Method for producing organoids, organoids produced with this method, and use thereof

Technical Field

The invention relates to a method for producing organoids which ensures the production of organoids with a predetermined size and shape. The invention also relates to organoids produced with this method, and use thereof. The invention is applicable to 3D bioprinting, tissue engineering, transplantology, and biomedical research.

Prior Art

There are a number of existing protocols describing a bioprinting method and system for three-dimensional organs and organoids, including document **US10513691B2,** where a method for printing an organoid comprises: providing a media chamber with a medium comprising a plurality of cells and one or more polymer precursors; and then using at least one energy beam to generate an organoid/organ. The patent does not disclose specific numerical parameters for bioprinting and a purification step using hexane from silicone oil.

Document **CN104411318A** discloses organoids comprising or consisting of placental vascular cells devoid of extracellular matrix and methods for their production. The methods for producing organoids include the bioprinting of one or more cell types on or within a decellularized placental vascular scaffold. Synthetic materials and ECM are used to produce a hybrid scaffold. The above document does not disclose a purification step using hexane from silicone oil.

European patent application **EP3993843A1** in turn discloses a hydrogel capsule comprising a cell, a protein, and a cross-linking agent; wherein the cell is within a first core layer comprising the protein; and wherein the first core layer is surrounded by a second layer comprising the protein and the cross-linking agent. The hydrogel capsules of the invention are particularly useful for encapsulating pancreatic islet cells. With regard to the application described, there are differences in the purification process, in which spheroids are purified by washing them with a medium, whereas the use of hexane from silicone oil is not disclosed.

Document **CN116330639A** relates in turn to the technical field of biological materials, in particular to 3D printing of a fallopian tube, and to a preparation method and application thereof. It discloses a bioink composition, which includes a bio-hydrogel and fallopian tube cells, the bio-hydrogel raw materials including a natural polymer material, a synthetic polymer material, and a photoinitiator. The application does not disclose specific numerical parameters for bioprinting and a purification step using hexane from silicone oil.

The next described solution **CN111197024B** discloses a pancreatic-like structure consisting of vascularised cells and pancreatic-like tissue cells, which has the morphology, phenotypic characteristics, and physiological functions of natural tissues. In addition, the above document describes a construction method for the pancreatic-like structure, comprising: preparation of pancreatic-like cell clusters: inducing differentiation of stem cells and/or progenitor cells into pancreatic-like cell clusters *in vitro*; mixing vascularised cells and pancreatic cell clusters with hydrogel materials, which are mixed and subjected to bioprinting; secondary crosslinking can proceed in any manner, e.g. chemical crosslinking, physical crosslinking, enzyme crosslinking, photocrosslinking, etc., preferably chemical crosslinking; subsequently, the three-dimensional structure is cultured using a multi-cell culture medium and/or bioreactor to obtain the pancreatic-like structure. The above document does not disclose the description of a purification step using hexane from silicone oil.

Document **CN117549552A** discloses organoids, a preparation method and application thereof. The first step is the preparation of a bioactive ink, which includes a dispersion medium and a biological unit; subsequently, the 3D printing method is used for bioprinting so as to apply the bioactive ink onto the substrate to obtain an organoid structure. The parameter settings of 3D printing include: printing pattern, the height of the printing needle from the substrate, inkjet printer air pressure, unit dot inkjet time, substrate contact angle, and ink droplet printing accuracy. The design of the bioactive ink of this disclosure allows for obtaining an ink droplet with significantly improved printing accuracy and achieving nanolitre-level printing. The method according to the invention does not disclose a purification step using hexane from silicone oil.

International patent application **WO2017011854A1** in turn discloses a process for producing a three-dimensional tissue culture model by (a) printing a drop of bioink on a substrate; (b) printing a drop of an activator on the drop of bioink to form a hydrogel droplet; (c) repeating steps (a) and (b) in any order to form a hydrogel mould adapted to receive a drop containing cells; (d) printing the drop containing cells on the hydrogel mould; and (e) repeating steps (a) and (b) in any order to form the three-dimensional tissue culture model comprising the cells encapsulated in the hydrogel mould. The process is carried out at a temperature up to 37°C. W . The above document does not disclose a purification step using hexane from silicone oil.

Document **WO2018127850A1** discloses methods of forming a three dimensional tumour model by bioprinting, as well as systems in which the three dimensional tumour model can be perfused and fluidly connected to a medium containing immune cells and/or other cells and factors present in the tumour's microenvironment. The process involves (a) providing a composition comprising a plurality of cell types comprising malignant cells and non-malignant cells of the tumour and a thickening agent; (b) subjecting the composition to a hanging drop method so as to produce a spheroid, and subsequently (c) embedding

the spheroid in an extracellular matrix material. The application does not disclose specific numerical parameters for bioprinting and a purification step using hexane from silicone oil.

