

A bioprinted liver model, a method for manufacturing the liver model and the use thereof in cytotoxicity evaluation

Technical field

- 5 The invention relates to a three-dimensional, bioprinted, living, and functional model, including the vascular system, mimicking cancerous liver tissue. It also relates to a method for manufacturing this model. The invention has applications in evaluating the cytotoxicity of drugs, including doxorubicin, and the efficacy of anti-cancer therapies.

Prior art

- 10 In EP3065791B1, an innovative method has been proposed to create three-dimensional tissue prints with an embedded blood vessel system. The design consists of tissue templates with different cell types, connected by a network of vessels of varying sizes and a surrounding extracellular matrix. Multiple cell types can be placed programmatically, and the number of types can range from 2 to 300. The cells used for printing are mammalian cells, such as embryonic cells, somatic and stem cells. The extracellular
- 15 matrix can be made of various materials, including hydrogel, such as gelatine, fibrin or methacrylated gelatine. Additionally, the structure of the construct may comprise functional channels with an epithelial layer and vascular channels with an endothelial layer. These channels may have different properties, such as curvature, irregular diameter or branching. Chemical substances, such as drugs or proteins, can be introduced into the construct by inserting them into the cells of the printed filaments. A tissue
- 20 construct can have a solid structure, a porous structure and/or a hollow structure (e.g. tubular or non-tubular) and can be fabricated to mimic the morphology and function of a specific organ. For example, a tissue construct may be the size and shape of a liver.

- WO2021015572A9 relates to a three-dimensional model of the liver tubular system, otherwise a spheroid-based liver organoid characterized by a unique tubular arrangement and a lobular structure that
- 25 reflects the natural distribution of lobules in liver tissue. In addition to this, the document cited presents an innovative method for producing this three-dimensional model, enabling its use in clinical and non-clinical studies. Non-clinical studies may include tests of structural strength, stability, effects on liver function. The bioink used in the context of the hepatic organoid to be manufactured may consist of various components such as hepatocytes, vascular endothelial cells, hydrogel, cellular growth factors. It
- 30 is important that the bioink contains components that promote the normal growth and function of cells in the lobular structure. Organoids in this context refer to structures based on these liver spheroid clusters and the spheroids are provided by the bioink. In the document described here, spheroidal liver clusters with a uniform size of 300 μm are obtained, suggesting that the diameter of these structures is in

question. In one embodiment, the hydrogel may include substances such as alginate, fibrin gel, carboxymethylcellulose, heparin sulfate, hyaluronic acid, or collagen.

CN110551679B presents a method for the precise printing and construction of a liver system containing the structure of three lobular vessels: the hepatic artery, portal vein and central vein in the hepatic lobule.

5 The method involves several key steps. In the first step, a bioink containing liver-associated cells, such as hepatocytes and non-parenchymal cells, is prepared along with GelMa, collagen and gelatine. The next step is the construction of a microfluidic system using PDMS materials, including a liquid entry layer, a cell culture layer and a sample detection layer. The bioink is then loaded into a multi-mode three-dimensional bioprinter system, and the liver lobular structure is precisely printed onto the cell
10 culture area of the microchip. The resulting liver microchip comprises the lobular structure in the form of a reticular monolayer or multilayer. The final step is to carry out an *in vitro* perfusion culture on the liver microchip, resulting in a liver system with a three-vessel lobular structure. This method has applications in drug testing and drug toxicity detection, for example for substances such as paracetamol, ibuprofen and indomethacin.

15 In WO2014151921A1, an engineered living three-dimensional liver tissue construct consisting of at least one compartment with planar geometry is described. This compartment comprises parenchymal cells within the interior, as well as non-parenchymal cells at its edge. These cells have been coherently fused to form a complex three-dimensional structure of liver tissue. Significantly, at least one component of the construct was bioprinted. Among the parenchymal cells, different types were used, such as adult
20 mammary liver tissue, fetal mammary liver tissue, embryonic stem cells (ESC), or induced pluripotent stem cells (iPSC). Non-parenchymal cells include a variety of types, such as vascular cells, endothelial cells, fibroblasts, mesenchymal cells, or immune cells. The construct may consist of multiple layers, at least one of which is compositionally or architecturally distinct, forming a laminar geometry.

In addition, a lobular structure can be included, where parenchymal cells are arranged in a way that
25 allows separation of the lobular space. Applications of the construct include enhancing liver function in humans by implantation in an area of injury, disease or degeneration, as well as using it in a matrix of constructs for various *in vitro* studies such as drug discovery, toxicity testing, or preclinical studies. The method for manufacturing the construct involves the preparation of bioinks with parenchymal and non-parenchymal cells, their application to support and subsequent incubation, leading to a three-
30 dimensional liver tissue with at least one compartment. The presented construct is characterized by maintaining significant viability *in vitro* culture for at least 7 days, while retaining important liver features, including the lobular structure.

The Juan Cui et al 2018 publication 'Fabrication of perfusable 3D hepatic lobule-like constructs through assembly of multiple cell type laden hydrogel microstructures' Biofabrication (doi:
35 <https://doi.org/10.1088/1758-5090/aaf3c9>) reveals the printing of liver models with HepG2 and NIH/3T3 cells (not in GelMA) into gear-like structures. The liver models were cultured without flow

(simply on a dish, like organoids), and with flow by ‘overlapping’ the gear channel - thus the effect of medium flow on cell viability in the constructs was investigated. Cultures were maintained for a minimum of 15 days and a maximum of 21 days. The effect of drugs on these constructs was not investigated.

- 5 S. Lu et al 2018 ‘Development of a biomimetic liver tumor-on-a-chip model based on decellularized liver matrix for toxicity testing’ Lab Chip (doi: DOI: 10.1039/C8LC00852C) investigated the effects of hepatic dECM and GelMA in the constructs. The constructed system is divided into two channels - one for medium and drug delivery and the other with cells (HepG2). The essence of this invention is that the flow provides a constant nutrient requirement as well as the removal of metabolic products. The culture
10 was conducted for 21 days and the effects of drugs including Sorafenib were tested.

The publication of Y. Li et al 2019 ‘3D bioprinting of hepatoma cells and application with microfluidics for pharmacodynamic test of Metuzumab’ Biofabrication, 11, 034102 (doi: <https://doi.org/10.1088/1758-5090/ab256c>) reveals a system constructed with two channels but in this case, cancer cells were cultured in one of the channels (not HepG2 but some line of SMMC-7721) while
15 the other channel was populated by GFP-HUVEC. In this case, the main aim was to simulate angiogenesis and vascular connection to the tumor cells. In addition, the effect of the drug Methuzumab was tested.

