

Method of animal-derived tissue decellularization and bioink comprising animal-derived decellularized tissue

The invention relates to a method of obtaining extracellular matrix from liver tissue. The method of decellularizing liver includes homogenizing the tissue, treating the tissue with enzymes and
5 surfactant, and cleaning the tissue with a washing solution. The method removes almost all detergent from the decellularized matrix without the need to add other chemicals. In the second aspect, the invention relates to a bioink composition based on a decellularized extracellular matrix of suitable consistency and viscosity. The present patent application relates to the field of tissue engineering.

10 Liver diseases are responsible for approximately 2 million deaths annually worldwide accounting for 3.5% of all deaths (Asrani et al., 2019). Liver transplantation is widely regarded as the only effective therapy for end-stage failure of this organ (Dutkowski et al., 2011).

Previous advances in tissue engineering make it possible to develop treatment solutions for patients with liver failure, either by producing a piece of living tissue used to regenerate the
15 damaged organ or by producing a functional liver substitute that can be transplanted into human. These capabilities create a need to develop methods for producing artificial tissues that have both short-term and long-term utility. Artificial organs - organoids can also be used as a model for evaluating drug safety, in toxicology or virology studies (Chu and Lamers, 2024). Existing in vitro liver models are often insufficient to fully reflect the native features of the organ and the full
20 pathophysiology. Therefore, it is necessary to construct an in vitro liver model with properties closer to the human liver. Wider use of organoids in biomedical research would also limit the use of model animals (McGill and Jaeschke, 2019).

The main components of organs are the extracellular matrix (ECM) and the cells embedded in it. In a healthy liver, the extracellular matrix makes up <3% of the organ and is largely confined to the
25 Glisson's capsule, portal tracts and central veins (Bedossa et al., 2003). It is a complex network of hydrated proteins and polysaccharides, providing not only physical support to cells, but also participating in the processes of morphogenesis, cell growth and differentiation, tissue repair, maintenance of tissue homeostasis, and stimulation of the expression of liver-specific functions (Arriazu et al., 2014) by, among other things, becoming a reservoir of cytokines, growth factors and
30 bioactive peptides (McQuitty et al., 2020).

Recent advances in tissue engineering allow the use of ECMs as scaffolds that, when filled with cells, serve as a three-dimensional tissue model. One of the main challenges in obtaining an artificial liver is maintaining the native functional characteristics of this organ in vitro. There are several methods for obtaining functional artificial organs with applications in tissue engineering and regenerative medicine. One of them is 3D bioprinting. Bioprinting makes it possible to automatically embed living cells in material of synthetic origin, as well as natural polymers such as collagen, gelatin, alginate, hyaluronic acid, fibrin and polyethylene glycol, and create a three-dimensional tissue construct. Decellularized ECM (dECM) is a promising component of bioink. Upon solubilization, dECM can be transformed into a hydrogel - a highly absorbent substance that cross-links at physiological temperatures due to the self-organization mechanisms of collagen (Saldin et al., 2017). The hydrogel can be engineered to mimic the natural extracellular matrix, have a similar degree of elasticity to natural tissue, and create an environment that promotes cell growth, differentiation and proper function due to the presence of tissue-specific biochemical signatures of the extracellular matrix. The hydrogel is obtained by enzymatic digestion of the extracellular matrix in an acidic environment. The digestion solution is usually kept under constant agitation for a specified period of time at room temperature. Enzymes such as pepsin, trypsin or a combination of these are used. Cells are then embedded in the construct to produce a bioink that mimics functional tissue. The development of ECM-derived hydrogels opens the possibility of their commercial use for cell encapsulation, as an in vivo injection material or as a bioink for 3D bioprinting applications.

The dECM is obtained by decellularization, which involves the removal of cells and other immunogenic material, essentially leaving only the non-immunogenic structural materials (collagen, elastin, laminin, etc.) of the extracellular matrix. The inflammation that can occur due to other immunogenic factors can have a negative impact on the success of graft acceptance, hence the method of decellularization should ensure that cells are removed from the tissue as much as possible.

Different tissues or organ types require different decellularization protocols, depending on their architecture and ECM composition. The desired decellularization process begins with cell lysis and separation of the nuclear and cytoplasmic components from the ECM using physical, enzymatic, chemical or a combination of these methods. Typical decellularization methods include a series of washing of tissues in chemical solutions containing various substances to aid the process. To date, the most commonly used have been non-ionic detergents (Triton X-100), anionic detergents (sodium dodecyl sulfate - SDS, sodium deoxycholate - SD), enzymatic agents

(trypsin, DNase I) or chelating agents such as ethylenediaminetetraacetic acid (EDTA). Such a solution may contain one or more compatible surfactants (e.g., anionic surfactants such as SDS and non-ionic surfactants such as Triton X-100). Sodium dodecyl sulfate and Triton X-100, are the most commonly used chemicals in liver decellularization procedures (Pan et al., 2014; Mazza et al., 2015). Surfactants act by disorganizing the phospholipid cell membrane, thereby causing cell lysis (Parsi, 2015). Generally, organ perfusion or mixing of organ fragments in a solution that allows detergents and enzymes to penetrate deep into the tissue is used. The substrate for cell disruption is an aqueous solution of at least one detergent, alternatively in buffer (e.g., PBS - phosphate-buffered saline) to achieve osmotic compatibility with the cells. Non-ionic detergents are preferred because of their gentler effects on tissue structure. They are considered relatively nondenaturing because they affect lipid-lipid and lipid-protein interactions rather than protein-protein interactions (Seddon et al., 2004). Ionic detergents have a greater negative effect on tissue structure and are also more difficult to remove with typical neutral solutions. Enzymes such as proteases, esterases or nucleases are also used. Some procedures use a bioburden-reducing agent including penicillin, streptomycin, peracetic acid, ethanol or a combination of these. After decellularization, all cellular elements and residual chemicals must be thoroughly rinsed from the scaffold with a neutral solution, such as PBS.

