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Short Communication

Lignin model compound in alginate hydrogel: a strong antimicrobial agent with high potential in wound treatment

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ABSTRACT

Nowadays bacterial resistance to known antibiotics is a serious health problem. In order to achieve more efficient treatment, lately there is an effort to find new substances, such as certain biomaterials, that are non-toxic to humans with antibiotic potential. Lignins and lignin-derived compounds have been proposed to be good candidates for use in medicine and health maintenance. In this study, the antibacterial activity of the lignin model polymer dehydrogenate polymer (DHP) in alginate hydrogel (Alg) was studied. The obtained results show that DHP–Alg has strong antimicrobial activity against several bacterial strains and biofilms and does not have a toxic effect on human epithelial cells. These results strongly suggest its application as a wound healing agent or as an adjunct substance for wound treatments.

1. Introduction

Lignins, the second most abundant organic compound on the Earth's surface, provide mechanical support to plant tissues as well as protection from physical/chemical stress and disease resistance. In plants, lignin is produced by oxidative coupling of three phenolic alcohols/acids (coniferyl, p-coumaryl and synapyl) catalysed by the enzyme peroxidase [1]. The enzymatically synthesised lignin model compound dehydrogenate polymer (DHP) from coniferyl alcohol is the best lignin substitute used in various experiments [2]. Lignins and lignin-derived compounds have been proposed to be good candidates for use in medicine and health maintenance [3,4]. The antibacterial properties of different kinds of lignin (organosolv, sulphite, kraft) have already been reported [5,6].

The aim of this study was to observe the effects of DHP, synthesised from coniferyl alcohol, on different bacterial strains and bacterial biofilms both cultured in laboratory conditions as well as wild strains isolated from patients' wounds. In addition, its wound healing effect was tested in vivo on sterile wounds induced on rat skin. DHP was applied in the form of a suspension as well as in a gel-like structure obtained by mixing it with sodium alginate (Alg) and adding calcium salt.

2. Materials and methods

2.1. Synthesis of dehydrogenate polymer and preparation of suspension and gel

The DHP of coniferyl alcohol (Sigma-Aldrich, St. Louis, MO) was synthesised according to the procedure described by Radotíc et al [7,8]. The obtained polymer was air-dried and was dissolved in dimethyl sulphoxide (DMSO).

Sodium alginate (Sigma) (0.2 g) was dissolved in 9.5 mL of distilled water. Subsequently, 0.5 mL of DHP in DMSO was added, giving

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a final concentration of 1% (w/v) DHP. Solutions were mixed, giving a milky suspension. This suspension was further diluted to obtain different concentrations. $CaCl_2$ at a final concentration of 0.5% (w/v) was added to make the gel form.

2.2. Micro-organisms

The Gram-negative bacteria *Enterobacter cloacae* human isolate, *Escherichia coli* ATCC 35210, *Pseudomonas aeruginosa* ATCC 27853 and *Salmonella enterica* serovar Typhimurium ATCC 13311 as well as the Gram-positive bacteria *Bacillus cereus* clinical isolate, *Listeria monocytogenes* NCTC 7973, *Micrococcus flavus* ATCC 10240 and *Staphylococcus aureus* ATCC 6538 were used. The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research 'Siniša Stanković', University of Belgrade (Belgrade, Serbia). Another five isolates, including three *S. aureus* (S.a. #1, S.a. #2 and S.a. #3), *P. aeruginosa* P.a. #1 and *Serratia* sp., were human isolates obtained from patients at the Department of Microbiology, Clinical Center of Serbia (Belgrade, Serbia). Bacteria were cultured on Mueller–Hinton agar (Merck, Darmstadt, Germany) at 37 °C for 24 h.

2.3. Antibacterial activity

The dose dependence of DHP was determined (Supplementary Table S1) and the stock suspension containing 10 mg/mL DHP and 20 mg/mL Alg was used for bacterial treatments. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by the microdilution method [9,10]. The compound for testing was added in tryptic soy broth medium (100 μL) with bacterial inoculum (1.0×10^5 CFU/well) to achieve the required concentration plus Ca^{2+} to a final concentration of 0.5%. Antibacterial activity was determined in regard to the DHP concentration (mg/mL) in the microplate wells. Microplates were incubated in a rotary shaker (160 rpm) for 24 h at 37 °C. MICs obtained from the susceptibility testing of various bacteria to the tested compound DHP–Alg were also determined by a colorimetric microbial viability assay based on reduction of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium (INT) colour and compared with the positive control for each bacterial strain [9,10]. The lowest concentration with no visible growth (at the binocular microscope) was defined as the concentration that completely inhibited bacterial growth (the MIC). MBCs were determined by serial subcultivation of 2 μL into microtitre plates containing 100 μL of broth per well and further incubation for 24 h. The lowest concentration with no visible growth was defined as the MBC, indicating 99.5% killing of the original inoculum. The optical density at 655 nm of each well was measured using Microplate Manager® 4.0 (Bio-Rad Laboratories, Hercules, CA) and was compared with a blank and the positive control. The antibiotics streptomycin (Sigma) and ampicillin (Panfarma, Belgrade, Serbia) were used as positive controls (1 mg/mL in sterile physiological saline).

