

METHOD OF FUNCTIONALIZATION OF A BIOPOLYMER AND METHOD OF CROSS-LINKING THEREOF

Field of the invention

The invention relates to a method of functionalizing a biopolymer, wherein a biopolymer having -OH or -NH₂ groups is reacted with at least one compound containing at least one C=C or C≡C bond and a chromophore group absorbing UV-Vis radiation directly adjacent to this bond. The invention also relates to a method for obtaining a solid biopolymer material, in which the functionalized biopolymer obtained by the method specified above is subjected to reversible cross-linking when exposed to light in the UV-VIS range. The invention also relates to use of a functionalized biopolymer as a support material for 3D bioprinting, as well as the use of a functionalized biopolymer for the production of structures selected from spheroids, organoids, artificial organs, coatings, and tissue models. The invention is used in tissue engineering, transplantology and medical and pharmacological research.

Background art

- Chemical compounds that can take part in cycloaddition reactions are used in cross-linking reactions of natural polymers (Pellá MCG, Lima-Tenório MK, Tenório-Neto ET, Guilherme MR, Muniz EC, Rubira AF. Chitosan-based hydrogels: From preparation to biomedical applications. *Carbohydr Polym.* 2018, 196, 233-245, doi: 10.1016/j.carbpol.2018.05.033; Eva Mueller, Isabelle Poulin, William James Bodnaryk, and Todd Hoare, Click Chemistry Hydrogels for Extrusion Bioprinting: Progress, Challenges, and Opportunities, *Biomacromolecules* 2022 23 (3), 619-640, doi: 10.1021/acs.biomac.1c01105; Kerim M. Gattás-Asfura, Eric Weisman, Fotios M. Andreopoulos, Miodrag Micic, Bill Muller, Sanjeev Sirpal, Si M. Pham, and Roger M. Leblanc, Nitrocinamate-Functionalized Gelatin: Synthesis and "Smart" Hydrogel Formation via Photo-Cross-Linking, *Biomacromolecules* 2005 6 (3), 1503-1509, doi: 10.1021/bm049238w; Koshy ST, Desai RM, Joly P, Li J, Bagrodia RK, Lewin SA, Joshi NS, Mooney DJ. Click-Crosslinked Injectable Gelatin Hydrogels. *Adv Healthc Mater.* 2016, 5(5), 541-7, doi: 10.1002/adhm.201500757; Jasper Van Hoorick, Liesbeth Tytgat, Agnes Dobos, Heidi Ottevaere, Jürgen Van Erps, Hugo Thienpont, Aleksandr Ovsianikov, Peter Dubruel, Sandra Van Vlierberghe, (Photo-) crosslinkable gelatin derivatives for biofabrication applications, *Acta Biomaterialia* 2019, 97, 46-73, doi: 10.1016/j.actbio.2019.07.035.)
- However, there is still a need to provide new ways of functionalizing biopolymers that can be cross-linked (either during 3D bioprinting or during other techniques for forming solid biopolymer structures such as mold casting) solely upon exposure to light, i.e. without the use of initiating additives cross-linking, such as photoinitiators, metal salts, transition metal complexes and hypervalent iodine compounds, which constitute an undesirable contaminant in the target structure. It is particularly

desirable to eliminate photoinitiators which have the greatest cytotoxic effect on cells that may constitute a component of the biopolymer material subjected to cross-linking.

Moreover, there is a constant need to provide new functionalized biopolymers capable of cross-linking upon exposure to light of a higher wavelength (i.e. lower energy) due not only to reducing the energy consumption of the cross-linking process, but above all to limiting the negative impact of UV radiation on the viability of the cells contained in the biopolymeric material subjected to cross-linking. Furthermore, there is a constant need to provide new functionalized biopolymers capable of reversible cross-linking upon exposure to light, which in certain applications may significantly facilitate the modification of the formed biopolymer structures or a more detailed analysis thereof.

10 **Summary of the invention**

The subject of the invention is a method of functionalizing a biopolymer, wherein a biopolymer having -OH or -NH₂ groups is reacted with at least one compound containing at least one C=C or C≡C bond and a chromophore group absorbing UV-Vis radiation and a chromophore group absorbing UV-Vis radiation immediately adjacent to this bond. Immediate proximity in this context means that the chromophore group is attached to the carbon atom directly adjacent to the carbon atom of the multiple bond. Preferably, the compound containing at least one C=C or C≡C bond is a carboxylic acid derivative, preferably selected from active ester, anhydride and acid chloride. Preferably, the carboxylic acid derivative is selected from a coumarin-3-carboxylic acid derivative, an exo-5-nonbornene-carboxylic acid derivative and a trans-cinnamic acid derivative. Preferably, the active ester is N-hydroxysuccinimidyl ester, which is obtained by reacting a carboxylic acid containing at least one C=C or C≡C bond with N-hydroxysuccinimide in the presence of a coupling agent, preferably selected from 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), dicyclohexylcarbodiimide (DCC) and N,N'-diisopropylcarbodiimide (DIC).

Preferably, the biopolymer is selected from proteins and polysaccharides. In a preferred embodiment of the invention, the biopolymer is selected from gelatin, hyaluronic acid, alginate, chitosan, dextran, starch, cellulose, collagen, chitin, carrageenan, inulin, glycogen and heparin, and is preferably selected from gelatin, chitosan and hyaluronic acid, and most preferably it is gelatin.

The subject of the invention is also a method for obtaining a solid biopolymer material, wherein the functionalized biopolymer obtained by the method specified above is subjected to reversible cross-linking upon exposure to light in the UV-VIS range, preferably in the range of light with a wavelength of 280-800 nm, and most preferably with a wavelength selected from 365nm and 405nm.

The subject of the invention is also use of a functionalized biopolymer obtained by the method specified above as a support material for 3D bioprinting.

The subject of the invention is also use of a functionalized biopolymer obtained by the method specified above for the production of structures selected from spheroids, organoids, artificial organs, coatings, and tissue models.

Functionalized biopolymers obtained using the method according to the invention allow to obtain
5 fully cross-linking solutions in the concentration range from 0.5% to 50% without the addition of a photoinitiator such as lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) or 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959). A functionalized biopolymer, such as gelatin, hyaluronic acid, alginate and other polymers of natural or semi-synthetic origin, with a molecular weight ranging from 1,000 kDa to 500,000 kDa, after functionalization according to the
10 method according to the invention, has a degree of substitution from 10 to 100% with a substituent being a compound containing at least one C=C or C≡C bond and a chromophore group that absorbs UV-Vis radiation directly adjacent to this bond.

Functionalized biopolymers obtained by the method according to the invention retain full solubility (in volumes from 0.1 to 1000 ml) in an aqueous environment, including physiological saline solutions
15 or cellular media with a pH of 4 to 8, or other - depending on the application.

Functionalized biopolymers obtained by the method according to the invention are suitable for use in extrusion, volumetric printing and other 3D printing technologies requiring the use of a material with appropriate viscosity with a clear gel point in order to obtain a uniform and coherent fiber to maintain printing resolution. The printing temperature can range from 5°C to 50°C depending on the
20 concentration of the prepolymer solution used, and in this case the prepolymer should be understood as the functionalized biopolymer according to the invention before the cross-linking process begins. The printing pressure in extrusion printing typically ranges from 5 to 150 kPa. Print speeds typically range from 1 to 100 mm/s. The needle diameter typically ranges from 50 to 900 μm. The printed model can include any dimensions expressed in mm, as well as the degree of infill from 5% to 100%,
25 which means it is possible to obtain both solid and openwork prints.

The functionalized biopolymers obtained by the method according to the invention are fully cross-linked without the presence of a photoinitiator when exposed to light with a wavelength from 280 nm to 800 nm, with a light power ranging from 1 mW/cm² to 1000 mW/cm², for a time from 10 to 720 seconds.

