

Process for preparation methacrylated dECM

5 The subject of the invention is a process for preparation methacrylated dECM, the methacrylated dECM obtained by this process, and the use of the obtained methacrylated dECM.

There are known solutions in the state of the art for the methacrylation of compounds used subsequently in bioprinting processes, including the methacrylation of the
10 decellularized extracellular matrix (dECM) components.

Document WO2019122351A1 discloses a combination of ECM extracellular matrix and cellulose nanofibrils for application in 3D printing processes of human tissues. The disclosed solution is characterised by increased cell life and good printability. The ink used, called CELLINK®, according to the disclosure, consists of dispersed
15 nanofibrillated cellulose with the addition of a cross-linking component.

Document WO2021014359A1 describes a detergent-free method for preparing decellularised extracellular matrix in powder and liquid form. The invention also relates to a method for preparing a bioink containing dECM, used in 3D printing. According to one embodiment, the document discloses a method for preparing a bioink, comprising
20 the steps of:

- preparation of a paste containing 5-50% (w/v) dECM powder and 1-10% (w/v) dECM solution;
- incubation of the paste at a temperature of 7-10 °C for at least 24 hours;
- adding 1.46-7.32% (w/v) methacrylated gelatin, 0.15-1.10% (w/v) methacrylated
25 hyaluronic acid, 5-10% (w/v) glycerol and photoinitiator;
- gentle mixing.

Document WO2022180565A1 discloses a bioink containing silk fibroin for use in 3D bioprinting, and the production of models capable of supporting hematopoiesis and the production of platelets and blood cells. According to embodiments, the bioink contains
30 gelatin or a derivative thereof selected from the group consisting of, e.g. methacrylated gelatin.

5 Document WO2020081982A1 discloses bioinks that can be used for three-dimensional printing of structures. According to embodiments, the bioink composition may include gelatin methacrylate and collagen methacrylate.

Document IN201831038727A describes the composition of bioinks consisting of, e.g., silk fibroin, gelatin methacrylate, chitosan methacrylate, polyethylene glycol
10 dimethacrylate, and decellularised extracellular matrix.

Document US9623051B2 discloses methods for preparing decellularised extracellular matrix compositions. Such compositions may be used to coat media such as tissue culture media, osteogenic gels and medical devices.

Document US20210324336A1 describes a bioink used in the printing of three-
15 dimensional structures, comprising methacrylated hyaluronic acid and an extracellular matrix component, a biocompatible photoinitiator capable of cross-linking the methacrylated hyaluronic acid and an ECM component to form a hydrogel.

Document US20210138114A1 discloses an extrudable photocrosslinking hydrogel consisting of a biochemically modified extracellular matrix (ECM) with an embedded
20 electroconductive nanomaterial, a photoinitiator and a solvent. The hydrogel is used in the formation of tissues or organs printed *in situ* or *in vitro*. Biochemical modification of the ECM involves enriching the matrix with methacrylic functions, acrylic functions, or their mixtures. ECM is modified with methacrylic acid or methacrylic anhydride.

Document US20190106673A1 describes bioink compositions characterised by
25 elasticity similar to natural tissue. The disclosed composition includes thiolated hyaluronic acid, methacrylated collagen and water.

Document WO2021250186A1 discloses a biodegradable bioink with mechanical properties relevant to structural stability that has surface-modifiable functional groups. The described method includes the steps of: providing a (meth)acrylated, cross-linked
30 biocompatible hydrogel carrier derived from the extracellular matrix, and reacting the above-mentioned carrier with a functionalised peptide to obtain a cross-linked biocompatible hydrogel.

Publications by Ranjithkumar Ravichandran et al. ("Functionalised type-I collagen as a hydrogel building block for bio-orthogonal tissue engineering applications", 2016,

- 5 Journal of materials chemistry. B, (4), 2, 318-326. <http://dx.doi.org/10.1039/c5tb02035j>
and Mengxiang Zhu et al. („Gelatin methacryloyl and its hydrogels with an exceptional
degree of controllability and batch-to-batch consistency”, Scientific Reports, 2019.
<https://doi.org/10.1038/s41598-019-42186-x>) disclosed the methacrylation reaction of
collagen and gelatin, respectively, using methacrylic anhydride. Additionally, in the
10 second mentioned publication, methacrylation is carried out in a carbonate buffer
solution.

