

**RECOMBINANT PROTEINS, RECOMBINANT DNA SEQUENCES, VECTORS,
EUKARYOTIC AND PROKARYOTIC EXPRESSION SYSTEMS AND THEIR USE**

Field of the invention

The invention relates to recombinant RE15mR and EJ17zipR hybrid proteins having the structure
5 specified in the description, recombinant DNA sequences encoding the recombinant hybrid proteins,
vectors containing the recombinant DNA sequences, as well as eukaryotic and prokaryotic expression
systems transformed with the recombinant DNA sequences. The invention also concerns the use of
recombinant hybrid proteins as components of bioink intended for bioprinting, and also as
components of biomaterials. Furthermore, the invention relates to the use of the recombinant DNA
10 sequences, the vectors and the eukaryotic or prokaryotic expression systems for the production of the
recombinant hybrid proteins. The invention is used in bioprinting, medicine and cellular and tissue
engineering.

Prior art

Bioprinting and biomaterials are currently intensively researched and developed. Technical solutions
15 in these fields enable 3D printing of tissue structures, and ultimately treatment in regenerative
medicine. Thus, there are examples of natural and synthetic bioinks and biomaterials in the art, such
as the biopolymers disclosed in US 2022/0047706. Nevertheless, the materials used in bioinks and
biomaterials still need improving their effect on cell viability, proliferation, adhesion or cytotoxicity
(also in combination as a component of blends).

20 Aim of the invention

The aim of the invention was to propose a new, alternative recombinant hybrid protein useful in
particular as a component of bioink and a biomaterial. Such a protein should be universal, safe and
effective to use both in the laboratory environment, and ultimately in the patient. The aim of the
invention was also to overcome the technical problems found in the state of the art, in particular by
25 providing a protein with at least one parameter, such as the effect on cell viability, proliferation,
adhesion or cytotoxicity (also in combination as a component of blends), rheological parameters
(composite modulus, dynamic viscosity), printability (printability, resolution, continuity and stability
of the fiber), mechanical properties (compressive mechanical strength, Young's modulus) comparable
or better than in proteins currently used in the state of the art.

30 Summary of the invention

In the first aspect the subject of the invention is a recombinant RE15mR hybrid protein composed
sequentially of 3 resilin domains, 1 K+ domain, 3 resilin domains, 1 MMP domain, 3 elastin domains,
1 K+ domain, 4 elastin domains, 1 K+ domain, 3 elastin domains, 1 K+ domain, 4 elastin domains,
1 K+ domain, 3 elastin domains, 1 MMP domain, 7 RGD domains.

Preferably, the amino acid sequence of the recombinant RE15mR hybrid protein according to the invention contains 12-15% of proline and/or 32-38% of glycine.

Preferably, the molecular weight of the recombinant RE15mR hybrid protein of the invention is 26 kDa.

- 5 Preferably, the recombinant RE15mR hybrid protein of the invention has the amino acid sequence of SEQ #3.

Preferably, the recombinant RE15mR hybrid protein of the invention is a fragment of a recombinant protein.

- 10 Preferably, the recombinant RE15mR hybrid protein of the invention is at least partially methacrylated.

In a second aspect, the invention provides a recombinant DNA sequence selected from:

- a recombinant DNA sequence encoding the recombinant RE15mR hybrid protein as defined above,
- 15 a recombinant DNA sequence comprising a region containing a DNA sequence encoding the recombinant RE15mR hybrid protein as defined above, and
- a recombinant DNA sequence hybridizing to a DNA sequence encoding the recombinant hybrid protein RE15mR as defined above.

- 20 Preferably, the recombinant DNA sequence of the second aspect of the invention has a sequence that is at least 80% identical, more preferably at least 90% identical, and most preferably homologous to the recombinant DNA sequence of SEQ #1.

- 25 In a third aspect, the invention provides a vector including a recombinant DNA sequence comprising a region containing a recombinant DNA sequence according to the second aspect of the invention and/or a recombinant sequence comprising a portion of a region containing a recombinant DNA sequence according to the second aspect of the invention. Preferably, the vector according to the invention is a plasmid containing a promoter derived from phage T7, preferably selected from pET11a-d, pET15b, pET19b, pET28a-c(+), pET21a-d(+), pET22b(+), pET23a-d (+), pEt25b(+), pET44a-c(+), pET46Ek/LIC, and most preferably being pET11a.

- 30 In a fourth aspect, the invention provides a eukaryotic or prokaryotic expression system transformed with a recombinant DNA sequence containing a region comprising a recombinant DNA sequence according to the second aspect of the invention and/or a recombinant sequence containing a portion of a region containing a recombinant DNA sequence according to the second aspect of the invention. Preferably, the expression system is selected from a Chinese hamster ovary epithelial (CHO) cell and an Escherichia coli cell.

In the context of the present invention, the term "eukaryotic or prokaryotic expression system" also includes the so-called protoplasts derived from this expression system.

In the fifth aspect, the subject of the invention is a recombinant hybrid protein EJ17zipR composed of 1 ZIP domain, 56 elastin domains, 10 silk fibroin domains, 7 RGD domains, 56 elastin domains,
5 10 silk fibroin domains, 7 RGD domains.

Preferably, the amino acid sequence of the recombinant EJ17zipR hybrid protein of the invention contains 12-17% proline and/or 20-30% glycine.

Preferably, the molecular weight of the recombinant hybrid protein EJ17zipR according to the invention is 86 kDa.

10 Preferably, the recombinant EJ17zipR hybrid protein of the invention has the amino acid sequence of SEQ #4.

Preferably, the recombinant EJ17zipR hybrid protein of the invention is a recombinant protein fragment.

Preferably, the recombinant EJ17zipR hybrid protein of the invention is at least partially
15 methacrylated.

In a sixth aspect, the invention provides a recombinant DNA sequence selected from:

a recombinant DNA sequence encoding the recombinant hybrid protein EJ17zipR as defined above,

a recombinant DNA sequence comprising a region containing a DNA sequence encoding the
20 recombinant hybrid protein EJ17zipR as defined above, and

a recombinant DNA sequence hybridizing to a DNA sequence encoding the recombinant hybrid protein EJ17zipR as defined above.

Preferably, the recombinant DNA sequence of the sixth aspect of the invention has a sequence that is at least 80% identical, more preferably at least 90% identical, and most preferably homologous to
25 the recombinant DNA sequence of SEQ #2.

In a seventh aspect, the invention provides a vector including a recombinant DNA sequence containing a region comprising the recombinant DNA sequence according to the sixth aspect of the invention and/or a recombinant sequence containing a portion of a region comprising the recombinant DNA sequence according to the sixth aspect of the invention. Preferably, the vector
30 according to the invention is a plasmid containing a promoter derived from phage T7, preferably selected from pET11a-d, pET15b, pET19b, pET28a-c(+), pET21a-d(+), pET22b(+), pET23a-d (+), pEt25b(+), pET44a-c(+), pET46Ek/LIC, and most preferably being pET11a.

In an eighth aspect, the invention provides a eukaryotic or prokaryotic expression system transformed with a recombinant DNA sequence containing a region comprising a recombinant DNA sequence according to the sixth aspect of the invention and/or a recombinant sequence containing a portion of a region comprising a recombinant DNA sequence according to the sixth aspect of the invention.

- 5 Preferably, the expression system is selected from a Chinese hamster ovary epithelial (CHO) cell and an Escherichia coli cell.

In the ninth aspect, the invention provides the use of a recombinant hybrid protein selected from the RE15mR protein according to the first aspect of the invention and the EJ17zipR protein according to the fifth aspect of the invention as a component of a bioprinting ink.

- 10 In a tenth aspect, the invention provides the use of a recombinant hybrid protein selected from the RE15mR protein according to the first aspect of the invention and the EJ17zipR protein according to the fifth aspect of the invention as a biomaterial component.

- In an eleventh aspect, the invention provides the use of a recombinant DNA sequence according to the second aspect of the invention, or a vector according to the third aspect of the invention, or a eukaryotic or prokaryotic expression system according to the fourth aspect of the invention, for
15 producing a recombinant RE15mR hybrid protein according to the first aspect of the invention.

- In a twelfth aspect, the invention provides the use of a recombinant DNA sequence according to the sixth aspect of the invention, or a vector according to the seventh aspect of the invention, or a eukaryotic or prokaryotic expression system according to the eighth aspect of the invention, for
20 producing a recombinant hybrid protein EJ17zipR according to the fifth aspect of the invention.

Both proteins according to the invention, i.e. recombinant hybrid proteins RE15mR and EJ17zipR, are chimeric structural proteins containing sequences borrowed from elastin, i.e. reproducing natural elastin pentamers. Consequently, both of these proteins are classified as elastin-like proteins (ELPs).

Advantages of the invention

- 25 The invention is used in the bioprinting process. The produced proteins can be an addition to natural bioinks (e.g. based on dECM – decellularized extracellular matrix; mixtures of single proteins / natural compounds) and synthetic ones, as an additive that improves cell survival, proliferation and adhesion. Due to the individual domains in the designed proteins according to the invention, it is also possible to use them as a basic component of the bioink (and not only as an additive, as described
30 above).

Due to their structure (mainly collagen and elastin domains), the proteins according to the invention can be used in broadly defined regenerative medicine, which also uses other techniques (other than 3D bioprinting) for biomaterial production.

In addition to bioinks and biomaterials for the production of bionic organs and tissue systems, the proteins according to the invention can be used in regenerative medicine, e.g. as medical dressings or their components.

5 The proteins according to the invention will be used to create biomaterials in both methacrylated and non-methacrylated forms, which will definitely expand the possibility of their use in final products.

The proteins according to the invention have many benefits, the most important of which is the improvement of the physico-chemical and biological properties of bioprinted 3D tissue models, bionic organs and biomaterials used both in scientific research and regenerative medicine, e.g. by improving cell adhesion and growth, improving stiffness, degradation rate, elasticity or flexibility of
10 printouts.

Brief description of the figures of drawing

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

Fig. 1 shows a diagram of the RE15mR recombinant hybrid protein

15 Fig. 2 shows a diagram of the EJ17zipR recombinant hybrid protein

Fig. 3 shows tables with primer sequences for cloning and sequencing genes encoding recombinant hybrid proteins

Fig. 4 shows the DNA sequence SEQ #1 encoding the RE15mR recombinant hybrid protein

Fig. 5 shows the DNA sequence SEQ #2 encoding the EJ17zipR recombinant hybrid protein

20 Fig. 6 shows the amino acid sequence of the RE15mR recombinant hybrid protein

Fig. 7 shows the amino acid sequence of the EJ17zipR recombinant hybrid protein

Fig. 8 shows the map of the expression vector with the cloned gene coding the RE15mR hybrid protein.

25 Fig. 9 shows the map of the expression vector with the cloned gene coding the EJ17zipR hybrid protein.

Fig. 10 shows an image of the SDS-PAGE separation of samples taken from subsequent stages of obtaining the recombinant hybrid protein RE15mR

Fig. 11 shows an image of the SDS-PAGE separation of samples taken from subsequent stages of obtaining the recombinant hybrid protein EJ17zipR

30 Fig. 12A shows the ¹H NMR spectrum of the RE15mR protein before methacrylation

Fig. 12B shows the ¹H NMR spectrum of the RE15mR protein after methacrylation

Fig. 12C shows the ^1H NMR spectrum of the methacrylated RE15mR protein (from batch 15_E_003)

Fig. 12D shows the ^1H NMR spectrum of the EJ17mR protein before methacrylation

Fig. 12E shows the ^1H NMR spectrum of the EJ17mR protein after methacrylation

Fig. 13A shows the level of fluorescence in AlamarBlue staining of L929 cells on plates coated with RE15mR protein compared to fibronectin in the L929 cell proliferation assay

Fig. 13B shows the level of fluorescence in AlamarBlue staining of HUVEC cells on plates coated with RE15mR protein compared to fibronectin in the HUVEC cell proliferation assay

Fig. 14A shows the level of fluorescence in L929 cell cultures on plates coated with RE15mR protein compared to fibronectin in the L929 cell adhesion assay

Fig. 14B shows the level of fluorescence in HUVEC culture plates coated with RE15mR protein compared to fibronectin in the HUVEC cell adhesion assay.

