Contents lists available at ScienceDirect



International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac



Double layer bacterial nanocellulose - poly(hydroxyoctanoate) film activated by prodigiosin as sustainable, transparent, UV-blocking material

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ARTICLE INFO

Keywords: Prodigiosin Poly(hydroxyoctanoate) Bacterial nanocellulose Double-layer film

ABSTRACT

Synthetic materials alternatives are crucial for reaching sustainable development goals and waste reduction. Biomaterials and biomolecules obtained through bacterial fermentation offer a viable solution. Double-layer active UV-blocking material composed of bacterial nanocellulose as an inner layer and poly(hydroxyoctanoic acid) containing prodigiosin as an active compound was produced by layer-by-layer deposition. This study referred the new material consisted of the three components produced in sustainable manner, by bacterial activity: bacterial bio-pigment prodigiosin, bacterial nanocellulose and poly(hydroytoctanoate) - biopolymer obtained by microbial fermentations. Prior the final double layer film was produced, PHO films containing different PG concentrations as a layer in charge of the bioactivity (0.2, 0.5 and 1 wt%) was casted and systematically characterized (FTIR, DSC, XRD, wettability, SEM, transparency, mechanical tests) to optimize their properties. The formulation with the best UV-blocking properties and less toxicity effect tested using MRC5 cells was chosen as an outer layer in double-layer films production. Water contact angle measurements confirmed that hydrophilic – hydrophobic double layer film was obtained with the improved mechanical properties in comparison to the native BNC. Migration test indicated release of PG in all tested media as a consequence of bilayer formulation, while the PG release from PHO in 10 % ethanol was not detected. All findings from the study suggested this activated, UV-blocking material as a candidate with excellent potential in packaging industry.

1. Introduction

Favorable mechanical and physical properties, low cost and straightforward production make plastic materials an indispensable part of our lives [1]. However, the fact that these non-renewable petroleumbased materials are also, in the majority of cases, not biodegradable poses as a serious ecological problem. A significant contributor to the global plastic waste generation is the packaging industry of very short-lived products, commonly referred to as single-use plastics [2,3]. To address this, research efforts have been aimed at finding sustainable biodegradable alternatives like poly(lactic acid) (PLA) [4], pullulan [5], thermoplastic starch (TPS) [6], or polyhydroxyalkanoates (PHAs) [7–10]. Biomaterials and biomolecules obtained through bacterial fermentation offer a viable industrial solution [11]. Among the available contenders, PHAs have distinguished themselves as the most promising green materials to replace petrochemical plastics because they are characterized by a completely green life cycle: they are obtained via microbial biosynthesis during the fermentation process using renewable, not food-competing carbon sources, even waste streams, and they are inherently biocompatible and biodegradable [12–14].

From the standpoint of processing and application, the most interesting trait of PHAs is their structural heterogeneity, which translates into a wide range of physicochemical properties. The hydrophobic nature of PHAs biopolyesters gives them excellent water barrier-like properties [15], along with good barrier-like properties against CO_2 and O_2 [16,17]. These characteristics are important, seen as a packaging material needs to act as a barrier that protects the enclosed food from the environment and at the same time must preserve its organoleptic

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https://doi.org/10.1016/j.ijbiomac.2024.135087

Received 8 May 2024; Received in revised form 14 August 2024; Accepted 24 August 2024 Available online 26 August 2024

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properties. Therefore, PHAs as renewable, biodegradable and easily modified thermoplastic biopolymers have become increasingly studied as food and other products' packaging materials [8]. Although PHA has been approved by FDA to be used in food packaging applications [18], there are still some limitations in its wider application, andthe main drawback is the high production cost which can be overcome by using inexpensive carbon sources like wastes. Elastomeric medium chain length PHAs (mcl-PHAs) are generally inert, but offer the possibility to be further enhanced through incorporation of constituents like UVblocking agents, antioxidants [19] or antimicrobials [20,21]. These mcl-PHAs-based blends or composites exhibit improved structural and functional properties [22]. Another natural biopolymer, intensively investigated for a number of applications is the bacterial nanocellulose (BNC), an exopolysaccharide of β -D glucopyranose, which has outstanding mechanical properties [23,24]. BNC has also been recognized as a promising packaging material thanks to its sustainability, inertness, high specific surface area and highly porous threedimensional structure and biocompatibility [25].

Recently, multi-layer film packaging has been widely applied as an emerging technique that combines unique properties of individual polymer layer constituents resulting in the creation of a packaging material with improved properties in terms of barrier properties, durability and functionality [26,27]. Multi-layer active and colorimetric films can be produced using layer-by-layer deposition method [28].

Biodegradable and non-toxic PHAs films with excellent film-forming properties are usually transparent and require the addition of constituents that can improve the UV barrier properties, but remain promising candidates to produce the outer protective layer of colorimetric multilayer materials. One such constituent that can activate PHAs is prodigiosin (PG), a bio-pigment produced by both Gram-positive and Gramnegative bacteria [29]. PG belongs to a class of bacterial secondary metabolites, pyrrolylpyrromethene alkaloids, with an intensive color that can vary from red to purple due to the presence of a conjugated system of double bonds [29]. One of the main roles of PG is to protect the bacterial cells against the harmful effects of the UV radiation by absorbing it [30], however, PG also exhibits remarkable biological activities like anticancer [31], antioxidative [32] and antimicrobial [32,33]. As an attractive colorant with a dual function, PG has been used to design a couple of biopolymer-based active food packaging formulations, including antimicrobial bacterial cellulose/chitosan films [34] and pH-responsive sensors [35]. The microbial pigments are already gaining attention of the industry as suitable substitutes to synthetic dyes because they are sustainable and biodegradable [36]. Previously, PG has been incorporated into BNC for food packaging and biomedical applications [34,37] and into poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) for anticancer activity [38].