30 Functionalized biopolymers obtained by the method according to the invention are fully suitable for use in 3D cultures, tissue engineering and other applications with living cells in which UV-Vis light is the cross-linking factor. The technology does not require the use of an external initiator, which may have a potential cytotoxic effect.

Functionalized biopolymers obtained using the method according to the invention can be used as a single material in a bioink or as an addition to a mixture of other printable materials. This means that in the 3D bioprinting process, during which the cross-linking of functionalized biopolymers takes place, cross-linking reactions can occur not only between molecules of one type of functionalized biopolymer, but also between molecules of two or more different functionalized biopolymers. A functionalized biopolymer or a mixture of biopolymers functionalized with various compounds can constitute from 0.1% to 99.9% of the total blend.

Functionalized biopolymers according to the invention or their blends have potential applications in biological research using the reference L929 cell line in the initial phase of research, or in accordance with the ISO 10993-5 standard of replacement lines, provided that the same or similar MTT test results are obtained, i.e. CCL 1 (NCTC clone 929), CCL 163 (Balb/3T3 clone A31), CCL 171 (MRC-5) and CCL 75 (WI-38), CCL 81 (Vero) and CCL 10 [BHK-21 (C-13) and V -79 379A, and in subsequent phases of research using specific cell lines consistent with the application. Functionalized biopolymers according to the invention, their blends and materials with the addition of cell lines can be homogenized using the inter-syringe mixing technique or specialized cell mixers dedicated to biological applications.

Brief description of the drawing figures

The subject of the invention in an embodiment is illustrated, in a way that does not limit the scope of the invention, in the attached drawing, in which:

Fig. 1 shows the ^1H NMR spectrum of the material obtained in example 1 in the cross-linking process of gelatin functionalized with coumarin-3-carboxylic acid;

Fig. 2a shows the ^1H NMR spectrum of exo-5-nonbornenecarboxylic acid;

Fig. 2b shows the ^1H NMR spectrum of the material obtained in Example 2 in the crosslinking process of a mixture of a 15% solution of gelatin functionalized with coumarin-3-carboxylic acid and a 15% solution of gelatin functionalized with exo-5-nonbornenecarboxylic acid;

Fig. 3a shows the ^1H NMR spectrum of trans-cinnamic acid;

Fig. 3b shows the ^1H NMR spectrum of the material obtained in example 3 in the cross-linking process of a mixture of a 15% solution of gelatin functionalized with coumarin-3-carboxylic acid and a 10% solution of gelatin functionalized with trans-cinnamic acid;

Fig. 3c shows the ^1H NMR spectrum of the material obtained in example 3 in the cross-linking process of a mixture of a 15% solution of gelatin functionalized with coumarin-3-carboxylic acid and a 15% solution of gelatin functionalized with trans-cinnamic acid;

Fig. 4 shows photos of printed flake-like scaffolds with different cross-linking times;

Fig. 5 shows the ^1H NMR spectrum of a 15% solution of gelatin functionalized with coumarin-3-carboxylic acid (GelCM);

Fig. 6 shows the ^1H NMR spectrum of a 12.5% GelCM solution;

Fig. 7 shows the ^1H NMR spectrum of a 10% GelCM solution;

5 Fig. 8 shows the scheme of the g.code file [template.gcode];

Fig. 9 shows a diagram of a fiber bending test platform;

Fig. 10 shows microscopic images of constructs in the fiber bonding test;

Fig. 11 shows the percentage of fiber diffusion rate Dfr in the fiber bonding test;

Fig. 12 shows the printability of Pr in the fiber bonding test;

10 Figure 13 shows the Alamar blue test results for cells printed in GelCM 10%, 12.5% and 15% and methacrylated gelatin (GelMA) 10% constructs;

Fig. 14 shows microscopic images of printed constructs with L929 cells immediately after the printing process

Fig. 15 shows microscopic images of printed constructs with L929 cells on 3rd day of the experiment

15 Fig. 16 shows microscopic images of printed constructs with L929 cells on 7th day of the experiment

Fig. 17 shows photos of biomaterials with L-929 cells after transfer to 6-well plates with supplemented culture medium, where A - GELMA 10%, GelCM 12.5%, GelCM 15%, B - GelCM 10%

Fig. 18 shows the percentage of lactate dehydrogenase (LDH) release from L-929 cells as a result of interaction with the tested biomaterials for 1, 5, 7 and 14 days

20 Fig. 19 shows microscopic photos of cell cultures of the L-929 line exposed to biomaterials GelCM 10%, GelCM 12.5%, GelCM 15%, GELMA 10% after a 14-day incubation.

Detailed description of the invention

The invention relates to a method of functionalizing a biopolymer in which a biopolymer having -OH or -NH₂ groups is reacted with at least one compound containing at least one C=C or C≡C bond
25 (activated or non-activated), i.e. a bond capable of undergoing a cycloaddition reaction 2+2, and a chromophore group that absorbs UV-Vis radiation.

The main goal of the method according to the invention is to obtain appropriately functionalized derivatives of natural polymers (hereinafter described and illustrated as Helix/P) for use in tissue engineering, including 3D bioprinting, classical and 3D cell culture, formation of spheroids/organoids
30 and artificial (bionic) organs, formation of material coatings, printing of specific tissue models (normal and cancerous) including vessels and the specific mass of artificial organs using cell lines,

microorgans (including pancreatic islets), organoids, spheroids and other three-dimensional cellular structures.

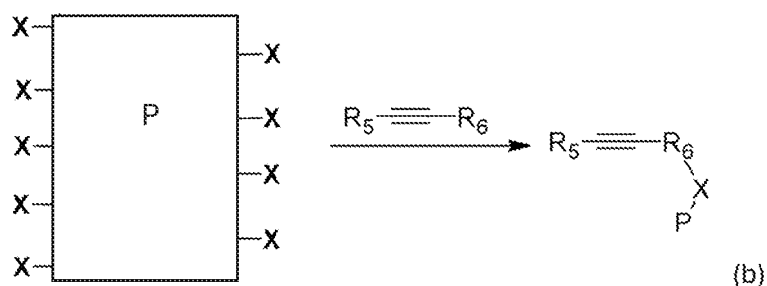
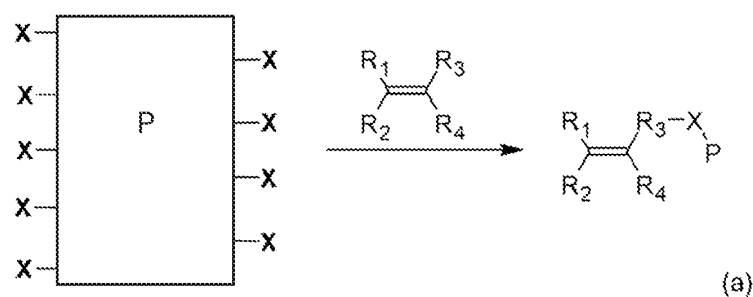
The proposed technology and the materials used for its implementation enable cross-linking/hardening of materials, mainly soft polymers, using UV-VIS radiation without the use of additives that initiate cross-linking, i.e. photoinitiators, metal salts, transition metal complexes and hypervalent iodine compounds. Cross-linking of the mentioned materials, i.e. functionalized biopolymers obtained by the method according to the invention, takes place due to a [2+2] cycloaddition reaction of compounds containing a carbon-carbon multiple bond and activated double or triple bonds, which are used as a separate source of the copolymer or constitute an integral part of the polymer within the same molecule. Illustrative diagrams of possible paths of the process of functionalization of biopolymers and their subsequent cross-linking are presented below. The paths presented are examples and cross-linking with the materials used can occur in any possible way resulting from the chemical reaction mechanism forming the basis of the proposed polymer cross-linking methods.

