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The membrane composite with silver nanoparticles for fibroblastic cell growth sustaining

Monika Drabik, Beata Kazimierczak, Anna Grzeczkowicz, Magdalena Antosiak-Iwanska, Angelika Kwiatkowska, Ludomira Granicka*

Nalecz Institute of Biocybernetics and Biomedical Engineering, Polish Academy of Sciences, Trojdena 4 st., 02-109 Warsaw, Poland, Tel. +48 22 592 59 00; emails: lgranicka@ibib.waw.pl (L. Granicka), mdrabik@ibib.waw.pl (M. Drabik), bkazimierczak@ibib.waw.pl (B. Kazimierczak), agrzeczkowicz@ibib.waw.pl (A. Grzeczkowicz), mantosiak@ibib.waw.pl (M. Antosiak-Iwanska), ankwiatkowska@op.pl (A. Kwiatkowska)

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ABSTRACT

The aim of the study was to develop the system of immobilized cells by using membranes with incorporated bactericidal component, which could be employed in biomedical applications. In this paper, we present assessment of the usability of a system of fibroblastic cells immobilized within composite membrane with commercial silver nanoparticles (AgNPs). We have assessed the basic properties of designed membrane such as water contact angle and permeability. It was observed that there was no significant difference between AgNPs-modified collagen membranes and the control–collagen membrane ($p > 0.05$). The mean contact angle value for all assessed composite membranes was equal to 84.13 ± 2.20. Moreover, we have evaluated the membrane influence on the selected fibroblastic cell line. The designed AgNPs composite membrane exhibited influence on cell mitochondrial activity, however, only the composite containing 25 ppm caused above 50% functionality decline. Designed material exhibiting transport properties allowing for permeation of nutrients and metabolites. The applied membrane composites with AgNPs at concentration 6.25 up to 12.5 ppm, may be recommended for biomedical applications, especially to sustain cell growth in the skin rebuilding bandages.

Keywords: Silver nanoparticles; Collagen I; Membrane properties; WEHI 164

1. Introduction

The nanoparticles of metals such as gold, copper or silver [1–4] are the most promising agents developed for bactericidal function considering multidrug resistance rising in microorganisms.

Especially, silver nanoparticles (AgNPs) have attracted much more attention than other metal nanomaterials, which could be attributed to their proven antimicrobial properties, high biocompability and low cytotoxicity. It is true to say that long before the discovery and patenting of antibiotics, silver has been used for medicinal purposes (e.g., wounds healing and infections treatment), also in the form of silver colloid [5]. According to the latest assessment of the United States Environmental Protection Agency, 0.005 mg of silver per kilogram of body weight per day is considered as non-toxic [6]. On the other hand, the daily allowed concentration of AgNPs in drinking water is 0.1 mg/L as reported by World Health Organization. Both European and American organizations (including United States Environmental Protection Agency and European Food Safety Authority) have increased their efforts to establish nanosilver safety standards, resulting in

* Corresponding author.

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) or the European Cosmetics Regulation [5]. It has been proven that AgNPs show a higher antibacterial activity than silver ions – presumably due to their larger surface area of potential interaction [1,4,7,8]. The surface area to volume ratio of the nanomaterials (structure of matter ranging in size from 0.1 to 100 nm) results in the dominance of surface phenomena and enhancement quantum mechanics role. Consequently, nanoparticles have different mechanical, magnetic, electrical as well as optical properties than the bulk materials [1,4,7,8].

In recent years, AgNPs have attracted great interest in such fields as pharmaceutical and food production, textile, household and cosmetic industries. Nevertheless, they have also been employed in medical and dental components and devices as well as applied in plant protection products [1–5,8,9]. The AgNPs have a potential to become commonly used in the field of biomedicine and biotechnology considering their properties. The increasing use of AgNP implies intensive studies on impact of the release of silver ions and AgNPs on the environment and living organisms. It is worth to notice that silver ions released from the nanoparticles present in the wastewater can react with chlorides and sulfides to form sparingly soluble salts [9].

