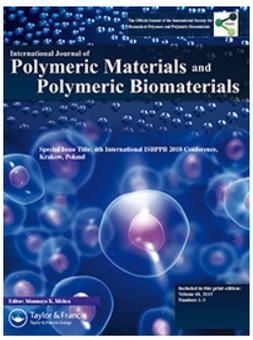

International Journal of Polymeric Materials and Polymeric Biomaterials

ISSN: 0091-4037 (Print) 1563-535X (Online) Journal homepage: https://www.tandfonline.com/loi/gpom20

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To cite this article: Elizaveta Akoulina, Andrej Dudun, Anton Bonartsev, Garina Bonartseva & Vera Voinova (2019) Effect of bacterial alginate on growth of mesenchymal stem cells, International Journal of Polymeric Materials and Polymeric Biomaterials, 68:1-3, 115-118, DOI: 10.1080/00914037.2018.1525730

To link to this article: https://doi.org/10.1080/00914037.2018.1525730

Published online: 19 Jan 2019.

INTERNATIONAL JOURNAL OF POLYMERIC MATERIALS AND POLYMERIC BIOMATERIALS
2019, VOL. 68, NO. 1–3, 115–118
https://doi.org/10.1080/00914037.2018.1525730

Effect of bacterial alginate on growth of mesenchymal stem cells

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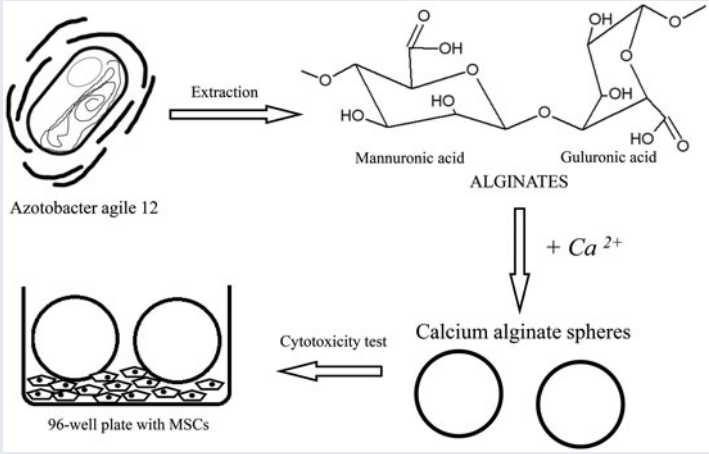
ABSTRACT

Capsular alginate was synthesized using bacteria *Azotobacter agile 12* in order to test its potential for use in biotechnology and tissue engineering. Capsular bacterial alginate was isolated and purified using EDTA treatment and dialysis. Calcium alginate spheres were produced and their effect on growth of mesenchymal stem cells was studied. Bacterial alginate shows significantly lower cytotoxicity than commercial alginate isolated from algae.

GRAPHICAL ABSTRACT



ARTICLE HISTORY
Received 18 June 2018
Accepted 15 September 2018

KEYWORDS
Azotobacter; bacterial alginate; capsular alginate; cytotoxicity; MSC

1. Introduction

Alginates (ALGs) are polysaccharides produced by brown algae and some bacteria (e.g. *Azotobacter sp.*). Being copolymers of α-L-guluronic acid and β-D-mannuronic acid, they are attractive for use in biotechnology. ALGs have numerous favorable qualities such as biocompatibility, biodegradation and because of its ability to form gel with bivalent ions like Ca^{2+}. Bacterial ALG differs from algae ALG by having acetylation at C2 and C3 atoms of mannuronic monomer[1]. Furthermore, monomers ratio and molecular weight of biopolymers can be achieved through bacterial biosynthesis which makes it more useful for different trends in biotechnology such as tissue engineering because it's impossible to control characteristics of ALG obtained from algae[2]. Bacterial ALG can be divided into two types: free polymer that bacteria produce in culture media and capsular polymer that form a capsule around bacteria cell. This capsular ALG has higher molecular weight and includes more of guluronic acid than the free polymer, which gives capsular ALGs capability to conglomerate with Ca^{2+} and form a gel that protects bacteria from oxygen and dehydration[3]. Because of this capsular ALG has a significant potential in biotechnology. ALGs from algae have been used for many years as a material for studies in various areas of biology and medicine and in treatment, for example, as drug carriers, wound coverings biomaterials for dental surgery, and, in some cases, in GIT diseases[4]. One of the advantages of ALG application as peroral formulations is their ability to maintain the gel state even under conditions of low gastric acidity. These peculiarities allow ALGs to perform efficient protection of GIT upon gastritis and other diseases. We discovered for the first time that *Azotobacter agile 12* from our collection is capable to high-performance synthesis of alginates, it is known that bacteria of this genus are capable to produce various extracellular polysaccharides[5]. In this work we compared cytotoxicity of algae and bacterial ALG using

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mesenchymal stem cells (MSC). MSCs are the most interesting cells line in contest of biotechnology because they have been highly investigated for use in tissue regeneration. Also, they have shown interesting data in cultivation within ALG constructs[6] so this approach can be a prospective strategy for tissue engineering.

