OPTIMIZATION OF THE PREPARATION PROCESS STAGES OF THE BIOINK COMPOSITIONS BASED ON SODIUM ALGINATE AND GELATIN TO IMPROVE THE VIABILITY OF BIOLOGICAL MATERIAL CONTAINED IN HYDROGEL 3D PRINTOUTS

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Abstract

Currently developing on a large scale, the opportunities for 3D printing represent more and more perspective solutions in the area of tissue engineering and personalized medicine. Due to their ability to reproduce the natural extracellular matrix and unique properties, hydrogels are popularly used materials to produce bioinks designated for 3D printing. Today, solutions based on sodium alginate and gelatin are frequently used compositions for this purpose. The high viability of the cells incorporated into bioink is the key parameter determining the application opportunities of printed structures. The parameters of the process used for the preparation of hydrogel compositions may have a direct impact on the viability of the cells incorporated within the printed structure. This study aims to develop a protocol for the preparation of hydrogel materials based on alginate and gelatin, providing the highest viability of the model osteoblast-like cell line Saos-2 incorporated directly into the bioink before the 3D bioprinting process. In the scope of this study, the analyzed process parameters of the preparation of the hydrogel bioinks are the method of combination of a polymer solution with biological material, the applied concentration, the cross-linking solution, and also the waiting time of the prepared hydrogel bioink for the 3D printing process. A key aspect of the study is the evaluation of the influence of 3D printing on changes in the survival rate of biological material directly after the manufacturing process and after individual incubation periods of the printouts in conditions reflecting the body's environment.

Keywords: hydrogel, bioink, 3D bioprinting, sodium alginate, gelatin, tissue engineering

Introduction

Regarding the application of hydrogel materials, they represent a highly versatile group of materials, which currently finds a wide range of applications, including biomedicine. The beginning of clinical applications of hydrogels came in the 60s of the last century, when these materials were characterized and described by O. Wichterle and D. Lím as materials used for the construction of contact and intraocular lenses [1-4]. Literature data show that hydrogels represent the first polymer biomaterials designed for clinical applications [4]. According to the definition, a hydrogel is described as a network of hydrophilic polymers forming a three-dimensional structure. The presence of hydrophilic functional groups results in the ability of this group of materials for the reversible process of absorption of vast amounts of water. The cross-linking of the hydrogels results in the fact that despite the absorption of large amounts of fluids and the swelling ability, they maintain their geometry [1,2]. In general, hydrogel materials are also characterized by high flexibility, porosity, and the possibility of response to external stimuli [5]. Currently, the literature describes numerous methods of hydrogel classification. The most commonly used factors that allow for systematic division of this group of materials are origin, polymer composition, degree of crystallinity, network electrical charge, cross-linking type, macroscopic evaluation, and degradation ability [1,2,5,6].

Due to their properties, hydrogel materials can provide an extremely beneficial environment for cell cultures in a 3D arrangement. Their biocompatibility, high fluid absorption, and the possibility of adjusting mechanical properties for the requirements of various applications mean that these materials can provide conditions close to the natural extracellular matrix [7]. The 3D printing capabilities expanding on a large scale create increasingly promising solutions for tissue engineering and personalized medicine. Additive manufacturing methods can be applied to form biomimetic constructs, which provide a perspective to design complete tissues and organs of the body [8]. We can distinguish several 3D printing techniques, among which the most commonly applied are extrusion-based printing, inkjet, laser printing, and stereolithography [9-11]. In general, as inks in 3D bioprinting techniques, hydrogel bioinks are used, consisting of polymer solution and biological material incorporated in it. Due to the possibility of applying a wide range of various polymer solutions with different viscosities as well as the application of high-density biological material, including cell aggregates, the 3D bioprinting technique based on extrusion of bioink is currently gaining high popularity [10,11]. Commonly applied materials for bioink production are hydrogels based on sodium alginate. It is a natural anionic polysaccharide consisting of linked residues (1-4)-linked β-d-mannuronic acid (M) and its C-5 epimer α-1-guluronic acid (G) [11,12]. Depending on the contents of G, M, and GM blocks, the physical properties of alginate may undergo modulation [13]. Due to the presence of G-blocks, the alginate has the ability to form gels, whereby through the presence of M and GM blocks, this biopolymer is characterized by flexibility [11]. Alginate undergoes ionic cross-linking, during which interactions between divalent cations, such as Ca^{2+}, Ba^{2+}, Mn^{2+}, Sr^{2+}, Zn^{2+}, and Mg^{2+}, and free carboxyl groups occur [14,15]. Alginate hydrogels are characterized by high biocompatibility, water and gas permeability, and mild conditions of gelation. They undergo limited, frequently uncontrolled degradation processes in the physiological environment. Those hydrogels can be modified to obtain desired properties designated for particular applications [15,16].

An important property of alginate-based hydrogels is the possibility of modifying the rheological properties, making them attractive materials used to produce hydrogel bioinks for tissue engineering. However, alginate hydrogels lack groups that stimulate the adhesion and proliferation of the cells in their structure, which results in their biological inertness [7,16,17]. To eliminate the disadvantages of alginate related to the lack of the biological groups binding cells in the hydrogel bioinks, its combination with the addition of gelatin is often used. It provides biocompatible, biodegradable protein obtained during the collagen hydrolysis process. Gelatin contains in its structure bioactive sequences of RGD collagen (arginine–glycine–aspartic acid), which actively promotes adhesion and the growth of the cells incorporated into the hydrogel [7,17-19]. Gelatin undergoes a sol-gel transition reaction during cooling in the range of temperatures of 20-30°C, which is a kind of thermo-reversible physical cross-linking [19]. The rheological properties of alginate- and gelatin-based hydrogels at room temperature make these materials ideal substrates for bioinks applied in additive technologies because they minimize the risk of damage to the cells incorporated into the bioink during the bioprinting process [7]. In order to achieve high viability and promote the proliferation of the cells encapsulated in a hydrogel bioink based on the polymer solutions mentioned above, it is recommended to apply lower alginate concentrations, which can form soft gels [18,20].

Important aspects of printability using a designed hydrogel bioink are shape compatibility, resolution, biocompatibility of the bioink, and the ability for cellular stimulation [13]. Designing and selecting appropriate physical properties of alginate-based hydrogels for particular applications is possible by analyzing available cross-linking methods, application of molecules with different chemical structures, or by change of cross-linking functionality [21]. The method and parameters used in the preparation process of alginate- and gelatin-based hydrogel bioinks may directly influence the quality of the obtained print and the viability of the cells incorporated into its structure. Before the bioprinting process, optimizing the individual stages of preparation of the hydrogel bioinks designated to be combined with the biological material is essential. The high viability of the cells incorporated into the volume of the hydrogel bioink and the printed structures are the key factors determining the application possibilities of the discussed material.