AgNPs can be obtained by physical (e.g., laser ablation, sonochemical methods and photochemical), chemical (e.g., reduction and nanoemulsions) and biological (e.g., psychrophilic bacteria culture) methods, among which chemical reduction is the most frequently applied [1,2,4,7,9]. It ought to be noted that the latter approach avoids the formation of nanoparticle agglomerates. After reduction of metal ions, synthesized nanoparticles are often stabilized by coating with capping agents, such as polysaccharides, salts of inorganic acids, amino or organic acids, polypeptides, flavones, phenolic compounds, surfactants or materials such as organic films, membranes, fibers and nanomaterials [1,2,8].

Various supports applied to sustain cell growth have been previously reported by many authors.

A great number of both natural and synthetic biomaterials have been already tested for their usefulness and cooperation with the cells [10–13]. Nevertheless, the bacteriostatic features of modern materials are not yet excellent and should be still improved. Moreover, such supports should be strictly tested regarding the complex requirements of the cell cultures sustaining, due to the living organisms' sensitivity as well as possible influence of the external environment. The aim of the study was to develop the system of immobilized cells by using membranes with incorporated bactericidal component designed for biomedical applications, especially to support cell growth in the skin rebuilding bandages.

The commercial AgNPs colloid in water has been selected for the preparation of nanocomposite membranes. Considering their unique properties – it seemed to be natural choice. These nanoparticles show bactericidal effects in the range of concentration 25–5 ppm against strains of *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Enterococcus hirae* – in most cases reducing their viability even after several hours of contact [14]. It can be noted that the membrane, joining this two features – bacteriostatic influence and cell growth sustaining has not been reported before.

AgNPs have attracted much more attention than other metal nanomaterials due to their proven antimicrobial properties, high biocompatibility and low cytotoxicity. In our study, we have designed AgNPs-modified membrane ensuring both eukaryotic cells growth and bacteriostatic activity of the material. To provide the high biocompatibility of the surface and support adherent cells differentiation [15], we have applied collagen I as a membranes matrix. We have assessed the basic properties of the membrane and evaluated their impact on chosen eukaryotic cells.

The function of targeted WEHI 164 cells immobilized within the composite membranes – collagen with AgNPs in concentrations ranging from 6.25 to 25 ppm was assessed during 72 h culture by flow cytometry and MTT test. Moreover, the AgNPs ability to influence membrane properties was examined.

The proposed membrane based on the collagen support and AgNPs forming the material bearing bactericidal properties could be potentially applied in systems with immobilized cells in bandages for sustaining cell growth especially directed toward skin rebuilding.

2. Experimental

2.1. Materials

Reagents: Collagen type I solution from rat tail (Sigma, EU), AgNPs used in concentration 50 ppm (Nano-Koloid, EU), propidium iodide (PI; Sigma, EU), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, EU), dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA). Media: RPMI 1640 (Biomed, EU) and fetal bovine serum (FBS; Sigma, EU).

Culture media: RPMI 1640 supplemented 10% FBS (RPMI 1640/10% FBS).

Cell lines: WEHI 164 – mouse fibrosarcoma cell line.

2.2. Methods

2.2.1. Preparation of the membrane film

Collagen solution was diluted to a working concentration of 0.02% using deionized water. To obtain the collagen composite with AgNPs, the 0.02% collagen solution in deionized water was added to AgNPs solution at concentration 50 or 25 or 12.5 ppm in proportion 1:1, obtaining four different 0.01% collagen solutions with 6.25 ppm AgNPs, with 12.5 ppm AgNPs and with 25 ppm AgNPs. The collagen membrane was prepared using 0.01% collagen solution in deionized water. The obtained solutions were slowly cast on a plate and left for 2 h at 4°C. After that time, the membranes were washed twice with the distilled water and then dried and UV sterilized.

