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Biochanin A formulation with electrospun poly(vinylpyrrolidone) fibers and possible applications

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Abstract: The aim of this paper is to examine the possibility of using electrospun poly(vinylpyrrolidone) (PVP) fibers as a carrier of the phytoestrogen biochanin A (BCA). PVP fibers were prepared with different BCA content by using electrospinning method at specific process parameters. Produced electrospun PVP––BCA fibers were characterized by chemical, physical-mechanical and biological methods. SEM, DSC and FTIR analyzes showed that there are no strong interactions between PVP and BCA molecules, neither thermal changes in tested temperature range (50–250 °C) and that equal distribution of BCA in the PVP electrospun fibers was achieved. Physico-mechanical tests showed that the physical properties and wetting angle of PVP change in the presence of BCA. Testing of electrospun PVP fibres with and without BCA on L929 fibroblasts in direct contact assay *in vitro* revealed a significant effect on proliferation and migration of fibroblasts. Biological tests confirmed that the system of electrospun PVP–BCA fibers can become suitable for the treatment of complicated wounds, where in the first stage of treatment the damaged tissue should be removed by the activity of electrospun PVP–BCA fibers. Another possibility of using electrospun PVP––BCA fibers is for the treatment of facial skin for the exfoliation intent.

Keywords: polymer; drug delivery; carriers; electrospinning; phytoestrogen.

INTRODUCTION

In this paper, the continuation of the research of the system with controlled release of biochanin A from the carrier based on electrospun polymer fibers is presented. The research on the production, characterization and release of biochanin A from electrospun poly(lactide) fibers was published in previous work.[1]

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Biochanin A (5,7-dihydroxy-3-(4-methoxyphenyl)chromen-4-one) belongs to the group of isoflavones, which due to its structural similarity to the female sex hormone 17-β-estradiol, have an effect as a phytoestrogen. Phytoestrogens are natural selective modulators of estrogen receptors, which can achieve both agonistic (estrogenic) and antagonistic (antiestrogenic) effects, depending on the concentration and the target site of action.[2] The mechanism of action of biochanin A includes its binding to other receptors, such as PPARγ, as well as the modification of some signaling pathways, such as NF-κB and MAPK. The most valuable natural sources of biochanin A are plants from the leguminous family, such as red clover, soybeans, chickpeas, alfalfa and peanuts.[3]

Biochanin A has numerous pharmacological activities, such as anticancer, antioxidant, anti-inflammatory, antidiabetic, antimicrobial, hepatoprotective and neuroprotective activity.[3,4] In addition to systemic application, biochanin A can also be used locally in the treatment of various skin inflammations, hyperpigmentation and wound healing.[5–7]

Despite numerous positive effects on human health, the therapeutical application of biochanin A is limited by its low solubility in water and physiological mediums, pronounced liver first-pass effect and with all that associated low bioavailability. The solutions for solving the mentioned limitations are the application of lyophilized and non-lyophilized pH- and temperature-sensitive copolymer hydrogel poly(*N*-isopropylacrylamide-co-acrylic acid) as a carrier for the modified release of biochanin A,[8] complexation of biochanin A with cyclodextrins[9] or by producing electrospun fibers based on poly(lactide).[1]

Electrospinning is an effective and simple method of producing structured polymer fibers whose diameter can vary from micrometer to nanometer sizes. The specific structure of polymer fibers, made like this, produce a large surface-to--volume ratio and high porosity. The morphology and size of the electrospun fibers depend on the properties of the polymer solution (relative molecular weight and concentration of the polymer, that is solution viscosity, conductivity, surface tension and polarity of the solvent), process parameters (voltage, solution flow rate and distance of the needle from the collector), as well as on the environment conditions (temperature, pressure and humidity). Polymers used for the production of electrospun fibers can be of natural or synthetic origin, and their mutual combinations or combinations with inorganic substances can also be used.[10,11] In biomedicine, electrospun fibers can be used for enzyme immobilization, wound treatment and tissue engineering, but also as carriers in drug delivery systems for oral, transdermal, ocular, nasal, rectal and vaginal application.[10,12]

By using electrospun nanofibers, all kind of profiles of active substance release can be achieved - immediate, continuous, delayed, release on demand, multiphase, but also the release of several active substances at the same time.[1,13] Nevertheless, the rapid release of poorly soluble active substances from electrospun nanofibers

rises a particular interest, because the dissolution of poorly soluble substances is a major challenge for the pharmaceutical industry.

Poly(vinylpyrrolidone), PVP, is obtained by polymerization of *N*-vinylpyrrolidone monomers. The amphiphilicity of PVP comes from the presence of two different functional groups in the structure, a polar lactam group and a non-polar part of methylene. PVP is used in both conventional and modern drug delivery systems due to its solubility, availability, ability to form films, complex forming ability, solubilizing, binding, stabilizing, suspending and thickening capabilities.[12] PVP is an important synthetic polymer which has: good adhesion and complexation properties, low toxicity, high hydrophilicity, biodegradability, biocompatibility and good solubility in water and various organic solvents.[11] Due to the extraordinary properties of PVP and electrospinning itself, electrospun fibers based on PVP are often used in the production of carriers for drug delivery of various active substances.