An important feature of the described methodology is the possibility of using light in the UV-Vis range with a wavelength of 280-800 nm, depending on the structure and absorption properties of the chromophore substituent located at the multiple bond undergoing 2+2 cycloaddition. Shifting the wavelength range towards higher wavelengths (lower energy) not only has a practical utility aspect (reducing the energy consumption of the process), but also has a significant impact on the use of materials created in this technology for use in broadly understood tissue engineering. Radiation close to the visible range has a much smaller impact on causing damage to cells that may be part of the hardened material.

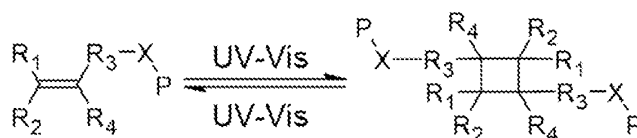
The biopolymer functionalization method according to the invention uses compounds containing at least one C=C or C≡C bond, i.e. a bond capable of undergoing a 2+2 cycloaddition reaction. Examples of such compounds are compounds containing a coumarin moiety in their structure, for example coumarin-3-carboxylic acid and coumarin-6-carboxylic acid. When exposed to light of an appropriate wavelength, these compounds undergo [2+2] cycloaddition reactions.

The scheme for obtaining functionalized biopolymers according to the invention and then cross-linking them is presented below:

(i) functionalization of the biopolymer with compounds containing carbon-carbon multiple bonds



(ii) reversible cross-linking of the functionalized biopolymer - for variant (a)



wherein

- 5 Helix = P represents a biopolymer selected from oligopeptides, proteins, polysaccharides, such as gelatin, hyaluronic acid, alginate, chitosan, dextran, starch, cellulose, collagen, chitin, carrageenan, inulin, glycogen, heparin;

X is -OH or -NH₂;

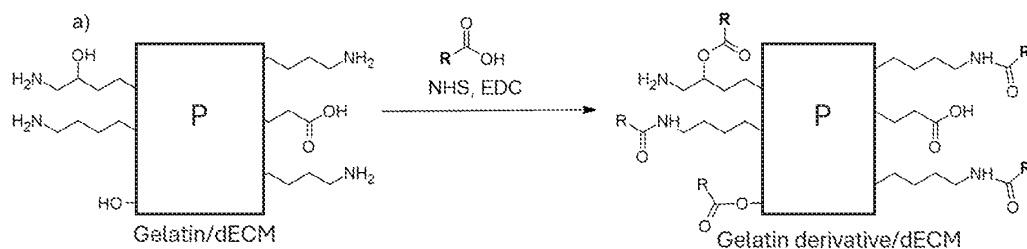
- 10 R₁, R₂, R₃, R₄, R₅, R₆, each independently of the others, is H, C₁-C₃₀-alkyl, C₂-C₃₀-alkenyl, C₂-C₃₀-alkynyl, C₆-C₁₀-aryl or C₅-C₁₀-heteroaryl containing from 1 to 3 heteroatoms selected from N, S and O.

Cross-linking in step (ii) is carried out using a light beam with a wavelength of 280-800 nm, with preferred wavelength values being 365 nm or 405 nm. The exposure time is 1-6000 s, preferably 10-360 s. The power of the light source used is 1-3000 mW/cm².

- 15 An important advantage of the functionalization of biopolymers according to the invention, illustrated in point (i) in the scheme above, is the possibility of cross-linking the hydrogel based on an appropriately functionalized polymer soluble in water or buffer without the need to use an initiator

and the possibility of reversing the cyclization reaction at an appropriately selected wavelength, as illustrated in point (ii) in the scheme above.

One of the preferred biopolymers for functionalization using the method according to the invention is gelatin. It is a natural polymer made of protein chains. In its structure gelatin has such amino acids as: lysine, hydroxylysine, proline and hydroxyproline, which play a key role in functionalization reactions, because the amino and hydroxyl residues of these amino acids take part in the reactions illustrated in the scheme below:



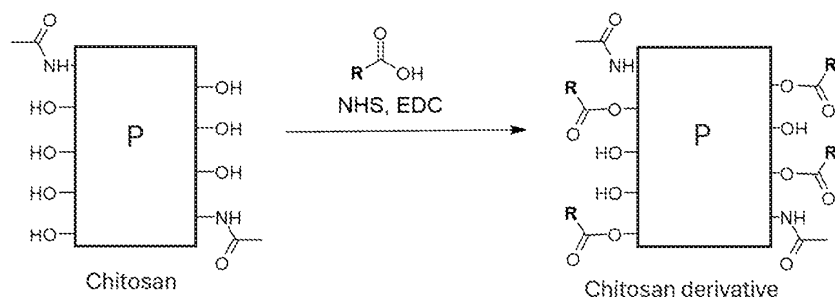
dECM – decellularized extracellular matrix [due to the gelatin content in dECM, it can be assumed that the dECM cross-linking mechanism is similar to the gelatin cross-linking mechanism]

R = R1 defined above

NHS - N-hydroxysuccinimide

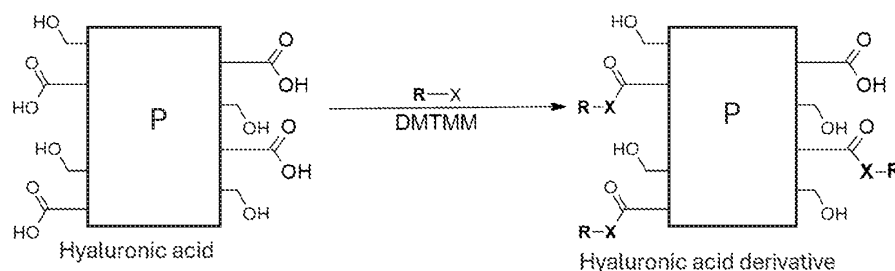
EDC - 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

Another preferred biopolymer for functionalization according to the invention is chitosan. This polysaccharide is a derivative of chitin produced in the deacetylation process. Chitosan is composed of β -glucosamine molecules connected by β -1,4-glycosidic bonds. Chitosan functionalization reactions involve free hydroxyl groups present in deacetylated β -glucosamine units. The functionalization of chitosan, like the functionalization of gelatin and dECM, preferably occurs using active esters of the appropriate carboxylic acids, as illustrated in the scheme below:



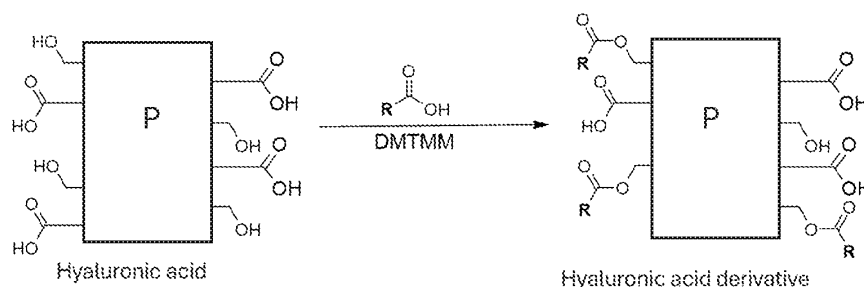
Another preferred biopolymer for functionalization using the method according to the invention is hyaluronic acid. This polysaccharide from the glycosaminoglycan group is composed of D-glucuronic acid and N-acetyl-D-glucosamine units, which are connected by β -1,4-glycosidic and β -1,3-

glycosidic bonds. Functionalization of hyaluronic acid takes place using a coupling reagent, DMTMM (4-(4,6-dimethoxy[1.3.5]triazin-2-yl)-4-methylmorpholine chloride). DMTMM is obtained by reacting CDMT (2-chloro-4,6-dimethoxy-1,3,5-triazine) with morpholine. Functionalization of the biopolymer can take place in two ways. The first one involves the activation of carboxyl groups of D-glucuronic acid units and then the reaction of the activated carboxyl group with compounds containing amino or hydroxyl groups, as illustrated in the scheme below:



X – as defined above

The second method of functionalizing hyaluronic acid involves activating the carboxyl groups of any acids with DMTMM and then reacting the activated acid with the hydroxyl groups of N-acetyl-D-glucosamine units, as illustrated in the scheme below:



As already mentioned above, one of the preferred examples of the method of functionalization of biopolymers according to the invention is a two-step reaction using a carboxylic acid derivative constituting an active ester. For this purpose, the carboxylic acid is reacted with NHS and a condensing reagent such as EDC, DCC or DIC. The active N-hydroxysuccinimidyl ester of the carboxylic acid used in this way is reacted with the biopolymer. The active ester reacts with free amino and hydroxyl groups present in the structure of the natural polymer, resulting in an appropriate functionalized biopolymer. Instead of the active ester, the anhydride or acid chloride of the selected carboxylic acid can be used.

