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Biodegradable zein/PEG nanofibers incorporated with natural antimicrobial compounds for eco-friendly food packaging

Pavel Pleva [a], Lucie Bartošová [a], Magda Janalíková [a], Martina Polášková [c,d], Alena Opálková Šišková [e,f], Lucie Matošková [b], Ondřej Krejčí [c], Jana Sedlaříková [b,*]

[a] Department of Environmental Protection Engineering, Faculty of Technology, Tomas Bata University in Zlín, Vavreckova 275, Zlín 760 01, Czech Republic
[b] Department of Fat, Surfactant and Cosmetics Technology, Faculty of Technology, Tomas Bata University in Zlín, Vavreckova 275, Zlín 760 01, Czech Republic
[c] Department of Polymer Engineering, Faculty of Technology, Tomas Bata University in Zlín, Vavreckova 275, Zlín 760 01, Czech Republic
[d] Centre of Polymer Systems, Tomas Bata University in Zlin, Trida Tomase Bati 5678, Zlín 760 01, Czech Republic
[e] Polymer Institute of the Slovak Academy of Sciences, Dubravska cesta 9, Bratislava 845 41, Slovakia
[f] Institute of Materials and Machine Mechanics of the Slovak Academy of Sciences, Dubravska cesta 9, Bratislava 845 13, Slovakia

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ABSTRACT

Nanofibrous zein/PEG based membranes incorporated with natural antimicrobial compounds were fabricated by electrospinning method. Structural and thermal analysis of prepared nanofibers revealed that the applied processing technique did not significantly affect the structure of pristine zein polymer. Morphological characterization showed a higher degree of polydispersity in the fibers modified with eugenol, thymol, nisin, or their combinations, and an average fiber diameter in the range from 300 to 390 nm. Nanofibrous samples with eugenol and thymol prevented the growth of *Escherichia coli* and *Staphylococcus aureus*, while the nisin or its mixtures with phenols proved a high antibacterial effect against Gram-positive *Listeria ivanovii*. Zein/PEG membranes with bioactive molecules significantly eliminated biofilm formation, with the most pronounced effect of zein/PEG/Eug/Thy combination. Biodegradability testing of bioactive membranes revealed no significant slowdown of degradation process in comparison to control sample. Zein/PEG hydrophilic nanofibers enriched with phenol/nisin combinations demonstrated a high potential for development of sustainable packaging to improve the shelf-life and quality of foods.

Introduction

Food quality and safety are the key parameters directly affecting the health of consumers. Several strategies have been developed to prevent microbial spoilage and biofilm formation. Antimicrobial natural compounds can be incorporated to ensure the prolonged shelf-life of foods. Essential oils belonging to the complex, volatile mixtures based on terpenoid, phenolic and aromatic structures have significant biological properties [1–3]. Eugenol and thymol, as the basic components in clove and thyme oil, respectively, have been applied in different carrier systems for their antioxidant, antibacterial and antifouling properties. Nisin is a low molecular weight bacteriocin produced by *Lactococcus lactis* strains, and it is effective primarily against Gram-positive microorganisms, such as *Listeria monocytogenes*. This bioactive peptide molecule is FDA-approved for food products and is often used to enhance their shelf life [4]. Worse stability and activity in an alkaline environment can limit the practical applications of nisin, which could be improved by its combination with other antimicrobials. The mixtures of nisin and essential oil components have been studied by Hossain et al., who investigated their effects against *Listeria monocytogenes* biofilm on food-contact surfaces [4]. However, the potential synergic properties of these compounds incorporated in biopolymer-based carriers have not been previously investigated.

Zein belongs among the main maize proteins containing about 50 % of total protein concentration, rich in non-polar amino acids, including leucine, isoleucine, proline, alanine, valine, etc [5]. Zein has been investigated due to its beneficial characteristics, including biodegradability, biocompatibility, and non-toxicity. This versatile material represents an ideal candidate as a carrier of hydrophobic bioactive compounds applied in the biomedical or food industry. Zein can be processed easily in the form of coatings, nanoparticles, nanoribbons, or nanofibers [6]. Nanofibers have received much attention for their

* Corresponding author.
E-mail address: sedlarikova@utb.cz (J. Sedlaříková).

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Table 1
Designation of prepared zein/PEG-based samples with incorporated active compounds.

Sample	Concentration of active compounds
Zein/PEG	-
Zein/PEG/Eug	2 wt% eugenol
Zein/PEG/Thy	2 wt% thymol
Zein/PEG/Nis	2 wt% nisin
Zein/PEG/Eug/Nis	2 wt% eugenol and 2 wt% nisin
Zein/PEG/Thy/Nis	2 wt% thymol and 2 wt% nisin
Zein/PEG/Eug/Thy	2 wt% eugenol and 2 wt% thymol

application in active food packaging due to their high specific surface area, high porosity, and high loading capacity of active substances. Electrospinning is the most commonly used method to produce nanofibers. In this technique, polymers in the form of a solution or melt are formed into continuous fibers by applying an electric field [7,8].

Zein nanofibers, however, suffer from poor ductility and high brittleness as they are prepared in alcohol solvents, which limits the zein protein unfolding and following tighter packaged structure formation [6]. Thus, combining other polymers and plasticizers is recommended to optimize their mechanical and physico-chemical properties [9]. Polyethylene glycol (PEG), as a hydrophilic, water-soluble, non-toxic material, can be applied to improve the physical stability of zein and the functionality of the resultant membrane [10]. Moreover, EFSA 2006 concluded that consuming polyethylene glycol (PEG 6000) and its use as a plasticizer in the film-coating formulations for food supplement tablets or capsules is not of safety concern [11]. Polyethylene oxide-based/biopolymer systems were produced and characterized to obtain diverse types of smart food packaging systems. Curcumin loaded chitosan/PEO nanofibers were formulated by Yildiz et al. to monitor the freshness of chicken meat during storage. Zein/polyethylene oxide-based blends incorporated with nisin were fabricated in the study of Yu et al., which prevented microbial growth in chicken breasts. Neither of these studies has focused on investigating the complex blend nanofibrous system based on zein and incorporated phenol/antibacterial peptide mixtures [12,13].