Document **US20230357685A1** discloses a method for the fabrication of multi-spheroid tissues with precise spatial control over spheroid positioning. A 3D bioprinter is disclosed, comprising an electromagnet and dual printheads comprising a first nozzle and a second nozzle, wherein the first nozzle extrudes magnetic ink, and the second nozzle manipulates the electromagnet to provide spatial control over the construction of a multi-spheroid tissue. The resulting organoids are washed three times with DPBS. The document does not disclose bioprinting parameters and a purification step using hexane from silicone oil.

Document **KR1020210076153A** describes a method for producing a three-dimensional tissue *in vivo*, involving the deposition of one or more cell-containing compositions on a surface. In another aspect, the invention discloses an *in vivo* three-dimensional tissue comprising the deposition of at least one cell-containing composition and at least one composition containing an extracellular matrix (ECM) on a surface. The cells and/or liquid ECMs (and additional components) used in the creation of the three-dimensional tissue *in vivo* are printed on a surface, e.g. the cells and ECMs are subjected to bioprinting (e.g. using an inkjet printer). The above document does not disclose a purification step using hexane from silicone oil.

Patent **WO2015123183A1** in turn discloses micro-organoids, referred to as functional physiological units (FPUs), that are capable of replacing or augmenting one or more physiological functions in a patient. The micro-organoids are printed using 2D or 3D inkjet printing. The above document does not disclose a purification step using hexane from silicone oil.

In spite of a dynamic development in tissue engineering, there is still a continuing search for solutions which would enable the production of organoids that would be stable in culturing and at the same time functional. Therefore, the purpose of the present invention is to develop a technology for producing organoids with a defined size not exceeding 1200 μm, which would simultaneously be stable over time and efficient in use. The purpose of the invention is primarily to provide a controlled method for producing organoids.

This objective has been achieved by the invention according to claim 1.

The essence of the invention is a method for producing organoids, wherein:

- a hydrogel containing cells and optionally a photoinitiator is subjected to a bioprinting process at a temperature within a range of 20–30°C, a pressure within a range of 40–75 kPa, and a constant extrusion rate of 10 mm/s,

wherein,

the printed hydrogel is directed into a vessel containing oil,
and then the resulting hydrogel capsule in an oil layer is transferred to a sieve and washed with hexane and a cell medium.

Preferably, the hydrogel comprises a methacrylic natural polymer and/or methacrylic hyaluronic acid and/or a decellularized extracellular matrix.

Preferably, the hydrogel comprises GELMA (0.5%–20%), CHIMA (0.05%–1.5%), ALGMA (0.1%–7%), HAMA (0.1%–5%), dECM (0.5%–5%), or a mixture thereof.

Preferably, the step of printing biomaterial to the oil is followed by crosslinking with UV-Vis light.

Preferably, the photoinitiator is LAP.

Preferably, the cells are selected from: αTC-1, βTC-tet, HUVEC, L929, HEPG2, THLE-2, HDFa, and HUVEC-GFP.

Preferably, the cell content of the hydrogel is between 0.5 million/ml and 40 million/ml, preferably 10 or 20 million/ml.

Preferably, the washing is initially done three times with hexane, and subsequently with the cell medium.

The invention also relates to organoids produced with the method.

Preferably, the diameter of the organoids is below 1200 μm.

In a further aspect, the invention relates to a hydrogel capsule consisting of:

- an inner hydrogel layer containing polymerised methacrylated natural polymers and/or methacrylated hyaluronic acid and/or a decellularized extracellular matrix and cells suspended in the hydrogel, and

-an oil layer, wherein the oil layer is no more than 100 μm thick.

In a further aspect, the invention relates to the application of organoids in 3D bioprinting and tissue engineering.

The advantage of the invention is a controlled method for producing organoids of a given size, which is made possible by a sequence of operations: printing with given parameters into oil, cross-linking/fixing the structure, purification (including with hexane) of the oil layer, and ultimately their transfer.

Short Description of the Drawings

The invention is presented in the Drawing, in which:

Fig. 1 presents a schematic for the washing out of pancreatic organoids from silicone oil.

Fig. 2 presents the concentration of insulin dependent on the type of glucose administered in the organoids and control samples (GSIS test).

Fig. 3 presents the concentration of glucagon secreted by alpha cells located in the produced organoids and control samples during 28 days of incubation.

Fig. 4 presents microscopic imaging of organoids with α and β cells. The photographs were taken with an Olympus IX83 microscope in a bright field (BF), using fluorescent light: green (FITC—fluorescein isothiocyanate) and red (TRITC—tetramethylrhodamine), on day 0 of the experiment (on the day of printing).

Fig. 5 presents microscopic imaging of organoids with α and β cells. The photographs were taken with an Olympus IX83 microscope in a bright field (BF), using fluorescent light: green (FITC—fluorescein isothiocyanate) and red (TRITC—tetramethylrhodamine), on day 1 of the experiment.

Fig. 6 presents microscopic imaging of organoids with α and β cells. The photographs were taken with an Olympus IX83 microscope in a bright field (BF), using fluorescent light: green (FITC—fluorescein isothiocyanate) and red (TRITC—tetramethylrhodamine), on day 5 of the experiment.