Control of pH, temperature, concentration and amount of substrates allows control of the degree of substitution of derivatives in the range of 20-100%. The value of the degree of substitution affects the mechanical properties of the obtained material.

EXEMPLARY EMBODIMENTS**EXAMPLE 1 – material synthesis***Step 1. Synthesis of the active ester*

1.3324 g of coumarin-3-carboxylic acid (CAS: 531-81-7) (4.8 equivalents) and 0.9674 g of N-hydroxysuccinimide (4.8 equivalents) were placed in a round-bottom flask equipped with a stirring element. Everything was dissolved in 12.5 mL of DMF. N,N'-dicyclohexylcarbodiimide (DCC, 4 equivalents) was then added to the flask in portions while stirring (1000 rpm). The reaction was left for 4 hours at room temperature (25°C, 1000 rpm). After this time, the post-reaction mixture containing the active ester was filtered on a Schott funnel (G5) and used for the next stage of the synthesis.

Step 2. Functionalization of gelatin

A three-necked round-bottom flask equipped with a mixing element was placed in the heating block on a magnetic stirrer. 50 mL of carbonate buffer (CB, pH 9.55) was poured into it using a funnel and heated to 50°C. Then, 5 g of gelatin (1 equivalent) was added to the buffer, stirring continuously (1000 rpm). The mixture was left to dissolve completely (50°C, 1000 rpm). Then, a solution of the active ester in DMF was added dropwise to the solution. The mixture was left for 24 hours (50°C, 1000 rpm). After this time, the post-reaction mixture was diluted with 200 mL of phosphate-buffered saline (PBSx1, pH 7.40) and poured into 50 mL falcons. The mixture was centrifuged (10,000 rpm, 30 min), and the upper layer was filtered through a 0.22 µm filter. The clear solution was dialyzed (12-14 kDa tubes) at 40°C for 3 days, changing the water 3 times a day. The purified solution was frozen and freeze-dried (10°C, 48 h, 0.01 mbar). The finished product was subjected to 1H NMR analysis to determine the degree of substitution.

Synthesis optimization:

In order to optimize the synthesis, a series of experiments were carried out, changing parameters such as: buffer pH, type of solvent in stage 1 (DMF, DMSO, CHCl₃), type of condensing reagent (EDC and DCC), reaction time (2 h, 4 h, 8 h, 16 h, 24 h, 48 h), the ratio of reactants in the reaction.

¹H NMR analysis:

The solution for analysis was prepared by weighing an appropriate amount of sample (typically about 5 mg), dissolving it in 600 µL of deuterated water with the addition of 0.0916 mmol of tetramethylsilylpropanoic acid (TMSP, quantitative and chemical shift standard), and placing it in 5 mm NMR tubes. The samples were then placed in an NMR spectrometer (Agilent DirectDrive2 700 MHz). The temperature was set to 60°C. After the temperature stabilized, the samples were mixed, the probe was tuned, the pulse was measured, and the magnetic field inhomogeneity was corrected.

Then the ^1H spectrum was measured (measurement parameters: number of scans 8, repetition time 15 s, pulse time $45^\circ 2.5 \mu\text{s}$). The obtained ^1H NMR spectrum of the material obtained in this example in the cross-linking process of gelatin functionalized with coumarin-3-carboxylic acid is shown in Fig. 1.

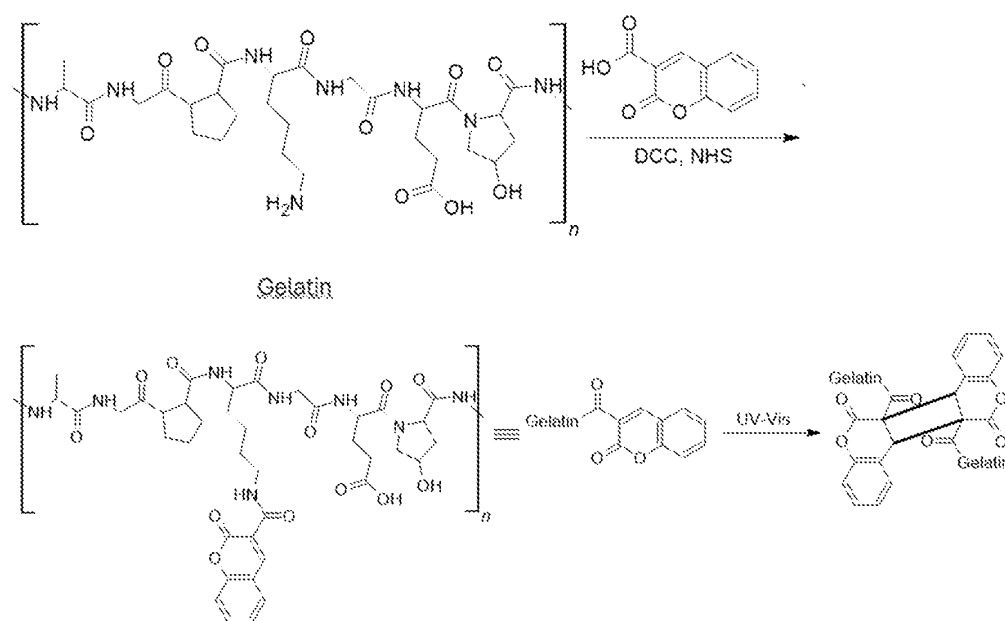
5 Degree of substitution:

The ^1H NMR spectrum of the material was analyzed using the NMRGlue package in the Python environment. After importing the data, exponential weighting (line broadening: 2 Hz), Fourier transform, phasing and baseline correction were performed for the regions of $8.85 \text{ ppm} \div 8.6 \text{ ppm}$, $1.05 \text{ ppm} \div 0.8 \text{ ppm}$, $0.1 \text{ ppm} \div -0.1 \text{ ppm}$. Then, the integrals of the peaks in the region of $8.85 \text{ ppm} \div 8.6 \text{ ppm}$ (corresponding to the proton in the double bond) were calculated and the peak in the region of $1.05 \text{ ppm} \div 0.8 \text{ ppm}$ (the peak of the proton coming from the mere) was integrated. Based on these parameters, the DS_{NMR} value (degree of substitution) was calculated using the formula:

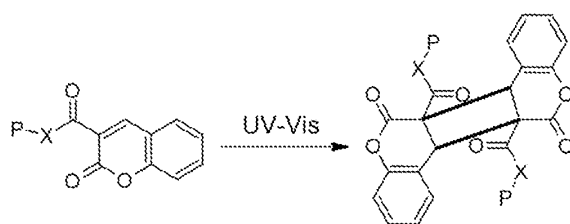
$$\text{DS}_{\text{NMR}} = \frac{\int \text{peak } (8.85 - 8.6 \text{ ppm})}{\int \text{peak } (1.05 - 0.8)} \cdot 9.96 \cdot 100\%$$

15 The ^1H NMR spectrum is shown in Figure 1.

The functionalization scheme of gelatin is as follows:



Simplified cross-linking scheme using gelatin functionalized with coumarin-3-carboxylic acid (last step of the process described above, with P-X- = Gelatin):



EXAMPLE 2 – material synthesis

The procedure was analogous to example 1, but in step 1, regardless of the synthesis of the active ester using coumarin-3-carboxylic acid, a parallel synthesis of the active ester was carried out using
 5 exo-5-nonbornenecarboxylic acid (CAS: 934-30-5) (in the same amount in terms of molar equivalent). As a result, two different active esters were obtained, which were then used - also in parallel processes in stage 2 (functionalization of gelatin) - to obtain appropriately functionalized gelatin derivatives.