The main objective of this study was to produce novel biodegradable, renewable, attractively colored multi-layer food packaging materials with improved UV-blocking properties, based on the constituents synthesized by various bacteria, namely PHAs, PG and BNC. Before the final double-layer material was produced, the optimization of the outer layer consisting of PHO and PG was performed to assess its toxicity but with the preserved UV-blockage ability was performed. Therefore, PHOPG films, with the variation in the PG concentration incorporated into the PHO (0.2, 0.5 and 1 wt%), were casted and briefly evaluated (FTIR, DSC, XRD, SEM, wettability). The influence of the PG incorporation on the obtained materials properties was investigated, followed by the cytotoxicity testing, while and the UV-blockage ability was held as crucial in the selection of the final PG concentration that was casted onto the BNC thus forming an active UV-blocking double layer material.

2. Materials and methods

2.1. Materials

Poly(hydroxyoctanoate), PHO was produced using Pseudomonas

putida KT2440 strain and the octanoic acid as a substrate [39]. Prodigiosin (PG) was produced in a sustainable manner via microbial fermentation using *Serratia marcescens* ATCC 27117 (American Type Culture Collection, ATCC, Manassas, VA, USA) and a variety of food wastes as a substrate [40].

2.2. Production of the PHO films with incorporated prodigiosin

PHO/PG films were produced using the solvent casting method according to a procedure described in our previous study [38]. In brief, PHO and PG were dissolved in CHCl₃ (magnetic stirrer, 30 min, room temperature, protected from the sun) and the obtained film solution was cast into a glass Petri dish (7 cm in diameter). The samples were dried for 14 days in the dark at room temperature. To investigate the effect of the PG content on the obtained films properties, multiple films solutions with fixed PHO content (750 mg) and increasing PG concentration (0.2, 0.5 and 1 % relative to the biopolymer weight) were made and cast. The control was a pure biopolymer film, prepared as described above, without the addition of PG. The obtained samples were denoted as PHO, PHOPG1, PHOPG2 and PHOPG3 for the control and the samples with an increasing PG content, respectively (Table 1). The yield of the obtained solvent casted films was calculated following the Eq. (1):

Yield (%) =
$$\frac{m_1}{m_t} \times 100\%$$
 (1)

where m_1 refers to the physical mass of the produced formulation and m_t is the theoretical mass of PHO and PG in feed.

2.3. Bacterial nanocellulose – poly(hydroxyoctanoate) – prodigiosin film (BNC-PHOPG2)

Bacterial nanocellulose (BNC) was produced in a Hestrin–Schramm (HS) medium supplemented with glucose as the carbon source following a previously adopted procedure [41,42]. After characterization of all prepared PHO/PG formulations (see Section 2.4), the selection was made for casting PHOPG2 onto the BNC film aiming to produce a double-layer material. To produce the BNC-PHOPG2 film, the PHO containing PG was prepared by dissolving 1.0 g of PHO and 5.0 mg of PG in 10 mL of CHCl₃ resulting in overall 0.5 wt% of PG. The prepared PHO/PG solution was casted onto the dry BNC films and left to evaporate overnight resulting in the double-layer BNC-PHOPG2 film that was further examined.

2.4. Material characterization

2.4.1. Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra were recorded using an IR-Affinity spectrometer (Thermo Fisher Scientific, NICOLET iS10, Waltham, MA, USA) in the attenuated total reflection (ATR) mode. Measurements were performed in the range of 4000 to 400 cm⁻¹ at room temperature and a resolution of 4 cm⁻¹, with the fixed number of scans at 32.

2.4.2. Optical microscopy

An Olympus SZX10 microscope (Olympus, Tokyo, Japan) equipped with a digital imaging system was used to examine overall surface morphology of the film samples.

2.4.3. Scanning electron microscopy (SEM)

Morphology od the samples was investigated by SEM using a JEOL JSM-6390LV SEM (JEOL USA Inc.) operated at 25 keV. The internal structure was observed using cryo-fractured samples. Prior to the analysis, the samples were coated with a conducting layer of gold. The film thickness was measured at ten random points of each sample formulation using a digital micrometer (0.001 mm accuracy) (S00014, Mitutoyo Corporation, Kawasaki, Japan).

Sample codes and compositions.

Sample	Biopolymer, mg	Prodigiosin, mg	CHCl ₃ , mL	% wt of PG	Yield, %	Thickness, µm
РНО	750	-	5	-	99.3	137 ± 0.01
PHOPG1		1.5		0.2	97.0	127 ± 0.01
PHOPG2		3.75		0.5	93.9	130 ± 0.03
PHOPG3		7.5		1.0	97.9	147 ± 0.01

2.4.4. Differential scanning calorimetry and thermogravimetric analysis (DSC/TGA)

The DSC and TGA were performed on a TA Instruments SDT Q600 instrument. The measurements were performed in nitrogen atmosphere with a heating rate of 10 $^{\circ}$ C/min, and a temperature range of 25–600 $^{\circ}$ C. The samples weight was about 5 mg. The melting enthalpy obtained from the DSC thermograms was determined from the area under the endotherms using the TRIOS Software TA Universal Data Analysis.

