DOI: 10.15825/1995-1191-2021-1-60-74

MATERIALS FOR CREATING TISSUE-ENGINEERED CONSTRUCTS USING 3D BIOPRINTING: CARTILAGINOUS AND SOFT TISSUE RESTORATION

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3D Bioprinting is a dynamically developing technology for tissue engineering and regenerative medicine. The main advantage of this technique is its ability to reproduce a given scaffold geometry and structure both in terms of the shape of the tissue-engineered construct and the distribution of its components. The key factor in bioprinting is bio ink, a cell-laden biocompatible material that mimics extracellular matrix. To meet all the requirements, the bio ink must include not only the main material, but also other components ensuring cell proliferation, differentiation and scaffold performance as a whole. The purpose of this review is to describe the most common materials applicable in bioprinting, consider their properties, prospects and limitations in cartilage restoration.

Keywords: regenerative medicine, tissue engineering, cartilage tissue, biomaterials, hydrogel, 3D bioprinting, scaffold.

INTRODUCTION

The cartilaginous tissue of the musculoskeletal system is exposed to great mechanical stress, is easily damaged, and due to the lack of blood and lymphatic vessels in it, it is slow to recover. Cartilage defects are often caused by trauma, age-related metabolic disorders, congenital diseases, and a number of other factors, in particular, endocrine pathologies and malignant neoplasms. Restoration of damaged cartilage remains a major medical problem, and modern tissue engineering can provide new solutions to it.

In recent years, 3D bioprinting has become increasingly common in tissue engineering. The advantage of the technology lies in the ability to form tissue-engineered constructs (scaffolds) with a given geometry and structure. Among the main methods of 3D bioprinting are extrusion, inkjet and laser. The most used technology today is extrusion-based 3D bioprinting. One of its main advantages is the ability to produce high cell-density constructs and the use of several components in printing [1–4], which became possible thanks to the emergence of 3D bioprinters with multiple printheads (dispensers).

A special class of biomaterials, bio-inks, is used to manufacture scaffolds through bioprinting. The concept "bio-ink" was first used in 2003 [5] and currently means a solution or hydrogel with cells [4, 6]. Bio-ink components are classified based on their role in scaffold creation [7, 8]. So sacrificial (support) materials are needed to support the construct during printing until the base material is completely polymerized, in particular when channels and cavities are formed in the scaffold. Other groups are the structural components (give the scaffold additional rigidity, modify porosity, etc.). And finally, functional components, which provide conditions for cell proliferation, differentiation, and synthetic activity.

The development of materials suitable for use as bio-ink is a special challenge. These materials must be suitable for both the printing process and for subsequent maturation of the scaffold with incorporated cells. For these purposes, a number of natural biomaterials have already been tested, including alginate [9–16], gelatin [17–23], collagen [24–30], hyaluronic acid (HA) [17, 31–34], silk fibroin [20–22], chitosan [31, 35, 36] and agarose [37, 38]. Synthetic materials such as polycaprolactone [9, 22, 39–42] and polylactide [43–45] are also widely used.

The main role of a biomaterial in tissue regeneration is to support cell function. Thus, materials for creating a scaffold must provide transport of gases, nutrients, and regulatory factors in order to make cell survival, proliferation, and differentiation possible. Besides, they must undergo biological degradation at a controlled rate close to the rate of regeneration of the tissue being replaced and be non-toxic and non-immunogenic. Finally, they should not only serve as a supporting structure for cells, but also provide mechanical strength of the tissue const-

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ruct as a whole and make its fixation in the implantation zone possible.

An ideal example of such a material is natural extracellular matrix (ECM), whose basic properties should be mimicked by scaffolds. The ECM microenvironment provides not only physical support for cell adhesion, but also signals regulating the life cycle, metabolism, and their differentiated state. The ECM is the main source and conductor of biochemical and biomechanical signals to ensure the organization and functioning of the tissue as a whole [46]. ECM is a multicomponent system of matrix macromolecules, composition and structures specific to each tissue type. The main ECM components are fiber-forming proteins such as collagens, elastin, fibronectin, laminins, glycoproteins, proteoglycans, and glycosaminoglycans [47]. In most tissues, the main fibril-forming component of the ECM is type I collagen, and in cartilaginous tissue, type II collagen [47].

