solutions.⁶⁻⁸ This combination of immiscible fluids is not ideal for cell culturing because water soluble nutrients and drug compounds are unable to diffuse through the oil phase into the aqueous droplets where the cells reside. Additionally, biomolecules such as protein may be disrupted by denaturation upon exposure to the oil phase.⁹ Therefore, introducing alternative droplet fluidic methods to create uniform droplet hydrogels for biocompatible 3D cell culturing applications is necessary.

Summary

[002] In general, the present disclosure provides methods and apparatus for acoustically assisted droplet generation. The methods and apparatus utilize water-in-water systems and the resulting droplets may be used, for example, to produce cell culturing microgels.

[003] According to a more specific embodiment, the present disclosure provides a new method of synthesizing size-controlled dextran-alginate droplets via acoustic modulation integration with microfluidics. According to a still further embodiment, the present disclosure provides a microcapillary-based, acoustically assisted, microfluidic system for differential aqueous phase infusion and coupling of one or more entrapped cells in a pre-gel phase. According to yet another embodiment, the method and/or apparatus may be used in 3D cell culturing applications. According to still another embodiment, the method and/or apparatus may be used in 3D bioprinting applications. According to yet another embodiment, the method

can be used for phase separation for component biologicals such as drugs or biological molecules like complex sugars, nucleic acids, proteins, amino acids etc.

[004]

Brief Description of the Drawings

[005] Fig. 1 is a schematic illustration of an exemplary embodiment of the system for producing the 3D microgels of the present disclosure.

[006] Fig. 2 is a graph showing frequency vs flow of droplets without cells.

[007] Fig. 3 is graph showing the monodisperse size of droplets without cells.

[008] Fig. 4 is an image of monodisperse droplets without cells.

[009] Fig. 5 is an image of monodisperse droplets with cells.

[010] Fig. 6 is a graph showing frequency vs flow of droplets with cells.

[011] Fig. 7 is graph showing the monodisperse size of droplets with cells.

[012] Fig. 8 is an image of polydisperse droplets without cells.

[013] Fig. 9 is an image of polydisperse droplets with cells.

[014] Fig. 10 is a graph showing viscosity vs. shear rate of droplets with no, low, and high cell numbers in both DEX and HEPES.

[015] Fig. 11 is a graph showing the DEX Index of refraction.

[016] Fig. 12 is an image showing no droplet formation without cells.

[017] Fig. 13 is an image showing no droplet formation with cells.

[018] Fig. 14 is a schematic illustration of 3-D bioprinting applications using the methods and/or apparatus of the present disclosure.

Detailed Description

[019] In general, the present disclosure provides methods and apparatus for acoustically assisted droplet generation. The methods and apparatus utilize water-in-water systems and the resulting droplets may be used, for example, to produce cell culturing microgels.

[020] According to a more specific embodiment, the present disclosure provides a new method of synthesizing size-controlled dextran-alginate droplets via acoustic modulation integration with microfluidics. According to a still further embodiment, the present disclosure provides a microcapillary-based, acoustically assisted, microfluidic system for differential aqueous phase infusion and coupling of one or more entrapped cells in a pre-gel phase. According to yet another embodiment, the method and/or apparatus may be used in 3D cell culturing applications. According to still another embodiment, the method and/or apparatus

may be used in 3D bioprinting applications. According to yet another embodiment, the method can be used for phase separation for component biologicals such as drugs or biological molecules like complex sugars, nucleic acids, proteins, amino acids etc.

[021] Fig. 1 is a schematic illustration of an exemplary embodiment of the system of the present disclosure. It will be understood that the system shown in Fig. 1 could take the form of a low-cost system using components that are readily available in a laboratory setting (as described in greater detail in the Examples section below), or could be a wholly integrated device comprising components that are designed specifically for their intended purpose in the system. In general, the system 10 includes a microfluidic channel (or device) 12, which is fluidly connected to first and second fluid reservoirs 14 and 16, respectively. An acoustic wave generator 18 is positioned between the second fluid reservoir 16 and microfluidic device 12. Reservoir 24 collects droplets produced in the microfluidic device.

[022] According to an embodiment, the first and second fluid reservoirs may contain aqueous polymer solutions which, when combined under suitable conditions, such as using the system described herein, form three-dimensional (3D) hydrogels having an inner phase and an outer phase.

[023] Accordingly, in practice, first reservoir 14 may contain the outer phase fluid while second reservoir 16 may contain the inner phase fluid. The inner phase fluid from reservoir 16 flows through fluid line 20 and encounters acoustic waves produced by acoustic wave generator 18. The acoustic wave generator oscillates across a small frequency range to modulate droplet formation. The inner phase fluid droplets then encounter the outer phase fluid flowing through fluid line 22 and enter microfluidic channel (or device) 12. The resulting droplets are then collected in reservoir 24, which may contain a suitable media, including, for example CaCl_2 or any other suitable crosslinking agent.

[024] Fluid reservoirs 14 and 16 may take the form of or include syringe pumps or other automated or hand operated mechanisms for controlling fluid flow from the reservoirs.

[025] Acoustic wave generator 18 may take the form of a standard household speaker or other suitable device. While the presently described embodiments primarily utilize an acoustic wave generator to modulate the droplet formation of the inner phase solution, it will be understood that other suitable mechanisms could be employed and are contemplated by the present disclosure. Other suitable well-known mechanisms/devices for modulating droplet formation include, but are not limited to, sonicators, vibrating rods, piezoelectric systems, and the like.

[026] It should be noted that while the present disclosure and accompanying drawings disclose two reservoirs designed to produce two phases, the presently described techniques can also be used to produce more than two phases.

[027] According to an embodiment aqueous polymer solutions are dextran (DEX) and polyethylene glycol (PEG). According to a more specific embodiment the inner phase fluid is a DEX aqueous solution and the outer phase fluid is a PEG aqueous solution.

[028] When dissolved in water, certain multicomponent polymer solutions exhibit phase separation related to thermal equilibrium and polymer properties that include density.¹⁰⁻¹³ Of these polymers, dextran (DEX) and polyethylene glycol (PEG) exhibit phase separation at specific mass percent concentrations. This phase separation of DEX and PEG enables the creation of immiscible droplets of either phase after the solution is physically mixed. Previous work has shown that incorporating these fluids in a microfluidic device can form droplets after a secondary physical force is applied to break up the fluidic jet.^{9,14} This secondary force overcomes the low interfacial tension of the two aqueous phases, enhancing droplet breakup in a co-flowing system. The present disclosure provides a novel system that uses an acoustic force to break-up an aqueous two-phase fluidic system into monodispersed droplets for 3D hydrogel formation and cell culture.

[029] Oil-water interfacial tensions are on the order of tens of millinewtons/meter (mN/m), meaning stable phase separation is favorable.^{15,16} In contrast, the DEX and PEG aqueous solutions exhibit a very low interfacial tension on the order of hundreds of micronewtons/meter ($\mu\text{N/m}$),⁹ requiring higher input energy to favor separation in microfluidic systems. We found that a secondary force supplied by an amplified signal from a speaker facilitates the breakup of two solutions into droplets. By coupling the fluidic inlet of the inner phase (composed of 25% w/w 550 kDa Dextran, 0.5% w/w sodium alginate and a range of concentrations of EMT6 mouse mammary carcinoma model cells) with a direct attachment to a speaker, an acoustic oscillation force enables droplet breakup of a co-flowing fluidic jet. Controlling the input frequency and inner phase flow rates manipulates the size of the hydrogel droplets across a range of 60 to 120 μm diameter for drops without cells (Fig. 2).

[030] As described in greater detail below, the effect of droplet size variation by frequency modulation is observed when enriched PEG (outer phase) is co-flowed with enriched DEX (droplet phase) and monodispersed DEX drops are formed.