The aim of this study was to prepare novel zein/PEG-based nanofibrous membranes enriched with food-safe bioactive compounds by a versatile, low-cost electrospinning technique and to investigate their potential for eco-friendly sustainable active food packaging.

Materials and chemicals

Zein and polyethylene glycol 6000 (PEG) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Ethanol (EtOH) was supplied from Lach-Ner (Neratovice, Czech Republic). Antibacterial compounds - thymol (Thy), eugenol (Eug) and nisin A from *Lactococcus lactis* (Nis) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification.

All used bacterial strains (Gram-negative bacteria *Escherichia coli* ATCC 25922 and Gram-positive bacteria *Staphylococcus aureus* ATCC 25923 and *Listeria ivanovii* ATCC 49954) were supplied by Czech Collection of Microorganisms (Brno, Czech Republic). They were cultivated on Mueller Hinton agar (MH) and MH broth (both HiMedia, Mumbai, India) at 37 °C/24 hours.

For cytotoxicity assay, the mouse embryonic fibroblasts NIH/3T3 cell line (ATCC CRL-1658 NIH/3T3, Marlboro, MA, USA) were used. The compound 3-(4,5-Dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was sourced from Calbiochem (Merck Millipore, Darmstadt, Germany).

Methods

Preparation of polymer solutions

Firstly, zein solution was prepared at a concentration of 30 wt% and dissolved in 80 % EtOH at laboratory temperature using a magnetic stirrer Heidolph (Schwabach, Switzerland) (500 rpm, 2 h). It followed with the preparation of PEG solution at a concentration of 20 wt% in 70 wt% ethanol at laboratory temperature using the magnetic stirrer (500 rpm, 1 h). These solutions were mixed in a ratio of 5:1 (zein:PEG). Subsequently, potential antibacterial compounds thymol, eugenol, nisin and their combinations were added into polymer solutions to their final concentrations of 2 wt%, see Table 1.

These compounds were quantitatively added to homogeneous, clear, viscous zein/PEG solutions and stirred on a magnetic stirrer Heidolph (250 rpm, 25 °C, 30 min). Seven samples of zein/PEG solutions with incorporated compounds were prepared.

Electrospinning

Electrospinning was conducted under ambient conditions (temperature of 23 ± 1 °C and a relative humidity of 39 ± 1 %). The setup adopted a horizontal spinning configuration, and the voltage was supplied by a Spellman SL-150W high-voltage power supplier with positive polarity, set at 15 kV. The solution was delivered using a single-syringe ERA Pump System, model NE-1000, at a feeding rate of 1 $mL.h^{-1}$. A flat–end needle with a 0.8 mm inner diameter was employed for mat preparation, maintaining a working distance of 12 cm between the needle's top and the aluminum collector.

Scanning Electron Microscopy (SEM)

The morphology of the prepared nanofibers, antibiofilm activity and biodegradability were observed by scanning electron microscope (SEM), JEOL JSM-6610 (Tokyo, Japan) at accelerated voltage 15 kV. The samples were coated with a thin conductive gold layer by sputter coater Balzers SCD 040 (Balzers Union Limited, Balzers, Liechtenstein). The average diameter of fibers was assessed by the Image J software (LOCI, University of Wisconsin, Madison, WI, USA) from 270 measured values.

Fourier Transform Infrared Spectroscopy (FTIR)

The chemical composition of the prepared films was analyzed using Fourier transform infrared spectroscopy (FTIR) conducted on a Nicolet 6700 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The instrument was set to attenuated total reflectance (ATR) mode and featured a diamond crystal equipped with OMNIC Paradigm software. The measurement conditions involved 64 scans at a resolution of 2 cm^{-1} within the range of 4000–400 cm^{-1}.

The wettability of zein/PEG nanofibers

The surface properties of zein/PEG nanofibers were investigated using the sessile droplet method on an Attension Theta tensiometer (Biolin Scientific, Västra Frölunda, Sweden) coupled with the OneAttension software under ambient conditions. Distilled water, with a droplet volume of 3 μL, served as the reference liquid. The values of the nanofibers' wettability were evaluated after 3 s of a droplet contact with the sample surface. All measurements were conducted at least in triplicate.

Differential Scanning Calorimetry (DSC)

The thermal analysis was performed using differential scanning calorimetry on a DSC 700/1 device (Mettler Toledo, USA) placed in the temperature range of 0 °C to 200 °C at a heating rate of 10 °C/min under N_2 atmosphere; the samples weighed approximately 5 mg. The results

