Abstract

Background. Open wound treatment requires a use of bandage material to prevent the development of pathogenic microflora and to provide the necessary conditions for tissue regeneration.

The aim of the study was to compare the effectiveness of polyacrylamide (PAA) and dextran-graft-polyacrylamide (D-PAA) hydrogels loaded with silver nanoparticles (AgNPs), antibiotics, and photosensitizers for the treatment of bacterial infection of open wounds.

Materials and Methods. PAA and D-PAA hydrogels with AgNPs, methylene blue (0.001%) without (MB) and with red light irradiation (660 nm) (MB+L), chlorhexidine (0.05%) and cefuroxime (0.1%) were used. There were tested in vitro and in vivo (a rat model) antibacterial activities against wild-type Staphylococcus aureus, Escherichia coli, antibiotic-resistant Escherichia coli and Klebsiella pneumoniae strains obtained from the wound. Clinical investigations were performed in patients with chronic venous ulcers of the lower extremities with no response to traditional treatments.

Results. S. aureus, E. coli, and K. pneumoniae strains were sensitive to PAA and D-PAA hydrogels with AgNPs, chlorhexidine, and cefuroxime. Antibiotic-resistant E. coli was not inhibited by the hydrogels with cefuroxime. This strain was less sensitive to chlorhexidine and MB+L. There were no differences between unloaded PAA and D-PAA hydrogels; the antibacterial properties of the dressing were determined by an antibacterial component loaded into the hydrogel. The use of unloaded D-PAA hydrogels in vivo helped reduce the size of the wound by 28.6% and 42.8% three and five days after wound modeling, respectively. Similar results were obtained for D-PAA hydrogels loaded with cefuroxime, chlorhexidine, and MB+L. D-PAA hydrogel with AgNPs reduced wound size by 50% and 62.5% three and five days after wound induction, respectively, demonstrated greater antibacterial activity and was selected for clinical investigations. In a patient, 14 days after bandage application, the fibrin membrane disappeared, the ulcers were covered with pink granulations, marginal epithelialization appeared.

Conclusions. PAA and D-PAA hydrogels can be loaded with the antibacterial compounds of various types. The type of polymer does not affect the antibacterial properties of the final hydrogels. The hydrogels with chlorhexidine and MB+L can be potentially used to treat bacterial contamination of wounds and ulcers. Nevertheless, their disadvantage is the inability to absorb or precipitate tissue breakdown products that interfere with normal regeneration and inflammation. D-PAA/AgNPs are the best option for treating ulcers due to the ability to control the properties of the hydrogels and nanoparticles, as well as multiple mechanisms of antibacterial action.

Keywords
Antibacterial Hydrogel; Argentum Nanoparticles; Wounds; Ulcers; Chronic Venous Ulcers

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Skin is a mechanical barrier which protects the internal organs from adverse factors. Mechanical injuries, burns, along with risk factors such as diabetes mellitus, malignant tumors, etc., disrupt normal skin functioning. Incorrect wound treatment can lead to oxidative stress, inflammation, and impaired angiogenesis [1]. In open wounds, the most common strains are the genera *Staphylococcus*, *Pseudomonas*, *Klebsiella*, *Acinetobacter*, *Aeromonas*, *Escherichia*. They differ in physiological properties, optimal temperature, pH, tolerance to oxygen, toxin and adhesine production, sensitivity to antibiotics, etc. [2] A variety of strains in infected wounds create unfavorable conditions for treatment and increase antibiotic resistance of individual bacteria. Biofilm formation and the presence of antibiotic-resistant strains significantly impair treatment efficacy. The problem arises in the treatment of both ulcers associated with metabolic syndrome and combat injuries [3–5]. One of the promising treatment methods involves the use of hydrogels as carriers of different antibacterial agents [6–8]. The hydrogels consist of hydrophilic cross-linked polymer chains of natural, synthetic, or hybrid origin [9]. Natural polymers such as polysaccharides and proteins have strong biocompatibility, biodegradability, and cell adhesion but they are more quickly destroyed by bacteria and fungi, and therefore, require special storage and sterilization conditions [10–12]. Synthetic and hybrid polymers are more resistant to environmental conditions and decay. They are obtained by polymerization of acrylamide, ethylene glycol, acrylic acid, hydroxyethyl methacrylate, etc. [13] The structure and properties of such polymers are easy to control. The combination of synthetic and natural polymers significantly increases hydrogel biocompatibility. Investigations show high efficacy of collagen/polyacrylamide [14], chitosan/polyacrylamide [15], alginate/polyacrylamide [16] combinations to treat open wounds. To provide the desired properties, the hydrogels can be loaded with drugs and nanoparticles, including biologically active small molecules, antibiotics, peptides, metal ions and their oxides, etc. [17–19]