In the aspect of 3D bioprinting, most natural polymers have insufficient mechanical properties and weak degradation. In contrast, synthetic polymers have good mechanical properties, but do not contain macromolecules normally found in living tissues. Therefore, various combinations of these materials are promising. Synthetic polymers are often added to gels in the form of granules or microfibers. At the same time, many authors have noted that simultaneous printing with natural and synthetic polymers is difficult due to the incompatibility of optimal temperatures: the printing temperature is in the range of 100 to 240 °C for synthetic polymers, and 4 to 30 °C for biogels [9, 15, 25].

The objective of this review is to highlight biomaterials and their combinations used primarily for cartilage repair. Meanwhile, the presented materials can be used for repair and regeneration of most soft tissues.

1. MAIN NATURAL COMPONENTS OF BIO-INK

Natural polymers such as agarose, alginate, hyaluronic acid, gelatin, collagen, fibroin, and chitosan are the most common as the main component of bio-ink, due to a certain similarity with ECM.

Agarose. It is a polysaccharide derived from red and brown algae, which consists of alternating residues of beta-D-galactopyranose and 3,6-anhydro-alpha-L-galactopyranose. It is widely used in molecular biology and tissue engineering due to its reversible gelling properties. In this case, the sol-gel and gel-sol transition temperature, as in the case of most hydrogels, depends not only on the concentration of the initial solution, but also on the molecular weight of the polymer [48]. Disadvantages of agarose-based bio-ink include a lack of conditions for maintaining cell growth [49, 50] and a low biodegradation rate [48]. Therefore, agarose is recommended to be used only as a sacrificial material, for example, for creating microchannels during scaffold vascularization [38].

Alginate. It is a polysaccharide derived from brown algae. It consists of guluronic and mannuronic acids [51]. This polymer supports cell growth well [52] and is relatively inexpensive. The material is readily soluble in water and polymerizes with divalent cations such as calcium and barium, as a result of ion exchange reactions [10, 44]. However, the biocompatibility of alginate is lower than that of natural polymers of animal origin, such as gelatin [53]. Alginate hydrogels degrade by releasing cross-linking gel cations or by decomposing the main chain through glycoside bond hydrolysis [54]. The main disadvantage of alginate is considered to be its low biomechanical properties, which complicate the printing process [16].

Chitosan. Chitosan is a natural polysaccharide derived from alkaline N-deacetylated arthropod chitin [55]. Chitin microfibrils are the main structural components in the exoskeleton of crustaceans and insects. It is also a part of the cell walls of fungi and yeast [56]. The hydrophilic structure of chitosan promotes adhesion and proliferation of almost all cell types [57]. The degradation rate of chitosan in comparison with natural polymers of animal origin, such as collagen, gelatin, and fibrin, is relatively low [57] and depends on both the degree of its deacetylation and its molecular weight [58]. In general, the half-degradation time in the body exceeds 30 days [59]. It is also known that this polymer is biocompatible, has antimicrobial properties, low toxicity, and immunogenicity, and, consequently, is of interest as a scaffold material [60–62].

Hyaluronic acid. Hyaluronic acid (HA) is a non-sulfated glycosaminoglycan consisting of repeating disaccharide fragments of N-acetyl-d-glucosamine and d-glucuronic acid [63]. It is found in almost all types of connective tissue [64]. In the body, it supports a number of biological processes such as cell growth, migration, and differentiation [65]. It is obtained by extraction from animal tissues (typically rooster combs) or biotechnologically as a product of the synthesis of modified bacteria of the genus *Streptococcus* or *Pasteurella* [64]. Due to the high content of carboxyl and hydroxyl groups, HA is a highly hydrophilic compound; therefore, it is capable of forming a gel-like structure in aqueous solutions as a result of intermolecular interaction of linear macromolecules [63]. However, as a 3D printing material, HA has limitations due to its weak mechanical properties, slow gelation, and very short biodegradation period [66, 67]. Therefore, in bio-inks, it is usually used in combination with other materials, such as alginate [68], gelatin [33], and collagen [34].

Collagen. Collagen is the main structural protein in most connective tissue types, maintaining the biological and structural integrity of ECM. Collagen has low immunogenicity, good biocompatibility, biodegradability, and regulatory functions in relation to cell adhesion, migration, and differentiation [69]. At 37 °C, it forms a

hydrogel with a triple helix structure [70]. Collagen is characterized by relatively low mechanical properties; but due to its high biocompatibility, it is one of the most frequently used scaffold components [26–29]. However, most of the commercial collagen preparations are immunogenic, which requires the use of its highly purified variants for tissue engineering.