The publication by F. Sharifi et al 2020 ‘A hepatocellular carcinoma-bone metastasis-on-a-chip mode; for studying thymoquinone-loaded anticancer nanoparticles’ Bio-Design and Manufacturing (doi: <https://doi.org/10.1007/s42242-020-00074-8>) reveals a model of hepatic cancer metastasis on bone. The
20 essence is the culture of HepG2 in one chamber, where the other chamber contained hydroxyapatite - as a signal from bone cells for HepG2 to metastasize to bone. No drugs were administered, but the model was run for up to 21 days.

The aim of the invention is to create a three-dimensional, bioprinted, living and functional model
25 including the vascular system to mimic tumor liver tissue. Such a model is intended for evaluating the cytotoxicity of drugs inter alia doxorubicin and the efficacy of anti-cancer therapies. The bioprinted tissue model is used to develop more precise and efficient research methods, for example in anticancer drug research. The 3D bioprinted liver tissue model makes it possible to produce conditions replicating those in native tissue, in order to increase the efficiency of research into drug toxicity and the efficacy
30 of anticancer therapies with respect to conventional, commonly used methods using 2D in vitro cell cultures.

The three-dimensional model can be used in flow-model studies, and tissue-model studies, and can serve as a model for controlled supplementation. Recent studies suggest that cells in 3D models respond differently to the drugs being tested than in traditional cell cultures, allowing a more precise assessment

of response to proposed therapies and predicting the development of resistance to the drug used. Models are also an alternative to the increasingly criticized use of animals for testing in pre-clinical research on ethical grounds.

Three-dimensional models contribute to the development of more precise and efficient research methods (e.g. in anti-cancer drug research) and also allow a tumor to be better reflected.

From the resulting tumor models, the necessary slice or fragment of cultured tissue can also be taken and submitted for histological analysis. Depending on the need, this may be an entire piece of tissue divided into appropriate fragments or a small section of it - a biopsy.

The object of the invention is a bioprinted liver model with a vascular system consisting of layers and comprising at least one flow channel in which:

the first two layers forming the base of the model are 100% filled with bioink, and wherein layer 1 is positioned at an angle of 0° to the flow channel and layer 2 is positioned at an angle of 90° to the flow channel,

the inner layers of the model are filled from 20% to 100% with bioink, wherein the inner layers comprise a centrally located at least one flow channel, the perimeter of which is printed with 100% filling and in which every inner layer is arranged at an angle of 60° with respect to the previous layer,

the last two layers enclosing the model: the penultimate and the last layer are 100% filled with bioink and wherein the penultimate layer is arranged at an angle of 0° with respect to the flow channel and the last layer is arranged at an angle of 90° with respect to the flow channel,

wherein the bioink used is based on natural polymers, synthetic polymers, methacrylated derivatives of natural polymers or mixtures thereof.

Preferably, the liver model consists of 14 layers.

Preferably, the liver model comprises two flow channels.

Preferably, every layer of the model is surrounded by an outer contour with a thickness equal to the thickness of one pathway.

Preferably, the height of every layer is 0.15 mm.

Preferably the diameter of the flow channel is 1.5 mm.

Preferably, the flow channel is populated with fibroblast cells line (e.g. RFP-HDF-a) and endothelial cells (e.g. GFP-HUVEC) in a ratio of 2:1, respectively.

The invention also refers to a method for manufacturing a liver model, comprising the following steps:

- a) printing a flat layer that forms the base of the model, wherein layer 1 is laid at an angle of 0° in relation to the flow channel,
 - b) printing of the inner layers, wherein every inner layer is laid at an angle of 60° to the previous layer, wherein at least one flow channel is printed in the inner layers,
 - 5 c) printing the closing layer, being the top cover of the model at an angle of 90° to the flow channel,
 - d) curing of the printed model by UV light, visible light, thermally or enzymatically,
 - e) optionally, supplying the flow channel with culture medium with endothelial cells (e.g. GFP-HUVEC) and fibroblasts (e.g. RFP-HDF-a)
- 10 wherein bioinks based on natural polymers, synthetic polymers, methacrylated derivatives of natural polymers or mixtures thereof are used to bioprint the model,
- wherein when printing the flow channel, two nozzles are used: one for layering the bioink and the other for forming a temporary support bioink in the area of the channel, wherein the support material is removed after printing is completed, wherein the support bioink is Pluronic or alginate hydrogel,
- 15 wherein the printing temperature of the layers is between 15 and 20 °C, at a pressure of 10-60 kPa and a printing speed of 8-12 mm/s.
- Preferably, the natural polymers are selected from the group comprising collagen, hyaluronic acid, alginate, gelatin, decellularized extracellular matrix or chitosan.
- Preferably, the synthetic polymers are selected from the group comprising PEGDA, PCL, and Pluronic.
- 20 Preferably, the methacrylated derivatives of natural polymers are selected from the group comprising: methacrylated gelatin, methacrylated hyaluronic acid, methacrylated chitosan, methacrylated dECM.
- Preferably, cells from the hepatocyte line (e.g. HepG2, HepRG, THLE2) are suspended in the bioink.
- Preferably, a photoinitiator selected from the LAP or Irgacure group is added to the bioink.
- Preferably, the bioink further comprises bioactive factors such as, for example, VEGF, FGF or EGF.
- 25 Preferably the bioink comprises at least one additive selected from the group of: hydroxyapatite, silver nanoparticles, glycerol, dextran, vitamin C, glutathione, RGD peptides, laminin, PEGDA, trypan blue, fluorescent green dyes, NaCl, HEPES.
- The invention also refers to the use of the bioprinted liver model to assess drug cytotoxicity and the efficacy of anti-cancer therapies.
- 30 Preferably the drug is doxorubicin.

A model was manufactured, allowing the preservation of a structure resembling a liver lobule, within which successive layers are shifted by 60° for each other, showing a high surface-to-volume ratio. This approach allowed efficient distribution of nutrients and oxygen to cells suspended in the biomaterial, leading to their proliferation inside the bioconstruct, where cells formed numerous spheroids. High cell viability was noted during the conduct of the experiment.

In addition, the study showed a high durability of the model (21 days), the structure of the construct did not degrade, retaining similar properties to the day the experiment was set up.