2.4.5. X-ray diffraction (XRD)

The XRD analysis was performed using a X'Pert PRO (PANalytical B. V.) diffractometer operated at 40 kV and 30 mA by Ni-filtered Cu Ka radiation. The measurements were performed at room temperature in the 2θ range of $10-60^{\circ}$ with a step size of 0.02° . The crystallite sizes of the investigated films were calculated based on the measurement of FWHM (full width at half maximum) of reflections applying the Scherrer equation, which was done by using the X'Pert HighScore software with the use of the PeakFit mode. The crystallographic parameters in the crystal system were calculated based on three representative reflections. Also, the degree of crystallinity of the biopolymer in the neat and the PG-containing samples was evaluated using PeakFit 4.12 software.

2.4.6. Mechanical properties

The mechanical properties of the samples were evaluated by the tensile method using the AG-Xplus Universal Testing Machine (Shimadzu Corporation, Kyoto, Japan), equipped with a 100 N load cell at ambient temperature and the test speed was set at 5 mm/min. The mechanical properties including the tensile strength, elongation at break and Young's modulus of elasticity were evaluated for both the pure biopolymer and the PG-containing films. Five measurements were performed for each sample and the average values \pm standard deviation (SD) were presented.

2.4.7. Contact angle and surface free energy measurements

The contact angle and the surface free energy of the film samples was evaluated using a Krüss DSA100M Drop Shape Analyzer optical contact angle measuring instrument (Gmbh, Hamburg, Germany) equipped with an optical microscope and a digital camera (20 fps) that takes highspeed images and uses a digital image processing algorithm to calculate the contact angle of the droplet based on the Young-Laplace equation. Stainless steel syringe needles (NE 44, Krüss Gmbh, Hamburg, Germany, with an outer diameter of 0.5 mm) were used for each analysis and the volume of the drop were 11.5 mm³ for water and 2.5 mm³ for diiodomethane. For all investigated samples, the dynamic contact angles between the films and water, as a polar liquid, as well as with diiodomethane, as a non-polar liquid, were determined. During the measurements, the environmental chamber temperature was controlled using a thermostatic water bath, which maintained a constant humidity and a constant temperature (22.0 \pm 0.3 °C). For each sample, at least five successive measurements were performed and the final results were presented as the average values \pm SD. In addition, the surface free energy was also calculated by the Owens-Wendt method [43], generally accepted as one of the best suitable for polymer materials evaluations. The same methodology was applied both for polar and non-polar solvents (i.e., pure water $\delta = 72.30$ mN/m (Millipore Q, 18.60 m Ω /cm), diiodomethane $\delta = 50.80 \text{ mN/m}$).

2.4.8. Transparency and UV-blockage

The transparency of the samples was determined using a UV/Vis spectrophotometer cell (HALO DB-20S, Dynamica Scientific Ltd., United Kingdom). The film strips $(1 \times 4 \text{ cm}^2)$ were placed perpendicularly to the light beam, the transmission was measured in the ultraviolet and visible spectra (at wavelengths between 200 and 800 nm) and the results were expressed as percentage transmittance. The measurements were done in triplicate and the representative average spectra was presented. Additionally, the transparency at 600 nm (T_{600}) was calculated applying the following Eq. (2) [44]:

$$T_{600} = -\log\% T/L \tag{2}$$

where %T is the transparency of films measured at 600 nm and L is the film thickness in mm. In addition, the qualitative assessment of the samples contact transparency was conducted visually.

2.4.9. Migration tests

The migration of PG from the film samples into the food simulant media: 10 %, 50 %, and 95 % aqueous ethanol solutions (ν/ν) was evaluated according to the European Commission Regulations [45,46]. The media without film samples, plain PHO and plain BNC films were tested as controls. The film samples were immersed in triplicates into the media (6 dm² of contact film surface per kg of media) and incubated for 10 days at 20 °C in the dark under static conditions. After incubation, the media were taken out and analyzed for released PG by measuring the absorbance at 535 nm using a UV–Vis spectrophotometer (HALO DB-20S, Dynamica Scientific Ltd., United Kingdom). The concentration of the released PG (mg PG/kg medium) was determined according to the *Lambert-Beer* law using the extinction coefficient of 51,300 L/(mol × cm) [29,47].

2.4.10. Cytotoxicity assessment

To assess cytotoxicity, the standard MTT assay [48] and methods suitable for materials testing were used as previously described [49]. Human fibroblasts cells line (MRC5) was obtained from the ATCC culture collection. For all materials, the samples extracts were prepared by incubating the material samples (10 mg/mL) in the RPMI-1640 medium for 72 h at 37 °C, which were then tested in serial dilutions (100 %, 50 %, 25 % and 12.5 % ν/ν). After 48 h of treatment with the sample extracts, cell proliferation was determined using the MTT reduction assay by measuring the absorbance at 540 nm and 670 nm on the Epoch multiplate reader (BioTek Instruments, Inc., Winooski, USA). The results were presented as percentage of the control (untreated cells) that was arbitrarily set to 100 %.

2.5. Statistical analysis

GraphPad Prism 9.4.1 software (GraphPad Prism 9.4.1, La Jolla, CA, USA) was used to perform a one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. The results are presented as mean \pm standard deviations (SD). The difference was statistically significant at $p \leq 0.05$.