2.2.2. Cell line culture

WEHI 164 cell line (American Type Culture Collection, Rockville, MD, USA) was maintained in RPMI 1640 supplemented with 10% FBS, 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 1% penicillin and streptomycin. Cells were cultured to over 80% confluence and then washed with phosphate buffered saline, without

Ca^{2+} and Mg^{2+}. After that the cells were collected with 0.25% trypsin Ethylenediaminetetraacetic acid (PAA Cell Culture Company, EU). Moreover, cells were counted using hemocytometer (Scepter™ 2.0 Cell Counter, Merck Millipore, EU). After the passage, the cells were cultured within membrane films of composite collagen with incorporated AgNPs or within collagen membrane films. The collagen without nanoparticles was used as the standard negative control. During 72-h culture the cells after trypsinization were examined using flow cytometry with PI or MTT test.

2.3. Adsorption of polyelectrolyte layers on the support

The polypropylene flat membranes as a support at dimension of 1 cm × 1 cm were washed in ethanol and then in physiological saline. Then, the support was incubated in the solution of collagen or modified collagen with AgNPs at concentration 6.25 or 12.5 or 25 ppm for 4 min at both sides. After incubation in the respective solutions the support was washed out with 0.9% NaCl to remove unabsorbed polymer and then dried. The following polyelectrolyte membrane layers were adsorbed: collagen or collagen with 6.25 ppm of AgNPs (6.25 ppm AgNPs), collagen with 12.5 ppm of AgNPs (12.5 ppm AgNPs) and collagen with 25 ppm of AgNPs (25 ppm AgNPs).

2.4. Wettability and work of adhesion evaluation

The wettability and work of adhesion for water of polyelectrolyte membranes adsorbed on the support: collagen or 6.25 ppm AgNPs or 12.5 ppm AgNPs or 25 ppm AgNPs or the support alone (as a control) were analyzed in surface energy analyzer Phoenix 150 Surface Electro Optics (Haas, EU) in dedicated software – SEO Software-IMAGE XP. The fluid was dispensed from a manually controlled syringe. The position of the sample stage was precisely adjusted along the *x*-, *y*- or *z*-axis for fine image analysis. The images were analyzed automatically by software applying analysis algorithm using a wave function. All measurements were performed at room temperature.

2.5. Assessment of the transport properties of the membranes

To estimate transport properties of the membrane we have applied a particular measuring system. Alginate spherical cores (prepared of 1.5% alginate solution in 0.1 M NaCl) were coated with the evaluated membrane and next placed into the solution of model particles. As the model particles we applied Dextrans of molecular weight 70 and 150 kDa. Then the concentration of the indicator was spectrophotometrically analyzed.

Diffusive permeability was evaluated using a thermodynamic description of diffusive mass transport across a homogenous membrane (Fick's law) and a two compartment model [11]. The uncoated alginate spherical cores served as a negative control.

2.6. Flow cytometry

To examine the impact of the designed membrane on cell viability we have applied flow cytometry. Moreover, the method was used to determine whether the AgNP compound shows the cytotoxic effect on the cells. Data acquisition was performed on Canto II flow cytometer (Becton Dickinson Immunocytochemistry Systems, USA). The results were processed by the FACS Diva software system (Becton Dickinson, USA).

2.7. MTT assay

The MTT assay to assess cellular mitochondrial activity was performed after 72 h of culture. Shortly, the cells were seeded on the membrane films. After 72 h of culture the MTT solution was added at the concentration of 5 g/L to the culture in a 1:10 dilution of the medium. Next, the cells were incubated for 2 h at 37°C, 5% CO_2. Afterwards, the solution was discarded and DMSO was added to each well. After 15 min of shaking an absorbance was measured at 550 nm using spectrophotometer (HP 8452 diode-array spectrophotometer).

2.8. Statistical analysis

Mean values and standard deviations as well as significance of difference were calculated in the Statistica 7.1 software. The values of $p < 0.05$ were assumed as significant.

3. Results and discussion

3.1. Water contact angle measurements

The surface wettability of adsorbed on the support membranes built of collagen or collagen with incorporated 6.25 ppm AgNPs or 12.5 ppm AgNPs or 25 ppm AgNPs was analyzed. The wettability of examined membranes declined as compared with the polypropylene hydrophobic support (Fig. 1).

