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Antimicrobial activity of new materials based on the blends of collagen/chitosan/hyaluronic acid with gentamicin sulfate addition

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ABSTRACT

In this study polymeric blends based on collagen, chitosan and hyaluronic acid in the form of thin films with the addition of gentamicin sulfate were obtained. Surface morphology of films was evaluated based on atomic force microscopy images. Surface free energy was measured and its polar and dispersive components were calculated. Moreover, oxygen and water vapor permeability through the material were measured as well as the water content in materials was studied. Thermal stability was determined by differential scanning calorimetry. Microbiological tests were performed to evaluate the diffusion of the drug from matrixes. The results showed that thin films based on collagen, chitosan and hyaluronic acid enriched in gentamicin sulfate inhibit the growth of both, Gram negative bacteria (*E. coli* and *P. aeruginosa*) and Gram positive ones (*S. aureus*).

1. Introduction

Skin is one of the most important organs in a vertebrate's organism, the area occupied by skin is ca. 2 m^2 and by weight amounts one-tenth of body mass [1]. This organ takes part in homeostasis, protects the organism from environmental factors including the prevention of invasion by microorganisms [2]. Many types of trauma like burns, cuts or even surgical procedures can cause the loss of skin integrality [3]. For many ages wound dressings were applied to support wound healing. Traditionally, they were made from materials which absorb fluids. Such materials can adhere to the desiccated wound surface and cause trauma during wound dressing removal [4]. Moreover, a place of trauma is prone to microbiological infection. Antimicrobial topical drugs need to be applied 1–2 times per day to reduce infection. This has some disadvantages, changing the wound dressing may cause patients discomfort and result in a greater workload for nurses [5]. Novel wound dressing materials should provide a moist environment around the wound to promote the healing process by keeping wound fluids and the growth factor in contact with the wound [4,6]. Nowadays, wound dressing materials can be enriched with active agents such as polyphenols [7], essentials oils [8], drugs [9] or metal nanoparticles [10].

Drug release from various matrixes is currently one of the most rapidly advancing areas of science. Chemists, chemical engineers and doctors are contributing to human health care [11]. Those advantages have led to the development of drug delivery systems. Comparing them to conventional dosage forms have numerous advantages including improved efficiency, reduced toxicity and improved patient compliance [12]. Depending on the application of the drug delivery systems, degradable or non-degradable matrixes can be used [13]. Natural and some synthetic polymers such as drug delivery matrixes offer biocompatibility which is beneficial for their use as biomaterials. Materials with the drug addition can be obtained as 3D porous forms or thin films depending on their potential application. The release of drugs occurs firstly from the matrix surface and then from the inert parts as a result of the swelling properties of the material. Water molecules flow into the 3D structure and the drug is released from the inert parts of the matrix. This release forms thin films which occurs during the dissolution of the polymeric layer. The concentration of released drugs can be manipulated by the use of various matrixes contents [14].

Gentamicin is an aminoglycoside antibiotic, commonly used for treating both bone and soft tissue infections [15]. Gentamicin can be used against serious infection especially caused by Gram – negative bacteria strain [16]. The mechanism of action is blocking synthesis of bacteria proteins [17] via electrostatic binding with negatively charged phospholipids' head groups [18]. Gentamicin is bonded to specific ribosomal proteins leading to the creation of nonfunctional complexes resulting in mRNA misreading [19].

Collagen, chitosan and hyaluronic acid are promising for medical application because of ability of film forming structure, bioactivity, biocompatibility and biodegradability [20]. Collagen is a protein

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naturally occurs in human body, as component of dressings is able to interacts with cells and regulates cell anchorage, migration, proliferation and survival [21]. Moreover, collagen is able to promote tissue regeneration. Hyaluronic acids with collagen are components of extracellular matrix (ECM). Hyaluronic acid provides a moist environment and protects the wounded tissue surface from dryness and encourages healing. Moreover, it enhances collagen secretion at the wound site by fibroblast proliferation and have an optimistic effect in scarless wound healing [22]. Chitosan is a linear polysaccharide obtained during deacetylation of chitin. Chitosan used as component of wound dressing can prevent wound dehydration, can accelerate tissue regenerate as well as can inhibit infection by stimulating the macrophages to release cytokines [23].

The aim of the study was to obtain polymeric blends based on chitosan, collagen and hyaluronic acid in the form of thin films with the addition of gentamicin sulfate. The polymers ratios were determined by miscibility studies [24]. Various analyses were made and several properties of materials were characterized. Moreover, microbiological tests were performed to evaluate the antimicrobial activity of the obtained films with added gentamicin.