3. Results and discussion

In this work, double layer film consisted of BNC as a hydrophilic layer and PHO containing the active compound PG as the hydrophobic layer was produced by a straightforward casting method. In the production of multi-layer films, a commonly used casting method consists of pouring out each film-forming solution one-by-one [50]. Prior to the final formulation of the double-layer film, three formulations of different concentrations of PHO containing PG (0.2, 0.5 and 1.0 wt%) were prepared by the solvent casting method (Table 1). The polymer film containing the medium PG concentration (0.5 wt%) was selected as a model to cast onto the BNC film, forming a new double-layer material (Fig. 1). The obtained yields of the PHO formulations (PHOPG1, PHOPG2 and PHOPG3) were high, rated from 94 to 99 %, while the final formulation of the double-layer film, BNC-PHOPG2, yielded in 98 %. Thickness of the control biopolymer PHO was 137 $\mu m,$ while the incorporation of the active compound PG resulted in the decrease in thickness after the addition of 0.2 and 0.5 wt% (127 µm and 130 µm, respectively), while the formulation with the 1.0 wt% of PG had a thickness of 147 µm, significantly higher in comparison to control. This could be explained by the better compatibility and stronger intermolecular interactions established between PG and PHO, resulting in a more compact structure when lower concentration of the active compound was added. The thickness of the BNC as a hydrophilic layer was higher, $482 \pm 0.04 \ \mu m$ (yield of 6.0 g/L), but after the second layer formation by casting, the final material had a thickness of 967 \pm 0.05 µm (the obtained yield was 98 %).

When all the structural, morphological, thermal and wettability properties of the three PHO/PG formulations with different PG content were done and evaluated, PHOPG2 was selected as a model formulation for double layer BNC material production, as it was the most promising in terms of the best UV-blockage ability, but lowest toxicity.

3.1. FTIR analysis

To confirm the incorporation of PG into the PHO biopolymer and the successful preparation of PHOPG films (PHOPG1, PHOPG2 and PHOPG3), FTIR-ATR spectroscopy was employed. Spectra of the synthesized PHO, pure PG and the appropriate PHOPG films containing 0.2, 0.5 or 1.0 wt% of PG were collected, and the results are shown in Fig. 2. All absorption peaks inherent to a polyester build from monomeric units

(mixture of C6 and C8 chain lenght) were detected in the FTIR spectrum of PHO. In the PHO spectrum, medium bands appear at 2955 cm⁻¹, 2930 cm⁻¹, 2872 cm⁻¹ and 2859 cm⁻¹, corresponding to the C—H stretching modes of methine, methylene and methyl groups [51]; the most prominent absorption band at 1726 cm⁻¹ is attributed to the stretching vibration of the ester carbonyl group [52]; weak bands from 1467 to 1438 cm⁻¹ are attributed to the assymmetric deformations of the terminal methyl group and methylene groups; a weak band at 1379 cm⁻¹ comes from the symmetric deformations (bending and wagging) of the terminal methyl group; strong to medium bands at 1161–1097 cm⁻¹ originate from the asymmetric stretching of saturated ester (C–O–C) bonds; weak bands from 987 to 969 cm⁻¹ and at 725 cm⁻¹ are attributed to the skeletal vibrations of the methylene groups [53].

In the FTIR spectrum of PG, medium bands are present in the region from 3300 to 3000 cm⁻¹ which can be atributed to heteroaromatic N—H bond deformation vibration out-of-plane, while bands ranged from 2950 to 2850 cm⁻¹ corresponded to the –CH₂ stretching vibrations. Further, bands at 1634, 1607 and 1575 cm⁻¹ are atributed to aromatic –C=C– and –C=N– streching; bands at 1543 cm⁻¹ and 1510 cm⁻¹ are atributed to aromatic –C–C– vibrations in heterocycles; weak bands from 1470 to 1320 cm⁻¹ are attributed to the assymmetric deformations of the terminal methyl group and methylene groups; various medium bands from 1290 to 900 cm⁻¹ come from deformation vibrations in-plane of the aromatic = C–H and –C=C– bonds. The FTIR spectrum of PG confirmed the characteristic conjugated tripyrrole structure [54].

The spectra of the three PHOPG samples (PHOPG1, PHOPG2 and PHOPG3) include all mentioned bands from the PHO polymer, and only the PHOPG3 (containing 1.0 wt% of PG) has a small variation in the absorbance in the range from 1700 to 1500 cm⁻¹, as labeled in Fig. 3. The lack of the wavenumber shifts indicated that PG was not chemically bound to PHO, but only physically trapped inside the polymer. All other characterization techniques used in the present study confirmed the incorporation of PG into all three PHOPG1, PHOPG2 and PHOPG3 films, but changes in their FTIR-ATR spectra were hardly detectable due to the low percentage of PG used to produce formulations. In our previous work, significant changes in the FTIR spectra were only observed when PG loading was higher than 5 wt% [38].



Fig. 1. Overview of preparation of the PHO-PG films and BNC-PHO-PG formulation.



Fig. 2. FTIR analysis of: a) PG and PHOPG samples with increasing PG content and b) double-layer BNC-PHOPG material.

The formation of the double layer film was confirmed by comparing the pure BNC spectrum with the BNC-PHOPG2 double layered film (Fig. 2b). In the FTIR spectrum of BNC, the stretching vibration of the –O–H groups of the polymer is distinguished as a broad band from 3500 to 3200 cm⁻¹; the weak band at 3000–2800 cm⁻¹ is attributed to the cyclohexane stretching vibrations; medium bands at 1750–1500 cm⁻¹ and 1450–1200 cm⁻¹ are atributed to deformation in-plane –O–H vibrations; a variety of strong bands at 1200–750 cm⁻¹ are attributed to C—O streching vibrations of both primary and secondary hydroxyl groups, as well as the the asymmetric stretching of saturated ether (C–O–C) bonds od the biopolymer; out-of-plane –O–H vibration bands are visible below 750 cm⁻¹.

The iIncreased intensity of the bands at $3500-3200 \text{ cm}^{-1}$, as well as $1450-1200 \text{ cm}^{-1}$ in BNC-PHOPG2, suggest that free –OH groups are involved in the binding of PHOPG2 to BNC. Furthermore, the decreased intensity of bands at 1624, 1394 and 1369 cm⁻¹ and the flattening of the peak at 828 cm⁻¹ further implied the formation of hydrogen bonds

between PHOPG2 and the BNC biopolymer.