This study aims to perform an analysis of the influence of different parameters of the hydrogel preparation process of bioinks based on the selected composition of sodium alginate and gelatin (2% w/v alginate and 9% w/v gelatin) containing model osteoblast-like cell line *Saos-2*. The research conducted aims to develop a protocol of alginate-gelatin bioinks preparation, ensuring the highest cell viability of the model line contained in the polymer solutions directly before the 3D bioprinting process. A key aspect of this study is to evaluate the influence of 3D printing on the viability of biological material directly after the manufacturing process and to analyse the changes in cell survival rate in the printouts after individual periods of their incubation under conditions reflecting the body environment.

Materials and Methods

In the present work, the effect of the preparation method of hydrogel composition based on sodium alginate (Alg) and gelatin (Gel), methods of combining them with biological material, the type of cross-linking solution based on calcium chloride, and its concentration on the survival rate of the *Saos-2* line cells contained inside the material were analysed. The cell survival rate was also assessed as a function of the length of waiting time of prepared hydrogel bioink for the 3D printing process. The final component of the study was to evaluate the effect of the 3D printing process on changes in the survival rate of the biological material during subsequent incubation periods of the printouts under standard conditions, i.e., the temperature of 37ºC, at 100% relative humidity, in 5% carbon dioxide and 95% of the air.

Materials

A hydrogel material composition containing 2% w/v sodium salts of alginic acid (Alg) from brown algae (Sigma Aldrich, USA) and 9% w/v gelatin (Gel) from porcine skin type B (Sigma Aldrich, USA) was used in the study. The hydrogel solutions were prepared in the McCoy's 5A culture medium (Capricorn Scientific GmbH, Germany) supplemented with 15% of FBS (Fetal Bovine Serum) (Sigma Aldrich, USA) and 1% of P/S (Penicillin/Streptomycin Solution) (Capricorn Scientific GmbH, Germany). Each time before the preparation process of the hydrogel solutions, appropriate amounts of sodium alginate and gelatin powders underwent UV sterilization for 1 hour. Calcium chloride (Sigma Aldrich, USA) was used for cross-linking the polymer solutions. After the cross-linking process, the samples were rinsed with PBS buffer (Phosphate-Buffered Saline) (Capricorn Scientific GmbH, Germany). Cell survival rate was assessed using live/dead staining (Biotium, CA, USA).

Cell viability assessment through live/dead assay

For the preparation of the hydrogel bioinks model line of immortalized *Saos-2* osteoblast-like cells (ATCC, USA) was used. The cultivation was conducted using complete McCoy's 5A culture medium (Capricorn Scientific GmbH, Germany) supplemented with 15% of FBS (Fetal Bovine Serum) (Sigma Aldrich, USA) and 1% P/S (Penicillin/Streptomycin Solution) (Capricorn Scientific GmbH, Germany) in an incubator under standard conditions. Cell passages were performed after 70-80% confluence was achieved.

The analysis of cell viability in bioinks and hydrogel printouts was carried out with live/dead staining using the mixture of dyes: calcein-AM applied for green labeling of live cells and ethidium homodimer (EthD) responsible for red labeling of dead cells. The cells incorporated in bioinks and hydrogel printouts were imaged using a fluorescent microscope (Nikon Eclipse LV100ND, Tokyo, Japan). The images of live and dead cells were taken in 10 randomly chosen areas of the analyzed samples. Based on the recorded images, the number of live and dead cells was calculated using ImageJ (LOCI, University of Wisconsin, USA). To determine the viability of the *Saos-2* line cells, the following relation was used:

$$\text{Viability [\%]} = \frac{\text{the number of living cells}}{\text{the number of living cells + dead cells}} \cdot 100\%$$

The results presented in the following sections show the calculated average viability of the cells incorporated into bioinks or hydrogel printouts.

Hydrogels preparation

Optimization of the cross-linking solution - selection of the concentration of the cross-linking solution based on CaCl₂ and the type of solvent used for its preparation

Sodium alginate and gelatin were dissolved independently in McCoy's 5A culture medium dedicated to *Saos-2* line cells with the addition of 15% FBS and 1% P/S for 1 hour at a temperature of 37ºC with the speed of 150 rpm to obtain solutions with concentrations of 4% w/v Alg and 18% w/v Gel. The prepared polymer solutions were combined in a 1:1 ratio, obtaining a composition of 2% w/v Alg and 9% w/v Gel. The obtained binary polymer solution was combined directly with *Saos-2* line cell pellet. The biological material was stirred with the polymer composition for 1 min using a serological pipette with a capacity of 25 ml. The density of biological material in the hydrogel solution was $1 \cdot 10^6$ cells/ml. The hydrogel bioink prepared this way was poured onto 24-well plates forming samples of equal height (FIG. 1). The samples were cross-linked with various calcium chloride solutions, as shown in TABLE 1. The cross-linking time of each of the samples was 10 min. The cross-linking process was carried out at room temperature. After the cross-linking process, each sample was rinsed with PBS buffer three times. The evaluation of cell survival rate in hydrogel bioinks directly after the individual processes of cross-linking and rinsing of the samples was carried out using the live/dead assay.
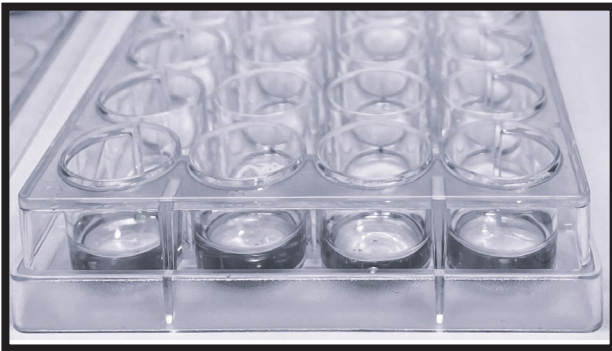
FIG. 1. An example picture of alginate- and gelatin--based hydrogel bioinks placed on 24-well plates.

TABLE 1. Compositions and concentrations of used cross-linking CaCl₂-based solutions.