Then, the cross-linking process of both functionalized gelatin derivatives was carried out together.

10 A simplified cross-linking scheme of a mixture of coumarin-3-carboxylic acid-functionalized gelatin and exo-5-nonbornenecarboxylic acid-functionalized gelatin solutions is shown below, with P-X-gelatin:

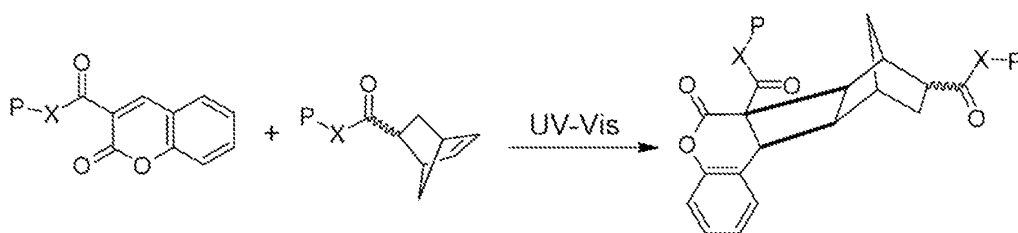


Fig. 2a shows the ^1H NMR spectrum of exo-5-nonbornenecarboxylic acid, and Fig. 2b - the ^1H NMR
 15 spectrum of the material obtained in this example in the cross-linking process of a mixture of a 15% solution of gelatin functionalized with coumarin-3-carboxylic acid and 15% solution of gelatin functionalized with exo-5-nonbornenecarboxylic acid.

In both this example and Examples 3 and 4 below, solutions of functionalized gelatin were prepared in PBS.

20 EXAMPLE 3 – material synthesis

The procedure was analogous to example 1, but in step 1, regardless of the synthesis of the active ester using coumarin-3-carboxylic acid, a parallel synthesis of the active ester using trans-cinnamic acid (CAS: 140-10-3) was carried out (in the same amount in terms of molar equivalent). As a result,

two different active esters were obtained, which were then used - also in parallel processes in stage 2 (functionalization of gelatin) - to obtain appropriately functionalized gelatin derivatives.

Then, the cross-linking process of both functionalized gelatin derivatives was carried out together.

A simplified cross-linking scheme of a mixture of solutions of gelatin functionalized with coumarin-3-carboxylic acid and gelatin functionalized with trans-cinnamic acid is shown below, with P-X-gelatin:

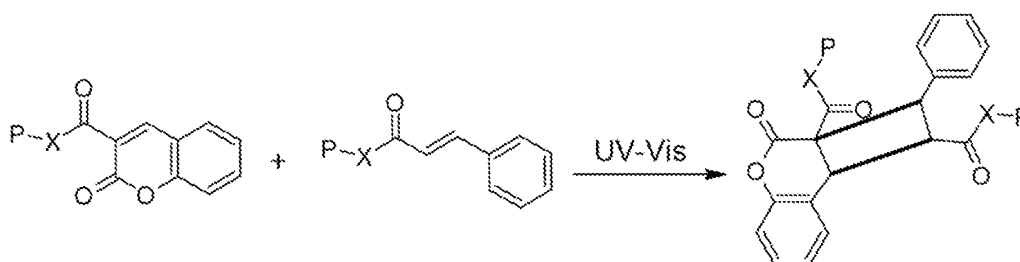


Fig. 3a shows the ^1H NMR spectrum of trans-cinnamic acid, and Fig. 3b - the ^1H NMR spectrum of the material obtained in this example in the cross-linking process of a mixture of a 15% solution of gelatin functionalized with coumarin-3-carboxylic acid and a 10% solution of gelatin functionalized with trans-cinnamic acid.

EXAMPLE 4 – material synthesis

The procedure was analogous to example 3, but instead of a mixture of a 15% solution of gelatin functionalized with coumarin-3-carboxylic acid and a 15% solution of gelatin functionalized with trans-cinnamic acid, a mixture of a 15% solution of gelatin functionalized with coumarin-3-carboxylic acid and 10% solution of gelatin functionalized with trans-cinnamic acid was crosslinked.

Fig. 3c shows the ^1H NMR spectrum of the material obtained in this example in the cross-linking process of a mixture of a 15% solution of gelatin functionalized with coumarin-3-carboxylic acid and a 15% solution of gelatin functionalized with trans-cinnamic acid.

EXAMPLE 5 – determining the cross-linking profile

The experiment included preparation of the material, digestion of the material using a 0.5% solution of type II collagenase, carrying out the freeze-drying process and ^1H NMR analysis for the following variants:

Variant 1: printing of four-layer 10x10 mm flake-like scaffolds with different cross-linking times (time: 60 s, 120 s, 240 s, 360 s, lamp power: 13.0 mW/cm², wavelength: 365 nm) for a solution with a concentration of 15%

Variant 2: printing of single-layer 10x10 mm flake-like scaffolds with different cross-linking times (time: 120 s, 240 s, 360 s, lamp power: 13.0 mW/cm², wavelength: 365 nm) for solutions with a concentration of 10% and 12.5 %

Preparation of solutions

- 5 To prepare a 15% GelCM solution, 1.0515 g of GelCM (coumarin-3-carboxylic acid-functionalized gelatin obtained in Example 1 above) (AZ-041-9) was weighed into a 50 ml bottle using an analytical balance. Then, 5.959 ml of PBSx1 was added using an automatic pipette. The bottle with the solution, wrapped in aluminum foil, was transferred to a thermoblock (40°C, 400 rpm) for approximately 30 minutes to dissolve the substance.
- 10 To prepare a 12.5% GelCM solution, 250.0 mg of GelCM (AZ-049-9) was weighed into a 5 ml bottle using an analytical balance. Then, 1.750 ml of PBSx1 was added using an automatic pipette. The bottle with the solution, wrapped in aluminum foil, was transferred to a thermoblock (40 °C, 400 rpm) for approximately 30 minutes to dissolve the substance. Then, the resulting solution was adjusted to pH = 7.37 with 5 µL of 5M NaOH solution.
- 15 To prepare a 10% GelCM solution, 200.0 mg of GelCM (AZ-049-9) was weighed into a 5 ml bottle using an analytical balance. Then, 1,800 ml of PBSx1 was added using an automatic pipette. The bottle with the solution, wrapped in aluminum foil, was transferred to a thermoblock (40 °C, 400 rpm) for approximately 30 minutes to dissolve the substance. Then, the resulting solution was adjusted to pH = 7.31 with 5 µL of 5M NaOH solution.

20 *Printing of flake-like scaffolds*

The BioX CELLINK bioprinter and the Polbionica UV-Vis lamp were used for testing.

(a) 15% GelCM solution

- 4 four-layer flake-like scaffolds 10x10 mm were printed from the 10x10x4_05_G4S10 file. Appropriate printing parameters (13.5-14 °C, 10 mm/s, 175 kPa) and material cross-linking were also selected:
- 25

P1. 360s; 365nm; 13.0 mW/cm²; P2. 240 s; 365nm; 13.0 mW/cm²; P3. 120s; 365nm; 13.0 mW/cm²; P4. 60s; 365nm; 13.0 mW/cm²

A 580 µm needle (pink, plastic needle) was used for printing.