The aim of the present invention is to produce methacrylated dECM that can be used
in therapeutic applications, e.g. in regenerative medicine.

- The present invention relates to the dECM methacrylation process, including the
15 following steps:

- a) placing 1M carbonate buffer in the reaction vessel and heating to 50 °C,
- b) adding dECM to the carbonate buffer from step (a) to obtain a dECM solution
with a concentration of 4% (w/v),
- c) sterilizing the solution obtained in step (b) by irradiating with UV radiation for
20 15 minutes,
- d) adding methacrylic anhydride in the amount of 0.5 mL/1g dECM,
- e) after adding methacrylic anhydride, the reaction is carried out at 50 °C for 1
h,
- f) adding phosphate-buffered saline solution to obtain a 5-fold dilution of the
25 mixture from step (e),
- g) placing the solution from step (f) in the dialysis tube, and then placing the
dialysis tube in deionised water, and this step is carried out at a temperature
of 40 °C for no longer than 4 days,
- h) after completing step (g), the solution in the dialysis tube is transferred to
30 aluminium trays and then placed at -80 °C for at least 3 hours,
- i) freeze-drying the frozen solution from step (h) under the following conditions:
shelf temperature 10 °C, pressure 0.1 mbar, freeze-drying time 48 hours.

- Preferably, methacrylic anhydride is added in step (c) using a syringe pump over a
period not longer than 3 hours or in portions at 15-minute intervals, over a period not
35 longer than 3 hours.

- 5 Preferably the deionised water is replaced at least 11 times in step (g).

Preferably, after completing step (g), the solution in the dialysis tubes is concentrated on a rotary evaporator to 25-35% of the initial volume, with the volume of the concentrated solution being not less than 150 mL.

- 10 The invention also relates to methacrylated dECM obtained according to the above process,

wherein the degree of substitution with methacrylic groups is determined by ^1H NMR at 60°C using the equation:

$$DS_{NMR} = \frac{\int(5.8 - 5.6)}{\int TMSP} \div \frac{\int(1.05 - 0.8 \text{ ppm})}{\int TMSP} \cdot 9.96 \cdot 100\%$$

TMSP – internal standard, ranges from 75 to 135%

- 15 In a further aspect, the invention relates to the use of methacrylated dECM obtained by the process of the invention in a bioprinting process.

- 20 Preferably, the bioprinting temperature is in the range from 10 to 35 °C, the pressure is in the range of 5-50 kPa, the printing rate is from 1 to 30 mm/s, and the needle diameter is from 100 to 900 µm, cross-linking occurs at a wavelength from 365 nm to 405 nm, from 5 to 360 s, at a power of up to 1 to 100 mW/cm², with the bioink containing from 1 to 15% (w/v) of methacrylated dECM and from 0.1 to 0.5% (w/v) LAP.

- 25 The advantage of the invention is the improvement of mechanical properties and stability of the ECM scaffold derived from the pancreas. Pancreas-derived methacrylated dECM serves as an advanced 3D framework with tailored properties, making it particularly useful for tissue engineering and 3D culture applications. This biomaterial is used to produce bioinks for 3D bioprinting, offering a biocompatible and bioactive environment that supports tissue-specific cell adhesion, migration and regeneration. Its adaptability makes it a valuable tool for constructing scaffolds that closely mimic the native pancreatic tissue microenvironment, promoting cell proliferation and differentiation for therapeutic interventions. Thanks to the methacrylation process, dECM gains the ability to cross-link without the addition of other cross-linking agents such as GELMA, HAMA, etc., which allows for even better
- 30

- 5 mapping of the natural environment for cells and reduces the cytotoxicity of the entire bioink.