3.2. DSC analysis

The thermal properties of the prepared films were defined by DSC analysis and the recorded thermograms are shown in Fig. S1. The neat PHO had a single melting peak at 52 °C, while the melting peak of PG-containing formulations appeared at lower temperatures, ranged from 50.6 to 51.1 °C. The characteristic values estimated from the DSC analysis are listed in Table 2. The incorporation of PG into the biopolymer matrix disrupted the well-organized crystalline structure, which is reflected through the decrease in the PHOPG T_m values. The characteristic melting temperature of PG, expected at 200 °C [38], was not detected in the PHOPG films, pointing out that PG was incorporated in an amorphous form. Contrary to the observed melting temperatures, the estimated melting enthalpies suggested the enhancing effect of PG addition to PHO crystallization and higher values of ΔH_m for the



Fig. 3. a) XRD patterns of the PHOPG films, b) The average size of crystallites calculated for samples based on reflections at around 18.0°, 19.7° and 21.8° of 20.

PHOPG1 and PHOPG2 were observed. Regardless of PG affecting the PHO chain mobility and organization, this phenomenon was not gradual, i.e. a further increase in the PG content had no influence on the thermal properties (the formulation with the highest PG amount had lower $T_{\rm m}$ and $\Delta H_{\rm m}$ values compared to neat PHO). The degree of crystallinity, $X_{\rm c}$, for the prepared polymer films could not be precisely calculated according to the DSC data, due to the deficiency of a theoretical value for 100 % crystalline pure PHO [39].

3.3. XRD analysis

The XRD patterns gave an insight into the crystal structure of the prepared PHOPG films. From the presented XRD diffractograms, a structural similarity for all recorded samples was observed (Fig. 3a), with three characteristic Bragg peaks, 2θ , at around 18.0° , 19.7° and 21.8° . PHO, known as a semi-crystalline polymer, has three crystalline peaks typical for mcl-PHAs; 2θ at 18.0° corresponds to (120) plane, 2θ at 19.7° corresponds to (080) and the peak at 2θ of 21.8° is attributed to (140) plane [39,55]. A small, shoulder like peak, detected at 2θ value of 24.4° for PG-containing formulations might be attributed to the active

Table 2

The Differential scanning calorimetry (DSC) analysis results (melting temperature, T_m , melting enthalpy, ΔH_m) and X-ray diffraction (XRD) analysis results.

Sample	DSC			XRD		
	T _m , °C	$\Delta H_{\rm m}$, J/g	$X_{\rm c}^{\rm XRD}$, %	2 <i>θ</i> , [°]	$d_{ m hkl}$	FWHM ^a
PHO	52.0	9.8	51.6	18.000	4.928	0.570
				19.720	4.496	1.040
				21.790	4.076	0.830
PHOPG1	50.8	12.4	49.3	18.050	4.908	0.670
				19.760	4.490	1.150
				21.890	4.055	0.760
PHOPG2	51.1	10.2	47.3	18.060	4.895	0.660
				19.760	4.484	1.080
				21.862	4.062	0.760
PHOPG3	50.6	9.6	45.4	18.060	4.895	0.720
				19.719	4.498	1.070
				21.820	4.068	0.900

^a Full width at half maximum.

compound PG as was previously reported [38]. After the activation of PHO with PG, the shifting of the characteristic 2θ peaks at 18.0° and 21.8° to the lower values for PHOPG3 confirmed the contraction of the unit cell. This results directly points to the influence of the PG concentration on the crystallization properties of PHO, but also to the concentration limit (from 1 wt% and above) that can cause variations in the crystallization properties. Moreover, the average size of crystallites was calculated for characteristic reflections and the sizes of approximately 8–16 nm was observed. The introduction of PG into the PHO biopolymer reflected on the crystallite size and the crystallite sizes of all reflections was lower for the PG-containing formulations, which was associated with the decrease of the *d* distance, undoubtedly confirming the contraction of the crystal unit cell in PHOPG formulations.

The degree of crystallinity calculated from the XRD diffractograms for neat PHO was 51.6 %, while X_c values of films containing PG were lower (Table 2). Therefore, PHOPG1 with the lowest PG content had a degree of crystallinity of 49.3 %, while the highest concentration of the incorporated active compound in PHOPG3, indicated the lowest X_c value at 45.4 %,

3.4. Transparency and UV-barrier properties

Transparency is one of the key features of food packaging materials, while the packaging should also attract the attention of consumers, be transparent in the visible spectrum to allow visual inspection, and at the same time act as a UV-barrier to prevent photodegradation of thepacked products. To evaluate optical properties of the obtained PHOPG, their transmittance was measured in the UV (200–400 nm) and VIS (400–700 nm) range (Fig. 4). The most commonly used wavelength (600 nm) to define *T* values was selected as a midpoint of the visible range [56].

The addition of PG made the obtained samples attractively colored and the intensity of the color was proportional to the PG loading. Regarding the transparency in the visible spectrum, where transparency at 600 nm detected as the midpoint of the visible range (T_{600}) for this specific PHO/PG based materials, a decrease in the films' transparency was observed with the addition of active compound PG (20.0 \pm 0.15 for PHO, 19.1 \pm 0.09 for PHOPG1, 17.1 \pm 0.08 for PGOPG2 and 7.94 \pm 0.10 for PHOPG3), however, it did not affect the possibility to visually inspect the enclosed product. The decrease in T_{600} values of the PHOPG formulation was proportional to the concentration of the active compound, which can be correlated with the intensive coloration and also with the film thickness (the highest thickness was measured for the PHOPG3 formulation) as one of the highly affecting parameter [56]. Finally, PHO and the two formulations with 0.2 and 0.5 wt% of PG were clear and completely see-through, while the PHOPG3 film with the highest PG content was more opaque.