Formulations with fast-dissolving polymers such as PVP have more importance in recent years. Namely, such formulations can dissolve or disintegrate within a few seconds or several minutes in contact with a wet surface, which enables their application without liquids or chewing. Fast release leads to quick start of effect, and thus the bioavailability of the incorporated active substances can be increased. This kind of formulations can be particularly useful in immobile patients, the elderly and children, in the treatment of sore throat and oral ulcers.[14]

Ultrafine PVP K30 fibers are made by electrospinning with ibuprofen as an active substance. The results of physicochemical testing showed a good compatibility of ibuprofen and polymer, as well as that ibuprofen in the fibers is in an amorphous form. An in vitro dissolution test showed that the fibers dissolve within 10 s by a controlled dissolution mechanism of polymers.[15] Fast-dissolving membranes for the delivery of poorly water-soluble drugs were prepared by electrospinning, whereas PVP was used as a polymer matrix and feruloyl-oleyl-glycerol as a model substance. By using PVP in a concentration of 5 % and a voltage of 14 kV, uniform, smooth fibers with a diameter of 700–800 nm were obtained. The rapid dissolution of these fibers, with an average dissolution time of 2±1.5 s was confirmed by wetting time assays.[16] Electrospun fibers based on PVP were used as a carrier for poorly soluble cholecalciferol (vitamin D3). The diameter of the electrospun fibers of PVP with cholecalciferol was 0.2–2.9 μm. The amount of released cholecalciferol within the first 20 s was 82.1 %, *i.e.*, 51.9 % from fibers in which the ratio of the remedy and PVP was 1:4, *i.e.*, 1:2.[17]

The antibacterial electrospun nanofibers for oral use are made of PVP K90 polymer with propolis extract (5 %). Smooth and uniform fibers were obtained, which increase the solubility of propolis in water while simultaneously reduce the adhesion of bacteria to smooth surfaces, thus increasing the antibacterial activity of propolis.[18] Fast-dissolving formulations of electrospun PVP nanofibers with

ornidazole for the treatment of gingivitis were developed. The mechanical and mucoadhesive tests showed that the optimal formulation contains 15 % of PVP nanofibers. The release of ornidazole from the electrospun PVP fibers formulation was more efficient than the gel and solution formulations with ornidazole, with the complete amount of ornidazole released in 5 min.[19]

Due to its biocompatibility, non-toxicity, hydrophilicity and bioadhesiveness, PVP K90 was used as a carrier for the preparation of sublingual fast-release carvedilol formulations. The produced nanofibers had a smooth, cylindrical, crosslinked structure, and the average diameter was 745±57 nm. The release and permeability of the drug were significantly higher compared to the just common physical mixture. The results of the *in vitro* release test showed that up to 80 % of carvedilol is released within 30 min, which is contributed by the amorphous structure of the drug in the electrospun nanofibers, the hydrophilicity of PVP and the large surface area of the fibers.[13] By fitting the inclusion complex of resveratrol with hydroxypropyl-*β*-cyclodextrin into electrospun PVP nano-fibers, the solubility of resveratrol was significantly increased. In addition, the antioxidant activity of resveratrol was increased, probably as a result of increased solubility. Also, its penetration through the stratum corneum into the deeper layers of the skin is increased, and its anti-inflammatory effect has been proved (reduction of the expression of inflammatory proteins COX-2 and MMP-9 in keratinocytes).[20]

Electrospun fibers based on poly(caprolactone) (PCL) and PVP in a ratio of 70:30 were used as a carrier for trans-anethole and its effect on osteogenesis was tested. The diameter of the fibers prepared like this was 242±36 nm. The addition of *trans*-anethole did not affect the diameter of fibers, nor the swelling properties, protein adsorption, degradation and biomineralization of the fibers. Continuous release of *trans*-anethole from nanofibers was achieved and its effect on osteoblasts differentiation at the cellular and molecular level was proven. The release of *trans*-anethole from the nanofibers was monitored for 25 days, after which 44.37, 49.72 and 60.23 % was released from the fibers with 5, 10 and 20 μM *trans*-anethole, respectively. The starting faster release of *trans*-anethole due to PVP erosion is followed by a slower release by Fickian diffusion which release rate is directly proportional to the concentration gradient.[21]

The objective of this work is to develop a formulation based on PVP fibers with a different biochanin A content by the using electrospinning method in order to perform characterization and its effect on wound healing and the proliferation of L929 fibroblasts *in vitro*. The formulation of biochanin A with electrospun microfibers can be applied in various pharamceutical forms for systemic and local administration.

EXPERIMENTAL

Reagents

Biochanin A (BCA), purity of 98 % (Sigma Aldrich); potassium bromide (KBr) for IR spectroscopy, ≤100 % (Merck KGaA); Hanks' buffered solution pH 7.4 GmbH (PAA Laboratories, Pasching, AUT); 2-propanol, purity of 99.5 % (Centrohem, Belgrade, RS) and 3-(4,5--dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), purity of ≥97.5 % (Sigma Aldrich) were used. Poly(vinyl-pirrolidone) (PVP) K85-95, *MW* ~ 1300000 (Acros Organics, Geel, Belgium) were used for preparation of electrospun polymer matrices. Ethanol, purity of 95.5 % (Acros Organics, Geel, Belgium) was used for the preparation of polymer-based solutions for electrospinning. All chemicals were used as received.

Preparation of solutions for electrospinning

For electrospinning, PVP-based solutions were prepared by dissolving appropriate amount of PVP in ethanol, so that the final concentration of polymer was 12 wt. % by a method published earlier.[22] Active materials were prepared by adding of 2 and 5 % of biochanin A (calculated on the polymer weight) to basic polymer solutions. All solutions were mixed 24 h prior to electrospinning on magnetic stirrer at room temperature. Viscosity was measured on MYR viscometer, ver. V0, model 3000, which is in accordance with ISO 2555/ASTM method. Production of fiber cariers was done on electrospinning machine Fluidnatek LE-10 (manufacturer Bioinicia, Paterna, Spain) and process parameters were adjusted for each prepared solution. The list of samples with electrospinning process parameters is presented in Table I.

TABLE I. Samples and voltages of electrospinning; needle-to-collector distance: 10 cm, flow rate: 2 cm^3/h

Sample name	Voltage, kV
PVP	13
PVP-BCA-2%	14
PVP-BCA-5%	14.5

Stretching of the material

The mechanical properties of the prepared samples were examined using a tensile testing machine EZ-LX Test (Shimadzu, Kyoto, JPN). The obtained materials were cut in rectangular shaped strips, thickness and width were measured, and the samples were stretched with load of 1 mm/min. Stress (N/mm^2) and stroke-strain (%) were followed in maximum and break point.