The solvent used to prepare CaCl₂ cross-linking solution	CaCl₂ concentration
Deionized water	0.75%
Deionized water	1.5%
Deionized water	5%
McCoy's 5A	0.75%
McCoy's 5A	1.5%
McCoy's 5A	5%

Optimizing the method of combining alginate-gelatine solutions with the cell pellet

Three separate solutions of hydrogels were prepared in a volume ratio by mixing alginate and gelatin powders simultaneously in McCoy's 5A culture medium dedicated to *Saos-2* line cells with the addition of 15% FBS and 1% P/S for 1 hour at a temperature of 37°C at a speed of 150 rpm.

The following methods of combining the cell pellet with hydrogel material were analyzed:
- a direct combination of the hydrogel with the cell pellet,
- the initial combination of the cell pellet with 2% of the volume of culture medium used to prepare the hydrogel, followed by a combination of the cell suspension with the hydrogel material,
- the initial combination of the cell pellet with 8% of the volume of culture medium used to prepare the hydrogel, followed by a combination of the cell suspension with hydrogel materials.

The obtained binary polymer solutions were combined directly with the cell pellet or the corresponding cell suspensions of the *Saos-2* line according to the data given in TABLE 2. The biological material was stirred for 1 min with the alginate-gelatine solution using a serological pipette of 25 ml capacity. The obtained density of biological material in the hydrogel bioink was $1 \cdot 10^6$ cells/ml.

TABLE 2. Tested methods of combining biological material with the polymer solution.

The amount of culture medium subtracted initially from the prepared hydrogel	The composition of the hydrogel mixture used
0%	1 g Alg + 4.5 g Gel + 50 ml McCoy's 5A
2%	1 g Alg + 4.5 g Gel + 49 ml McCoy's 5A
8%	1 g Alg + 4.5 g Gel + 46 ml McCoy's 5A

The prepared hydrogel bioinks were poured onto 24-well plates forming samples of equal height (analogous to FIG. 1). Each sample was cross-linked using the 5% CaCl₂ solution prepared in McCoy's 5A culture medium. The cross-linking time of each of the samples was 10 min. The cross-linking process was carried out at room temperature. After the cross-linking process, each sample was rinsed in PBS buffer three times. Assessment of the cell survival rate in hydrogel materials directly after individual cross-linking and rinsing processes of the samples was carried out using the live/dead assay.

Evaluation of rheological properties of polymer solution containing 2%Alg 9%Gel (w/v) intended for 3D printing process

According to the results of the preceding studies, which have allowed us to select the bioink preparation method providing the highest cell survival rate, a polymer solution with a final concentration of 2%Alg 9%Gel in a volume ratio was prepared by mixing together alginate and gelatin powders, initially subtracting 8% of the culture medium from the mixture, which finally was added to the cell pellet of the *Saos-2* line. Polymer solution was mixed at a temperature of 37ºC with a speed of 150 rpm for 1 hour. The rheological properties of the polymer solution prepared in an optimized method were evaluated using an MCR502 rheometer (Anton Paar, Graz, Austria). For the measurement, a parallel plate geometry with a diameter of 20 mm was used. The viscosity test was conducted at 34ºC, which is analogous to the conditions of the 3D bioprinting process. The measurement was carried out in the shear rate range of 1-1000 1/s. Before the test, the prepared polymer solution was thermostated for 10 min to achieve an even temperature of the material in the entire volume.

The effect of bioink waiting time for 3D printing and evaluation of the effect of the 3D bioprinting process itself on the viability of the cells contained in hydrogel printouts

An appropriate amount of the hydrogel providing a seeding density equal to $1 \cdot 10^6$ cells/ml was added to the cell suspension. The biological material was mixed with the polymer composition described in the previous section using a serological pipette with a capacity of 25 ml. The prepared bioink was divided into three parts, which were placed in separate sterile syringes with a capacity of 5 ml. Two parts of the bioink were subjected to incubation under standard conditions for respective time periods - 30 and 45 min before the printing process. The bioink not subjected to the incubation process was the reference - it was used to make the printouts right after combining the hydrogel with the biological material. The prepared hydrogel bioinks were used for direct printing of tube structures, which were divided using a surgical scalpel into identical samples with a length of 5 mm and inner diameter of 6 mm (FIG. 2A-C).

The 3D bioprinting process was performed using an own design bioprinter (FIG. 3) in which the material is pneumatically extruded, and the printing takes place on the horizontal shaft using the cone nozzle. The set print height refers to the final wall thickness of the obtained tubular structure. The height of the print layer was set to 250 µm. During the 3D bioprinting process, the prepared bioink was heated up to the temperature of 34ºC.

The printed samples were cross-linked using a 5% $CaCl_2$ solution prepared in McCoy's 5A culture medium. The cross-linking time of each of the samples was 10 min. The cross-linking process was carried out at room temperature. After the cross-linking process, each sample was rinsed with PBS buffer three times. The viability assessment of the cells contained in the printouts was carried out using the live/dead assay.

The effect of incubation time of 3D printouts on the viability of the cells contained in them