Gelatin. This protein is a product of collagen denaturation and does not differ from the latter in terms of its amino acid composition [20]. Gelatin can be obtained from bones, tendons, or skin of animals by acidic or basic hydrolysis [71]. Despite its chemical composition similar to collagen, it lacks antigenic and immunogenic properties [72]. In vivo degradation time of gelatin crosslinked with glutaraldehyde, according to some data, is about 3 weeks [73]. Gelatin is often used in bioprinting as the main component or in combination with other biomaterials [20, 22, 33]. The most widespread are its modified forms, such as gelatin methacrylate (GelMA), which polymerizes quickly enough under the influence of UV, allowing full use of 3D-printing capabilities [17, 33, 37].

Silk fibroin. It is a natural macromolecular protein polymer with good biocompatibility and mechanical properties suitable for printing, and biodegradability [74]. Fibroin protein forms layers of antiparallel beta sheets [75]. Fibroin molecular composition and structure can vary depending on the silk source. For instance, silk formed by silkworm consists of two main proteins – sericin and fibroin. Fibroin is the structural center of silk, and sericin is the surrounding sticky component [75]. Gelation of silk fibroin can be induced in its aqueous solutions by high temperature, lowering of pH, sonication, and freezing; its electrogelation with formation of the β-structure conformation, which physically crosslinks and stabilizes the gel, has also been described [74]. Modification of silk fibroin with methacrylate has also been obtained [76]. Silk is degraded in vivo by proteolytic enzymes slowly (usually over a year) [77] and has good mechanical properties in terms of bioprinting [78].

The materials described above are actively used in biomedical research worldwide, as evidenced by the analysis of publications available in the PubMed database (Fig. 1). It should be noted that the bulk of the experimental work on scaffolds for cartilage replacement was performed using collagen: it has been very frequently used in the first 15 years of the 21st century. However, the situation has changed in the last 5 years: authors give preference to alternative variants of the main component of tissue-engineered constructs, specifically chitosan and fibroin (Fig. 2). One should also pay attention to the decrease in the frequency of agarose use in recent years, which may be related to its weak matrix properties for cells and extremely low rate of biodegradation. A similar trend may become characteristic of HA and alginate in the next 5 years. In general, it can be noted that the materials presented in Fig. 2 (with the exception of agarose) have been used with approximately the same frequency in the last 5 years – from 6.3 to 8.3% of the total number of studies.

2. MULTICOMPONENT BIO-INK

Obviously, the use of only one material as a bio-ink cannot provide all the mechanical and functional properties that are required to complete tissue-engineered constructs (TECs); so in recent years, scaffolds have been formed using a combination of several materials.

A silk fibroin and gelatin combination is quite often used [20, 21, 22, 79]. Silk fibroin acts as a structural material providing the mechanical properties of the gel and its biodegradation, while gelatin gives the viscosity (required for bioprinting) to the initial solution and elasticity to the scaffold after polymerization. In terms of ease of extrusion, gel strength in combination with its cytocompatibility, the following ratio of components of various silk and gelatin grades showed good results:

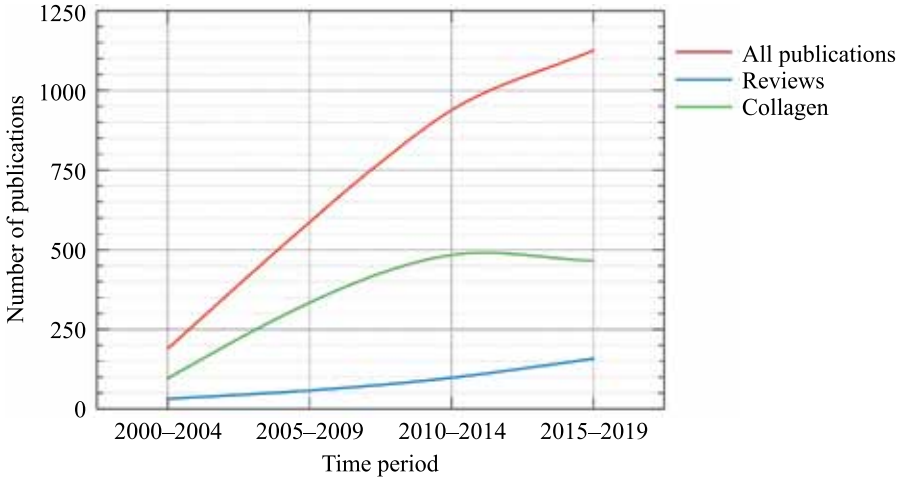

Fig. 1. Number of publications (from 2000 to 2019) on creation of tissue-engineered constructs for cartilage replacement