Fig. 4 shows photos of printed flake-like scaffolds with different cross-linking times.

30 (b) 12.5% GelCM solution

3 flake-like scaffolds measuring 10x10x5 mm were printed. Appropriate printing parameters (14°C, 81-87 kPa, 20 mm/s and 15°C, 63-68 kPa, 20 mm/s) and material cross-linking were also selected:

Q5. 360s; 365nm; 13.0 mW/cm²; P6. 240 s; 365nm; 13.0 mW/cm²; P7. 120s; 365nm; 13.0 mW/cm²

(c) 10% GelCM solution

3 flake-like scaffolds measuring 10x10x5 mm were printed. Appropriate printing parameters (14°C, 33-40 kPa, 20 mm/s and 15°C, 21-27 kPa, 20 mm/s) and material cross-linking were also selected:

5 P8. 360s; 365nm; 13.0 mW/cm²

P9. 240 s; 365nm; 13.0 mW/cm²

P10. 120s; 365nm; 13.0 mW/cm²

Digestion of flake-like scaffolds

The printed flake-like scaffolds were placed in 24-well plates. Each flake-like scaffold was poured
10 with 1 mL of 0.5% collagenase type II solution and placed in an incubator for 48 h at 37°C.

Freeze-drying

The digested flake-like scaffolds were frozen at -80°C. Then it was freeze-dried for 48 h (15% variant) and 24 h (12.5%, 10% variants). Lyophilization conditions: 10°C, 0.01 mbar.

¹H NMR analysis

15 In order to perform ¹H NMR analysis, the lyophilized product was weighed into 10 2 mL Eppendorf falcons, 590 µL of deuterated water and 10 µL of TMSP standard solution with a concentration of 0.95 mg/mL were added. The samples were placed in a thermoblock (40°C, 400 rpm) and heated for 10 minutes to dissolve the substance. Then the samples were submitted for ¹H NMR analysis (Subcontractor-Spektrino). Material weighings: P1. 6.0 mg; P2. 5.7 mg; P3. 5.9 mg; P4. 5.4 mg; Q5.
20 10.7 mg; P6. 11.5 mg; P7. 11.6 mg; P8. 12.0 mg; P9. 15.5 mg; P10. 17.4 mg.

RESULTS

Analytical signals of the digested flake-like scaffolds are found in the ¹H NMR spectrum in the range of 8.85 ÷ 8.6 ppm and 3.95 ÷ 4.0 ppm. The peak at 8.85 ÷ 8.6 ppm corresponds to protons at the double bond in the coumarin moiety. As the cyclization reaction of this moiety progresses, this signal
25 disappears and at the same time a signal appears at 3.95 ÷ 4.0 ppm, which in turn corresponds to the protons at the cyclobutane ring formed in the cyclization reaction of the coumarin moiety. The presence of signals in the range of 3.95 ÷ 4.0 ppm proves that the GelCM material cross-links under the influence of UV-Vis radiation.

The results of ¹H NMR measurements were analyzed and the percentage of unreacted coumarin-3-
30 carboxylic acid groups (DS of the cross-linked material) was determined, taking into account the mer content (lysine residues) in the sample according to the following formula:

$$DS_{NMR} = \frac{\int peak (8.85 - 8.6)}{\int peak (1.05 - 0.8 ppm)} \cdot 9.96 \cdot 100\%$$

The percentage of unreacted species was converted to the degree of cross-linking of the material, taking into account the degree of substitution of the non-cross-linked material (DS of the starting material). The results are summarized in the table below:

5 **Table 1:** Summary of ¹H NMR analysis results - values of the degree of substitution (DS) of the cross-linked material

Concentration	Sample no.	DS of cross-linked material, [%]	DS of starting material, [%]	Cross-linking degree, [%]
15% GelCM	P1	11.8	27	56.3
	P2	21.9		18.9
	P3	24.7		8.5
	P4	27.8		~0
12,5% GelCM	P5	5.7	29	80.3
	P6	12.4		57.2
	P7	19.2		33.8
10% GelCM	P8	4.1	29	85.8
	P9	7.4		74.5
	P10	11.0		62

Fig. 5 shows the ¹H NMR spectrum for a 15% GelCM solution (a), Fig. 6 - for a 12.5% GelCM solution (b), and Fig. 7 - for a 10% GelCM solution (c).

Conclusions

10 ¹H NMR analysis indicates that the longer the cross-linking time, the higher the degree of cross-linking of the material for each concentration variant. The highest degree of cross-linking was obtained for the cross-linking parameters: 360 s, 13.0 mW/cm², 365 nm. These parameters were used in subsequent material tests.

EXAMPLE 6- printability

15 Preparation of solutions

(i) 15% (w/w) GELCM

2.5 ml of a 15% (w/w) GELCM solution (AZ-057-9, DS = 50%) was prepared.

Using an analytical balance, 378.6 mg of GELCM lyophilisate was weighed on a weighing vessel. Then, the weighed lyophilisate was transferred to a 5 ml falcon and supplemented with 2.145 ml of PBSx1. The GELCM solution was left in the thermoblock (50°C, 400 rpm) for 30 min. After complete
5 dissolution of GELCM, the pH of the solution was checked (pH0 = 6.55), adjusting its value to pH = 7.39 (5 µl of 5M NaOH and 1.5 µl of 5M HCl were added). The prepared solution was filtered using a 0.22 µm syringe filter into a sterile, aluminum foil-wrapped falcon with a capacity of 5 ml.

(ii) 12.5% (w/w) GELCM

2.5 ml of a 12.5% (w/w) GELCM solution (AZ-057-9, DS = 50%) was prepared.

10 Using an analytical balance, 318.8 mg of GELCM lyophilisate was weighed on a weighing vessel. Then the weighed lyophilisate was transferred to a 5 ml falcon and supplemented with 2.232 ml of PBSx1. The GELCM solution was left in the thermoblock (50°C, 400 rpm) for 30 min. After complete dissolution of GELCM, the pH of the solution was checked (pH0 = 6.58), adjusting its value to pH = 7.39 (4 µl of 5M NaOH and 0.5 µl of 5M HCl were added). The prepared solution was filtered using a
15 0.22 µm syringe filter into a sterile, aluminum foil-wrapped falcon with a capacity of 5 ml.

(iii) 10% (w/w) GELCM

2.5 ml of a 10% (w/w) GELCM solution (AZ-057-9, DS = 50%) was prepared.

Using an analytical balance, 254.3 mg of GELCM lyophilisate was weighed on a weighing vessel. Then the weighed lyophilisate was transferred to a 5 ml falcon and supplemented with 2.289 ml of PBSx1.
20 The GELCM solution was left in the thermoblock (50°C, 400 rpm) for 30 min. After complete dissolution of GELCM, the pH of the solution was checked (pH0 = 6.66), adjusting its value to pH = 7.43 (3 µl of 5M NaOH and 0.5 µl of 5M HCl were added). The prepared solution was filtered using a 0.22 µm syringe filter into a sterile, aluminum foil-wrapped falcon with a capacity of 5 ml.

(iv) 10% (w/w) GELMA + 0.25% (w/w) LAP

25 2.5 ml of a 10% (w/w) GELMA solution (P10-01, DS = 81/86%) was prepared.

Using an analytical balance, 318.8 mg of GELMA lyophilisate was weighed on a weighing vessel. Then, the weighed lyophilisate was transferred to a 5 ml falcon and supplemented with 2.291 ml of PBSx1. The GELMA solution was left in the thermoblock (50°C, 400 rpm) for 30 min. Then, after dissolving GELMA, 6.5 mg of LAP weighed on a weighing bottle was transferred to the falcon with the
30 solution. After complete dissolution of GELMA with LAP, the pH of the solution was checked (pH0 = 7.33). The prepared solution was filtered using a 0.22 µm syringe filter into a sterile, aluminum foil-wrapped falcon with a capacity of 5 ml.