The polymer solution with a final concentration of 2%Alg 9%Gel was prepared in a volume ratio by mixing together sodium alginate and gelatin, initially subtracting 8% of the culture medium from the mixture, which subsequently was used to suspend the cell pellet. The alginate-gelatin solution was mixed at a temperature of 37ºC with a speed of 150 rpm for 1 h. The missing amount of McCoy's 5A medium was added to the *Saos-2* line cell pellet. An appropriate amount of the hydrogel providing a seeding density equal to $1 \cdot 10^6$ cells/ml was added to the cell suspension. The biological material was stirred for 1 min with the polymer composition using a serological pipette of 25 ml capacity. The prepared hydrogel bioink was used for direct printing of structures in the form of tubes, which were divided using a surgical scalpel into identical samples (analogously to the process of preparation of the printouts described in previous section). Each sample was cross-linked using the 5% $CaCl_2$ solution prepared in McCoy's 5A culture medium. The cross-linking time was 10 min. The cross-linking process was carried out at room temperature. After the cross-linking process, each sample was rinsed in PBS buffer three times. The printouts were placed in 24-well plates supplemented with 1.5 ml of McCoy's 5A culture medium + 15% FBS + 1% P/S and subjected to respective periods of incubation of 1 and 3 days under standard conditions. The assessment of the viability of the cells contained in printouts directly after the manufacturing process and after each incubation period was carried out using the live/dead assay.
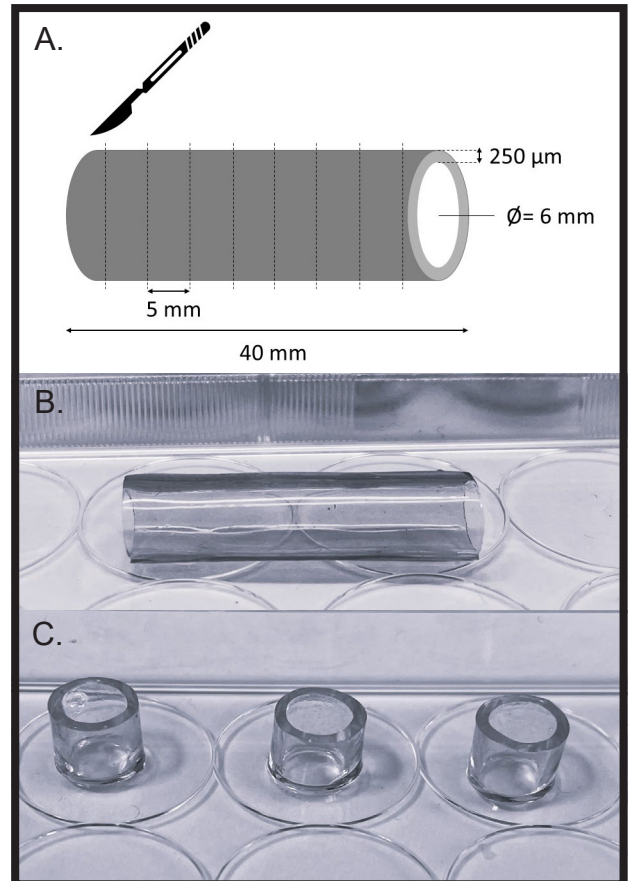

FIG. 2. A. Schematic of preparation of the hydrogel printouts samples; B. Example picture of a single hydrogel printout; C. Photo of samples obtained by cutting the hydrogel printout.
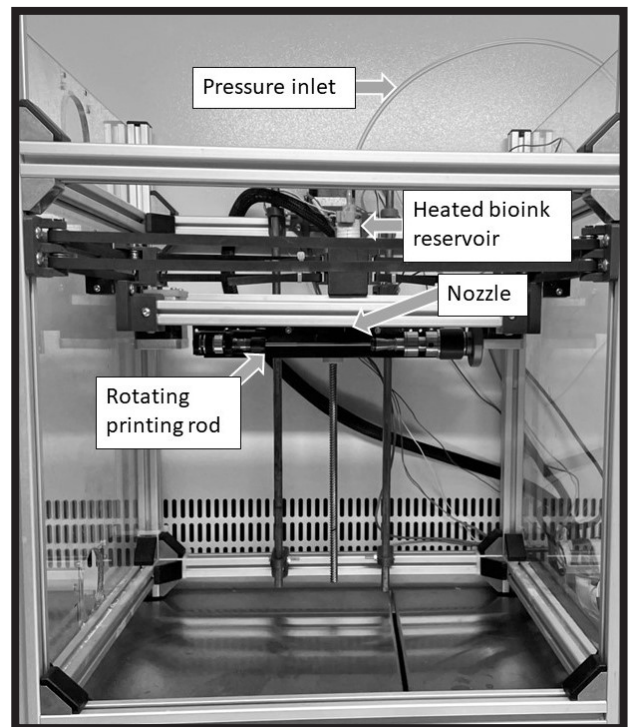

FIG. 3. A picture of a 3D bioprinter of own design which was used to print samples.

Statistical analysis

Each experiment was carried out in three repetitions for each sample. The results of survival rates are presented as mean ± standard deviation. Statistical significance was assessed using a one-way ANOVA analysis of variance. Statistical significance was marked in the graphs with an asterisk when $p < 0.05$.

Results and Discussion

Hydrogels preparation

Results of optimization of the cross-linking solution - selection of a concentration of $CaCl_2$-based cross-linking solution and the type of solvent used for its preparation

The test results of the effect of the concentration of the $CaCl_2$-based cross-linking solution and the type of solvent used for its preparation are presented in FIG. 4. The study showed that the hydrogel samples cross-linked with $CaCl_2$ solutions prepared in McCoy's 5A culture medium are characterized by a higher survival rate of the *Saos-2* line cells compared to the hydrogels cross-linked with aqueous $CaCl_2$ solutions. The highest cell survival rate, equal to 88.2 ± 2.0%, is observed for hydrogels cross-linked with 0.75% $CaCl_2$ solution in culture medium and hydrogels cross-linked with 5% $CaCl_2$ solution prepared in culture medium (88.0 ± 2.1%). The lowest survival rate is observed for the samples cross-linked with a 5% aqueous $CaCl_2$ solution, for which the cell viability is 76.6 ± 1.4%. According to ISO standard 10993 - Part 5 [22], materials that do not demonstrate cytotoxic effects are considered those for which the cell viability reaches the value of ≥ 70%. Thus, for all the materials analyzed, biocompatibility was obtained. Calcium ions are used in eukaryotic cell culture. They are essential for the normal function of vital cells. Literature data indicate that the endoplasmic reticulum stores calcium, but a low calcium concentration (2 mM) is required in the cytoplasm to maintain normal cellular functions [23]. The use of higher concentrations of calcium chloride extracellularly can lead to osmotic stress, due to disruption of cellular electrolytes, resulting in cell membrane damage [23-25]. However, the degree of cellular destruction varies, depending on the used concentration of calcium ions and the duration of exposure of the biological material to their action. Damage of cells may preserve their functional activity and proliferative capacity for regeneration or damage them completely. The applied calcium ion concentration should balance the osmotic stress. The study conducted by N. Cao's team and collaborators indicated that the rate of cell proliferation is higher in hydrogels with high calcium concentrations, which may be a result of calcium ion exchange, which is an important second transmitter signal in cell metabolism, between cells and their extracellular environments [23]. Research conducted by the L. Fischer team demonstrated that the physiological concentration of calcium ions in the hydrogel can increase cell survival after 3D bioprinting, because they can improve the ability of cells to overcome shear stress-induced deformation and plasma membrane damage during the extrusion process [26]. Higher concentrations of the cross-linking solution allow for obtaining a more stable structure of the gel by the increased availability of ions cross-linking the material. Higher Ca^{2+} concentrations are responsible for the formation of gels with higher cross-linking densities. The mechanical properties of alginate hydrogels are mainly controlled by the cross-linking density [27].
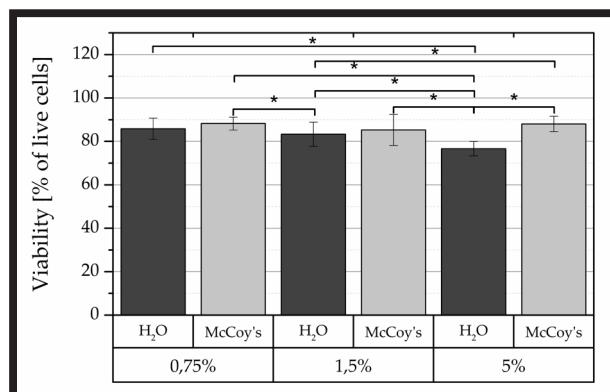
FIG. 4. Evaluation results of the degree of the Saos-2 line cell survival rate depending on the cross-linking solution concentration of the $CaCl_2$-based hydrogel and the type of solvent used for its preparation (H_2O, culture medium McCoy's).