Bombyx mori 1.5%, Philosophamia ricini 1.5% and gelatin 7% [22]. The authors noted that more than 9% gelatin content and more than 2% silk content created very high viscosity and excessive printing pressure. Gelatin content below 5% provided insufficient viscosity, while silk fibroin content less than 1% resulted in very slow gelation. The silk to gelatin ratio 1:2 (6.9%) provided optimal mechanical properties (in terms of compression modulus), degradation rate, and microenvironment for cell proliferation, differentiation, and formation of cartilage tissue [20]. In changing the percentage ratio of silk fibroin in a gelatin-based hydrogel (30%) and nano-hydroxyapatite-based hydrogel (3%), Wu et al. found that 10% silk fibroin provides better mechanical properties to the scaffold (tensile modulus was 10.6 MPa) [21]. With increased silk fibroin content, the number of hydrogen bonds between molecules and, as a consequence, the degree of crosslinking of fibrils increased; biodegradation rate in this case naturally decreased. It should be noted that, according to Ke et al. [39], native human cartilage has a 14.7 MPa modulus of elasticity, which is close to the values obtained in the above work.

Combinations of gelatin with HA were investigated by Sakai et al. [17, 33]. The authors showed that GelMA and methacrylated HA content determined the behavior of cells in the scaffold. Thus, in scaffolds with a gelatin content of 1% and 2% versus 3% and 5%, a more pronounced suppression of cell growth was observed. In these works, only modified versions of gelatin and HA were used. Addition of methacrylate groups made the material suitable for rapid cross-linking, and, despite a rather low gelatin content, created a hydrogel structure stable at physiological temperatures, close in mechanical properties to those of native hyaline cartilage. A combination of thiolated HA with methacrylated collagen was investigated in a similar way [34]. The optimal for bioprinting, according to the authors, is a collagen/HA ratio of 3:1 with 6% and 2% content, respectively. Although other formulations (2:1 and 4:1) showed similar mechanical properties and were able to maintain cell viability in the same way as the 3:1 gel ratio. However, with such component ratios, the gels also exhibited certain drawbacks. For example, at a 4:1 ratio, formation of collagen bundles in the solution was observed already at room temperature, which, according to the authors, was associated with excessive collagen concentration. The 2:1 formulation, on the other hand, was characterized by insufficient amount of this material for cell interaction.

The chitosan-collagen pair is a frequently tested combination [24, 25]. An in vitro study showed the biocompatibility of scaffolds made with these materials: they supported the adhesion of mature chondrocytes, their spread over the surface and within TECs, providing a high level of their viability. In addition, it was shown that the amount of chitosan in the scaffold composition is the parameter directly affecting the pore size and its morphology [24]. Inclusion of hyaluronic acid in chitosan scaffold enhanced ECM cartilage production, chondrocyte proliferation, and cell adhesion to the scaffold surfaces [31].

Alginate-based scaffolds remain one of the most accessible and studied options [9, 37]. Research by Daly et al. [37] showed that alginate and agarose hydrogels supported hyaline-like cartilage formation to a greater extent than GelMA, as evidenced by the pronounced staining of newly formed tissues for type II collagen. On the other hand, GelMA promoted the formation of fibrous cartilage to a greater extent, as evidenced by the detection of higher amounts of type I collagen in the scaffolds. High levels of cell viability (~80%) were retained in all scaffolds after printing when the above components were used as bioinks. GelMA showed the best printability in this work, creating structures with greater accuracy than alginate and agarose bio-ink. Algi-