Considering that PG has UV-blocking properties, the potential of the film samples to act as a UV-barrier was also investigated. The presence of PG has decreased the transmission in the UV region, however, only the



sample with the medium PG loading (PHOPG2) has completely blocked the UV light, while samples PHOPG1 and PHOPG3 had a similar visual transparency, yet less effective thanPHOPG2. These results could point to the limit in the incorporated amount of the active UV-blocking agent that would affect and express the UV-blocking ability, but it should be taken into account that these differences were not pronounced between the films (approximate difference in *T*% was 5 %). The obtained results are in accordance with the findings from a study with *Vibrio* sp. where it was shown that PG acts as an UV screen and its protective role is correlated with the pigment concentration in the bacterial cell [30].

A dual role of PG as an appealing pigment with biological activity has attracted the attention in the field of food packaging. So far, several active, antimicrobial [34] and smart [35] food packaging materials have been developed. Moreover, finding new UV-blocking components or agents for biomaterials suitable for potential use in food packaging, opens new possibilities to develop alternative materials that may address the shortcomings of aluminum/metalized foils currently in use [57]. In this study, we have shown that PG can be used as an UV-barrier component of a potential food packaging material.

3.5. Cytotoxicity evaluations

The cytotoxicity assessment was performed by the MTT assay using the MRC5 cell line and filtered active material extracts. According to the results presented in Fig. S3, the pure PHO polymer, but also PHOPG1 and PHOPG2 formulations were not toxic at all tested material extract concentrations (100 %, 50 %, 25 % and 12.5 %), with the cell viability higher than 80 %. The incorporation of the active compound PG at the concentrations of 0.2 and 0.5 wt%, resulted in a similar cell viability as the treatment with pure PHO, while a higher concentration of the incorporated PG resulted in lower cell viability, but only for the cells treated with the highest concentration of the extract (100 %), which further served as an elimination factor in the PG-containing biopolymer formulation optimization. In addition, BNC alone and the final doublelayer PG-containing material was also investigated. While the BNC supported the cell growth at all tested concentrations, for the doublelayer BNC-PHOPG2 material, safety was confirmed for the concentrations of 12.5 and 25 %, while mild toxicity was observed at the tested concentration of 50 %. Finally, the viability of around 60 % was noticed for the highest extract concentration of BNC-PHOPG2, which could be explained by the hydrophilic-hydrophobic nature of the material that further initiated a higher PG release into the tested medium.

3.6. Film morphology

The solvent casting method was used to produce the PHOPG films. The obtained samples were uniform in terms of the overall appearance and color (no phase separation or pigment agglomeration was detected). In comparison to the control sample of neat PHO, the most obvious characteristic of the PG-containing films was their color, with an intensity that increased with the PG content.

In Fig. 5, the optical and SEM micrographs of the surface morphology and the cryo-fractured internal structure of the film samples are shown. It can be observed that, besides being colored, the samples with the incorporated PG also exhibit other morphological differences compared to PHO. The mmacroscopic spherulite biopolymer crystals were visible on the surface of all films, however, there was a difference in spherulites' sizes between the different formulations. The spherulites in PGcontaining samples appeared larger when compared to the pure PHO. The internal structure observed by cross-section SEM analysis indicated that PG-containing films appeared more structured and heterogeneous.

The morphology of the cross-section of the BNC and the double layer BNC-PHOPG2 films is presented in Fig. 6. The FESEM images of the blank BNC indicated the entangled fibrous network as expected [58], while after the top layer formation, the surface of the films became smoother, more compact and with a clearly visible layer-to-layer





Fig. 5. Morphology of the neat biopolymer (PHO) and the samples with incorporated prodigiosin (PHOPG1, PHOPG2 and PHOPG3): Optical micrographs at 1.6 \times magnification (first column). SEM micrographs of the samples surface (second column, 100 \times) and corresponding cross-section (third column, 600 \times).



Fig. 6. FESEM images of BNC and BNC-PHOPG2 cross section view at different magnification (500 \times and 5000 \times). The purple compartment (cones) point to the enlarged interface layer between BNC and PHOPG2.

microstructure. More importantly, the homogeneous and uniform morphology, with no delamination and disjunction of the layers, was observed by FESEM (the presented cross section captions of the double layer film with the interface layer emphasized by the purple cones on the enlarged FESEM images). No separation was detected between the BNC and PHOPG2 layers, indicating their good adhesion (Fig. S4). The natural adhesive properties without the need for additional adhesives were reported for the electrospun polymer materials [59]. The observed good adhesion between the hydrophilic BNC and the hydrophobic PHOPG could be explained by the possible formation of hydrogen bonds and intermolecular interactions between –OH groups available on the BNC surface and the PHOPG biomaterials [60].