Fig. 15 shows the level of fluorescence in the culture of L929 cells on plates coated with RE15mR protein compared to fibronectin in the cytotoxicity test of RE15mR protein against L929 cells.

Fig. 16 shows photos of samples in the fluorescence study of HUVEC and HDF α cells stained with calcein AM and ethidium homodimer after 24, 48 and 96 hours in the contact study of the RE15mR protein blend.

Fig. 17 shows the results of absorbance measurement in the MTT assay in the study of cytotoxicity of the RE15mR protein against cells of the reference line L929

Fig 18A shows the level of fluorescence in AlamarBlue staining of L929 cells on plates coated with EJ17zipR protein compared to fibronectin in the L929 cell proliferation assay

Fig18B shows the level of fluorescence in AlamarBlue staining of HUVEC cells on EJ17zipR protein-coated plates compared to fibronectin in the HUVEC cell proliferation assay

Fig 19A shows the level of fluorescence in L929 cell cultures on plates coated with EJ17zipR protein compared to fibronectin in the L929 cell adhesion assay.

Fig 19B shows the level of fluorescence in HUVEC culture plates coated with EJ17zipR protein compared to fibronectin in the HUVEC cell adhesion assay.

Fig. 20 shows the level of fluorescence in the culture of L929 cells on plates coated with the EJ17zipR protein compared to fibronectin in the cytotoxicity test of the EJ17zipR protein against L929 cells.

Fig. 21 shows photos of samples in the fluorescence study of HUVEC and HDF α cells stained with calcein AM and ethidium homodimer after 24, 48 and 96 hours in the contact study of the blend with the EJ17zipR protein.

Fig. 22 shows the results of absorbance measurement in the MTT assay in the study of cytotoxicity of the EJ17zipR protein against cells of the reference line L929

Fig. 23 shows the results of rheological measurements of the tested materials based on GelMa containing the RE15mR recombinant protein, where A - gel point, B - complex modulus, C - dynamic viscosity

Fig. 24 shows the result of assessing the printability of GelMa-based biomaterials containing the RE15mR recombinant protein, where A – results of the fiber fusion test, B – results of the fiber collapsing test

Fig. 25 shows the mechanical parameters of bioinks based on 10%GelMa containing the RE15mR protein, where A - mechanical strength, B - Young's modulus, C - conventional yield strength

Fig. 26 shows the result of determining the degree of water absorption of GelMa-based materials containing the RE15mR recombinant protein.

Fig. 27 shows the results of rheological measurements of the tested material containing dECM and RE15mR protein, where A - gel point, B - complex modulus, C - dynamic viscosity

Fig. 28 shows the result of the assessment of the printability of biomaterials containing dECM and the RE15mR recombinant protein, where A – results of the fiber fusion test, B – results of the fiber collapse test

Fig. 29 shows the mechanical parameters of bioinks containing dECM and recombinant RE15mR protein, where A - mechanical strength, B - Young's modulus, C - conventional yield strength

Fig. 30 shows the result of determining the degree of water absorption of the tested material containing dECM and RE15mR protein

Fig. 31 shows the results of rheological measurements of the tested materials based on GelMa containing the EJ17zipR recombinant protein, where A - gel point, B - complex modulus, C - dynamic viscosity

Fig. 32 shows the result of the assessment of the printability of GelMa-based biomaterials containing the EJ17zipR recombinant protein, where A – results of the fiber fusion test, B – results of the fiber collapse test

Fig. 33 shows the mechanical parameters of bioinks based on 10%GelMa containing the EJ17zipR protein, where A - mechanical strength, B - Young's modulus, C - conventional yield strength

Fig. 34 shows the determination of the degree of water absorption of GelMa-based materials containing the EJ17zipR recombinant protein.

Fig. 35 shows the results of rheological measurements of the tested material containing dECM and EJ17zipR protein, where A - gel point, B - complex modulus, C - dynamic viscosity

Fig. 36 shows the result of the assessment of the printability of the biomaterial containing dECM and the EJ17zipR protein, where A – results of the fiber fusion test, B – results of the fiber collapse test

Fig. 37 shows the mechanical parameters of bioinks containing dECM and EJ17zipR protein, where A - mechanical strength, B - Young's modulus, C - conventional yield strength

- 5 Fig. 38 shows the result of determining the degree of water absorption of the tested material containing dECM and EJ17zipR protein

Detailed description of the invention

The invention is related to recombinant structural proteins obtained by genetic engineering using an eukaryotic or prokaryotic expression system, preferably the epithelium of the Chinese hamster ovary
 10 CHO or Escherichia coli. The recombinant structural proteins according to the invention are composed of domains of the structural proteins resilin, elastin and silk fibroin. Preferably, the resilin domains in the invention are represented by the sequences: SDTYGAPGGGNGGRP, GGRPSDSYGAPGGGN, GGRPSDSF(or M)GAPGGGN, PGGGNGGRPSTDYGA, GGRPSSSYGAPGQGN, GGRPSDSFGAPGGGN, GAPAQTPSSQY, AQTTPSSQYGAP, and most
 15 preferably GGRPSDSYGAPGGGN. Elastin domains are preferably represented by the sequences: VPGXG (wherein X = V, I, A) or JGGZG (wherein J, Z = V, L, A), and most preferably VPGIG and VPGAG. Silk fibroin domains are preferably represented by the sequences: GAGAGS. The recombinant hybrid proteins of the invention are enriched with functional domains, preferably:

- the RGD motif of the fibronectin cell adhesion sequence; most preferably AVTGRGDSPASS, and
 20 optionally a shortened GRGDSP or an extended TVYAVTGRGDSPASS;
- an MMP motif recognized by matrix metalloproteinases, most preferably GPQGIWQG;
- lysine-rich K⁺ cross-linking domains enabling chemical modifications of recombinant structural proteins; most preferably GGKGGKGGKGG;
- ZIP domains encoding the stabilizing supramolecular structure of the leucine zipper; most
 25 preferably

VGGGGGKENQIAIRASFLEKENSALRQEVADLRKELGKCKNILAKYEAGGGGG.

Without departing from the scope of the present invention, one skilled in the art is able to make quantitative or qualitative changes to the functional domains to adjust the properties of the recombinant hybrid proteins of the invention.

- 30 Enriching the sequence of recombinant hybrid proteins with RGD domains binding integrins, derived from fibronectin, promotes the adhesion of many types of endothelial cells, smooth muscle cells and fibroblasts, so that the biomaterials enriched with recombinant hybrid proteins have a positive effect on the growth of cells having physical contact with them. Metalloproteinase-sensitive MMP

sequences, derived from the human alpha(I) collagen chain, were added to recombinant structural proteins to promote proteolytic degradation associated with the possibility of extracellular matrix rearrangement by proliferating cells. The presence of the K⁺ motif is intended to functionalize the peptides with selected chemical groups to facilitate the controlled cross-linking of biomaterials enriched with recombinant hybrid proteins, desired in bioprinting. Enrichment of recombinant structural proteins with a ZIP sequence from the hepatic leukaemia factor (HLF) dimerization domain is expected to provide structural stability during printing, due to the ability to form amphiphilic alpha helical structures based on hydrophobic interactions.

The use of the described domain combinations according to the invention allows to utilize such developed protein in the polymeric biomaterial remodelling by cells, but also due to its general importance as a collagenase substrate: to mediate cell invasion, migration, proliferation, and growth of new tissues. The obtained recombinant hybrid proteins will enable modulating cell growth by fine-tuning the RGD density and introducing MMP-sensitive domains in the created hydrogels, which are to simulate the extracellular matrix (ECM).

15 EXAMPLES

EXAMPLE 1

The recombinant RE15mR hybrid protein is composed sequentially of 3 resilin domains, 1 K⁺ domain, 3 resilin domains, 1 MMP domain, 3 elastin domains, 1 K⁺ domain, 4 elastin domains, 1 K⁺ domain, 3 elastin domains, 1 K⁺ domain, 4 elastin domains, 1 K⁺ domain, 3 elastin domains, 1 MMP domain, 7 RGD domains.

Fig. 1 shows a diagram of the RE15mR recombinant hybrid protein, Fig. 4 shows the SEQ #1 DNA sequence encoding the RE15mR recombinant hybrid protein, and Fig.6 shows the SEQ #3 amino acid sequence of the RE15mR recombinant hybrid protein.

According to a preferred embodiment, the amino acid sequence of the RE15mR recombinant hybrid protein contains 12-15% proline and 32-38% glycine, and the molecular weight of the protein is approximately 26 kDa.

EXAMPLE 2

The recombinant hybrid protein EJ17zipR is composed of 1 ZIP domain, 56 elastin domains, 10 silk fibroin domains, 7 RGD domains, 56 elastin domains, 10 silk fibroin domains, and 7 RGD domains. Fig. 2 shows a diagram of the recombinant EJ17zipR hybrid protein, Fig. 5 shows the DNA sequence of SEQ #2 encoding the recombinant hybrid protein of EJ17zipR, and Fig. 7 shows the amino acid sequence of SEQ #4 of the recombinant hybrid protein of EJ17zipR.

According to a preferred embodiment, the amino acid sequence of the recombinant hybrid protein EJ17zipR contains 12-17% proline and 20-30% glycine, and the molecular weight of this protein is about 86 kDa.

The protein should oligomerize into molecules larger than 1 mDa (verification method - polyacrylamide gel electrophoresis in native conditions).

EXAMPLE 3

To obtain recombinant hybrid proteins according to the invention, a plasmid vector containing a promoter derived from phage T7, namely pET11a, was used. Sequences encoding the recombinant hybrid proteins of the invention, namely, DNA sequence SEQ #1 encoding the recombinant hybrid protein RE15mR (Fig. 4) or DNA sequence SEQ #2 encoding the recombinant hybrid protein EJ17zipR (Fig. 5), were incorporated into the vector using methods known in the art techniques of molecular cloning methods using selected restriction sites.

In particular, the restriction sites were NdeI and BamHI. The cloned sequences were amplified using selected primers with the sequences given in Fig. 3. An expression vector was obtained enabling efficient and stable expression of recombinant proteins in E. coli cells from the BLR(DE3) strain.

The map of the expression vector with the cloned gene coding for the RE15mR hybrid protein is shown in Fig. 8, and the map of the expression vector with the cloned gene coding for the EJ17zipR hybrid protein is shown in Fig. 9.