2. Materials and methods

2.1. Sample preparation

Collagen (Coll) was extracted in our laboratory from rat tail tendons [25]. Chitosan, hyaluronic acid and gentamicin sulfate were purchased from the Sigma-Aldrich Company, Poland. Chitosan (CT) powder (degree of deacetylation DD = 77% $M_v = 5.4 \times 10^5$ g/mol) was used without further purification. Hyaluronic acid (HA) powder with a viscosity average molecular weight of 1.8 × 106 g/mol was used [24]. Chitosan and collagen were prepared as 1% (w/w) concentration solution in 0.1 M acetic acid separately. Hyaluronic acid was prepared as 1% (w/w) concentration solution in 0.1 M hydrochloric acid. Chitosan and collagen mixtures were prepared by mixing the two solutions in different weight ratios 50/50 and hyaluronic acid was added in ratio 1, 2 and 5% (w/w). During the mixing on a magnetic stirrer, gentamicin sulfate (Gen) was added in ratio 0.4 mg per 1 cm^2 of film with the thickness 0.011–0.013 mm. The homogenized solutions were split on the glass plate and put in the incubator at 37 °C for the solvent evaporation. Films without gentamicin sulfate were also obtained as control samples.

2.2. Atomic force microscopy (AFM)

Topographic images were obtained using a multimode scanning probe microscope with a Nanoscope IIIa controller (Digital Instruments, Santa Barbara, CA) operating in the tapping mode, in air, at room temperature. Surface images were acquired at fixed resolution (512 × 512 data points) with a scan rate of 1.97 Hz. Silicon tips with spring constant 2–10 N/m were used. Roughness parameter such as the root mean square (R_q) and roughness average (R_a) were calculated from 1 μm × 1 μm scanned area using Nanoscope software.

2.3. Contact angle measurement

Surface free energy (S), its polar (P) and dispersive (D) components can be calculated by the contact angle measurement in which non-covalent forces between the liquid and film surface are formed by Owens-Wendt method [26]. The contact angles of two liquids: glycerol and diiodomethane were measured at a constant temperature using a goniometer equipped with a system of drop shape analysis (DSA 10 Control Unit, Krüss, Germany).

2.4. Water content

The water content of the film was determined by drying samples in an oven at 105 °C until constant weight. Results were expressed as a percentage of water content in a dry sample weight.

2.5. Differential scanning calorimetry (DSC)

Differential scanning calorimetry measurements were made by DSC equipment (NETZSCH Phoenix DSC 204 F1). Heating rate was 10 °C/min, from 20 to 250 °C in nitrogen atmosphere with flow 40 ml/min. The weight of samples was 1.0–1.5 mg and was measured using aluminum measuring pans.

2.6. Water vapor permeation rate (WVPR)

The WVPR of films was investigated using the method described by Phaechamud et al. with slight modifications [6]. Dried calcium chloride (with known weight) as a desiccant was placed in to the plastic container (diameter of 40 mm). The desiccant was prepared by drying it at 105 °C for 24 h before use. Films were placed on top of containers and sealed tightly. Each kind of sample was tested in duplicate. Containers with calcium chloride without a cover were left as control samples. After 24 h, films were removed and the weight gain was determined.

2.7. Oxygen permeability

The oxygen permeability of polymer films was evaluated by measuring the dissolved oxygen in the distilled water [4]. Deionized water was boiled 10 min to remove dissolved oxygen. A plastic container, with a diameter of 4 cm, was filled with 50 ml of oxygen free deionized water. Polymer film was sealed tightly by tape and parafilm on top of the container. Control samples were not covered by film. Samples were left in room condition for 24 h. Each kind of sample was evaluated in triplicate. The dissolved oxygen was measured using Winkler's method [4]. Results are presented as a percent of detainee oxygen by film compared to uncovered samples.

2.8. Antimicrobial activity

The antimicrobial activity of polymeric films with gentamicin was tested against three bacterial species: *Staphylococcus aureus* ATCC6538, *Escherichia coli* ATCC8739 and *Pseudomonas aeruginosa* ATCC15442 by using diffusion method. Bacterial liquid culture was prepared in a medium containing bacteriological peptone (5 g/l) and yeast extract (3 g/l). After incubation overnight at 37 °C the bacterial cultures were centrifuged. Bacterial cells pellet were suspended in saline salt to obtain optical density 0.5 in McFarland scale. Inoculum were spread on agar plate with solid nutrient agar (bacteriological pepton - 5 g/l, yeast extract – 3 g/l, agar 15 g/l). Each kind of film was cut into 1 × 1 cm pieces and placed on top of an agar plate. Then, samples were placed into a refrigerator at 4 °C for 2 h to allow the diffusion of gentamicin from matrixes. After 2 h samples were incubated for 24 h at 37 °C. After incubation inhibition zones without bacteria growth on the agar plates were measured. Each sample was tested in duplicate. Samples without gentamicin addition were tested as ~~a~~ control samples.

3. Results and discussion

3.1. Atomic force microscopy (AFM)

The roughness of surface is an important parameter in considering the suitability of biomaterial for use in tissue engineering [27,28]. It has been reported that surface roughness has an influence on the cells attachment on the polymeric thin films [29]. The smooth surface allow for the biofilm formation instead of cell adhesion. It is necessary to get