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Structure and stability of composite gels based on collagen and carboxymethylcellulose

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The creation of biocompatible gels, based on type I collagen is studied. To improve the mechanical properties, 10−30% carboxymethylcellulose (CMC) was added to the collagen gel. It is shown that with an increase in the CMC content up to 30%, the stability of composite gels increases. With the help of scanning electron microscopy it is shown, that the ability of collagen to form native fibrils decreases with the addition of CMC. It was also shown by electrophoresis that the presence of CMC increases the rate of degradation of the composite collagen gel.

Keywords: type I collagen, carboxymethylcellulose, composite collagen gels, degradation.

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Introduction

The extracellular matrix (ECM) forms the microenvironment of cells and participates in the regulation of their vital activity [1]. Depending on the type of tissue (organ), ECM imparts certain mechanical and biochemical properties to tissues and influences the physiology of cells [2]. The behavior and development of cells are determined by a number of ECM properties described below.

When cells are cultivated in a gel, they are distributed throughout its volume, changing its mechanical properties, while hydrolysis, dissolution and enzymic degradation of the entire material occur [3]. The rate of the latter depends on the number of available cleavage sites in the polymer and on the degree of enzymic load [4]. Understanding of the degradation rates is necessary when timely and controlled release of biologically active molecules from a matrix is required, for example, for drug targeted delivery. The importance of degradation is due to the use of matrices as a carrier for transplanted cells. In this case, the rate of regeneration increases, since the destruction of the hydrogel stimulates vascularization and fusion of donor cells with host tissues [5]. However, it is especially important that the decomposition products of the matrix are not toxic.

According to the source of origin hydrogels can be divided into natural and synthetic. Proteins and polysaccharides [3] belong to the natural ones. These polymers are characterized by good biocompatibility, bioactivity and low toxicity [3,6]. However, since they are obtained from natural sources, it is difficult to maintain the stability of characteristics in different batches of supplied materials, which negatively affects the reproducibility of experimental results. The literature contains data about too rapid degradation or deformation of matrices based on such materials [6], as well as difficulties in monitoring gel characteristics (for example, stiffness and porosity) and accurate determination of the molecular structure of the gel.

Collagen is structural protein, which is the main component of connective tissue [7]. Today, up to 30 different types of this protein are known, structurally all of them consist of a right-handed triple helix of three polypeptide chains [2]. Collagen type I is the most common and well-studied, widely represented in bones, dermis, ligaments and tendons, as well as in the cornea of the eye [8].

It was shown that collagen gel promotes proliferation, migration, and osteogenic differentiation of mesenchymal stem cells (MSCs), providing high level of cell survival [9]. Since it is a natural component of ECM, it is biologically active, non-toxic and biodegradable [8]. Besides, collagen has a relatively low immunogenicity, which makes it possible to use a gel based on it in regenerative medicine.

The disadvantages of collagen gels are low mechanical properties and rather weak resistance to enzymic degradation. Cells during cultivation in gels synthesize their own ECM proteins, which contributes to the contraction of the collagen matrix. It was found that the most significant contribution to this is the secretion of fibronectin [10].

In order to improve the mechanical properties of the collagen gel can be modified. The most common way is the formation of crosslinks (intra- and intermolecular bonds) by physical methods (UV radiation, heating to a high temperature) or by chemical agents (glutaraldehyde, formaldehyde) [11]. However, such methods are often destructive or toxic to cells and are suitable for first obtaining a three-dimensional scaffold, and only then populating it with cells.

If it is possible to first distribute the cells over the substrate and then start the gel formation process, the system can be used in the form of injections [12]. This approach limits the use of a variety of materials and their modifications, but allows for a tighter contact between the transplanted material and the tissues of the recipient, accelerating and facilitating regenerative process. The method also makes it possible to more evenly distribute the cells over the gel volume [13]. These factors have a positive effect on the reproducibility of the results, in particular, when using the obtained three-dimensional system as a platform for drug testing.

Besides, the addition of other polymers, in particular, carboxymethyl cellulose (CMC), which already proved itself as a non-toxic and inexpensive material that enhances the stability of composite structures, can be a reasonable way to improve the mechanical properties of collagen gel.