Determination of the wetting angle

Surface properties were determined using a contact angle goniometer (Ossila, Sheffield, UK) with water as a wetting medium. The drop (5 μl) was dripped onto the surface of material and contact angle was measured.

Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) was used for the examination of thermal properties of the obtained materials. A small amount of sample (5 mg) was put into a pan and heated in one cycle from room temperature to 250 °C at the speed of 10 °C/min in a nitrogen atmosphere. The TA Instruments Q20 differential scanning calorimeter (TA Instruments, New Castle, DE, USA) was used for these tests.

Fourier transform infrared spectroscopy (FTIR)

The electrospun PVP fibers with and without biochanin A were ground to powder in an amalgamator (WIG-L-BVG, 31210-3A, Dentsply RINN, a Division of Dentsply International Inc., York, PA, USA). FTIR spectra of the biochanin A, electrospun PVP fibers, PVP-BCA-2% and PVP-BCA-5% were recorded using the technique of thin transparent pastilles, by vacuuming and pressing under the pressure of about 200 MPa. The pastilles were prepared by mixing 150 mg of KBr and 0.7 mg of the sample. FTIR spectra were recorded in the wavenumber range of 4000–400 cm^{-1} on a Bomem Hartmann & Braun MB-series FTIR spectrophotometer (Bomem Hartmann & Braun, Quebec, Canada). The obtained spectra were analyzed using the Win-Bomem Easy software.

Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was used to examine the morphology of the electrospun PVP fibers with and without biochanin A. The samples were sprayed by an alloy of gold and palladium (85/15) under vacuum in a Fine Coat Jeol JFC-1100 Ion Sputter (Jeol Ltd., Tokyo, JPN). The metalized samples of electrospun PVP fibers were scanned using a Jeol scanning electron microscope JSM-5300 (Jeol Ltd., Tokyo, JPN), under a magnification of 10,000 times, voltage 20 kV, vacuum 1.33×10^{-5} Pa.

Modified release of biochanin A from electrospun PVP fibers

The samples of electrospun PVP fibers (2.5–3.5 mg) with 2 and 5 % of biochanin A were soaked in with 10 cm^3 of Hanks' buffer (pH 7.4). The samples were stirred (120 rpm) and thermostated in a water bath at 37 °C. The release of biochanin A was monitored by sampling 200 µl of the solution at certain time intervals and diluting with 800 µl of methanol. All samples were filtered on the Econofilter with the pore diameter of 0.45 µm and analyzed by using the HPLC method. The dissolution of biochanin A in Hanks' buffer was previously reported.[1] For the construction of the calibration curve, a series of standard solutions of biochanin A in methanol (1–100 µg/cm^3) were prepared. The dependency of peak area on biochanin A concentration is linear, with a correlation coefficient $R^2 = 0.999$, and is represented as (this is example 1 of an equation):

$$A = 30.03 + 129.53C \quad (1)$$

where C (µg/cm^3) is the concentration of biochanin A.

High performance liquid chromatography (HPLC)

The high performance liquid chromatography (HPLC) method was applied for the quantitative analysis of biochanin A released from electrospun PVP fibers, as well as for solubility testing of biochanin A in Hanks' buffer. A mobile phase (800 µl) was added to every sample (200 µl) taken at a certain time interval. All samples were filtered on the Econofilter with the pore diameter of 0.45 µm and analyzed on Agilent Technologies 1100 Series HPLC device under the following conditions: column: Zorbax Eclipse XDB-C18 (4.6 mm×250 mm, 5 µm); mobile phase: methanol; flow rate: 1 cm^3/min; detection: DAD detector Agilent Technologies 1200 Series, $\lambda = 265$ nm; temperature: 30 °C; injected sample volume: 20 µl.

Cell proliferation assay

The L929 fibroblast cell line (mouse skin fibroblasts) was used for *in vitro* studies of wound healing activity of electrospun PVP fibers with and without biochanin A. L929 fibroblasts were cultivated in Dulbecco's Modified Eagle Medium (DMEM) containing 10 % fetal bovine serum (FBS), 2 mM stable glutamine, and antibiotic–antimycotic solution (complete

DMEM), at 37 °C in a humidified environment containing 5 % CO_2. All cell culture reagents were purchased from Capricorn Scientific GmbH, Germany.

For the cell proliferation assay, L929 cells were seeded in standard 24 well plates (Greiner Bio-One, Germany) at a density of 1×10^4 cells per well. Twenty-four h after the cultivation of cells, samples of electrospun PVP fibers were added to the cells (direct contact assay). The dimensions of the tested samples were 1 cm×1 cm. The cells incubated only with the medium without the tested material (complete DMEM) were used as a control cell culture (untreated cells). Each tested sample was examined in three replicates, and so was the control culture. The cells were incubated with the tested samples or control medium for the next 72 h. After the incubation period ended, an MTT test was performed according to the previously established protocol.[1,23]

The MTT test is widely used for assessment of cell proliferation and is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (tetrazolium salt MTT) by mitochondrial dehydrogenases of living cells, resulting in formazan crystals formation that corresponds to the number of cells. The cells were washed with phosphate buffer saline and then 300 µl of MTT solution per well (concentration 1 mg/ml) was added to the cells. The cells were incubated with MTT solution for the next three h followed by formazan crystals dissolution with 2-propanol. The absorbance of dissolved formazan was measured on a Multiskan Ascent photometric plate reader (Thermo Labsystems, Helsinki, Finland) at a wavelength of 540 nm with correction wavelength of 650 nm. The mean absorbance values were calculated for each tested sample, as well as for the control cell culture. The cell proliferation rate was calculated according to the following formula:

$$\text{Cell proliferation} = 100\frac{\text{Absorbance value of cells treated with fibers}}{\text{Absorbance value of untreated cells}} \quad (2)$$