EXAMPLE 4

Recombinant hybrid proteins RE15mR and EJ17zipR were obtained by a method comprising the following steps:

1. Culture of E. coli bacterial cells of the BLR(DE3) strain [optional BL21(DE3)] transformed with a plasmid encoding recombinant hybrid proteins with the addition of an appropriate antibiotic (preferably ampicillin 50-200 µg/ml). The method uses conventional culture media, selected depending on the host strain used. For the BL21(DE3) and BLR(DE3) strains used, it can be a standard, rich LB medium, additionally supplemented with proline and glycine. Cultures in bioreactors use mineral medium, the composition of which was originally developed during the experiments leading to the development of the invention. The composition of the medium used is shown in Table 1 below:

Table 1: Composition of culture media

<i>Enriched mineral substrate:</i>	
MgSO ₄ ·7H ₂ O	0.20 g/L
K ₂ HPO ₄	10.0 g/L
NaH ₂ PO ₄ ·4H ₂ O	4 g/L

Acetic acid	0.16 g/L
CaCl ₂ ·2H ₂ O	0.04 g/L
Glucose	0.2 %
Trace salts	3 ml/L
<i>Composition of trace salts:</i>	
Ammonium iron(III) citrate	0.15 g/L
MnSO ₄ ·H ₂ O	1.00 g/L
ZnSO ₄ ·7H ₂ O	2.78 g/L
CoCl ₂ ·6H ₂ O	1.86 g/L
Na ₂ MoO ₄ ·2H ₂ O	2.00 g/L
CaCl ₂ ·6H ₂ O	4.50 g/L
CuSO ₄ ·5H ₂ O	1.85 g/L
H ₃ BO ₃	0.50 g/L
HCl	3.2 %
<i>Amino acids and aromatic compounds:</i>	
Proline	0.2-0.8 mg/mL
Glycine	0.5-1.0 mg/mL

Growing cultures using this medium, especially large-scale cultures in a bioreactor, presents the advantage of relatively low cost, while ensuring a satisfactory growth level (comparable to the level of growth in media used in laboratory-scale cultures). Bacterial biomass is produced in the first phase of culture, and culture parameters for this phase are: temperature: 30 °C, agitation: 150-700 rpm, aeration: 5-10 LPM (litres per minute), DO (dissolved oxygen, i.e. the amount of oxygen dissolved in a fluid): >20%, pH: 7.1 +/-1. Culture is carried out until the optical density OD₆₀₀ of 0.7-0.9 is reached. Next, the induction of expression of recombinant hybrid proteins is carried out by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a concentration of 0.4-1.0 mM, or lactose to a concentration of 5-20 mM, and the culture is carried out under the following conditions: temperature: 37 °C, agitation: 500-700 rpm, aeration: 7-10 LPM, DO: >20%, pH: 7.1 +/-1 for 5-7 hours until an optical density of OD₆₀₀ 3.0 - 9.0 is reached.

2. At this stage, the bacterial biomass is separated from the culture medium by centrifugation. The bacterial cells are then suspended in a lysis buffer formulated for this purpose, shown in Table 2 below.

Table 2: Lysis buffer composition

NaCl	0.5 M
Tris HCl pH 7,5	50 mM
EDTA pH 8,0	10 mM
β -mercaptoethanol	5 mM
Lysozyme	0.35 mg/ml
PMSF	1%
Benzamidine	10 mM

Bacterial biomass disintegration carried out using a high-pressure flow disintegrator, with a set pressure of 800-900 bar. Over the course of work, it is preferable to perform 3 to 4 disintegration rounds and add 0.4% (w/v) polyethyleneimine (PEI) to the suspension to precipitate the host DNA.

3. Separation of insoluble protein fractions, non-disintegrated bacterial cells and precipitated DNA
5 from the supernatant containing recombinant hybrid proteins by centrifugation.

4. Incubation of the supernatant containing the recombinant hybrid proteins at 85-95°C for 15-30 min, and centrifugation of the denatured proteins. During the conducted experiments, it was also shown that it is advantageous to add a protease inhibitor cocktail to the recombinant protein solution at this stage (preferably cOmplete, EDTA-free, Roche, Cat. 05056489001) in the amount of 1 tablet
10 per 50 mL of solution.

5. Precipitation of recombinant protein from solution by adding ammonium sulfate to 10% saturation (for EJ17zipR protein) or 20-40% saturation (for RE15mR protein) at room temperature, followed by centrifugation of precipitated proteins and dissolution in 20-40 mM TRIS buffer with 10 mM EDTA pH 8.0.

15 6. Dialysis of the recombinant protein suspension into 20-40 mM TRIS buffer with 10 mM EDTA pH 8.0 for 24-48 hours at 4 °C.

7. Protein purification on Macro-Prep High Q Media (Bio-Rad). The resin-filled column was equilibrated with a calibration buffer with the following composition: 20-50 mM TRIS buffer pH 8.0. The protein solution obtained as a result of salted-out protein dissolution was applied to the
20 column equilibrated as such. The separation was carried out by FPLC (Fast Protein Liquid Chromatography). Proteins not bound to the matrix were washed away with the calibration buffer. The proteins bound to the resin were eluted with an elution buffer composed of 20-50 mM TRIS buffer pH 8.0 + 1 M NaCl. A flow of 1-2 mL/min was used during the separation, and fractions above the absorbance of 0.05 AU (absorbance units) were collected. The concentration of eluted protein
25 was determined by the Bradford and BCA method (Pierce BCA protein Assay Kit). The recombinant hybrid proteins RE15mR and EJ17zipR according to the invention did not bind to the resin and were eluted from the column with the calibration buffer.

8. Endotoxin removal, preferably using Pierce High-Capacity Endotoxin Removal Resin columns according to the manufacturer's instructions.

30 9. Dialysis of the recombinant protein suspension to ddH₂O for 24h-48h at 4 °C.

10. Lyophilization of recombinant hybrid protein.

The method described above is characterized in that the obtained recombinant RE15mR protein is able to gel at a concentration above 200 mg/mL at 4°C, with the addition of 0.5M NaCl. The recombinant EJ17zipR protein does not form hydrogels. The yield of this method is 20-70 mg of

recombinant RE15mR and EJ17zipR protein from 1 litre of culture. The course of the purification process for the recombinant RE15mR structural protein in terms of the product profile, compared to impurities in SDS-PAGE electrophoresis under denaturing conditions, is shown in Fig. 10, which shows an image of the SDS-PAGE separation of samples taken from subsequent stages of obtaining the recombinant hybrid protein RE15mR, where in image A the numbers indicate: 1 - E. coli BLR(DE3) culture before induction; 2 - E. coli BLR(DE3) culture after IPTG induction; 3 - LMW protein mass standard; 4 - supernatant after sonication; 5 - precipitate after sonication; 6 - supernatant after sonication and incubation at 90°C; 7 - sediment after sonication and incubation at 90°C; 8 - supernatant after sonication, incubation and salting-out with ammonium sulfate 0-20% saturation; 9 - sediment after sonication, incubation and salting-out with ammonium sulfate 0-20% saturation; 10 - supernatant after sonication, incubation and salting-out with ammonium sulfate 20-40% saturation; 11 - sediment after sonication, incubation and salting-out with ammonium sulfate 20-40% saturation; 12 - supernatant after dialysis I; 13 - precipitate after dialysis I, and in image B the numbers mean: 1 - LMW protein mass standard; 2 - entry to the Macro-Prep High Q Media deposit; 3 - fractions not related to the Macro-Prep High Q Media resin 4 - fractions bound to the Macro-Prep High Q Media resin 5 - fractions entering the Pierce High-Capacity Endotoxin Removal Resin deposit; 6 - fractions eluted from the High-Capacity Endotoxin Removal Resin 7, 8, 9, 10 - final protein solution intended for freeze-drying.

The course of the purification process for the recombinant structural protein EJ17zipR In terms of the product profile against impurities in SDS-PAGE electrophoresis under denaturing conditions is illustrated in Fig. 11, which shows an image of the SDS-PAGE separation of samples taken from subsequent stages of obtaining the recombinant hybrid protein EJ17zipR, where in the image A the numbers mean: 1-- E. coli BLR(DE3) culture before induction; 2-- E. coli BLR(DE3) culture after induction; 3-- Broad Multi Color protein mass standard; 4-- supernatant after sonication; 5-- precipitate after sonication; 6-- supernatant after sonication and incubation at 90°C; 7-- sediment after sonication and incubation at 90°C; 8-- supernatant after sonication, incubation and salting-out with ammonium sulfate 0-10% saturation; 9 - sediment after sonication, incubation and salting-out with ammonium sulfate 0-10% saturation; 10- supernatant after dialysis I; 11 - precipitate after dialysis I, and in image B the numbers indicate 1 - LMW protein mass standard; 2 - entry to the Macro-Prep High Q Media resin 3 - fractions not related to the Macro-Prep High Q Media resin 4 - fractions bound to the Macro-Prep High Q Media resin; 5 - fractions entering the Pierce High-Capacity Endotoxin Removal Resin ; 6 - fractions eluted from the High-Capacity Endotoxin Removal Resin; 7, 8, 9, 10 - final protein solution intended for freeze-drying.

EXAMPLE 5

Recombinant hybrid proteins of the invention RE15mR and EJ17zipR were subjected to the methacrylation process. In a preferred embodiment, the method comprises a method comprising the following steps:

- 5 1. The recombinant hybrid protein according to the invention from the batch to be subjected to the methacrylation process was weighed on an analytical balance into a reaction vessel with a capacity of X mL.

Note: All masses should be recorded to 3 significant numbers. The selection of the capacity of the reaction vessel (X mL) depends on the volume of the final reaction mixture, which should not exceed
10 60% of the total capacity of the vessel.

2. The reaction vessel was equipped with a mixing element and placed over a magnetic stirrer. Using an automatic pipette, X mL of PBSx1 buffer was dispensed into it to obtain a 2% (w/v) solution. The stirrer speed was set in the range from 200 to 1500 rpm, in the preferred embodiment at approximately 1000 rpm. The mixture was left to stir continuously at room temperature until the
15 substrate was completely dissolved. (usually about 10 minutes).

Note: Instead of PBSx1, any buffer that maintains the pH at neutral pH or above the protein isoelectric point can be used.

3. After the substrate has completely dissolved, the reaction vessel is placed in an ice-water bath (crystallizer filled with water and ice to half its volume to maintain the lability of the liquid phase)
20 and protected from light. (for example by wrapping the whole thing in aluminum foil). Expected bath temperature $\leq 4^{\circ}\text{C}$.

4. X mL of methacrylic anhydride (MMA) or other electrophilic reagent was measured using an automatic pipette and added to the reaction.

Note: 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 anhydride (MMA) used for
25 the reaction.

In the case of protein methacrylation reactions, it is necessary to select the appropriate amount of methacrylic anhydride (or other nucleophilic reagent) each time, taking into account the series of a given protein. To do this, you need to read the amino acid sequence of the protein and read how many
30 lysine residues are in the protein chain. Then, taking into account the total mass of 1 mole of protein and the mass weighed into the reaction, calculate how much methacrylic anhydride should be used at the ratio of free lysine amino groups to anhydride of $n_{\text{NH}_2}:n_{\text{MMA}} = 1:1$. The following formula should be used in calculations - if the nucleophilic reagent is in a liquid state:

$$v_{Nu} = \frac{\frac{m_{protein}}{M_{protein}} \cdot n_{lysine} \cdot M_{Nu}}{d_{Nu}} \cdot 1000$$

wherein:

v_{Nu} – nucleophilic agent volume [μ l]

$m_{protein}$ – mass of protein weighed for the reaction [g]

5 $M_{protein}$ – protein molar mass [$\frac{g}{mol}$]

n_{lysine}

– the number of lysine residues found in the amino acid chain of 1 mole of protein

M_{Nu} – molar mass of the nucleophilic reagent [$\frac{g}{mol}$] methacrylic anhydride 154.16 $\frac{g}{mol}$

d_{Nu} – density of the nucleophilic reagent [$\frac{g}{ml}$]; methacrylic anhydride 1.035 $\frac{g}{ml}$

10 or from the formula below - if the nucleophilic reagent is in the solid state:

$$m_{Nu} = \frac{m_{protein}}{M_{protein}} \cdot n_{lysine} \cdot M_{Nu} \cdot 1000$$

wherein:

m_{Nu} – nucleophilic agent volume [mg]

$m_{protein}$ – mass of protein weighed for the reaction [g]

15 $M_{protein}$ – protein molar mass [$\frac{g}{mol}$]

n_{lysine}

– the number of lysine residues found in the amino acid chain of 1 mole of protein

M_{Nu} – molar mass of the nucleophilic reagent [$\frac{g}{mol}$]

The amount of methacrylic anhydride used is a parameter controlling the degree of substitution.