Decellularized tissue can be treated with stabilizing compounds, such as glutaraldehyde, to cross-link and stabilize the components of the decellularized tissue. The decellularized material should be subjected to sterilization, such as radiation.

To date, the use of Triton X-100 and SDS in liver decellularization has been described several times. One of the most commonly used detergents for this purpose, Triton X-100, has been added to the European Chemicals Agency's (ECHA) Candidate List as a substance of concern due to its degradation into endocrine disruptors (ECHA, 2022).

There are several protocols describing liver decellularization including US patent application **US20230016722A1** where tissue is subjected to several cycles of freezing and thawing, and rinsed with PBS solution. Then, the tissue is incubated with a detergent such as SDS, SDC or Triton X-100. The tissue can then be treated with a DNase solution with streptomycin. The above document does not disclose the use of Tergitol for liver decellularization.

Another US patent, **US11648335B2**, discloses a method for decellularizing liver tissue, comprising perfusing the tissue with an ionic or non-ionic detergent, followed by washing with a

buffer. In addition, treatment of the tissue with a solution of DNase and betadine is provided. The patent does not disclose the use of Tergitol for decellularization.

EP3027235A1 is a European patent application disclosing a method of decellularizing liver tissue by treating the tissue with a solution that is hyper- or hypotonic relative to the interior of the cells.

- 5 The solution also contains an emulsifier or surfactant. In addition, the solution may contain trypsin, a protease inhibitor such as EDTA, and an antibiotic. The document does not disclose the use of Tergitol.

A study by Hussein et al, (2024) is a review comparing 25 liver decellularization methods of various origins, but none of the cited articles mention the use of Tergitol.

- 10 To date, Tergitol has been used in the successful decellularization of porcine aortic valves (Faggioli et al., 2022), porcine pulmonary valves (Tondato et al., 2023), porcine pericardium (Todesco et al., 2022) and small intestinal submucosa (Casarin et al., 2022).

EP3095469A1 describes the use of Tergitol for decellularization, but does not disclose a method regarding decellularization of liver tissue.

- 15 Patent **WO2011057174A1**, on the other hand, discloses that an exemplary detergent used for decellularization of liver tissue may be Tergitol. The application does not disclose the use of Tergitol in a step gradient concentration.

Tergitol was also mentioned in an application **EP3328992**. It was used for liver decellularization along with other optional decellularization reagents. For example, enzymes including trypsin with
20 EDTA were claimed. Samples were washed with PBS, again, however, the method of producing the decellularized scaffold does not reveal the use of Tergitol in a step gradient concentration for decellularization of porcine liver.

- Also revealed is a method for obtaining extracellular matrix isolated from amphibians -
WO2023154873A3. The process involves washing a tissue sample with a buffer solution followed
25 by detergent and incubating the sample with at least one protease and nuclease to obtain the isolated ECM. An example of a buffer solution can be PBS. The detergent used could be Tergitol. Streptomycin is listed as an example antibiotic, and sample enzymes may be trypsin and DNase. The above document does not disclose the method of decellularization of liver tissue.

- On the other hand, in international patent application **WO2023235832A1**, describing a bioink
30 composition for 3D printing, a hyaluronic acid derivative was used as a cleavable polymer

precursor, and methacrylated gelatin was given as a non-cleavable polymer precursor. LAP was used as an exemplary photoinitiator. The bioink does not contain decellularized dECM, and no sonicated bioink is used.

5 In the next described solution **CN115634316B**, acetic acid and pepsin are added to powdered dECM obtained from dental follicle tissue, followed by NaOH. The dECM thus produced and dissolved is mixed with GelMA.

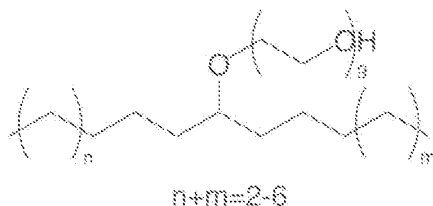
EP4003439A1 is a European patent application describing a method for making bioink.

10 The first step in the preparation of the bioink is to obtain a solution of dECM. The procedure involves dissolving the dECM powder in pepsin and hydrochloric acid. The solution is neutralized with NaOH and PBS. Then, mixing the dECM powder and solution produces a paste, which is mixed at a later stage with GelMA, HAMA, LAP, glycerol, growth factors, vitamins, cells and islets of Langerhans. The solution provides for dECM sonication. With respect to the described application, there are differences in the proportions of bioink components, one of which is glycerol, which is not present in the tested composition. HepG2 cells were also not used.

15 **EP4175688A1** is another European patent application, disclosing a composition of biocompatible hydrogels that can contain dECM, GelMA, HAMA and LAP. The document does not disclose the method of decellularization and instructions on how to use the obtained dECM to produce a bioink.