E. Abdolahinia et al [14] demonstrated by computer simulation that CMC interacts with collagen amino acid residues. During the interaction of polymers, predominantly hydrogen and van der Waals bonds are formed.

M. Zhang et al [15] found that the strongest interaction between polymers in solution occurs at a dry matter mass ratio of CMC and succinylated collagen 1 : 1. CMC molecules have −COOH, −COONa groups and initial (unsubstituted) hydroxyl groups of cellulose. Collagen in this case, for the most part, has carboxyl groups and, to a lesser extent, hydroxyl groups. It was shown that the mass ratio of polymers in solution influences the predominance of certain types of interactions. Thus, when the CMC content is 50% maximum, the hydrogen bonds predominate in the mixture, which favorably affects the mixing of polymers and the stability of the resulting mixture. With CMC fraction over 70%, electrostatic repulsion becomes the main one. Thus, the study by M. Zhang can be useful for preliminary selection of the ratios of CMC and collagen in order to obtain the most stable gel compositions.

C. Ding et al [16] found that CMC reduced the rate of collagen fibril formation *in vitro*, while the diameter of the fibrils themselves decreased. Besides, the polysaccharide addition improves the thermal stability of collagen gels.

There are developments of two-layer scaffolds based on collagen and CMC, intended for the reconstruction of skin tissues [16], as well as for the prevention of adhesive processes after operations on the abdominal organs [17]. CMC in this case is a layer that prevents cell adhesion. The aim of this work is to study the structural properties of composite collagen gels and their stability under *in vitro* conditions.

1. Experimental part

1.1. Materials Gels formation

Type I collagen (obtained at the Center for Cell Technology at the Institute of Cytology of the Russian Academy of Sciences by acid extraction) was brought to a concentration of 2 mg/ml in the final volume. Collagen was mixed with 1% (w/v) solution of CMC in a buffer solution (phosphate buffered saline, PBS) (10, 20, 30% of the final gel volume, no CMC was added to the control samples). Then 10X 199 medium was added in amount of 10% of the final volume. A 4M aqueous solution of NaOH was added to the resulting mixture until the color changed from yellow to pink, indicating a transition from an acidic medium to a neutral weakly alkaline one. The solution was brought up to final volume with PBS.

After adding each component, the mixture was well mixed on a vortex (Biosan, Latvia), and the manipulations were carried out on ice to avoid premature gelation. The mixture was added in equal volume to the wells of the plate and incubated for 15 min at 37°C to form a gel, after which the samples were flooded with PBS.

When preparing gels for fetMSC culturing in them, a suspension was added so that the number of cells was $25 \cdot 10^4$ in 1 ml of gel, and stirred using an automatic dispenser. The mixture was adjusted to final volume with DMEM/F-12 medium.

1.2. Research methods

1.2.1. Evaluation of hydrolytic degradation of gels

The degree of the matrices degradation was assessed by quantitative determination of the collagen exit from the gels according to the Lowry−Hartree method [18]. The gels were prepared according to the above method in 24-well plate in a volume of 300 μl per well. Scaffolds were incubated in PBS (1 μl per well) at 37°C for 1, 7, and 21 days. To avoid the liquid evaporation the wells were sealed with film Parafilm (Bemis, United States), the plate was additionally wrapped in food wrap.

At the end of the incubation 500 μl of liquid were taken from each well and transferred into microcentrifuge tubes, centrifuged for 10 min at 10 000 rpm. We took 300 μl of the supernatant liquid into glass test tubes, added 700 μl PBS. 1000 μl PBS was used as a control. Then 900 μl of reagent A was added, the reagent contains sodium-potassium tartrate, sodium carbonate and NaOH. The contents of the test tubes were mixed on the vortex, after which they were heated for 10 min at 37°C in a water bath. Next, 100 μl of reagent B containing sodium-potassium tartrate, copper (I) sulfate, and NaOH were added to the test tubes. The solutions were again mixed on vortex and incubated for 10 min at room temperature. Then, 3 ml of reagent C containing Folin's reagent and water in the ratio of 1 : 15 was added to each tube. They were stirred, heated for 10 min at 37°C in a water-bath.