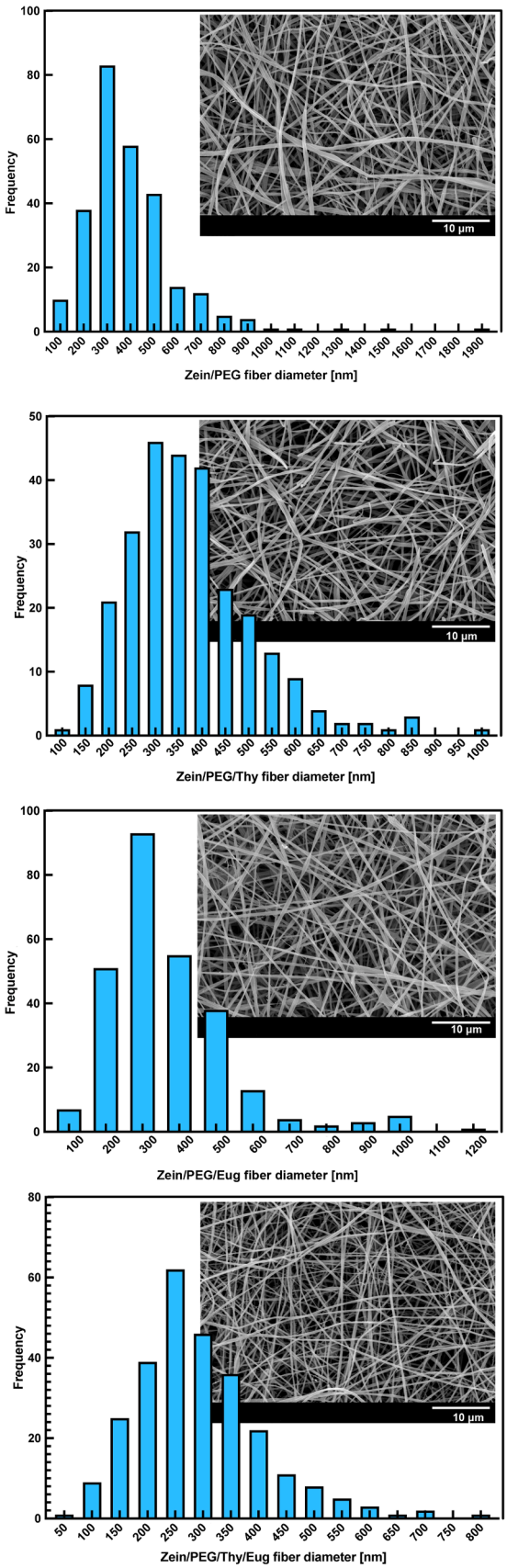

Fig. 1. SEM images and diameter distribution curves of zein/PEG nanofibers.

were evaluated from the first heating cycle of the samples.

Thermogravimetric analysis

Thermogravimetric analysis was performed on a STA 449F1 Jupiter (Netzsch, DEU). Measurements were performed in a nitrogen atmosphere at the flow rate of 50 mL/min with temperature ranging 25 ± 400 °C. A typical heating rate of 10 K/min was applied. The sample weight ranged from 4 mg to 8 mg.

Antibacterial activity—disk diffusion method

The antibacterial activity of the nanofibers was assessed against *Escherichia coli*, *Staphylococcus aureus* and *Listeria ivanovii* by disk diffusion method. At first, the disks (9 mm in diameter) were cut from the fabricated nanofibers. One mL of a 0.5 McFarland bacterial suspension in saline solution was spread over the entire surface of MH plate. The excess suspension was removed, and the plate was let to dry. Then the disks of nanofibers were carefully placed on the Petri dishes. All dishes were then incubated at 37 °C for 24 hours. The antibacterial activity was determined by ruler measuring the diameter of an inhibition zone including the disk. The whole experimental procedure was repeated in triplicate.

Antibiofilm activity

The biofilm formation was determined in glass tubes containing 3 mL of BHI broth (Brain Heart Infusion, Himedia, Mumbai, India) enriched with 5 wt% sucrose (Himedia, Mumbai, India), which were inoculated by 60 µL of bacterial suspension (0.5 McFarland turbidity). The nanofiber disks, along with one strain of tested bacteria (*E. coli*, *S. aureus*, *L. ivanovii*), were incubated at 37 °C for 72 hours. Following incubation, the nanofiber samples were thoroughly rinsed by sterile distilled water to remove planktonic cells. Antibiofilm activity test was performed using fluorescence microscopy according to Pleva *et al.* (2022) and it was evaluated according to McFadden et al. (2023) [14,15]. This measurement was complemented by scanning electron microscopy using a JEOL JSM-6610 microscope (Tokyo, Japan) at accelerated voltage 15 kV. After withdrawal, rinsing and dehydration, the sample was placed on a target and frozen (-80 °C). The samples were coated with a thin conductive gold layer by sputter coater Balzers SCD 040 (Balzers Union Limited, Balzers, Liechtenstein).

The nanofiber disks after cultivation with bacteria and rinsing with water were transferred onto a glass slide, stained with fluorescent dye (SYTO 9 and propidium iodide) for 10 seconds and covered with a square coverslip. The Olympus BX53 (Olympus, Tokyo, Japan) fluorescence microscope equipped with the Microscope Digital Camera DP73 (Olympus, Tokyo, Japan) and the Cell Sens Standard 1.18 (Olympus, Tokyo, Japan) software was used for analysis. This method relies on the absorption of live (green) and dead (red) bacterial cells based on the dye's interaction with the cell membrane. Fluorescence microscopy extends the optical capabilities of light microscopy to intrinsic or added fluorescent light emission [16]. The ImageJ software, developed by the National Institutes of Health (NIH, U.S.), was utilized to measure the areas covered by green and red fluorescence. The result was expressed as percentage of live/dead cells after direct counting, which was carried out according to modified procedure by McFadden et al. (2023) [15]. The results were related to the control, in terms of the percentage decrease in biofilm formation compared to the bacterial growth and biofilm production on zein/PEG nanofibers without added antimicrobial components.

Cytotoxicity of Zein/PEG nanofibers

Samples were tested according to ISO 10993–5. Cytotoxicity of the extract was measured using an MTT assay and an NIH/3T3 cell line was used. On the first day of the experiment, cells were seeded in a

Table 2
The mean diameter of zein/PEG nanofibers.