3.7. Mechanical properties

The mechanical properties of the PHOPG films, but also BNC and BNC-PHOPG2 double layer films, were tested and the stress-strain curves are presented in Fig. S3a, while the estimated tensile strength, Young's modulus and elongation at break are summarized in Table 3. Young's modulus, referring to the stiffness or the rigidity of the material, was the lowest for PHO, while the incorporation of PG caused its increase. The highest values of the Young's modulus were calculated for the PHOPG2 (0.095 MPa), while after the further addition of the active compound, Young's modulus lowered, but it was still higher in comparison to PHO. The highest capacity before breaking (stress at break) was observed for the lowest incorporated amount of PG in PHOPG1. Finally, the elongation at break of neat PHO was the highest in the tested series at 425 %, whereas PG-containing films possessed significantly lower values that were directly proportional to the PG content (with the increase in PG wt%, the elongation at break increased). Conversely, the native BNC sample demonstrated an increased brittleness and a lower degree of hardness compared to all PHOPG films, with the tensile strength of 3.5 MPa(Fig. S3b), which was in line with previously reported studies [61]. It is important to note that the BNC was subjected to mechanical tests after freeze-drying, as lyophillization can significantly alter its mechanical properties. These properties are highly dependent on the bacterial strain used for its production, the composition of the culture medium and the method of drying, but as also influenced by whether it is tested wet in the form of a gel [62,63]. However, the formation of the double-layer BNC-PHOPG2 films led to improved mechanical properties, resulting in a more flexible and ductile material. Notably, the elongation at break of the novel material doubled compared to that of the BNC alone. Despite the mechanical properties of BNC-PHOPG2 not being on par with those of PHOPG films, the resulting double-layer material boasted the desirable and quite satisfactory mechanical characteristics for BNC-based materials. Additionally, it offered some enhanced benefits in terms of the UV-blockage and non-toxicity.

3.8. Hydrophilicity and wettability evaluation

Contact angle measurements were performed to estimate the hydrophilicity and the wettability of the prepared PHOPG polymer films surface, but also of the final double-layer BNC-PHOPG2 film material. Two different solvents were used, water as the polar and diiodomethane as the non-polar, and the measured contact angles are presented in Fig. 7, while the evaluated surface free energy parameters are shown in Table 3. The PHA-based polymers indicated a mostly hydrophobic surface nature with the water contact angles (WCAs) above 80° [64], while the WCA increased with the number of carbon atoms in the PHA monomer units, hence the PHO appeared as more hydrophobic in comparison to the PHBV [10]. The WCA of the PHO was close to 80°, indicating quite a hydrophobic nature of the biopolymer, but after the incorporation of the also hydrophobic active compound PG, the WCA gradually increased with the increase in the PG content, reaching the highest value of 90° for the PHOPG3 formulation. The combination of the two hydrophobic components PHO and PG resulted in a highly hydrophobic surface of the obtained films that could be explained by a synergistic effect of the good compatibility of the compounds and the formation of hydrogen bonds and intermolecular interaction, which are reflected on the wettability properties. Contrary to water, dropping the diiodomethane as a non-polar solvent resulted in the lower values of contact angles, ranged between 60 and 70°, but with not that prominent difference in the series considering the SD. Consecutively, the surface free energies of the PHO and PHOPG formulations were preferentially dictated by the contribution of the dispersive component γ_s^L , rather than by the polar component $\gamma_s^{SP},$ which was negligible for the PHOPG2 and PHOPG3 polymer films. More importantly, the double-layer BNC-PHOPG2 film possessed both hydrophobic and hydrophilic surface wettability, depending on the layer exposed to the tested solvents. By combining BNC as a hydrophilic biopolymer (WCA of $<20^{\circ}$ and high diiodomethane angle - above 60°) and PHOPG as a hydrophobic component, the formed double-layer material appeared to be highly hydrophobic on the PHOPG2 side (red one) with the WCA of 90°, while the first BNC layer implied hydrophilic surface properties. The obtained results are very promising in terms of creation of new packaging material with combined properties, which was the goal of the research.

3.9. PG migration into the food simulant media

Active food packaging materials devised to maintain or even improve the food quality and extend its shelf life can be generally classified as migratory and non-migratory. The efficiency of the migratory active packaging relies on the material's ability to release the active compounds [65]. To this end, the capability of the developed films to release PG into the food simulant media was evaluated. Aqueous ethanol solutions at concentrations of 10 %, 50 %, and 95 % (ν/ν) served as the simulant media. These percentages were selected to mimic the environments of hydrophilic substances (10 %), mixtures with both hydrophilic and lipophilic components, alcoholic mixtures, and oil-in-water emulsions (50 %), as well as substances with predominantly lipophilic characteristics and those with free fats on the surface (95 %). The test was performed by total immersion of the samples in the food simulants for 10 days at 20 °C, representing the worst foreseeable conditions of use during 3 to 30 days at temperatures between 5 and 20 °C [45,46].

All film samples preserved their integrity over the entire incubation period regardless of the media. The results in Table 4, however, showed that the media type did affect the release of PG from the films. No PG release from the PHOPG films occurred into 10 % ethanol. On the other hand, there was a PG release from the PHOPG films into 50 % and 95 % ethanol, which increased with the ethanol concentration of the media. As expected, a higher PG release occurred from the films with a higher incorporated PG content. As the release of the active compounds from the polymeric matrix also implies the solvent diffusion into the polymeric matrix, matrix relaxation and active compounds diffusion through the relaxed polymeric matrix, it could be influenced by the solvent polarity, solubility and affinity between the polymer, the active compound and the solvent [66,67]. Accordingly, the release of PG from the PHOPG films lines with the above-discussed contact angle measurement. The hydrophobic character of the PHO matrix, PG alone and the corresponding PHOPG films leads to their higher chemical affinity towards the less polar media. As a result, the less polar media, i.e. with higher ethanol contents, will have the higher ability to penetrate the PHO matrix and induce its relaxation, increasing the free volume of the polymeric chains and allowing PG release. The release behavior influenced by the surface hydrophilic/hydrophobic character is confirmed for the film formulation BNC-PHOPG2. Owing to the hydrophilicity of BNC, PG release from BNC-PHOPG2 was detected in 10 % ethanol. There was an increasing trend of PG release from BNC-PHOPG2 with the increase in the ethanol content of the media, which is in line with the results for the PHO films, as the hydrophobic character of PG promoted its release into the less polar media. However, the BNC hydrophilic layer decreased the PG release into the 95 % ethanol compared to the PHO film with the same PG content.