20 The **CN109054496A** Chinese patent application, on the other hand, reveals the preparation of a bioink, consisting of a cell-free matrix hydrogel, a crosslinking curing agent and bioactive molecules. Listed as crosslinking curing agents are PEG polyether ester, collagen, gelatin, chitosan, agar, hyaluronic acid or products of the above substances modified with unsaturated double bonds. The method according to the invention does not produce a sonicated hydrogel.

25 The subject matter of the invention is a method of enzyme-detergent decellularization of liver, particularly porcine liver tissue, using a less toxic, biodegradable nonionic detergent - Tergitol 15-S-9 with a molecular weight of 596 g/mol in a stepwise concentration gradient.



Tergitol 15-S-9 structural formula.

The purpose of the invention is to develop a composition for a biomaterial containing dECM obtained by decellularizing animal liver and enriching it with other factors that improve its functional characteristics. The goal was also to develop a way to produce a biomaterial with unique properties in such a way that it meets the following conditions:

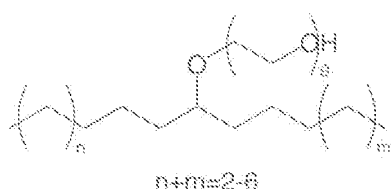
- ensuring optimal rheological properties of the developed liver bioinks: viscosity in the range of 130-1700 mPa-s, storage modulus (G') 2-130 Pa and loss modulus (G'') 2-15 Pa
- ensuring optimal mechanical parameters of objects printed with the developed bioinks: mechanical strength in the range of 50-650 kPa, elasticity 100-200 kPa
- ensuring adequate fiber integrity, allowing continuous printing from 2-3 ml of material while maintaining printing parameters safe for the cellular material: temperature 5-35°C, pressure 5-40 kPa, printing speed 5-45 mm/s.

The invention relates to a method for decellularization of animal tissue, comprising the steps of:

- a) mechanical homogenization of tissue of animal origin,
- b) incubating the homogenized tissue from step a) in a solution of trypsin with a concentration in the range of 0.005 - 0.5% and EDTA at a concentration of 0.002 - 0.2% at 37°C for 1-3 h,
- c) removing the trypsin and EDTA solution and subjecting the filtered tissue to incubation in a PBS buffer solution at a pH of 7.2-7.6 containing a non-ionic detergent at a concentration of 4 - 2% and NH₄OH at a concentration of 0.2 - 0.08%, wherein the incubation is carried out at a temperature below room temperature for 24-48 h,

- d) rinsing the tissue with PBS buffer solution containing an antimicrobial agent, the rinsing is carried out at a temperature below room temperature, for at least 72h,
- e) incubation in deoxyribonuclease solution of 0.001 to 0.003% (w/v) for 8 hours at 37°C,
- f) rinsing the decellularized tissues obtained in steps a)-e), with a PBS buffer solution containing an antimicrobial agent, the rinsing taking place at a temperature below room temperature, for at least 72 h,
- 5 in which

- the non-ionic detergent is a compound with a molecular weight of 596 g/mol of formula:



- 10 - step c) is followed by additional steps:
- c1) incubation of the tissue in a PBS buffer solution with a pH in the range of 7.2 - 7.6 containing non-ionic detergent at a concentration of 1.5 - 0.5% and NH_4OH at a concentration of 0.2 - 0.08%, wherein the incubation is carried out at a temperature below room temperature, for 36-48h, and
- 15 c2) incubation of the tissue in a PBS buffer solution with a pH in the range of 7.2 - 7.6 containing non-ionic detergent at a concentration of 0.75 - 0.25% and NH_4OH at a concentration of 0.2 - 0.08%, wherein the incubation is carried out at a temperature below room temperature, for 36-48h.

Advantageously, the tissue of animal origin is liver tissue.

- 20 Advantageously, in step c), c1) and c2) during incubation, a PBS buffer solution containing non-ionic detergent and NH_4OH is exchanged three times.

Advantageously, steps c), c1) and c2) are carried out at 4°C.

Advantageously, the antimicrobial agent is streptomycin at a concentration of 0.01% (w/v).

Advantageously, during the rinsing in step d), the PBS buffer solution containing the antimicrobial agent is exchanged eight times.

Advantageously, during rinsing in step f), the PBS buffer solution containing the antimicrobial agent is exchanged five times.

In a second aspect, the invention relates to a bioink containing decellularized tissue of animal origin, at a concentration of 0.5-10%(w/v), preferably 5%(w/v).

Advantageously, the bioink comprises HAMA solution of 0.5-2%(w/v), GELMA solution of 5-20% (w/v) and LAP solution of 0.15- 0.5%(w/v).

10

The invention is shown in the Drawing, where:

Fig. 1 Showing the appearance of the liver before and after decellularization.

Fig. 2 Showing electrophoretic separation of DNA in a 1% agarose gel. At number 12 is DNA isolated from ECM obtained by decellularization in a Tergitol gradient.

15 Fig. 3 Cells after exposure to bioink extracts prepared in ten different variants. K- (negative control), K+ (positive control).

Fig. 4 Temperature dependence of modules for liver bioink.