The surfaces of all evaluated membranes exhibited the water contact angle below 90° indicating their hydrophilic character. The representing pictures of the water droplets showing the contact angles for evaluated composite membranes are presented in Figs. 2(A)–(E). There was no

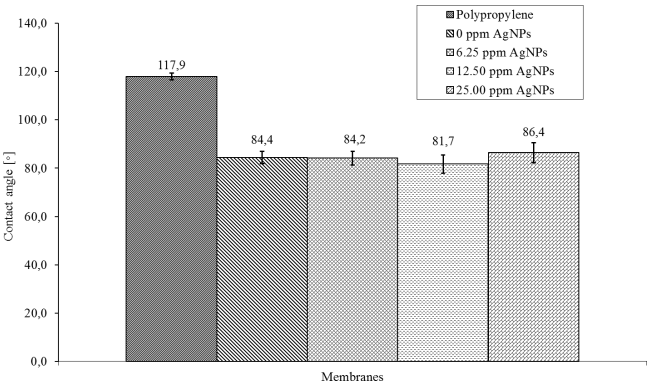

Fig. 1. Water contact angle. Polypropylene – polypropylene support; collagen – polypropylene support with adsorbed collagen; 6.25 ppm AgNPs – polypropylene support with adsorbed collagen with 6.25 ppm of AgNPs; 12.5 ppm AgNPs – polypropylene support with adsorbed collagen with 12.5 ppm of AgNPs; 25 ppm AgNPs – polypropylene support with adsorbed collagen with 25 ppm of AgNPs. The values are presented as mean ± SD.

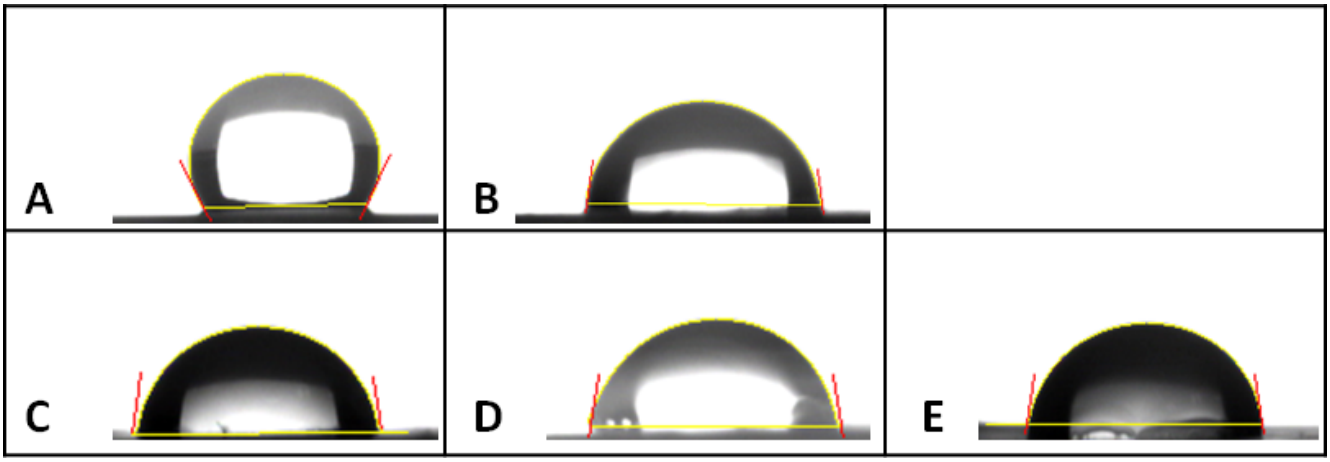

Fig. 2. The representing pictures of the water droplets showing the contact angles for evaluated composite membranes. (A) Polypropylene support, (B) polypropylene support with adsorbed collagen, (C) polypropylene support with adsorbed collagen with 6.25 ppm of AgNPs, (D) polypropylene support with adsorbed collagen with 12.5 ppm of AgNPs and (E) polypropylene support with adsorbed collagen with 25 ppm of AgNPs.

