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The membrane composite scaffolds with antithrombotic features for adherent cells function sustention

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ABSTRACT

The aim of the study was to design the scaffold encompassing bacteriostatic and antithrombotic properties as the elements of the dressing systems which could find biomedical applications. In this paper, we present the scaffolds produced of polysaccharide and/or protein involving the constituents allowing to obtain desired features. We have assessed physicochemical properties of designed membranes using Fourier transform infrared spectroscopy and water contact angle measurements. The mean contact angle value for the scaffolds with the antithrombotic constituent was equal to 18.0 ± 7.2, whereas the scaffolds without this component showed meanly 68.7 ± 0.6. Moreover, we evaluated the interaction of scaffolds with fibroblastic cell line evaluating the cell functions. The differences in percentage of viable cells cultured in the presence of designed scaffolds did not exceed 20%. All the designed membrane scaffolds based on alginate composite with properties that favored adhesion or weakened it without impairing the function of the cells would possibly find a biomedical application. However, the microscopic assessment showed the increased proliferation of cells immobilized within membrane scaffold, which was built of bilayer constructed of alginate with incorporated silver nanoparticles and heparin. This membrane encompassing bacteriostatic and antithrombotic features could be recommended as an element of dressing systems.

Keywords: Bacteriostatic and antithrombotic features; Alginate; Silver nanoparticles; Membrane scaffold

1. Introduction

Regenerative medicine is a dynamically developing discipline. Its idea is to repair or restore dysfunctional fragments of a tissue by replacing diseased or damaged cells with healthy ones. This discipline is becoming more and more important due to the shortage of transplantable organs in relation to the number of patients awaiting for transplantation. This problem touches the whole world. Therefore, alternative methods of damaged structures reconstruction have been explored.

Information provided by organizations monitoring the state of health, statistical data and reports prepared by companies carrying out pharmaceutical market surveys demonstrate that due to the progressive number of civilization-related diseases, the demand for innovative medical materials will continue to grow in the coming years. Nowadays used bandages or skin substitutes do not suffice to prevent inflammatory processes, which in the wound lead to dysregulation of a balance between formation and inactivation of reactive forms of oxygen, in consequence damaging living cells [1]. Hence, the research has been concentrated on achieving a wound care bandage or a skin substitute that provide an effective and a scar-free wound healing. Moreover, blood contacting surfaces may activate platelets and result in serious medical problems, such as thrombosis. Although a lot of biomaterials are used for medical devices – implants and

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coatings, there still exists the need for better polymer systems to regulate biological processes at the blood–material interface. Rational design of nanostructured multilayer coatings can direct cellular processes at interfaces [2].

The layer-by-layer (LbL) method can be applied in a multilayer production. Since its introduction in the early 1990s, LbL self-assembly of films technique has been widely used in the fields of nanoelectronics, optics, sensors, surface coatings, and controlled drug delivery. LbL films are created through the sequential deposition of biomolecules in solution containing functional groups that drive self-assembly [3,4]. Most techniques rely on electrostatic interactions between oppositely charged polyelectrolytes during a sequential deposition; however, a variety of other chemical interactions are also harnessed by LbL techniques, including hydrogen bonding [5], biomolecules recognition [6], click chemistry [7], sol–gel reactions [8], or combined processes [9].

Concurrently, the research interest focused on material nanotechnology products application [10–17] creates security concern about nanoparticles usage. This entails an increase in the number of studies on the nanoparticles impact on the environment, living organisms and, in particular, human health observed in recent years.

The aim of this work was to design eukaryotic cells supporting scaffolds as the bandage elements encompassing the properties allowing for achievement of antithrombotic and bacteriostatic functions for biomedical or biotechnological purposes, especially for dressing wounds.

The silver nanoparticles (AgNPs) exhibiting bactericidal effects in the range of concentration 5–25 ppm against strains of *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli,* and *Enterococcus hirae* were applied in the experiments [18]. Sodium alginate, heparin, and collagen type I materials were selected for a multilayer membrane scaffold construction. The selection of protein and polysaccharide was related to their properties and wide variety of medical applications. Heparin, as an active substance, combines antithrombotic, anti-inflammatory, and anti-edema properties. Collagen is present at every stage of wound healing process. Therefore, it is widely used in the construction of skin substitutes including several forms of scaffolds [19,20].