Fig. 7 presents microscopic imaging of organoids with α and β cells. The photographs were taken with an Olympus IX83 microscope in a bright field (BF), using fluorescent light: green (FITC—fluorescein isothiocyanate) and red (TRITC—tetramethylrhodamine), on day 14 of the experiment.

Fig. 8. Microscopic imaging of organoids with α and β cells. The photographs were taken with an Olympus IX83 microscope in a bright field (BF), using fluorescent light: green (FITC—fluorescein isothiocyanate) and red (TRITC—tetramethylrhodamine), on day 28 of the experiment.

Fig. 9 presents sample photographs of organoids with spheroids formed after one month of culturing. Microscopic imaging of cells. The photographs were taken with an Olympus IX83 microscope in a bright field (BF).

Fig. 10 presents sample photographs of organoids with the spheroids formed, forming clusters on day 21 of culturing. Microscopic imaging of cells. The photographs were taken with an Olympus IX83 microscope in a bright field (BF).

Fig. 11 presents microscopic imaging showing organoids stained using the H&E method. Photographs taken with an Olympus IX83 microscope (shown in microscopic images at 4x, 10x, and 20x magnification).

Fig. 12 presents an immunohistochemical analysis of pancreatic organoids (staining of paraffin sections). The colour pink indicates the presence of insulin. Photographs taken with an Olympus IX83 microscope (shown at 4x, 10x, and 20x magnification).

Fig. 13 presents an immunohistochemical analysis of pancreatic organoid sections. The colour blue indicates the presence of glucagon. Photographs taken with an Olympus IX83 microscope (shown at 4x, 10x, and 20x magnification).

Fig. 14 presents an immunohistochemical analysis of pancreatic organoid sections. The colour blue indicates the presence of endothelial cells. Photographs taken with an Olympus IX83 microscope (shown at 4x, 10x, and 20x magnification).

Fig. 15 presents an immunohistochemical analysis of pancreatic organoid sections. The colour pink indicates the presence of fibroblasts. Photographs taken with an Olympus IX83 microscope (shown at 4x, 10x, and 20x magnification).

Fig. 16 presents an example of an organoid with the formation of spheroids. A: vital staining observations. B: histological preparation imaging.

Fig. 17 presents the cytotoxicity level of 2D cell cultures and organoids, illustrating the sensitivity of cells in 2D culturing and organoids to given drug concentrations A. during the experiment, and B. during administration of the doxorubicin drug, represented by measuring the activity of lactate dehydrogenase secreted into the culture medium. HepG2: 2D culture of the HepG2 cell line, Version 1: Organoid culture: 100% HepG2, HDFa:HUVEC-GFP: 2D culture of the HDF-a and HUVEC-GFP cell lines, Version 3: Organoid culture: 33% HepG2, 33% HDFa, 33% HUVEC-GFP, Version 5: Organoid culture: 33% HepG2, 17% THLE-2, 33% HDFa, 17% HUVEC-GFP. Error bars represent SE ± 3.

Fig. 18 presents the cytotoxicity level of 2D cell cultures and organoids, illustrating the sensitivity of cells in 2D culturing and organoids to given drug concentrations A. during the experiment; B. during administration of the doxorubicin drug, represented by measuring the activity of lactate dehydrogenase secreted into the culture medium. Version 2: Organoid culture 100% THLE-2, HDFa:HUVEC-GFP: 2D culture of the HDF-a and HUVEC-GFP cell lines, Version 4: Organoid culture 33% THLE-2, 33% HDFa, 33% HUVEC-GFP, Version 6: Organoid culture: 17% HepG2, 33% THLE-2, 33% HDFa, 17% HUVEC-GFP. Error bars represent SE ± 3.

Fig. 19 presents: A. Analysis of the concentration of AFP secreted into the culture medium during the experiment and B. during the administration of doxorubicin. HepG2: 2D culture of the HepG2 cell line, Version 1: Organoid culture 100% HepG2, HDFa:HUVEC-GFP: 2D culture of the HDF-a and

HUVEC-GFP cell lines, Version 3: Organoid culture 33% HepG2, 33% HDF-a, 33% HUVEC-GFP, Error bars represent SE ± 2.

Fig. 20 presents FDA/Pi staining, version: 1 (100% HepG2) liver organoids throughout the experiment. Bright Field—bright field, Merged—FDA and PI channels superimposed. Scale: 500 μm.

Fig. 21 presents FDA/Pi staining, version: 2 (100% THLE-2) liver organoids over the course of the experiment. Bright Field—bright field, Merged—superimposed FDA and Pi channels. Scale: 500 μm.

Fig. 22 presents FDA/Pi staining, version: 3 (33% HepG2, 33% HDF-a, 33% HUVEC-GFP) liver organoids over the course of the experiment. Bright Field—bright field, Merged—superimposed FDA and Pi channels. Scale: 500 μm.

Fig. 23 presents FDA/Pi staining, version: 4 (33% THLE-2, 33% HDF-a, 33% HUVEC-GFP) liver organoids over the course of the experiment. Bright Field—bright field, Merged—superimposed FDA and Pi channels. Scale: 500 μm.