After the emergence of antibiotic-resistant strains of microorganisms, searching for alternative ways to combat bacterial infections began. Nanoparticles of silver, gold, copper, zinc oxide, titanium oxide, etc., are effective antibacterial agents with multiple mechanisms of action [20]. Physical methods of bacterial inactivation play an important role as well. First, this is a photoinactivation with the use of photosensitizers [21]. These substances are low-toxic and photodissociate after light irradiation of certain wavelengths with the formation of free radicals, including singlet oxygen, which damage bacterial cells.

Based on this, the aim of our investigations was to compare the effectiveness of polyacrylamide (PAA) and dextran-graft-polyacrylamide (D-PAA) hydrogels loaded with silver nanoparticles (AgNPs), antibiotics, and photosensitizers for the treatment of bacterial infection of open wounds.

**Study settings**

PAA and D-PAA-based hydrogels with different loaders (chlorhexidine, methylene blue without (MB) and with light irradiation (MB+L), cefuroxime, and AgNPs) were used. The research was divided into three stages: *in vitro*, *in vivo*, and clinical stage. At each stage, the best variants of hydrogels with loaders were determined and their effectiveness was tested at the next stage.

**Hydrogels**

PAA and D-PAA hydrogels were used; dextran (D) with \( M_w = 20,000 \text{ g/mol} \). The hydrogels were obtained by free radical polymerization using cerium (IV) ammonium nitrate as an initiator in the presence of a cross-linker reagent N,N’-methylene-bis-acrylamide. A detailed method for obtaining the hydrogels was described in our previous work [22]. Cross-linking agent concentration was 0.4 wt%. The hydrogels were washed in distilled water for 48 h to remove unreacted chemicals; they were not toxic to pluripotent cells, fibroblasts, breast and prostate cancer cells.

Samples of the hydrogels measuring 1x1x1 cm (1 g) were used. The washed hydrogels were incubated in 0.1 M AgNO\(_3\) aqueous solution for 24 h. Photochemical reduction of Ag\(^+\) ions into AgNPs in hydrogel matrices was performed using a halogen lamp at a wavelength of 350 nm with a power of 250 watts for 10 min.

MB (0.001%) was used as a photosensitizer; chlorhexidine (0.05%) and cefuroxime (0.1%) were used as an antibacterial compound. The hydrogels were incubated in aqueous solutions of these compounds for 24 h.

**In Vitro Antibacterial Activity**

Wild-type *Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella pneumoniae* strains were isolated from open wounds of patients in the diagnostic laboratory of the O.S. Kolomiychenko Institute of Otolaryngology of National Academy of Medical Science of Ukraine. The obtained strains of microorganisms were tested for antibiotic sensitivity: cefuroxime, cefazolin, ceftriaxone, amoxicillin, azithromycin, amikacin, gentamicin, chloramphenicol, norfloxacin. The isolated strains were sensitive to a list of antibiotics. There was used one antibiotic-resistant *E. coli* strain, partly sensitive only to norfloxacin. The sensitivity of bacteria to the hydrogels was tested by the disc-diffusion method on Mueller-Hinton agar. Samples of the hydrogels with a diameter of 5 mm and a height of 1 mm were used. The Petri dishes were kept at 37°C for 24 h. The diameter of inhibition zone for nanocomposites with AgNPs and antibacterial substances was measured. The hydrogels with MB were kept on agar for 30 min after which they were irradiated by red light at a wavelength of 660 nm (Lika-Led, Cherkasy, Ukraine) at a dose of 20 J/cm\(^2\), light power 0.1 J/s. *In vitro* experiments were repeated three times.