[031] According to another embodiment of the present disclosure, this methodology is used to generate monodispersed hydrogels for cell culturing by incorporating a crosslinkable matrix or other gelling substance into at least one of the aqueous phases. According to a specific

embodiment, alginate may be used as the crosslinkable matrix. Varying the weight percent concentrations of alginate drastically changes the viscosity of the already highly viscous DEX droplet phase. Specifically, high concentrations of alginate can inhibit the breakup of the inner phase even with incorporation of an amplified signal to the speaker. To overcome this, it was found that reducing the amount of alginate to 0.5% in the DEX droplet phase enabled droplet generation after increasing the amplitude of the applied acoustic force generated from the speaker. A matrix of ideal frequencies, controlled by an Agilent Technologies 33250A Function/Arbitrary Waveform Generator, across varying droplet phase flow rates can be seen in Figs. 2-13. In general, a decrease in droplet size with increasing acoustic frequency and decreased flow rates was observed.

[032] As stated above, flowing droplets are collected in a suitable media within a reservoir. According to a specific embodiment, the flowing droplets are collected into a CaCl_2 bath, which allows for rapid crosslinking of the DEX-alginate gels, resulting in hydrogels with an almost perfect aspect ratio.

[033] According to a still further embodiment, the presently described methodology is used for 3D cell culturing. Specifically, a single cell suspension of a desired cell line is incorporated into the DEX-alginate aqueous phase prior to droplet formation.

[034] A well-known challenge with the interface of microfluidic droplet generation and cell culture is the necessity for low volumetric flow rates, where cells in a biomaterial suspension may be out of ideal culture conditions for up to hours at a time. As demonstrated by experiments described in greater detail below, the presently described methodology resulted in no quantitative statistically significant difference between a live control group and the experimental (cells in DEX-alginate) group for necrosis. There was a qualitative increase in the necrosis of the cells in the DEX-alginate. However, this is likely due to rigorous dilution, washing, and centrifugation protocol necessary to recover the cell population from the DEX-alginate pre-gel and not due to exposure to the biomaterial nor the time for device operation. Furthermore, the viability of these microgels proved exceptional after 9 days of suspension culture in spinner flask bioreactors. Qualitatively, it appears that there is a lag phase in the proliferation rate of the cells from initial encapsulation to day 3 which is likely caused by a low cell encapsulation concentration (~10 cells/droplet) and the cells being isolated from one another. Once cell-cell contact is established, the growth rate appears to become more exponential and cellular aggregation as in tumor spheroid formation is observed.

[035] Acoustically assisting water/water droplet generation for 3D cell culturing hydrogels provides a low cost and reproducible method to grow and study cell systems across multiple

applications. This technology provides a robust method to generate 3D hydrogels for cell culture and has the potential to be used alongside drug discovery platforms or for 3D printing applications. Our initial work proves to be a platform with feasibility for scaling up by incorporating higher volume microfluidics with an acoustic actuator to yield larger, uniform populations of 3D cell encapsulating hydrogels. This method of encapsulating biological components can be used to trap Giant Unilamellar Vesicles (GUVs) which have a notoriously short lifespan in vitro. Finally, using this gentle method to encapsulate biological components may prove to aid in study for future cancer therapeutics or other drug discoveries since the alginate matrix provides a more biomimetic 3D matrix for cell growth.

[036] According to a still further embodiment, the present disclosure provides a method of 3D bioprinting. Specifically, the aqueous two phase microspheres of the present disclosure can be used to shield cells from shear forces produced during the printing process.

[037] 3D printing has emerged as an ideal solution for tissue engineering and simulated environments for disease study and could allow design of multicellular architectures. A key technical hurdle preventing the widespread use of cell-based additive patterning is the lack of cell-compatible “bio-inks” that have the required properties for scalable 3D printing.²⁰ Additionally, printing is hindered by low cell viability of approximately 40-80%,²¹ attributed to high shear force on the cells as they are extruded through the printer nozzle.

[038] Accordingly, the aqueous two phase system derived microspheres with encapsulated cells described herein could serve as ‘toner’ that can be dispersed within a secondary continuous hydrogel liquid for controlled spatial deposition of a cell-containing “bio-ink” via extrusion printing. (See Fig. 14) The secondary continuous liquid can include crosslinkable biological or synthetic materials used in previous cell printing studies to enable consolidation by crosslinking of the printed liquid microfeatures. Moreover, polypeptides or other additives can be included in the phase separated gel droplet and confer specific function through bioactive domains within the material matrix to direct cell outcome and behavior.

Exemplary Materials and Methods

[039] The description below provides a specific example of specific embodiments of the presently described invention. However, the examples below should not be considered as being limiting as to the scope of the invention as a whole.

[040] *Device setup, construction and coupling with loud speaker.* For the construction of our microfluidic glass microcapillary device, we use a Sutter Instrument co. P-97 micropipette puller to make our 100 μm injection tip and 200 μm collection tip. The diameters are filed with

fine sand paper to exact measurements. The diameter of the unpulled regions of the World Precision Instruments borosilicate glass capillaries is 1.0 mm OD. The injection and collection capillaries are housed in a 1.5 mm OD Harvard borosilicate glass square capillary tube. The ends of the square capillary are covered with Probe Needles M919 plastic-steel syringe tips normal to the surface of the horizontal orientation of the glass capillaries. A square pattern is cut on either side of the syringe tips to snugly cover the capillary components of the device. The syringe tips are then covered and glued down with Deycon Epoxy to prevent leaking over a plain 25 x 75 x 1 mm cm Swiss glass slide. The capillaries are aligned using a Zeiss Axio brightfield microscope at 10X magnification. The capillary ends and syringe tips are coupled to Scientific Commodities, Inc. LDPE 1.3208 mm OD Micro Medical Tubing.

[041] The microfluidic device is placed on the microscope stage for droplet synthesis. The injection tubing, coupled to the injection capillary for inner phase infusion, is taped directly to a bare household loudspeaker surface. The loudspeaker is connected to a Fosi Audio 50W 4 ohms, 20 Hz - 20 kHz, 0.04% THD) Stereo Audio Amplifier Mini Hi-Fi Professional Amp for Home Speakers approximately 10 cm from the microfluidic device. The frequency input signal's source is an Agilent Technologies 33250A Function/Arbitrary Waveform Generator connected directly into the amplifier. The input peak-to-peak voltage is set to 10 V for all of the experiments. We vary the frequency in 5 Hz intervals from 10 Hz to 60 Hz to generate the matrices seen in Figs 2 - 13. The injection tubing is connected to a Becton Dickson 10mL plastic syringe and Harvard Apparatus, PHD 200 Infuse/Withdraw, CAT#: 70-2001, FREQ(HZ) 50/60 Syringe Pump approximately 74 cm from the microfluidic device. The injection syringe contains the $\sim 10^7$ cells/mL of EMT6 mouse mammary carcinoma cells in 5% enriched 550 kDa Dextran 25mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150mM NaCl aqueous solution from Sigma Aldrich with 0.5% by weight alginate from Sigma Aldrich at pH7.4. The tubing connected to the outer phase infusion syringe tip is connected to a Becton Dickson 60mL plastic syringe on another Harvard Apparatus Syringe Pump approximately 80 cm from the microfluidic device. The 60mL syringe contains the outer phase solution of 17% enriched 8 kDa Poly(ethylene glycol) 25mM HEPES, 150mM NaCl aqueous solution at pH 7.4 from Sigma Aldrich. The second syringe tip distal to the injection side is coupled with the same Micro Medical Tubing and connected to a Becton Dickson 10 mL plastic syringe for excess outer phase waste approximately 20 cm from the device. The collection capillary is connected with a short 2.38 mm OD Tygon tubing to another 1.0 mm OD $\sim 90^\circ$ bent World Precision Instruments borosilicate glass capillary that is submerged in a

20 mL Scintillation vial half-filled with a solution of 1M CaCl₂, 150 mM NaCl, 25 mM HEPES, and pH 7.4 crosslinking solution.