In vitro *wound healing assay*

To examine the effects of electrospun PVP fibers without and with 2 and 5 % biochanin A on wound healing in vitro, we performed a "scratch" assay according to our previously published protocol.[24,25] Briefly, L929 fibroblasts were seeded in a sterile 48 well plates and incubated under the standard cell culture conditions (37 °C, 5 % CO_2 and in humidified environ ment). After reaching the 100 % confluence, a wound ("scratch") was created in a cell monolayer, in the middle of each well. The cells were then washed with buffer and samples of electrospun PVP fibers with and without biochanin A, or complete DMEM, were added. Each sample, as well as the control one, was tested in three replicates and the experiment was performed twice under the same conditions. The fibroblasts were incubated with the samples of electrospun PVP fibers without and with 2 and 5 % biochanin A and the effect on wound's closure was monitored after three days of incubation. A microscopic analysis of the wound's closure was performed on an inverted light microscope, an Axio Observer.Z1 (Carl Zeiss, Oberkochen, Germany) and morphometric measurements were made in ZEN 2 (blue edition) software (Carl Zeiss, Oberkochen, Germany) after imaging. The extent of wound closure was determined by measuring the width of the wound area before incubation with electrospun PVP fibers with and without biochanin A and three days after the incubation with the samples as well as with complete medium (control), and is expressed as the percentage of wound closure.

Statistical analysis

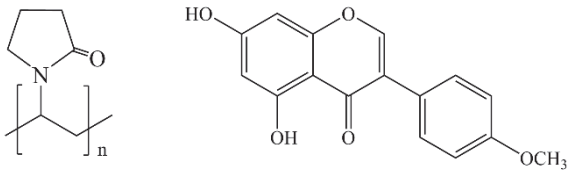
The results of the MTT proliferation assay as well as the in vitro wound healing assay of at least two independent experiments were analyzed using one-way analysis of variance (ANOVA). MTT test results were expressed as a percentage of cell proliferation with relative

standard deviation calculated according to the control culture of cells for which the cell proliferation rate was considered to be 100 %. As statistically significant differences, we considered those for which $p < 0.05$. The data analysis was performed using the software package SPSS Statistics version 20.0 (IBM).

RESULTS AND DISCUSSION

Preparation and characterization of electrospun PVP fibers with and without biochanin A

Electrospun PVP fibers were prepared using 9 wt. % solution of PVP in ethanol. The structure of poly(vinylpyrrolidone) is shown in Fig. 1a. In addition to that, the prepared solutions have an appropriate viscosity of 380 mPa·s, which is a very important parameter for the morphology of obtained fibers. The fibers with biochanin A were prepared by electrospinning of PVP polymer solutions with 2 % or 5 % of biochanin A. The structure of biochanin A is shown in Fig. 1b.



(a) (b)

Fig. 1. Structure of: a) poly(vinylpyrrolidone) and b) biochanin A.

The electrospinning process is performed at room temperature which is very important for thermosensitive active substances, such as biochanin A, because thermal degradation of biochanin A is prevented and its stability is maintained.

Mechanical properties of electrospun PVP fibers

Mechanical properties of the samples are summarized in Table II. Measuring was conducted on five samples and the mean value was calculated. When comparing the mechanical properties of the samples within a PVP-based series of materials, it can be concluded that the presence of biochanin A induced a decrease of maximum stress, from 6.87 N/mm^2 for PVP to 5.38 N/mm^2 for the samples with 5 % of biochanin A. Also, elongation was reduced and at the point of maximum stress it was 20.54 % for pure PVP and 11.07 % for PVP-BCA-5%, which is almost a half of the value.

Since it is poorly soluble in water, miscibility of biochanin A and PVP might be poorer compared to its miscibility with PLA,[1] what could be the reason for decrease of values for all followed mechanical parameters along with the increase of concentration of biochanin A. The presence of biochanin A had a higher influence on elongation of materials, decreasing the elasticity with the concentration

increase. Besides all, electrospun PVP fibers can be used as a carrier in formulations for controlled delivery of biochanin A.

TABLE II. Mechanical properties of electrospun PVP fibers with and without biochanin A

Sample name	Max. stress N/mm^2	Max. stroke-strain %	Break stress N/mm^2	Break stroke-strain %
PVP	6.87±0.24	20.54±0.31	6.36±0.19	25.80±0.033
PVP-BCA-2%	6.047±0.21	17.38±0.25	5.86±0.17	18.72±0.24
PVP-BCA-5%	5.38±0.15	11.07±0.21	1.48±0.04	14.19±0.23

Surface properties of electrospun PVP fibers

Table III shows the values of the contact angle of the samples of electrospun PVP fibers with and without biochanin A. The hydrophobic properties of biochanin A affected the surface properties of the obtained active materials compared to pure polymer materials. The content of 2 % biochanin A in the electrospun fibers based on PVP increases the contact angle from 28.12 to 57.61°, which is an increase of almost 30°, while the contact angle for PVP-BCA-5% is 69.71°, *i.e.*, compared to PVP it increases by more than 40°. This shows that biochanin A has more influence on the contact angle change in PVP-based electrospun fibers than PLA.[1]

TABLE III. Contact angle of electrospun PVP fibers with and without biochanin A

Sample name	Contact angle, °
PVP	28.12±0.51
PVP-BCA-2%	57.61±1.21
PVP-BCA-5%	69.71±1.67

Thermal properties of electrospun PVP fibers

The results of the DSC analysis are shown in Fig. 2. Due to good incorporation of BCA into the electrospun PVP fibers, the DSC curves of PVP-BCA-2% and PVP-BCA-5% do not have a peak corresponded to melting temperature of BCA at 216 °C. Also, for DSC curves that corresponded to PVP, PVP-BCA-2% and PVP--BCA-5% there were no any thermal changes in temperature range from 50 to 250 °C. Based on all the above, it can be concluded that the incorporation of biochanin A into a PVP matrix do not affect its thermal properties.