**In Vivo Antibacterial Activity**

A rat model was used to evaluate infected wound treatment. White outbred male rats (240–260 g) were kept in the standard conditions of the vivarium in the O.S. Kolomiychenko Institute of Otolaryngology of the National Academy of Medical Science of Ukraine. The research was divided into three stages: *in vitro*, *in vivo*, and clinical stage. At each stage, the best variants of hydrogels with loaders were determined and their effectiveness was tested at the next stage.
Institute of Otolaryngology of National Academy of Medical Science of Ukraine. For each hydrogel+loader combination, five animals were selected. The experiment was repeated three times. A skin area of 7-8 mm in diameter was removed from the rat’s back. The wound was infected with a mixture of previously obtained wild-type \textit{S. aureus}, \textit{E. coli}, \textit{E. coli res.}, and \textit{K. pneumoniae} strains. The number of bacteria in the suspension was $10^8$ CFU/ml (colony-forming unit/ml). The infected wounds were kept open for 30 min; they were then covered with a standard fabric material (gauze dressing), unloaded hydrogels, and hydrogels with AgNPs, chlorhexidine, cefuroxime, MB. Samples with photosensitizer were incubated on the wound for 30 min and irradiated by red light at a wavelength of 660 nm (Lika-Led, Cherkasy, Ukraine). Irradiation dose was 20 J/cm$^2$, light power was 0.1 J/s. After 24 hours, the dressings were removed, and wound bacteria were sown on selective yolk-salt and Endo-agar media. The wounds were covered again for 24 hours. The condition of the wound and the healing process were monitored visually; the wound diameter was measured on third and fifth days after wound modeling.

**Clinical Research**

Clinical studies were carried out in the hospital of the Ivan Frankivsk National Medical University (Ukraine) according to the type of dependent data, that allowed for excluding the individual characteristics of each patient and evaluating the effectiveness of using the tested hydrogels. The study involved 9 patients with chronic venous insufficiency of the lower extremities and active ulcers in the lower third of the legs. The ulcers were initiated treated using conventional methods (solutions of chlorhexidine, decasan, betadine, furaciline, dressings with antiseptics, antibiotics, and hormonal preparations) with no positive effect.

All the patients were divided into 2 groups: Group 1 (4 patients) – unloaded hydrogel; Group 2 (5 patients) – hydrogel with AgNPs. The ulcers were washed with a Cyteal solution diluted 20 times with 0.9% sodium chloride. The ulcers were covered with hydrogel samples and fixed with a sterile gauze bandage. Bandages were changed every three days for 14 days. The change in ulcer size was not measured due to the individual characteristics of each patient. The healing process was evaluated visually.

**Statistical Analysis**

Statistical data processing was performed using OriginLab 8.0. Normality of variables was evaluated using the Shapiro-Wilk test. The descriptive statistics of bacterial growth inhibition and wound diameter were presented as Mean ± Standard Deviation (M ± SD). The ANOVA method and the Scheffé test were applied to compare the data between groups.

**Results**

**In Vitro Antibacterial Activity**

The obtained wild-type \textit{S. aureus}, \textit{E. coli}, and \textit{K. pneumoniae} strains were sensitive to cefuroxime, cefazolin, ceftiraxone, amoxicillin, azithromycin, amikacin, gentamicin, chloramphenicol, norfloxacin. In addition, antibiotic-resistant strain of \textit{E. coli res.} was isolated, which was partly sensitive to norfloxacin only. The presence of such microorganisms significantly reduced the effectiveness of conventional treatment methods. Bacterial \textit{S. aureus}, \textit{E. coli}, and \textit{K. pneumoniae} strains were sensitive to PAA and D-PAA hydrogels with AgNPs, chlorhexidine and cefuroxime (Table 1).