According to the Commission Regulation (EU) No 10/2011, PG is not among substances with a provided specific migration limit [45]. In that case, a generic specific migration limit of 60 mg/kg shall apply along with the risk assessment by the European Food Safety Authority. The PG release in all tested media was below this limit, being in line with the Regulation. The obtained results generally suggested the potential of the developed films for application as releasing active food packaging materials, especially for foods with less polar character, such as alcoholic

Table 3

Results of mechanical tests of PHOPG films and surface energy results (γ_s^{L} - dispersive, γ_s^{SP} - polar and γ_s - total surface free energy).

Sample	Young's modulus, MPa	Stress at break, MPa	Elongation at break, %	Surface free energy		
				γ_s^L , mJ/m ²	$\gamma_s^{SP},mJ/m^2$	$\gamma_s, mJ/m^2$
РНО	0.075 ± 0.01	7.83 ± 1.00	425.0 ± 48.9	21.1	10.2	31.3
PHOPG1	0.087 ± 0.01	8.61 ± 0.84	312.6 ± 45.4	24.5	6.83	31.3
PHOPG2	0.095 ± 0.01	$\textbf{7.15} \pm \textbf{0.43}$	324.8 ± 32.5	21.7	4.9	26.6
PHOPG3	0.083 ± 0.01	$\textbf{8.05} \pm \textbf{0.45}$	396.0 ± 14.4	27.0	3.1	30.1
BNC	_	3.49 ± 1.36	7.51 ± 1.56	11.9	60.3	72.2
BNC-PHOPG2	-	3.45 ± 0.50	14.5 ± 2.11	36.6	0.71	37.3



Fig. 7. Contact angle measurements of PHOPG films and BNC-PHOPG2 (** diiodomethane destroyed the BNC layer).

Table 4 Migration of PG from the film samples into the food simulant media: 10 %, 50 %, and 95 % aqueous ethanol solutions (ν/ν).

Sample	10 % ethanol	50 % ethanol	95 % ethanol
PHOPG1	n.d. ^a	0.10 ± 0.02 0.26 \pm 0.01	1.62 ± 0.38
PHOPG3	n.d.	0.20 ± 0.01 1.54 ± 0.41	4.17 ± 0.39 13.1 ± 1.78
BNC-PHOPG2	$\textbf{0.04} \pm \textbf{0.03}$	0.24 ± 0.02	1.55 ± 0.63

^a n.d. - not detected.

beverages, oil-in-water emulsions (for example, dressings, sauces), and fatty food products. Also, the performed test highlighted the ability to tailor the release of actives by forming bilayer films with different hydrophilic/hydrophobic surfaces.

A recent study has addressed the immobilization of PG onto BNC [37] and its biomedical potential including the antimicrobial and the antiadhesive abilities. Also, the multilayer PVA-BNC-PG formation produced by electrospinning the hydrophilic PVA-chitosan layer was also suggested as a potential active packaging material [34], while the BNC-PHBV composite layered material with improved barrier properties using different plasticizers was also recognized as a material of interest in food packaging [68]. Despite the BNC-PHAs type of the material as a candidate has already been investigated for packaging applications, no reports addressing the migration tests and food tests have been published so far. In our study, novel materials with excellent properties were obtained without any plasticizers, further adding to their sustainability and safety.

4. Conclusion

The presented research effectively developed a double-layer, UVblocking, non-toxic biopolymer film comprising of PHO, BNC and the sustainably produced active compound PG through a layer-by-layer deposition method and without the use of plasticizers. The preliminary optimization of the PHOPG formulation to determine the ideal PG concentration was essential for attaining the targeted properties, such as significant UV-blocking capability and the absence of toxicity. The manufacturing process validated the material's dual functionality, as indicated by the WCA analyses, that highlighted the distinct hydrophilic/hydrophobic character of the double-layer film. Additionally, the evaluations of mechanical properties showed improvements of BNC in the BNC-PHOPG2 configuration. The investigation illustrated the regulated release of PG into a variety of food simulants, exhibiting a selective release behavior that preferred more lipophilic environments. These outcomes emphasize the role of PG as a critical component in the UV-blocking PHA-BNC based formulations, paving the way for the development of novel materials for the packaging technologies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2024.135087.

Funding

This research was funded by financial support from the Science Fund of the Republic of Serbia, IDEJE (No 7730810, BioECOLogics), European Union's Horizon 2020 Research and Innovation Programme (grant agreement No 870292, BioICEP) and European Union's Horizon Europe EIC Pathfinder programme (grant agreement No 101046758, EcoPlastiC).

CRediT authorship contribution statement

Ivana Malagurski: Writing – original draft, Validation, Methodology, Formal analysis. Jelena Lazic: Writing – original draft, Validation, Formal analysis. Tatjana Ilic-Tomic: Writing – original draft, Methodology, Formal analysis. Ana Salevic: Writing – original draft, Methodology, Formal analysis. Maciej Guzik: Writing – review & editing, Writing – original draft, Methodology, Formal analysis. Maciej Guzik: Writing – review & editing, Writing – original draft, Methodology, Formal analysis. Jasmina Nikodinovic-Runic: Writing – review & editing, Writing – original draft, Methodology, Formal analysis. Jasmina Nikodinovic-Runic: Writing – review & editing, Writing – original draft, Supervision, Conceptualization. Marijana Ponjavic: Writing – review & editing, Writing – original draft, Validation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Data availability

The data presented in this study are available on request from the corresponding author.

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