Sample	Mean ± SD [nm]
Zein/PEG	400 ± 210[a]
Zein/PEG/Eug	370 ± 180[a]
Zein/PEG/Thy	380 ± 140[a]
Zein/PEG/Nis	310 ± 150[b]
Zein/PEG/Eug/Nis	320 ± 160[b]
Zein/PEG/Thy/Nis	390 ± 150[a]
Zein/PEG/Eug/Thy	300 ± 120[b]

concentration of 5×10^5 cells per mL of cultivation medium. All samples were cut into squares with an area of 12 cm^2 and covered with 2 mL of medium. Samples were put into Incucell for 24 hours at 37 °C. After 24 hours, extracts were filtered and added to cells at different concentrations (100 %, 75 %, 50 %, 25 %, 10 %, 1 %). Cells with extract were incubated for 24 hours, at 37 °C in humidified air. MTT assay was accomplished on the third day of the experiment. The cultivation medium with extract was changed with 90 µL of fresh medium and 10 µL of MTT diluted in H_2O at a 5 mg/mL concentration. Absorbance was measured after 4 hours (measured absorbance at 570 nm, reference 690 nm). Results were evaluated as relative cell viability depended on the used extract concentration. A cytotoxic effect is observed if relative cell viability is lower than 0.7.

Biodegradability of zein/PEG nanofibers

In this study, aerobic composting biodegradation tests were conducted using a modified method based on ISO 14855 [17,18,19] The tests were carried out in 500 mL biometric flasks equipped with septum stoppers. The substrate for these experiments consisted of mature commercial garden compost Argo (Argo CS a.s., Česká Skalice, Czech Republic). The biodegradation process occurred at a temperature of 58 ± 2 °C. Each flask was filled with 2.5 g of dry-weight compost, 1.5 g of perlite, and 1 mL of mineral medium. The water content of the substrate mixture was adjusted to 50 % by the addition of sterile water. Each sample flask received 100 mg of the sample, cut into 5 × 5 mm fragments. Three flasks were prepared for each sample, along with three additional reference flasks and four blank flasks. Cellulose (Sigma Aldrich, USA) served as a reference standard. The CO_2 produced in the blank flasks was consistently subtracted from the sample measurements to determine the net sample mineralization. Throughout the experiment, headspace gas samples were periodically collected through the septum using a gas-tight needle and were then analyzed using a mass spectrometer HPR-40 DSA (HIDEN Analytical, 2020, Warrington, United Kingdom) to quantify the amount of released CO_2.The mineralization percentage (D_t) was calculated as:

$$D_t = \frac{(CO_2)_t \times (CO_2)_b}{ThCO_2} \quad (1)$$

where $(CO_2)_t$ is the accumulated CO_2 released by each sample, $(CO_2)_b$ is the accumulated CO_2 released by the blank flasks, and $ThCO_2$ is the theoretical CO_2 from the sample. For each carbon content sample, a flash elemental analyzer 1112 (Thermo Fisher Scientific, Waltham, MA, USA) was used. For the biodegradability determination, a scanning electron microscopy was performed as in case of the antibiofilm activity testing.

Statistical data analysis

The results of zein/PEG-based nanofibers characterization (fibers diameters and wettability) and determination of antibacterial activity were presented as mean ± standard deviation (SD). Statistical analysis was performed by one-way ANOVA followed by a Tukey test, utilizing Statistica software version 10 from StatSoft, Inc. (Tulsa, OK, USA), with a significance level set at $p < 0.05$.

Results and discussion

Morphology of zein/PEG nanofibers

The morphology of prepared zein/PEG and zein/PEG/active molecules based nanofibrous structures is illustrated in Fig. 1 and Fig. S1. The mean fiber diameter of the zein/PEG sample was 400 nm, while the addition of various active molecules or their combination caused the reduction of the final average fiber diameter, ranging from 300 to 390 nm (Table 2). The incorporation of active substances into polymer solution can influence the charge density leading to the change in fibrous morphology, which was also supported by SEM analysis (Figs. 1, S1). The lowest fiber diameter was observed with the combination of eugenol and thymol (Fig. 1, Table 2). In zein/PEG membrane containing no active molecules, a continuous beadless homogeneous structure was observed. On the other hand, a more diverse polydisperse structure with a number of smaller fibers appeared in membranes modified with eugenol, thymol, nisin, or their combinations. The conditions during the electrospinning process, such as voltage, collector distance, and the physicochemical properties of electrospun solutions should also be considered principal factors affecting the final size of fibers in the membrane structure [20,21].

Fourier Transform Infrared Spectroscopy (FTIR)

In the spectrum of the zein/PEG membrane (Fig. 2), the peaks characteristic for zein polymer predominate that were shown at 3290 cm^{-1}, 1647 cm^{-1}, 1535 cm^{-1}, corresponding to -NH_2, C═O, and N-H, C-N, respectively. A random coil conformation characteristic for zein structure was confirmed by the peak at 1647 cm^{-1} ascribed to C═O stretching of primary amide [21]. Modification of zein/PEG membranes with eugenol, thymol, nisin, or their combinations did not reveal any considerable structural variations, except for the minor peaks in the range from 1720 to 1870 cm^{-1} and 3560–3650 cm^{-1}. The absence of

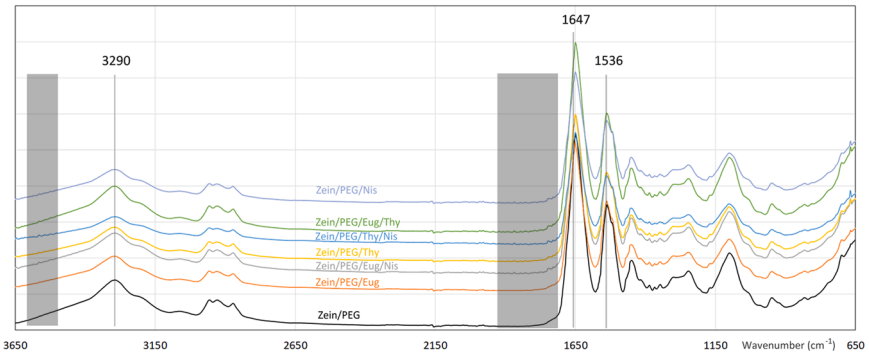

Fig. 2. FTIR spectra of the zein/PEG-based nanofibers.

Table 3
Contact angles for zein/PEG nanofibers.