20 Depending on the expected degree of methacrylation, it is possible to use a 1- to 10-fold excess of anhydride relative to the free amino groups of lysine. For this purpose, the volume of anhydride calculated from the above formula should be multiplied by a number in the range 1-10.

In addition to the amino groups of lysine, other amino acid units such as tryptophan, tyrosine, lysine, threonine, serine or arginine may also undergo methacrylation, provided they are present in the amino acid sequence. When calculating the degree of protein substitution, remember to take them into account. Based on the calculations made, in order to methacrylate individual proteins (taking into account the ratio of methacrylic anhydride to lysine amino groups 1:1), the following should be added dropwise:

- RE15mR protein RE15mR ($M_{protein} = 25670 \frac{g}{mol}$; $n_{lysine} = 7$): 0.0406 μ l of methacrylic acid anhydride per 1 mg of protein
- 30 • EJ17zipR protein EJ17zipR ($M_{protein} = 85940 \frac{g}{mol}$; $n_{lysine} = 6$): 0.0104 μ l of methacrylic acid anhydride per 1 mg of protein

5. The reaction was carried out under the given conditions ($T \leq 4^{\circ}\text{C}$, stirring in the range from 200 to 1500 rpm, in the preferred embodiment at approx. 1000 rpm) until reaction, preferably for 24 h.
6. After the required reaction time, the PBSx1 solution was added to the mixture in portions until the post-reaction mixture was diluted 5 times (1:4 mixture:PBSx1).
- 5 7. The resulting solution was poured into dialysis tubing according to the following procedure.
- a) Measure and cut the appropriate length of dialysis tube
- b) Place the tubes in a beaker with demineralized water and leave for 10 minutes, then replace the water with fresh water, soak and leave for 10 minutes, and finally soak in a 40% ethanol-water solution for 10 minutes.
- 10 c) Gently remove the soaked tube from the beaker and tie it at one end. Place a funnel in its open end and then pour a small amount of water to check the tightness of the tube.
- d) If there are no leaks, remove the water from the tube and then quantitatively transfer the solution to it, washing the reaction vessel with 3x1 mL of deionized water.
- e) Tie the tube and secure it with a clip on both sides, and then place it in a 5 L beaker.
- 15 f) Fill the beaker with deionized water (approx. 4 L) and place it in the refrigerator.
8. The dialysis process was carried out at a temperature of 4°C for 2 days, changing the water once a day.
9. After the dialysis process was completed, the solution from the dialysis tubes was transferred quantitatively to a large beaker, and then approximately 5 mL (using an automatic pipette) to glass
- 20 vials.
- Note: Each empty glass vial should be weighed and its mass recorded as m_1 . Then transfer the solution to it, weigh it again and describe the mass as m_2 .
10. The vials of solution were placed in a freezer at -80°C and frozen, preferably for a minimum of 3 hours. Then it was freeze-dried according to the following parameters:
- 25 a) shelf temperature: 0°C
- b) pressure: 0.100 mbar
- c) duration: 48 h
11. The vials with the obtained lyophilisates were weighed and the mass was described as m_3 . Then, to determine the mass of the lyophilisate in each vial, $\Delta m = m_3 - m_1$ was calculated.
- 30 12. Vials of material were stored at -20°C .
13. The final product was analyzed for the degree of substitution using ^1H NMR spectroscopy.

Results of analysis of the degree of substitution with methacrylic groups using ^1H NMR spectroscopy for the RE15mR protein

The approximate degree of substitution was determined for the RE15mR protein. Fig. 12A shows the ^1H NMR spectrum of the protein before the methacrylation process, where no signals are observed in the 5 ppm-6.5 ppm chemical shift region. Fig. 12B shows the ^1H NMR spectrum of a protein after methacrylation, in which a 2-fold excess of methacrylic acid anhydride relative to the lysine amino groups was used for methacrylation. The degree of substitution was determined by comparing the signal integration from the tryptophan aromatic ring located at 7.47 ppm. The signal integration is 0.09 and corresponds to 2 amino acids per mole in the protein chain. The integration for the methacrylic group attached to tryptophan is also 0.09, which means that the tryptophan has been 100% substituted. Taking into account that there are 6 tyrosine, 7 lysine, 7 threonine, 31 serine and 13 arginine units per mole in the protein chain, the signals from the methacrylic groups attached to individual amino acids were integrated, compared to the tryptophan signals and on this basis the degree of substitution (DS) was calculated) methacrylic groups. The spectrum shows the presence of methacrylic groups attached to tryptophan (6.34 ppm and 5.90 ppm) DS ~ 100%, tyrosine (5.66 ppm and 5.42 ppm) DS ~ 100% and lysine (5.81 ppm and 5.53 ppm) DS~100%. Moreover, in the area of 5.68-5.76 ppm and 5.44-5.50 ppm, overlapping signals of protons of methacrylic groups attached to threonine, serine and arginine are observed. However, the degree of substitution for individual amino acids cannot be determined unambiguously due to overlapping signals and low spectral resolution caused by the low degree of functionalization of the remaining amino acids and effects related to the size of the protein molecule.

Fig. 12C shows the ^1H NMR spectrum of the methacrylated RE15mR protein (from batch 15_E_003), in which a 10-fold excess of methacrylic acid anhydride relative to the lysine amino groups was used for methacrylation. The spectrum shows methacrylic groups attached to the following amino acids: tryptophan (6.34 ppm and 6.01 ppm), tyrosine (5.66 ppm and 5.41 ppm), lysine (5.81 ppm and 5.53 ppm), threonine (5.90 ppm and 5.68 ppm), serine (5.64 ppm and 5.31 ppm) and arginine (6.11 ppm and 5.73 ppm).

Results of analysis of the degree of substitution with methacrylic groups using ^1H NMR spectroscopy for the EJ17zipR protein

Fig. 12D shows the ^1H NMR spectrum of the protein before the methacrylation process, where no signals are observed in the 5 ppm-6.5 ppm region. Fig. 12E shows the ^1H NMR spectrum of a protein after methacrylation, in which a 2-fold excess of methacrylic acid anhydride relative to the lysine amino groups was used for methacrylation. In the spectrum, one can observe the presence of methacrylic groups, signals coming from protons at the double bond at a shift of 5.73 ppm and 5.39 ppm, and protons from the methyl group at a shift of 1.18 ppm. At 7.27 ppm and 7.21 ppm, signals

of aromatic protons of phenylalanine located in the protein chain are observed. The integral of 2.48 of this signal corresponds to 145 aromatic protons, which are located in the rings of 29 phenylalanine units per mole in the protein structure. The signal integration of methacrylic protons is 0.12. Taking into account the number of protons coming from phenylalanine, it was determined that 6 methacrylic groups were attached to the protein chain per 1 mole of protein. However, it is not possible to clearly determine which of the amino acids the methacrylic groups were attached to due to the complexity of the obtained spectrum and its low resolution in the allyl region. Therefore, it is not possible to determine the exact degree of substitution specifying the site of functionalization. However, due to the chemical shift, it was assumed that the result obtained corresponds to 100% substitution of lysine amino groups.

EXAMPLE 6

The biological activity of the recombinant hybrid proteins according to the invention was tested, including their effect on cell viability, proliferation, adhesion and cytotoxicity. Protocols, reagents and equipment routinely used in this type of research were used. The control in the study was fibronectin used for coating in an amount of 1 µg/cm². The negative control was the absence of any protein coating of the well.

Composition of the culture medium

DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS, 4 mM L-glutamine, 4.5 g/l glucose, 1 mM sodium pyruvate, 1500 mg/L sodium carbonate, and 50 I.U./ml penicillin and 50 µg/ml streptomycin.

Ready-made culture medium: Endothelial Cell growth kit – VEGF, ATCC Primary Cell Solutions, catalog number PCS-100-041

Dulbecco's Modified Eagle's medium - high glucose, Sigma, D6429

Cell lines used in the tests

L929 - mouse fibroblasts, adherent cells growing in a monolayer, ATCC cat. no. CCL-1

Human Umbilical Vein Endothelial Cells (HUVEC), after passage II, ATCC cat. no. PCS-100-010

Human Dermal Fibroblasts HDFα, ATCC code no. PCS-201-012

AlamarBlue staining reagent

AlamarBlue™ Cell Viability Reagent (Invitrogen, Cat. No. DAL1100); resazurin, active ingredient of the reagent, non-toxic blue compound; after entering living cells in a reducing environment, the conversion of resazurin to resorufin, which is red and strongly fluorescent, is induced. Indicator of the number of living, metabolically active cells.

Viability/cytotoxicity testing using the commercial LIVE/DEAD™ kit for mammalian cells (Invitrogen, cat. no. L3224)

Calcein AM and EthD-1 staining

- 5 Assessment of cell viability - calcein derivative staining. Non-fluorescent acetylmethoxycalcein (calcein AM) freely penetrates cell membranes and is enzymatically converted in the cell by intracellular esterases to the intensely fluorescent green calcein.

EthD-1 ethidium homodimer penetrates cells through damaged membranes and upon binding to nucleic acids, bright red fluorescence is enhanced forty times, which allows the detection of dead cells in the population.

- 10 The use of calcein AM / EthD-1 staining allows not only to qualitatively and quantitatively assess the share of live and dead cells in the population, but also to preserve their shape and location, because, unlike the MTT method (reference method with staining with formazan salts), it does not require cell disintegration to measurements (absorbance/fluorescence) and allows for microscopic observation in situ.

15 **6.1 Biological activity of the RE15mR protein**

Effect of RE15mR protein on cell proliferation

- 20 The RE15mR protein obtained from the E. coli DE3 BLR strain culture, after being purified and lyophilized was used to coat 96-well plates intended for culturing eukaryotic cells in suspension (unmodified surface). An aqueous solution of RE15mR protein was applied to the plates in the amount of 1 and 5 $\mu\text{g}/\text{cm}^2$ and dried. The control in the study was fibronectin used for coating in an amount of 1 $\mu\text{g}/\text{cm}^2$. The negative controls were wells not coated with protein. Cells of the L929 or HUVEC line were seeded on the prepared plates in an amount of 5×10^3 /well. Cells were incubated in dedicated culture medium for 2, 24 and 48 hours. After this time, the culture wells were washed with sterile phosphate-buffered saline (PBS buffer) to remove dead and non-adherent cells, the cells
25 were stained with alamarBlue reagent for three hours and fluorescence was measured at light wavelengths of 530 nm (excitation) and 590 nm (emission).

Fig. 13A shows the level of fluorescence in AlamarBlue staining of L929 cells on plates coated with RE15mR protein compared to fibronectin in the L929 cell proliferation assay. Fig.13B shows analogous results from a study using HUVECs.

- 30 The increase in fluorescence after 48 hours compared to 2 and 24 hours, taking into account the division time for the L929 cell line (Fig. 13A), indicates active cell proliferation both in the positive control and in the wells coated with the tested protein. It can therefore be concluded that the RE15mR protein does not interfere with the rate of cell proliferation used in the cell-line study. The same observation applies to the HUVEC study (Fig. 13B), but only for a coating of 1 $\mu\text{g}/\text{cm}^2$.

Effect of RE15mR protein on cell adhesion

The RE15mR protein obtained from the E. coli DE3 BLR strain culture, after being purified and lyophilized was used to coat 96-well plates intended for culturing eukaryotic cells in suspension (unmodified surface). An aqueous solution of RE15mR protein was applied to the plates in the amount of 1 and 5 $\mu\text{g}/\text{cm}^2$ and dried. The control in the study was fibronectin used for coating in an amount of 1 $\mu\text{g}/\text{cm}^2$. The negative control was the absence of any protein coating of the well. Cells of the L929 or HUVEC line were seeded on the prepared plates in an amount of 1×10^4 /well. Cells were incubated in dedicated culture medium for 2, 4 and 24 hours. After this time, the culture wells were washed with sterile phosphate-buffered saline (PBS buffer) to remove dead and non-adherent cells. In this case, washing was intended to remove non-adherent cells. Cells were stained with alamarBlue reagent for three hours and fluorescence was measured at light wavelengths of 530 nm (excitation) and 590 nm (emission).