significant difference in water contact angle between the collagen and the AgNP composite membrane layers ($p > 0.05$). Considering the concentration of AgNPs of 6.25, 12.5 and 25 ppm, there was $p = 0.91$, $p = 0.27$ and $p = 0.42$, respectively. The results proved that AgNPs involvement into the collagen membrane did not influence its wettability. Moreover, there was no statistical significant difference in the contact angle between various collagen composites 6.25 ppm AgNPs or 12.5 ppm AgNPs or 25 ppm AgNPs ($p > 0.05$). There was, respectively, $p = 0.33$, $p = 0.40$, $p = 0.13$ comparing Ag 6.25 ppm with Ag 12.5 ppm or Ag 6.25 ppm with Ag 25 ppm or Ag 12.5 ppm with Ag 25 ppm. Simultaneously, what is shown in Fig. 3, the work of adhesion for all evaluated membranes increased as compared with unmodified substrate. Likewise, there was no significant difference in work of adhesion between the collagen membrane and the collagen with incorporated AgNPs composite membrane.

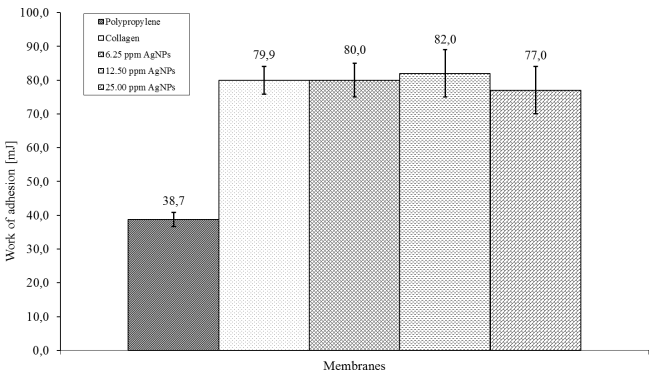

Fig. 3. Work of adhesion. Collagen – polypropylene support with adsorbed collagen or collagen; 6.25 ppm AgNPs – polypropylene support with adsorbed collagen with 6.25 ppm of AgNPs; 12.5 ppm AgNPs – polypropylene support with adsorbed collagen with 12.5 ppm of AgNPs; 25 ppm AgNPs – polypropylene support with adsorbed collagen with 25 ppm of AgNPs. The values are presented as mean ± SD.

3.2. Assessment of the transport properties of the membranes

All evaluated membranes exhibited comparable changes in the concentration of model particles during the examination time (Figs. 4 and 5). The changes for individual time intervals of permeability and time product indicated membrane permeating ability for examined solutes of molecular weight 70 and 150 kDa (Figs. 6 and 7). The calculated permeability (P) of the AgNPs-modified membranes increased for both the group with AgNP concentration of 12.5 ppm and the one with the AgNPs concentration of 25 ppm comparing with the group of AgNPs concentration of 6.25 ppm (Fig. 8).

However, the significant influence of different collagen modifications on collagen membrane hydrophilicity was not observed, the influence on P has been revealed.

It can be noted that the permeability (P) is affected not only by the physicochemical features of the membrane material, but also by the interaction between the membrane and the evaluated solute. Thus, the membrane material interaction with negatively charged Dextran solute may imply the P changes. The solute size cannot be neglected as well. We observed the permeability increase in case of the Dextran marker of 70 kDa for 12.5 and 25 ppm AgNPs share in composite comparing with unmodified collagen and AgNPs 6.25 ppm composite. It can be explained by stronger attraction between Dextran 70 and the AgNPs present in the membrane at higher concentration than in membrane composite 6.25 ppm AgNPs. On the other hand, the discrepancy was observed in case of the permeability of membrane with AgNPs concentration of 6.25 ppm for Dextran 150. The influence of MW of solute as well as the oxidative dissolution of AgNPs cannot be excluded. The small amount of the AgNPs may undergo oxidative dissolution causing Ag^+ formation [16]. This leads to electrostatic interaction between the formed cations with the Dextran 150 resulting in appearing of spherical barrier around the alginate core. As a consequence, the solutes permeating are blocked resulting in P decline. The higher concentrations of the AgNPs undergo oxidative dissolution in a small extent and in this case the share of hydrogen