2. Experimental

2.1. Materials

Reagents: Alginic acid (sodium alginate-low viscosity Sigma-Aldrich, EU), collagen type I solution from rat tail (Sigma-Aldrich, EU), heparin (5,000 U/mL, Polfa S.A., Poland), AgNPs used in concentration 50 ppm (Nano-Koloid, EU), propidium iodide (PI; Sigma-Aldrich, EU), acridine orange (AO; Sigma-Aldrich, EU), Hoechst stain solution (Hoechst33258 Sigma-Aldrich, EU), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, EU), dimethyl sulfoxide (DMSO; Sigma-Aldrich, EU), phosphate buffered saline (PBS; Gibco, EU), trypsin/EDTA (Invitrogen, USA).

Culture media: RPMI 1640 (Biomed, EU) supplemented 10% fetal bovine serum (FBS; Sigma-Aldrich, EU) (RPMI 1640/10% FBS).

Cell lines: WEHI 164 – mouse fibrosarcoma cell Line (American Type Culture Collection, Rockville, MD, USA).

2.2. Methods

2.2.1. Preparation of the membrane scaffold

The membrane scaffolds were formed using the LbL method. The following monolayer or bilayer composite membrane scaffolds were prepared:

- Alginate and collagen I composite layer (AC)
- Alginate and collagen I composite/heparin bilayer (AC/H)
- Alginate and heparin composite layer (AH)
- Alginate and heparin composite/collagen I bilayer (AH/C)
- Alginate and heparin composite/collagen I with incorporated silver nanoparticles bilayer (AH/CAg)
- Alginate with incorporated silver nanoparticles layer (AAg)
- Alginate with incorporated silver nanoparticles/heparin bilayer (AAg/H)

The membranes were deposited directly on the surface of the culture plate as a support. As the first layer: alginate solution in 0.1 M NaCl at concentration 2 mg/mL and pH 7.2 or alginate solution in 0.1 M NaCl at concentration 4 mg/mL and collagen solution in PBS free from calcium ions, magnesium ions (PBS) at concentration 2 mg/mL solution, ratio 1:1 and pH 7.2 or alginate solution in 0.1 M NaCl at concentration 4 mg/mL and heparin solution in PBS at concentration 150 U/mL solution, ratio 1:1 and pH 7.2 or alginate solution in 0.1 M NaCl at concentration 4 mg/mL and AgNPs solution in PBS at concentration 12.5 ppm, ratio 1:1 and pH 7.2 were applied. After 2 h deposition time, the plate was washed twice with physiological saline and dried.

Next, in case of bilayer membranes, the second layer was deposited. As the second layer: heparin solution in PBS at concentration 75 U/mL at pH 7.2 or heparin solution in PBS at concentration 150 U/mL and AgNPs solution in PBS at concentration 12.5 ppm, ratio 1:1 and pH 7.2 or collagen solution in PBS at concentration 1 mg/mL at pH 7.2 or collagen solution in PBS at concentration 2 mg/mL and AgNPs solution in PBS at concentration 12.5 ppm, ratio 1:1, at pH 7.2 were applied.

The final concentration of the substrates in the membranes was as follows: alginate – 2 mg/mL; heparin – 75 U/mL; collagen – 1 mg/mL; and silver nanoparticles – 6.25 ppm.

The physicochemical properties of the designed membranes were analyzed using the Fourier transform infrared (FTIR) spectroscopy and the water contact angle evaluation.

2.2.2. Culture of WEHI 164 cells

WEHI 164 cells were maintained in standard RPMI medium (Gibco, EU) supplemented with 10% FBS and 1% antibiotics. The cells were seeded on the designed membranes and cultured (37°C in 5% CO_2) for 11 d.

The proliferation and viability of cells cultured at standard conditions on the prepared flat composite membrane scaffolds were examined. The cells cultured without the membranes served as a reference. After 3, 8, and 11 d of culturing the cell function was examined by flow cytometry (FACS Canto II flow cytometer, Becton Dickinson Immunocytochemistry Systems, USA) with the use of PI, and by the MTT tests. Moreover, the degree of colonization

of tested membrane scaffold surfaces by adherent cells was analyzed using microscopic observations: light inverted microscope and scanning electron microscope (SEM).

2.3. FTIR analysis

The membranes were analyzed by infrared FTIR spectroscopy using a Digilab Excalibur FTS 3000 Mx spectrometer equipped with a reusable ZnSe crystal re-amplifier and a transmission adapter.