Fig. 14A shows the level of fluorescence in L929 cell cultures on plates coated with RE15mR protein compared to fibronectin in the L929 cell adhesion assay. Fig. 14B shows analogous results from a study using HUVECs.

The increase in fluorescence during incubation (Fig. 14A) indicates the adhesion of cells to the plastic used, both in the positive control and in the wells coated with the tested protein. It was observed that the RE15mR protein promotes cell adhesion used in the cell line study to an extent comparable to fibronectin - a commercially available protein used to coat surfaces intended for eukaryotic cell culture. The same observation applies to the HUVEC study (Fig. 14B), but only for a coating of 1 $\mu\text{g}/\text{cm}^2$.

Cytotoxicity of the RE15mR protein against L929 cells

The RE15mR protein obtained from the E. coli DE3 BLR strain culture, after being purified and lyophilized was used to coat 96-well plates intended for culturing eukaryotic cells in suspension (unmodified surface). An aqueous solution of RE15mR protein was applied to the plates in an amount of 1 $\mu\text{g}/\text{cm}^2$ and dried. The control in the study was fibronectin used for coating, also in an amount of 1 $\mu\text{g}/\text{cm}^2$. Cells of the L929 line were seeded on the prepared plates in the amount of 2×10^4 /well, 1×10^4 /well and 5×10^3 /well. Cells were incubated in dedicated culture medium for 24, 48 and 72 hours. Then, the medium was removed from selected wells, the cells were flooded with 70% methanol and incubated for 30 minutes, obtaining a negative control in the form of a population of 100% dead cells. Cells were stained with calcein AM (1 μM) and ethidium homodimer (EthD1, 2 μM) for 30 min, and fluorescence was measured at light wavelengths of 485 and 530 nm (excitation) and 530 and 645 nm (emission).

Fig. 15 shows the level of fluorescence in the culture of L929 cells on plates coated with RE15mR protein compared to fibronectin in the cytotoxicity test of RE15mR protein against L929 cells. Based

on the increase in fluorescence during incubation (Fig. 15), it can be concluded that the RE15mR protein used to coat the culture plastic is not cytotoxic to L929 line cells to an extent comparable to fibronectin, commonly used for this purpose, regardless of the culture time.

Cytotoxicity of blends containing RE15mR protein against HUVEC cells

- 5 The cytotoxicity of the blends was tested using the "contact" method by seeding HUVEC cells mixed with HDF α in a 1:2 ratio onto wells covered with cross-linked material containing the recombinant blend protein.

After lyophilization, the RE15mR protein was used to prepare blends based on a 10% methacrylated gelatin solution with the addition of the tested protein in the amount of 0.5 or 0.1 mg/ml.

- 10 In the contact test, the obtained blend was coated on 48-well plates intended for culturing eukaryotic cells. A mixture of cell lines was sown on the prepared plates in a total amount of 2.5×10^5 /well, 1.25×10^5 /well and 0.625×10^5 /well. Cells were incubated in dedicated culture medium for 24, 48 and 96 hours.

- Cells were stained with calcein AM (1 μ M) and ethidium homodimer (EthD1, 2 μ M) for 30 minutes and green (live cells) and red (dead cell nuclei) fluorescence was observed using an Olympus IX83 microscope with CellSens software enabling photo documentation. A summary of photos for individual blends showing the fluorescence of stained cells at subsequent time points is shown in Fig. 16. The control were cells seeded on TC-modified plastic (plastic adapted for adherent cultures) and GelMa blends, further included photos from cultures on the surface of blends containing 0, 5 mg/ml RE15mR protein (B15H) and blends containing 0.1 mg/ml RE15mR (B15L). The photos were taken at 4x and 10x lens magnification.

- In a contact test performed on HUVEC and HDF α cells growing on the surface of blends with the addition of RE15mR protein, no cytotoxic effect of the tested protein was found at both tested concentrations. The cells were actively dividing, the number of dead cells was negligible and directly proportional to the total number of growing cells.

Cytotoxicity of the RE15mR protein towards L929 cells – absorbance measurement in the MTT test

- The cytotoxicity of the RE15mR protein towards L-929 cells (measurement of absorbance in the MTT test) was tested in accordance with the ISO 10993-5:2009(E) standard: Biological assessment of medical devices. Part 5: In vitro cytotoxicity studies. Depending on the planned exposure time, cells were seeded into 96-well plates at densities of 1×10^5 /ml and 5×10^4 /ml, respectively, 100 μ l per well.

Culture was carried out overnight under standard conditions (5% CO₂ and 37°C) in supplemented DMEM medium so that the fibroblasts had a chance to spread to the bottom of the culture vessel. The test was performed using the direct method, adding a solution of purified protein reconstituted

in the medium to the culture. After checking the confluence and population status, a protein solution in the medium was placed on the plates at a concentration of 1, 0.5 and 0.1 mg/ml in an amount of 100 μ l. The plate with cells at a density of 1×10^5 /ml was incubated for 24 hours, and the culture at the initial density of 5×10^4 /ml was exposed to the RE15mR protein for 48 hours.

- 5 After this time, the cells were incubated with the MTT reagent solution for 2 hours, then all the fluid above the cells was removed and the formed formazan salt crystals were dissolved with DMSO. The amount of colored product formed, proportional to the number of living cells, was determined by measuring absorbance at light wavelengths of 570 and 650 nm. According to the standard, the expected result is that the viability of cells exposed to a cytotoxic agent is not less than 70% of the
- 10 viability of untreated cells in the negative control.

Based on the results obtained, it was concluded that the RE15mR protein in the tested concentration range (0.1 - 1 mg/ml) at 24- and 48-hour exposure is not cytotoxic to the L-929 fibroblast line (Fig. 17).

6.2 Biological activity of the EJ17zipR protein

15 Effect of the EJ17zipR protein on cell proliferation

- The EJ17zipR protein obtained from the E. coli DE3 BLR strain culture, after being purified and lyophilized was used to coat 96-well plates intended for culturing eukaryotic cells in suspension (unmodified surface). An aqueous solution of the EJ17zipR protein was applied to the plates in the amount of 1 and 5 μ g/cm² and dried. The control in the study was fibronectin used for coating in an
- 20 amount of 1 μ g/cm². The negative control was the absence of any protein coating of the well. Cells of the L929 or HUVEC line were seeded on the prepared plates in an amount of 5×10^3 /well. Cells were incubated in dedicated culture medium for 2, 24 and 48 hours. After this time, the culture wells were washed with sterile phosphate-buffered saline (PBS buffer) to remove dead and non-adherent cells, the cells were stained with alamarBlue reagent for three hours and fluorescence was measured
- 25 at light wavelengths of 530 nm (excitation) and 590 nm (emission).

Fig. 18A shows the level of fluorescence in AlamarBlue staining of L929 cells on plates coated with EJ17zipR protein compared to fibronectin in the L929 cell proliferation assay. Figure 18B shows analogous results from a study using HUVECs.

- The increase in fluorescence after 48 hours compared to 2 and 24 hours, taking into account the division time for the L929 cell line (Fig. 18A), indicates active cell proliferation both in the positive
- 30 control and in the wells coated with the tested protein. The slightly lower signals obtained for the tested protein are probably the result of lower cell adhesion to the EJ17zipR protein than to fibronectin. Nevertheless, it can be concluded that the EJ17zipR protein does not interfere with the rate of cell proliferation used in the cell line study. The same observation applies to the HUVEC
- 35 study (Fig. 18B), but only for a coating of 1 μ g/cm².

Effect of the EJ17zipR protein on cell adhesion

The EJ17zipR protein obtained from the E. coli DE3 BLR strain culture, after being purified and lyophilized was used to coat 96-well plates intended for culturing eukaryotic cells in suspension (unmodified surface). An aqueous solution of the EJ17zipR protein was applied to the plates in the amount of 1 and 5 $\mu\text{g}/\text{cm}^2$ and dried. The control in the study was fibronectin used for coating in an amount of 1 $\mu\text{g}/\text{cm}^2$. The negative control was the absence of any protein coating of the well. Cells of the L929 or HUVEC line were seeded on the prepared plates in an amount of 1×10^4 /well. Cells were incubated in dedicated culture medium for 2, 4 and 24 hours. After this time, the culture wells were washed with sterile phosphate-buffered saline (PBS buffer) to remove dead and non-adherent cells. In this case, washing was intended to remove non-adherent cells. Cells were stained with alamarBlue reagent for three hours and fluorescence was measured at light wavelengths of 530 nm (excitation) and 590 nm (emission).

Fig. 19A shows the level of fluorescence in L929 cell cultures on plates coated with EJ17zipR protein compared to fibronectin in the L929 cell adhesion assay. Fig. 19B shows analogous results from a study using HUVEC cells.

The increase in fluorescence during incubation (Fig. 19A) indicates the adhesion of cells to the plastic used, both in the positive control and in the wells coated with the tested protein. It was observed that the EJ17zipR protein promotes cell adhesion used in the cell line study, although compared to fibronectin, the number of adhering cells is lower. The effect of the concentration of the protein used on the adhesion of the cells used is clearly visible - as the protein concentration increases, the number of adhering cells increases after just 24 hours of culture. The same observation applies to the HUVEC study (Fig. 18B), but only for a coating of 1 $\mu\text{g}/\text{cm}^2$.

Cytotoxicity of the EJ17zipR protein against L929 cells

The EJ17zipR protein obtained from the E. coli DE3 BLR strain culture, after being purified and lyophilized was used to coat 96-well plates intended for culturing eukaryotic cells in suspension (unmodified surface). An aqueous solution of the EJ17zipR protein was applied to the plates in an amount of 1 $\mu\text{g}/\text{cm}^2$ and dried. The control in the study was fibronectin used for coating, also in an amount of 1 $\mu\text{g}/\text{cm}^2$. Cells of the L929 line were seeded on the prepared plates in the amount of 2×10^4 /well, 1×10^4 /well and 5×10^3 /well. Cells were incubated in dedicated culture medium for 24, 48 and 72 hours. Then, the medium was removed from selected wells, the cells were flooded with 70% methanol and incubated for 30 minutes, obtaining a negative control in the form of a population of 100% dead cells. Cells were stained with calcein AM (1 μM) and ethidium homodimer (EthD1, 2 μM) for 30 min, and fluorescence was measured at light wavelengths of 485 and 530 nm (excitation) and 530 and 645 nm (emission).

Fig. 20 shows the level of fluorescence in the culture of L929 cells on plates coated with the EJ17zipR protein compared to fibronectin in the cytotoxicity test of the EJ17zipR protein against L929 cells. Based on the increase in fluorescence during incubation (Fig. 20), it can be concluded that the EJ17zipR protein used to coat the culture plastic is not cytotoxic to L929 cells, although compared to fibronectin, commonly used for this purpose, we observe a significantly lower fluorescence signal after 72 hours of cultivation. breeding.

Cytotoxicity of blends containing the EJ17zipR protein towards cells

The cytotoxicity of the blends was tested using the "contact" method by seeding HUVEC cells mixed with HDF α in a 1:2 ratio onto wells covered with the cross-linked blend.

After lyophilization, the EJ17zipR protein was used to prepare blends based on a 10% methacrylated gelatin solution with the addition of the tested protein in the amount of 0.5 or 0.1 mg/ml.