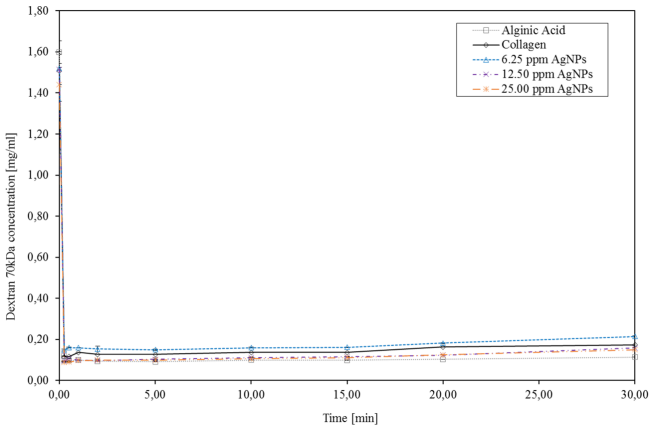

Fig. 4. Dextran 70 concentration in function of time during permeation through the membrane. Alginic acid – the core of alginic acid; collagen – the core covered with collagen; 6.25 ppm AgNPs – the core covered with collagen composite with 6.25 ppm AgNPs; 12.5 ppm AgNPs – the core covered with collagen composite with 12.5 ppm AgNPs; 25 ppm AgNPs – the core covered with collagen composite with 25 ppm AgNPs. The values are presented as mean ± SD.

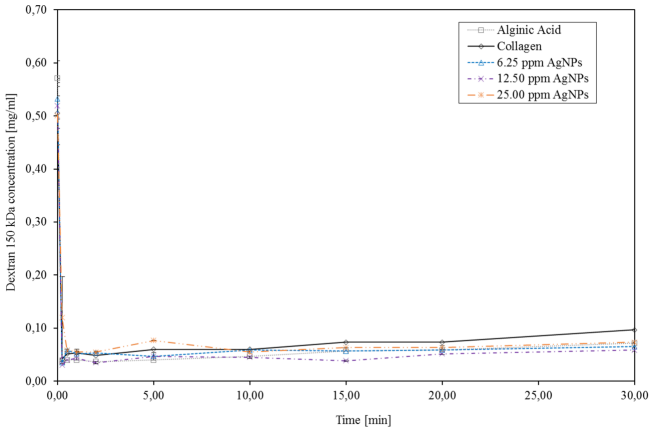

Fig. 5. Dextran 150 concentration in function of time during permeation through the membrane. Alginic acid – the core of alginic acid; collagen – the core covered with collagen; 6.25 ppm AgNPs – the core covered with collagen composite with 6.25 ppm AgNPs; 12.5 ppm AgNPs – the core covered with collagen composite with 12.5 ppm AgNPs; 25 ppm AgNPs – the core covered with collagen composite with 25 ppm AgNPs. The values are presented as mean ± SD.

interactions between the membrane and Dextran 150 solutes predominate, hence the permeability achieved higher values similar to the negative control. Nevertheless, the transportation of the markers is still possible.

3.3. Evaluation of functioning of cells within different membranes

Flow cytometry analysis was used to determine the function of cells cultured within the prepared membranes. The results showed that there was no statistical difference in cell viability between the cells grew with collagen films without AgNPs and composite collagen membranes containing AgNPs at a concentration of 6.25 or 12.5 or 25 ppm

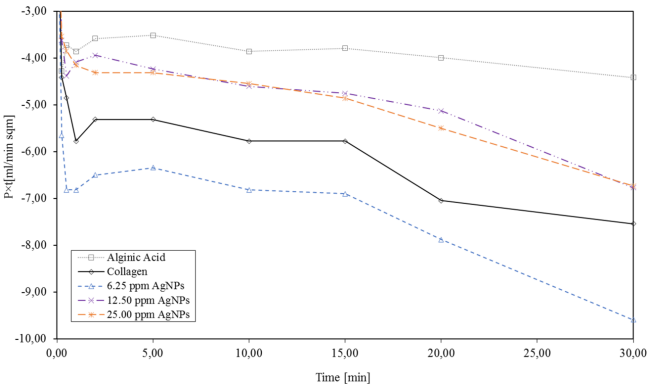

Fig. 6. Membrane permeability and time product during individual time intervals permeation for Dextran 70. Alginic acid – the core of alginic acid; collagen – the core covered with collagen; 6.25 ppm AgNPs – the core covered with collagen composite with 6.25 ppm AgNPs; 12.5 ppm AgNPs – the core covered with collagen composite with 12.5 ppm AgNPs; 25 ppm AgNPs – the core covered with collagen composite with 25 ppm AgNPs. The values are presented as mean ± SD.