The analysis performed in the scope of the present study showed that the application of a cross-linking solution based on $CaCl_2$ of McCoy's 5A culture medium as a solvent, analogous to the solvent used to prepare the polymer composition, leads to an increase in the viability of the cells incorporated into cross-linked hydrogels. This phenomenon may result from an interaction of calcium ions with components contained in the culture medium.

With the purpose of obtaining hydrogel structures with a higher degree of cross-linking, potentially greater stability, and better strength properties, with simultaneous preservation of a high cell survival rate, for further testing, the cross-linking solution with the concentration of 5% prepared in McCoy's 5A culture medium was used.

The results of the live/dead assay given in FIG. 5, presenting images of the cells incorporated inside the cross-linked hydrogel bioinks, prove that the biological material was uniformly distributed in the whole volume of the hydrogel. In each of the samples, the cells adapt the spherical morphology for all the analyzed variants of cross-linking solutions, which is commonly observed for the biological material contained inside sodium alginate-based hydrogels [23].

Optimization results of the combining method of alginate-gelatin solutions with the cell pellet

The results of the analysis of the effect of the combination method of hydrogel materials with *Saos-2* line cell pellet are shown in FIG. 6. The study showed that the highest survival rate of osteoblast cells, equal to 90.9 ± 1.6%, was observed in the samples in which cells before combining with hydrogel were suspended in an 8% volume of culture medium used to prepare the hydrogel and slightly lower in those suspended in 2% volume of culture medium used to prepare the hydrogel. The lowest survival rate was observed in the samples in which the cell pellet was directly combined with the hydrogel material - in this case, the cell viability was 71.1 ± 1.5%. The results obtained showed that the amount of the culture medium used to prepare the cell suspension before its combination with the polymer solution has a crucial role in ensuring the high survival rate of the cells incorporated into the bioink. With the increasing volume of the culture medium used for suspension of the cell pellet, the viability of the cells in the produced hydrogel structures increases. This phenomenon may be caused by a reduction of the shearing forces acting on the biological material previously placed in the cell suspension during the process of its combination with the hydrogel compared to the direct mixing of the cell pellet with the polymer solution.
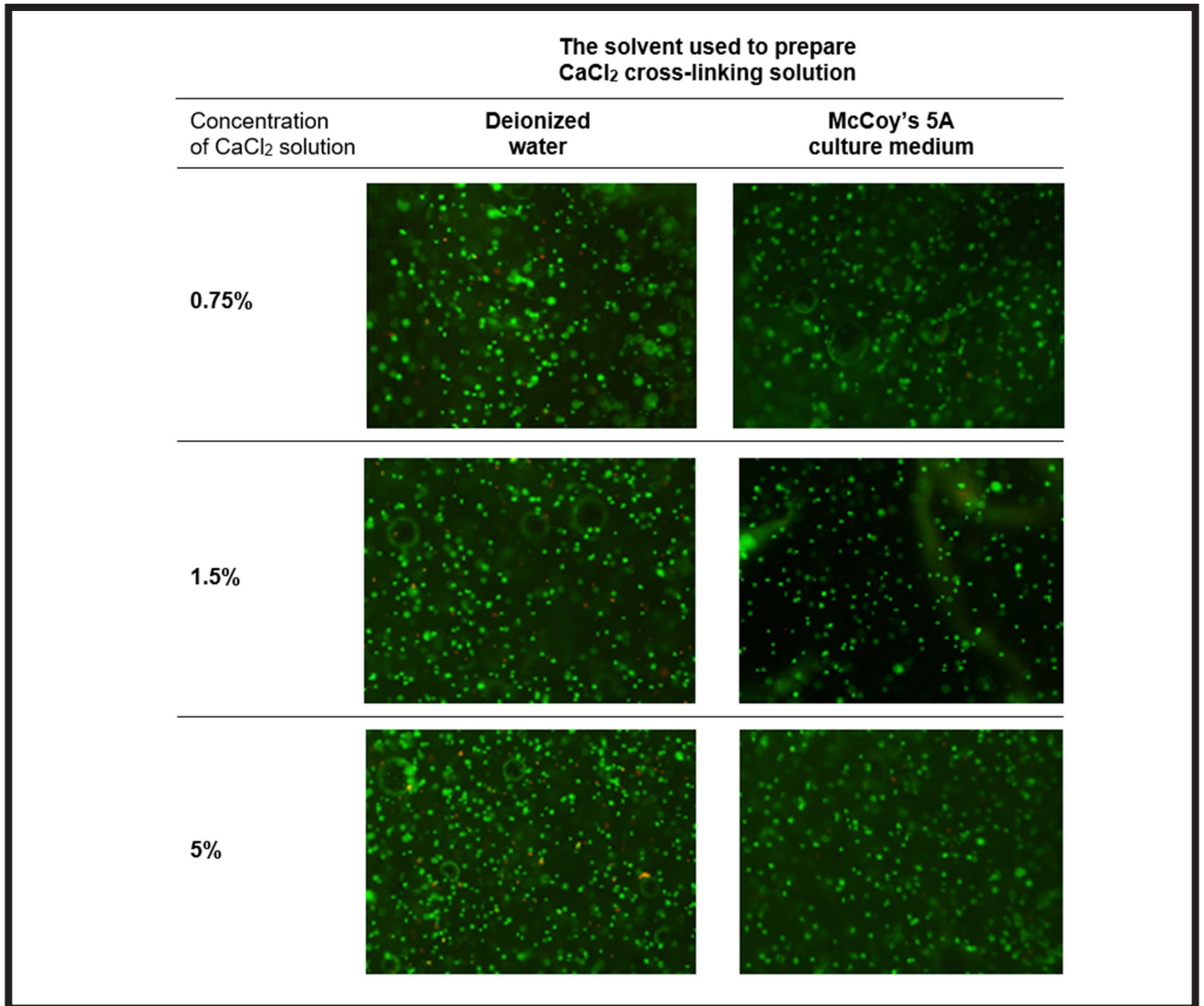
FIG. 5. Examples of the images obtained in the live/dead survival rate assessment of the *Saos-2* line cells for the samples after cross-linking with different CaCl$_2$ solutions.
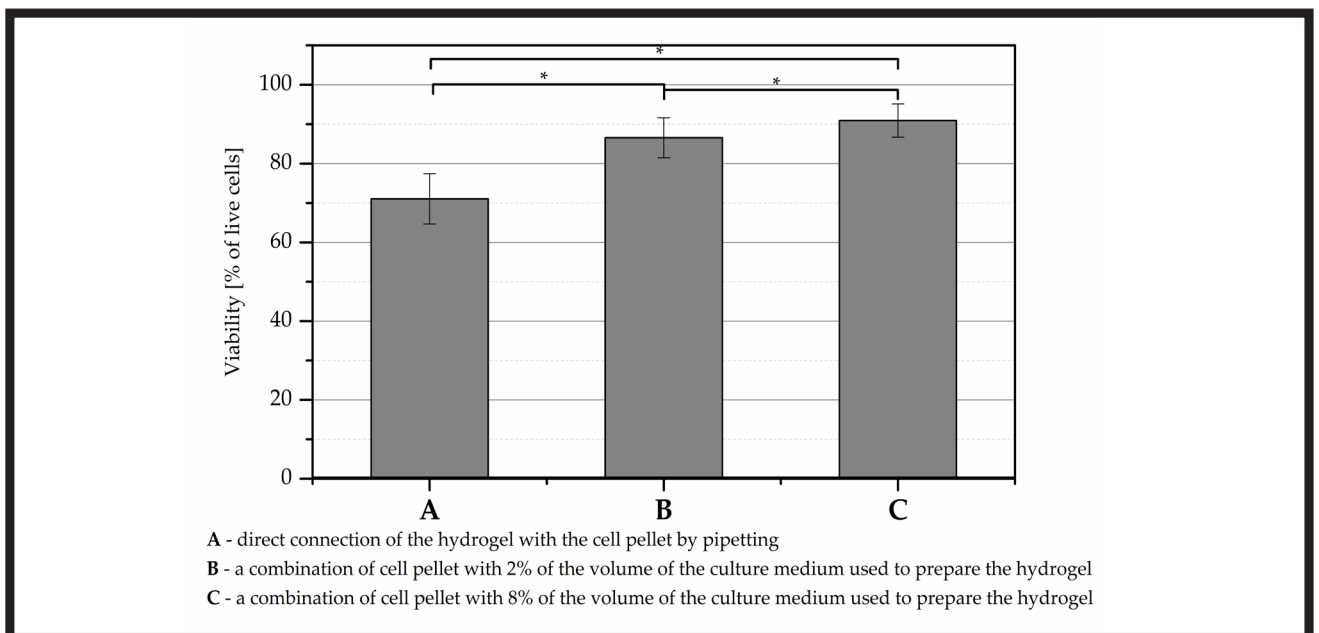

