Preparation and Characterization of Bacterial Cellulose Composites with Silver Nanoparticles

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Bacterial cellulose (BC) is widely used in medicine as a dressing material due to its good biological properties – high biocompatibility, low adhesion and the ability to absorb wound exudate. The BC does not have antimicrobial activity itself, which limits the use of products in infected wounds, and also treatment of wounds in hospitals. A method for producing a two-component composite material based on bacterial cellulose (BC) synthesized in culture of Komagataeibacter xylinus B-12068 with silver nanoparticles [BC/AgNps], by hydrothermal synthesis of AgNO₃ in the layer of BC at different temperatures and concentrations of AgNO₃, is proposed. The antibacterial activity of BC/AgNps samples against Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, and Staphylococcus aureus has been confirmed in vitro with the disc diffusion method. It is shown, that the antibacterial activity of samples is mostly expressed in cultures of P. aeruginosa and S. aureus.

Keywords: bacterial cellulose, wound dressings, silver nanoparticles, composites, antibacterial activity.

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**Способ получения и характеристики композитов бактериальной целлюлозы и наночастиц серебра**

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Бактериальная целлюлоза (БЦ) широко используется в медицине в качестве перевязочного материала благодаря своим биологическим свойствам – высокой биосовместимости, низкой адгезионности и способности поглощать раневой экссудат. Собственно БЦ не обладает антибактериальной активностью, что ограничивает использование изделий при инфицированных ранах, а также при лечении ран в стационарах. Предложен вариант способа получения двухкомпонентного композитного материала на основе бактериальной целлюлозы (БЦ), синтезированной культурой Komagataeibacter xylinus В-12068, с наночастицами серебра [БЦ/AgNps] путем гидротермального синтеза AgNO₃ в толще пласта при различных температурных режимах и концентрациях AgNO₃. С использованием дискодиффузионного метода подтверждена антибактериальная активность образцов БЦ/AgNps в отношении Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae и Staphylococcus aureus. Показано, что антибактериальная активность образцов композита наиболее выражена по отношению P. aeruginosa и S. aureus.

Ключевые слова: бактериальная целлюлоза, наночастицы серебра, композиты, бактерицидная активность.

**Introduction**

Bacterial cellulose (BC) is a biopolymer, synthesized by microorganisms, similar in chemical structure to plant cellulose, but possessing unique physico-mechanical and chemical properties. BC is characterized by high biocompatibility, does not show cytotoxicity or allergic reactions. The BC demonstrates no adhesion towards opened soft tissues, so it can be an ideal material for the wound dressings and other medical devices, intended for the contact with inner medium of human body (Ul-Islam et al., 2013). Gel-films of BC have an ordered structure and represent a three-dimensional network structure, consisting of evenly distributed ribbon-like microfibrils. Complex of these properties in a combination with cheapness and simplicity of the synthesis make BC very popular for the investigations as a material for the restoration of skin, bones, connective tissues of different structure, blood vessels, the basis for drug deposition and delivery, etc. (Saska et al., 2011; Castro et al., 2012; Petersen, Gatenholm, 2011; Keshk, 2014).

Compositions of BC with various materials – chitosan (Lin et al., 2013), collagen (Wen et al., 2015), sodium alginate, gelatin, polyethylene glycol (Shah et al., 2013) are
produced to improve the physico-mechanical properties and functionality of the resulting biomaterial. Bacterial cellulose itself does not possess antimicrobial activity. However, taking into account its favorable structure, it is possible to use it in a composition with “incrusting the surface” heavy metal nanoparticles, wherein they will act as a local agent for directed kill of bacteria, thereby possessing conditions for faster wound healing (Czaja et al., 2004; Yang et al., 2012).