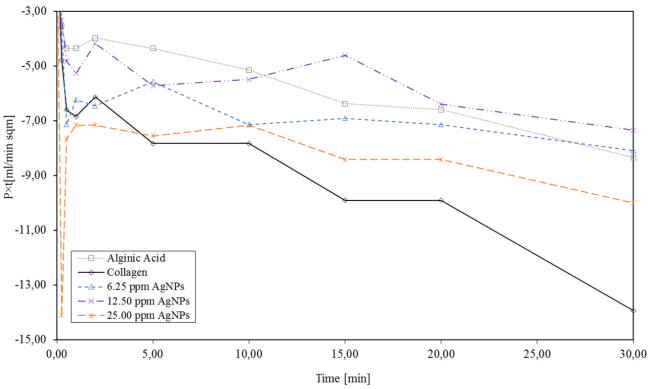

Fig. 7. Membrane permeability and time product during individual time intervals permeation for Dextran 150. Alginic acid – the core of alginic acid; collagen – the core covered with collagen; 6.25 ppm AgNPs – the core covered with collagen composite with 6.25 ppm AgNPs; 12.5 ppm AgNPs – the core covered with collagen composite with 12.5 ppm AgNPs; 25 ppm AgNPs – the core covered with collagen composite with 25 ppm AgNPs. The values are presented as mean ± SD.

(Fig. 9). Nevertheless, MTT examination revealed lower mitochondrial activity of cells for all composite collagen membranes comparing with collagen membrane (Fig. 10). The decline was meanly 35% for membranes modified with AgNPs of the concentration of 6.25 and 12.5 ppm. The membrane with the highest applied concentration of AgNPs (25 ppm) caused over 50% mitochondrial activity decline.

The toxicity of AgNPs, with high probability, is based on the disruption of cell membranes and silver ions release. As a result, the reactive species of oxygen are formed. Those changes inhibit proteins, and thereby induce mitochondrial dysfunction and chromosomal aberrations [1,3,5,8].

The membrane composites of the AgNPs concentration of 6.25, 12.5 and 25 ppm were selected on the basis of support usability, considering toxicity toward the eukaryotic cells.

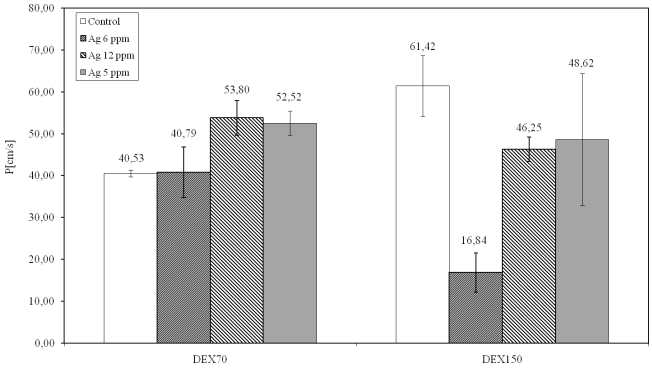

Fig. 8. Membrane permeability for collagen composite membranes for Dextran 70 or Dextran 150. Control – the core covered with collagen; 6.25 ppm AgNPs – the core covered with collagen composite with 6.25 ppm AgNPs; 12.5 ppm AgNPs – the core covered with collagen composite with 12.5 ppm AgNPs; 25 ppm AgNPs – the core covered with collagen composite with 25 ppm AgNPs. The values are presented as mean ± SD.