2. Experimental methods

2.1. Culturing

Bacteria - *Azotobacter agile*[7–9] strain 12 isolated from soddy-podzolic soils of the Moscow region. The strain was taken from a collection from Russian State Agrarian University - Moscow Timiryazev Agricultural Academy and for a long time is maintained at the Laboratory of the Biochemistry of Nitrogen Fixation and Metabolism of the Bach Institute of Biochemistry, Federal Research Centre "Fundamentals of Biotechnology" of the Russian Academy of Sciences. The strain had the following morphological features: large, slimy colonies; oblong, motile (with flagella), gram-negative cells that reside individually or are arranged in pairs and chains; and strict aerobes. This strain was never investigated for alginate production. Bacterial culture was maintained in solid Ashby's medium with the following composition per liter: 1.05 g K_2HPO_4, 0.2 g K_2HPO_4, 0.2 g $MgSO_4$, 0.2 g NaCl, 0.006 g Na_2MoO_4, 5 g $CaCO_3$, 20 g sucrose, and 20 g agar. Bacterial culture was cultivated in liquid Burk's medium with low concentration of phosphates in750 mL flasks containing 100 mL of medium at 250 rpm[10].

2.2. ALG extraction and purification

After 3 days of cultivation (250 rpm, 28 °C) cultural media was centrifuged and biomass was isolated. Then biomass was processed with 0.9% NaCl and 50 mM EDTA to isolate capsule ALG and after that the samples were centrifuged for 30 min at 15,000 g. Supernatant was isolated and supplemented with a threefold volume of 96% ethanol in order to get capsular ALG. This capsular ALG was purified by repeated precipitation with 96% ethanol. Then dialysis was conducted against 0.5% NaCl for 24 h. Effectivity of dialysis was detected by measuring conductivity of ALG solution in 0.9% NaCl using S30 SevenEasy^{TM} conductivity, Mettler Toledo.

The molecular mass of ALG was determined by viscosimetry. The viscosity of ALG solution in 0.2 M NaCl was measured at 25°C using standard glass capillary kinematic viscometer with 0.56 mm capillary diameter on RT RheoTec viscosimeter (RheoTec Messtechnik GmbH, Germany). The specific viscosity was calculated according to the formula

$$\eta_{sp} = (t - t_0)/t_0$$

where t_0 is the flow time of the solvent (s) and t is the flow time of the polymer solution (s) with approximate flow time of solvent (0.2 M NaCl) – equal to 97 s.

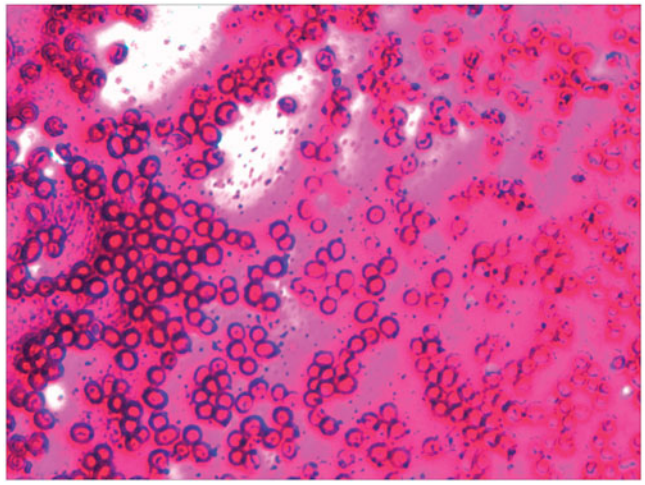

Figure 1. Azotobacter agile 12 surrounded by capsular alginate visualized by fuchsine staining. Picture was taken by light microscopy on a Biomed-1 microscope (Biomed, Russia) with a digital camera. Magnification 1000×.