Metallic silver and its forms and compounds have a pronounced bactericidal effect, inhibiting the development of a lot of pathogenic microorganisms. Silver ions interact with thiol groups of cell wall proteins, disrupting bacterial respiration and transporting substances through the cell membrane (Percival et al., 2005). Composite biomaterials “polymer/metallic nanoparticles” can be prepared in several ways: by mechanically mixing the polymer with metal nanoparticles in the form of powder or suspension, with the polymerization of the monomers in situ in the presence of metal nanoparticles, or by chemical reduction, for example sodium borohydride (Yang et al., 2012a; Wua et al., 2015). In the latter case, the plurality of hydroxyl groups on microfibrils of bacterial cellulose serve as anchor areas for silver ions, limiting the growth of the particles within the pores, realizing their controlled agglomeration and narrowing the size distribution as a result.

The purpose of this work is to obtain and to characterize bacterial cellulose composites with silver nanoparticles.

Materials and methods

Bacterial cellulose films were synthesized in culture Komagataeibacter xylinus B-12068 with the method of surface culturing in static conditions. To remove remnants of bacterial cells and components of grow medium BC films were treated with 1.0 M NaOH at 70 °C, with following streamed washing in deionized water. For BC composites with silver nanoparticles a hydrothermal method was used, without the utilization of any catalysts, using the discs of BC layer as a reducing and stabilizing agent (Yang et al., 2012). Cleaned wet BC films were cut into discs with an average diameter of 10 mm, then were placed in a flask with 0.001 M AgNO₃ solution and heated for 60 minutes to 30, 60 and 90 °C. Also, the effect of different concentrations of AgNO₃ in the reactionary medium was estimated for 0.0001, 0.001 and 0.01 M, with the selected temperature in the system 90 °C.

Parameters of resulting silver nanoparticles were investigated with a Zetasizer Nano ZS particle analyzer (Malvern, U.K.), employing dual angle dynamic light scattering. Composite films of BC with silver nanoparticles [BC/AgNps] were lyophilized at -40 °C and pressure of 0.12 mbar for one day in a unit ALPHA 1-2/LD (“MartinChristGmbH”, Germany). The microstructure of samples was examined using scanning electron microscopy with TM-3000 microscope with Hitachi X-ray analysis system and QUANTAX 70 program.

Thermal analysis of samples was performed using an STA 449 Jupiter synchronous thermal analyzer (NETZSCH, Germany) for simultaneous thermogravimetry and differential scanning calorimetry (DSC), combined with a QMS 403 Aelous quadrupole mass spectrometer for analysis of gases, and evolved by the heating specimens. X-ray structure analysis and determination of crystallinity of films (Cₓ) were performed using a D8 ADVANCE X-ray spectrometer (“Bruker”, Germany), with graphite monochromator on a reflected beam.

To estimate the antimicrobial activity of BC/AgNps discs, they were preliminary autoclaved with 120 °C at 0.5 atm. The direct inhibitory
effect of BC/AgNps was tested on cultures of *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* using disc diffusion method in agar on Petri dishes. The diameter of the growth inhibition zones and the distance from the edge of the film to the end of the zone were measured in photographs of dishes, using the Image J program. The results were processed using the Microsoft Excel application package. The arithmetic mean and standard deviation were calculated.

**Results and discussion**

Two modes of hydrothermal preparation of the bacterial cellulose composite with silver nanoparticles [BC/AgNps] were used: a change in the temperature and AgNO₃ concentration in the reaction solution; this provided a series of samples (Fig. 1). The change in the temperature of the medium with the AgNO₃ concentration of 0.001 M had a different effect on the size of the silver nanoparticles formed. After incubation of BC films in a solution of silver nitrate at 30 °C, there appeared to be an aggregation of the silver particles formed and their average size was recorded at 757 nm. When the temperature of the reaction solution was increased to 60 and 90 °C, the particle size was significantly reduced, to 45 and 13 nm, respectively. The use of AgNO₃ solutions of different concentrations (0.0001, 0.001 and 0.01 M) with stabilization of the medium temperature at 90 °C provided the

![Fig. 1. An appearance of the reaction medium (A) and size distribution of Ag-nanoparticles in the system (B) during the synthesis of composite films in three regimes of temperature (left) and with varied concentrations of AgNO₃ in the reaction solution (right).](image-url)
formation of silver nanoparticles with an average size of 13, 23 and 12 nm, respectively.