Fig. 24 presents FDA/Pi staining, version: 5 (33% Hep2G, 17% THLE, 33% HDF-a, 17% HUVEC-GFP) liver organoids over the course of the experiment. Bright Field—bright field, Merged—superimposed FDA and Pi channels. Scale: 500 μm, except for Day 1 and Day 7 panels, where the scale is 1 mm.

Fig. 25 presents A. FDA/Pi staining, version: 6 (17% HepG2, 33% THLE-2, 33% HDFa, 17% HUVEC-GFP) liver organoids over the course of the experiment. Bright Field—bright field, Merged—superimposed FDA and Pi channels. Scale: 500 μm.

Embodiments of the Invention

Embodiment 1 Development of a composition and a method for producing a homogeneous biomaterial

The electrohydrodynamic direct printing technology was used to develop a method for 3D printing of the cell-filled organoids according to the invention. The experiment tested natural methacrylated polymers, including: alginate, gelatine, chitosan, and hyaluronic acid, as well as decellularized extracellular matrix (dECM). In order to optimise the composition of biomaterials and at the same time obtain proper biological, chemical and mechanical properties, a series of experiments were conducted with solutions containing varying amounts of individual components, including: GELMA (0.5%–20%), CHIMA (0.05%–1.5%), ALGMA (0.1%–7%), HAMA (0.1%–5%), dECM (0.5%–5%), a photoinitiator (preferably LAP) (0.05%–5%).

According to the embodiment, after the bioprinting step, the organoids present in the oil layer are cross-linked using UV-Vis light, under controlled conditions, to fix and preserve their shape.

According to the invention, the production method uses oil-based media selected so as to enable the production of organoids with a spherical shape and having no deformations. The resulting organoids have a specific size and retain their stiffness. Furthermore, optimisation of their purification method enables the diffusion of nutrients into the organoids.

The following commercially available oil media were applied: Silicone oil AP 150, 3M™ NOVEC™ 7500 Engineered Fluid, 3M™ NOVEC™ 7200 Engineered Fluid, Silicone oil (378348), and Silicone oil (CL00-1918.1000).

The best results were produced when using:

Silicone oil (378348): the resulting organoid had a diameter of 921.42 (± 68.51) μm, and its oil layer thickness was 116.58 (± 47.82) μm.

Silicone oil (CL00-1918.1000): the resulting organoid had a diameter of 931.38 (± 30.64) μm, and its oil layer thickness was 35.46 (± 12.89) μm.

However, organoids of better quality—without deformations—are produced using Silicone oil (CL00-1918.1000).

The organoids, subjected to a purification process, can be transferred into a culture chamber/medium/dish/vessel by the formation of negative pressure at the tip of a needle. This solution allows for the transfer of individual organoids and enables their precise dosage.

Examples of hydrogels tested:

1. **5% (w/v) GelMA + 0.25% (w/v) LAP**: prepared by diluting a 20% GelMA solution with 1xPBS, ultimately using 0.5 ml of the 20% (w/v) GelMA solution, 1.5 ml PBSx1, 5 mg LAP;
2. **10% (w/v) GelMA + 0.25% (w/v) LAP**: prepared by diluting a 20% GelMA solution with 1xPBS, ultimately using 1 ml of 20% (w/v) GelMA, 1 ml PBSx1, 5 mg LAP;
3. **10% (w/v) GelMA + 0.4% CHiMA + 0.25% LAP**: prepared by combining 1 ml of 20% (w/v) GelMA + 1 ml of 0.8% (w/v) CHIMA, 5 mg LAP;
4. **10% (w/v) GelMA + 1% (w/v) HAMA + 0.25% (w/v) LAP**: prepared by combining 1 ml of 20% (w/v) GelMA + 1 ml of 2% (w/v) HAMA, 5 mg LAP;

5. **10% (w/v) GelMA + 0.4% CHiMA + 0.25% LAP**: prepared by combining 100 µl of 20% (w/v) GelMA + 100 µl of 0.8% (w/v) CHIMA + 0.5 mg LAP + 20 µl (8 million) of L929 cells

6. **10% (w/v) GelMA + 0.4% CHiMA + 0.25% LAP**: prepared by combining 100 µl of 20% (w/v) GelMA + 100 µl of 0.8% (w/v) CHIMA + 0.5 mg LAP + 20 µl (8 million) of INS-1E cells

7. **10% (w/v) GelMA + 1% (w/v) HAMA + 0.25% (w/v) LAP**: prepared by combining 100 µl of 20% (w/v) GelMA + 100 µl of 1% (w/v) HAMA + 0.5 mg LAP + 20 µl (8 million) of L929 cells

8. **10% (w/v) GelMA + 1% (w/v) HAMA + 0.25% (w/v) LAP**: prepared by combining 100 µl of 20% (w/v) GelMA + 100 µl of 1% (w/v) HAMA + 0.5 mg LAP + 20 µl (8 million) of INS-1E cells

9. **10% (w/v) GelMa + 0.5% (w/v) HAMA + 0.5% (w/v) dECM + 0.25% (w/v) LAP**: prepared by combining 1 ml of 20% (w/v) GelMA + 500 µl of 2% (w/v) HAMA + 500 µl of **2% (w/v) dECM**, 0.5 mg LAP

Preparation of solutions and hydrogel

Preparation of a solution of 20% (w/v) GelMA + 0.5% (w/v) LAP.