A - direct connection of the hydrogel with the cell pellet by pipetting
B - a combination of cell pellet with 2% of the volume of the culture medium used to prepare the hydrogel
C - a combination of cell pellet with 8% of the volume of the culture medium used to prepare the hydrogel

FIG. 6. Evaluation results of the *Saos-2* line cells survival rate depending on the combining method of the biological material with the hydrogel.

Results of evaluation of rheological properties of the polymer solution containing 2%Alg 9%Gel (w/v) used for 3D printing process

The curve of viscosity dependence as a function of shear rate, presented in FIG. 7, developed for a polymer solution containing 2%Alg 9%Gel (w/v) prepared in a method optimized during the experiments described in the previous sections, indicates that the viscosity of the developed polymer solution changes as a function of the shear rate. The obtained dependence proves that the material is a non-Newtonian fluid. With increasing shear rate, the viscosity of the polymer solution decreases in the range from the initial maximum values of 5.2551 Pa·s to 0.4786 Pa·s. The course of the curve proves the ability of the material to shear thinning. This is a very important characteristic of the polymer solution during 3D bioprinting processes, which makes it possible to reduce the force needed to extrude the material and decrease the shear stress during the printing process. The use of too high shear stresses related to the extrusion of the bioink can cause mechanical damage of the biological material contained in the printed structures [28,29].

Evaluation results of the effect of the waiting time of the bioink for 3D printing and the effect of the 3D bioprinting process itself on the viability of the cells contained in the hydrogel printouts

The results of the analysis of the effect of the 3D direct printing process on the survival rate of the cells contained in the bioink are presented in FIG. 8. The study showed that the 3D bioprinting process does not reduce the cell survival rate. Available literature data have also confirmed the non-destructive character of 3D printing toward biological material [20]. For each of the hydrogel bioink variants tested, the viability of the *Saos-2* line cells in final printouts is higher than 97%. The results of the live/dead assay (FIG. 9) prove that the biological material was uniformly distributed throughout the volume of the manufactured structure. The 3D bioprinting process allowed to obtain a higher survival rate of the biological material than in the case of the above-described studies leading to the production of the samples without using 3D printing. This phenomenon can be the result of the production of the structures with a layer thickness of about 250 µm during the 3D printing, which was not possible in the case of the samples poured on the surface of test plates. The high thickness of the samples can significantly limit the access of nutrients and gas exchange to the biological material incorporated inside the gel, which may result in a reduction in its viability [30].
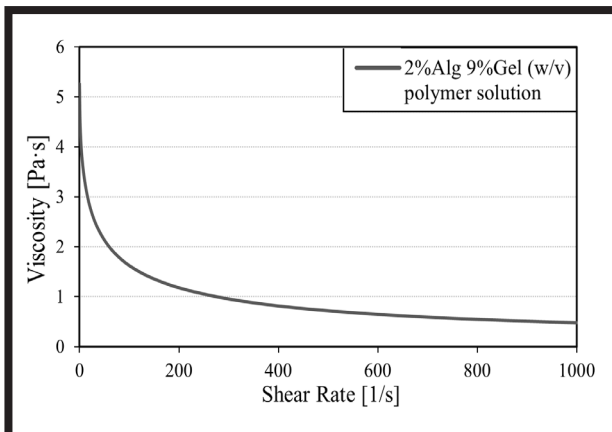

