Structure of gels layers with cells

B G Pokusaev¹, S P Karlov¹, A V Vyazmin¹,², D A Nekrasov¹, N S Zakharov¹, D P Khramtsov¹, D A Skladnev³ and D V Tyupa³

¹ Moscow Polytechnical University, Department of Chemical Engineering, ul. Staraya Basmannaya 21/4, Moscow, 105066, Russia
² Scientific Research Institute of Rubber Industry, poselok NIIRP, Sergiev Posad, 141312, Russia
³ Federal Research Centre «Fundamentals of Biotechnology» RAS, Leninsky prospect, 33, build. 2, 119071, Moscow, Russia

E-mail: pokusaev2005@yandex.ru

Abstract. The structure of two-layer agarose gels containing yeast cells is investigated experimentally by spectrometry, to shed a light on the theoretical foundations for the development of bioreactors by the method of 3D bioprinting. Due to division, cells overcome the layer of the dispersion phase separating successively applied layers of the agarose gel. However a gel layer of 100 μm thick with a high concentration of silver nanoparticles completely excludes the infiltration of yeast cells through it. A special sort of agarose is suggested where the concentration of silver nanoparticles formed by cells from salt of silver can serve as an indicator of the state of the yeast cells in the volume of the gel.

1. Introduction
The idea of growing tissues and organs in vitro using stem cells is not new. However, in order to implement this idea, it is necessary to create special bioreactors which would be capable of maintaining the required temperature, pH-level, osmotic pressure, supplying cells with nutrients and oxygen, removing their metabolic products as well as fulfilling many other requirements, which provides necessary physiological conditions for immobilized cells [1]. The deviation from the optimal level of most of the listed factors leads to inhibition of metabolic activity, deterioration of the physiological state of cells and, ultimately, to a retirement of their growth [2-4].

The use of the method of 3D additive manufacturing to create such tissues and organs from stem cells seems to be very promising [5]. Currently gels are considered a high-potential structure-forming material for formation of artificial tissues from immobilized cells using the method of additive manufacturing. In the construction of 3D-bioreactors the gels can be used in the capacity of a neutral coat of gels in which biological microscopic objects are cultivated. They allow nutrients and oxygen to penetrate into the growing medium and remove metabolic products and carbon dioxide.

Agarose gel is a typical gel which is formed from the solution when the temperature is lowered. It is widely used in microbiology for growing of microbiological objects. Such gels have been thoroughly investigated (see, for example, [6]), including by the optical methods [7]. The following aspects have been studied: structure of links which are formed in the disperse phase of gel formation, physical-chemical properties of gels; also, technology of generation for various uses has been described [8]. Nevertheless, many properties of agarose gels which determine whether they may be
used for the creation of bioreactors with the help of additive manufacturing require additional studying.

Observation of biogenic nanoparticles growth (OBNG) methodology is used to assess the physiological state of living cells immobilized in an agarose gel [9, 10]. The idea is that within a few minutes only metabolically active cells are able to form nanoparticles of recover silver (Ag⁺) from the solution of a silver salt in the liquid medium with cells. Respectively, the formation of nanoparticles is sharply inhibited with the weakening of the physiological state of the cells. In the present work, the OBNG methodology is used to analyze the state of living cells immobilized in agarose gels.

The aim here is to carry out an investigation the "thin" structure and adhesion between gel layers applied upon one another in presence of immobilized cells. The peculiarities of the displacement of yeast cells are studied in layered agarose gels intended for the formation of artificial biological structures using 3D-bioprinting technology. The directional restriction of growth of immobilized cells is considered due to the creation of layers of nanoparticles.

2. Materials and methods

In experiments related to immobilization in gels yeast cells *Yarrowia lipolytica* were used as model eukaryote biological object [11]. The cultivation of yeast immobilized cells in the gel is carried out under optimal temperature conditions - 28°C. Immobilization of cells is carried out by placing them into a gel of 1 mm³ volume containing a 10% complete growth medium LB. A suspension of fresh yeast cells is introduced into an agarose gel at a temperature of 42°C with a concentration of 5×10⁶ cells/mm³ when studying the structure of gel layers with cells and 10⁷ cells/mm³ in experiments with nanoparticles. In all experiments, the yeast gel is placed on top of the sterile gel to ensure a better aeration of the cells.