The structure of the model allowed microscopic observations at every level of the bioconstruct and efficient penetration of FDA and Pi stain deep into the biomaterial, enabling assessment of cell viability, but observations under white light showed poor translucency of the biomaterials, probably due to the presence of dECM.

In the experiments carried out, the model construction was shown to have a favorable effect on cell culture, and the possibility of assessing the viability of the bioconstruct using FDA/Pi staining

For the liver bioink models, high cell viability was achieved, which was maintained for 22 days of the experiment. Spheroids were formed in the 20% and 100% filled models, but only in the 20% filled models was it possible to carry out survival observations and FDA/Pi staining deep into the bioconstruct.

H&E staining in the 20%-filled model showed that the cells proliferate extensively in spaces that mimic liver lobules. On day 8 of culture, cells form clusters in the above spaces, which by day 15 take the shape of spheroids, which increase in size on day 22. H&E staining in the 100% filled model showed that cells suspended in the biomaterial do not proliferate. Cells were shown to be able to proliferate only in the area near the surface of the bioconstruct, where they form cell clusters, and at the surface, where they form spheroids.

Liver bioink is the optimal biomaterial for culturing constructs using hepatocellular carcinoma cells. It stimulates cell proliferation and allows the formation of spheroids. In addition, the 20%-filled model appears to be a better environment for cell culture and observation, as only in this case proliferation occurs for 22 days across the entire surface of the bioconstruct, including in the deeper parts, and observations can be made across the entire bioconstruct area.

Brief description of the figures

Fig. 1 3D model with elements mimicking liver lobules and perfusion channels, a: first layer, b: Layer 3 - first layer of lobules and first layer of canal, c: Layer 5 - third and final layer of lobules, d: Layer 6 - first layer with altered material, e: Layer 7 - we are halfway through the channel print, f: Layer 12 - last layer with channel, g: Layer 13 - first layer on the channel, h: Layer 20 - the last layer of the print.

Fig. 2 3D model comprising two flow channels, a: Layer 1, b: Layer 4 - the beginning of the channel and the middle element, c: Layer 8 - layer where we are halfway through printing the channel and middle element, d: Layer 13 - the last layer of the channel and middle element print, e: Layer 14 - the first layer after the channel and middle element are closed, f: Layer 20 - the last print layer.

Fig. 3 Overview figure of the liver tissue model with flow channel in layers 3-12, 100% fill for the perimeter of the model and perimeter of the flow channel, layers 1-2 and 13-14 and 20% fill for layers 3-12, based on the G-code file named GP_lh15inf20_T0T1.

Fig. 4 Overview figure of a liver tissue model with 14 layers surrounded by a contour and 100% filling, with a flow channel. Cells distributed in only 3 layers surrounding the channel. Channel printed with 5% sonicated hydrogel. Based on a G-code file named kanalyGP3x14.gcode.

Fig. 5. shows the project of the printed models. Printing was carried out on a 3D bioprinter. An external UV-Vis lamp, Polbionica, was used to crosslink every layer, (a) model with 100% fill, (b) model with 30% fill.

Fig. 6 Lobular structure printout.

Fig. 7 Figure showing a 3D bioprinted liver tissue model of a lobular structure with 20% filling.

Fig. 8 The figures show the *in vivo* observations of the models. The images show the formation of spheroids from HepG2 cells in a 20% filled model after a 21-day incubation. Observations carried out under light brightfield (BF) using an Olympus IX83 inverted microscope.

Fig. 9. The figures show the *in vivo* observations of the model. The images show the formation of spheroids from HepG2 cells in a 100% filled model after a 21-day incubation. Observations carried out under light brightfield (BF) using an Olympus IX83 inverted microscope.

Fig. 10 Evaluation of the morphological structure of the bioconstruct by microscopic imaging of H&E staining slides. Observations carried out using an Olympus IX83 inverted microscope, brightfield technique, in white balance. Histological image of liver models: in the histological image of the bioconstruct, a structure imitating tissue lobules is visible. Numerous small spaces are present in the construct.

Fig. 11 Evaluation of the morphological structure of the bioconstruct (day 21) based on microscopic imaging of H&E stained slides. Spheroidal structures of HepaRG cells are visible in the spaces mimicking the lobules. In the main channel, the presence of cells populating it is observed.

Fig. 12 Graph showing AFP concentration. The model was incubated in a bioreactor for 21 days without drug administration, then on day 21 the appropriate dose of doxorubicin (100 mg/m² liver model area or 50 mg/m² liver model area) was administered to the model.

Fig. 13 Graph showing cytotoxicity based on the LDH test performed. The model was incubated in a bioreactor for 21 days without drug administration (DAY1.C - DAY21.M), then on day 21 the appropriate dose of doxorubicin (50 mg/m² or 100 mg/m² of liver model area) was administered to the model. After 24 hours and 7 days, LDH levels were re-tested. The graph shows the presumed cellular response to the administered drug dose (increase in LDH levels). In addition, there was an almost 2-fold increase in LDH after a 2-fold higher dose. After 7 days, there is stabilization in the enzyme concentration tested.

Fig. 14 Dosage charts

Functional liver models with different cellular compositions were printed. These included the following cell lines: HepG2, HepRG, THLE2, which were inserted into the bioink structure that constitutes the body of the construct: and the cell lines used to populate the channel of the construct: RFP-HDF, GFP-HUVEC.

After incubation of the bioconstructs in the ResearchLine 2.5 incubator (Polbionica), which provides tissue models and bionic organs with in vitro culture conditions lasting no less than 21 days, the drug doxorubicin was administered to the models at baseline concentrations of 20 - 200 mg/m² of the model area.

The models tested were characterized by a fill rate ranging from 20% - 100% and the presence of a main flow channel that was populated with cells of the RFP-HDF-a and GFP-HUVEC lines at a 2:1 ratio (5,333·10⁶/mL and 2,666·10⁶/mL), respectively. Cells of the HepG2 line were suspended in a whole volume of bioink, the final concentrations of which ranged from 4·10⁶/mL do 12·10⁶/mL of bioink, depending on the test variant. The bioink composition was 10% (w/v) GELMA: 0.5% (w/v) HAMA: 1% (w/v) liver dECM, with a LAP concentration of 0.19% (w/v).

The viability, proliferative capacity and functionality of the cell lines were assessed, as well as the morphology of the bioconstruct after administration of the anticancer drug, doxorubicin. The bioconstruct was cultured for a period of 21 days after which the first dose of the drug was administered to the model.