Using an analytical balance located under a laminar flow cabinet, 31.5 mg of LAP and 1.25 g of GelMA were weighed out on weighing dishes. The reagents were transferred to a Falcon tube (protected from light with aluminium foil). Using an automatic pipette, 6.25 ml of PBSx1 were added to the prepared Falcon tube. The Falcon tube with the solution was then placed in a heating block (50°C, 400 rpm) until the lyophilisate dissolved. The dissolved lyophilisate was neutralised to pH in a range of 7.2–7.4 using neutralising agents. The prepared solution was filtered through a 0.22 um syringe filter into a sterile Falcon tube, which was protected from light. The solution was stored in a refrigerator until use.

Preparation of a 2% (w/v) HAMA solution.

Using an analytical balance located under a laminar flow cabinet, 122 mg of HAMA were weighed out on a weighing dish. The reagents were transferred to a 25 ml Falcon tube (protected from light with aluminium foil). Using an automatic pipette, 6.1 ml of PBSx1 were added to the prepared Falcon tube. The Falcon tube with the solution was then placed in a heating block (8°C, 1000 rpm) until the lyophilisate dissolved. The dissolved lyophilisate was neutralised to pH in a range of 7.2–7.4 using neutralising agents. The prepared solution was filtered through a 0.22 um syringe filter into a sterile Falcon tube, which was protected from light. The solution was stored in a refrigerator until use.

Preparation of a 2% (w/v) dECM solution produced in a decellularization process of the pancreas

10 ml of 0.01 M HCl solution were quantitatively transferred into a bottle 25 ml in volume using a serological pipette. Then, using an analytical balance, 10 mg of pepsin were weighed out on a weighing dish and transferred to the HCl solution. The solution was stirred on a magnetic stirrer for 20 mins. (until the pepsin dissolved) at a rate of 500 rpm at room temperature. Subsequently, 200 mg of ECM from decellularization of the pancreas were weighed out and quantitatively transferred to the pepsin solution. The hydrogel was left on the magnetic stirrer for 72 h at a stirring speed of 500 rpm, on a thermostatted metal platform heated up to 30°C. After dissolution of the dECM powder, neutralisation of the resulting hydrogel to pH within a range of 7.2–7.4 was performed using neutralising agents. The prepared solution was stored in a refrigerator until use.

Preparation of a 2% (w/v) dECM solution produced in a decellularization process of the liver

10 ml of 0.1 M HCl solution were quantitatively transferred into a bottle 25 ml in volume using a serological pipette. Then, using an analytical balance, 20 mg of pepsin were weighed out on a weighing dish and transferred to the HCl solution. The solution was stirred on a magnetic stirrer for 20 mins. (until the pepsin dissolved) at a rate of 500 rpm at room temperature. Subsequently, 200 mg of ECM from decellularization of the liver were weighed out and quantitatively transferred to the pepsin solution. The hydrogel was left on the magnetic stirrer for 48 h at a stirring speed of 500 rpm, on a thermostatted metal platform heated up to 30°C. After dissolution of the dECM powder, neutralisation of the resulting hydrogel to pH within a range of 7.2–7.4 was performed using neutralising agents. The prepared solution was stored in a refrigerator until use.

Preparation of a 0.8% (w/v) ChiMA solution

Using an analytical balance located under a laminar flow cabinet, 80 mg of sterile ChiMa lyophilisate were weighed on a weighing dish. The reagent was transferred to a 25 ml Falcon tube (protected from light with aluminium foil). Using a serological pipette, 10 ml of 1% (v/v) acetic acid were added to the prepared Falcon tube. The Falcon tube with the solution was then placed in a heating block (50°C, 500 rpm) until the lyophilisate dissolved. The dissolved lyophilisate was neutralised to pH in a range of 7.2–7.4 using neutralising agents. The solution was stored in a refrigerator until use.

Preparation of hydrogel: 10% (w/v) GelMA + 1% (w/v) HAMA + 0.25% (w/v) LAP.

Using an automatic pipette, 1.5 ml of a prepared 20% (w/v) GelMA + 0.5% (w/v) LAP solution and 1.5 ml of a prepared 2% (w/v) HAMA solution were added to a 5 ml Falcon tube (protected from light with aluminium foil).