An agarose-based gel "Chemapol" was used as a main gel during the experiments. It is known that gel properties can vary depending on their manufacturer. The gels used in the experiments were obtained by mixing agarose with distilled water with heating up to 90°C by convectional and UHF methods. Agarose gels with 0.6–1.5% of agarose are used in the experimental study of the layered structure of a gel with cells. The concentration of agarose gel in experiments with nanoparticles is 0.4% of agarose, both providing and immobilizing cells, and their volumetric proliferation. In these experiments, two types of agarose are used with differing in the level of electroendoosmosis (EEO).

For investigation two-layer gels system with immobilized cells the spectroscopic method is used. This method analyses the form of spectrum of transparent or reflected light of the sample. The growth and the displacement of yeast cells are investigated by recording the increase in the optical density of the samples in agarose gels of various densities using the optical fiber optical spectrometric system shown in figure 1.

![Figure 1. Fiber-optic spectrometric system for recording changes occurring in multilayer gels used for evaluation of diffusion processes and the control of the silver nanoparticles formation](image-url)
The formation of biogenic silver nanoparticles Ag\(^\circ\) is performed by the OBNG method. As a source of silver cations, a solution of silver nitrate salt AgNO\(_3\) is used, introduced into a molten agarose gel (at a temperature of 42°C) immediately before its cooling at a final concentration of 5 µg/ml.

The registration of spectral properties and changes occurring in multilayer gels is carried out using an optical fiber spectrometric system based on equipment from OceanOptics (USA) for evaluation of diffusion processes and the control of the formation of silver nanoparticles. The system allows measurements at a 2 mm\(^2\) point along the entire height of the cuvette.

3. Results and discussions

It was important to determine how gel of various thicknesses influences growth of cells in different conditions, practically in conditions of different aeration. Due to formation of layered gels with additive technologies, it is important to know the basic regularities in the behavior of immobilized microorganisms within the gel and in close proximity to border between gel layers, filled only with the dispersion medium. Here is the fundamental answer to the question of whether immobilized gels spontaneously spread through border between gel layers, formed of agarose gels, including different concentrations.

3.1. Gels layers with cells

Comparison photos of the two-layer gel system are presented, consisting of both sterile agarose gels with concentration of 0.6%, and the same system, when the upper layer contains encapsulated yeast cells (figure 2). Photos were taken 48 hours after the formation of two-layer systems. It is possible to notice that the gel with cells is much more turbid. Border between gel layers is also filled with the turbid substance and is blurred in the direction of the gel with the cells, whereas for a sterile system, it remains clear.

![Figure 2](image.png)

Figure 2. Comparison photos of the two-layer agarose gels without and with yeast cells. Notations: 1 – the interface of the gels; 2 – agarose gel with a concentration of 0.6%; 3 – the same gel with yeast cells

Using a specially developed thin cuvette by visual observation it was found that the density of the yeast growth remains unchanged in the volume of the agarose gel with a concentration of 0.6% to a depth of 11–12 mm. The density of yeast growth increased with continued incubation cuvettes up to 48 hours and its concentration is 10 times more compared with initial concentrations. It should be noted that during the cultivation of yeast in special cuvettes with layered top layer agarose gel with a concentration of 0.3% by weight (during modeling of usage of protection layer of liquid gel) the entire volume of the upper, the source of sterile liquid gel, also gradually filled with yeast cells. This indicates the ability of cells to overcome pre-formed boundary between gel layers in the direction of concentration changes for agarose gel of 0.6% to 0.3% by weight. This circumstance will require additional studies of the movement of specific living cells, designed specifically for 3D-bioprinting in the gels used for their cultivation, variety, and speed changing density.
Figure 3 shows the results of spectral sensing region adjacent to the boundary between gel layers for two-layer gel system formed by the gels with agarose concentration of 0.6% if the top layer of yeast cells (for gel cells distance is measured in the positive region). The measurements were carried out as one hour after the formation of such a system, and in 48 hours, which allows to trace the spectral changes caused by the condition of the gel and immobilized yeast cells. Intensity values of light transmission were treated to an appropriate amount of sterile gel of the same concentration, measured immediately after its formation. A layer of gel containing the cells has a lower ability for passing light compared with sterile gel, due to the increasing light scattering on yeast cells which size is about 10 µm.