It was shown that 0.001% MB did not inhibit the growth of microorganisms, but in combination with irradiation by red light at a wavelength of 660 nm (MB+L), it provided an antibacterial effect. Antibiotic-resistant \textit{E. coli res.} was not inhibited by the hydrogels with cefuroxime. In addition, this strain was less sensitive to chlorhexidine and MB+L. The hydrogels with MB were excluded from \textit{in vivo} studies due to a lack of antibacterial activity.

**In Vivo Antibacterial Activity**

Rats’ open wounds were infected with four bacterial strains, including antibiotic-resistant \textit{E. coli res.} Wound microbiota was identified after 24 hours. The relative CFU count of the wounds covered with the hydrogels decreased. There were no differences between PAA and D-PAA hydrogels. The antibacterial properties of the dressing were determined by an antibacterial component loaded into the hydrogel. The following \textit{in vivo} studies were carried out with D-PAA hydrogels, since they are a priority in the preparation of AgNPs and have the best kinetic characteristics of small molecule desorption.

A significant number of \textit{Staphylococcus spp.} CFUs

| Table 1. Sensitivity of wild-type \textit{S. aureus}, \textit{E. coli}, \textit{E. coli res.}, and \textit{K. pneumoniae} strains to the hydrogels with different antibacterial components. |
|-----------------------------------------------|
| **Antibacterial component** | \textit{S. aureus} | \textit{E. coli} | \textit{E. coli res.} | \textit{K. pneumoniae} |
| | PAA | D-PAA | PAA | D-PAA | PAA | D-PAA | PAA | D-PAA |
| Control (unloaded hydrogel) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| AgNPs | 22±2 | 26±2 | 20±1 | 22±3 | 19±2 | 23±3 | 21±2 | 23±2 |
| Chlorhexidine | 15±1 | 19±2 | 21±2 | 22±2 | 17±2 | 19±1 | 19±1 | 22±1 |
| Cefuroxime | 26±2 | 25±3 | 23±2 | 25±3 | 0 | 0 | 24±1 | 23±2 |
| MB | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| MB+L | 14±1 | 13±2 | 16±2 | 14±2 | 12±2 | 11±3 | 17±2 | 14±2 |
Figure 1. Isolation of *Staphylococcus* spp. on yolk-salt agar after removing wound bandages: gauze, unloaded hydrogel (D-PAA), hydrogels with chlorhexidine, AgNPs, cefuroxime (CFR), methylene blue with light absorption at 660 nm (MB+L).

Figure 2. Isolation of *E. coli* and *K. pneumoniae* on Endo-agar after removing wound bandages: gauze, unloaded hydrogel (D-PAA), hydrogels with chlorhexidine, AgNPs, cefuroxime (CFR), methylene blue with light absorption at 660 nm (MB+L).

Figure 3. Healing process of artificially infected rat wounds using different materials: gauze bandage (1), unloaded D-PAA hydrogel (2), D-PAA hydrogels with cefuroxime (3), chlorhexidine (4), MB+L (5), AgNPs (6). A - bandaging, B - 3 days after wound induction, C - 5 days after wound induction.

were isolated from the wounds covered with gauze bandages (Fig. 1).

The vast majority of colonies belonged to the genus *Staphylococcus*, including *S. aureus*, as well as there was a slight presence of *E. coli*, including *E. coli* res. and *K. pneumoniae* (Fig. 1, 2).

Far fewer *Staphylococcus* spp. CFUs were found in the wounds covered with unloaded hydrogel and hydrogel with MB+L. However, CFUs of all four strains were identified. In cases of using the cefuroxime-loaded hydrogels, there were detected a few CFUs of *Staphylococcus* spp. and *E. coli* res. in the wounds. A slight presence of *Staphylococcus* spp. CFUs was detected when using the hydrogels with AgNPs and chlorhexidine. Thus, the hydrogels with AgNPs, chlorhexidine, and MB+L reduced CFU count in the infected wounds, including *E. coli* res.

Fig. 3 shows the healing process of the infected wounds using different dressings. No significant changes in wound size were detected 24 hours after wound induction.

After 3 days, the size of the wounds covered with gauze bandage did not change significantly, and after 5 days, it reduced twice (Table 2).