Fig. 5 Results of viscosity testing of liver bioink material

Fig. 6 Dependence of conservation modulus and loss modulus on shear stress of liver bioink

20 Fig. 7 Schematic of the gcode file - *template.gcode*

Fig. 8 Schematic of the fiber collapse test platform

Fig. 9 Percentage of fiber diffusion rate *Dfr* - fiber fusion test

Fig. 10 Printability *Pr* - fiber fusion test

Fig. 11 Photo of the constructs in the fiber collapse test

Fig. 12 Fiber collapse ratio Cf - fiber collapse test

Embodiments

Embodiment 1 decellularized hepatic ECM

Materials

- 5 Pork livers were obtained from a local slaughterhouse and stored at -20°C. Organs were previously frozen, thawed, rinsed with betadine solution (2% v/v) and then with streptomycin solution (0.1mg*ml⁻¹) dissolved in PBS. After rinsing, the organs were fragmented using scissors while macroscopic removal of visible fat, vessels and other contaminants.

Quantitative results are presented as means ± standard deviation. All analyses were performed in
10 triplicate.

Tergitol™ 15-S-9 with a molecular weight of 596 g/mol, CAS: 84133-50-6

Method of decellularization of liver tissues

Activities specified as being conducted at a temperature below room temperature are preferably conducted at a temperature of 4 °C.

- 15 Fragmented organs were mechanically fragmented using a jug blender. The fragmented and mixed material was weighed and placed in bottles and incubated in a trypsin-EDTA solution (0.05% (w/v) trypsin (Gibco, USA) and 0.02% (w/v) EDTA (Sigma Aldrich, USA)) together with an antimicrobial agent, preferably at a concentration of 0.01% (w/v), for 1.5 h at 37°C. After this time, the material from the bottles was transferred to a stainless steel sieve and filtered.
- 20 The unfiltered material was poured back into the bottles. The minced liver tissue was flooded with a 3% solution of non-ionic detergent - Tergitol 15.S.9 (Sigma-Aldrich, USA) in a PBS solution with pH 7.4 and 0.1% NH₄OH. The bottles with the material were placed in an incubator with a stirring function at 150 rpm at 4°C for 24-48 h, preferably for 17-19 h. After this time, the decellularization solution was changed 1-3 times a day for 12 days. After three exchanges of the 3% Tergitol
- 25 solution, the detergent concentration was changed to 1% Tergitol + 0.1% NH₄OH in PBS. After four exchanges of the 1% solution, the next four exchanges were performed using 0.5% + 0.1% NH₄OH in PBS. At each exchange of the decellularization solution, the material was fragmented with scissors. Tissue purification was performed using a neutral washing buffer – PBS without Ca²⁺ and Mg²⁺ with the addition of streptomycin (0.1mg*ml⁻¹). After nine changes of the washing solution,

the decellularized tissue was ground using a blender. Then, washing was carried out with a solution of nuclease-DNase I dissolved in PBS with the addition of Ca²⁺ and Mg²⁺ for 8 hours with the function of mixing at a speed of 150 rpm at the temperature optimal for DNase efficiency in order to remove dispersed DNA fragments after the detergent treatment. After 8 hours of incubation of the material in the DNase I solution, the material was filtered on a stainless steel strainer and placed back in the bottle. Then the tissues were washed again with PBS with the addition of streptomycin (0.1mg*ml⁻¹) for another 2 days at three-hour intervals three times a day. A single decellularization process lasts 12 days (the procedure diagram is provided in Table 1).

10

15 Table. 1 Table showing the scheme of the decellularization procedure (12 days)

<u>Day 1</u>	<u>Day 2</u>	<u>Day 3</u>	<u>Day 4</u>	<u>Day 5</u>	<u>Day 6</u>	<u>Day 7</u>
-Material processing -Incubation with trypsin (0.05%) and EDTA (0.02%) in PBS (37°C, 1.5h)	-3x rinsing in a solution of Tergitol 3% in PBS (4°C)	-3x rinsing in a solution of Tergitol 1% in PBS (4°C)	-1x rinsing in a solution of Tergitol 1% in PBS (4°C)	-2x rinse in Tergitol 0.5% solution in PBS (4°C) -1x rinse in PBS with streptomycin (4°C)	-1x rinse in PBS with streptomycin (4°C)	-1x rinse in PBS with streptomycin (4°C)

-Incubation in a solution of Tergitol 3% in PBS (4°C)			-2x rinsing in 0.5% Tergitol solution in PBS (4°C)			
Day 8	Day 9	Day 10	Day 11	Day 12		
-3x rinsing in PBS with streptomycin (4°C)	-3x rinsing in PBS with streptomycin (4°C)	- Incubation with DNAase (10mg/l) in PBS (37°C, 8h). -1x rinse in PBS + strep. (4°C)	-3x rinse in PBS + strep. (4°C)	-1x rinse in PBS + strep. (4°C) -Pooling -Precipitation of material in liquid nitrogen (preparation for lyophilization)		

After decellularization, the material was prepared for lyophilization. A small amount of tissue was ground on the walls of a mortar cooled with liquid nitrogen. dECM was broken into small fragments and stored at -80°C until lyophilization. The material was then lyophilized. The freeze-dried material obtained was ground in a cryogenic mill to obtain a powder with a particle diameter of less than 100µm. The material was subjected to radiation sterilization (25 kGy).

5

DNA from powdered dECM was isolated using the Qiaamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Briefly, samples were digested using proteinase K

and digestion buffer. Samples were applied to columns for DNA uptake. After washing steps, DNA was precipitated and eluted. DNA concentration was measured spectrophotometrically (Nanodrop, Thermo Scientific, USA). The average DNA content per mg of tissue was determined. Electrophoresis on a 1% agarose gel was performed to separate and determine the size of DNA fragments in dECM.