A photo of bacterial cellulose composite films with silver nanoparticles is shown in Fig. 2. The presence of silver in the BC-films was confirmed with the scanning electron microscopy and X-ray spectra analysis (Table 1). It was shown, that the average mole percentages of silver in composite

![Fig. 2. Photos of raw (A, C) and lyophilized (B) BC/AgNPs films synthesized in three regimes of temperature (A, B) and with varied concentrations of AgNO₃ in the reaction solution (C)](image)

Table 1. Influence of synthesis conditions on the element composition of composite BC films with silver nanoparticles

| Samples          | O     | C     | N     | Ag   |
|------------------|-------|-------|-------|------|
| BC film          | 59.8  | 40.1  | -     | -    |
| Influence of temperature at 0.001 M AgNO₃ |       |       |       |      |
| 30 °C            | 52.9  | 42.5  | 1.1   | 3.0  |
| 60 °C            | 51.0  | 40.3  | 0.6   | 3.3  |
| 90 °C            | 52.3  | 41.7  | 1.5   | 3.7  |
| Influence of AgNO₃ concentration at 90 °C |       |       |       |      |
| 0.0001 M         | 51.0  | 43.9  | 1.4   | 1.0  |
| 0.001 M          | 54.2  | 41.7  | 0.6   | 3.6  |
| 0.01 M           | 50.2  | 40.7  | 1.2   | 5.3  |
samples depended on the temperature in system insignificantly, and was 3.08, 3.32 and 3.71 at 30, 60 and 90 °C, respectively. In contrast, increasing of concentration of AgNO₃ in the reaction solution from 0.001 to 0.01 M was accompanied by an increasing of silver content in the composites from 1.08 to 5.32 %.

To identify the appearance of possible structural changes in the composite, X-ray diffraction analysis and differential scanning calorimetry were involved. In BC samples without inclusion of Ag the degree of crystallinity was measured as 75 and 72 %. For samples, obtained at the highest concentration of AgNO₃ in the system and the high content of silver in the composite (5.3 %) with an average size of nanoparticles 12 nm, the degree of crystallinity decreased up to 61 % (Fig. 3). With a decrease in the content of silver in the composite to 3.6 % and 1 %, the degree of crystallinity increased to 83 and 86 %, respectively, that was higher than the value of pure BC. It should be noted, that the Cₓ of BC is significantly influenced by the type and conditions of the biosynthesis and the carbon substrate, and, according to the literature data and our own results, may be from 45 to 90 % (Shah et al., 2013; Shao et al., 2015).

The results of differential scanning calorimetry showed that the samples of pure cellulose did not have clearly defined peaks in the field of thermal degradation. Therefore, an important parameter for this natural polymer is the temperature of the onset of destructive processes (Tₒ.d.). Unlike BC, BC composites with silver nanoparticles obtained at different regimes had some differences and were characterized by greater thermostability (Fig. 4).

Thus, composites obtained at different temperatures, but with a similar content of silver in BC films (approximately of 3 %), had, in comparison with the BC, more pronounced peaks in the thermal degradation region (415-425 °C); the onset of the decomposition temperature for the samples was in the region of 320-325 °C.

![Fig. 3. X-ray patterns of the BC composite with silver nanoparticles obtained at different concentrations of AgNO₃ in the reaction solution: 1 – 0.01 M, 4 – 0.001 M, 5 – 0.0001 M; the degree of crystallinity (Cₓ) 61, 83 and 86 %, respectively; 2 and 3 – the starting cellulose (without silver), the degree of crystallinity 72 and 75 %.](image-url)
(Fig. 4A); this is significantly higher (almost 60-80 °C) than in the BC samples. The composites obtained by stabilizing the temperature at the level of 90 °C, but at different concentrations of AgNO₃ in the reaction medium, also showed higher thermal stability than the BC (Fig. 4B, curve 1). In all composite samples, the regions of onset of thermal degradation were shifted to the right with regard to the BC; at the same time they were characterized by the presence of two peaks with a gap between them from 40 to 120 °C (Fig. 4B, curves 2-4). In general, the filling of cellulose films with silver nanoparticles increased the thermal stability of BC.