FIG. 7. Viscosity curve for a polymer solution containing 2%Alg 9%Gel (w/v) prepared in a method optimized during previous experiments that ensures the highest viability of the biological material before the 3D bioprinting process.
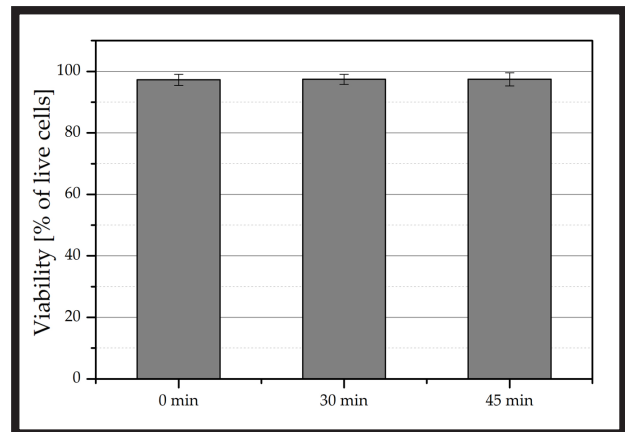

FIG. 8. Evaluation results of the degree of the survival rate of the Saos-2 line cells contained in the hydrogel printouts as a function of the waiting time of the bioink for the 3D printing process.
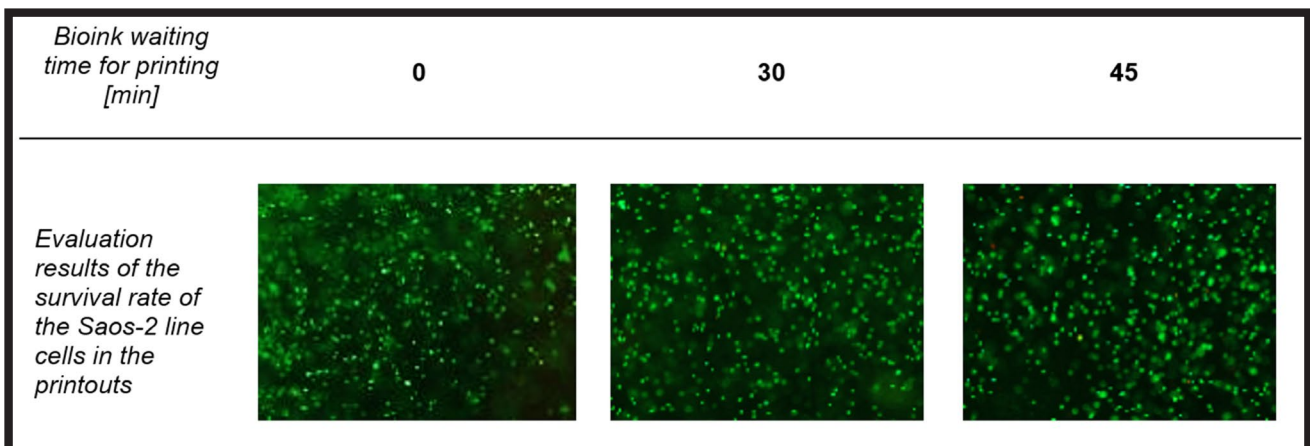

FIG. 9. Examples of the images obtained in the live/dead survival rate assessment of the *Saos-2* line cells in the hydrogel printouts in relation to the bioink waiting time used for the 3D bioprinting process.

The analysis also demonstrated that the waiting time of the hydrogel bioink for 3D printing, prolonged even up to 45 min, does not reduce the cell survival rate in the final printouts. This observation provides an important aspect of the printouts preparation process. Using the manufacturing parameters mentioned earlier, the 45-minute time is sufficient for printing the structures with the entire volume of the hydrogel bioink placed in a syringe with a capacity of 5 ml typically applied in bioinks extrusion. One of the key parameters responsible for achieving high cell viability in the printouts is the incubation temperature of the prepared hydrogel bioink. The results of the study conducted by the team of L. Ouyang and collaborators indicate that an extended 3D bioprinting process at temperatures below 30°C for up to 40 min results in a significant decrease in the viability of cells in the obtained structures [31]. This phenomenon is a result of the gelatin sol-gel transition, which is the thermal crosslinking at a temperature of approximately 20-30°C. Literature data indicate that the melting point of gelatin-based hydrogels is about 28°C [19]. The use of a lower temperature during the 3D bioprinting process results in the necessity to increase the shear forces applied to the biological material during bioink extrusion. The results obtained during our experiment prove that a decrease in cell viability inside the hydrogel bioink after its incubation for 45 min at 37°C is not observed because the prepared hydrogel bioink did not undergo a process of thermal cross-linking of gelatin. Previous studies conducted by members of our team prove that with increasing temperature, the viscosity of the tested hydrogel is lower [32]. The 3D bioprinting process carried out in the current experiment was conducted at a temperature above the crosslinking of gelatin, which did not generate the need to increase the pressure during extrusion, and consequently to apply higher shear forces acting destructively on the biological material in the printouts.

Evaluation results of the effect of incubation time of 3D printouts on the viability of the cells contained in them

The analysis of the changes in the viability of the *Saos-2* line cells contained in the hydrogel printouts after individual periods of their incubation in McCoy's 5A culture medium presented in FIGs. 10 and 11 demonstrate that together with the increasing incubation time of the printouts, cell survival rate decreases. However, in all the cases, it was higher than 70%. Directly after the 3D bioprinting process, the viability of the cells in the hydrogel printouts was nearly 97%.