During the experiment, viability observations of the model were carried out using an Olympus IX83 optical microscope, observations were made using white light (in white balance) and fluorescence.

Due to this phenomenon, it is only possible to observe that liver cells have proliferated, forming small clusters deep within the biomaterial. However, conducting an effective observation of proliferating cells on the surface of the biomaterial under white light is effective and has made it possible to observe that cells proliferate on the surface of the bioconstruct, forming spheroids that show the ability to fuse together.

After the experiment, a section of the model was treated with FDA/PI stain, from which the viability of the bioconstruct and the LDH test were assessed.

FDA and PI stain effectively penetrate the bioconstruct, allowing the viability of cells suspended in the biomaterial and included in the spheroids to be imaged, demonstrating a high cell viability of ~80%.

It is possible to make observations of the construct at different heights.

Liver bioink is the optimal biomaterial for culturing constructs using hepatocellular carcinoma cells. It stimulates cell proliferation and allows the formation of spheroids. In addition, the 20%-filled model appears to be a better environment for cell culture and observation, as only in this case proliferation occurs for 22 days across the entire surface of the bioconstruct, including in the deeper parts, and observations can be made across the entire bioconstruct area.

The remaining sections of the slides were placed in a 4% formaldehyde solution and transferred to prepare histological slides of H&E stain, IHC and IF dyed slides to assess bioconstruct morphology after doxorubicin administration. The functionality of the model was assessed by medium samples collected during the experiment, subjected to ELISA for the tumor marker alphafetoprotein (AFP) and proangiogenic factors (VEGF, vWF). And the expression of selected genes (e.g. AFP, VEGF-A) was determined using qPCR.

The following HCC marker proteins were selected for IHC and IF dyeing: Ki-67, GPC-3, OPN, GOLPH2 (GP73), CDH1:

- Ki-67: The nuclear protein Ki-67 is widely used as a proliferative and prognostic marker in hepatocellular carcinoma (HCC). High levels of Ki-67 in HCC indicate the potential progression of HCC and the poor prognosis of patients.
- GPC-3: Glypican-3 protein (GPC-3), a member of the heparan sulphate proteoglycan group, binds to the cell membrane and its elevated levels are observed in hepatocellular carcinoma (HCC). GPC-3 is not detected in normal liver tissues and benign liver changes. Therefore, GPC-3 is currently being used as a diagnostic biomarker and HCC-specific positron emission tomography probe to identify HCC in liver tissues.
- OPN: Osteopontin protein (OPN), an extracellular matrix protein, is one of the prognostic factors in hepatocellular carcinoma (HCC), wherein it is not a typical oncogene. It exists in a

mutated form in active tumor cells and is regulated by various pathways including signaling, receptors and growth factors.

- GOLPH2 (GP73): GP73 is a type II transmembrane glycoprotein located in the Golgi membrane. It is expressed only in biliary epithelial cells and has a low detectable level of expression in healthy liver hepatocytes. GP73 expression in hepatocytes is significantly increased in liver diseases including during hepatitis C virus (HCV) infection, HBV- and HCC-related cirrhosis, alcoholic hepatitis, and autoimmune hepatitis. High levels of GP73 expression in patients with HCC suggest its role in liver carcinogenesis. Elevated GP73 levels in serum have been observed in patients with HCC, making it a sensitive and specific biomarker for the early diagnosis of HCC. Furthermore, serum GP73 levels are positively associated with HCC progression and negatively associated with clinical outcomes.
- CDH1: E-cadherin (CDH1) is an adhesion molecule of the adhesin group that has been implicated in the development of malignant tumors. Adhesins are trans-membrane glycoproteins involved in cell-cell adhesion in tissue and responsible for cell-cell contact with the extracellular matrix. With mutations that inactivate E-cadherin, tumor cell adhesion is more easily lost in the primary tumor, leading to metastasis. E-cadherin, as an adhesion molecule, is a prognostic factor during the promotion and progression phase, as it is the loss of cell-cell contact with the tumor mass that is one of the conditions for the formation of distant metastasis.

A liver tissue model with a 20% filled vascular system has been shown to be the most efficient and creates a more optimal environment for cell proliferation than a 100% filled model. The 20%-filled model containing structures that mimic liver lobules creates an optimal environment for cell culture, allowing spheroids to form throughout the model. The structure used improves the efficiency of distribution of the culture medium throughout the model by increasing nutrient availability, stimulating liver cell proliferation and enabling the formation of spheroidal structures in the spaces mimicking liver lobules.

Preferred Embodiments of the Invention:

BIOINKS:

Bioinks based on natural polymers:

- Collagen (3-10 mg/mL): Promotes cell adhesion and differentiation, often used in combination with hyaluronic acid (1-2% w/v) or alginate (1.5-2.5% w/v).
- Hyaluronic acid (0.5-2% w/v): Hydrophilic polysaccharide to promote hepatocyte proliferation, also available in a methacrylated version. Being in a mixture with collagen (1:3) or gelatine (5-10 mg/mL).

- Alginate (1-3% w/v): A natural polysaccharide with gelling properties, often combined with gelatine (5-10 mg/mL) or dECM (3-8 mg/mL).
- Gelatin (5-15 mg/mL): A protein polymer that promotes cell adhesion, particularly popular when mixed with hyaluronic acid (1-2% w/v) or alginate (1.5-2.5% w/v).
- 5 • Decellularized extracellular matrix (dECM, 3-8 mg/mL): Bioactive matrix containing hepatocyte support factors, often used with hyaluronic acid (0.5-1% w/v) or alginate (1-2% w/v).
- Chitosan (1-3% w/v): A biodegradable polysaccharide with antimicrobial properties, often used in combination with alginate (1-2% w/v) or PEGDA (10-15% w/v).
- 10 Bioinks based on synthetic polymers:
 - PEGDA (10-20% w/v): Synthetic hydrogel with the ability to crosslink in a controlled manner, often used in a mixture with collagen (3-5 mg/mL) or dECM (3-8 mg/mL).
 - PCL: Biodegradable polyester for mechanical stability. Combined with PEGDA for improved elasticity or with alginate (1-3% w/v) for gelling properties.
- 15 • Pluronic (15-30% w/v): A block copolymer, used as a carrier material, often in combination with methacrylated derivatives of natural polymers such as GelMA or HAMA.