The invention is presented in the Drawing, wherein:

Fig. 1 shows a diagram of the reaction set used in the dECM methacrylation process

Fig. 2 shows a graph illustrating the degree of substitution measurement (DSNMR)

- 10 Fig. 3 shows the ECMMA preparation scheme

Fig. 4 shows the design of the model printed using a 3D printer

Fig. 5 shows a visualisation of the flake bioprinting process

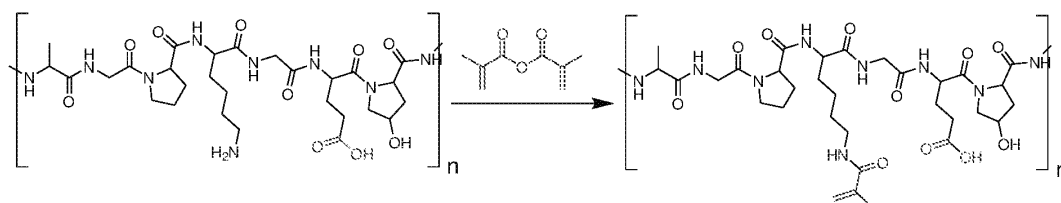
Fig. 6 shows solutions subjected to cross-linking

Fig. 7 shows the results of the cross-linking tests performed

- 15 Fig. 8 shows a diagram illustrating the functionality analysis of pancreatic islet beta cells in dECMMA bioink with 3 concentrations

Fig. 9 shows a graph illustrating cell viability depending on the concentration of the extract

- 20 ECM (extracellular matrix) is the extracellular matrix, which is a complex protein structure produced by cells. The extracellular matrix fills the space between cells. Its building blocks are most often proteins and related polysaccharides. The molecules that make up this structure can basically be divided into three main classes: collagens, proteoglycans and integrin-binding proteins, with different quantitative proportions that determine its properties. The main components of the extracellular matrix of animals
- 25 are collagens, which, depending on the type, differ in chain structure and the content of individual amino acids. In the general amino acid composition of collagen, 33% is glycine, 10% proline, 10% 4-hydroxyproline, as well as 3-hydroxyproline (<0.5%), 5-hydroxylysine (1%) and lysine. The presence of numerous free amino groups allows the functionalisation of this material through simple chemical transformations, such as
- 30 the methacrylation reaction, which involves replacing the amino groups with methacrylic anhydride, according to the reaction below.



Preparation of methacrylated dECM

1. A three-necked flask with a capacity of 5-5000 mL, equipped with a mixing element, was placed in a heating block on a magnetic stirrer. The diagram of the reaction system is shown in Fig. 1.

The selection of the reaction flask size depends on the starting solution volume, the amount of which cannot exceed 60% of the flask volume. An overview of the reaction vessel selection is presented in Table 1 below.

Table 1. Selection of the appropriate volume of the reaction vessel.

No.	Amount of CB solution [mL]	ECM mass [g]	Flask size [mL]
1	50	2	100
2	100	4	250
3	250	10	500

Then 1 M carbonate buffer (CB) in a volume ranging from 2.5 to 3000 mL was measured using a graduated cylinder, filtered through a 0.22 μm filter and poured into the flask. The excess carbonate buffer was transferred to a glass bottle and illuminated with an UV lamp for 15 minutes.

2. The necks of the flask were secured with a rubber septum, and a thermocouple (previously wiped with ethanol) was installed in one of the lateral necks,

5 so that the sensor was immersed in the solution but did not impede mixing. A vent needle was placed in the middle neck. The flask was protected from light with aluminium foil and heated to a set temperature of 50 °C.

3. Next, ECM was weighed on a scale, and its mass should range from 5 to 10,000 mg. In the next step, ECM was added in small portions to the flask with CB
10 buffer, resulting in a 4% solution (w/v). At the moment of adding the substrate to the flask, the septum with the vent needle was removed, and it was dispensed through the middle neck, taking care to prevent the substrate from settling on the grinding. If necessary, rinse with a small amount of demineralised water and reinstall the septum with the needle. The mixture was left to stir continuously at 1000-1200 rpm until the
15 substrate was completely dissolved.