[042] *Aqueous polymer phase separation (polymer phase enrichment).* The Dextran (DEX) and Poly(ethylene glycol) (PEG) solutions are “enriched” prior to hydrogel synthesis. 5% w/w of a 50mL aqueous solution of 550 kDa DEX in 25mM HEPES buffer, 150 mM NaCl at pH of 7.4 is dissolved thoroughly. A 17% w/w 50mL aqueous solution of 8 kDa (PEG) 25mM HEPES buffer, 150mM NaCl at pH of 7.4 is dissolved as well. The two clear solutions are added to a 125 mL glass separatory funnel and mixed thoroughly. The two aqueous polymer phases are given 48-hours to separate into distinct phases. The higher density DEX phase separates into the bottom of the funnel and the lower density PEG remains as the top phase. A Refractometer was used to verify the bottom phase as DEX. Each phase is then collected into separate vials. 0.5% w/w alginate is added to the collected DEX and stirred for 24 hours for complete mixing. The DEX-alginate and PEG solutions are then both filtered using a 0.2 µm Nalgene bottle top filter to sterilize for cell culture.

[043] Four pure dextran weight percent solutions were mixed in the polymer buffer, used for the cell encapsulation experiments, containing millipore filtered water, 150 mM NaCl, and 25 mM Hepes pH controlled at 7.4. The solutions were prepared at 5% w/w, 10% w/w, 20% w/w, and 30% w/w of DEX in polymer buffer. The solutions were measured for their index of refraction values 5 times each with a standard deviation smaller than the area of the points on the graph. A 17% PEG and 5% DEX in aqueous polymer buffer solution was enriched as described previously; the bottom phase was collected for interrogation. The value of the unknown bottom phase was plotted against the known indexes of refraction and interpolated to the fit quadratic curve to determine the mass %. Based on the total volume the bottom phase, ~20 mL, relative to the total volume of partitioned PEG and DEX, 200 mL, the index of refraction value (red) agrees with the theoretical calculated number; 24.6% to 25% respectively.

[044] *Three-dimensional cell culturing.* EMT6 mouse mammary carcinoma cells are maintained using standard sterile cell culture conditions (37°C, 5%CO₂) in 150 mm tissue culture treated polystyrene dishes (Corning) with 20 mL of Minimal Essential Media supplemented with 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin (Sigma-Aldrich) to grow up for encapsulation. In general, if desired cell/droplet occupancy is 10 cells/100 µm DIA droplet then 2×10^7 cells/mL is required for the encapsulation protocol. This concentration is easily adjusted by changing the initial cell encapsulation concentration. Cells at ~80%

confluence in 150 mm dishes (depending on the desired final concentration) are washed with 3 mL of 0.5% Trypsin and then incubated in 5 mL of 0.5% Trypsin (Sigma-Aldrich) for up to 30 minutes to digest and release attached cells. The cells are then washed off the dish with gentle pipetting by adding 5 mL of media to the 5 mL of trypsin and cells. This 10 mL cell suspension is transferred into a 50 mL centrifuge tube (and combined with cells recovered from additional 150 mm dishes if applicable), a sample is taken for cell counting using a Z Series Coulter Counter and centrifuged at 1500 RPM for 10 minutes to pellet. The cell concentration to be used is determined, the cell pellet is resuspended into 20 mL physiological buffer (25 mM HEPES, 150 mM NaCl, pH 7.4), and the volume corresponding to the desired final cell concentration is transferred to a fresh 50 mL centrifuge tube. This adjusted cell suspension is then centrifuged at 1500 RPM for 10 mins to wash and pellet. The physiological buffer supernatant is then aspirated and the cell pellet is resuspended into 500 μ L of sterile filtered DEX-alginate by gentle pipetting using a 1000 μ L pipette. The 500 μ L of DEX-alginate cell suspension is drawn up into an 18G needle/10 mL syringe (BD) and then added dropwise to 2.5 mL of DEX-alginate in a 50 mL centrifuge tube while vortexing. The 3 mL cell suspension is then passed through a 40 μ m cell strainer test-tube filter insert (BD Falcon) into a fresh 50 mL centrifuge tube to produce the final single cell suspension in DEX-alginate. A sample is taken from this solution to count the final cell concentration as there is some cell loss during the protocol due to the highly-viscous nature of the DEX-alginate and the filtering out of any cell clumps. This dropwise-vortex mixing and filtering of the DEX-alginate and cells guarantees a single cell suspension which is paramount to avoiding clogs in the microfluidics device. The cell suspension is loaded into a 10 mL syringe for droplet synthesis in the microfluidics system.

[045] Over the course of droplet synthesis (about once/hour), crosslinked droplets are removed from the setup and allowed to settle in the 1 M CaCl_2 buffer so that the excess Ca^{2+} may be removed and the cell encapsulating droplets can be transferred by gentle pipetting into a temperature equilibrated 125 mL spinner flask bioreactor (Corning) with 125 mL of complete media adjusted to pH 7.4 with 25 mM HEPES. For the t_0 initial time point, a 20 μ L sample of droplets is pipetted onto a microscope slide (VWR) and placed onto a brightfield microscope for imaging using a Nikon camera and SPOT Basic software. Once the total cell encapsulating droplet population has been transferred into the spinner flask bioreactor, the head space is backfilled with 5% CO_2 and placed onto a slow stir plate in a warm room maintained at 37°C for the duration of the time course (9 days). At days 3, 5, 7, and 9 samples are taken from the

culture for imaging as described above and the culture media is changed. The spinner flask bioreactor is removed from the stir plate, allowing the droplets to settle to the bottom of the flask. Once settled, the media is aspirated being careful to leave about 20 mL at the bottom so as to not aspirate droplets and then refilled to a final volume of 125 mL with temperature equilibrated complete media and backfilled as before.

[046] On day 9, the total droplet population is collected and the cells are recovered as well as counted for comparison to the initial cell concentration. To collect the droplet population, the droplets are allowed to settle and the media is aspirated until 20 mL of media and droplets remain in the spinner flask. The 20 mL of droplets and media is pipetted into a 50 mL centrifuge tube and the droplets are allowed to settle once more (~10 minutes). The remaining media is carefully aspirated and 20 mL of a droplet dissolving buffer (0.5% trypsin, 50 mM sodium citrate, 25 mM HEPES, 150 mM NaCl pH 7.4) is added to the droplets in the 50 mL tube. The tube is incubated at 37°C for 30 minutes with vortexing every 5 minutes to encourage dissociation of cell aggregates. The recovered cells in dissolving buffer are then centrifuged at 1500 RPM for 10 minutes, resuspended into 1 mL of physiological buffer and counted using the Coulter Counter.