Methacrylated derivatives of natural polymers:

- Methacrylated gelatine (GelMA, 5-15 mg/mL): Allows photo-curing using UV or visible light in the presence of photoinitiators. Promotes cell adhesion and proliferation..
- 20 • Methacrylated hyaluronic acid (HAMA, 0.5-2% w/v): Hydrophobic, bioactive material with UV light cross-linking capability.
- Methacrylated chitosan (ChiMA, 1-3% w/v): Biodegradable polysaccharide with antimicrobial properties, suitable for crosslinking.
- Methacrylated dECM (3-8 mg/mL): Bioactive extracellular matrix for high biological
- 25 compatibility with photo-curing capability.

And other derivatives, including metacrylic, of other natural polymers which enable the curing of materials in the following ways.

WAYS OF CROSS-LINKING FRAMEWORKS:

- Photo-curing: Used in methacrylated derivatives (GelMA, HAMA, ChiMA, methacrylated
- 30 dECM). Using UV or visible light in the presence of photoinitiators such as Irgacure 2959.

- Ionic cross-linking: Used in alginates with Ca^{2+} or Ba^{2+} ions.
 - Enzymatic cross-linking: Applied to natural polymers such as collagen or gelatine, using enzymes such as genipin or transglutaminase.
 - Chemical cross-linking: Use of reactants such as aldehydes to reinforce bioconstruct structures.
- 5 • Thermal cross-linking: Use of temperature, e.g. for Pluronic, enabling controlled gelation.

When preparing bioinks for printing bionic models, the reactants can be mixed in any volume ratio, which is most often expressed as a percentage of the total volume of the mixture. For example, volume ratios ranging from 1:9 (10% of one component and 90% of the other) to an even ratio of 1:1 can be used, depending on the required mechanical, rheological or biological properties of the bioink.

- 10 • For natural polymers such as collagen and hyaluronic acid, ratios of 1:4 or 3:7 are usually used to achieve a balance between viscosity and bioactivity.
- For combinations of natural and synthetic polymers (e.g. alginate and PEGDA), a ratio of 2:3 to 3:2 can be used to ensure adequate mechanical properties and to maintain biocompatibility.
- 15 • For mixtures containing methacrylated derivatives (e.g. GelMA or HAMA), an even ratio of 1:1 or a higher concentration of methacrylated derivative (e.g. 7:3) is usually used to facilitate cross-linking and stabilization of the structure.

The final ratio of components should be tailored to the required properties of the bioink, such as viscosity, cross-linking ability or biocompatibility with cultured cells.

- 20 Bioinks can be supplemented with bioactive factors such as VEGF, FGF or EGF, which promote cell differentiation, proliferation and angiogenesis. These are particularly useful in liver models, where they stimulate hepatocyte function or promote blood vessel development.

- 25 To improve the properties of bioinks, nanoparticles are often used, such as hydroxyapatite, which promotes mineralization and gives the bioconstruct better mechanical properties, or silver nanoparticles, which have an antibacterial effect, protecting the bioink from microbial contamination. The addition of rheology-altering agents, such as glycerine or dextran, allows the viscosity of bioinks to be regulated, which is key when using different printing technologies such as extrusion. These make bioinks more stable and easier to use. Bioinks can also contain antioxidants, such as vitamin C or glutathione, which protect cells from oxidative stress caused, for example, by UV light during the printing process or bioconstruct maturation

- 30 Adhesion-enhancing agents such as RGD peptides or laminin are used to improve the interaction of cells with the bioconstruct material. These facilitate cell adhesion and proliferation, especially in synthetic materials such as PEGDA, which have limited biocompatibility in their original form. Biological dyes,

such as trypan blue or fluorescent green dyes, can also be introduced into bioinks to enable visualization of printing processes or assessment of cell viability in the bioconstruct. Fluorescence can also support real-time studies of tissue development. The addition of osmolytics and pH regulators, such as NaCl or HEPES, maintains physiological conditions in the bioink. NaCl stabilizes the osmotic balance, while HEPES regulates pH, which provides an optimal environment for cells.

GEOMETRY AND DESCRIPTION OF THE PRINTING METHOD

Bioprinting a 3D model of the liver, which incorporates liver lobule-like elements and perfusion channels, offers significant advantages over simpler, static models. Liver lobules are the basic functional units of the liver, where key metabolic, detoxification and protein synthesis processes take place.

Mapping their structure allows for a more faithful reproduction of the organization of hepatocytes around the central vein, oxygen and nutrient gradients and differences in cell function depending on their location in the lobule. The addition of perfusion channels, which allow the culture medium to flow through the model, further enhances its realism. The flow-through environment dynamically delivers oxygen and nutrients, allows for efficient removal of toxins and metabolic products, and generates metabolic gradients, characteristic of natural lobules. This architecture makes the model better reflect physiological conditions, resulting in higher hepatocyte functionality, longer life span and the possibility of long-term studies

Flow models allow the mechanisms of liver disease, such as steatosis, fibrosis or necrosis, to be studied in a more realistic environment and allow drugs to be tested under conditions that mimic their actual distribution and metabolism in the body. Furthermore, perfusion channels allow real-time sampling, which provides the opportunity to monitor dynamic processes such as changes in metabolite, enzyme or toxin concentrations in response to administered substances. Such functionality opens up new possibilities for both disease research and the precise assessment of drug efficacy and toxicity. In addition, flow models allow different cell types to coexist in their natural proportions and interactions, increasing the realism of the liver microenvironment. As a result, they can be easily adapted to research on individual patients, creating personalized liver models, which is particularly important in precision medicine.

The 3D model, with its liver lobule-like elements and perfusion channels, represents an advanced research tool that not only reproduces physiological conditions more faithfully *in vivo*, but also enables long-term studies, dynamic monitoring of biological processes and the development of personalized therapies. With its complexity and functionality, the model sets a new standard in liver research and drug development (Fig. 1).