4. After the substrate was completely dissolved, the pH of the resulting solution was measured, labelled as pH₁. The flask with the solution was then irradiated with UV light for 15 minutes to sterilise the reaction mixture. When measuring the pH of a solution with a pH meter, extreme care must be taken when introducing the electrode
20 into the reaction mixture.

5. Methacrylic anhydride was measured into a syringe equipped with a needle according to the following procedure:

- a) an empty syringe with a tube and a needle was weighed (m1);
- b) the target volume of methacrylic anhydride (MMA) was measured into the
25 syringe so that there were no air bubbles in the system and weighed again (m2);
- c) after adding MMA, the dosing system was weighed again to determine the exact amount of MMA added to the reaction mixture (m3).

The key parameter describing the efficiency of the methacrylation reaction is the degree of **DS** substitution, which is controlled by adjusting the amount of methacrylic
30 anhydride (MMA) used for the reaction. It should be noted that the substitution degree depends largely on the characteristics of the substrate itself, i.e. the content of collagen and fat in the starting ECM. The relationship between the ratio of MMA used per 1 g of ECM and the target substitution degree of the final product is shown below.

- 5 Table 2. Selecting the amount of MMA relative to DS.

No.	Amount of MMA/ 1 g ECM	DS
1	0.3 mL	20-30%
2	0,5 mL	40-60%
3	0,6 mL	75-85%
4	0.8 mL	95-100%

6. The syringe with MMA was wrapped in aluminium foil and placed in the syringe pump, and the needle with the connected tube was inserted into the septum in the lateral neck of the flask, so that the dosed anhydride did not flow down the wall, but directly into the reaction mixture. The appropriate flow rate was set and dosing was performed until the solution in the syringe was completely exhausted, controlling the mixing efficiency and viscosity of the reaction mixture from time to time. If necessary, increase the mixer speed. The rate of MMA instillation should be adjusted according to its volume, maintaining that the total MMA instillation time should be 3 h. For the variant without a syringe pump, MMA can be added in portions at 15-minute intervals, so that the total dosing time is also 3 hours.

7. After adding all the measured MMA dropwise, weigh the syringe again and calculate the exact amount of MMA added to the reaction (see item 5). The reaction was carried out under the given conditions ($T = 50\text{ }^{\circ}\text{C}$, stirring 1000-1200 RPM) for another 1 h.

8. Then, measure the pH of the post-reaction mixture (pH_2). If the process is not continued on the same day, fill the flask with the mixture with demineralised water (depending on the volume of the flask) and leave it in the refrigerator overnight. If you need to leave the mixture for a longer time (2-3 days), freeze the flask with the mixture at $-20\text{ }^{\circ}\text{C}$ without adding water first.

5 9. In order to determine the amount of methacrylic acid, 2-3 mL of the mixture was taken into a Falcon device so that the foam formed in the mixture did not distort the collected volume.

 10. Next, the PBSx1 solution was added to the mixture portion-wise, until achieving 5-fold dilution of the reaction mixture (1:4 mixture/PBSx1).

10 11. The obtained solution was poured into previously prepared dialysis tubes according to the following procedure:

 a) the appropriate length of the dialysis tube was measured and cut off - the length of the dialysis tube was selected so that one dialysis vessel contained no more than 1,500 mL of post-reaction solution (approx. 80 cm of the tube holds approx. 500-
15 600 mL of solution, taking into account the space to tie the ends; one beaker holds 3 such tubes). The MWCO 1000 Da dialysis tube is immersed in sodium azide solution,

 b) the tube were placed in a beaker with demineralised water and left for 10 minutes, then the water was replaced with fresh, they were soaked and left for 10 minutes, the water was replaced with fresh again and left for 10 minutes,