2.4. Water contact angle evaluation

The membrane water contact angle was measured using Phoenix 150 (Surface Electro Optics Haas, EU). The analyzed membranes were placed on the cover glass as a support. A syringe of 10 mL volume was used to manually control fluid dispensing during the contact angle measures. The distance between the apex of the needle and the sample surface was maintained at 15 mm. After a water droplet was formed on the analyzed membrane surface, the contact angle was monitored by magnifying camera and dedicated software – SEO Software-IMAGE XP. The images were analyzed automatically by software involving an analysis algorithm using a wave function. All measurements were performed at room temperature.

2.5. SEM analysis

Visualization of the membrane's surfaces to assess their ability to adhere cells after 8 d of culturing was performed using the SEM (TM 1000, Hitachi, Japan). The sample was dehydrated by the standard method, then a thin layer of gold was deposited on the cell surface by a sputtering system.

2.6. Flow cytometric analysis

Flow cytometry (FACS Canto II, Becton Dickinson Immunocytochemistry Systems, USA) FACS Diva software system (Becton Dickinson, USA) was used to examine the cells.

2.7. Microscopic observations

Cell morphology studies were performed as a qualitative analysis of cells using fluorescent dyes: AO and PI.

The viability of cells cultured on the membranes was evaluated by staining the samples with fluorescent markers. After 8 d of culturing the cells were stained with a mixture containing PI (5 μg/mL) and AO (0.5 μg/mL). The cells were analyzed in Olympus IX 70 fluorescence microscope using a 495/635 nm filter. Images showing the cell morphology were captured using an inverted fluorescence microscope (Olympus IX71) and analyzed with CellP software (Olympus, Warsaw, Poland).

2.8. MTT mitochondrial activity assay

The viability of the WEHI 164 cells seeded on tested membrane scaffolds was evaluated in 3-, 8-, and 11-d culturing periods using the MTT assay (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, EU). The MTT assay is based on the cleavage of yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells [21]. Cells were seeded at a density of 1×10^4 cells per well in a 96-well plate. The solution of MTT at concentration of 5 mg/mL was added to the culture at the ratio of 1:10 and incubated in a culture medium (5% CO_2, 37°C) for 2 h. Subsequently, the wells were centrifuged (1,600 rpm, 10 min) and poured into the precipitate. Next, 200 μL of DMSO was added for the formazan crystals dissolution. After 10 min of shaking, the absorbance was measured in a spectrophotometer at the wavelength 540 nm.

3. Results and discussion

3.1. FTIR spectrometer analysis

Analyzing the alginate–collagen composite membrane, the peaks representing N–H group absent in alginate membrane appeared: at $\nu = 1{,}200$ and at $\nu = 1{,}340$, and the latter was translocated in reference to collagen membrane (at $\nu = 1{,}360$). The peak indicating C=O bond presence at $\nu = 1{,}620$ and $\nu = 1{,}740$ in alginate and collagen membrane, respectively, was translocated to $\nu = 1{,}720$ (Fig. 1).

The involvement of a heparin as a layer or as a composite layer constituent in AC/H or AH/C membrane resulted in translocation of the peak representing C=O bound from $\nu = 1{,}720$ to $\nu = 1{,}760$ and N–H group peak from $\nu = 1{,}360$ to $\nu = 1{,}370$ (Fig. 1).

Alginate–heparin composite membrane analysis showed the peak representing N–H group presence at $\nu = 1{,}360$ (absent in alginate membrane). The peak representing C=O was observed at $\nu = 1{,}720$, whereas the alginate and heparin membranes exhibited the peak at $\nu = 1{,}620$ and $\nu = 1{,}740$, respectively (Fig. 1).

3.2. Water contact angle evaluation

The surface properties of polymer materials such as free surface energy and contact angle are of great importance in polymer applications. Wettability of the surface of the material is a parameter influencing on the biological interactions between the material and the living organisms. Some authors assume that the water contact angle between 60° and 75° [22] is the best for conducting cell cultures on membranes. The designed membranes water contact angle values are presented in Fig. 2. The alginate membrane was used as a reference. All the examined membranes except for the membranes involving heparin are characterized with the contact angle c. 68%.

The heparin presence as a layer or as a constituent of the composite membrane layer caused about four times water angle decline, which corresponded to the membranes wettability increase. The highest water angle decrease was observed for membranes with no collagen or AgNPs as the constituent. This effect could be caused by changes of adhesive joints presence. The correlation between the bacteriostatic component – AgNPs presence in the scaffold and the scaffold contact angle value was not observed.