After 24 hours of the printouts incubation, the cell viability was equal to 93.0 ± 2.0%, whereby after three days, the viability was 84.0 ± 0.6% compared to the cell viability of the samples that were not subjected to the degradation process. The decrease in the viability of the cells incorporated into printouts recorded during the study may result from the release of calcium ions cross-linking the hydrogel. Sodium alginate-based hydrogels cross-linked with calcium ions in physiological conditions undergo reactions of the exchange of divalent Ca^{2+} ions to monovalent Na^+ ions present in the degradation medium [21]. This process leads to a gradual de-cross-linking of the hydrogel. The literature data have shown that the release of calcium ions from the printouts occurring in high amounts during discussed processes may demonstrate a cytotoxic effect [33]. The cause of the reduction in cell viability may also be the process of gelatin release from the hydrogel structures. At temperatures above 25°C, gelatin starts to form a liquid, which dissociates at a temperature of incubation of 37°C. Under such conditions, the gelatin not bound to the cross-linked blocks of alginate undergoes liquefaction, which in turn leads to its gradual washing out from the material [20,33]. Gelatin has RGD peptide sequences in its structure, promoting adhesion, migration, and differentiation of the cells.
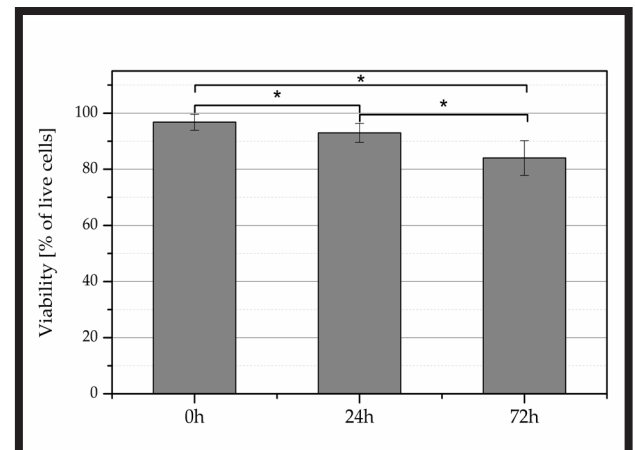

FIG. 10. Evaluation results of the degree of the *Saos-2* line cells survival rate contained in the hydrogel printouts right after manufacturing (0 h) and after 24 h and 72 h incubation in standard conditions.
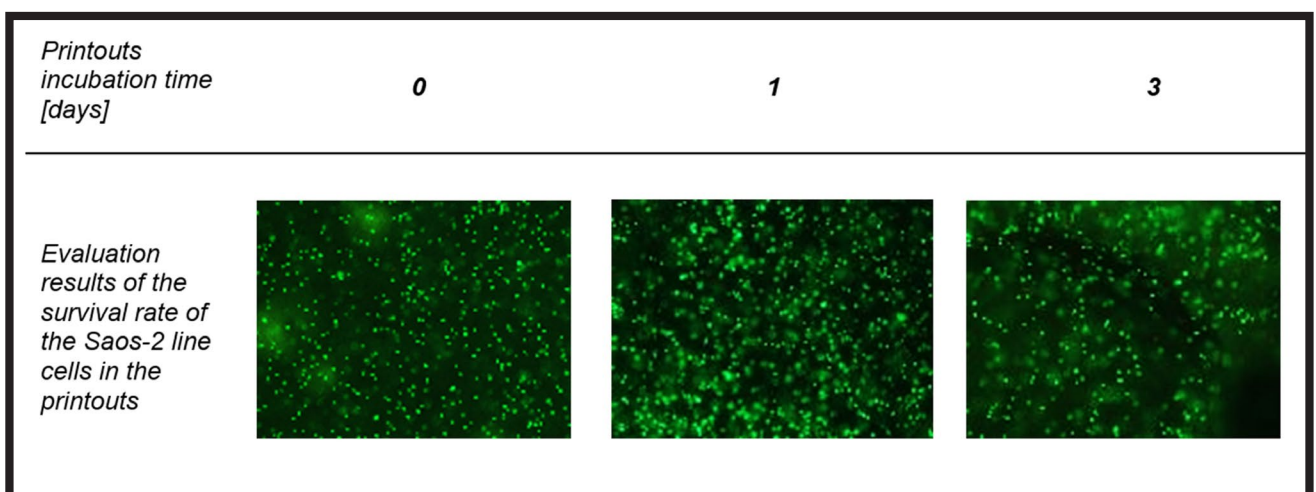

FIG. 11. Examples of the images obtained in the live/dead survival rate assessment of the *Saos-2* line cells in the hydrogel printouts after individual incubation periods in standard conditions.

Therefore, as a result of the gelatin release, a reduction in the number of biological groups promoting the viability of the cells may occur. The survival rate of the cells contained in the hydrogel material may also undergo a reduction as an effect of the application of overly high concentrations of the polymer solutions forming a hydrogel. Higher concentrations of bioink components prevent cell migration and nutrient diffusion due to the entangled polymer network, which results in a high rigidity of the material [20]. In order to analyze the possibilities of improvement of the survival rate of the cells contained in the printouts during incubation processes, it is necessary to perform further research. It should focus on a material composition characterized by reduced structure rigidity with a lower content of the alginate, of which the rheological properties will enable the 3D bioprinting process. It is also essential to try to reduce the thickness of the hydrogel print path with the aim to ensure higher permeability of the structure for the nutrients contained in the culture medium, metabolic product removal, and enhance the efficiency of gas exchange [28,34].

Conclusions

The research conducted within the framework of this study proves that the 3D bioprinting process itself does not affect the cell survival rate. One of the factors determining the survival rate of the biological material at the stage of the hydrogel bioink preparation is the method of combining the biological material with the polymer solution. The analysis demonstrated that the preparation of the cell pellet suspension in the largest possible volume of the culture medium directly before the mixing procedure with hydrogel solution, provides the highest viability of the cells in the bioink. The obtained results also indicated that an important parameter determining the viability of the cells in the hydrogel printouts is the solvent used for the preparation of the $CaCl_2$ cross-linking solution. It was demonstrated that the preparation of the cross-linking solution based on the culture medium provides a higher survival rate of the cells in the hydrogel printouts with respect to commonly used aqueous solutions. In addition, the performed tests confirmed that the prolonged 3D printing waiting time of the hydrogel bioink up to 45 min does not reduce the cell survival rate in the final manufactured structures.

The selection of the individual manufacturing process parameters of the alginate- and gelatin-based hydrogel bioinks, such as the combining method of the polymer solution with the biological material, concentration, and the solvent used to prepare the cross-linking solution based on $CaCl_2$, and also the waiting time of the prepared hydrogel bioinks for the 3D bioprinting process have the critical impact on the survival rate of the biological material contained in the hydrogel printouts. The results of the experiments performed demonstrate that with the application of the most optimal process parameters of the hydrogel bioinks preparation directly after the 3D printing, the survival rate of the model *Saos-2* line cells every time reaches values exceeding 96%. The observations of the changes in the survival rate of the osteoblast-like cells in the hydrogel printouts for individual periods of time indicate that with increasing incubation time of the samples, the survival rate of the cells undergoes a gradual reduction, which may be the result of the release of the gelatin and calcium ions from the hydrogel or potentially overly high rigidity of the material's structure.

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