The results of the experiments were the basis for the invention. It was shown that after the decellularization process, the color of the tissue changed from the initial red-pink color of native liver tissue to white. At the same time, the removal of the cellular component was detected based on the DNA content. The DNA concentration in the powdered tissue decellularized using Tergitol at three different concentrations - the step gradient was $13.73 \text{ ng/mg} \pm 2.76$. Which indicates the removal of more than 99% of the DNA of the native tissue, where the DNA content was estimated at $4646.2 \pm 339.04 \text{ ng/mg}$. DNA analysis by agarose gel electrophoresis (Fig. 3) confirmed the removal of DNA to below detectable levels. Crapo et al. proposed minimum criteria for DNA residues that the authors believe would be sufficient to confirm the effectiveness of the decellularization process. The proposed criteria suggest that cell-free dECM should contain less than 50 ng dsDNA per mg dECM (dry weight) with fragments smaller than 200 pz (Crapo et al., 2011). According to the results, the reproducibility of the technique can be observed as it respects the established limits of DNA detection.

Fat measurement

Soxhlet extraction was used to measure free fat content. 0.5g of liver powder (dry weight) was placed in cellulose thimbles, covered with cotton wool and weighed. Lipids were extracted with 70 ml of n-hexane for 4 hours, with a cycle time of about 10 minutes. The weight content of the extracted lipids relative to the weight of the test sample was determined.

Results

The fat content of the native organ averaged $18.03\% \pm 7.78$ and showed large fluctuations. The method described above reduced the percentage of fat to $1.46\% \pm 1.16$. An extracellular matrix with a lower fat content improves the properties of the final product, i.e. increases viscosity and improves printability.

Measurement of the content of sulfated glycosaminoglycans (sGAGs)

The content of sulfated glycosaminoglycans (sGAGs) in native cryo-grinded and cell-free tissues was quantified using the Blyscan GAG Assay Kit (Biocolor, UK). Briefly, 20-50 mg of dry tissue was weighed and placed in a 1.5-ml tube containing 1 ml of papain solution and incubated at 65°C for 16 hours with occasional stirring. Portions of each sample were mixed with 1,9-
5 dimethylmethylene blue dye and reagents from the kit. Absorbance at 565 nm was measured with a microplate reader and compared with standards made from bovine chondroitin sulfate to determine absolute sGAG content.

Results

Glycosaminoglycans were present in all native tissue samples, but their content decreased
10 dramatically after decellularization. In liver, the sGAG content decreased from 7.91 ug/mg $\pm$ 1.07 to a level of 0.47 ug/mg $\pm$ 0.12. Decellularization or matrisome enrichment methods lead to significant losses in soluble molecules associated with the extracellular matrix in various organs (Krasny et al., 2016). The effect of sGAG deficiency on the durability of implanted decellularized scaffolds requires further study.

15 Measurement of collagen content

Quantitative determination of collagen was carried out using a commercially available fluorescent assay (Collagen Assay Kit, Sigma-Aldrich, USA). Samples of powdered matrix dissolved in 0.5M acetic acid were applied to a 96-well plate. The acid-soluble collagen is then enzymatically digested into peptides. Peptides containing glycine at the N-terminus then react
20 with the dye to form a fluorescent complex. The fluorescence intensity of this product, measured at $\lambda_{ex}/\lambda_{em}$ = 375/465 nm, is directly proportional to the concentration of collagen in the sample.

Results

The decellularization method has less effect on collagen content, and the preservation of this key component in dECM was confirmed. An approximately 10-fold enrichment of the final material
25 was observed in collagen compared to native tissue (654.3 ug/mg $\pm$ 74 vs. 58.6 $\pm$ 38.6).

Cell viability studies in contact with dECM and bioink produced on the basis of this component

The MTT test was used to assess cell viability, which is based on the reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide - MTT) to purple
30 formazan crystals by metabolically active cells. The cell line L-929 (ATCC®, cat no: CCL-1™) was

used, which was cultured in DMEM with 10% FBS and 1% antibiotics. Ten variants of fresh bioinks were placed in inserts in the culture medium according to the principle of 0.1 g of cross-linked bioink per 1 ml of DMEM. The extracts were incubated for 24 hours in complete culture medium at 37°C. Extracts from under the inserts were applied to the cells. The plates were incubated at 24-, 48-, 72h intervals. Then the MTT solution was removed and 100 µl of DMSO was added. The plates were shaken until the crystals were completely dissolved. Absorbance was measured at 570 nm and 650 nm. The positive control was a culture incubated with 0.1% Triton X-100.

Results

Cell viability is greater than 70% for all bioink extracts and at all time points (24, 48 and 72h) relative to L-929 cells, which means that bioinks based on the obtained dECM allow maintaining high cell viability. Figure 3 shows L-929 line cells after exposure to biomaterial extracts.

Detergent content

The residual detergent content – Tergitol was determined using a UHPLC/HPLC Ultimate 3000RS system (Thermo Scientific) with a Corona CAD corona discharge detector. The separation was achieved on an Acclaim C8 120A column (150 x 4.6 mm; 3 µm) (Dionex) at a column temperature of 30°C. Mobile phase A was 0.1% HCOOH and mobile phase B was methanol.

Results

The concentration of non-ionic detergent in the final products was low, below 5 ng/mg. This was achieved by repeated washing of the tissue in a neutral PBS solution. The residual detergent content is important when such a material (dECM) is to be used in the bioprinting process with living cells. Although Tergitol is considered to be less toxic, it is still important to minimize the detergent residue in the final product as much as possible.