3.3. Flow cytometric analysis

The obtained results showed that after 11 d of culturing, the percentage share of viable cells cultured with composite

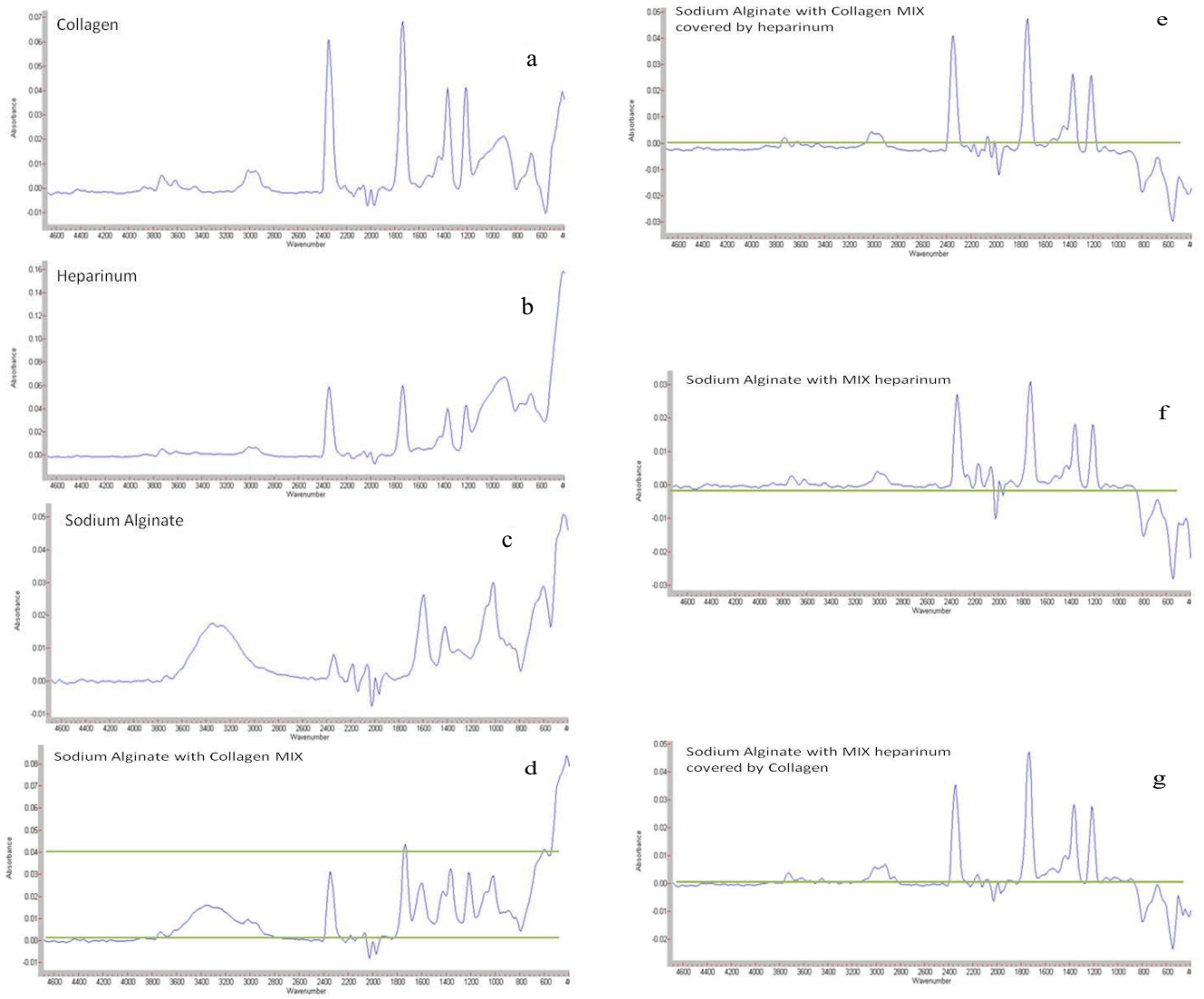

Fig. 1. (Fourier transform infrared-attenuated total reflectance) spectra (range of ν = 600–4,800 cm^{-1}) of membranes: (a) collagen, (b) heparin, (c) alginate, (d) alginate–collagen, (e) alginate–collagen/heparin, (f) alginate-heparin, (g) alginate–heparin/collagen.