20 c) the soaked tube was gently removed from the beaker and tied at one end. A funnel was placed in its open end and a small amount of water was poured to check the tightness of the tube,

 d) if no leaks were noticed, the water should be removed from the tube and then the solution was quantitatively transferred to the tube, washing the reaction vessel with
25 3x10 mL of deionised water,

 e) the tube was tied and secured on both sides with a clip, and then placed in a 5 L beaker sprayed with ethanol (previously equipped with a "sun" type mixing element and a dialysis stand sprayed with ethanol),

 f) the beaker was filled with deionised water (approx. 4 L) and placed on a
30 magnetic stirrer. The entirety was protected against light with aluminium foil,

 12. The dialysis process was carried out at 40 °C (400 rpm) for 4 days, changing the water 3 times a day (usually at 8:00 a.m., 12:00 p.m. and 04:00 p.m.). The number

- 5 of dialysis days can be modified due to possible time constraints. It is important that the number of transfers is at least 11, one can change the water e.g. 4 times a day.

13. After completing the dialysis process, 2-3 mL of solution was taken from the dialysis tube (taking care of the foam, which may distort the collected volume) in order to determine methacrylic acid. If a methacrylic acid signal was observed, dialysis was
10 continued (another 6 transfers, procedure as in item 11). If the signal is not visible, repeat the measurement in splitless mode. If the presence of methacrylic acid is confirmed – repeat dialysis, if there is no signal – go to item 14.

14. The solution from the dialysis tubes was quantitatively transferred to a large beaker, and then to a 1000 mL round-bottom flask with approximately 600 mL of
15 solution each, and concentrated on a rotary evaporator to approximately 25-35% of the initial volume (not less than 150 mL). The concentrated solution was transferred to a glass bottle and kept in the refrigerator or freezer. If the volume of the reaction mixture after dialysis is less than 1500 mL, one can skip the concentration process and go to item 15.

20 The final concentration parameters are:

- a) bath temperature: 40 °C
- b) pressure: 30 mbar
- c) rotations: 150 rpm
- d) initial temperature of the cooler: -8°C

25 The final parameters were achieved by gradually reducing the pressure, starting from 140 mbar. At a pressure of 90 mbar, evaporate the solution for about 20 minutes (until foam stops being released), then gradually reduce the pressure again. Reducing the pressure too quickly may result in the solution foaming and part of the contents of the flask being transferred straight into the receiver.

30 15. After the concentration process is completed, transfer approx. 150-180 mL of the solution (using a smaller beaker) to aluminium trays (previously sprayed with ethanol and signed with the name of the sample and the tray number) placed in plastic

5 stands. The exact amount of solution added to each tray was weighed and marked as m_4 .

16. The trays with the solution were placed in a $-80\text{ }^{\circ}\text{C}$ freezer and frozen for a minimum of 3 hours. N, the frozen samples were transported in a Styrofoam box for freeze-drying. Freeze-drying parameters:

- 10 a. shelf temperature: $10\text{ }^{\circ}\text{C}$
b. pressure: 0.100 mbar
c. duration: 48 hrs

17. The obtained lyophilisates were transferred to a previously weighed plastic container (record as m_5 on the side of the container), the entirety (container + product, 15 recorded on the container as m_6) were weighed and these two values were subtracted from each other to obtain the weight of the product (recorded on the container as Δm).

18. The container with the material was stored at $-20\text{ }^{\circ}\text{C}$.

19. The obtained product was checked for cross-linking and gelation. For this purpose, a 10% (mass-mass) solution of the product was prepared in water, then LAP 20 was added in such an amount that its concentration in the entire solution was 0.25%. 1 mL of the obtained solution was taken, transferred to the mold and irradiated using a wavelength of 365 nm or 405 nm (at the highest power) for 1 minute.

20. The water of the obtained product after freeze-drying was determined. For this purpose, calculations were made according to the formula:

25
$$M\%_{\text{H}_2\text{O}} = \frac{m_6 - m_5}{m_4 - m_5} \cdot 100\%$$

21. After freeze-drying, approximately 15 mg of dry product was weighed into a vial with a rubber stopper, marked with the name of the sample and the approximate amount of weighed material and placed in a freezer ($-20\text{ }^{\circ}\text{C}$).