Morphology of the electrospun PVP fibers

The morphology of the electrospun PVP fibers was examined using scanning electron microscopy (SEM). The SEM images of PVP fibers with and without biochanin A are shown in Fig. 3. Sample obtained from pure PVP (Fig. 3a) represents a network of fibers that are round and smooth without visible irregularities in the structure. Fibers keep this look even if they contain biochanin A in amounts of 2 and 5 % (Fig. 3b and c, respectively) and are a continuous network of fibers on

which surface the presence of crystals of the active substance is not observed. This indicates that biochanin A is incorporated in fibers of PVP polymers. The diameter of electrospun PVP fibers with dispersed biochanin A is in the range of 0.1–1 μm.

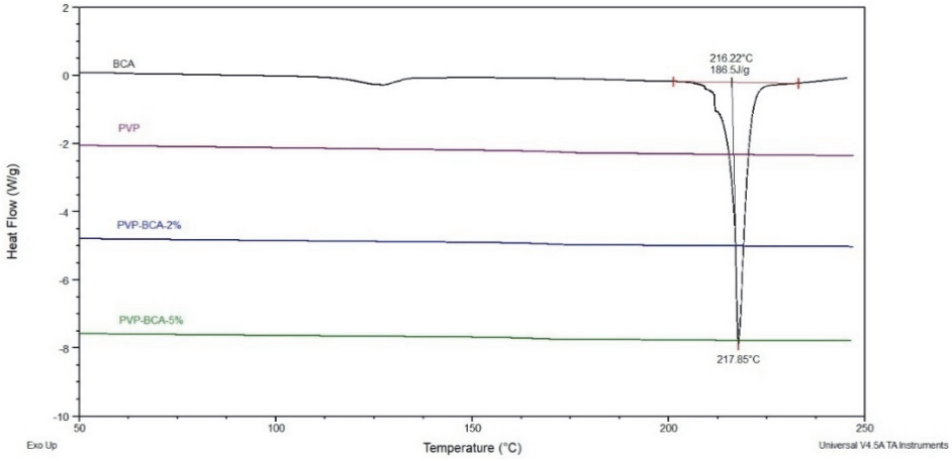
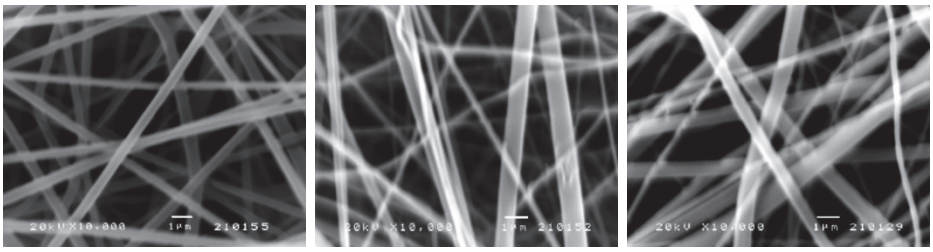


Fig. 2. DSC curves of: BCA, electrospun PVP fibers, PVP-BCA-2% and PVP-BCA-5%.



(a) (b) (c)

Fig. 3. SEM images of electrospun fibers of: a) PVP, b) PVP-BCA-2% and c) PVP-BCA-5% (bar 1μm; magnification 10,000×).

FTIR analysis

The structural characterization of biochanin A, PVP and PVP-BCA-5% is performed using the FTIR method. In the FTIR spectrum of biochanin A (Fig. 4a), wide, intensive absorption band with maximum at 3259 cm^{-1} originates from valence vibrations of phenolic OH groups, ν(OH). Characteristic valence vibrations of the phenolic C–O bond, ν(C–O)Ar, give intensive bands in the range of 1260––1000 cm^{-1}, and this band is present in the spectrum of biochanin A at 1176 cm^{-1}. Planar deformation vibrations of hydroxyl groups, δ(OH), give a low-intensity band with a maximum at 1323 cm^{-1}. The strong absorption band at 1661 cm^{-1} is a result of valence vibrations of the carbonyl group, ν(C=O). The characteristic absorption bands at 1625, 1585 and 1515 cm^{-1} come from valence vibrations of

aromatic double bonds, ν(C=C)Ar. The asymmetric valence vibrations of ether C–O–C bond, ν_{as}(C–O–C), give two bands at 1258 and 1237 cm^{-1}.