![Figure 3](image.png)

Figure 3. The time variation of the relative intensity of light transmittance of the layered gels with agarose concentration of 0.6% in the border areas of the distance to the boundary in the presence of the top layer of yeast cells. Visually observing the interface between the layers of the gels in the beginning of the experiment corresponds to a value of 0, with minus – without yeast (bottom layer), with a plus - with yeast (top layer). Notation of curves: 1 – within one hour after formation. 2 – after 2 days

We had some differences in the dependency of relative intensity of light transmitted from a distance to border between gel layers. In a two-layer gel system with cells one hour after its formation is practically absent the minimum intensity of transmitted light, which is detected for distinct two-layer sterile system. The reason for this is to that space between gel layers is filled by yeast cells in the process of formation. With increasing time of cell cultivation, such a minimum appears and becomes more and more noticeable, while its width increases significantly in the part of the layer, in which yeast cells were introduced originally. The behaviour of curve 2 in figure 3 resembles the change in concentration of a substance in a stationary environment when the diffusive mass transfers through the interface. It can be assumed that the mechanism of migration of cells from one environment to another via boundary between gel layers is presented.

It is important to note that the relative intensity of light transmittance through the layers of the gel after 48 hours it becomes lower than at the beginning of the process, and the gradient of its change from the sterile gel increases. The decrease in the intensity of light transmission explained from the standpoint of the self-seal dispersion medium in the gel when it is aging. This process takes place in both layers of the gel. However, the decrease in relative intensity for sterile gel is significantly more than that of the layer containing cells. This suggests that the observed pattern is caused by the penetration of yeast cells into sterile gel and the beginning of their reproduction and growth in it. Moreover, large gradient of relative intensity of light passing near the boundary shows that, initially, the yeast cells begin their reproduction and growth under sterile gel side boundary between gel layers.

3.2. Formation of nanoparticles in agarose gels by cells

For yeast cells grown in a complete growth medium the addition of a solution of silver nitrate AgNO₃ leads to the formation of silver nanoparticles 25–70 nm in size within 20–30 minutes, as it was previously found by [9, 10]. The formation of nanoparticles was observed both in photographs (with the transmission electron microscope) and by spectrometry (a specific peak close to the wavelength of the light 400 nm). In a sterile growth medium (that is, an environment that does not contain live yeast
nanoparticles were not formed at the same time, since the reduction of cations ceased at a stage not larger than clusters of silver atoms (up to 0.5–2 nm). Such clusters are detected using a transmission electron microscope, but they are not determined spectrometrically.

To study the formation of nanoparticles by cells in gels, the silver salt solution was introduced into a molten 0.4% agarose gel and into the same gel containing fresh yeast cells at a concentration of $10^7$ cells/ml. Control cuvette with agarose gel did not contain cells. The cuvettes were incubated for 30 minutes at room temperature, after which the spectrum of both preparations was determined. As expected, a characteristic peak is observed indicating the presence of silver nanoparticles in cuvette with yeast cells. However, in a cuvette with a sterile agarose gel, such a peak is quite pronounced (see figure 4). The magnitude of the peak in the presence of yeast cells is higher than in the sterile gel. However, the difference is not significant as to evaluate the physiological state of the cells the gel using these data. Thus, it is established that silver nanoparticles are formed in a 0.4% agarose gel when the silver nitrate solution interacts with the gel itself.

![Figure 4. Transmission spectra during the formation of silver nanoparticles in agarose gels with different levels of EEO, including in the presence of yeast cells. The composition of the samples: 1 - aqueous solution of AgNO$_3$, 2 - ordinary agarose gel with AgNO$_3$, 3 - low EEO agarose gel with AgNO$_3$, 4 - ordinary agarose gel with AgNO$_3$ and cells, 5 - low EEO agarose gel with AgNO$_3$ and cells.](image)

To prevent the formation of silver nanoparticles in agarose, another substance was used with a low level of EEO. Figure 4 shows that the formation of nanoparticles in the presence of agarose with low EEO is insignificant. In further experiments concerning the interaction of living cells and silver salts (as indicators of the physiological state of cells), only low EEO agarose was used.