^1H NMR measurements of the substitution degree

30 To measure the degree of substitution by ^1H NMR, a 5 mg sample was dissolved in 600 μL of D_2O with 0.00916 mmol of TMSP (quantitative standard - 3-(trimethylsilyl)

5 propionic acid) and placed in a 5 mm NMR tube. The samples were then placed in an NMR spectrometer (Agilent DirectDrive2 700 MHz). The temperature was set to 60°C . Once the temperature had stabilised, the samples were mixed, the probe was tuned, the pulse was measured, and the magnetic field inhomogeneity was corrected. Next, the ¹H spectrum was measured (measurement parameters: number of scans 8, 10 repetition time 15 s, pulse time 45° 2.5 μs). The NMRGlue package in the Python environment was used for spectrum analysis. After importing the data, weighting was performed with the exponential function (line widening: 2 Hz), Fourier transform, phasing and baseline correction for 6.7 ppm areas: 6.45 ppm, 1.05 ppm: 0.8 ppm, 0.1 ppm: -0.1 ppm. Next, the peak integrals in the 6.7 ppm region were counted: 6.45 ppm 15 (corresponding to the proton in the double bond) and an integrated peak in the 1.05 ppm region: 0.8 ppm (corresponding to a proton from merium). Based on these parameters, the DS. NMR value was calculated (degree of substitution) using the formula:

$$DS_{NMR} = \frac{\int(5.8 - 5.6)}{\int TMSP} \div \frac{\int(1.05 - 0.8 ppm)}{\int TMSP} \cdot 9.96 \cdot 100\%$$

20 The calculation results are presented in Table 3 and in the graph in Fig. 2. .

Table 3. Degree of substitution calculated by NMR analysis.

ECMMA	dECM	Degree of substitution NMR, [%]
T6 (12-14 kDa)	M64	87
T7 (12-14 kDa)	M61	85
T8 (3.5 kDa)	M44+M45	75
T11 (3.5 kDa)	M102	122
T11 (1 kDa)	M102	122
T12 (3.5 kDa)	M102	74
T12 (1 kDa)	M102	73

T13 (3.5 kDa)	M102	109
T13 (1 kDa)	M102	113
T15 (1 kDa)	M102	134

5 **Hydrogel preparation 10% ECMMA + 0.25% LAP**

15 mL of PBSx1 was quantitatively transferred into a 50 mL dark glass bottle with a serological pipette. Using analytical scales, LAP (photoinitiator - lithium phenyl-2,4,6-trimethylbenzoylphosphinate) was weighed in a weighing vessel, and then 39.4 mg of LAP was quantitatively transferred to a bottle with PBSx1. The bottle with the solution
10 was transferred to a water bath (30°C) and stirred at 500 rpm for 15 min (until the photoinitiator dissolved). The LAP solution was then filtered into a sterile 50 mL falcon. 9.1 mL of filtered LAP solution was added to a second sterile 50 mL bottle. Then, the ECMMA lyophilisate (previously bleached with an UV lamp) was weighed and added to the photoinitiator solution in an amount of 1.01 g. The ECMMA solution was left in a
15 water bath (30°C) until fully dissolved, and the mixing rate was selected at 100-200 rpm.

Table 4. Reagents used to prepare the hydrogel

Reagents	
PBSx1 [mL]	LAP [mg]
15	39.4
LAP solution [mL]	ECMMA [g]
9.1	1.01

The ECMMA preparation scheme is shown in Fig. 3.

20 **Bioprinting of scaffold**

5 Scaffold measuring 10 x 10 x 3 mm were printed, according to the programmed design (Fig. 4), Table 5 contains detailed information about the model. The printing was carried out on a 3D bioprinter – CELLINK BioX. An external UV-Vis lamp, Polbionica, was used to cross-link each layer. 10% ECMMA hydrogel was used to print the scaffold. The scaffolds were printed on a Petri dish. Detailed information about the model is
10 presented in Table 5.