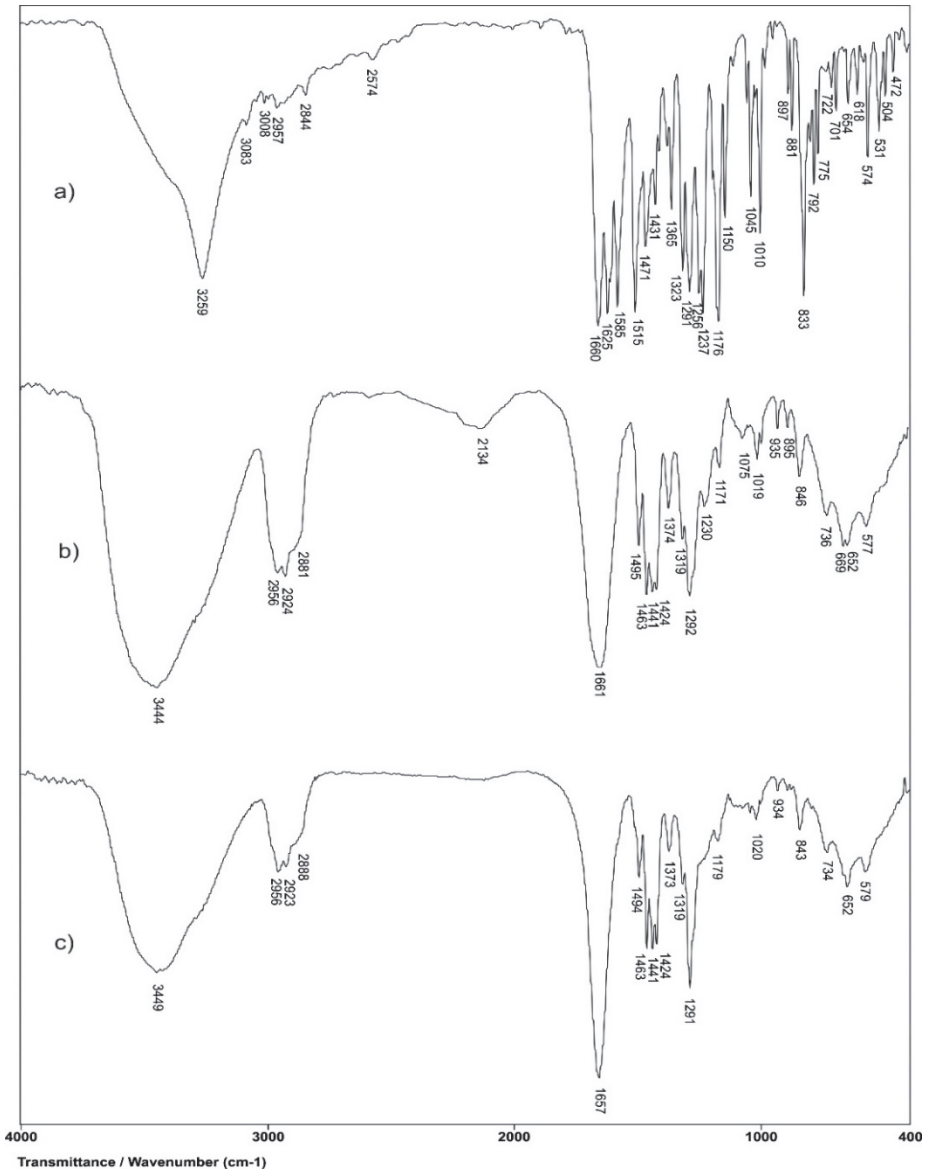


Fig. 4. FTIR spectra of: a) biochanin A, b) PVP and c) electrospun PVP-BCA-5% fibers.

It was ascertained that, depending on the production conditions, the PVP polymer chain has an OH-group at the one side of the molecule, and the other side of the molecule is hydrolyzed so that the pyrrolidone molecule is getting segregated

and an aldehyde group is forming. The valence vibration coming from the OH--group is observed in the FTIR spectrum of PVP with a maximum at 3444 cm^{-1}. In poly(vinylpyrrolidone), the peak which is coming from the valence vibrations of C=O appears at 1661 cm^{-1}. The valence vibrations of –CH appears at 2956 and 2924 cm^{-1}. The medium intensity band at 1292 cm^{-1} comes from C–N valence vibrations. By comparing the values of the vibration frequencies in the FTIR spectrum of PVP and PVP with 5 % biochanin A, it can be observed that there are no significant differences in frequencies, the differences are non or up to 5 cm^{-1}. Based on this, it can be concluded that the mentioned groups from PVP do not contribute to the formation of strong intermolecular bonds with biochanin A and that the release from PVP will be accomplished by diffusion. If PVP with biochanin A gets into contact with a polar solvent (water, physiological media, *etc*.) the PVP will dissolve and thereby release the entire content of biochanin A in a short period of time.

In order to examine interactions between biochanin A and PVP, FTIR spectra of PVP with and without biochanin A were analyzed and vibration frequencies of the significant groups are shown in Table IV.

TABLE IV. Comparative values of the vibration frequencies in the FTIR spectra of PVP and PVP-BCA-5%

Vibration	Peak in the spectrum of PVP, cm^{-1}	Peak in the spectrum of PVP-BCA-5%, cm^{-1}
ν(O–H)	3444	3449
$\nu_{as}(C–H_2)$ from pyrrole ring	2956	2956
$\nu_{s}(C–H_2)$ from pyrrole ring	2924	2923
ν(C=O)	1661	1657
ν(C–N–C)	1441	1441
δ(C–H)	1374	1373
ν(C–N)	1292	1291
$\delta(CH_2)$ rock	1019	1020
ν(C–C)	935	934
$\delta(CH_2)$	846	843
δ(N–C=O)	577	579
δ(C–N–C)	652	652

By comparing the values of the vibration frequencies in the FTIR spectra of PVP and PVP-BCA-5%, it can be observed that there are no significant differences (0 – 5 cm^{-1}). The results of this analysis showed that the mentioned groups from PVP do not contribute in the formation of any intermolecular bonds with biochanin A.

Modified release of biochanin A from electrospun PVP fibers

The amount of biochanin A released over time from PVP-BCA-2% and PVP--BCA-5% in Hanks' buffered solution pH 7.4 during time is shown in Fig. 5a and b, respectively.

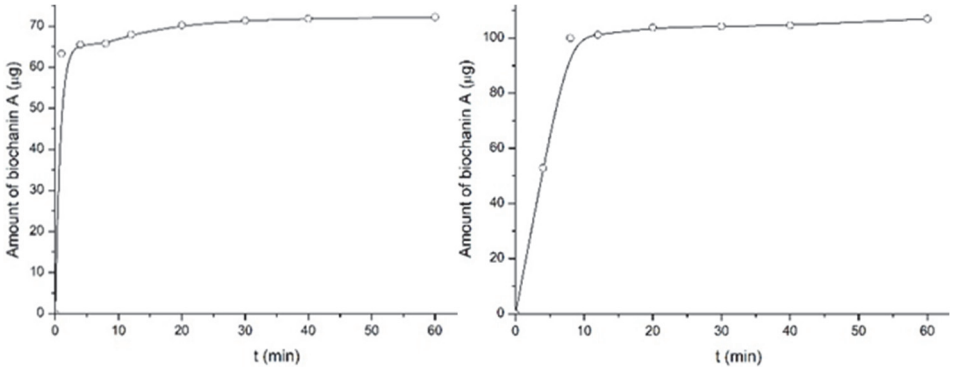


Fig. 5. Amount of biochanin A released from electrospun: a) PVP-BCA-2% and b) PVP-BCA-5% fibers in a buffer at pH 7.4 and temperature of 37 °C.