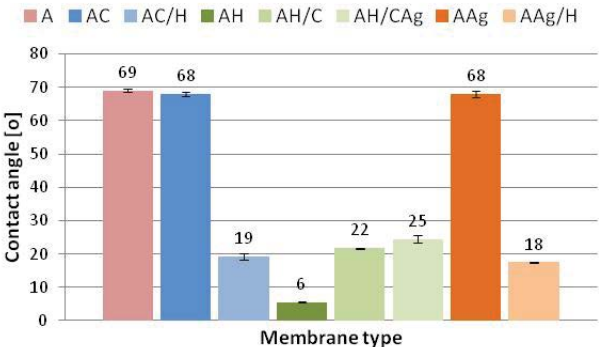

Fig. 2. Water contact angle values of the designed membranes: alginate (A), alginate–collagen (AC), alginate–collagen/heparin (AC/H), alginate–heparin (AH), alginate–heparin/collagen (AH/C), alginate–heparin/collagen with silver nanoparticles (AH/CAg), alginate with silver nanoparticles (AAg), and alginate with silver nanoparticles/heparin (AAg/H).

membranes involving AgNPs: AAg, AAg/H, and AH/CAg was comparable. There were meanly 95% of alive cells in the examined cell population. The cells cultured within other membranes exhibited lower living cells percentage share, however the viability decline did not exceed 20%. The correlation between the antithrombotic component presence in the scaffold and the percentage of viable cells cultured within the scaffolds was not observed. The results of cytometric measurements are shown in Fig. 3.

3.4. Microscopic observations

On the basis of the microscopic observation of the cells cultured on the bilayer membranes involving heparin: AC/H, AH/C, AH/CAg, and AAg/H, it was found that the cells grown on the AAg/H membrane reproduced at the same rate as the control cells (Control), reaching the logarithmic growth phase. In case of the cells cultured on the AH/CAg membrane,

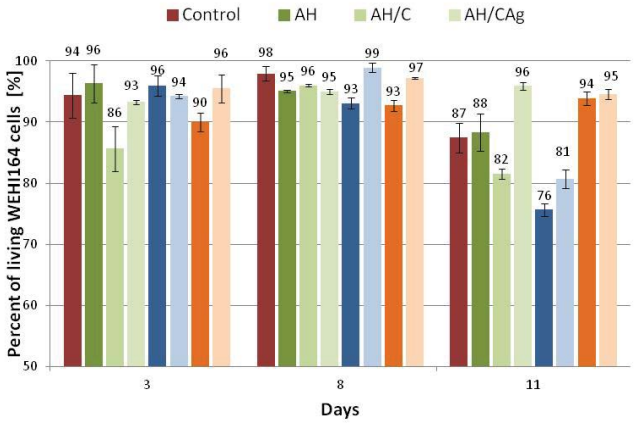

Fig. 3. Results of cytometric measurements: percentage of alive WEHI 164 cells, seeded on tested membranes: alginate–heparin (AH), alginate–heparin/collagen (AH/C), alginate–heparin/collagen with AgNPs (AH/CAg) after 3, 8, and 11 d of culture. The cells cultured without a membrane support served as a control reference (Control). The values are presented as mean ones ± SD (n = 6).

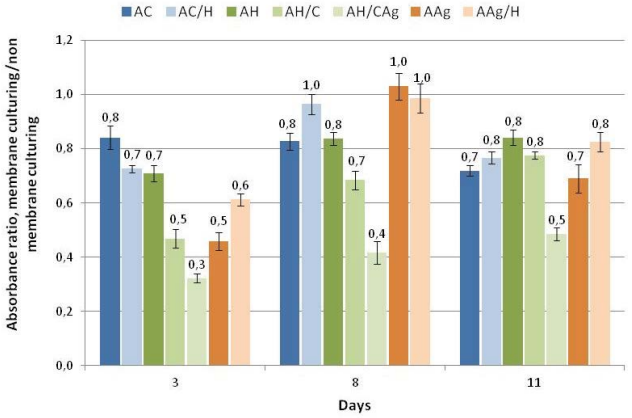

Fig. 5. The mitochondrial activity of WEHI 164 cells expressed by formazan production, presented in the form of the ratio of average production of formazan by cells grown on membranes: alginate–heparin (AH), alginate–heparin/collagen (AH/C), alginate–heparin/collagen with AgNPs (AH/CAg), alginate with AgNPs (AAg), and alginate with AgNPs /heparin (AAg/H) – to the average production of formazan by control cells after the 3, 8, and 11 d of culturing. The cells cultured without a membrane support served as a control. The values are presented as mean ones ± SD (n = 6).

slower proliferation was observed – the cells remained in the stationary growth phase whereas the control cells went from the logarithmic phase to the death phase (Fig. 4).