The optical density was measured in quartz cuvettes on a UV spectrophotometer PE-5400 (Ekroskhim, Russia) at a wavelength of $\lambda = 650$ nm. The protein weight was found according to the equation for the calibration curve obtained

earlier in the laboratory:

$$D = 0.002x + 0.003367,$$

where x — weight of collagen in μg, D — optical density.

Next, the percentage of protein yield was found using the formula

$$Protein\ desorption = \frac{m_{measured}}{m_{initial}} \cdot 100\%.$$

Based on the analysis results a graph of the dependence of collagen dissolution on the amount of CMC in the gel was plotted at different incubation periods.

1.2.2. Investigation of the surface morphology of matrices

The gels were prepared according to the above method by applying to coverslips. The samples were then washed twice with PBS in deionized water and air dried. Images of the matrix surface morphology were obtained using a scanning electron microscope JSM-7001F (Jeol, Japan) in the secondary electron mode at an accelerating voltage of 5 kV and a beam current of 10 pA. Preliminarily, a layer of gold 20 nm thick was deposited on the samples by magnetron sputtering using unit Emitech K950 (UK).

1.2.3. Collagen in matrices resistance to enzymatic degradation

Type I collagenase dissolved in TESCA buffer (contains calcium necessary for enzyme operation) was added to the studied gel samples at the rate of 12 U per 1 mg of collagen, to the control — buffer only. Incubated on a thermoshaker for 1 h at a temperature of 37°C. Next, the solution was transferred into microcentrifuge tubes, centrifuged for 10 min at 10 000 rpm. The supernatant liquid was carefully collected, 80 μl of sample application buffer was added to the sediment. Then the samples were heated for 5 min at 99°C.

Electrophoresis was carried out in 7.5% polyacrylamide gel, putting 10 μl of sample into a pocket. After process completion the gel was stained with Coomassie dye for 30 min and washed in hot water with the addition of glacial acetic acid until the protein bands were clearly visible.

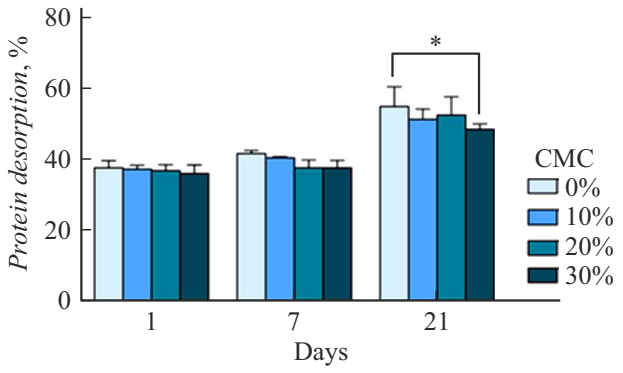

Figure 1. Percentage of collagen yield from the gel vs. amount of CMC in the matrix at three incubation periods (two-way ANOVA, Tukey's test: $p < 0.05$).

1.2.4. Statistical processing of the result

Calculations were carried out in Microsoft Excel and GraphPad Prism programs. To compare the samples, one-way and two-way analysis of variance (ANOVA) was used.

2. Results and discussion

2.1. Evaluation of hydrolytic degradation of gels

Because the cells are cultured in a liquid nutrient medium, collagen matrices are subject to hydrolytic degradation. Stability is an important characteristic of matrices, since it is often necessary to know at what rate certain substances are released from them. In this regard, the degradation of gels in phosphate buffer at 37°C (to simulate physiological conditions) was evaluated at three incubation periods (1, 7 and 21 days).