The results show that after wetting the PVP-BCA-2% electrospun fibers and PVP-BCA-5%, BCA is getting released in a short period of time: from PVP-BCA--2% more than 90 % is released in about 2 min and from PVP-BCA-5% about 98 % in about 5 min. The reason for the fast release profile obtained is probably because PVP dissolves in aqueous mediums and, practically, the dissolution rate of PVP dictates the release rate of biochanin A. The obtained rapidly degradable electrospun poly(vinylpyrrolidone) nanofibers with biochanin A, in contact with moisture, may have practical applications as suitable carriers for topical application to the skin and mucous membranes that provide fast, modified delivery. Transdermal application is an alternative route that can achieve initial therapeutic concentrations of medicinal substances in the systemic circulation in a short period of time, avoiding the interaction of the drug with the gastrointestinal tract, eliminating the influence of food, pH and enzymes on its stability and absorption and metabolic degradation in the liver.

Biological testing

Cell proliferation. The effects of electrospun PVP fibers with biochanin A (PVP-BCA-2% and PVP-BCA-5%) and without biochanin A (PVP) on fibroblasts' proliferation are shown in Fig. 6 whereas the look of L929 untreated cells and cells treated with samples of electrospun fibers PVP, PVP-BCA-2% and PVP--BCA-5% is shown in Fig. 7.

The concentration-dependent effect of biochanin A, released from PVP fibers on the proliferation of L929 fibroblasts, was noticed in the direct culture system used. Slight anti-proliferative activity of PVP pure foil was noticed while PVP--BCA-2% and PVP-BCA-5% foils acted extremely anti-proliferative on L929 fib-

roblasts, which is probably due to immediate dissolution of foils into the cell culture medium at the moment of adding them to the cells when the whole amount of biohanin A was released, from the foils, immediately.

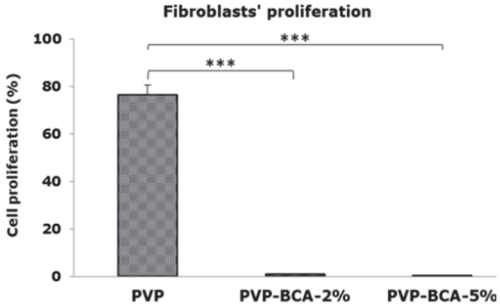


Fig. 6. Results of MTT test showing the effect of examined electrospun PVP fibers with 2 and 5 % BCA and without BCA on proliferation of L929 fibroblasts; (*) $p < 0.05$, (***) $p < 0.001$.

In Fig. 7 it is obvious that cell number is reduced and it corelates with the results of MTT test presented in Fig. 6. It can also be seen that with the use of PVP--BCA-5%, a smaller number of cells survived compared with the PVP-BCA-2% sample (Fig. 7).

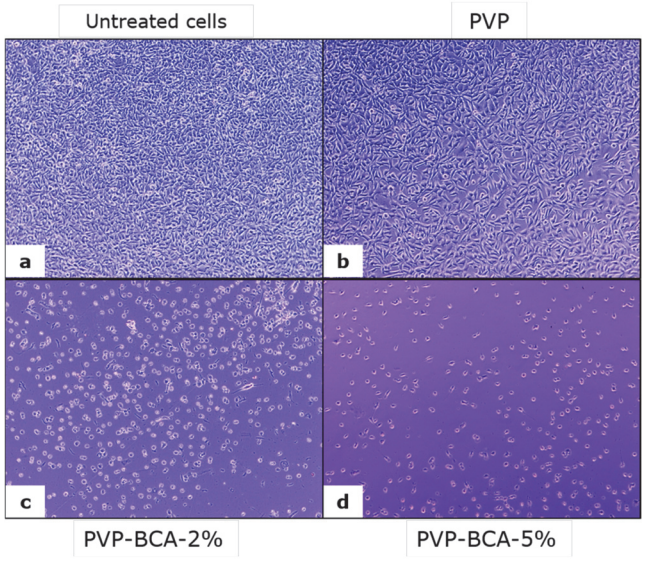


Fig. 7. L929 cells after three days of incubation with standard cell culture medium (untreated cells) without electrospun fibers; a) PVP, b) PVP-BCA-2%, c) PVP-BCA-5% and d) electrospun fibers.

In vitro *wound healing activity*. In vitro wound healing activity was examined using the *in vitro* "scratch" assay. The wound (scratch) was created in a confluent

cell monolayer which was followed by incubation with the electrospun fibers or complete medium (control).

The results of in vitro wound healing activity of PVP foils, as well as appearance of the wounds after three days of incubation are presented in Fig. 8. Complete wound closure was achieved with PVP foil only without addition of BCA. However, cells were apoptotic, as it was noticed in proliferation assay, and no wound healing activity was seen when cells were treated with PVP-BCA foils due to cytotoxic activity of used concentrations of BCA.