In the contact test, the obtained blend was coated on 48-well plates intended for culturing eukaryotic cells. A mix of cell lines was seeded on the prepared plates in a total amount of 2.5×10^5 /well, 1.25×10^5 /well and 0.625×10^5 /well. Cells were incubated in dedicated culture medium for 24, 48 and 96 hours.

Cells were stained with calcein AM (1 μ M) and ethidium homodimer (EthD1, 2 μ M) for 30 minutes and green (live cells) and red (dead cell nuclei) fluorescence was observed using an Olympus IX83 microscope with CellSens software enabling photo documentation. A compilation of photos for individual blends showing the fluorescence of stained cells at subsequent time points is shown in Fig. 21. The control were cells seeded on TC-modified plastic (plastic adapted for adherent cultures) and GelMa blends, further included photos from cultures on the surface of blends containing 0.5 mg/ml EJ17zipR protein (B17H) and blends containing 0.1 mg/ml EJ17zipR (B17L). The photos were taken at 4x and 10x lens magnification.

In a contact test performed on HUVEC and HDF α cells growing on the surface of blends with the addition of the EJ17zipR protein, no cytotoxic effect of the tested protein was found at both tested concentrations. The cells were actively dividing, the number of dead cells was negligible and directly proportional to the total number of growing cells.

Cytotoxicity of the EJ17zipR protein towards L929 cells - absorbance measurement in the MTT test

The cytotoxicity of the EJ17zipR protein towards L-929 cells (measurement of absorbance in the MTT test) was tested in accordance with the ISO 10993-5:2009(E) standard: Biological assessment of medical devices. Part 5: In vitro cytotoxicity studies. Depending on the planned exposure time, cells were seeded into 96-well plates at densities of 1×10^5 /ml, 5×10^4 /ml and 2.5×10^4 /ml, 100 μ l per well.

Culture was carried out overnight under standard conditions (5% CO₂ and 37°C) in supplemented DMEM medium so that the fibroblasts had a chance to stick to the bottom of the culture vessel. The test was performed using the direct method, adding a solution of purified protein reconstituted in the medium to the culture. After checking the confluence and population status, a protein solution in the medium was placed on the plates at a concentration of 1, 0.5 and 0.1 mg/ml in an amount of 100 µl. The plate with cells at a density of 1×10^5 /ml was incubated for 24 hours, the culture at an initial density of 5×10^4 /ml was exposed to the EJ17zipR protein in the medium for 48 hours, and the cells with an initial density of 2.5×10^4 /ml were exposed to EJ17zipR solutions for 72 hours.

After this time, the cells were incubated with the MTT reagent solution for 2 hours, then all the fluid above the cells was removed and the formed formazan salt crystals were dissolved with DMSO. The amount of colored product formed, proportional to the number of living cells, was determined by measuring absorbance at light wavelengths of 570 and 650 nm. According to the standard, the expected result is that the viability of cells exposed to a cytotoxic agent is not less than 70% of the viability of untreated cells in the negative control.

Based on the results obtained, it was concluded that the EJ17zipR protein in the tested concentration range (0.1 - 1 mg/ml) at 24-, 48- and 72-hour exposure is not cytotoxic to the L929 fibroblast line (Fig. 22).

Summary of biological activity studies

The results obtained in biological tests indicate that the recombinant proteins subject to the invention may constitute a valuable component of bioinks for 3D printing. Possible wide range of applications: coating culture vessels in the culture of adherent cells (alternative to fibronectin or commercial matrices); as a component of bioinks for printing organs, organoids, spheroids; a factor promoting cell adhesion, proliferation and migration in three-dimensional tissue models and complex vascular systems.

EXAMPLE 7: Usefulness of a biomaterial containing the RE15mR protein in 3D bioprinting technology

Assessment of the usefulness of a material containing methacrylated gelatin (GelMa) and RE15mR protein

The RE15mR protein obtained from the culture of the E. coli BLR DE3 strain and the purified RE15mR protein after lyophilization were used to create a bioink with high utility in the technology of three-dimensional bioprinting of constructs. The bioinks were prepared as a composition of two main components: (i) recombinant RE15mR protein with a mass of 26 kDa, containing mainly resilin and elastin domains, synthesized as previously described, (ii) methacrylated gelatin (GelMa DS 80; Polbionica Ltd., Poland) with a concentration in the range of 5-20% (w/v) in 1xPBS with LAP with a final concentration not higher than 0.5% for biomaterials mixed with cells or in the range of 0.1%

to 2% for materials without cells (optimally 0.5 %) (lithium phenyl-2,4,6-trimethylbenzoylphosphinate) (Polbionica Sp. z o. o., Poland) as a photoinitiator. The recombinant protein was used at four concentrations: 0.1, 0.5, 1.0 and 1.5 mg/ml. The reference sample was 10% GelMa with LAP without the addition of recombinant protein.

5 The rheological properties of the developed material were tested using an Anton Paar MCR 72 rheometer. Three rheological parameters were measured. The measurement of the complex modulus depending on the temperature change was performed under conditions of 30% deformation, 1Hz frequency and in the temperature range 10-40°C. The measurement of the complex modulus depending on the change in the set strain of 0.01-100% was performed at a temperature of 20°C at a
10 frequency of 1 Hz. The rotational measurement of dynamic viscosity was performed at a constant temperature of 20°C and a constant shear rate of 2 1/s. All tests performed for the tested materials were carried out using a plate with a diameter of 25 mm, and the table with samples was set at a distance of 1 mm from the plate. The characterized protein was tested for the possibility of using it as a component of a hydrogel or bioink with high utility in 3D bioprinting technology. Based on the
15 results of rheological measurements, the sol-gel phase transition point, the dependence of the storage modulus and loss modulus on shear stress, and the average viscosity at a given temperature at a constant shear rate were determined. Fig. 23 shows the results of rheological measurements of the tested materials based on GelMa containing the RE15mR recombinant protein, where A - gelation point, B - complex modulus, C - dynamic viscosity.

20 The use of the recombinant RE15mR protein in the hydrogel does not affect the temperature of the sol-gel phase transition point, which was 17°C for both the reference sample and the tested material. However, changes in the modulus values were found due to changes in the concentration of the RE15mR protein. The use of a high concentration of recombinant RE15mR protein in the hydrogel does not affect the dependence of the storage modulus on shear stress; the value of the loss modulus
25 is higher than the value of the storage modulus, which indicates the advantage of viscous properties over elastic ones. The use of a relatively low protein concentration leads to an initial increase in the value of the storage modulus compared to the value of the loss modulus for low shear stresses.

The addition of recombinant RE15mR protein to the hydrogel resulted in an increase in dynamic viscosity; the higher the protein concentration, the higher the viscosity of the biomaterial, which is
30 important when using this material in bioprinting technology.

There was no effect of the addition of recombinant RE15mR protein to the bioink containing dECM on the dynamic viscosity and the sol-gel phase transition point compared to the reference bioink without the addition of protein.

35 The use of the RE15mR protein at a relatively low concentration of 0.1 mg/ml results in a significant increase in the values of the complex module components compared to the reference bioink. An

increase in the modulus value indicates an intensification of the elastic or viscous properties of a given material.

The printability of the developed biomaterials was tested. For this purpose, a specially developed procedure of a three-stage assessment system was used: a fusion test of fibers printed in the form of a template, a collapse test of a fiber printed on a three-dimensional platform and an assessment of fiber continuity during continuous bioink printing in a volume of 3 ml. The prepared materials for printing were incubated at an appropriate temperature in the range of 21-25°C in the thermoblock and in the BIOX Cellink printer head immediately before starting the test (optimally 15 min). In order to select appropriate material printing parameters, a series of extrusion tests were performed under various temperature (10-30°C) and pressure (10-70 kPa) conditions.

Based on the data obtained, the most optimal printing parameters for a given material were selected, including pressure in the range of 35-55 kPa, temperature in the range of 21-23°C and printing speed of 8-20 mm/s.

In order to carry out the fiber fusion test, a print model was designed in which two layers were printed 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 BIO X™ extrusion printer (Cellink, Sweden). The prepared print follows a pattern in a 0°–90° pattern, which reproduces the 2D effect and increases the distance between the fibers (FD). The distance between the fibers was in the range of 1–5 mm with 1 mm increments. The printing speed, needle diameter and printing distance used in the test are 20 mm/s, 21G (0.609 mm) and 0.8 mm, respectively. The print was cross-linked with an external UV-Vis lamp of 365 or 405 nm, with a power ranging from 13 to 28.5 W/cm², for 15-20 s. Based on the results, two parameters were determined, i.e. the percentage of the diffusion rate (the rate of material spreading) (Dfr) and printability (Pr). The pore diffusion rate without material spreading is 0 (i.e. At = Aa) and for a perfect model representation the printability is 1.

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

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

wherein:

Dfr - pore diffusion rate

Pr – printability of the material

At - theoretical pore surface

Aa - actual pore surface

L – pore circumference.

Fig. 24 shows the result of assessing the printability of GelMa-based biomaterials containing the RE15mR recombinant protein, where part A concerns the results of the fiber fusion test as a measure of the material's printability and resolution. Based on the results, it is concluded that the diffusion rate decreases and the printability increases with the increase of the pattern pore size. The material containing the RE15mR recombinant protein is characterized by high printing resolution, better than material without the addition of protein. The material as an addition to bioink can be used when it is necessary to print relatively small objects with a lot of detail, due to the high printing resolution and the lack of uncontrolled spread of the material. The material may be used in bioprinting of controlled drug dosing systems or tissue models with a vascular system used for testing new active substances or oncological drugs. The ability to print models with high resolution allows for printing a model with a complex, branched vascular system, and thus enables bioprinting of organs. and 3D models used in regenerative medicine and transplantology, ensuring faster vascularization of the manufactured constructs and ensuring optimal gas exchange and the supply of nutrients even with large 3D constructs.

The mid-span deflection of the suspended fiber was analyzed to determine the material's collapse affinity. In order to carry out the experiment, a special platform was designed and 3D printed, 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 a platform using bioprinting technology using a BIO X™ extrusion printer (Cellink, Sweden), and a photo of the print was immediately taken. The print was made at a speed of 20 mm/s using a 21 G (0.609 mm) nozzle. The collapse area factor (C_f), which is the percentage of the actual area after the suspended fiber has been deflected from the theoretical area, was calculated using the following equation:

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

wherein:

C_f - collapse area factor

A_a^c - actual area under the curve

A_t^c - theoretical area under the curve.

If the material is too viscous and cannot make the fiber stay between two pillars, the actual area is zero and the collapse factor is 0. On the other hand, if the fiber does not collapse and forms a straight bridge between successive pillars, then $A_t^c = A_a^c$ and the factor is 100%.

Fig. 24, part B, shows the results of the fiber collapse test as a measure of its stability. Materials containing RE15mR recombinant proteins are characterized by fiber continuity and stability within the optimal range of printing parameters. The collapse rate is over 80%. The use of recombinant RE15mR protein as an addition to the hydrogel or bioink improves parameters indicating high resolution and stability of printable fibers.

Fiber continuity when printing 2-3 ml of the tested bioink was assessed using the 0/1 system, with the value being 0 when the fiber breaks and 1 when it is pulled. Materials containing the RE15mR recombinant protein are characterized by fiber continuity and stability in the optimal range of printing parameters. The tested materials are printable in the following range of printing parameters: temperature: 10-30°C and pressure 10-70 kPa, extrusion speed 8-25 mm/s, however, the optimal printing parameters are in the following temperature ranges: 21-23°C, pressure 35-55 kPa and printing speed 8-20 mm/s.