Printability tests

Printability tests were carried out using a BioX CELLINK bioprinter. The printing parameters of each material are included in Table 2 below. The parameters used allowed to obtain a uniform, compact fiber. The procedure for conducting printability tests was 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).

Table 2. Printing parameters in printability tests of pre-cross-linked materials

Variant	Pressure range [kPa]	Temperature [°C]	Speed [mm/s]
15% (w/w) GELCM	60-70	17	10
12.5% (w/w) GELCM	40-50	17	10
10% (w/w) GELCM	30-40	17	10
10% (w/w) GELMA + 0.25% (w/w) LAP	30-40	17	10

The presented technology includes extrusion printing, volumetric printing and other printing technologies that require appropriate material viscosity in order to obtain a uniform and coherent fiber to maintain printing resolution. The printing temperature may vary from 5 °C to 50 °C depending on the material used. Printing pressure for extrusion printing ranges from 5 to 150 kPa. Print speed ranging from 1 to 100 mm/s. The needle diameter ranges from 50 to 900 µm. The printed model can include any dimensions expressed in mm, as well as the degree of filling from 5% to 100%, thus obtaining openwork prints.

Fiber bonding test

An appropriate g-code was prepared for the fiber bonding test: template.gcode, which assumes printing two layers one after the other using the tested material without the use of cross-linking with an external lamp between them. Prints were made using a BioX CELLINK printer. The print follows the pattern in a 0°–90° pattern, which gives a 2D effect and increases the distance between the fibers. The distance between the fibers was in the range of 1–5 mm with 1 mm increments. 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 within the appropriate range of pressures and temperatures given in Table 2 above. The print was cross-linked with an external UV-Vis lamp, Polbionica, using the following parameters: wavelength 365 nm, time 360 s with a power of 13 mW/cm² in the case of GELCM and wavelength 405 nm, cross-linking time 30 s, power 28.5 mW/cm² in the case of GELMA. After printing, microscopic photos were taken. Processing of photos was carried out using ImageJ software. Based on the results, two parameters described by the

following equations were determined, i.e. the percentage of 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 a perfect model representation the printability is 1.0.

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

5

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

A_t – theoretical pore surface area,

A_a – actual pore surface,

L – actual circumference of the pore.

Fig. 8 shows the scheme of the g.code file [template.gcode]

10 Fiber bending test

The mid-span bending of the suspended fiber was analyzed to determine the collapse of the material. In order to carry out the experiment, a special platform was designed consisting of seven pillars spaced from each other by known distances of 1, 2, 3, 4, 5, 6 mm. The dimensions of the five posts placed inside the structure are $2 \times 10 \times 6 \text{ mm}^3$, and the dimensions of the two edge posts are $5 \times 10 \times 6 \text{ mm}^3$. A single fiber of the tested material was deposited on the platform according to the g-code: MR_test1.gcode, and then a photo of the print was immediately taken. Processing of photos was carried out using ImageJ software. During the process, the temperature and pressure conditions were adjusted depending on the tested material, and the print itself was made at a speed of 10 mm/s using a 25 G (0.250 mm) needle. Collapse area coefficient C_r , i.e. the percentage of the actual area after bending the suspended fiber in relation to the theoretical area.

20

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

A_a^c – theoretical area under curve,

A_t^c – real area under curve.

Fig. 9 shows a diagram of the fiber bending test platform

25 Results

For each material variant, 3 fiber bonding tests were performed. Then, microscopic photos of the obtained constructs were taken - shown in Fig. 10. On the basis of the obtained measurements, the percentage of fiber diffusion rate Df_r and printability P_r were calculated - the results are presented in the graphs shown in Figs. 11 and 12, respectively.

Conclusions

All tested materials showed continuous, compact fibers, which allowed for obtaining prints with good resolution. It was not possible to print pores of 1x1 mm in any of the tested materials (the exception is the 2nd attempt to print 15% (w/w) GELCM, which produced a small pore). For each material
5 variant, a printability above 0.8 was achieved, as well as a diffusion rate percentage below 50%. The diffusion rate decreases with increasing pore size. Printability for each pore size, except for 1x1 pores, is at the level of 0.8-0.9 for all materials. The best printability was shown by 15% (w/w) GELCM and 10% (w/w) GELMA + 0.25% (w/w) LAP. The lowest percentage of diffusion for pores 4x4 and 5x5 was shown by 12.5% GELCM, while the lowest percentage of diffusion for pores 2x2 and 3x3 was
10 shown by the reference material 10% (w/w) GELMA + 0.25% (w/w) LAP.

EXAMPLE 4 – study of proliferation rate and toxic effects in the cell

Alamar blue test

This method is based on the conversion of a compound called alamar blue (resazurin) into the resorufin compound together with living cells. Resazurin is known as an oxidative redox blue dye that
15 passes freely through the cell membrane to enter the cell, where it is reduced and converted to fluorescent pink resorufin. Dead cells cannot reduce resazurin and are unable to generate a fluorescence signal due to loss of metabolic activity. The resulting signal is detected using fluorometers, and the intensity increases as the number of viable cells increases.

The results of the Alamar blue test for cells printed in the Gelcm 10%, 12.5% and 15% and Gelma
20 10% constructs are shown in Fig. 13. The control consisted of L929 cells seeded directly on the plate, without biomaterial. The measurement was performed at 4 time points: on the day of printing and on the 3rd, 7th and 14th day after printing.

Conclusions

Cells in the 10% and 12% Gelcm biomaterials proliferated at a similar level. The degree of cell
25 proliferation (decrease in the population growth rate) for 15% Gelcm was much lower than for cells in other biomaterials. The results indicate that the degree of cell proliferation in 10% Gelm was the highest in the first hours of the experiment, with time the cells divided less easily compared to K or 10% and 12.5% Gelcm.

Microscopic observations

I. Imaging of biomaterials with cells in the bright field

30

After printing, the constructs were transferred to a multiwell plate and imaged under a bright field Olympus microscope using a 10x objective.

II. Imaging of biomaterials with cells after FDA/Pi staining

Cell viability was assessed based on FDA/PI staining in accordance with a developed procedure in which cell staining (to distinguish dead from living cells) is performed using two fluorescent dyes: propidium iodide (PI) and fluorescein diacetate (FDA). Fluorescein diacetate can penetrate the cell membrane. After entering the cell, FDA is hydrolyzed by intracellular esterases to fluorescein, which exhibits fluorescent properties. Living cells can accumulate this compound, which allows them to emit intense green fluorescence. Propidium iodide has an electrical charge and does not penetrate intact cell membranes. It stains cells with necrotic or late apoptosis red. Samples were suspended in 1 x PBS (stained with FDA/PI and immediately observed under a fluorescence microscope. Two solutions were prepared for staining: FDA (5 mg/ml in acetone) and PI (2 mg/ml in PBS).

Fig. 14 shows microscopic images of printed constructs with L929 cells immediately after the printing process, Fig. 15 - microscopic images of printed constructs with L929 cells on 3rd day of the experiment, and Fig. 16 - microscopic images of printed constructs with L929 cells on 7th day of the experiment

Summary

In the tested groups: 10% Gelcm, 12.5% Gelcm and 10%Gelcm, L929 cells showed a high level of viability. In the following days, a high level of viability was observed, above 90%, and proper development of cells printed in the biomaterial: 10% Gelcm, 12.5% Gelcm and 10% Gelma (as a reference material).

Assessment of cytotoxicity of GelCM biomaterials using the LDH release test

The aim of the experiments was to assess the cytotoxicity of biomaterials GelCM 10%, GelCM 12.5%, GelCM 15% (batch GelCM AZ-050-9). The cytotoxicity of the bioconstructs was tested using a test based on the analysis of lactate dehydrogenase (LDH) activity of the mouse fibroblast cell line: L-929 (ATCC®, cat no: CCL-1™).