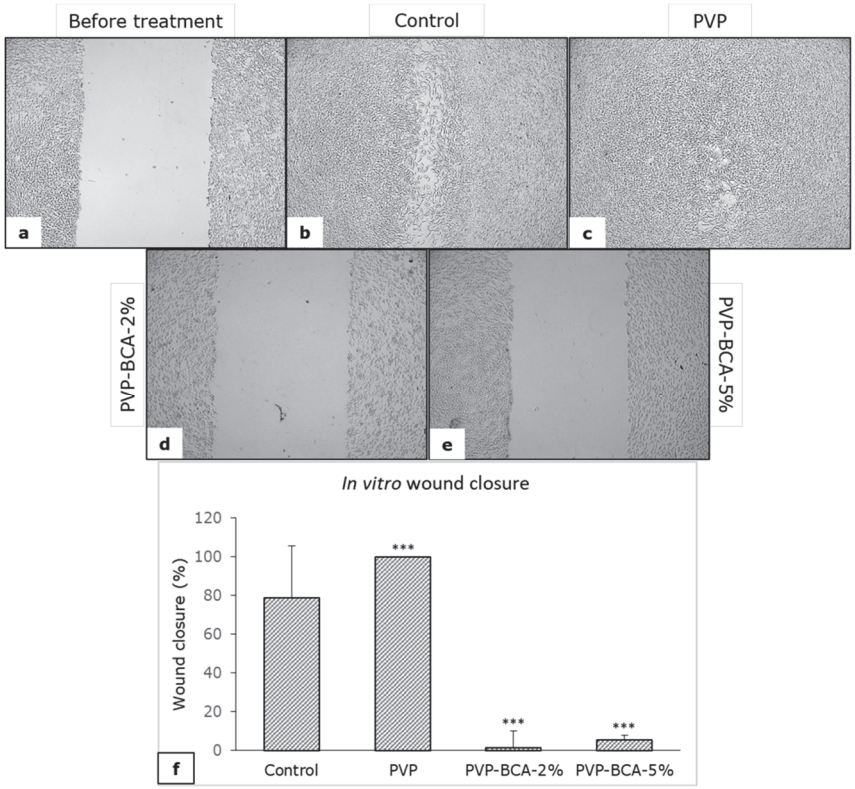


Fig. 8. Appearance of *in vitro* created "wounds": a) before, b) three days after incubation with complete medium (control), c) PVP, d) PVP-BCA-2%, e) and PVP-BCA-5%; f) percentage of wound closure; (**) $p < 0.01$.

Although the electrospun PVP-BCA fibers showed to be an inadequate system for the release of BCA used *in vitro* model, there are cases where they may be potentially applicable in clinical practice. Electrospun PVP–BCA nanofibers represent a suitable system for the treatment of complicated skin wounds. Electrospun PVP fibers with BCA can provide antimicrobial activity in damaged skin.[26,27] Biochanin A can protect damaged skin by inhibiting the expression of COX-2

proteins, *e.g.*, in UV-induced damage to keratinocytes. In those cases, PVP electrospun fibers with BCA can act therapeutically.[28] One of the possible ways of action is to reduce the growth of keratinocyte cells suffering from psoriasis. Namely, the release of cytokines linked to psoriasis (IL-17A and IL-23) were significantly reduced upon BCA treatment. Furthermore, findings demonstrated that BCA treatment alleviated the psoriasis-like symptoms via modulating NF-κB and MAPK signaling pathways.[29] Also, this BCA formulation with electrospun PVP fibers can be easily applied to skin with hyperkeratosis or to psoriasis-affected skin, which will reduce keratinocyte cell growth and thus remove cells. At the same time, BCA reduces skin inflammation in those places. The possible mechanism of action was published by Lv *et al.* that showed that BCA is effective in the treatment of psoriasis by activating the Nrf2/HO-1 pathway.[30]

However, in order to further develop a potential formulation, some *in vivo* analyses are necessary, *e.g.*, animal experimentations.

CONCLUSION

Electrospun PVP-BCA fibers were obtained from the solution by which uniform distribution of BCA in the polymer matrix was ensured. The characterization of electrospun PVP–BCA fibers was performed by FTIR, DSC and SEM analysis, examination of mechanical properties, measurement of contact angle, monitoring of BCA release from electrospun PVP–BCA fibers and accomplishment of biological tests. The results showed that the surface properties of the fibers and their mechanical properties change in the presence of BCA. The results of chemical analyses show that there are no strong interactions between the BCA and PVP molecules, which enables the rapid separation of BCA from the PVP matrix, especially when the electrospun PVP–BCA fibers get wetted. That is why this system of electrospun PVP–BCA fibers can become suitable for the treatment of complicated wounds, at which in the first stage of treatment the damaged tissue should be removed by the activity of electrospun PVP–BCA fibers. Simultaneously present biochanin A would exert antimicrobial activity, which would ensure a complete treatment. Another potential use of electrospun PVP–BCA fibers is for facial skin exfoliation.

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ИЗВОД

ФОРМУЛАЦИЈЕ БИОХАНИНА А СА ЕЛЕКТРОСПИНОВАНИМ ВЛАКНИМА ПОЛИ(ВИНИЛПИРОЛИДОНА) И МОГУЋЕ ПРИМЕНЕ

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Циљ овог рада је испитивање могућности коришћења електроспинованих поли(винилпиролидонских) (PVP) влакана као носача фитоестрогена биоханина А (BCA). PVP влакна су припремљена са различитим садржајем BCA коришћењем методе електроспиновања при специфичним процесним параметрима. Произведена електроспинована PVP–BCA влакна су окарактерисана хемијским, физичко-механичким и биолошким методама. SEM, DSC и FTIR анализе су показале да нема јаких интеракција између молекула PVP и BCA, нити термичких промена у тестираном температурном опсегу (50––250 °C) и да је постигнута једнака расподела BCA у електроспинованим PVP влакнима. Физичко-механички тестови су показали да се физичка својства и угао квашења PVP мењају у присуству BCA. Тестирање електроспинованих PVP влакана са и без BCA на L929 фибробластима у директном контактном тесту *in vitro* показало је значајан ефекат на пролиферацију и миграцију фибробласта. Биолошки тестови су потврдили да систем електроспинованих PVP–BCA влакана може постати погодан за лечење компликованих рана, где у првој фази лечења оштећено ткиво треба уклонити деловањем електроспинованих PVP-BCA влакана. Друга могућност коришћења електроспинованих PVP–BCA влакана је за третман коже лица у сврху ексфолијације.

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