Finally, the tested materials were characterized in terms of their mechanical parameters. The mechanical compressive strength of the samples was tested using a static compression test. For this purpose, an apparatus was designed and assembled consisting of the following elements: a computer with installed Axis FM software, Pronterface, drivers for the force gauge and a tripod, a tripod with an electric drive and control, an Axis FB50 force gauge (maximum force 50 N) mounted on the tripod, a printed head compressive. In order to carry out the experiment, cylindrical samples with dimensions: d - diameter 10 mm and h - height 5 mm (100% filling, cross-linking with an external UV-Vis lamp after each layer) were designed and printed on a BIO X™ 3D extrusion printer (Cellink, Sweden). All samples were initially loaded with a force from 0 to 0.05 N. The samples were compressed at a constant speed of 10 mm/min at room temperature until 80% deformation was obtained, with points collected every 0.025 s. After the measurement, the data and a graph were saved (force dependence from the time of measurement). Based on the results, the mechanical strength of the samples was calculated as the maximum stress (ratio of force to the surface of the printed sample) and Young's modulus as the slope coefficient of the simple relationship between stress and deformation of the sample in the deformation range of 0.1-0.5. The conventional yield strength R0.01 is the value of the stress that causes permanent deformation in the tested sample (after unloading the sample) with a value equal to 0.01% of the measurement height.

The measurement results are presented in Fig. 25. The use of the recombinant RE15mR protein in the bioink leads to significant changes in the mechanical parameters of the printed constructs. An increase in protein concentration in the biomaterial leads to an increase in the mechanical strength of the structure. The addition of the RE15mR protein at a low concentration to the biomaterial leads to a decrease in the Young's modulus of the printed construct compared to the reference sample, while an increase in the concentration of the RE15mR protein leads to another increase in the value of this parameter. The use of the RE15mR protein as a hydrogel component leads to an increase in the

mechanical parameters of the printed constructs, so it can be used in the production of 3D models. Depending on the concentration of the added protein component used, we can produce a material with different properties. Due to its high affinity for endothelial cells, the biomaterial with the addition of the RE15mR protein is a biomaterial that is used in the process of producing vessels, vascular prostheses produced by bioprinting or entire tissue models and bioprinted organs with a vascular system. The material enriched with the RE15mR recombinant protein, thanks to its properties, provides an ideal substrate for the cells forming the vascular system and ensures high mechanical strength of the printed construct, which is extremely important in flow systems. Additionally, this biomaterial is characterized by high printing resolution, which significantly translates into a reduced level of clotting in contact with blood, because the vessel lumen is characterized by a smooth internal surface.

The water absorption capacity of the tested material was tested. For this purpose, 200 µl of biomaterial was poured onto weighed dishes and cross-linked with light with a wavelength of 365 or 405 nm, a power of 13 - 28.5 mW/cm² and a time of 20 s in 3 repetitions. The sample was weighed again to obtain the mass of the material after cross-linking. 10 ml of deionized water were added to the dishes and parafilm. It was left at room temperature for 24 h. After 24 h, the water was removed and the dish was dried, weighed again, and then 10 ml of water was added again, parafilm and left for another 24 h at room temperature. Weighing and replacing deionized water was repeated after 48 and 72 h. The degree of water absorption (water content per mg of cross-linked material) can be calculated using the following formula:

$$\text{Water absorption degree} \left[\frac{mg_{H_2O}}{mg_{biomaterial}} \right] = \frac{W_N - W_M}{W_M}$$

wherein:

W_N – biomaterial mass after soaking at a given time point

W_M – biomaterial mass after pouring and gelling

The results of the water absorption measurement are presented in Fig. 26. The use of a low concentration of the RE15mR recombinant protein resulted in an increase in the degree of water absorption compared to the reference sample. However, a 3-fold increase in protein concentration significantly reduced the degree of absorbability of the biomaterial compared to the reference sample.

Assessment of the usefulness of material containing dECM and RE15mR protein

The RE15mR protein obtained from the culture of the E. coli BLR DE3 strain and the purified RE15mR protein after lyophilization were used to create a bioink with high utility in the technology of three-dimensional bioprinting of constructs. The analyzed material was a bioink based on cell-free extracellular matrix dECM obtained from the pancreas, enriched with the recombinant RE15mR protein. In addition to dECM-based bioink (81.27 mg dECM/ml bioink), the tested material contains

methacrylated gelatin (37.15 mg/ml) and methacrylated hyaluronic acid (5.57 mg/ml) and LAP (2.32 mg/ml). The experiment used the recombinant RE15mR protein with a mass of 26 kDa, containing mainly resilin and elastin domains at two concentrations: 0.1 and 1.5 mg/ml. The recombinant RE15mR protein was dissolved in methacrylated hyaluronic acid at the appropriate concentration.

5 The reference sample was dECM-based material without the addition of recombinant protein.

The rheological properties of the developed material were tested using an Anton Paar MCR 72 rheometer. The measurement of the complex modulus depending on the temperature change was performed under conditions of 5% deformation, frequency of 1 Hz and in the temperature range of 10-35°C. The measurement of the complex modulus depending on the change in the set strain 1-100% was performed at a temperature of 20°C at a frequency of 1 Hz. The rotational measurement of dynamic viscosity was performed at a constant temperature of 25°C and a constant shear rate of 100 1/s. All tests performed for the tested materials were carried out using a plate with a diameter of 25 mm, and the table with samples was set at a distance of 1 mm from the plate. Based on the results, the sol-gel phase transition point, the dependence of the storage modulus and loss modulus on shear stress, and the average viscosity at a given temperature at a constant shear rate were identified. The results of rheological tests are presented in Fig. 27, where A - gelation point, B - complex modulus, C - dynamic viscosity. The obtained results indicate no effect of the addition of the RE15mR recombinant protein on the dynamic viscosity and the sol-gel phase transition point compared to the reference bioink A. The addition of the above protein at a concentration of 0.1 mg/ml causes a significant increase in the values of the complex modulus components compared to the reference bioink A.

The printability of the developed biomaterials was tested. For this purpose, a specially developed procedure of a three-stage assessment system was used: a fusion test of fibers printed in the form of a template, a collapse test of a fiber printed on a three-dimensional platform and an assessment of fiber continuity during continuous bioink printing in a volume of 3 ml. The detailed methodology for testing the printability of the assessed materials has been described above. Based on the data obtained, optimal printing conditions were selected for a given material, including pressure in the range of 35-45 kPa, temperature in the range of 23-25°C and printing speed of 8-20 mm/s. Fig.28, part A, shows the results of the filament fusion test as a measure of the material's printability and resolution. Based on the results, it is concluded that the diffusion rate decreases and the printability increases with the increase of the pattern pore size. The material containing the RE15mR recombinant protein is characterized by high printing resolution, better than the material without the additive.

Fig.28, part B, shows the results of the fiber collapse test as a measure of its stability. Materials containing the RE15mR recombinant protein are characterized by fiber continuity and stability. The

collapse rate is over 80%. The tested materials are characterized by fiber continuity and smoothness in the optimal range of printing parameters: temperature: 23-25°C and pressure 35-45 kPa.

Finally, the tested materials were characterized in terms of their mechanical parameters. The mechanical compressive strength of the samples was tested using a static compression test. Similarly
5 as before, based on the results, the mechanical strength of the samples was calculated as the maximum stress (ratio of force to the surface of the printed sample) and the Young's modulus as the slope coefficient of the simple relationship between stress and deformation of the sample in the deformation range of 0.1-0.5. The conventional yield strength $R_{0.01}$ is the value of the stress that causes permanent deformation in the tested sample (after unloading the sample) with a value equal
10 to 0.01% of the measurement height. The measurement results are presented in Fig. 29.

The use of the recombinant RE15mR protein in the bioink leads to significant changes in the mechanical parameters of the printed constructs. The addition of the RE15mR protein at a relatively low concentration of 0.1 mg/ml to the biomaterial leads to an increase in the mechanical strength of the printed construct compared to the reference sample. As before, it is recommended to use the
15 RE15mR protein as an addition to the bioink used in the bioprinting of hard tissue models (e.g. bones, cartilage) and tissue models with a vascular system exposed to significant stresses and shear forces (e.g. the vascular system in 3D bioprinted models and organs; models of vessels and other structures exposed to variable effects of flow, pressure or forces forcing the flexibility of the model).

The water absorption capacity of the tested material was tested according to the procedure described
20 earlier. The results of the water absorption measurement are presented in Fig. 30. The obtained results show that the addition of the recombinant RE15mR protein causes an increase in the degree of water absorption compared to the reference bioink A. The ability to absorb water by the construct is important in the case of e.g. bone scaffolds, because it reflects the efficiency of fluid absorption, body and transport of nutrients to cells.

25 **EXAMPLE 8: Usefulness of a biomaterial containing the EJ17zipR protein in 3D bioprinting technology**

Assessment of the usefulness of a material containing methacrylated gelatin (GelMa) and EJ17zipR protein

The purified EJ17zipR protein obtained from the culture of the E. coli BLR DE3 strain and the
30 purified EJ17zipR protein after lyophilization were used to create a bioink with high utility in the technology of three-dimensional bioprinting of constructs. The bioinks were prepared as a composition of two main components: (i) recombinant EJ17zipR protein of 86 kDa, containing mainly elastin and silk fibroin domains, synthesized independently as described earlier, (ii) methacrylated gelatin (GelMa DS 80; Polbionica sp. z o.o., Poland) with a concentration in the range
35 of 5-20% (w/v) in 1xPBS with LAP (lithium phenyl-2,4,6-trimethylbenzoylphosphinate) with a final

concentration not higher than 0.5% for biomaterials mixed with cells or in the range 0.1% to 2% for materials without cells (optimally 0.5%) (Polbionica sp. z o. o. Poland) as a photoinitiator. The recombinant protein was used at four concentrations: 0.1, 0.5, 1.0 mg/ml. The reference sample was 10% GelMa with LAP without the addition of recombinant protein.

- 5 The rheological properties were tested in a similar manner as in the case of the GelMa-based hydrogel enriched with the RE15mR recombinant protein.

Based on the results, the sol-gel phase transition point, the dependence of the storage modulus and loss modulus on shear stress, and the average viscosity at a given temperature at a constant shear rate were identified. Fig. 31 shows the results of rheological measurements of the tested materials based
10 on GelMa containing the recombinant protein EJ17zipR, where A - gelation point, B - complex modulus, C - dynamic viscosity. The obtained results indicate that the addition of the recombinant protein has no effect on the temperature of the sol-gel phase transition point, which was 17°C for both the reference sample and the tested material. The addition of recombinant protein EJ17zipR, regardless of concentration, changed the dependence of the complex modulus on shear stress. For
15 the reference sample, in the entire strain range tested, the loss modulus is higher than the storage modulus. However, for biomaterials containing the EJ17zipR recombinant protein, initially the storage modulus is higher than the loss modulus, increasing the set deformation causes a change in the relationship, the viscous properties (G'') begin to outweigh the elastic properties (G'). The addition of recombinant protein reduces the dynamic viscosity compared to the reference sample.

- 20 The printability of the developed biomaterials was tested according to the previously described three-step procedure. Based on the data obtained, optimal printing conditions were selected for a given material, including pressure in the range of 35-50 kPa, temperature in the range of 20-22°C and printing speed of 8-20 mm/s. Fig. 32, part A, shows the results of the filament fusion test as a measure of the material's printability and resolution. Based on the results, it is concluded that the diffusion
25 rate decreases and the printability increases with the increase of the pattern pore size. The material containing the EJ17zipR recombinant protein is characterized by high printing resolution, better than the material without the additive, therefore the EJ17zipR recombinant protein is recommended as an addition to biomaterials used in 3D bioprinting technology. Fig. 32, part B, shows the results of the fiber collapse test as a measure of its stability. Materials containing EJ17zipR recombinant proteins
30 are characterized by fiber continuity and stability. The collapse rate is over 80%. The tested materials demonstrate fiber continuity and smoothness in the optimal range of printing parameters: temperature: 20-22°C and pressure: 35-50 kPa.