[047] *Flow cytometry analysis.* Upon completion of droplet synthesis, we assay the cells from the remaining cell suspension in DEX-alginate for apoptosis by annexin V and necrosis by propidium iodide (also known as LIVE/DEAD). The DEX-alginate cell suspension is compared to LIVE and DEAD control populations. The LIVE cells are processed with trypsinization into a single cell suspension and remain in complete media on ice during the experiment while the DEAD control cells are treated by suspension in 50% ethanol for 60 minutes at 37°C and 5% CO₂ in the incubator. Once the DEX-alginate cell suspension has been in ambient conditions for the duration of droplet generation (~2 hours), it is transferred to a 15 mL centrifuge tube and diluted by the addition of physiological buffer to dilute the viscosity of the solution. The suspension is centrifuged at 3000 RPM for 10 minutes. In this first wash, the cells form a band towards the bottom of the test tube but have not yet been fully pelleted so ~5 mL of the diluted DEX-alginate is removed by aspiration and 5 mL of physiological buffer is added and vortexed. The tube is centrifuged at 3000 RPM for 10 minutes. This wash/centrifugation protocol is repeated once more to pellet the cells at the bottom of the tube and remove as much residual DEX-alginate solution as possible. The DEX-alginate-buffer supernatant is aspirated and the cells are washed in 10 mL of physiological buffer followed by aliquoting into 4, 15 mL test tubes and centrifugation at 2000 RPM for 10 minutes. The staining

protocol is then followed as described in Alexa Fluor 488® annexin V/Dead cell apoptosis Kit with Alexa Fluor 488® annexin V and PI for flow cytometry from Invitrogen. The samples are analyzed (100,000 events per group) using an Attune NxT flow cytometer.

[048] *Rheology*. A Discovery Hybrid Rheometer HR-3 with the 40 mm 2.0 cone plate, peltier plate steel geometry has been used to do the rheology analysis. Each sample was loaded two times on the rheometer and the experiment was repeated for three times for each load. The rheological characteristic of the samples has been analyzed with two different methods. The first one is the frequency sweep with the angular frequency from 628 to 0.1 rad/s and the second one is the flow ramp with shear rate from 0.01 to 1000 1/s. The young and storage modulus and complex viscosity can be obtained from the frequency sweep test and the viscosity can be obtained from the flow ramp test (used for Figure 2). Each measurement has been repeated three times for consistency.

[049] The results obtained for the following solutions of PEG and DEX are listed below. We chose these solutions to observe whether cell concentration affects viscosity which therefore affects the droplet generation capabilities of our microfluidic device.

1. DEX+PBS (no, low, and high cell)
2. ALG+PBS (no, low, and high cell)
3. DEX+PBS (no, low, and high cell)
4. ALG (no, low, and high cell)
5. DEX (no, low, and high cell)

[050] The specific methods and compositions described herein are representative of preferred embodiments and are exemplary and not intended as limitations on the scope of the invention. Other objects, aspects, and embodiments will occur to those skilled in the art upon consideration of this specification, and are encompassed within the spirit of the invention as defined by the scope of the claims. It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. The invention illustratively described herein suitably may be practiced in the absence of any element or elements, or limitation or limitations, which is not specifically disclosed herein as essential. The methods and processes illustratively described herein suitably may be practiced in differing orders of steps, and that they are not necessarily restricted to the orders of steps indicated herein or in the claims.

[051] References: All patents and publications referenced or mentioned herein are indicative of the levels of skill of those skilled in the art to which the invention pertains, and each such

referenced patent or publication is hereby incorporated by reference to the same extent as if it had been incorporated by reference in its entirety individually or set forth herein in its entirety. Applicants reserve the right to physically incorporate into this specification any and all materials and information from any such cited patents or publications.

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What is claimed is:

1. A method for synthesizing monodispersed populations of aqueous droplets comprising:
 - providing first and second aqueous phase solutions;
 - delivering an acoustic force to the first aqueous phase solution;
 - co-flowing the first and second aqueous phases through a microfluidic device to form phase separated droplets; and
 - collecting the droplets.
2. The method of claim 1 wherein at least one of the first aqueous phase solutions includes a cross-linkable matrix.
3. The method of claim 2 wherein at least one of the first aqueous phase solutions includes a viable cell suspension.
4. The method of claim 3 wherein the aqueous phase solution containing the viable cell suspension also includes an additive that interacts with the cells.
5. The method of claim 1 wherein the first or second aqueous phase solution is dextran (DEX).
6. The method of claim 1 wherein the first or second aqueous phase solution is polyethylene glycol (PEG).
7. The method of claim 1 wherein one of the aqueous phase solutions is DEX and the other is PEG.
8. The method of claim 2 wherein the cross-linkable matrix is alginate.
9. A population of monodisperse biocompatible three-dimensional (3-D) hydrogels incorporating a crosslinkable matrix suitable for cell encapsulation.
10. The population of monodisperse biocompatible 3-D hydrogels of claim 9 comprising an encapsulated cell culture.

11. The population of monodisperse biocompatible 3-D hydrogels of claim 9, wherein the hydrogels are formed by:

- providing first and second aqueous phase solutions wherein the first aqueous phase solutions includes a cross-linkable matrix and a viable cell suspension;
- delivering an acoustic force to the first aqueous phase solution;
- co-flowing the first and second aqueous phases through a microfluidic device to form phase separated droplets; and
- collecting the droplets.

12. The population of monodisperse biocompatible 3-D hydrogels of claim 11 wherein at least one of the first or second aqueous phase solutions includes an additive that interacts with the cells in the viable cell suspension.

13. An ink for three-dimensional (3D) bio-printing comprising the population of monodisperse biocompatible three-dimensional (3-D) hydrogels of claims 10, 11, or 12.

14. A device for forming a monodisperse population of biocompatible three-dimensional (3D) hydrogels comprising:

- means for applying an acoustic force to a first aqueous phase solution;
- means for co-flowing the first and second aqueous phase solutions to a microfluidic channel, wherein the microfluidic channel produces droplets from the co-flowed first and second aqueous phases; and
- means for collecting the droplets.

15. A method for producing a three-dimensional (3D) biostructure incorporating viable cell cultures comprising:

- providing a bioink comprising a population of monodisperse biocompatible three-dimensional (3-D) hydrogels incorporating a crosslinkable matrix encapsulating a viable cell culture; and
- extruding the bioink into a three-dimensional shape through a printer nozzle.

16. The method of claim 15 wherein the bioink is produced by:

- providing first and second aqueous phase solutions wherein the first aqueous phase solutions includes a cross-linkable matrix and a viable cell suspension;

delivering an acoustic force to the first aqueous phase solution;
co-flowing the first and second aqueous phases through a microfluidic device to form phase separated droplets; and
collecting the droplets.

17. The method of claim 16 wherein at least one of the first or second aqueous phase solutions includes an additive that interacts with the cells in the viable cell suspension.