The use of unloaded hydrogels helped reduce the size of the wound by 28.6% and 42.8% three and five days after wound modeling, respectively. Similar results were obtained for the hydrogels loaded with cefuroxime, chlorhexidine, and MB+L. The use of the hydrogels with AgNPs helped reduce wound size by 50% and 62.5% three and five days after wound induction, respectively. Black and brown spots of silver oxide (Ag$_2$O) and AgNPs were found at the contact point of the nanocomposite and the skin. Thus, D-PAA hydrogels with AgNPs have greater antibacterial potential. The use of dextran in the basis of the copoly-
Table 2. Wound size after covering them with the hydrogels containing different antibacterial components.

| Variant               | Wound diameter, mm (M±SD) | Initial size | 1 day | 3 days | 5 days |
|-----------------------|---------------------------|--------------|-------|--------|--------|
| Gauze bandage         | 8±1                       | 8±1          | 7±1   | 4±2*   |        |
| Unloaded D-PAA        | 7±2                       | 7±2          | 5±2   | 4±1*   |        |
| D-PAA/cefuroxime      | 7±1                       | 7±1          | 4±2*  | 4±2*   |        |
| D-PAA/chlorhexidine   | 8±1                       | 8±1          | 6±1   | 4±2*   |        |
| D-PAA/MB+L            | 8±1                       | 7±1          | 5±2*  | 4±1*   |        |
| D-PAA/AgNPs           | 8±2                       | 8±1          | 4±1*  | 3±1*   |        |

Note: *p<0.05 relative to the initial size

mer structure allows for controlling the physicochemical properties of the final hydrogel. The multiple mechanisms of AgNP antibacterial action, the reduction in inflammation [23], and the ability to control their size and quantity by changing the polymer structure potentially allows for using D-PAA/AgNPs composites to prevent and treat bacterial infection of wounds. At the next stage, D-PAA and D-PAA/AgNPs hydrogels were chosen for clinical investigation.

Clinical Investigations

Patients noted pain and discomfort relief after applying unloaded hydrogels and hydrogels with AgNPs to their ulcers. Marginal epithelialization and pink granulation of the ulcers was detected on the eighth day after the start of treatment for D-PAA hydrogels and on the sixth day for D-PAA/AgNPs hydrogels.

Smaller ulcers (about 20-30 mm) were almost completely epithelialized on the 14th day after applying D-PAA/AgNPs. For D-PAA, such changes were detected 16-18 days after the start of treatment. The size of larger ulcers (up to 40-50 mm) remained almost unchanged during treatment with the hydrogels but similar changes in granulation and epithelialization were registered as well. The ulcers were covered with dry crusts 14 days after hydrogel application. After using D-PAA/AgNPs hydrogels, a dark coating was found on the surface and edges of the ulcers. Similar changes were recorded on the wounds during in vivo stage. These were the consequences of silver deposition after contact with atmospheric oxygen.

For example, the dynamic process of patient’s ulcer healing after using D-PAA/AgNPs hydrogels is shown.

Case Report

A 73-year-old female patient B. suffered from iliac vein thrombosis 16 years ago. She developed chronic venous insufficiency of both lower extremities, with periodically appearing ulcers on the legs and feet. For treatment, various methods were used, which were often accompanied by allergies. Then ulcers, growing and painful, appeared simultaneously on both lower extremities (Fig. 4, 5).

Pain relief was noted when applying the bandages. After their removal, dark fragments of gel with silver remained on the tissues. After 14 days, lateral left foot ulcer epithelialized; on the right leg, ulcer size reduced by half. The patient is active.

Discussion

Hydrogels contain a lot of water, which allows for creating the necessary conditions for tissue regeneration [24].

PAA and D-PAA hydrogels differ in structure and sorption characteristic [25]. The 3D-structure of polymers determines the kinetic and capacitive properties of hydrogels, as well as the size and number of AgNPs [26]. There are

Figure 4. Lateral left foot ulcers. A – ulcers on admission, B – D-PAA/AgNPs hydrogel bandage, C – 8 days after bandage application, D – 14 days after bandage application.