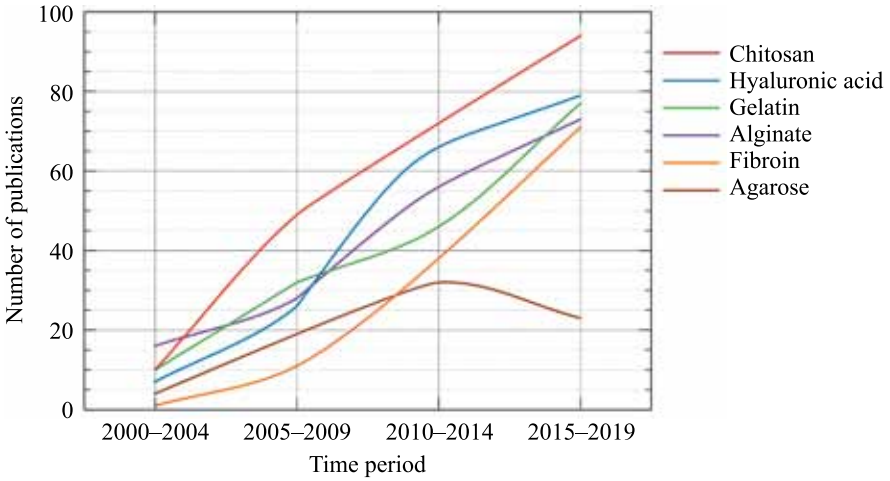

Fig. 2. Number of publications (experimental studies only, from 2000 to 2019) on the use of a range of biomaterials for creation of cartilage scaffolds

nate- and collagen-based TECs showed a homogeneous distribution of chondrocytes, increased expression of cartilage-specific genes, namely Acan, Sox9, and Col2a1, and decreased Col1a1 expression, proving that the chondrocyte phenotype is preserved [9].

Decellularized ECM can be used as bio-ink components, providing a natural microenvironment for cells. The advantages of such a component include the presence of biochemical signals of the original native ECM, correct protein proportions, and ability to selectively retain the adhesion and proliferation of cells of a particular tissue or organ. In a recently published paper by Basok et al., a microdispersed tissue-specific matrix was obtained from decellularized porcine articular cartilage, which retained the morphofunctional properties of ECM [80, 81]. The authors showed that such a matrix is capable of supporting the adhesion, proliferation, and chondrogenic differentiation of mesenchymal stromal cells.

3. MATERIALS THAT REINFORCE SCAFFOLD STIFFNESS

3.1. Scaffold materials

Scaffold materials serve to stiffen the construct. Moreover, they must be biocompatible, or at least bioinert and have a low degradation rate in the body. Synthetic polymers such as polycaprolactone (PCL), polylactide (PLA), polyglycolic acid (PGA), copolymer of lactic and glycolic acids (PLGA) are commonly used as scaffold materials [39, 40, 82, 83].

PCL is the most commonly used polymer for 3D porous scaffolds. It is a linear aliphatic polyester obtained by ring-opening polymerization of ε-caprolactone [84]. It is biodegradable, but more stable than PLA, since it is semi-crystalline and hydrophobic [85, 86]. Pati et al. [82] used PCL to support decellularized adipose tissue encrusted with mesenchymal stem cells. The volume of the structure remained constant for a long time due to the fact that the PCL scaffold retained its structure during the tissue remodeling process. Shim et al. [87] also used PCL support to create a scaffold with atelocollagen and supramolecular HA for the reconstruction of osteochondral defects in rabbit knee joints. PCL has already received FDA approval for clinical use [88].

PLA is a thermoplastic complex polyester that is derived from corn, sugarcane, wheat, or rice, making it affordable and inexpensive [89]. PGA is a synthetic polymer of glycolic acid [90]. PGA is more crystalline than PLA because it does not contain a methyl side chain; however, PLA is more hydrophobic [91].

Another scaffold material is the synthetic copolymer PLGA (usually 75% lactic acid and 25% glycolic acid) [92]. It is also a biocompatible material that degrades to non-toxic products (H_2O and CO_2 [93]). Like PCL, PLGA has already received FDA approval for clinical use [88].

The main disadvantage of the above-described synthetic polymer materials in terms of 3D printing is the need to maintain a high temperature when printing them (from 100 to 230 °C), which makes it difficult to use them together with hydrogels with cells. One of the options for creating composite scaffolds is the two-stage printing tactic: first, plastic, and then hydrogel. For instance, in a recent study, Kaye et al. [83] used a system with two dispensers – for printing separately PCL and alginate/collagen hydrogel with chondrocytes: hydrogel was printed into PCL channels after the latter had cooled. Thus, a tracheal tissue construct was obtained, which was implanted in New Zealand rabbits. The authors showed that such a scaffold induces cartilage formation while maintaining its integrity. It should be noted that the authors separated the hydrogel with chondrocytes from tracheal lumen with an intermediate membrane. In the absence of such separation, there was a tendency for inflammation, cartilage growth limitation and stenosis. PCL and hydrogel were used in another work on tracheal scaffold fabrication [39]. The authors obtained scaffolds that had mechanical properties similar to native tracheal cartilage and smooth muscle tissue. Izadifar et al. [10] formed constructs from a cell-containing alginate hydrogel in channels created between PCL strands in each layer. This approach demonstrated the possibility of creating a scaffold with the required geometry and high level of cell survival. The work of Romanazzo et al. was similar in printing method. [40]. Cell viability in the resulting scaffolds varied from 70 to 90% [10, 40].