The efficiency of the decellularization method is $0.22\% \pm 0.096$, which is due to the highly cellular nature of liver tissue. After the process is complete, the decellularized tissue segment can reduce its weight by more than 99%.

Embodiment 2 BIOINK

Preparation of bioink based on decellularized hepatic ECM

Preparation of 8 ml of hepatic dECM hydrogel 6.5% (w/v) - unsonicated.

195.2 mg of pepsin was transferred to 30 ml of 0.1 M HCl solution. The solution was stirred on a magnetic stirrer at 500 rpm until the enzyme was dissolved (approximately 20 min) at room temperature. Then 1951.2 mg of powdered decellularized liver tissue was transferred to the pepsin solution. The solution was left on a magnetic stirrer for 48 h at 500 rpm, in a metal bath at 23°C. After 48 h of continuous stirring, the resulting hydrogel was neutralized. For this purpose, 3.33 ml of PBSx10, 0.7 ml of 1xPBS and 0.6 ml of 5 M NaOH were added to the hydrogel. The pH of the solution was stabilized to 7.4 with NaOH. The solution after neutralization was used to create liver bioink and sealing bioink.

Preparation of dECM hydrogel 5% (w/v)

500.4 mg of hepatic dECM was weighed into a sterile falcon. Then 10 ml of PBSx1 was added to the weighed portion. The falcon with the suspension was placed in a thermoblock at 23°C and 500 rpm. Then the falcon with the hydrogel was placed in an ice bath and sonicated for 5 min at about 22°C and 60% amplitude. Before use, the hydrogel was heated in a thermoblock at 37°C with 800 rpm stirring to liquefy and deaerate.

Preparation of 15 ml of GELMA 20% (w/v) + LAP 0.38% (w/v) solution

78.1 mg of LAP was weighed into 20.0 ml of PBSx1. The solution was stirred at 500 rpm at 50°C for about 15 min until the photoinitiator dissolved. Then 4.0021 g of GELMA lyophilisate was added to the solution. The solution was left in a water bath (50°C) until completely dissolved, and the stirring speed was adjusted to the degree of foaming (100-200 rpm). The prepared solution was filtered using a syringe filter with a pore diameter of 0.22 µm. The filtered solution was stored in a refrigerator, protected from light.

Preparation of 10.0 ml of HAMA 2% (w/v) solution

A portion of HAMA lyophilisate in the amount of 201.8 mg was weighed into a 10.0 ml PBSx1 falcon. The solution was shaken in a thermoblock at 1000 rpm and 8°C for 1 h. After complete dissolution, the pH was stabilized with NaOH and HCl to 7.35. The solution was then filtered using syringe filters (0.22 µm). The filtered solution was stored in a refrigerator.

Preparation of 8 ml of GELMA 20% (w/v) + LAP 0.6% (w/v) solution

60.7 mg of LAP was added to 10 ml of PBSx1. The solution was transferred to a thermoblock and stirred at 500 rpm and 50°C for about 15 min (until the photoinitiator dissolved). Next, 2.0016 g of GELMA lyophilisate was added to the solution. The whole was left in the thermoblock (50°C) until

completely dissolved, and the stirring speed was selected from the degree of foaming (300-500 rpm). After completely dissolving GELMA, the solution was filtered using a syringe filter (0.22 µm). The filtered solution was stored in a refrigerator, protected from light.

Preparation of 10 ml of HAMA 2% (w/v) + LAP 0.5% (w/v) solution

- 5 50.6 mg of LAP was added to 10.0 ml of PBSx1. The solution was transferred to a thermoblock and mixed at 500 rpm and 50°C for 15 min (until the photoinitiator dissolved). Then 201.8 mg of HAMA lyophilisate was added to the solution and shaken in a thermoblock at 1000 rpm and 8°C for 1 h. After complete dissolution, the pH was adjusted to 7.3 using NaOH and HCl. The solution was then filtered using syringe filters (0.22 µm). The filtered solution was stored in a refrigerator.

10 **Preparation of liver bioink with added cells**

- 2 ml of non-sonicated hydrogel was taken with a syringe with an injection needle. A luer-lock adapter and a syringe without a plunger were screwed onto the syringe with the hydrogel, to which 2.5 ml of HAMA solution and 5.0 ml of GELMA + LAP 0.38% solution were administered. The whole was mixed between syringes and transferred to a thermoblock set at 12°C. The procedure was repeated the next day. Then, 150-180 µl of HepG2 cell suspension (2-12 million cells/ml of bioink) were added to the above portion of bioink and mixed between syringes. Such bioink can be transferred to a sterile cartridge and placed in the printer head.

The cells were counted using an automatic cell counter. Then, cells in the amount of 2-12 million cells/ml of bioink were suspended in the culture medium, topping up to 0.2 mL.

20 **Preparation of liver sealing bioink**

- 4.5 ml of liver hydrogel was taken into the syringe, to which 2.7 ml of HAMA 2% (w/v) + LAP 0.5% (w/v) solution and 1.8 ml of GELMA 20% (w/v) + LAP 0.6% solution were then added. The whole was mixed to obtain a homogeneous mixture of ingredients. Until use, the bioink was stored at room temperature without access to light.
- 25 Preferably, the bioink composition contains decellularized tissue of animal origin at a concentration of 0.5-10% (w/v), preferably 5% (w/v) and additionally contains HAMA 0.5-2% (w/v) solution, GELMA 5-20% (w/v) solution and LAP 0.15-0.5% (w/v).