Sample	[°]
Zein/PEG	49.7 ± 2.1[a]
Zein/PEG/Eug	36.7 ± 3.6[a, b, c]
Zein/PEG/Thy	33.8 ± 11.7[b, c]
Zein/PEG/Nis	46.3 ± 6.1[a, b]
Zein/PEG/Eug/Nis	40.9 ± 8.1[a, b, c]
Zein/PEG/Thy/Nis	39.7 ± 8.3[a, b, c]
Zein/PEG/Eug/Thy	32.8 ± 12.3[c]

changes in the frequency of peaks or their absorbance intensities indicates that original chemical structure of the polymer was preserved after the applied electrospinning process and incorporation of bioactive molecules.

The wettability of zein/PEG nanofibers

Different letters in the column indicate significant differences between the samples ($p < 0.05$).

The wettability of the samples was measured by the sessile drop method. It is known that zein polymer belongs among the hydrophobic materials, due to the high ratio of hydrophobic groups in this structure [21,22]. As can be seen from the data in Table 3, all the contact angles were below 90° indicating wettable surfaces, presumably as the result of the addition of a hydrophilic PEG to zein polymer. Zein/PEG system with no active substances exhibited the value of 50°, which is lower compared to the result (65°) obtained for the zein/PEO blend based electrospun membrane in the study of Surendranath et al. [6]. The contact angle values are strongly dependant on many factors including the fiber diameter, porosity, roughness and capillary action since each surface inequality of the membrane can act as the capillary promoting decline or increase of the liquid [21]. Considerable variations in contact angle values in time could also be expected regarding the nonhomogeneous structure of nanofibrous surface. The authors Santos et al. presented a significant drop of contact angle values of zein fibers from initial 94° to 26°after 3 s of the droplet contact with the measured surface [21]. The incorporation of active molecules into polymer matrix resulted in further decline of the contact angles, revealing a higher hydrophilicity. It can be the consequence of the disturbed fiber structure after the modification with active molecules, which was documented by the SEM analysis (see Fig. 1). Similarly, in the study of Santos et al. [21], an increase in hydrophilicity of zein fibers with the incorporation of phenolic Jambolan extract was reported. Several hydrophilic polymers, based on PEG, polysaccharides or some zwitterionics, have been developed. These materials proved the ability to bind water molecules instead of microorganisms, resulting in the reduction of biofouling process. An enhanced wettability of membranes could be also applied for better interactions of incorporated active molecules in the functional packaging [23,24].

Differential scanning calorimetry of zein/PEG nanofibers

DSC technique was used to analyse both reversible and irreversible thermal transitions of pristine materials and their combinations in samples prepared by the electrospinning process. Fig. 3 shows their thermograms obtained from first heating scan. In the case of pure zein powder, the denaturation (T_d) and glass transition temperature (T_g) can be detected. It is equal to 84 °C and 157 °C, respectively, which is in good accordance with the literature [25,26,6]. Concerning the neat PEG powder, the endothermic peak at 65 °C can be observed, representing the melting of the crystalline phase. This semicrystalline polymer is very often used in combination with natural polymers in the electrospinning process to reduce the process instability and broaden thus the processing window also to modify the resulting physical and mechanical fiber properties [27].

Then the zein/PEG thermograms that are composed by zein/PEG mixture enriched with antibacterial agents - thymol, nisin, eugenol and their combinations, can be compared. Concerning the fibers based just on the zein/PEG, with the absence of bioactive compounds, the denaturation peak around 90 °C with a shoulder that can be ascribed to the melting of PEG phase, can be seen. Thus, both polymers behave independently maintaining their thermal characteristics. The presence of a denaturation peak proved that the processing conditions for nanofibrous membranes fabrication, involving the preparation of solutions and the electrospinning process itself, does not destroy the native structure of the zein and provides structure that is even more thermally stable.

Additionally, Tg of 166 °C is observed, followed by a relaxation peak. The approximately 10 °C increase in Tg and the presence of the relaxation peak are likely linked to the rapid nature of electrospinning process, which immobilizes polymer chains in nonequilibrium state, potentially due to chain orientation and fast solvent evaporation.

In the case of fibers with a complex composition containing several types of antibacterial agents and their combinations, only the

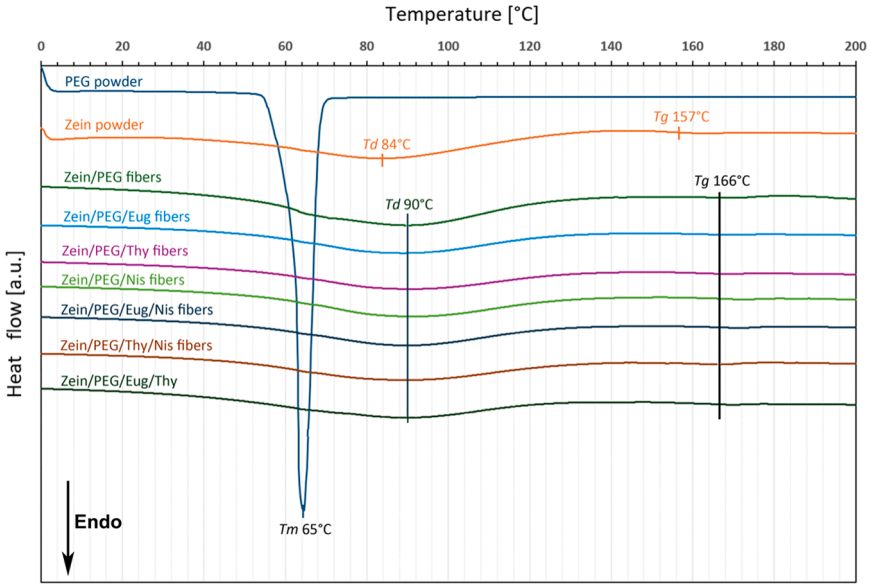

Fig. 3. Thermograms of pristine zein and PEG powders and fibers prepared by electrospinning process.