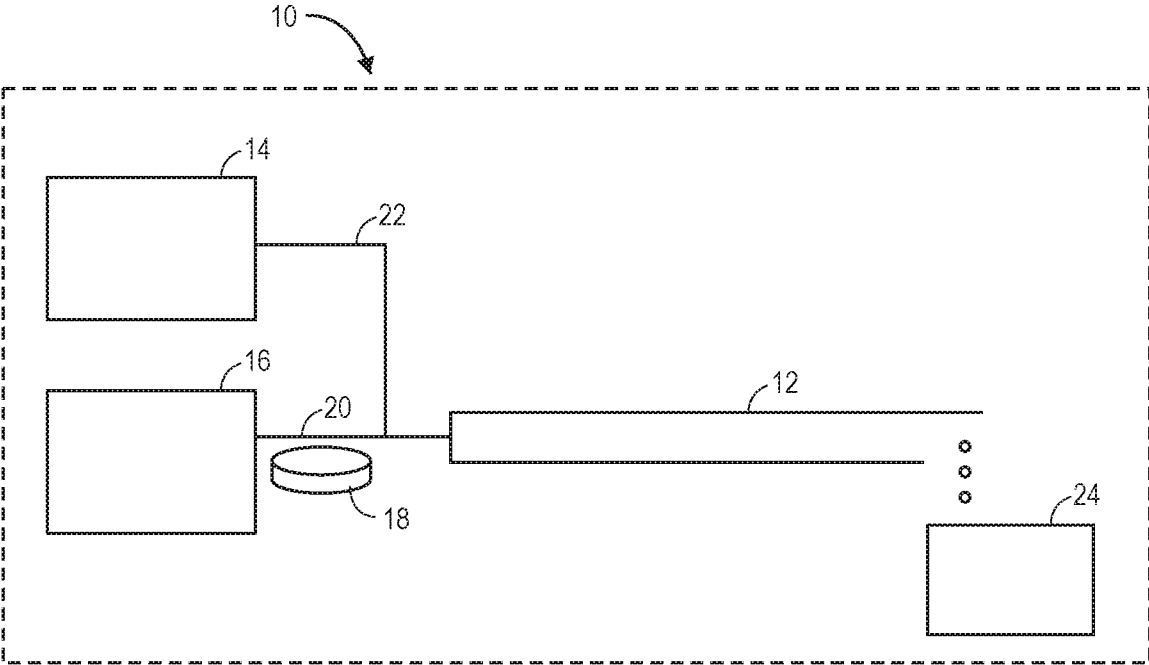


FIG. 1

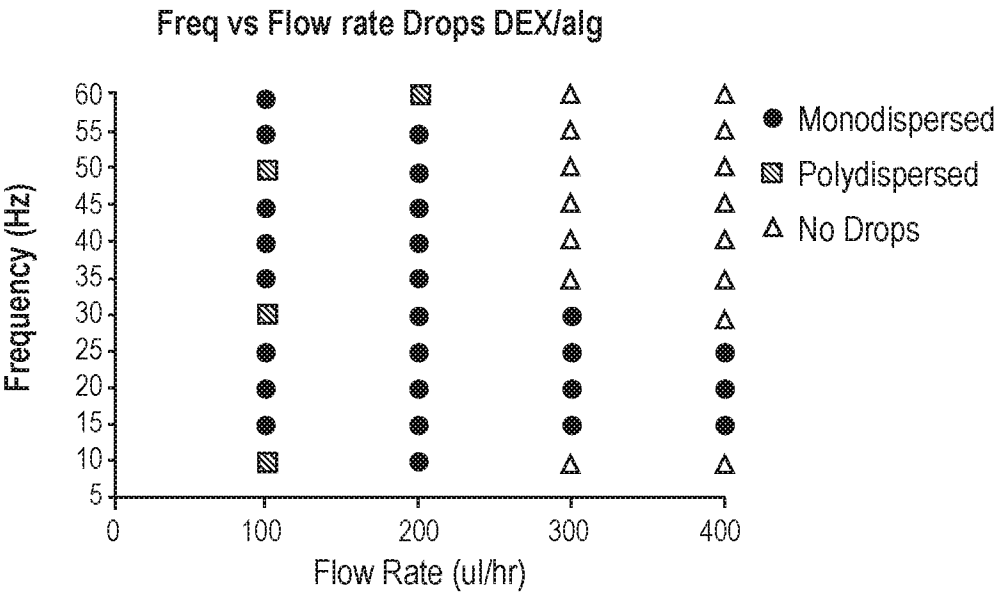


FIG. 2

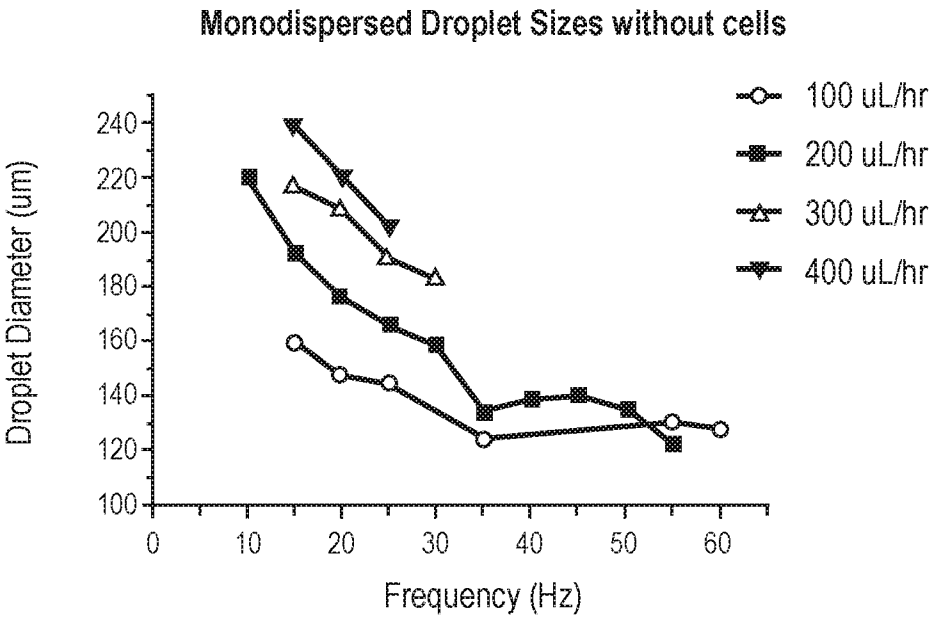


FIG. 3

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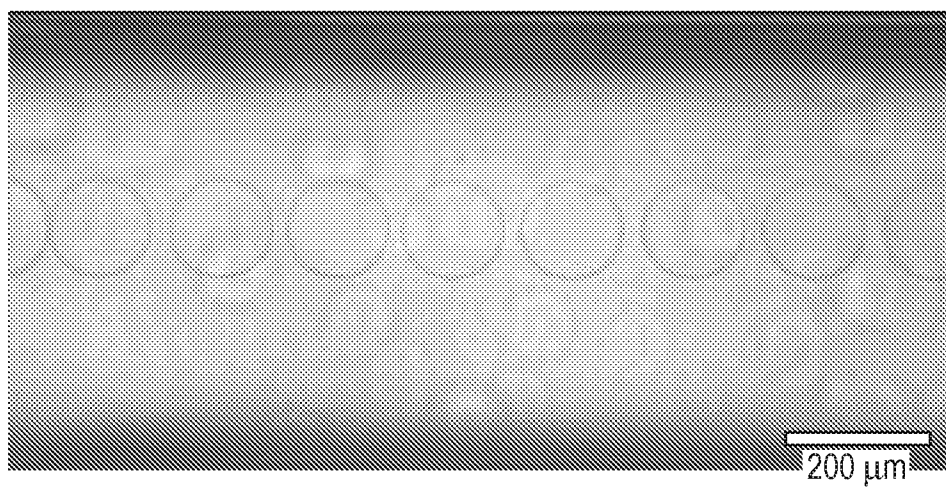