Other possibilities for optimizing the mechanical properties of scaffolds produced by 3D printing are also described. For example, the addition of various nanoparticles (nanosilicates, halloysite nanotubes, nanocellulose, graphene) to TECs increases their rigidity and biological activity [16, 94–96]. For instance, the addition of alginate, methylcellulose, and halloysite nanotubes to a hydrogel at 20 mg/mL to 40 mg/mL concentration increased the tensile strength proportionally twofold (from 164 to 381 kPa), and the compressive stress 1.5 times (from 426 to 648 kPa) [16].

The mechanical properties of bio-ink with different proportions of chitosan, gelatin, and hyaluronic acid increased with the addition of graphene [94, 97, 98]. It has been shown that a 0.06% graphene content is most conducive to the formation of a porous scaffold structure, as well as a high value of the compression modulus. It should be noted that dependence of the mechanical properties of the scaffold on graphene content turned out to be nonlinear. Graphene can also be used in powder form: Sayyar et al. [97] showed that the addition of 0.5% graphene increased the tensile strength and elastic modulus of methacrylated chitosan by more than 67% and 40%, respectively, and also improved the adhesion and proliferation of L929 fibroblasts. Xavier et al. studied GelMA-based bio-ink with the addition

of 2% nanosilicate [98]. Nanosilicate (in proportion to its concentration) increased the mechanical strength of the scaffold, and nanosilicate-laponite (decomposes into magnesium, orthosilicic acid and lithium readily removed by the body) facilitated the process of removing the scaffold biodegradation products.

Cellulose and methylcellulose are commonly used options for enhancing the stiffness of a bio-ink scaffold [11–13, 95, 96, 99]. Müller et al. [99] used commercial bio-ink based on sodium alginate and nanocellulose for cartilage 3D printing. Addition of nanocellulose improved the bioprinting quality. However, this component had a negative effect on cell proliferation. These data were confirmed in the publication on the use of nanocellulose hydrogels for auricular cartilage: the average cell viability after biofabrication did not exceed 68.5–76.9%. [95]. Adding methylcellulose to the hydrogels increased the scaffold elasticity and stability, as well as microporosity [13]. In addition, this proved to be one of the optimal approaches to achieve a higher elasticity of the hydrogel coming out of the printing needle, which opens up the possibility of printing large multilayer constructs [96].

Addition of PCL and PLA microfibers to printing hydrogels can be an additional option to improve the rigidity of finished constructs. For example, PCL microfibers have been successfully used by Daly et al. [34]. Narayanan et al. used bio-ink with PLA nanofibers (0.5%) in the design of meniscus tissues [44]. It can also be noted that PCL granules form clusters of cells around themselves, promoting their survival and proliferation in the scaffold [100].

3.2. Sacrificial components

The use of sacrificial components in scaffold formation is one of the key bioprinting techniques today. A combination of the base hydrogel with incorporated cells and the sacrificial material during printing allows both to provide temporary support of the base hydrogel until its complete polymerization, and to form niches and channels imitating blood vessels responsible for the access of gases and nutrients [30, 38, 101–106]. The main requirements for the sacrificial material are complete utilization from the scaffold within a specified timeframe and the absence of cytotoxicity of its degradation products. Various materials used for this purpose have been described in the literature. Lee et al. [105] used gelatin to form channels with a lumen of up to 1 mm in the collagen scaffold. Bertassoni et al. [38] developed a similar strategy for building vasculature using agarose gel. A number of studies have used the commercial product Pluronic F-127 as a sacrificial material [30, 101, 103]. In particular, using this component, it was possible to form macropores in a scaffold of nanofiber collagen [30]. Fitzsimmons et al. found that Pluronic F-127 has an advantage over gelatin as a sacrificial material for the creation of vascularized tissues due to the uniformity of the filament during printing and a higher compression modulus [101]. The use of filaments made of polyvinyl alcohol [102] and alginate [104] as a sacrificial material has been described. In addition to filaments, the sacrificial material can be in the form of microspheres, providing the scaffold with controlled microporosity [106, 107].