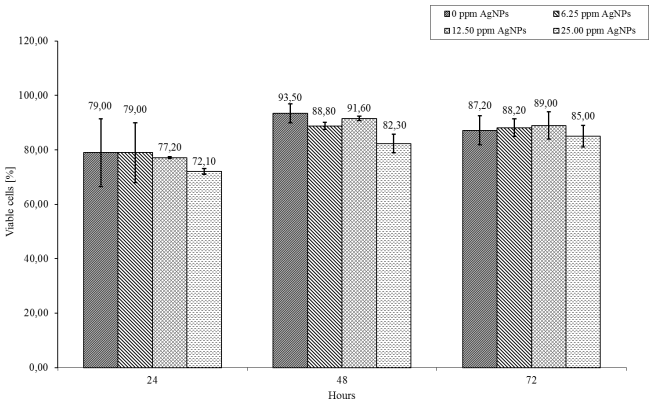

Fig. 9. The percentage of viable WEHI 164 cells during 72-h culture in the presence of collagen (0 ppm AgNPs) or composite collagen membrane (6.25 ppm AgNPs or 12.5 ppm AgNPs or 25 ppm AgNPs). The values are presented as mean ± SD.

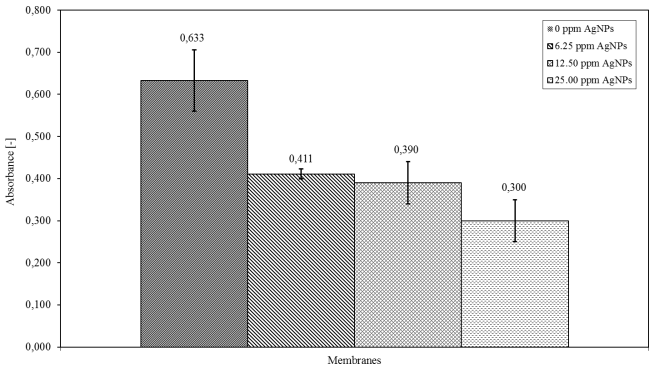

Fig. 10. Evaluation of mitochondrial activity represented by formazan production expressed by absorbance of WEHI 164 cells after 72-h culture in the presence of collagen (0 ppm AgNPs) or composite collagen membrane (6.25 ppm AgNPs or 12.5 ppm AgNPs or 25 ppm AgNPs). The values are presented as mean ± SD.

As upper limit we presumed about 50% decline in cell viability. Furthermore, the concentrations ensuring the sustaining of bactericidal properties were also considered [14]. The role of the constructed composites was to keep the balance between the necessity of maintaining cells viability and the requirement of the sustaining the effect of bacteriostatic elements. The cytotoxic impact on the cells in vitro was observed in case 25 ppm modification. Thus, the membrane composites with the AgNPs concentration of 6.25 and 12.5 ppm can be considered for further applications. However, it can be noted that in vitro studies often show higher AgNPs toxicity than in vivo ones [1,2]. The studies investigating the in vitro penetration and permeation of AgNPs through human skin indicated only penetration into the stratum corneum and the outermost surface of the epidermis [17]. Moreover, it is well known that the biocidal effects of nanosilver depend not only on the chemophysical properties of the nanoparticles (size, shape, charge, etc.), but also on the characteristics of the exposed organisms.

The analogous problems emerge in case of drug delivery systems; nonetheless, for now the data obtained do not show the clear image on the effect of nanocarriers. Especially, the penetration of such particles is a controversial topic [18].

4. Conclusion

The applied membrane composites with the bacteria growth inhibitors – AgNPs have exhibited transport properties allowing for permeation of nutrients and metabolites. The promising results were obtained for collagen films with AgNPs at concentration of 6.25 up to 12.5 ppm incorporated within. We highly recommend designed membranes for biomedical applications, particularly as a part of bandages supporting skin rebuilding during the process of wound healing. Nevertheless, cytotoxicity of AgNPs to the living organisms, especially human cells, is still unclear and required more complex studies, especially considering their possible biomedical applications. Such tests should also include an evaluation of the cytotoxicity of AgNPs composites with other materials.

Acknowledgments

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