Table 1. Conductivity of ALG solutions.

sample	conductivity, mS/sm
0.9% NaCl	17.48
Bacterial capsular ALG 0.2% before dialysis	19.15
Bacterial capsular ALG 0.2% dialysis 24 h	18.11
Bacterial capsular ALG 0.2% dialysis 30 h	18.07
Algae ALG 0.2%	18.10

The molecular mass was calculated according to the Mark-Houwink equation $[\eta] = K \times (M)^a$ with the following coefficients:

$$K = 7.3 \times 10^{-5}; a = 0.92; [\eta] = 7.3 \times 10^{-5} \times (M)^{0.92},$$

where M is the molecular mass and $[\eta]$ is viscosity[11].

2.3. IR spectroscopy

IR spectra were registered in the reflection mode in a Hyperion-2000 IR microscope connected with an IFS-66 v/s IR-Fourier spectrometer (Ge crystal, resolution of 2 cm^{-1}, a range of 4000–600 cm^{-1}, scan.-50) (Bruker, United States). For comparison, this method was also applied in a study of a commercial ALG sample isolated from algae (Sigma-Aldrich, Germany).

2.4. Cytotoxicity

Cytotoxicity of ALG was examined by one-week cocultivation experiment with mesenchymal stem cells (MSCs). MSCs were obtained using standard method with collagenase type I[12]. MSCs were isolated from the femurs of young (3–5 days old) Wistar rats and cultured for 2 weeks in DMEM (Dubecco's Modified Eagle Medium, PanEco, Russia) supplemented with 10% fetal calf serum (FCS, Biological Industries, Israel) and 100 U/mL penicillin. The approvals for all surgical experimental procedures and ethical guidelines were issued according to the ISO 10993-1:2009. MSCs were cultivated for 3 passages. The cells were

Table 2. Yield and characteristics of ALG different origin after extraction and purifying.

Alginate origin	Biomass dry	ALG yield after extraction	ALG yield after dialysis	Molecular weight	M/G	Acetylation degree
Azotobacter agile 12	1.83 g/l	3.41 g/l	1.72 g/l	450 kDa	72/28	22%
Sigma Aldrich	–	–	–	850 kDa	80/20	0%

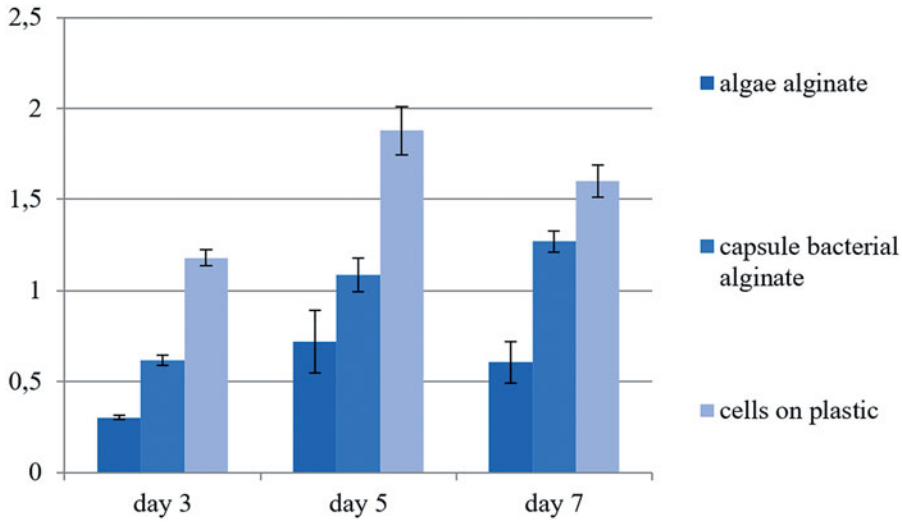

Figure 2. Cell growth on plastic with bacterial or algae ALG or without.

removed by incubation in a solution of trypsin-versen for 5 min, then counted using Goryaev chamber.

ALG powder was washed by ethanol 96% and lyophilized. 1%(wt) solution of ALG in 0.9% NaCl was made. After putting this solution drop by drop in 5% CaCl gell spheres were formed. The spheres were then washed in PBS until there was no sediment.