FIG. 3

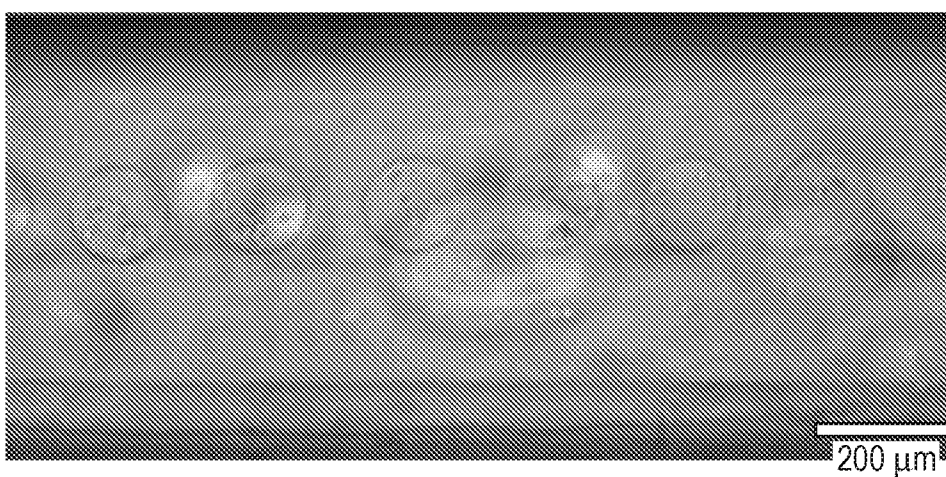
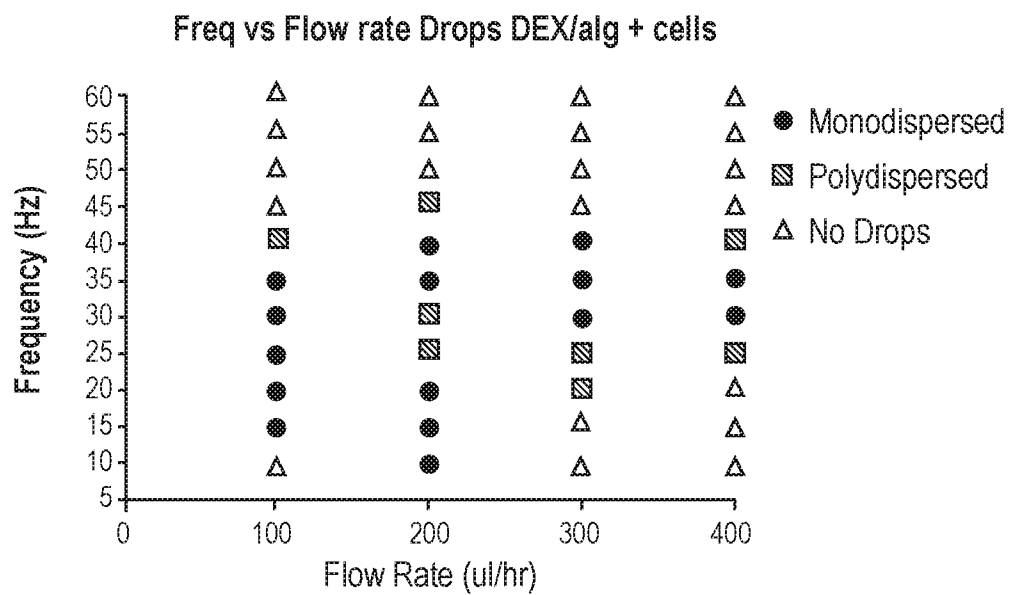
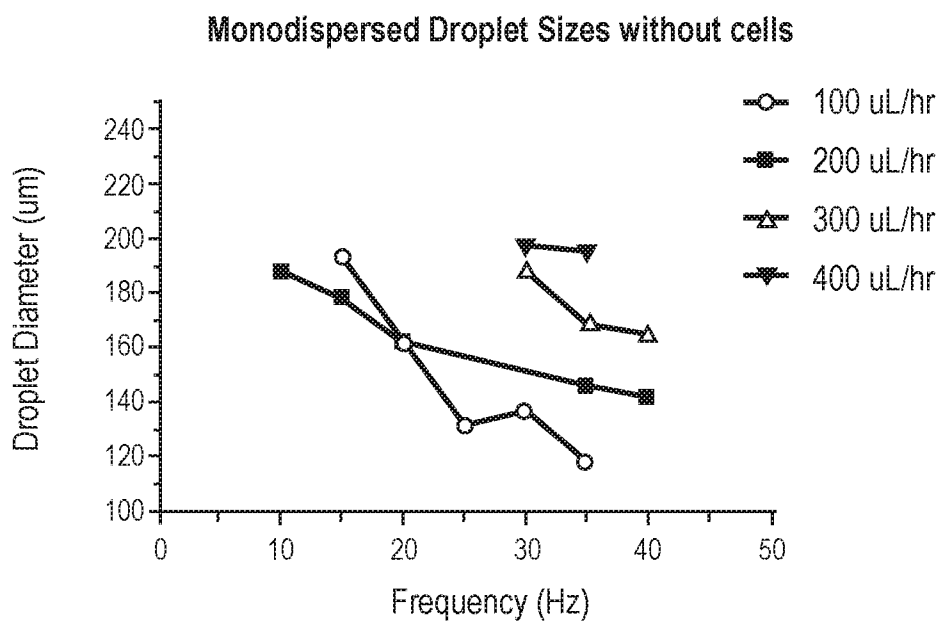


FIG. 4

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**FIG. 6****FIG. 7**

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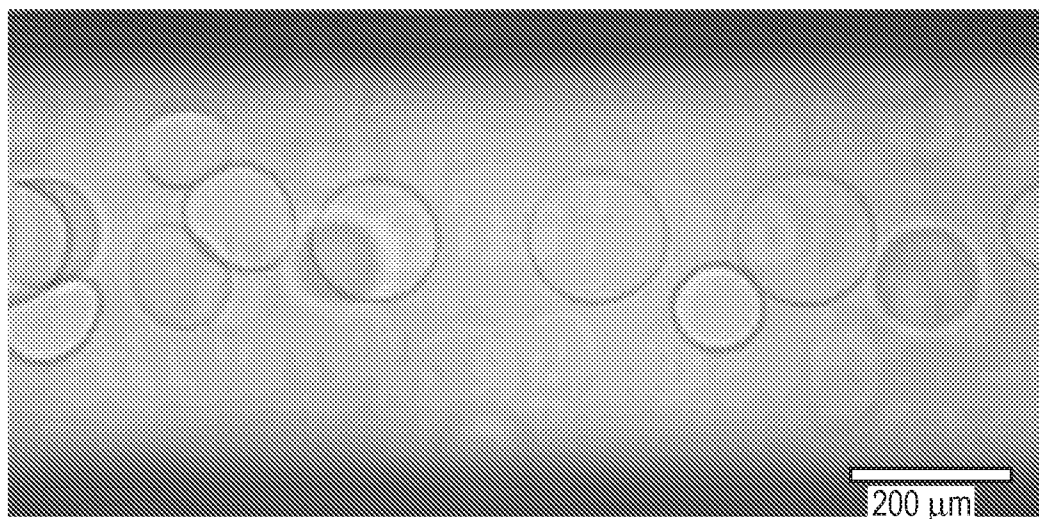