Lactate dehydrogenase (LDH) activity test – principle of the method

Cytotoxicity (cytotoxic activity) is the ability of a specific factor (chemical, physical or biological) to disrupt the functioning of cells. It involves, among others, inhibition of their growth, proliferation or induction of cell death [Abe K., Matsuki N., Measurement of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction activity and lactate dehydrogenase release using MTT, Neurosci Res. 2000; 38(4): 325–9.]. Currently, there are many tests used on the market to determine cell viability after exposure to the test substance, including the MTT test (MTT - 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide), neutral red test (NR) or measurement of the activity of the cytoplasmic enzyme lactate dehydrogenase (LDH) [Abe K., Matsuki N., Measurement of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction activity and lactate dehydrogenase release using MTT, Neurosci Res. 2000; 38(4): 325–9; Jost L.M., Kirkwood

J.M., Whiteside T.L., Improved short- and long-term XTT-based colorimetric cellular cytotoxicity assay for melanoma and other tumor cells, J Immunol Methods. 1992; 147(2): 153–65; Wang S., Yu H., Wickliffe J.K., Limitation of the MTT and XTT assays for measuring cell viability due to superoxide formation induced by nano-scale TiO₂, Toxicol Vitro. 2011; 25(8):2147–51.

- 5 Lactate dehydrogenase (LDH) is a soluble, stable cytosolic enzyme that is released into the culture medium upon disruption of the integrity of the cell's plasma membrane. The released enzyme can be detected by various methods, for example colorimetric, fluorometric or luminescence (the most sensitive). In our own research, the commercial LDH-Glo™ Cytotoxicity Assay kit from Promega was used, based on a bioluminescent method for quantitative determination of released LDH.
- 10 LDH released from damaged cells catalyzes the oxidation of lactate to pyruvate, with the simultaneous reduction of NAD⁺ to NADH. The reductase uses NADH and the reductase substrate to produce luciferin, which is converted to a bioluminescent signal by rLuciferase Ultra-Glo™. The generated luminescent signal is proportional to the amount of LDH present [Holmes RS, Goldberg E. Computational analyzes of mammalian lactate dehydrogenases: human, mouse, opossum and platypus LDHs. Comput Biol Chem. 2009 Oct;33(5):379-85; Khan AA, Allemailem KS, Alhumaydhi FA, Gowder SJT, Rahmani AH. The Biochemical and Clinical Perspectives of Lactate Dehydrogenase: An Enzyme of Active Metabolism. Endocr Metab Immune Disord Drug Targets. 2020;20(6):855-868]. The determination of LDH activity in the supernatant is a measure of the toxicity of the tested substance towards cells in culture. This method allows us to clearly determine whether a given
- 15 substance causes damage to the plasma membrane of cells and, consequently, their death.
- 20

Preparation of biomaterials for the LDH test

Four biomaterials were received from the Bioprint Team: GELMA 10% (Gelma batch P10-01), GelCM 10%, GelCM 12.5%, GelCM 15% (GelCM batch AZ-050-9) with printed mouse fibroblast cells. Each biomaterial was printed in three repetitions. 5x10⁶ cells were used for printing per 1 ml of a given

25 bioink. Bioconstructs were placed in a 6-well plate in DMEM culture medium (total volume of medium was 4 ml). Throughout the entire experiment, the constructs were stored in standard culture conditions, i.e. 37°C and 5% CO₂. The controls in the experiment were: L-929 cells alone - negative control, L-929 cells treated with Triton X-100 0.1% - positive control and a printout without cells. Fig. 17 shows photos of biomaterials with L-929 cells after transfer to 6-well plates with supplemented

30 culture medium, A - GELMA 10%, GelCM 12.5%, GelCM 15%, B - GelCM 10%.

LDH test procedure

Assessment of the degree of toxicity of the tested biomaterials GELMA 10%, GelCM 10%, GelCM 12.5%, GelCM 15%. against L-929 cells were performed using the LDH assay. The test was carried out at four time points - on days 1, 5, 7 and 14. For the test, samples of the culture medium were

collected (on days 1, 5, 7 and 14) and diluted in a ratio of 1:100 in sample storage buffer (LDH Storage Buffer). A single study consisted of 3 repetitions (1 biomaterial was printed in 3 repetitions). The samples were stored at -20°C until testing. Before starting the test, a reaction mix was prepared according to the manufacturer's recommendations: 50 µl LD Detection Enzyme Mix and 0.25 µl
5 Reductase substrate/sample. A dilution series of the LDH standard was also performed. The reaction mix was spotted onto a white 96-well plate in a 1:1 ratio with the test samples. It was incubated at room temperature for 60 min, and then the luminescence was read using a microplate reader.

LDH release analysis

Fig. 18 shows the percentage of lactate dehydrogenase (LDH) released from L-929 cells as a result
10 of interaction with the tested biomaterials for 1, 5, 7 and 14 days. LDH enzymatic activity results were expressed as percentage relative to the positive control (K+).

Fig. 19 - microscopic photos of cell cultures of the L-929 line exposed to biomaterials GelCM 10%, GelCM 12.5%, GelCM 15%, GELMA 10% after a 14-day incubation. The photos were taken using an Olympus IX83 microscope in a bright field (BF), magnification 4x, 10x, 20x.

Observations and conclusions:

15

All biomaterials cause an increase in the cytotoxic effect over time, i.e. with the length of incubation of cells in a given biomaterial, the level of LDH in the medium increases. 7-day exposure of GelCM 10%, GelCM 12.5%, GelCM 15% materials to cells causes the release of LDH at a level of less than 30%. Microscopic observation shows that until day 7 the cell morphology was normal (spindle shape,
20 no granules, normal confluence).

Claims

1. A method of functionalizing a biopolymer, characterized in that the biopolymer having -OH or -NH₂ groups is reacted with at least one compound containing at least one C=C or C≡C bond and a chromophore group absorbing UV-Vis radiation directly adjacent to this bond.
2. The method of claim 1, wherein the compound containing at least one C=C or C≡C bond is a carboxylic acid derivative, preferably selected from an active ester, an anhydride and an acid chloride.
3. The method of claim 2, wherein the carboxylic acid derivative is selected from a coumarin-3-carboxylic acid derivative, an exo-5-norbornene-carboxylic acid derivative and a trans-cinnamic acid derivative.
4. The method according to claim 2 or 3, wherein the active ester is N-hydroxysuccinimidyl ester, which is obtained by reacting a carboxylic acid containing at least one C=C or C≡C bond with N-hydroxysuccinimide in the presence of a coupling agent, preferably selected from 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), dicyclohexyl-carbodiimide (DCC) and N,N'-diisopropylcarbodiimide (DIC).
5. The method according to one of claims 1-4, wherein the biopolymer is selected from proteins and polysaccharides.
6. The method of claim 5, wherein the biopolymer is selected from gelatin, hyaluronic acid, alginate, chitosan, dextran, starch, cellulose, collagen, chitin, carrageenan, inulin, glycogen and heparin, and is preferably selected from gelatin, chitosan and hyaluronic acid, and most preferably it is gelatin.
7. A method for obtaining a solid biopolymer material, characterized in that the functionalized biopolymer obtained by the method specified in one of claims 1-6 is subjected to reversible cross-linking upon exposure to light in the UV-VIS range, preferably in the range of light with a wavelength of 280-800 nm, and most preferably with a wavelength selected from 365 nm and 405 nm.
8. Use of a functionalized biopolymer obtained by the method defined in one of claims 1-6 as a support material for 3D bioprinting.
9. Use of a functionalized biopolymer obtained by the method defined in one of claims 1-6 for the production of structures selected from spheroids, organoids, artificial organs, coatings, tissue models.