Table 4
Characteristic parameters of TGA analysis pertaining to the neat materials and prepared fibers.

Sample	T_{onset} (°C)	T_{max} (°C)
Zein	237	316
Thymol	96	162
Eugenol	109	191
Nisin	-	-
PEG	336	-
Zein/PEG	237	319
Zein/PEG/Thy	237	316
Zein/PEG/Eug	237	318
Zein/PEG/Nis	237	319
Zein/PEG/Eug/Nis	236	318
Zein/PEG/Eug/Thy	236	319
Zein/PEG/Thy/Nis	236	315

-not detected.

Table 5
The values of inhibition zones (mm) for tested bacteria (nanofiber disk diameter is 9 mm).

Sample	*E. coli*	*S. aureus*	*L. ivanovii*
Zein/PEG	ND^a	ND^a	ND^a
Zein/PEG/Eug	10.2 ± 0.4^b	10.5 ± 0.3^b	ND^a
Zein/PEG/Thy	9.5 ± 0.3^c	9.7 ± 0.1^b	ND^a
Zein/PEG/Nis	ND^a	ND^a	14.0 ± 0.3^b
Zein/PEG/Eug/Nis	9.7 ± 0.2^c	10.1 ± 0.3^b	15.2 ± 0.4^c
Zein/PEG/Thy/Nis	ND^a	ND^a	19.9 ± 0.7^d
Zein/PEG/Eug/Thy	9.4 ± 0.2^c	9.3 ± 0.2^c	ND^a

ND – nondetectable. Different letters in the column indicate significant differences between the samples ($p < 0.05$).

denaturation peak followed by the glass transition of zein was detected. Indeed, the addition of antibacterial agents suppresses the crystallization of PEG, since its melting peak is not apparent, and does not have a significant effect on the resulting thermal properties of zein.

Thermogravimetric analysis of zein/PEG nanofibers

Comparison was made as to the thermal behaviour of neat materials and fibers. Table 4 lists data on characteristic temperatures. The extrapolated onset temperature (T_{onset}), defined as the intercept of extrapolated starting mass with the tangent applied to the maximum slope of the TG curve, and T_{max}, corresponding to the temperature of the peak maximum on the DTG curve. Similar decomposition behaviours were observed for all prepared fiber mats with T_{onset} around 237 °C, which is similar to the thermal stability of pure zein powder. Analogously the T_{max} is detected, which means that the rate of degradation of the individual samples is almost the same, because their T_{max} is in the range of 315 – 320 °C. The lowest thermal stability was found for two types of antibacterial agents, namely thymol and eugenol. The third antibacterial agent, nisin, shows the highest thermal stability of all materials used, because even at 400 °C there was no noticeable weight change. Considering the characteristic temperatures determined, it seems that the antibacterial agents used have negligible effect on the thermal stability of the fibers. Indeed, the thermal stability is controlled by the zein used.

Antibacterial activity—disk diffusion method

The antibacterial activity of prepared samples gained by disk diffusion method against Gram-negative bacteria *Escherichia coli*, and Gram-positive bacteria *Staphylococcus aureus* and *Listeria ivanovii* are displayed in Table 5. Statistically significant results compared to the control Zein/PEG sample were observed in the case of all samples with bioactive compounds except zein/PEG/Nis and zein/PEG/Thy/Nis against *E. coli* and *S. aureus*. Only nanofibers with incorporated nisin were strongly active against *L. ivanovii*.

Active molecules of essential oils, such as thymol and eugenol, are referred to have widespread antibacterial activity against different microorganisms [1]. On the other hand, nisin, as a natural bacteriocin produced by *Lactococcus lactis*, shows antibacterial activity against a majority of Gram-positive foodborne bacteria [28].

The study of Han et al., 2017 [29] investigated the antimicrobial properties of the coaxial fibers based on polyvinylpyrrolidone (PVP)/nisin coated by hydrophobic poly(ε-caprolactone) shell. The results revealed the antimicrobial activity was decreased by 3 log orders of magnitude after further 24 hours. Therefore, the materials modified with nisin are more suitable for applications requiring the more significant release in the initial phase. The release of nisin and inhibiting *Listeria ivanovii* strain was proved in our study.

The resistance mechanism exhibited by microorganisms against nisin includes cell wall thickening, change in surface charge, changes in membrane phospholipid and fatty acid composition, enzymatic degradation of nisin, DNA mutation, and differential gene expression, especially in Gram-positive bacteria such as *S. aureus* or *Listeria monocytogenes* [30].

Functional nanofibers with incorporated eugenol, thymol, and their combination showed significant antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. What more, nanofibers enhanced with nisin and its combination with thymol and eugenol exhibited high antibacterial activity against the Gram-positive bacteria *Listeria ivanovii*. Zein/PEG/Eug nanofibers were most efficient against *E. coli* (10.2 ± 0.4 mm) and *S. aureus* (10.5 ± 0.3 mm), whereas no inhibition was found against *L. ivanovii*. Elsewhere, eugenol was successfully

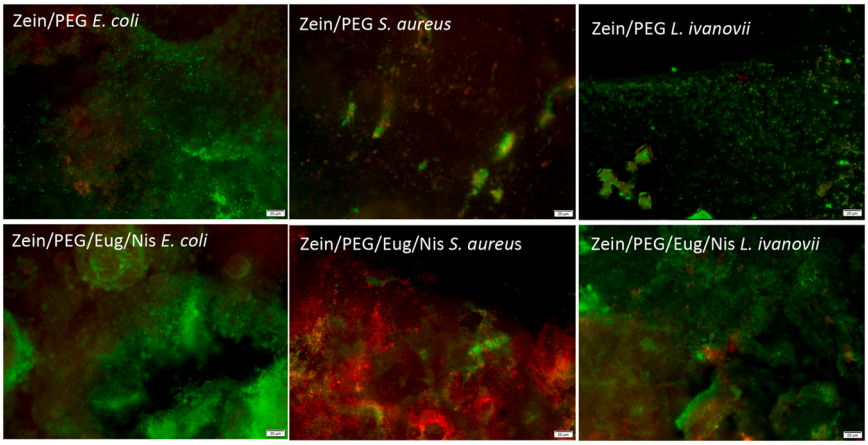

Fig. 4. Biofilm formation evaluation for zein/PEG nanofibers after 72 h.