FIG. 8

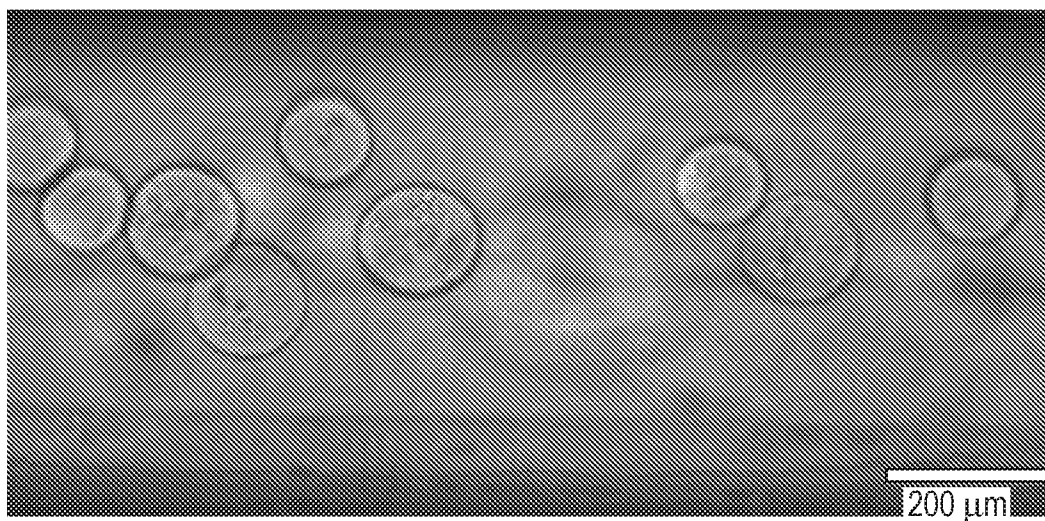


FIG. 9

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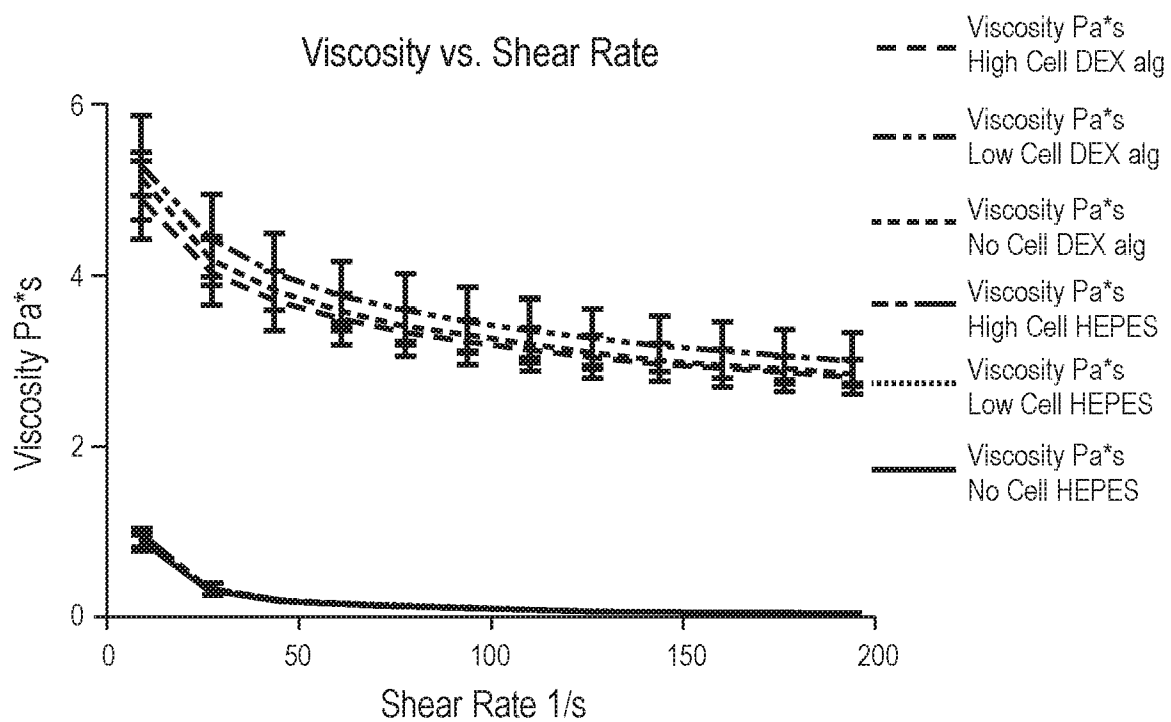


FIG. 10
DEX Index of Refraction

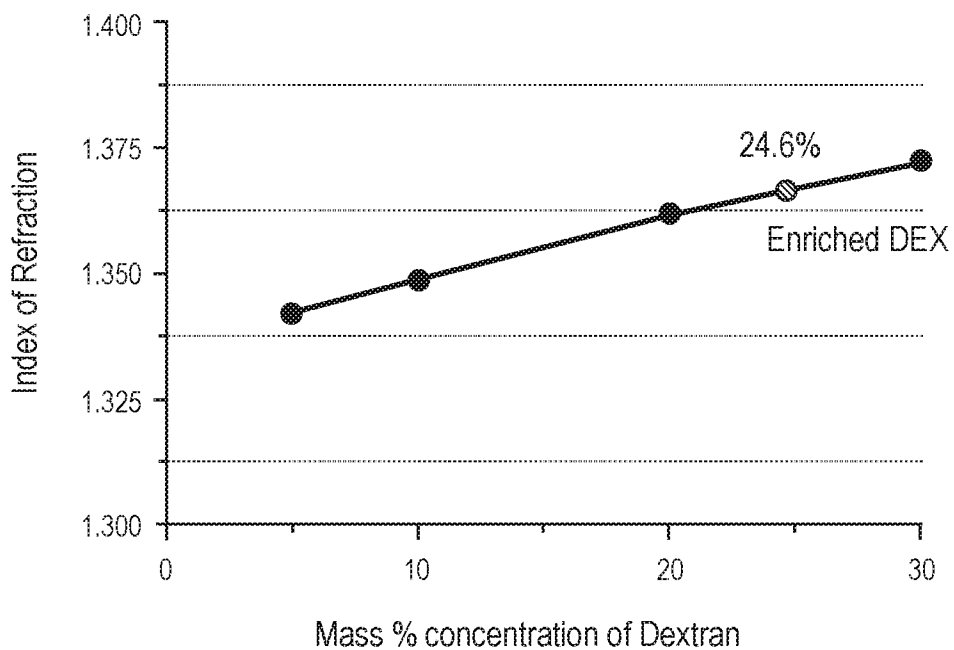


FIG. 11

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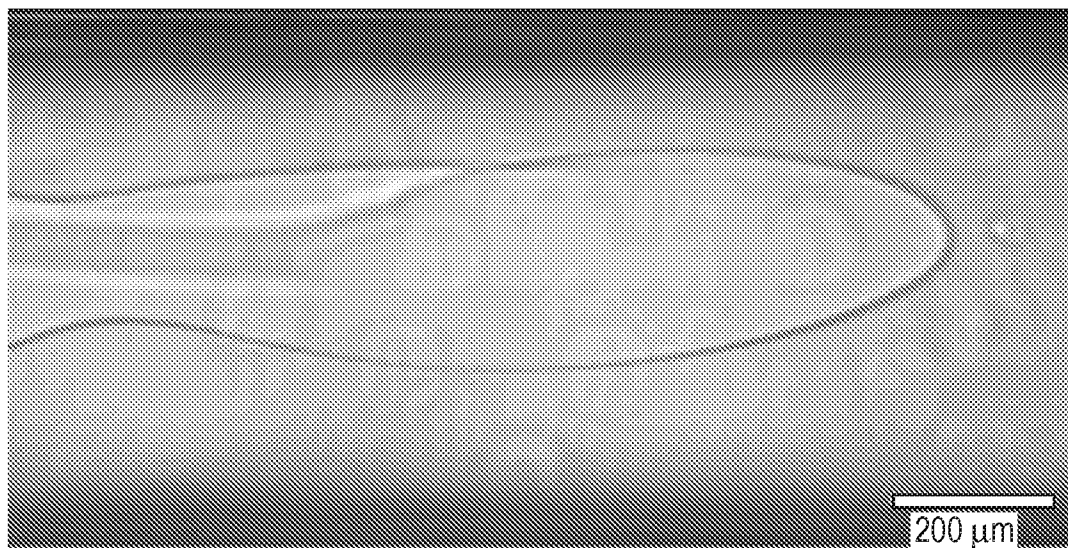