- Finally, the tested materials were characterized in terms of their mechanical parameters determined using a static compression test, the procedure of which was described earlier. Based on the results,
35 the mechanical strength of the samples was calculated as the maximum stress (the ratio of force to

the surface of the printed sample) and the Young's modulus as the slope coefficient of the simple relationship between stress and deformation of the sample in the deformation range of 0.1-0.5. The conventional yield strength $R_{0.01}$ is the value of the stress that causes permanent deformation in the tested sample (after unloading the sample) with a value equal to 0.01% of the measurement height.

5 The measurement results are presented in Fig. 33.

The use of the recombinant EJ17zipR protein in the hydrogel leads to significant changes in the mechanical parameters of the printed constructs. An increase in protein concentration in the biomaterial leads to an increase in the mechanical strength of the structure compared to the structure made of the reference material. Moreover, the material containing the EJ17zipR protein at a concentration of 1 mg/ml was not destroyed under the influence of the applied force. The hydrogel containing the EJ17zipR protein showed significant elasticity. The material containing the EJ17zipR protein is a recommended addition to the bioink used in the bioprinting technology of tissue models requiring significant mechanical strength and elasticity, i.e. cartilage, bones, but also blood vessels, which are exposed to variable pressure and shear forces of variable intensity in a small time range.

10

15 Moreover, this material can be successfully used to print tissue models with a functional vascular system exposed to relatively high stresses, especially in the case of research conducted on cells inhabiting the vascular system. The produced protein is also a solution for tissue models / models of the heart and other organs, the so-called soft, requiring the vascular system (e.g. pancreas, liver, etc.)

The water absorption capacity of the tested material was tested according to the previously described procedure. The results of the water absorption measurement are presented in Fig. 34. The obtained results indicate that the use of a low concentration of the recombinant EJ17zipR protein does not affect the degree of water absorption, while the use of a higher concentration causes an increase in the water absorption degree compared to the reference biomaterial.

20

Assessment of the usefulness of material containing dECM and EJ17zipR protein

25 The purified EJ17zipR protein obtained from the culture of the E. coli BLR DE3 strain and the purified EJ17zipR protein after lyophilization were used to create a bioink with high utility in the technology of three-dimensional bioprinting of constructs. The analyzed material was a bioink based on cell-free extracellular matrix dECM obtained from the pancreas, enriched with the recombinant EJ17zipR protein. In addition to dECM-based bioink (81.27 mg dECM/ml bioink), the tested material contains methacrylated gelatin (37.15 mg/ml) and methacrylated hyaluronic acid (5.57 mg/ml) and LAP (phenyl-2,4,6-lithium trimethylbenzoylphosphinate) (2.32 mg/ml). The experiment used the recombinant EJ17zipR protein with a mass of 86 kDa, containing mainly elastin and silk fibroin domains at two concentrations: 0.1 and 1.0 mg/ml. The recombinant EJ17zipR protein was dissolved in methacrylated hyaluronic acid at an appropriate concentration. The reference sample

30

35 was material without the addition of recombinant protein.

The rheological properties of the developed dECM-based material were tested using the previously described procedure. Based on the results, the sol-gel phase transition point, the dependence of the storage modulus and loss modulus on shear stress, and the average viscosity at a given temperature at a constant shear rate were identified. The results of measuring the rheological properties of the dECM-based material enriched with the EJ17zipR recombinant protein are presented in Fig. 35, where A - gelation point, B - complex modulus, C - dynamic viscosity. The obtained results indicate no effect of the addition of the EJ17zipR recombinant protein on the dynamic viscosity and the sol-gel phase transition point compared to the reference bioink A. The addition of the above protein causes an increase in the values of the complex modulus components compared to the reference bioink A.

The printability of the developed biomaterials was tested according to the previously described procedure. Based on the data obtained, optimal printing conditions were selected for a given material, including pressure in the range of 35-40 kPa, temperature in the range of 23-25°C and printing speed of 8-20 mm/s. Fig. 36, part A, shows the results of the filament fusion test as a measure of the material's printability and resolution. Based on the results, it is concluded that the diffusion rate decreases and the printability increases with the increase of the pattern pore size. The material containing the EJ17zipR recombinant protein is characterized by high printing resolution, better than the material without the additive. The material as an addition to bioink can be used when it is necessary to print relatively small objects with a lot of detail, due to the high printing resolution and the lack of uncontrolled spread of the material. The material may be used in bioprinting of controlled drug dosing systems or tissue models with a vascular system. Fig. 36, part B, shows the results of the fiber collapse test as a measure of its stability. Materials containing recombinant proteins are characterized by fiber continuity and stability. The collapse rate is over 70%. The tested materials show the continuity and smoothness of the printed fiber in the optimal range of printing parameters: temperature: 23-25°C and pressure 40 kPa.

Finally, the tested materials were assessed in terms of their mechanical parameters determined using a static compression test. Similarly as before, based on the results, the mechanical strength of the samples was calculated as the maximum stress (ratio of force to the surface of the printed sample) and the Young's modulus as the slope coefficient of the simple relationship between stress and deformation of the sample in the deformation range of 0.1-0.5. The conventional yield strength $R_{0.01}$ is the value of the stress that causes permanent deformation in the tested sample (after unloading the sample) with a value equal to 0.01% of the measurement height. The results of measuring mechanical parameters are presented in Fig. 37.

The use of the recombinant EJ17zipR protein in the bioink leads to significant changes in the mechanical parameters of the printed constructs. The use of the EJ17zipR protein at a concentration of 0.1 mg/ml leads to an increase in the mechanical strength of the printed structure compared to the

reference sample. The use of the recombinant EJ17zipR protein does not introduce significant changes in the Young's modulus of the printed structure compared to the reference sample.

- 5 The water absorption capacity of the tested material was tested according to the previously described procedure. The results of the water absorption measurement are presented in Fig. 38. The addition of the recombinant protein EJ17zipR causes an increase in the degree of water absorption compared to the reference bioink A. The ability to absorb water by the construct is important in the case of e.g. bone scaffolds, because it reflects the efficiency of absorption of body fluids and the transport of nutrients to cells.

Claims

1. A recombinant RE15mR hybrid protein consisting of 3 resilin domains, 1 K+ domain, 3 resilin domains, 1 MMP domain, 3 elastin domains, 1 K+ domain, 4 elastin domains, 1 K+ domain, 3 elastin domains, 1 K+ domain, 4 elastin domains, 1 K+ domain, 3 elastin domains, 1 MMP domain, 7 RGD domains.
2. The RE15mR recombinant hybrid protein according to claim 1, wherein the amino acid sequence contains 12-15% proline and/or 32-38% glycine.
3. The recombinant RE15mR hybrid protein according to claim 1 or 2, wherein the molecular weight of the protein is 26 kDa.
4. The recombinant RE15mR hybrid protein according to one of claims 1-3, having the amino acid sequence of SEQ #3.
5. The recombinant RE15mR hybrid protein according to one of claims 1-4, constituting a fragment of the recombinant protein.
6. The recombinant RE15mR hybrid protein according to one of claims 1-5, which is at least partially methacrylated.
7. A recombinant DNA sequence selected from:
 - a recombinant DNA sequence encoding the recombinant RE15mR hybrid protein defined in one of claims 1-4,
 - a recombinant DNA sequence comprising a region comprising the DNA sequence encoding the recombinant RE15mR hybrid protein as defined in one of claims 1-4, and
 - a recombinant DNA sequence hybridizing with a DNA sequence encoding the recombinant RE15mR hybrid protein defined in one of claims 1-4.
8. The recombinant DNA sequence according to claim 7, having a sequence that is at least 80% identical, more preferably at least 90% identical, and most preferably homologous to the recombinant DNA sequence of SEQ #1.
9. A vector including a recombinant DNA sequence comprising a region containing the recombinant DNA sequence as defined in claim 7 or 8 and/or a recombinant sequence comprising a portion of a region containing the recombinant DNA sequence as defined in claim 7 or 8.
10. The vector according to claim 9, being a plasmid containing a promoter derived from phage T7, preferably selected from pET11a-d, pET15b, pET19b, pET28a-c(+), pET21a-d(+), pET22b(+), pET23a-d (+), pEt25b(+), pET44a-c(+), pET46Ek/LIC, and most preferably being pET11a.

11. A prokaryotic expression system transformed with a recombinant DNA sequence containing the region comprising the recombinant DNA sequence as defined in claim 7 or 8 and/or a recombinant sequence containing a portion of a region comprising the recombinant DNA sequence as defined in claim 7 or 8.
12. The expression system of claim 11, which is Escherichia coli cells.
13. A recombinant hybrid protein EJ17zipR composed of 1 ZIP domain, 56 elastin domains, 10 silk fibroin domains, 7 RGD domains, 56 elastin domains, 10 silk fibroin domains, 7 RGD domains.
14. The recombinant EJ17zipR hybrid protein according to claim 13, wherein the amino acid sequence contains 12-17% proline and/or 20-30% glycine.
15. The recombinant EJ17zipR hybrid protein according to claim 13 or 14, wherein the molecular weight of the protein is 86 kDa.
16. The recombinant EJ17zipR hybrid protein according to one of claims 13-15, having the amino acid sequence of SEQ #4.
17. The recombinant hybrid protein EJ17zipR according to one of claims 13-16, constituting a fragment of the recombinant protein.
18. The recombinant hybrid protein EJ17zipR according to one of claims 13-17, which is at least partially methacrylated.
19. A recombinant DNA sequence selected from:
 - a recombinant DNA sequence encoding the recombinant hybrid protein EJ17zipR defined in one of claims 13-16,
 - a recombinant DNA sequence comprising a region containing the DNA sequence encoding the recombinant hybrid protein EJ17zipR as defined in one of claims 13-16, and
 - a recombinant DNA sequence hybridizing with a DNA sequence encoding the recombinant hybrid protein EJ17zipR as defined in one of claims 13-16.
20. The recombinant DNA sequence according to claim 19, having a sequence that is at least 80% identical, more preferably at least 90% identical, and most preferably homologous to the recombinant DNA sequence of SEQ #2.
21. A vector including a recombinant DNA sequence containing a region comprising the recombinant DNA sequence as defined in claim 19 or 20 and/or a recombinant sequence containing a portion of a region comprising the recombinant DNA sequence as defined in claim 19 or 20.

22. The vector according to claim 21, being a plasmid containing a promoter derived from phage T7, preferably selected from pET11a-d, pET15b, pET19b, pET28a-c(+), pET21a-d(+), pET22b(+), pET23a-d (+), pEt25b(+), pET44a-c(+), pET46Ek/LIC, and most preferably being pET11a.
23. A prokaryotic expression system transformed with a recombinant DNA sequence containing a region comprising the recombinant DNA sequence as defined in claim 19 or 20 and/or a recombinant sequence containing a portion of a region comprising the recombinant DNA sequence as defined in claim 19 or 20.
24. The expression system of claim 23, which is Escherichia coli cells.
25. Use of a recombinant hybrid protein selected from the RE15mR protein defined in one of claims 1-6 and the EJ17zipR protein defined in one of claims 13-18 as a component of a bioink for bioprinting.
26. Use of a recombinant hybrid protein selected from the RE15mR protein as defined in one of claims 1-6 and the EJ17zipR protein as defined in one of claims 13-18 as a biomaterial component.
27. Use of the recombinant DNA sequence as defined in claim 7 or 8, or the vector as defined in claim 9 or 10, or the prokaryotic expression system as defined in claim 11 or 12, for the production of a recombinant RE15mR hybrid protein as defined in one of claims 1-6.
28. Use of the recombinant DNA sequence as defined in claim 19 or 20, or the vector as defined in claim 21 or 22, or the prokaryotic expression system as defined in claim 23 or 24 for the production of the recombinant hybrid protein EJ17zipR as defined in one of claims 13-18.