Printability

Printability tests were conducted using a **bioprinter**. Printing parameters using liver biomaterial are included in **Table 2**. The applied parameters allowed obtaining a uniform, compact fiber. The assessment of the possibility of using a given material for printing was carried out according to a specially developed procedure based on a literature review (Ahasan Habib, Venkatachalem Sathish, Sanku Mallik, Bashir Khoda, 3D Printability of Alginate-Carboxymethyl Cellulose Hydrogel Materials (Basel) 2018 Mar 20;11(3):454. doi: 10.3390/ma11030454). As part of the work carried out, a number of tests were performed, in which the following parameters were assessed: material spreading rate - percentage of diffusion rate, printability, collapse factor of the printed fiber, and fiber continuity and smoothness.

Table 2. Printing parameters in the printability tests of pre-crosslinked materials

Variant	Pressure range [kPa]	Temperature [°C]	Speed [mm/s]
Liver bioink	5-45	5-35	10-35

The presented technology includes extrusion printing requiring the appropriate viscosity of the material in order to obtain a uniform and coherent filament to maintain the print resolution. The printing temperature can vary from 5 °C to 37 °C depending on the material used. The printing pressure in the case of extrusion printing is in the range of 5 to 200 kPa. The printing speed is maintained in the range of 1 to 50 mm/s. During printing, needles with a diameter of 50 to 900 µm are used. The designed 3D model for printing can be characterized by any geometry, adapted to the needs of the experiment, as well as a degree of filling in the range of 5% - 100%, thus obtaining openwork prints.

Fiber fusion test

For the fiber fusion test, an appropriate g-code was prepared: *template.gcode*, which assumes printing two layers one after the other using the tested material without using external lamp cross-linking between them. The prints were made using a **bioprinter**. The print follows a pattern in a 0°–90° arrangement, which reflects the 2D effect and increases the distance between the fibers. The distance between the fibers was in the range of 1–5 mm, with an increment of 1 mm. The template pattern is shown in Fig. 7. The printing speed, needle diameter and extrusion width used in the

test are **10 mm/s, 25G (0.250 mm) and 0.3 mm**, respectively. During the test, the material was dosed in the appropriate pressure and temperature range, adapted to the tested material. The prints were cross-linked using an external UV-Vis lamp (Polbionica, Warsaw) using the following parameters: **wavelength 405 nm, time 30 s, power 28.5 mW/cm²**. After printing, microscopic photos were taken. The photos were analyzed using AxioVision software. Based on the results, two parameters described by the following equations were determined, i.e. the percentage of the diffusion rate Df_r (material spreading rate) and printability P_r . The pore diffusion rate without material spreading is 0 (i.e. $A_t = A_a$) and for an ideal reproduction of the model, printability is 1.

$$Df_r = \frac{A_t - A_a}{A_t} \cdot 100\%$$

10

$$P_r = \frac{L^2}{16 \cdot A_a}$$

A_t – theoretical pore area,

A_a – actual pore area,

L – actual pore circumference.

15 **Fiber collapse test**

The collapse at mid-span of the suspended fiber was analyzed to determine the degree of material collapse. In order to conduct the experiment, a special platform was designed consisting of seven pillars spaced apart by known distances of 1, 2, 3, 4, 5, 6 mm. The dimensions of five pillars placed inside the construct are $2 \times 10 \times 6 \text{ mm}^3$, and the dimensions of two edge pillars are $5 \times 10 \times 6 \text{ mm}^3$.

20 The platform design is shown in Fig. 8. A single fiber of the tested material was deposited on the platform according to the g-code: **MR_test1.gcode**, after which a photo of the printout was immediately taken. The photos were analyzed using AxioVision software. During the test, the temperature and pressure conditions were adjusted depending on the tested material. The printout was made at a speed of **10 mm/s** using a **25 G (0.250 mm)** needle. The collapse area

25 factor Cf , which is the percentage of the actual area after **collapse** of the suspended fiber in relation to the theoretical area.

$$C_f = \frac{A_a^c}{A_t^c} \cdot 100\%$$

A_a^c – theoretical area under the curve,

A_t^c – actual area under the curve.

Results:

5 Fiber fusion test

- For each material variant, **3 attempts of the fiber fusion test** were performed. Then, microscopic photos of the obtained constructs were taken. Based on the measurements, the percentage of the fiber diffusion rate *Dfr* and printability *Pr* were calculated - the results are presented in the graphs in **Figures 9 and 10**. The liver bioink is characterized by a continuous and compact fiber, which allowed for obtaining prints with good resolution,
- Using the tested materials, it was not possible to print pores of size 1x1,
- For each material variant, printability above 0.8 was obtained, as well as a percentage of the diffusion rate below 75% (except for the pore of size 1x1),
- The diffusion rate decreases with increasing pore size,
- Printability for each pore size, except for 1x1 pores, is 0.88-0.98 for all materials.

Fiber collapse test

For each material variant, 3 fiber **collapse** tests were performed. Then, a photo of the obtained fiber printed on the platform was taken (**Fig. 11**). Based on the obtained measurements, the fiber collapse factor *Cf* was calculated, the results are shown in **Fig. 12**.

- 20 The fiber collapse factor shows a value above 80%.