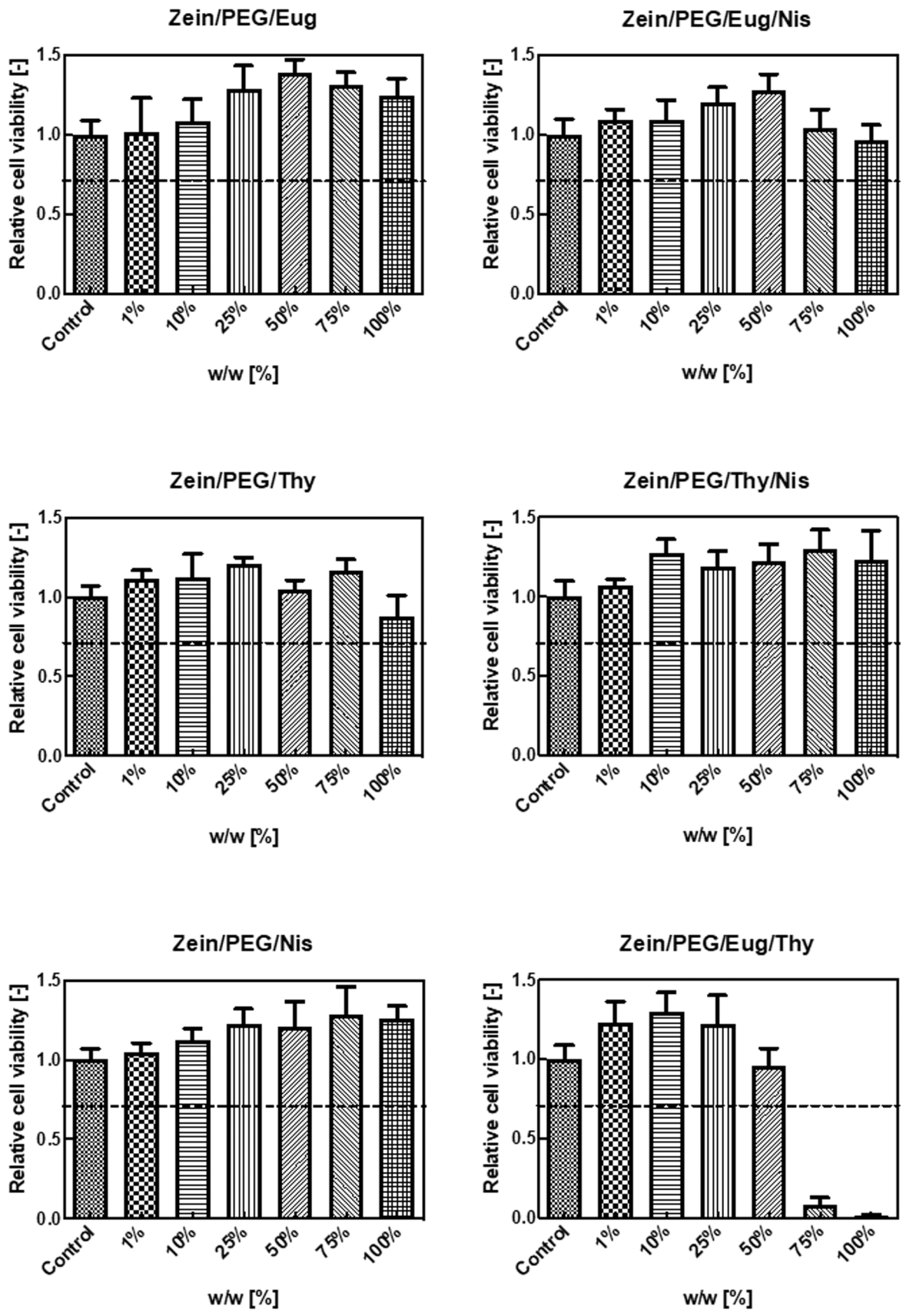

Fig. 5. Relative cell viability for zein/PEG-based nanofibers.

incorporated into the gelatin-based nanofibers by Yilmaz et. al., antibacterial observation confirmed inhibitory efficiency against tested bacteria [31]. On the other hand, the growth of *Listeria ivanovii* was reliably reduced only by zein/PEG nanofibers with incorporated nisin and its combinations. Related results were obtained by Yu et al., who fabricated zein/PEO nanofibers with incorporated nisin in different concentration (1, 5, 10 and 15 wt%). They monitored visible inhibition zones against tested bacteria, *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes* and *Salmonella* Typhimurium [12]. According to the results of this study, the best antibacterial activity was proved by zein/PEG/Eug/Nis; this mat was significantly active against all tested bacteria.

Antibiofilm activity of zein/PEG nanofibers

The evaluation of biofilm formation is demonstrated in Figs. 4, S2, and S3. All three tested bacterial strains were able to form biofilm on neat zein/PEG nanofibers after 72 hours of cultivation. Several factors are involved in the biofilm formation, either external such as temperature, pH, surface hydrophobicity, or internal like microbial virulence, *quorum sensing*, eDNA, extracellular polymeric substance, and cellular motility. They can apply with various intensity in distinct stages of biofilm production, e. g., cellular motility is the most principal factor for *L. monocytogenes* in attachment during the initial stage [32].