Table 5. Parameters of the bioprinted module

Model	Bioink volume [mL]	The number of layers	Layer height [mm]
10x10x3	0.3	6	0.5

Printing parameters were selected on an ongoing basis during the printing process, depending on the given portion of bioink, the parameter ranges used are presented in
15 Table 6. Fig. 5 shows photos of finished prints.

Table 6. Scaffold printing parameters

Printing temperature [°C]	Pressure [kPa]	Print rate [mm/s]	Needle	Crosslinking
17.0	39-45	15	metal, blue 609 μm	wavelength 405 nm; time 20 sec; power 28.5 mW/cm ²

Each flake was weighed, then transferred to a dish and sterile PBS with antibiotics was poured over.

Solutions for the cross-linking test were then prepared by sequentially adding the
20 following to 15-mL vials:

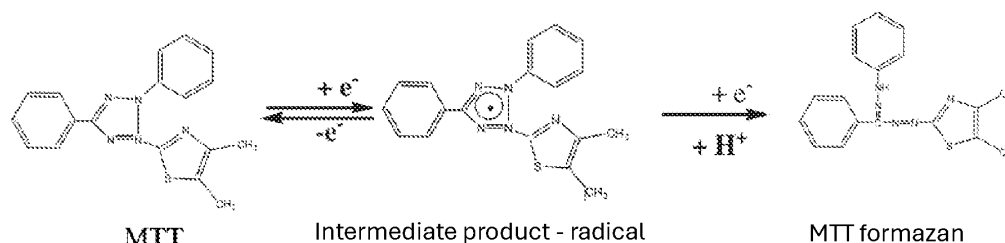
-375 mg ECMMA, 18.75 mg LAP and 7106.25 μ l PBSx1 for a 5% solution.

10 The cross-linked solutions are shown in Fig. 6. The results of the cross-linking tests
are shown in Fig. 7.

The obtained bioink enables full diffusion of insulin and glucagon in the bioprinted constructs. In order to confirm this, the analysis of pancreatic islet beta cell functionality was performed in dECMMA bioink with 3 concentrations (Fig. 8). The obtained results confirm that dECMMA can be used in tissue engineering and regenerative medicine using pancreatic islet cells and other cell lines.

The aim of the procedure is to assess the metabolic activity of cells and check how long the cytotoxicity of the tested materials persists: 10% ECMMA, 12,5% ECMMA, 15% ECMMA, batch T18.

Cell viability and proliferation are related to the ability of enzymes – mitochondrial dehydrogenase – present in viable cells to convert the water-soluble tetrazolium salt – 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) – for water-insoluble purple formazan crystals. The need to draw the supernatant from above the
25 cells and formazan crystals before adding an organic solvent (e.g. isopropanol or dimethyl sulfoxide) causes errors (such as accidentally removing some of the crystals), which reduces the sensitivity of this test - therefore, caution is necessary when doing this.



5 Preparation of extracts

The mass of the samples is presented in Table 7.

Table 7. Mass of samples used to prepare extracts

Biomaterial	Sample mass	Extract recovered from 2.5 mL of added medium
ECMMA 10% 24h	499.2 mg	2.3 mL
ECMMA 10% 48h	509.2 mg	2.3 mL
ECMMA 10% 72h	506.1 mg	2.3 mL
ECMMA 12.5% 24h	500.3 mg	2.3 mL
ECMMA 12.5% 48h	508.9 mg	2.3 mL
ECMMA 12.5% 72h	2.35 mg	2.35 mL
ECMMA 15% 24h	499.7 mg	2.35 mL
ECMMA 15% 48h	500.5 mg	2.3 mL
ECMMA 15% 72h	503.6 mg	2.3 mL

The scaffold produced from the ECMMA biomaterial were crushed and transferred to sterile 5mL Eppendorf tubes. Culture medium was added to the biomaterials, and then the samples were placed in an incubator (37°C) for a specific time, depending on the experiment variant.

Preparing samples for the MTT test

The same procedure was followed for each time variant of 24, 48 and 72 hours. The extract was collected from above the biomaterial into a new Eppendorf tube as a 100% extract. A 50% dilution was also made of each extract.