3.5. MTT mitochondrial activity assay

More diversified results were obtained in the mitochondrial activity assay (MTT) where the metabolism of WEHI 164 cells, expressed by the production of formazan, was determined by the ratio of the absorbance of cells grown on the tested composite membranes to the control sample (non-membrane cells culturing) (Fig. 5).

Analysis of the mitochondrial activity of cells grown on the evaluated composite membranes after 11 d of culturing, showed up to 50% lower production of formazan in reference to the control sample. The lowest mitochondrial activity was observed for the AH/CAg membrane culture (50%). In other cases, the mitochondrial activity of the cells was at the mean level of 77% of the control samples value.

4. Conclusion

All the composite membrane scaffolds AC/H, AH/C, AH/CAg, AAg/H as well as the reference membranes A/C, A/H with properties that favored adhesion or weakened it without impairing the function of the cells could find biomedical application. The heparin involvement as a layer or as a

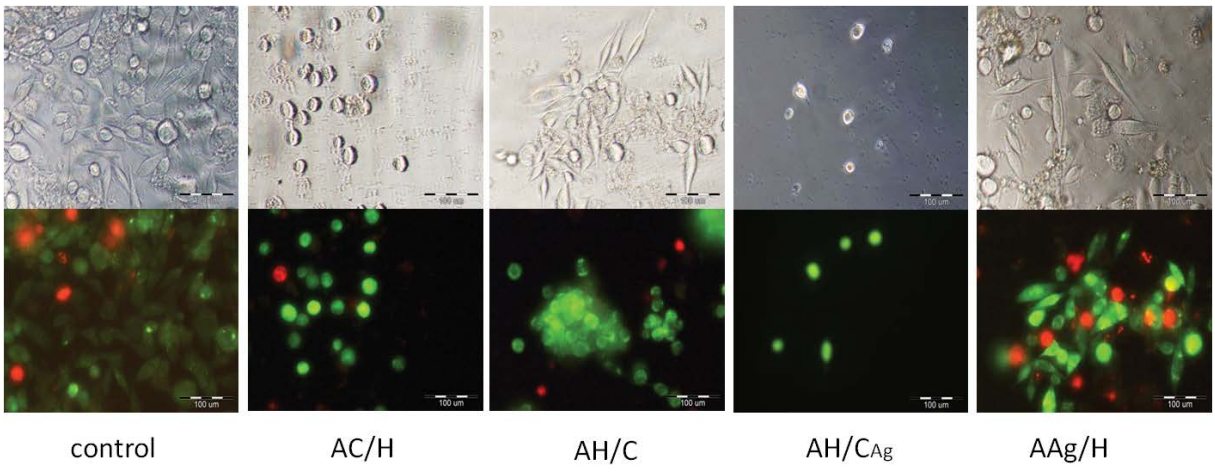

Fig. 4. Light microscopy observation after 8 d of culturing. The WEHI 164 cells seeded on tested membranes: alginate–collagen/heparin (AC/H), alginate–heparin/collagen (AH/C), alginate–heparin/collagen with AgNPs (AH/CAg), and alginate with AgNPs/heparin (AAg/H). The cells cultured without a membrane support served as a control reference (Control). The upper row – a visible light visualization; the lower row – fluorescence visualization with acridine orange and propidium iodide staining. Scale bar = 100 μm.

composite constituent caused wettability increase leading to the creation of antithrombotic properties. Based on microscopic observations of WEHI 164 cells cultured on prepared composite membranes, it could be noted that the cells cultured on membrane AAg/H reproduced faster comparing with the cells cultured on the other scaffolds. Moreover, there was no significant effect of 6.25 ppm AgNPs presence in AAg/H membrane on the percentage of viable cells after 11 d of culturing comparing with the A/H membrane and a control sample. Such scaffolds involving heparin allowing for wettability increase, especially composite AAg/H encompassing bacteriostatic substrate, may be recommended as the elements for dressings construction with antithrombotic properties for biomedical applications.

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