4. MECHANISMS OF POLYMERIZATION IN 3D PRINTING

Most of the materials used for bioprinting are initially in the state of solutions or suspensions, and must undergo the polymerization (cross-linking) stage (in order to form an elastic gel in the scaffold), which, depending on the experiment design, scaffold architecture and geometry, begins before printing, during printing or after formation of each layer. Controlled cross-linking of different materials is provided by different physical and chemical influences – light, temperature, ion concentration, pH, etc.

The most physiological for collagen is the "temperature" type of polymerization, which spontaneously occurs when the solution temperature rises to 20 °C [30]. In these cases, extrusion is performed with cold solution (+4 °C to +8 °C), and the platform on which the printing takes place is heated to 25–35 °C [108]. Collagen polymerization can also be induced by lowering the pH of the solution [109], but this can negatively affect cell viability in the formed scaffold [108, 110].

One of the new options for controlled polymerization of collagen with other materials is the use of genipin [1, 24, 25, 111, 112]. The genipin crosslinking mechanism is due to several nucleophilic substitution reactions involving different sites of collagen molecules [25]. In particular, it has been shown that to obtain optimal mechanical, structural and biological properties of a scaffold for replacing cartilage defects based on collagen and chitosan, a 1% genipin content is recommended [25]. After cross-linking, collagen and chitosan form a macroporous layer in which chondrocytes remain viable, mainly in the areas adjacent to the pores [24]. Genipin cross-linking is also possible for formation of gelatin- and silk fibroin-based scaffolds [20]. It is important to note that genipin is used due to шеы stable but long-term polymerization process of up to 1 hour [25, 111], which to some extent limits the use of this approach in the formation of large-sized scaffolds. In addition, some studies have shown delayed adverse effects of genipin, particularly in the degradation of the basic scaffold material [113]. An alternative to genipin is tannic acid, whose crosslinking mechanism is due to the formation of numerous hydrogen bonds between the two materials [112]. In Yeo et al., the optimum concentration of tannic acid for crosslinking was 2% [112]. However, Lee et al. observed improved mechanical properties even at 0.5% content [1].

Alginate solutions are characterized by the ability for ionotropic gelation under the action of such cations as Ca^{2+}, Ba^{2+}, Cu^{2+}, Al^{3+}, which act as crosslinking agents, interacting with the carboxyl groups of guluronate blocks of polysaccharide molecules; mannuronate blocks remain free in this case [14]. Calcium chloride is most often used as a crosslinking agent in alginate-based hydrogels [11, 12, 15].

In recent years, photocrosslinkable biomaterials have become increasingly common. This approach has several advantages over other crosslinking methods since it makes it easy to control printing by adjusting the rate and degree of cure of the resulting construct. Many natural biomaterials, such as gelatin [18, 19], silk fibroin [76], and collagen [114], are polymerized by acrylation under a UV lamp at 365 nm wavelength. Drzewiecki et al. demonstrated the use of photocuring of collagen methacrylamide as a fibrill-forming bio-ink for scaffold fabrication [114]. Photocrosslinking of HA methacrylate has been described by Onofrillo et al. when creating a cartilage scaffold [19]. Similarly, a gel based on silk fibroin methacrylate (SilMA) was prepared and studied, which, according to the authors, is biocompatible, biodegradable and has suitable biological and mechanical strength [76]. In contrast to genipin polymerization, in photocuring of methacrylate, polymerization of a single layer is completed in 5 minutes. However, some authors note that the disadvantage of acrylation is that the scaffolds have reduced biocompatibility, since unreacted acrylic groups are cytotoxic and, moreover, can cause local inflammatory reactions in vivo [115]. The frequently used photoinitiator Irgacure 2959, which is a source of free radicals required for polymerization reaction, has the same disadvantage [116]. Reactions with phenolic residues in natural biomaterials are another way to initiate cross-linking formation. For example, the mechanical properties of a hydrogel made from gelatin and HA modified with phenolic hydroxyl groups can be controlled by changing the concentrations of tris(bipyridine) ruthenium(II) dioxide and sodium-ammonium persulfate and the light irradiation time [17]. Riboflavin can also be used as a photoinitiator for collagen, which under a UV lamb causes the formation of covalent cross-links between amino acid groups in collagen chains [117]. The main advantage of riboflavin is that it is usually present in the body and, unlike other photoinitiators, is not cytotoxic. Riboflavin-induced photopolymerization of collagen hydrogel containing fibrochondrocytes did not change the scaffold shape, while increasing the expression levels of type II collagen and aggrecan genes in cells [70]. The optimal riboflavin level, increasing the elastic modulus, was 0.01% [70]. The broad utility of riboflavin has been shown by Batchelor et al. [118]. It should be noted that the use of riboflavin allows relatively rapid polymerization (from 10 seconds to 5 minutes) under visible light [70, 117].