To prepare L929 cells for the MTT assay, cells were seeded in 96-well plates for the collected extracts. According to the schedule, cells of the L929 line were

- 5 prepared for individual extracts. Cells at concentrations of 10^5 cells/mL were seeded in 96-well plates in columns of 100 μ L. PBS was added to the edge wells. The prepared plates were incubated at 37°C for 24 hours. Table 8 shows the viability of the seeded cells.

Table 8. Viability of seeded cells.

Seeding date	Total	Dead	Alive	Viability
29.01.2024	1.4×10^6	1.0×10^5	1.3×10^6	91%
30.01.2024	2.2×10^5	3.0×10^4	1.9×10^5	85%
31.01.2024	1.4×10^6	1.7×10^5	1.2×10^5	88%

10

Collecting extracts from biomaterials

Extracts were collected without the need for centrifugation. The colour of the medium was not changed at any time point.

Extracts of 100% and 50% for ECMMA 10%, 12.5% and 15% were applied to the plate.

- 15 Due to the obtained results, it was found that the material in the tested concentrations is not cytotoxic to cells, therefore it can be safely used in bioprinting tissue models and bionic organs.

Claims

1. A process for methacrylation of dECM, comprising the following steps:
 - a) placing 1M carbonate buffer in the reaction vessel and heating to 50 °C,
 - b) adding dECM to the carbonate buffer from step (a) to obtain a dECM solution with a concentration of 4% (w/v),
 - c) sterilizing the solution obtained in step (b) by irradiating with UV radiation for 15 minutes,
 - d) adding methacrylic anhydride in the amount of 0.5 mL/1g dECM,
 - e) after adding methacrylic anhydride, the reaction is carried out at 50 °C for 1 h,
 - f) adding phosphate-buffered saline solution to obtain a 5-fold dilution of the mixture from step (e),
 - g) placing the solution from step (f) in the dialysis tube, and then placing the dialysis tube in deionised water, and this step is carried out at a temperature of 40 °C for no longer than 4 days,
 - h) after completing step (g), the solution in the dialysis tubes is transferred to aluminium trays and then placed at -80 °C for at least 3 hours,
 - i) freeze-drying the frozen solution from step (h) under the following conditions: shelf temperature 10 °C, pressure 0.1 mbar, freeze-drying time 48 hours.
2. The process according to claim 1, characterised in that the addition of methacrylic anhydride in step (c) is done using a syringe pump over a period not longer than 3 h, or in portions at 15-min intervals over a period not longer than 3 h.
3. The process according to claim 1, characterised in that in step (g) the deionised water is replaced at least 11 times.
4. The process according to claim 1, characterised in that after completing step (g), the solution in the dialysis tubes is concentrated on a rotary evaporator to 25-35% of the initial volume, and the volume of the concentrated solution is not less than 150 mL.
5. Methacrylated dECM obtained by the process according to claims 1 to 5.

6. Methacrylated dECM according to claim 6, characterised in that the degree of substitution with methacrylic groups is determined by ^1H NMR at 60°C using the equation:

$$DS_{NMR} = \frac{\int(5.8 - 5.6)}{\int TMSP} \div \frac{\int(1.05 - 0.8 \text{ ppm})}{\int TMSP} \cdot 9.96 \cdot 100\%$$

TMSP – internal standard, ranges from 75 to 135%

7. Use of methacrylated dECM obtained by the process according to claims 1-5 in the bioprinting process.
8. The use according to claims 8, characterised in that the bioprinting temperature is in the range from 10 to 35 °C, the pressure is in the range of 5-50 kPa, the printing rate is from 1 to 30 mm/s, and the needle diameter is from 100 to 900 μm , cross-linking occurs at a wavelength from 365 nm to 405 nm, from 5 to 360 s, at a power of up to 1 to 100 mW/cm², with the bioink containing from 1 to 15% (w/v) of methacrylated dECM and from 0.1 to 0.5% (w/v) LAP.