Rheology:

In order to conduct the rheological study, 4.5 ml of liver bioink was prepared and then placed in a thermoblock at 35°C (at least 15 min before starting the measurements).

Rheological measurements were performed using the MCR 72 apparatus (Anton Paar, Poland).

- 25 The following relations were used to determine the desired rheological parameters:

- Dependence of the complex modulus on the temperature gradient in the range of 40-0°C – the gelation point of the material.

- Dependence of the complex modulus on the shear stress and strain.
- Variation of the viscosity value during the measurement at a constant temperature of 20°C at a shear rate of 100/s. Detailed measurement conditions are presented in Table 3:

Table 3 *Measurement parameters*

Parameter	Gelation point	Complex modulus	Viscosity
Temperature [°C]:	40-0	20	20
Shear rate [1/s]	-	-	100
Frequency [Hz]	1	0,01-10	-
Amplitude [%]	30	10	-
Spindle [-]	CP50		
GAP [mm]	0,112		
Sample volume [ml]	0,49		

5 Gelation point

Fig. 4 and 5 show the dependence of storage and loss modulus in the temperature gradient for liver bioink.

The gelation temperature of liver bioink was determined. The results are shown in Fig. 4, and the average values of the gelation temperature of the materials are listed in Table 4.

10

Table 4 *Gelation temperature of liver bioink.*

repetition	I	II	III	IV
T [°C]	18.81	19.25	18.81	-

The liver bioink has a gelation temperature in the range of 18.81-19.25°C.

- Viscosity

Fig. 5 shows the results of the liver material viscosity test at a constant temperature of 20°C at a constant shear rate of 100/s.

5 Table 5 shows the average viscosity value of the tested material.

Table 5 *Average viscosity value of the tested material*

repetition	I	II	III
η [mPas]	225.23 (± 78.56)	235.69 (± 80.4)	178.56 (± 58.25)

During the test, the viscosity of all materials increases with time.

- Complex modulus

10 Fig. 6 shows the dependence of the storage and loss modulus on the shear stress for the tested materials.

All tested materials are characterized by a storage modulus G' with a higher value than the loss modulus G'' , which indicates the advantage of the elastic properties of the material over the viscous ones. In both cases of the tested materials, no point of sample deformation was identified under the influence of the applied shear stress.

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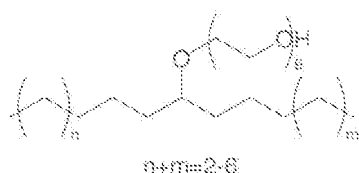
Claims

1. A method of animal-derived tissue decellularization, comprising the steps of:

- g) mechanical homogenization of tissue of animal origin,
- h) incubating the homogenized tissue from step a) in a solution of trypsin at a concentration of 0.005 - 0.5% and EDTA at a concentration of 0.002 - 0.2% at 37°C for 1-3 h,
- i) removing the trypsin and EDTA solution and subjecting the filtered tissue to incubation in a PBS buffer solution at a pH of 7.2-7.6 containing a non-ionic detergent at a concentration of 4 - 2% and NH_4OH at a concentration of 0.2 - 0.08%, wherein the incubation is carried out at a temperature below room temperature for 24-48 h,
- j) rinsing the tissue with a PBS buffer solution containing an antimicrobial agent, the rinsing is carried out at a temperature below room temperature, for at least 72 h,
- k) incubation in a deoxyribonuclease solution at a concentration of 0.001 to 0.003% (w/v) for 8 hours at 37°C,
- l) rinsing the decellularized tissues obtained in steps a)-e) with a PBS buffer solution containing an antimicrobial agent, the rinsing taking place at a temperature below room temperature, for at least 72 h,

characterized in that

- the non-ionic detergent is a compound with a molecular weight of 596 g/mol of the formula:



- step c) is followed by additional steps:

c1) incubation of the tissue in a PBS buffer solution with a pH in the range of 7.2-7.6 containing a non-ionic detergent at a concentration of 1.5 - 0.5% and NH_4OH at a concentration of 0.2 - 0.08%, wherein the incubation is carried out at a temperature below room temperature, for 36-48h, and

c2) incubation of the tissue in a PBS buffer solution with a pH in the range of 7.2-7.6 containing a non-ionic detergent at a concentration of 0.75 - 0.25% and NH_4OH at a concentration of 0.2 - 0.08%, wherein the incubation is carried out at a temperature below room temperature, for 36-48h.

2. The method according to claim 1, characterized in that the animal-derived tissue is liver tissue.
3. The method of claim 1, wherein in step c), c1) and c2) during incubation, the PBS buffer solution containing the non-ionic detergent and NH_4OH is exchanged three times.
4. The method of claim 1, wherein the incubation in steps c), c1) and c2) is carried out at a temperature of 4°C.
5. The method of claim 1, wherein the antimicrobial agent is streptomycin at a concentration of 0.01% (w/v).
6. The method of claim 1, wherein during rinsing in step d), the PBS buffer solution containing the antimicrobial agent is exchanged eight times.
7. The method of claim of claim 1, characterized in that during washing in step f) the PBS buffer solution containing the antimicrobial agent is exchanged five times.
8. Bioink comprising decellularized animal-derived tissue obtained by the method of claim 1 in a concentration of 0.5-10% (w/v), preferably 5% (w/v).
9. Bioink according to claim 8, characterized in that it further comprises a HAMA solution of 0.5-2% (w/v), a GELMA solution of 5-20% (w/v) and a LAP solution of 0.15-0.5% (w/v).