EXAMPLE 2—printability test

The organoid printability test was carried out using a BioX printer. The printing material used was hydrogel with 10% (w/v) GELMA + 1% (w/v) HAMA + 0.25% (w/v) LAP + cells at a specific concentration (e.g. fibroblasts, pancreatic cells: beta and alpha, endothelial cells, iPSCs, stem cells, liver cells, lung cells, and others). The printing tests were performed according to a programmed model—gcode file: 10 × 70 mm line. During the printing process, temperature and pressure conditions were adjusted to a given biomaterial. The following printing parameters were used during the test: temperature within a range of 20–30°C, pressure within a range of 40–75 kPa, post-flow within a range of 200–300 ms, with a constant extrusion rate of 10 mm/s and a pre-flow of 1 ms, wherein it is preferable to print at a temperature of 30°C while maintaining an extrusion pressure of 40 kPa and a speed of 10 mm/s, and a pre- and post-flow of 1 and 300 ms, respectively. Subsequently, the printed biomaterial was cross-linked using an external UV-Vis lamp (Polbionica S.A., Poland) with a light wavelength of 405 nm at an intensity of 28.5 mW/cm^2 for 20–30 s, preferably for 30 s. An EMD CELLINK head and a J02T2 nozzle were used for printing. The organoids were printed at a height of approx. 2 cm from the bottom of the dish (8 x 5 cm—going down when calibrating the head) filled with silicone oil (CL00-1918.1000, Chem-Lab). Subsequently, the cross-linked organoids were collected using a Pasteur pipette onto a 300 μm SyringeStrainer cell strainer. When all models with 1 ml of hydrogel were placed on the strainer, they were washed with 3 x 3 ml of hexane using a syringe, and in addition to that, with 1 ml of the cell medium at the end. Subsequently, approx. 3 ml of the dedicated cell medium were drawn into a syringe, and the syringe was screwed into the top of the 300 μm SyringeStrainer. The sieve was turned over, the collected cell medium was introduced thereinto, followed by manual agitation by a rotary motion in order to detach the organoids from the surface of the strainer (Fig. 1). Subsequently, the medium with the organoids was drawn into a syringe. The contents were transferred to a well on an insert plate. After printing all repetitions according to the embodiment, the plate was handed over for further analyses.

The purification process in hexane indicates easier removal of the oil layer around the printed organoids, the presence of which, as demonstrated during the analyses, also being influenced by the type of biomaterial used, as shown in Table 1.

Table 1. Size of the oil layer remaining after the organoid washing process, and the size of the organoids depending on the biomaterial used.

Hydrogel	Organoid size, diameter [μm]	Presence of oil layer, diameter [μm]
10% (w/v) GELMA + 0.25% (w/v) LAP	1130.76 (± 127.27)	5.75 (± 1.37)
1% (w/v) CHIMA + 0.25% (w/v) LAP	656.38 (± 25.46)	77.73 (± 14.74)

2% (w/v) HAMA + 0.25% (w/v) LAP	893.65 (± 50.18)	13.17 (± 4.98)
2% (w/v) ALGMA + 0.25% (w/v) LAP	860.00 (± 106.56)	27.59 (± 17.87)
10% (w/v) GELMA + 0.5% (w/v) CHIMA + 0.25% (w/v) LAP	1056.21 (± 25.08) μm	101.27 (± 15.82)
10% (w/v) GELMA + 1% (w/v) ALGMA + 0.25% (w/v) LAP	1375.92 (± 192.12)	11.19 (± 3.36)
10% (w/v) GELMA + 1% (w/v) HAMA + 0.25% (w/v) LAP—REF	931.38 (± 30.64)	35.46 (± 12.89)
1% (w/v) HAMA + 0.5% (w/v) CHIMA + 0.25% (w/v) LAP	780.09 (± 29.28)	Invisible
1% (w/v) HAMA + 1% (w/v) ALGMA + 0.25% (w/v) LAP	1014.32 (± 19.54)	28.86 (± 5.89)
0.5% (w/v) CHIMA + 1% (w/v) ALGMA + 0.25% (w/v) LAP	964.37 (± 97.58)	78.92 (± 35.71)

Normal cellular development and growth were also observed after only 7 days, and normal proliferation and morphology were observed in them. It can be therefore concluded that the oil layer removal step is essential, as it enables the diffusion of nutrients into the organoids, which directly contributes to cellular development.

The tests used printed organoids produced from a suitable bioink composition, enriched with different cell lines depending on the function they served. The cells originated from commercial cell lines and/or tissue-derived lines and stem cell lines. Organoids can consist of normal or tumour cell lines, or a mixture thereof.

Examples of organoid types:

a) pancreatic, prepared with the use of the following cells: αTC-1 (mouse alpha cells), βTC-tet (mouse beta cells), HUVEC (human endothelial cells), L929 (mouse fibroblasts) and others,

and

b) hepatic: HepG2 (human liver cancer cell line), THLE-2 (human left liver lobe epithelial cell line), HDFa (human fibroblasts), HUVEC-GFP (human endothelial cells) and others.

a) *PANCREATIC ORGANOIDS*

1. GSIS test and ELISA test to determine the level of secreted insulin

The GSIS test (Glucose *Stimulated Insulin Secretion*) assesses normal functioning of pancreatic islets under *in vitro* conditions. For mouse β-cells: low glucose concentration was 2.8 mM/100 ml, high glucose concentration was 16.7 mM/100 ml.

Glucose-stimulated insulin secretion mimics the process in which pancreatic β-cells release insulin in response to an elevated glucose level. It can therefore be concluded that the cells respond to glucose stimulation (Fig. 2)

2. ELISA test to determine the level of secreted glucagon

Based on the ELISA test results, it was observed that the pancreatic organoids produced exhibited α-cell functionality throughout the entire incubation period of the models. This is evidenced by the level of glucagon secreted. (Fig. 3).