FIG. 12

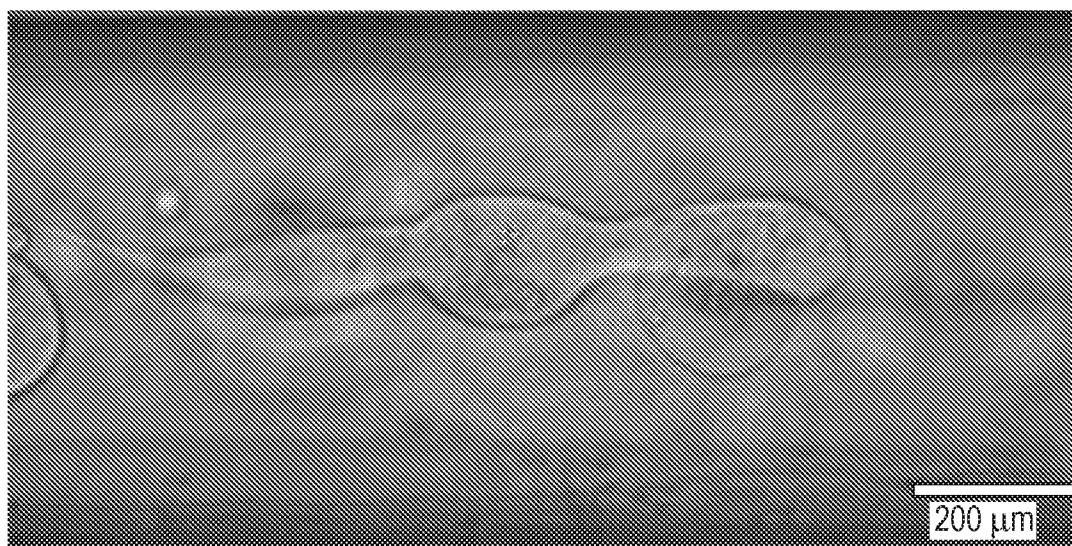
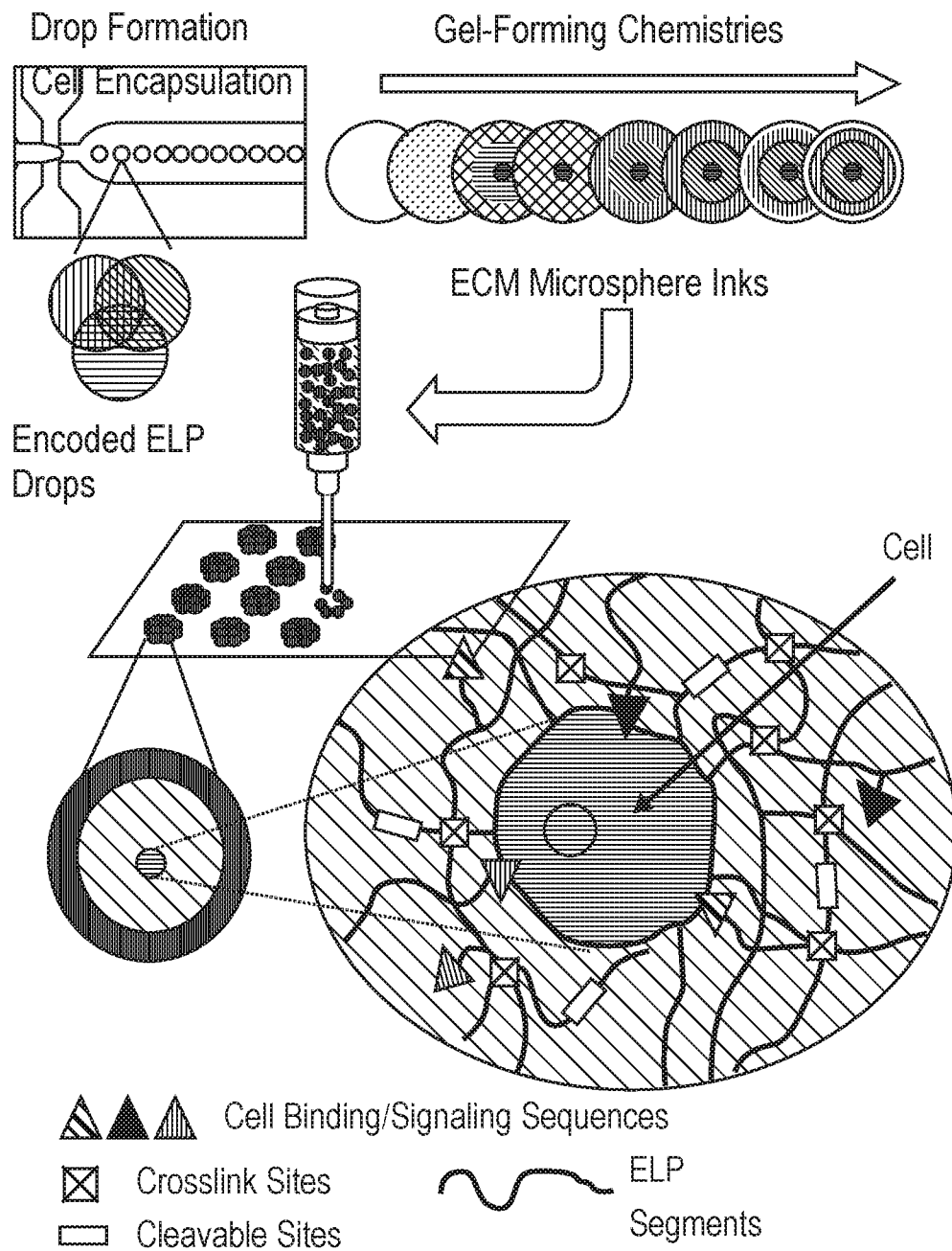


FIG. 13

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**FIG. 14**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2019/057243

A. CLASSIFICATION OF SUBJECT MATTER		
<i>C12N 5/02</i> (2010.01) <i>B33Y 30/00</i> (2015.01) <i>B33Y 70/00</i> (2015.01) <i>B33Y 80/00</i> (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)		
C12N, B33Y		
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)		
E-Library, Espacenet, Google Scholar, NCBI, PATENTSCOPE, PatSearch, PubMed, SCOPUS, USPTO, J-PlatPat		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MASTIANI M., et al. "High-Throughput Aqueous Two-Phase System Droplet Generation by Oil-Free Passive Microfluidics", ACS Omega. 2018-08-16; 3(8): 9296-9302; DOI: 10.1021/acsomega.8b01768	1-8, 11, 12, 14, 16, 17
Y	FRANKE T., et al. "Surface acoustic wave (SAW) directed droplet flow in microfluidics for PDMS devices", Lab on a Chip. 2009; 9(18):2625-2627; DOI: 10.1039/b906819h; especially page 2625, fig.1,2	1-8, 11, 12, 14, 16, 17
☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.		
*	Special categories of cited documents:	
"A"	document defining the general state of the art which is not considered to be of particular relevance	
"E"	earlier document but published on or after the international filing date	
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	
"O"	document referring to an oral disclosure, use, exhibition or other means	
"P"	document published prior to the international filing date but later than the priority date claimed	
	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
	"&"	document member of the same patent family
Date of the actual completion of the international search		Date of mailing of the international search report
13 December 2019 (13.12.2019)		30 January 2020 (30.01.2020)
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 V. Gorshkov-Cantacuzene Telephone No. (8-499) 240-25=91

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2019/057243

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	UTECH S., et al. "Microfluidic Generation of Monodisperse, Structurally Homogeneous Alginate Microgels for Cell Encapsulation and 3D Cell Culture", Advanced Healthcare Materials. 2015; 4:1628-1633; DOI: 10.1002/adhm.201500021	9-10
Y		8, 11- 13, 15-17
Y	DEVI M.G., et al. "A Review of Three-dimensional Printing for Biomedical and Tissue Engineering Applications", The Open Biotechnology Journal. 2018-09-07; 12:241-255; DOI: 10.2174/1874070701812010241; especially pages.241-246	13, 15-17