MODEL WITH TWO FLOW CHANNELS

A 3D bioprinted model of the liver composed of two flow channels and centrally placed liver lobules. The presented construction allows an even more faithful reproduction of the physiological conditions of the liver, especially in terms of its vascularization and metabolite gradients. Thanks to the presence of two perfusion channels, located on both sides of the lobules, it is possible to reproduce the arterial blood flow of the hepatic vascular system. The central location of the lobules between the channels allows better delivery of oxygen and nutrients to their entire structure, as well as efficient removal of metabolites. Compared to single-flow channel models, the dual channel arrangement allows for more pronounced oxygen and nutrient gradients in the lobules, which is crucial for studying the specific functions of hepatocytes in different metabolic zones. Such an arrangement allows a more accurate depiction of the functional diversity of hepatocytes, particularly in the context of drug metabolism, detoxification and protein synthesis. The central placement of the lobules between the channels also promotes an equal distribution of pressure and flow of the culture medium, further improving cell viability and functionality. The presence of two flow channels also enhances the research capabilities of the model. At the same time, the ability to sample from both channels offers the chance to monitor the dynamics of metabolites, enzymes and toxins in real time with even greater accuracy than in a single-channel model. Such a solution makes it possible to study the phenomena of interactions between different nutrients that more closely resemble natural conditions in the body. Furthermore, the presence of two flow channels enhances the realism of the liver microenvironment. This facilitates research into pathological mechanisms and the development of targeted therapies. The increased complexity of the model also allows for the integration of more liver cell types, such as endothelial, Kupffer or stellate cells, which can be positioned in specific regions in relation to the flow channels.

The bioprinting of a liver model using the extrusion method involves the precise deposition of layers of bioink to create a spatial, functional structure that mimics the architecture of the liver. Such a model can be printed with any bioink suitable for the extrusion method, which provides great flexibility in the choice of materials. Additionally, the model may or may not contain cells, allowing it to be used in both materials and biological research (Fig.2).

Model design and bioink preparation: The model was designed with a central flow channel, which acts as an imitation of blood vessels, allowing the flow of nutrient fluid (e.g. culture medium). Inside the structure, a multi-layered arrangement of fibers rotated at a fixed angle was planned to mimic the liver lobules - the basic functional unit of the liver, characterized by an arrangement of hepatocytes around vessels. The bioinks, adapted for extrusion printing, could consist of materials such as collagen, methacrylated gelatine (GelMA), hyaluronic acid (HAMA) or dECM, and could also contain additional bio-active agents, photoinitiators or structure enhancers.

Opening and closing layers: The bioprinting process started with the deposition of the opening layer, which provided a flat base for the model and ensured its stability. After building the inner layers (described below), the bioprinting was completed with a closing layer.

Layers mimicking liver lobules: The inner layers were designed as segments mimicking liver lobules. Every layer consisted of biomaterials that may or may not have contained cells, such as hepatocytes. The layered structure mimicked the radial arrangement of cells around the central vessel, which reflects the natural architecture of liver lobules. The bioinks used may have contained factors that promote bioactivity, such as RGD peptides or growth factors.

Flow channel: The flow channel was designed as the central element of the model, running the length of the model, with inlet and outlet holes. Two nozzles were used during the printing process: one to deposit the layered bioink and the other to form a temporary support material in the channel area (e.g. Pluronic F127 or alginate hydrogel). Once the printing was complete, the support material was removed, creating a free-flow space that could be perfused with culture medium or other test fluids.

Curing and maturation: After printing, the model was cured (in the case of photo-curable materials such as GelMA or HAMA) using UV or visible light to ensure the mechanical stability of the structure. In the case of models containing cells, they were placed in a flow bioreactor to ensure that the flow channel was dynamically fed with culture medium, mimicking blood flow under physiological conditions.

Functionality of the model: The model enabled the study of hepatocyte metabolism, substance exchange between the flow channel and lobular structures and cellular interactions. The layered structure made it possible to reproduce the actual nutrient gradient characteristic of the liver microenvironment.

With flexibility in bioink selection and the ability to use models both with and without cells, the bioprinted liver model provides an advanced platform for research into liver regeneration, drug metabolism and toxicology.

Preparation of G-code models for liver tissue models, model test.

VARIANT 1

For the liver tissue models, a G-code file was prepared for bioprinting purposes. A file named: kanalyGP_lh15inf20_T0T1 was prepared for the model with 20% fill. The layer height in the G-code file is 0.15 mm and the number of layers is 14. Layers 1-2 of every model have a 100% fill and are arranged at 0° and 90°. Layers 3-12 are arranged at 0°, 60° and 120° and have a fill of 20%, Layers 3-12 contain a centrally located flow channel, the perimeter of which is printed with 100% fill and has a diameter of 1.5 mm. Layers 13-14 are characterized by 100% fill and are arranged at 0° (layer 13 - above the channel) and 90° (layer 14) to the channel. Every layer is surrounded by an outer contour of one path

thickness. The volume for the 100% filled model is 1.1 mL of bioink. An overview drawing is shown in Figure 3.

Technical data:

- 5 • Head no. 1:
- full layers: 1,2,12,13
- contours
- Head no. 2:
- filling (20% 0,60,120 degrees)
- 10 • Head no. 3:
- Channel filling.
- Layer height 0.15mm

VARIANT 2

- 15 A file named *kanalyGP3x14.gcode* was prepared for the model with 100% fill. The layer height in the G-code file is 0.15 mm and the number of layers is 14. The layers are characterized by a 100% fill. Every layer is surrounded by an outer contour. A channel is located in the central part of the model. The cells are distributed only in the bioink located in the area of the 3 layers surrounding the channel. An overview drawing is shown in Figure 4.

Preparation of G-code models for liver tissue models, and model test.

- 20 A G-code file was prepared for every liver tissue model for the purpose of bioprinting:
- model with 30% fill, file named: *kanalyGP_lh015inf30*,
 - model with 100% fill, file named: *kanalyGP_lh015inf100*.
 - model with 20% fill, file named: *kanalyGP_lh15inf20_T0T1* and *GP4xx.gcode*

- 25 The layer height in the G-code file for every model is 0.15 mm and the number of layers is 14. Layers 1-2 of every model are characterized by 100% fill and are arranged at 0° and 90°. Layers 3-12 are arranged at 0°, 60° and 120° - these layers are printed with varying degrees of filling for the models: 20%, 30% or 100%. Layers 3-12 comprise a centrally located channel, the perimeter of which will be printed with 100% fill and will have a diameter of 1.5 mm. The channel will be printed with 5% sonicated hydrogel. For the models without a channel, layers 3-12 are arranged at 0°, 60° and 120° with a filling of 20%. Layers 13-14 are characterized by a 100% fill and will be arranged at 90° and 0°. Every
- 30

layer is surrounded by an outer contour of one path thickness. The volume of the 100%-filled model will be 1.1 mL of bioink.