3. Microscopic imaging of organoids

3.1 FDA/Pi staining

Cell viability was assessed by FDA/Pi staining. For this purpose, two dyes were used: propidium iodide (Pi) which stains dead cells, and fluorescein diacetate (FDA) which stains live cells. The organoids were suspended in a PBS solution, and both reagents were added in a 1:1 ratio. From the results, it was observed that the viability of organoid-forming cells was > 80% (Figs. 4–8). In addition, an increase in cell viability over time and their ability to form internal 3D structures were observed as a result of normal cell proliferation.

In the printed organoids, the growth of cells with normal morphology and their ability to form internal spheroids 90–160 μm in size were observed as early as on day 10 of the test. In addition, there was an observed increase in cell viability and stability over time (Fig. 9). Thus, it can be concluded that the resulting dimensions of the internal spheroids are particularly preferable when using pancreatic islets, which will provide the most preferable replication of the function of natural pancreatic islets.

Furthermore, it was observed that organoids with the resulting internal spheroids formed clusters on day 21 of the experiment by merging with each other, which led to the phenomenon of adhesion (Fig. 10).

3.2. H&E staining

The aim of the performed microscopic imaging of the prepared paraffin sections was to show the tissue structure and morphology of organoids containing α-, β- and endothelial and fibroblasts cells, as well as organoid fibroblasts with cells and organoids after one month of incubation fixed in 4% formalin. The making of the preparations and the performance of the test are aimed at confirming the presence of specific marker antibodies. The observations were carried out using an Olympus IX83 microscope.

The staining of the tissue with haematoxylin and eosin (H&E staining) enabled the evaluation of morphological changes within the biological material studied. It was observed that the organoids

exhibited well-preserved morphology, whereas the formed structures corresponded to the imaging of native pancreatic islets stained in pancreatic biopsy. (Fig. 11).

.2 Immunohistochemical staining

INSULIN

Pink staining of portions of organoids sections (mainly in the resulting spheroids) using an anti-insulin antibody (Santa Cruz sc-8033, 1:100) confirms the presence of insulin in the tissue. This demonstrated the secretory activity of the β-cells included in the organoids. (Fig. 12).

GLUCAGON

Blue staining of portions of organoids sections (mainly in the resulting spheroids) using an anti-glucagon antibody (Santa Cruz, sc-514592, 1:100) confirms the presence of glucagon in the tissue. (Fig. 13) This proves the secretory activity of the α-cells included in the organoids.

ENDOTHELIAL CELLS

Blue staining of portions of organoids sections (in the resulting spheroids) using the CD31 antibody (Abcam, ab76533) confirms the presence of endothelial cells in the tissue. Staining indicates that the endothelium is located primarily in the produced cell clusters (spheroids). No endothelial cell migration was observed (Fig. 14).

FIBROBLASTS

Pink staining of portions of organoids sections (in the resulting spheroids) using the vimentin antibody (Santa Cruz, sc-6260) confirms the presence of fibroblast cells in the tissue. The staining indicates that the fibroblasts are located: in the formed cell clusters (spheroids), and along the outer borders of the organoids (Fig. 15).

Microscopic observations confirm that the bioprinted pancreatic organoids exhibit the functionality of the listed cells: alpha, beta and endothelial cells, as well as fibroblasts (Figs. 11–15). There was a confirmed presence of proteins, i.e. insulin, glucagon, CD31 and vimentin, which proves normal functioning of the cells.

It was observed that the organoids and the formed spheroids maintained a stable structure throughout the entire incubation period. Smaller diameter spheroids were formed inside the 3D structures, their size varying from 40 to as much as 200 μm. Spontaneously formed spheroids consist of both alpha and beta cells, fibroblasts, and endothelium (Fig. 16).

b) ***LIVER ORGANOIDS***

In spite of the advantages of functioning 3D models, such as the bionic liver, 2D cultures are organoid liver bioconstructs with an advantage over the rest. They are characterised by a low level of structural complexity, which enables easier performance of experiments. The simple methodology of these models allows for a direct analysis of cell viability and the effect of cytostatics on tumour growth.

The tests used printed models enriched with the following cell lines:

- HepG2 cell line,
- THLE-2 cell line,
- HUVEC-GFP cell line,
- HDFa cell line.

Organoids of the following cell versions (compositions) were prepared for the tests, with $5*10^6$ cells/ml used for each version of liver organoids:

- version 1: 100% HepG2,
- version 2: 100% THLE-2,
- version 3: 33% HepG2, 33% HDFa, 33% HUVEC-GFP,
- version 4: 33% THLE2, 33% HDFa, 33% HUVEC-GFP,
- version 5: 33% HepG2, 17% THLE-2, 33% HDFa, 17% HUVEC-GFP,
- version 6: 17% HepG2, 33% THLE-2, 33% HDFa, 17% HUVEC-GFP.