Calcium ALG of different origin and concentration was added to MSCs while they grow on plastic. A number of living cells was determined by a standard XTT test[13]. To test cytotoxicity of calcium ALG, spheres 2 mm in diameter were produced. About two thousand MSCs per well were placed in the 96-well plate wells. After 2 h one sphere was added to each well. Plates were incubated for 3, 5 and 7 days. The control samples were examined for wells of 96-well plate without spheres. Cells viability was measured by the cell proliferation reagent XTT according to the manual (XTT Cell Proliferation Kit, Biological Industries, Israel). Before XTT test spheres of ALG were gently removed and 100 μL of fresh medium was added; then 50 μL of XTT reagent solution was added in each well. Multi-well plates were incubated at 37 °C for 4 h. Liquid was moved to a clean plate and absorbance measurements were conducted using a microplate spectrophotometer Zenyth 3100 Microplate Multimode Detector (Anthos Labtec Instruments GmbH, Austria) at 450 nm with reference wavelength at 640 nm[14].

Algae ALG (Sigma Aldrich) was taken as a control.

3. Results and discussion

The goal of this study was to obtain alginate with desired characteristics for its biomedical application. The first step was to cultivate the bacteria (Figure 1). *A. agile 12* was cultivated with low concentration of phosphates and high aeration for three days in order to get more alginate. After that media was obtained and biomass was processed with NaCl. At first, we processed biomass only with NaOH but, using that method, we didn't get enough alginate and its molecular weight was low. Than we started to use EDTA because of its ability to sequester Ca^{2+} and to form conjugates with it. Due to that EDTA facilitates ALG extraction from bacteria capsule. Then ALG was purified by several precipitations from supernatant by 96% ethanol and then lyophilized.

After the extraction of ALG from biomass, ALG solution in 0.1 M NaCl was examined for conductivity. Because of the method of extraction and purifying we suspected that there would be a lot of salt and EDTA in the ALG and it will affect cell growth and proliferation. Thus dialysis against 0.5% NaCl was conducted. The conductivity test showed (Table 1) that after dialysis conductivity of bacterial ALG solution was lower than before and it was similar to algae ALG conductivity. Further dialysis didn't reduce conductivity. Total amount of ALG after dialysis reduced two times (Table 2). This can be explained by the amount of salt and EDTA that were precipitated with ALG from culture media. Molecular weight of ALG after dialysis was 450 kDa. This bacterial alginate was tested via IR spectroscopy to register uniformity of polymer. The spectra of synthesized bacterial ALG are similar with algae ALG spectra and have common absorption bands with different intensities of bands at 1600 cm^{-1} (COO^-), 1720 cm^{-1} ($COCH_3$), 819 cm^{-1} (M-blocks), and 765 cm^{-1} (G-blocks). However, there are differences, which include the presence of bands at 1720 and 1245 cm^{-1} in bacterial ALG. These particular signals are characteristic of absorption bands of acetyl groups (1720

and 1245 cm^{-1})[10]. Produced bacterial ALG has 28% gulur-onic residues and 72% mannuronic residues and 22% groups are acetylated (Table 2).

Materials for bioengineering before in vivo experiments should be tested for biocompatibility. And one of the main test is cytotoxicity. The most common way to test cytotox-icity of this material is to add it into the media with grow-ing cells and to see the change of cell growth. Algae ALG is a common polymer for tissue engineering[15], however there is no information about cytotoxicity and biocompatibility of ALG of bacterial origin, especially, using MSCs as cells cul-ture. In this work we observed influence of ALG of different origin on MSCs growth. Figure 2 shows that both alginates either bacterial or algae origin have some cytotoxicity, but we can see that bacterial ALG has less effect on cell growth. The effect of alginate on MSCs growth can be connected not only with insufficient purifying but also to physicochem-ical properties of this biopolymer. Moreover, the growth of MSCs depends on their ability of differentiation, when pro-liferation of these cells is inhibited. In the future, we are going to see the mechanism of the effect of bacterial algi-nates properties on MSCs growth and proliferation.

4. Conclusion

In this work we obtained bacterial ALG from novel high-yield strain *Azotobacter agile 12* using improved purification methods. The isolated and purified ALG of bacterial origin had changed chemical structure, e.g. acetylated monomers, in comparison with algal ALG. We showed that ALG of bac-teria origin has a significantly lower effect on MSCs growth than commercial alginate from algae, which gives hope for use of bacterial alginate in biotechnology and biomedicine.

Funding

This work was supported by Russian Science Foundation, project # 17-74-20104 in all parts besides the part of A. agile 12 cultivation and growth maintaining that was support in the framework of the government task, registration #01201351358. The equipment of User Facilities Centers of M.V.Lomonosov Moscow State University (incl. the FACSAria SORP complex, Complex for production of micro - and nanoparticles of polymers) and Research Center of Biotechnology RAS was used in the work.

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