For scaffolds made from a silk-gelatin mixture, physical cross-linking can be performed under the influence of ultrasound [119], which induces "crystallization" of β-structures of fibroin as a result of increased molecular vibration, hydration of hydrophobic domains, and short-term increase in local temperature. "Cross-linking" of fibroin (and gelatin) can also be achieved by self-assembly using two different types of fibroin [22].

One of the options for maintaining a balance between printability and stiffness of the resulting construct is to use double polymerization of the material. The first stage involves selecting the viscosity of bio-ink (in the "gel-solution" boundary state) suitable for the printing process, and the second stage involves increasing the stiffness/elasticity (transition to the gel state) necessary to maintain the geometry immediately after after each layer is printed. Such an approach has been described in detail by Skardal A. et al. for the polymerization of acrylates and alkynes in the case of creating scaffolds based on collagen, HA, and gelatin [120]. Kajave et al. addressed the issues related to insufficient mechanical properties and rapid degradation of scaffolds obtained in this way, which is inherent in all TECs obtained using low concentrations of collagen [26]. The authors showed that sequential application of UV and genipin (0.5 mM) significantly improves the elasticity of scaffolds and increases their degradation time in the body both with incorporated cells and in cell-free variants.

5. COMMERCIAL INKS FOR 3D PRINTING

In recent years, commercial bio-ink preparations have appeared on the biotechnology market. For example, CELLINK (Sweden) developed bio-ink based on alginate, collagen, gelatin and chitosan – CELLINK's GelX series based on methacrylated gelatin and CELLINK Bioink based on nanofibrous cellulose and alginate, which can be modified with RGD peptides, tricalcium phosphate, laminins, and fibrinogen [121]. Their suitability for bioprinting has been demonstrated in a number of recent studies [11, 12, 95, 99]. Israeli company CollPlant chemically modified recombinant human collagen to create bio-ink (rhCollagen BioInk) suitable for a variety of printing technologies, including extrusion, inkjet printing, laser induced direct transfer and stereolithography. Advanced BioMatrix (USA) developed LifeInk 200 and LifeInk 240 bioinks for extrusion printing based on collagen, matacrylated collagen, gelatin, and HA, as well as thiolated HA [122]. The company also produces bioprinting ink. Biogelx produced synthetic bioinks that form a nanofiber network mimicking the extracellular matrix. These bio-inks can support cell growth and proliferation, signal transmission, and have rheological properties suitable for bioprinting [123]. It is worth noting that the cost of such bio-ink is quite high.

In addition to materials presently adapted for 3D bioprinting, a whole range of commercial cartilage re-

pair products are currently in clinical trials. They are either off-the-shelf scaffolds or hydrogels that polymerize rapidly at the implantation site. Among them are NOVOCART 3D, RevaFlex and MACI. RevaFlex is a tissue-engineered cartilage implant for knee cartilage repair and regeneration, containing allogeneic juvenile chondrocytes [124]. NOVOCART 3D is positioned as a personalized implant based on patient-derived chondrocytes, which are cultured on collagen scaffolds [125]. Similar is MACI, which contains autologous chondrocytes cultured on porcine collagen membrane and is designed to repair knee cartilage damage [126].

CONCLUSION

Publications of the last 5 years devoted to the use of various biomaterials in 3D-bioprinting of cartilaginous and soft tissues have been analyzed. We have discussed the advantages and disadvantages of the basic components of scaffolds, approaches to scaffold polymerization, including the types and features of the use of cross-linking agents, the ways of improving the properties of bio-ink, in particular by using additional components responsible for stiffness, porosity and other basic scaffold properties. Trends towards changes in the frequency of use of a number of materials have been analyzed. In general, despite a wide variety of basic biomaterials and a range of additional components used in the creation of TECs for replacement of cartilage and soft tissue defects, the search for new options for complete replacement of ECM continues.

The authors declare no conflict of interest.

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The article was submitted to the journal on 9.09.2020