When two-layer agarose gels are formed with agarose concentration of 0.4%, containing in the upper layer of the yeast cell, it is established that the yeast grows at an optimum temperature conditions pass through the interlayer boundary. After incubating the cuvette for 12 hours, the concentration of yeast cells in the upper layer increased approximately 10 times corresponding to approximately three divisions of a cell. Yeast cells also appear in the lower layer, but at a concentration much lower than in the upper layer. Hence the yeast cells pass through the boundary in double-layered gels as a result of their division.

In experiments on the evaluation of the interaction of agarose gels with different levels of EEO and a solution of silver nitrate, it is found that the formation of silver nanoparticles is much slower in low EEO agarose than in standard agarose. Formed nanoparticles show a high ability to diffuse through a 0.4% gel. The ability of the nanoparticles to pass through the gel makes it possible to use them to assess the state of the cells immobilized in the gel. In case of presence the agarose of fresh yeast cells and the silver salt solution in the upper layer of 0.4% low EEO gel, after the cultivation in the middle of the cuvette, two intermediate layers are formed. The upper one corresponds to the cell suspension on spectral characteristics; the lower one corresponds to the suspension of nanoparticles. The
concentration of nanoparticles at high diffusion rate can be considered homogeneous over the volume of the gel. This allows us to obtain the information on the state of the cells providing the reduction of cations from the parameters of the nanoparticles. This assumption requires further confirmation.

The above experiment demonstrated that concentrations of silver nitrate up to 5 µg/ml are useful for assessing the physiological state of the active cells, since the cells retain the same ability to divide, as without any silver substance.

In the experiment, it was checked whether a layer of nanoparticles could prevent the propagation of yeast cell growth in layers of 0.4% gel from low EEO agarose. The mature agent of silver nanoparticles is mixed with a small volume of gel and is applied as a separator between two layers of the gel with cells and without cells. The thickness of the separation layer is 100 µm. Yeast capable of growing in the upper layer does not overcome the gel layer containing the preparation of silver nanoparticles. Thus, it can be suggested to use layers with silver nanoparticles to limit the growth of bioobjects in gels during the realization of biotechnology of 3D-bioprinting.

4. Conclusions

Experimentally for cells of the yeast Yarrowia lipolytica, the possibility of their spontaneous transition from one layer agarose gel to another through the surface between gel layers filled dispersive medium was determined. This phenomenon occurs both when gel layers are of the same concentration and when concentration is drastically changes. It is found that the cells pass from one layer of gel to another through the boundary as a result of their division. Note that a gel layer of 100 µm thick containing concentrated silver nanoparticles does not allow cells to pass through themselves by the division.

It is observed that the addition of silver nitrate with a concentration lower than 5 µg/ml can be used as an indicator of the state of cells in the gel volume. Healthy and active yeast cells restore silver to form nanoparticles. Such nanoparticles readily diffuse through a 0.4% agarose gel. Their concentration outside the gel reveal the state of the yeast cells inside the gel.

Acknowledgments

This work was supported by the Russian Science Foundation (project no. 15-19-00177).

References

[1] Rodrígues C A V, Fernandes T G, Diogo M M, da Silva C L and Cabral J M S 2011 Biotech. Adv. 29 815
[2] Ott H C, Matthiesen T S, Goh S K, Black L D, Kren S M and Netoff T I 2008 Nat. Med. 14 213
[3] Placzek M R, Chung I M, Macedo H M, Ismail S, Mortera B T and Lim M 2009 J. R. Soc. Interface 6 209
[4] Lee M K, Rich M H, Baek K, Lee J and Kong H 2015 Sci. Rep. (Nature) 5 8948
[5] Marga F, Jakab K, Khatiwala C, Shepherd B, Dorfman S, Hubbard B, Colbert S and Gabor F 2012 Biofabr. 4 02200
[6] Duckworth M and Yaphe W 1971 Carbohydr. Res. 16 189
[7] Rees D A, Scott W E and Williamson F B 1970 Nature 227 390
[8] Santos G A 1990 A Manual for the Processing of Agar from Gracilaria (Manila: ASEAN/UNDP/FAO Regional Small-Scale Coastal Fisheries Development Project)
[9] Sorokin V V, Skladnev D A, Volkov V V, Tereshchenko E Y, Mulukin A L and Gal’chenko V F 2013 Dokl. Biol. Sci. 452 325
[10] Skladnev D A, Sorokin V V and Gal’chenko V F 2017 Microbiology 86 355
[11] Scioli C and Vollaro L 1997 Water Res. 31 2520