The yield of collagen from the gels was quantified by the Lowry–Hartree method. The principle of the method is that copper ions form complexes with peptide bonds, which interact with Folin's reagent, forming a colored reaction product [18]. The color intensity is directly proportional to the protein concentration and is measured by spectrophotometry. Fig. 1 shows the results of the study. It can be seen that there is a trend towards a decrease in the percentage of protein yield with increase in the CMC content in the gel. However, statistically significant ($p < 0.05$) differences were obtained after 21 days of incubation in phosphate buffer for a gel with 30% CMC content compared to pure collagen gel. However, this effect is weakly expressed, and it can be stated from the results that the CMC addition to the collagen gel does not affect the degradation rate of the composite gel.

Such increase in stability can be explained by the formation of complexes between collagen molecules and CMC. Based on the findings of E. Abdolahinia and M. Zhang et al [14,15], electrostatic, hydrogen, and van der Waals bonds appear between these polymers.

2.2. SEM study of the matrix surface

Fig. 2 shows images of the surface of the gels at three magnifications. It can be seen that large fibrils with a pronounced native structure are formed in pure collagen gels. As CMC is added, the number of fibrils and their diameter decrease, and nativeness disappears. In gels containing 30% CMC, no fibrils are formed.

The data obtained can be explained by the formation of complexes between collagen and CMC. The interaction of polymers (in particular, the formation of hydrogen bonds) prevents the assembly of fibrils, disrupting the natural structure of the protein. Earlier the study by C. Ding et