Assessment of cell damage using an LDH test, and an analysis of secreted metabolite levels

Assessment of cell damage and the levels of secreted metabolites was carried out using the following ELISA test kits, in accordance with the manufacturer's instructions:

a) LDH activity analysis—an LDH test (Promega, J2381) as an indirect indicator of cytotoxicity

The LDH test allowed for determining cell viability in the produced organoids . The test was carried out for samples collected on days 1, 2, 7, 10, 14 and 21 of incubation, as well as after administration of the drug doxorubicin. The control consisted of 2D culture cells maintained under standard conditions. The level of cytotoxicity (% cytotoxicity) was calculated based on the following equation:

$$\%\text{Cytotoxicity} = 100 \times \frac{(Released\ LDH\ in\ the\ samples - background\ medium)}{(Released\ LDH\ in\ the\ positive\ control - background\ medium)}$$

b) determine the level of alpha-fetoprotein (AFP) by means of the ELISA method (Thermofisher scientific, EHAFP)—a proliferation marker specific for hepatocyte tumour cells,

Alpha-fetoprotein is a protein produced, among others, by the very liver tumour cells . AFP expression in adults is often associated with an ongoing tumorigenesis process.

c) determine the level of von Willebrand factor (vWF) by means of the ELISA test (Thermofisher scientific, EHVWF)—an endothelial cell-specific marker of activity and proliferation,

d) assess the survival of liver organoids—the FDA/Pi test

The highest functionality of liver organoids is observed two weeks after the beginning of culturing. An analysis of the LDH and AFP levels indicates that during this period the cells cultured in the organoid structure exhibit low viability and high secretory activity, which proves their stability and optimal microenvironmental conditions (Fig. 17A, Fig. 18A, Fig. 19A).

An additional advantage is the high translucency ratio of the bioink used to form the organoids, enabling easy penetration by a light beam and efficient cross-linking, as well as precise microscopic observations of both the morphology and proliferation of cells in the 3D structure. The translucency ratio of the material also allows for the use of fluorescent staining, which facilitates a real-time analysis of cell viability and activity (Figs. 19–24).

Liver organoids may also be found useful in studies on the cytotoxicity of anti-cancer drugs, as evidenced by observations of the effect of the drug doxorubicin—after administration of the drug, there was an observed increase in the secretion of the enzyme LDH, and a decrease in the secretion of AFP (Fig. 17B, Fig. 18B, Fig. 19B).

It can therefore be concluded that the liver organoids produced according to the invention will become a valuable tool in preclinical tests, enabling more precise recreation of *in vivo* conditions compared to traditional 2D cultures.

Survival assessment: the FDA/Pi staining

Cell viability was assessed by FDA/Pi staining. For this purpose, two dyes were used: propidium iodide (Pi) which stains dead cells, and fluorescein diacetate (FDA) which stains live cells. The organoids were suspended in a PBS solution, and both reagents were added in a 1:1 ratio. The results of survival observations for the printed liver organoids (Figs. 19–25) showed a high level of viability and normal development of the printed cells in the organoids.

Claims

1. A method for producing organoids, wherein:
   - a hydrogel containing cells and optionally a photoinitiator is subjected to a bioprinting process at a temperature within a range of 20–30°C, a pressure within a range of 40–75 kPa, and a constant extrusion rate of 10 mm/s,
   wherein,
   the printed hydrogel is directed into a vessel containing oil,
   and then the resulting hydrogel capsule in an oil layer is transferred to a sieve and washed with hexane and a cell medium.
2. The method according to claim 1, wherein the hydrogel comprises a methacrylic natural polymer and/or methacrylic hyaluronic acid and/or a decellularized extracellular matrix.
3. The method according to claim 2, wherein the hydrogel comprises GELMA (0.5%–20%), CHIMA (0.05%–1.5%), ALGMA (0.1%–7%), HAMA (0.1%–5%), dECM (0.5%–5%), or a mixture thereof.
4. The method according to any one of claims 1 to 3, wherein the step of printing biomaterial for the oil is followed by crosslinking with UV-Vis light.
5. The method according to any of claims 1–4, wherein the photoinitiator is LAP.
6. The method according to any one of claims 1–5, wherein the cells are selected from: αTC-1, βTC-tet, HUVEC, L929, HEPG2, THLE-2, HDFa, and HUVEC-GFP.
7. The method according to claims 1–6, wherein the cell content of the hydrogel is between 0.5 million/ml and 40 million/ml, preferably 10 or 20 million/ml.
8. The method according to claims 1–6, in which the washing is initially done three times with hexane, and subsequently with the cell medium.
9. Organoids produced with the method according to claims 1–8.
10. Organoids according to claim 9, having a diameter of less than 1200 μm.
11. A hydrogel capsule consisting of:

- an inner hydrogel layer containing polymerised methacrylated natural polymers and/or methacrylated hyaluronic acid and/or a decellularized extracellular matrix and cells suspended in the hydrogel, and

-an oil layer, wherein the oil layer is no more than 100 μm thick.

12. Use of organoids according to claim 9 in 3D bioprinting and tissue engineering.