Model bioprinting

Models were printed - 30% and 100% fill. The models were printed according to the programmed project - G-code files: kanalyGP_lh015inf30.gcode and kanalyGP_lh015inf100.gcode. Fig. 5. shows the project of the printed models. The printing was carried out on a 3D bioprinter. An external UV-Vis lamp, Polbionica was used to crosslink every layer.

Table 1. Printing parameters of the model

Material	Printing temperature [°C]	Pressure [kPa]	Print speed [mm/s]	Needle	Cross-linking
bioink with cells	15.0-17.0	10-25	8-15	250 µm	wavelength 405 nm; time 20 sec; power 28.5 mW/cm ²
5% (w/v) ECM hydrogel - sonicated	17.0-18.0	15-30	8-15	250 µm	-

10 A liver model was printed with a single channel centrally located with a fill of 20%. The model consisted of 14 layers with a height of 0.15 mm, where the fibers of each layer were aligned with the next layer by 60°. The model was printed using a 250 µm needle, both filling the channel and the liver tissue mass. The model was printed according to the programmed project – G-code file: kanalyGP_lh15inf20_T0T1.gcode. Printing was carried out on a 3D bioprinter. An external UV-Vis
15 lamp, Polbionica, was used to crosslink every layer.

Table 2. Printing parameters of the model

Material	Printing temperature [°C]	Pressure [kPa]	Print speed [mm/s]	Needle	Cross-linking
bioink with cells	16.0-17.0	33-62	8-15	250 µm	wavelength 405 nm; time 20 sec; power 20 mW/cm ²
5% (w/v) ECM hydrogel - sonicated	19.0	30-60	5-15	250 µm	-

sealing bioink	RT	-	-	-	wavelength 405 nm; time 30 sec; power 28.5 mW/cm ²
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A 100% filled model with a single channel centrally located. The model consisted of 14 layers with a height of 0.15 mm, where the fibers of each layer were aligned with the next layer by 90°. The channel outlines constitute 3 paths. The model was printed according to the programmed project – G-code file: kanalyGP3x14.gcode.

- 5 Model with 20% filling without canal. Two lower and two upper layers with 100% filling. Successive layers of the model arranged at an angle of 60° to each other. The model was printed according to the programmed project – G-code file: GP4xxgcode.

Models were printed on a 250 µm needle, both canal filling and liver tissue mass. Printing was carried out on a 3D bioprinter. An external UV-Vis lamp, Polbionica, was used to crosslink every layer.

10 **Table 3.** Printing parameters

Material	Printing temperature [°C]	Pressure [kPa]	Print speed [mm/s]	Needle	Cross-linking
bioink with cells	17.5	33	12	250 µm	wavelength 405 nm; time 20 sec; power 20 mW/cm ²
5% (w/v) ECM hydrogel - sonicated	19	55	12	250 µm	-
sealing bioink	RT	-	-	-	wavelength 405 nm; time 30 sec; power 28.5 mW/cm ²

Models with 20% and 100% fill were printed. The petals consisted of 10 layers with a height of 0.15 mm, where the fibres of each layer were stacked 60° relative to the next layer. The petals were printed first with liver bioink and then with pancreatic bioink. Liver ECM bioink petals were printed using a 233 µm needle, pancreatic ECM bioink petals with 20% fill were printed using a 233 µm needle, and pancreatic ECM bioink petals with 100% fill were printed using a 250 µm needle. The petals were printed according to the programmed projects – G-code files: fi15x3_inf20_per1_ew03.gcode and fi15x10x015ew03inf100_0_60_120.gcode. Printing was carried out on a 3D bioprinter. An external UV-Vis lamp, Polbionica, was used to crosslink every layer.

- 15

Microscopic observations, FDA/PI staining, preparation of histological slides

Light brightfield (BF) vital microscopic observations; for this, the drains supplying the medium to the chamber were disconnected from the bottles and the medium was then pumped out of the system using a pump. After draining the system, the biochamber was disconnected from the drains and the channels were closed by using Venflon caps. The closed biochambers were placed on the microscope table of an Olympus IX83 optical microscope and observations were made using white light, in white balance (light brightfield (BF)). After the completion of the experiment, the models were stained using FDA/PI. For this purpose, a section of the preparation was transferred to the well of a 24-well plate and then injected sequentially with: 1 mL of 1xPBS, 5 μ L of PI at a concentration of 0.5 mg/mL and 5 μ L of FDA at a concentration of 5 mg/mL, gently pipetted and observed with a fluorescent lamp. The remaining sections of the slides were placed in a 4% formaldehyde solution and transferred to histological slides. The resulting slides were subjected to HE staining and immunofluorescence reaction.

Liver models were observed under an Olympus IX83 inverted microscope, using brightfield technique, in white balance (survival observations).

- Printout of the lobular structure: Observations under white light demonstrate the transparency of the biomaterial - it can be seen that they have proliferated, forming small clusters deep within the biomaterial. On the other hand, conducting white-light observations of proliferating cells on the surface of the biomaterial is effective and has made it possible to observe that the cells proliferate on the surface of the bioconstruct, forming spheroids that show the ability to fuse together.
- In addition, the FDA and PI stain effectively penetrate the bioconstruct, allowing the viability of cells suspended in the biomaterial and included in the spheroids to be imaged, demonstrating a high cell viability of ~80%.

It is possible to make observations of the construct at different heights.

BIOLOGICAL STUDIES ON THE RESULTING LIVER MODELS

- The printed models were characterized by:
- liver cells are distributed throughout the liver bioink volume,
 - fibroblast and endothelial cells filled the channel of the models,
 - main channel of the model with 100% filling, printed with 5% sonicated liver hydrogel,
 - duration of incubation of tumor models: 21 days - 34 days,
 - after this time the drug doxorubicin was administered to the models,
 - the following doses of the drug administered to the system were tested: 20 - 200 mg/m² model

Vital observation: HepG2 cells suspended in a bioprinted model using a 233µm-diameter metal needle, a 15mm-diameter model including a 100% filled perimeter, 10 layers 0.15mm high and 20% filled, arranged at a 60° angle to the previous layer.

Fig. 8 shows the vital observations of the models. The images show the formation of spheroids from HepG2 cells in the 20% filled model after 21 days incubation. Observations carried out under white light (BF) using an Olympus IX83 inverted microscope.

100% filled model, main channel:

Fig. 9 shows the vital observations of the model. In the images, the formation of spheroids from HepG2 cells can be observed both inside the model and on the surface of the model with 100% filling after a 21-day incubation. Observations carried out under white light (BF) using an Olympus IX83 inverted microscope.