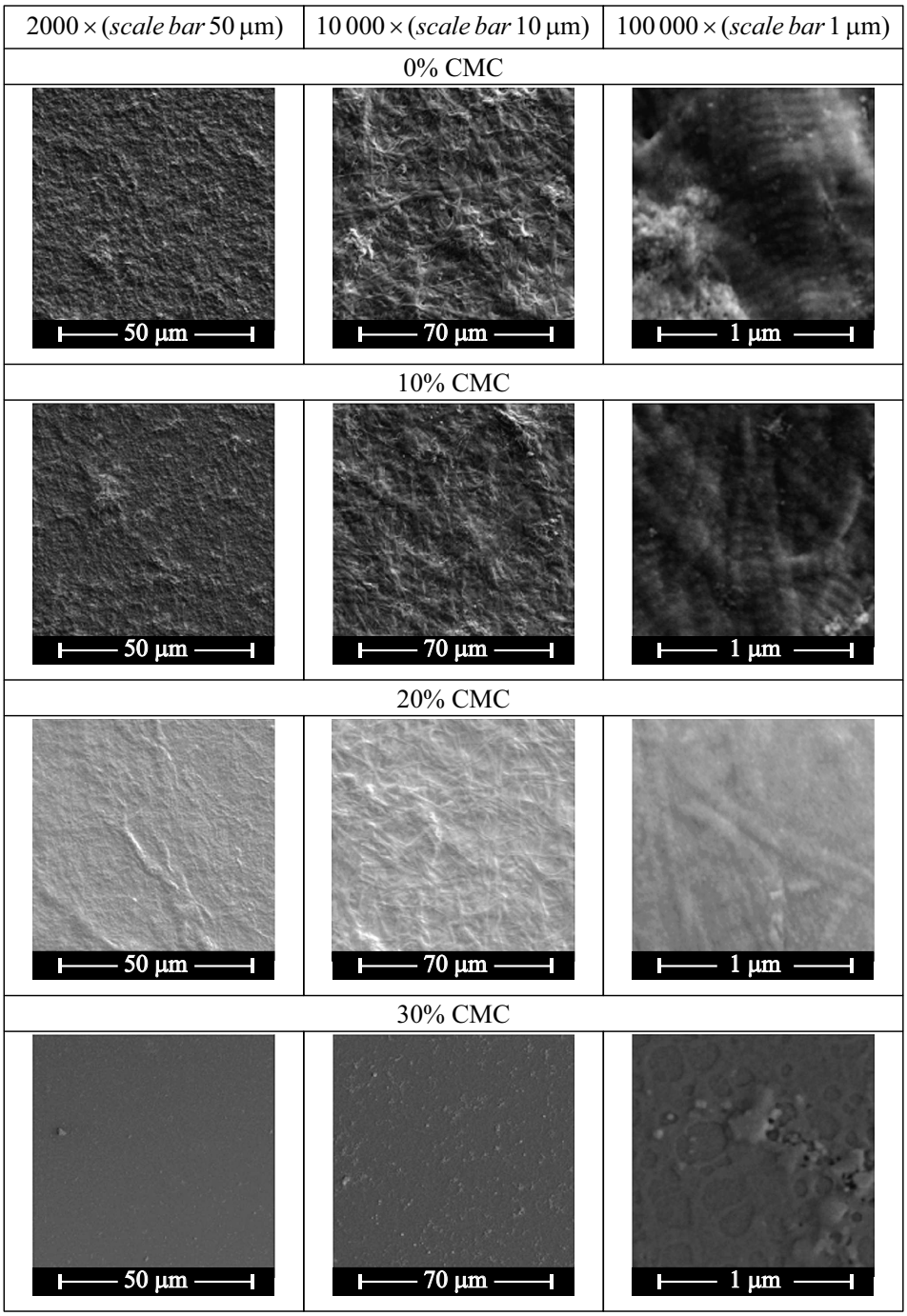

Figure 2. SEM images of matrix surfaces with different amounts of CMC at different magnifications.

al [15] showed that CMC reduces the rate of collagen fibrils formation and leads to their diameter decreasing.

2.3. Collagen in matrices resistance to enzymatic degradation

Besides hydrolytic degradation collagen gel is also subject to enzymatic degradation by proteases. Cells are able to secrete collagenases that break down collagen, thus changing their microenvironment [19]. Collagenases fragment the protein, and small fragments then diffuse into the surrounding liquid medium. Resistance to this type of degradation is another indicator of the gels stability.

To understand whether the addition of CMC affects the resistance of collagen to enzymatic degradation, the gels were incubated in TESCA buffer with collagenase addition. Then the gels together with the solution were centrifuged, and the precipitate was analyzed by electrophoresis.

Based on the analysis results, an electrophoregram typical for type I collagen was obtained: bands corresponding to α-

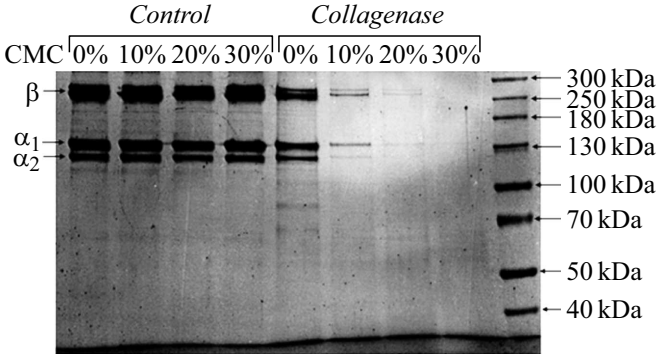

Figure 3. Electropherogram of matrices. The outer lane on the right corresponds to a protein marker containing components with a certain molecular weight (kDa).

chains and their dimers (β). When treated with collagenase, with an increase in the CMC content, thinning of the protein bands occurs, i. e., the amount of collagen in the gel decreases (Fig. 3). As the collagen degrades, small protein fragments leave the gel and remain in the supernatant after centrifugation. The more actively degradation occurs, the less protein is found in the sediment under study.

The results obtained are consistent with those presented above. CMC inhibits the formation of collagen fibrils, resulting in less dense protein packaging and more available sites for collagenase binding. Thus, CMC reduces the resistance of collagen gels to enzymatic degradation.

Conclusion

Collagen gels are promising carriers for cultivation and transplantation of cells used in regenerative medicine due to good cyto- and biocompatibility. However, insufficiently high mechanical properties lead to their rapid degradation and, consequently, to restrictions on their use as carriers for long-term cell cultivation. In the paper composite matrices based on collagen and CMC were studied. Adding CMC to collagen gel makes it more rigid and elastic. CMC can potentially increase the resistance of collagen gel to hydrolytic degradation due to the formation of complexes with the protein. Nevertheless, CMC reduces the resistance of collagen to enzymatic degradation by collagenase, preventing the formation of the fibril-native form of the protein.

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Conflict of interest

The authors declare that they have no conflict of interest.

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