2.4. Quantification of biofilm formation and effect of DHP–Alg on established biofilms

Five clinical isolates (three *S. aureus*, *P. aeruginosa* and *Serratia* sp.) were chosen for biofilm investigation experiments. Each isolate was characterised for biofilm-related properties as reported previously [11,12].

Biofilm biomass was quantified using a modification of a methodology described by Stepanovíc et al [13]. Briefly, 200 μL of an overnight bacterial suspension containing 1×10^8 CFU/mL was added to the wells of a sterile 96-well flat-bottomed microtitre plate (Sarstedt, Nümbrecht, Germany). Negative controls were represented by wells containing only 200 μL of the medium. *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853, known to form fully established biofilms, were added to each plate as a reference standard [14] and positive control, respectively. Incubation was performed at 37 °C for 24 h. Subsequently, the liquid was aspirated, the wells were washed and sessile cells were stained with crystal violet [15]. The absorbance was read at 620 nm [16]. The following classification was used: biofilm non-former (Doa ≤ Docn); weak biofilm-former (Docn < Doa ≤ 2 × Docn); moderate biofilm-former (2 × Docn < Doa ≤ 4 × Docn); and strong biofilm-former (4 × Docn < Doa); where Doa is the optical density of the biofilm and Docn is the optical density of the negative growth control. To evaluate the susceptibility of the bacterial biofilms to DHP–Alg, 200 μL of bacterial suspension was added to a flat-bottomed 96-well polystyrene microplate and was incubated at 37 °C for 48 h to allow biofilm formation. After washing the plates, DHP–Alg solution (200 μL) was added to the wells at concentrations ranging from 0.08 to 9 mg/mL. Ampicillin and streptomycin were used as positive controls. After 24 h at 37 °C, biofilm reduction was determined by following the staining process [10] and measuring ultraviolet absorbance at 620 nm. The MIC was defined as the minimum concentration of antimicrobial agent that inhibited further growth of the initial biofilm, and the MBC was defined as the concentration that resulted in a level of luminescence representing no bacterial growth (empty well).

2.5. Cytotoxicity testing

The cytotoxicity of DHP–Alg was assessed on human cervix carcinoma cells (Hep2c) and human conjunctival epithelial cells (HCjE) (kindly provided by Prof. Ilene Gipson, Schepens Eye Research Institute, Harvard Medical School, Boston, MA). Cells were seeded at a density of 20,000 cells/well (100 μL/well) in 96-well flat-bottomed tissue culture plates (Nunc, Roskilde, Denmark) and were incubated for 24 h with DHP–Alg at 37 °C in 5% CO_2. A stock solution of 10 mg/mL DHP–Alg in 5% DMSO was used for preparation of the starting solutions, each containing 200 μg/mL DHP–Alg filtered through 0.22-μm pore-size filters and further used for the preparation of double serial dilution samples. Upon addition of DHP–Alg-containing solutions (100 μL/well) to cell cultures, cells were incubated for an additional 48 h at 37 °C in 5% CO_2. Cells grown in the appropriate medium only were used as a reference. Preliminary experiments showed that DMSO at the concentration range used in experiment (<0.05%) had no impact on Hep2c and HCjE cell viability.

Viability of the Hep2c and HCjE cells was determined using a standard Cell Counting Kit-8 (a solution of WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt]) (Sigma). The rate of WST-8 transformation positively correlates with cell viability. Briefly, 10 μL/well of Cell Counting Kit-8 solution was added into the wells and 4 h later the absorbance was measured at 450/650 nm.

2.6. In vivo test of DHP–Alg on wounds and histological/stereological analysis

The methodology of the in vivo studies and the related histological analyses are presented in the Supplementary material.

2.7. Statistical analysis

In the antimicrobial activity tests, two samples for each species were used and all of the assays were carried out in duplicate. The results were analysed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$ and were expressed as the mean and standard error. This analysis was carried out using IBM SPSS Statistics for Windows v.22.0 (IBM Corp., Armonk, NY).