All samples of BC/AgNps composite, independently on the preparation conditions and content of silver nanoparticles, had a bactericidal effect, as shown in test cultures of pathogenic bacteria. Since inclusion of Ag-particles has a negative impact both, to the cell of tissues, in particular, actively dividing cells in wound regeneration zone, and to pathogenic bacteria, for the investigations of products, intended to the direct contact with wound medium, were taken samples with an average content of silver of medium value (about 3 %). Four strains were studied – three Gram-negative: *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and one – Gram-positive – *Staphylococcus aureus*, as they are the main representatives of the pathogenic flora of infected wounds.

Antibacterial effect of BC/AgNps composite in direct contact with the cultures was expressed in different degrees for the different strains. In general, *E. coli* and *K. pneumoniae* were less sensitive, *P. aeruginosa* and *S. aureus* more
sensitive. Also, significant differences were found towards the samples obtained at 60 and 90 °C (Fig. 5, Table 2). The observed differences in diameters of growth suppression zones can be explained by the physiological characteristics of strains, that causes their different sensitivity to the direct contact with silver ions in BC/AgNps composites (Ruparelia, 2008).

It is known, that the cytotoxic activity of Ag-nanoparticles depends on their size and concentration, whereas the minimum inhibiting concentration of Ag nanoparticles is

![Fig. 5. The grow suppression zones in cultures Escherichia coli (A), Klebsiella pneumoniae (B), Pseudomonas aeruginosa (C), and Staphylococcus aureus (D), with the diffusion test with: 1 – pure BC-films, 2, 3, and 4 – BC/AgNps, synthesized with different temperatures (30 °C, 60 °C, and 90 °C)](image)

| Bacterial test culture | Diameter of inhibition zones (mm) |
|------------------------|----------------------------------|
|                        | 30 °C   | 60 °C   | 90 °C   |
| *E. coli*              | 14±0.20 | 16±0.50 | 17±0.61 |
| *K. pneumoniae*        | 15±0.44 | 16±0.64 | 17±0.36 |
| *P. aeruginosa*        | 15±0.90 | 18±1.15 | 21±2.11 |
| *St. aureus*           | 15±0.73 | 19±0.28 | 20±1.58 |
0.05-0.1 mg/ml (Castro–Mayorga et al., 2014; Bindhu et al., 2015). We found, that with the concentration of the AgNO₃ solution of 0.001 M, the level of incorporated silver in BC films varied slightly, in range of 3.1-3.7 %. Composites obtained at higher temperatures showed a more pronounced inhibiting effect on bacteria. There is evident that the ambient temperature plays an important role in the antimicrobial activity of silver ions (Kulsky, 1980).

Thus, our results confirmed that the inhibition effect of BC/AgNps composite against pathogenic bacteria through the direct contact action in vitro was more noticeable in cases when composite samples synthesized at temperatures 60 and 90 °C were used. This phenomenon can be explained both with dimensional characteristics of the Ag-particles in composite discs formed at these temperatures and with a straight line relationship between the antibacterial activity and the content of Ag particles per unit of the surface of the samples.

Conclusion

The different modes of synthesis of composite bacterial cellulose wound coverings with silver nanoparticles intended for wounds infected with Gram-negative and Gram-positive bacteria were investigated. An inclusion of silver nanoparticles into the structure of bacterial cellulose layer with hydrothermal synthesis at 60 and 90 °C provides the formation of bio-active surface of BC/AgNps composite hydrogel films having antibacterial influence to the wound pathogenic flora, which is more noticeable for E. coli and K. pneumoniae.

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