Evaluation of the morphological structure of the bioconstruct (HepG2 cells suspended in a peripheral model with 20% filling) based on microscopic imaging of H&E staining slides. Observations carried out using an Olympus IX83 inverted microscope, brightfield technique, in white balance, with x10 and x20 objective.

Fig. 10 shows the evaluation of the morphological structure of the bioconstruct by microscopic imaging of H&E staining slides. Observations carried out with an Olympus IX83 inverted microscope, using brightfield technique, in white balance. Histological image of liver models: in the histological image of the bioconstruct, a structure imitating tissue lobules is visible. Numerous small spaces are present in the construct.

Fig. 11 shows an assessment of the morphological structure of the bioconstruct (day 21) based on microscopic imaging of H&E staining slides. Spheroidal structures of HepaRG cells are visible in the spaces mimicking the lobules. In the main channel, the presence of cells populating it is observed.

Below are the results for models using the following cell lines:

- HepG2 cell line, human hepatocellular carcinoma cell line (ATCC®, cat no: HB-8065™)
- HUVEC cell line, human umbilical vein endothelial cells (ATCC®, cat no: PCS-100-010™)
- HDFa cell line, human fibroblast line (ATCC®, cat no PCS-201-012™)

The models were printed using:

- liver bioink enriched with HepG2 cell line in an amount of 4×10^6 /mL of bioink,
- sealing bioink containing hepatic dECM,
- the model channel was filled with 5% (w/v) hydrogel of sonicated dECM

Fig. 12 shows a graph showing the AFP concentration. The model was incubated in a bioreactor for 21 days without drug administration, then on day 21 the appropriate dose of doxorubicin (100 mg/m² or 50 mg/m² liver model area) was administered to the model.

Fig. 13 shows a graph showing cytotoxicity based on the LDH assay performed. The model was incubated in a bioreactor for 21 days without drug administration (DAY1.C - DAY21.M), then on day 21 the appropriate dose of doxorubicin (50 mg/m² or 100 mg/m² liver model area) was administered to the model. After 24 hours and 7 days, LDH levels were re-tested. The graph shows the presumed cellular response to the administered drug dose (increase in LDH levels). In addition, there was an almost 2-fold increase in LDH after a 2-fold higher dose. After 7 days, there is stabilization in the enzyme concentration tested.

Claims

1. A bioprinted liver model with a vascular system consisting of layers and comprising at least one flow channel in which:

the first two layers forming the base of the model are 100% filled with bioink, and wherein layer 1 is positioned at an angle of 0° to the flow channel and layer 2 is positioned at an angle of 90° to the flow channel,

the inner layers of the model are filled from 20% to 100% with bioink, wherein the inner layers comprise a centrally located at least one flow channel, the perimeter of which is printed with 100% filling and in which every inner layer is arranged at an angle of 60° with respect to the previous layer,

the last two layers enclosing the model: the penultimate and the last layer are 100% filled with bioink and wherein the penultimate layer is arranged at an angle of 0° with respect to the flow channel and the last layer is arranged at an angle of 90° with respect to the flow channel,

wherein the bioink used is based on natural polymers, synthetic polymers, methacrylated derivatives of natural polymers or mixtures thereof.

2. The bioprinted liver model according to claim 1, wherein the liver model consists of 14 layers.
3. The bioprinted liver model according to claim 1, wherein the liver model comprises two flow channels.
4. The bioprinted liver model according to claim 1, wherein every layer of the model is surrounded by an outer contour with a thickness equal to the thickness of one pathway.
5. The bioprinted liver model according to claim 1, wherein the height of every layer is 0.15 mm.
6. The bioprinted liver model according to claim 1, wherein the diameter of the flow channel is 1.5 mm.
7. The bioprinted liver model according to claim 1, wherein the flow channel is populated with fibroblast cells line, preferably RFP-HDF and endothelial cells, preferably GFP-HUVEC, preferably in a ratio of 2:1, respectively.
8. A method for manufacturing a liver model, according to claim 1, comprising the following steps:
 - a) printing a flat layer that forms the base of the model, wherein layer 1 is laid at an angle of 0° in relation to the flow channel,
 - b) printing of the inner layers, wherein every inner layer is laid at an angle of 60° to the previous layer, wherein at least one flow channel is printed in the inner layers,
 - c) printing the closing layer, being the top cover of the model at an angle of 90° to the flow channel,
 - d) curing of the printed model by UV light, visible light, thermally or enzymatically,

- e) optionally, feeding the flow channel with culture medium with cells of the fibroblast line, preferably RFP-HDF and with endothelial culture, preferably GFP-HUVEC.

wherein bioinks based on natural polymers, synthetic polymers, methacrylated derivatives of natural polymers or mixtures thereof are used to bioprint the model,

wherein when printing the flow channel, two nozzles are used: one for layering the bioink and the other for forming a temporary support bioink in the area of the channel, wherein the support material is removed after printing is completed, wherein the support bioink is Pluronic or alginate hydrogel,

wherein the printing temperature of the layers is between 15 and 20 °C, at a pressure of 10-60 kPa and a printing speed of 8-12 mm/s.

9. The method according to claim 8, wherein the natural polymers are selected from the group comprising collagen, hyaluronic acid, alginate, gelatin, decellularized extracellular matrix or chitosan.
10. The method according to claim 8, wherein the synthetic polymers are selected from the group comprising PEGDA, PCL, Pluronic.
11. The method according to claim 8, wherein the methacrylated derivatives of natural polymers are selected from the group comprising: methacrylated gelatin, methacrylated hyaluronic acid, methacrylated chitose, methacrylated dECM.
12. The method according to claim 8, wherein cells from the hepatocyte line are suspended in the bioink, preferably HepG2, HepRG, THLE2.
13. The method according to claim 8, wherein a photoinitiator selected from the LAP or Irgacure group is added to the bioink.
14. The method according to claim 8, wherein the bioink further comprises bioactive factors such as VEGF, FGF or EGF.
15. The method according to claim 8, wherein the bioink comprises at least one additive selected from the group of: hydroxyapatites, silver nanoparticles, glycerol, dextran, vitamin C, glutathione, RGD peptides, laminin, PEGDA, trypan blue, fluorescent green dyes, NaCl, HEPES.
16. The use of the bioprinted liver model according to claim 1, to assess drug cytotoxicity and the efficacy of anti-cancer therapies.
17. The use according to claim 16, wherein the drug is doxorubicin.