Figure 5. Inner right leg ulcers. A – ulcers on admission, B – D-PAA/AgNPs hydrogel bandage, C – 8 days after bandage application, D – 14 days after bandage application.
many hydrophilic polymers that form hydrogels. Some of the materials, including those based on polyacrylamide, find industrial application in medicine [27].

Unloaded hydrogels, with some exceptions, do not have their own antibacterial activity. The determining factor of such materials is their ability to absorb, retain, and release biologically active substances. Materials with antibacterial components significantly accelerate wound healing [17]. Preventing bacterial growth in wounds significantly reduces the risk of serious complications, including sepsis. Our results demonstrate the antibacterial effect of PAA and D-PAA hydrogels with chlorhexidine, cefuroxime, MB+L, and AgNPs against gram-positive and gram-negative strains of microorganisms. However, antibiotic-resistant strains of microorganisms are becoming more common. In this case, the hydrogels with antibiotics were not effective. Unlike antibiotics, photosensitizers and metal nanoparticles act on several physiologically important targets for bacterial cells [21, 28]. In the presence of protons, AgNPs dissociate to form Ag\(^+\) [29]. AgNPs can penetrate bacterial cells or disrupt cell wall integrity. This mechanism prevents the rapid adaptation of microorganisms to their effect. Ag\(^+\) ions bind to bacterial cell components, disrupting their functions.

MB dissociates with the formation of free radicals when irradiated by red light with a wavelength of 660 nm. They disrupt the normal course of biochemical reactions and promote the accumulation of toxic products in bacterial cells [30–32]. Low concentrations of MB are less toxic to eukaryotic cells. Thus, the application of hydrogels in combination with MB+L can be used to treat open wounds.

Staphylococcus spp. were detected in wounds covered with various materials. We assume that these are representatives of the normal microflora of rat skin, but under certain conditions they can also interfere with normal wound healing [33]. The use of antibacterial hydrogels significantly reduces the presence of bacteria.

The hydrogels with AgNPs accelerate ulcer healing in patients with circulatory disorders. This is due to the physicochemical properties of the hydrogel, which forms the necessary microenvironment for tissue regeneration. Ag\(^+\) ions dissociated from AgNPs contribute to the precipitation of tissue breakdown products (e.g., proteins), reducing the load on the immune system and inflammation [23]. This is evidenced by pain relief reported by patients after the use of the hydrogels with AgNPs.

The density of the polymer network of hydrogels does not contribute to the sorption of large molecules of proteins, glycoproteins, etc. Therefore, the hydrogel itself cannot clean the wound, it only creates the necessary microenvironment for tissue regeneration. Saturation with antibacterial substances such as chlorhexidine or MB+L provides the material with antibacterial properties.

Thus, D-PAA/AgNPs are the best option for treating ulcers due to their ability to control the properties of the hydrogels and nanoparticles, as well as multiple mechanisms of antibacterial action, influence the inflammatory processes and clean up wounds from tissue breakdown products.

**Conclusions**

PAA and D-PAA hydrogels can be loaded with antibacterial compounds of various types. The type of polymer does not affect the antibacterial properties of the final hydrogels. The hydrogels with chlorhexidine and MB+L can be potentially used to treat bacterial contamination of wounds and ulcers. Nevertheless, their disadvantage is the inability to absorb or precipitate tissue breakdown products that interfere with normal regeneration and inflammation. D-PAA/AgNPs are the best option for treating ulcers due to the ability to control the properties of the hydrogels and nanoparticles, as well as multiple mechanisms of antibacterial action.

**Ethical Statement**

All in vivo experiments were carried out according to the Law of Ukraine “On the Protection of Animals from Cruelty” and the European Convention for the Protection of Vertebrate Animals Used for Research and Other Scientific Purposes. The design of the study was approved by the Ethics Commission of the Ivano-Frankivsk National Medical University (Decision No. 103, dated September 20, 2018). Written permission was obtained from each patient. The research was carried out according to the WMA Declaration of Helsinki “Ethical Principles for Medical Research Involving Human Subjects”.

**Informed Consent**

All patients who participated in the study signed a voluntary informed consent to participate.

**Conflict of Interest**

The authors declare that no conflicts exist.

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