Generally, the samples containing the antimicrobial compounds inhibited the bacterial biofilm formation. In the case of *Escherichia coli*, biofilm production was reduced both by the addition of individual components (eugenol, thymol) and active mixtures by more than 90 %, except for zein/PEG/Nis. The reason is that nisin itself is not effective against Gram-negative bacteria, such as *E. coli*, thus it has no antibiofilm effect against this type of microorganism. None of tested samples was able to prevent the biofilm formation by *Staphylococcus aureus*, except for the combination of eugenol and thymol, which suppressed the *S. aureus* biofilm formation, by up to 70 % compared to control fibers without antimicrobials. This could indicate the synergic effect of those two tested phenolic compounds. In terms of *Listeria ivanovii*, the most significant reduction of biofilm formation (up to 73 % compared to the control nanofibers without antimicrobials) was proved in the zein/PEG/Thy/Nis sample. Nisin is known for its antibacterial properties against Gram-positive bacteria such as *L. monocytogenes* [4]. The other tested nanofibers, both with the individual bioactive substance (eugenol, thymol, nisin) and their combinations, revealed a biofilm formation reduction by at least 40 %.

Eugenol itself has extremely high antibacterial and antifouling activity against a wide range of bacteria. That is the reason this active molecule is usually incorporated into biodegradable polymers with potential use as food package [33]. Otherwise, active molecule thymol is widely used as antibacterial and antifouling additive incorporated into the polymer matrix for the food industry. The antibacterial adhesion property was tested in the PLA-based fibers study Wang et al., against *Escherichia coli* and *Staphylococcus aureus*, their observations confirmed our findings [34].

The results of antibiofilm activity testing proved the complex interactions, especially in systems combining more antimicrobial substances. Their significant effect on the biofilm formation dynamics includes also bactericidal effect on sessile cells, due to gradual release in time (e.g. sample zein/PEG/Eug/Nis, against *S. aureus*, see Fig. 4).

Cytotoxicity of zein/PEG nanofibers

The MTT assay of fabricated samples was utilized to evaluate the cytotoxicity of zein/PEG-based nanofibers loaded with active compounds such as eugenol, thymol, and nisin. The viability of NIH/3T3 cells (%) after 24 hours was assessed, as shown in Fig. 5. The results reveal that almost all tested nanofibers were non-toxic to the tested cells. Zein/PEG/Eug/Thy extracts of 75 % and 100 % concentration exhibit

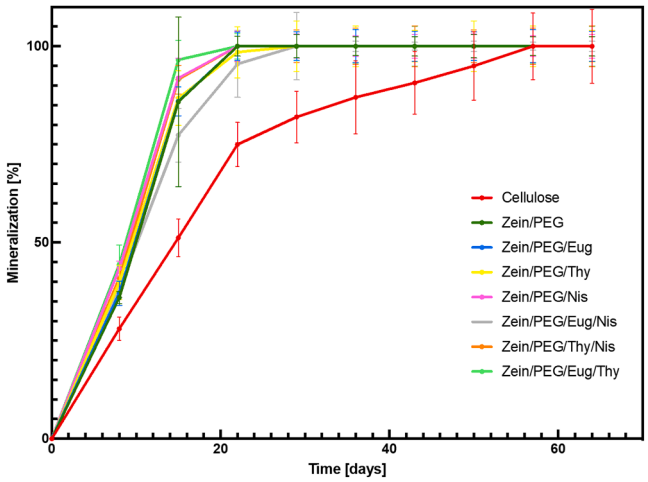

Fig. 6. Biodegradation of zein/PEG nanofibers in compost at 58 °C.

cell contact cytotoxic effects; these concentrations demonstrate cell viability below 10 %. Furthermore, nanofibers containing incorporated eugenol, thymol, nisin, and combination with nisin notably showed higher cell viability compared to the reference (zein/PEG). Elsewhere, nisin incorporated into bacterial cellulose also exhibits non-cytotoxic effect at concentration up to 250 μg/mL [35]. Furthermore, thymol and eugenol are also considered biocompatible active molecules at low concentrations [36]. Overall, results indicate that incorporated active compounds, except the combination of eugenol with thymol, do not have cytotoxic effects on NIH/3T3 cells.

Biodegradability of zein/PEG nanofibers

The biodegradability of zein/PEG nanofibers was observed under specific conditions of industrial compost. The measurement of CO_2 evolution served to monitor the mineralization process of the nanofibers during the experiment at 58 °C (Fig. 6). There is almost no difference between neat zein/PEG-based nanofibers and samples with incorporated bioactive compounds. After 7 days, there were visible defects on zein/PEG-based nanofibrous sample, which can be seen in Fig. 7. In the study by Mariotti et al., zein-based electrospun fibers indicated visible deformations on samples after 7 days of degradation study [37].

However, zein/PEG/Eug/Nis has emerged as the most effective sample at slowing down biodegradation by prolonging it for 8 additional days than the other samples. After 29 days, each sample, including the reference, was fully mineralized. It was described that polymer blends with zein may accelerate the degradation time [38]. Even though these functional nanofibers had incorporated antibacterial substances, such as eugenol, thymol, and nisin at the concentration of 2 wt%, that did not stop bacterial consortium present in tested compost from degrading fully every sample after 29 days.

Conclusions

The study dealt with the preparation of electrospun zein/PEG membranes incorporated with natural bioactive molecules, the phenolic monoterpenes eugenol and thymol, and bacteriocin nisin. Structural analysis revealed a rather physical interaction of antimicrobial compounds with polymer matrix, which was not significantly affected due to the used electrospinning technique. Antibacterial testing illustrated a high efficiency of prepared nanofibrous membranes with nisin against *Listeria* strain, while eugenol and thymol enriched fibers inhibited the growth of *E. coli* and *S. aureus*. Modifications of zein/PEG fibers with antimicrobial compounds showed no significant effects on their thermal properties and the biodegradability kinetics, on the contrary, a

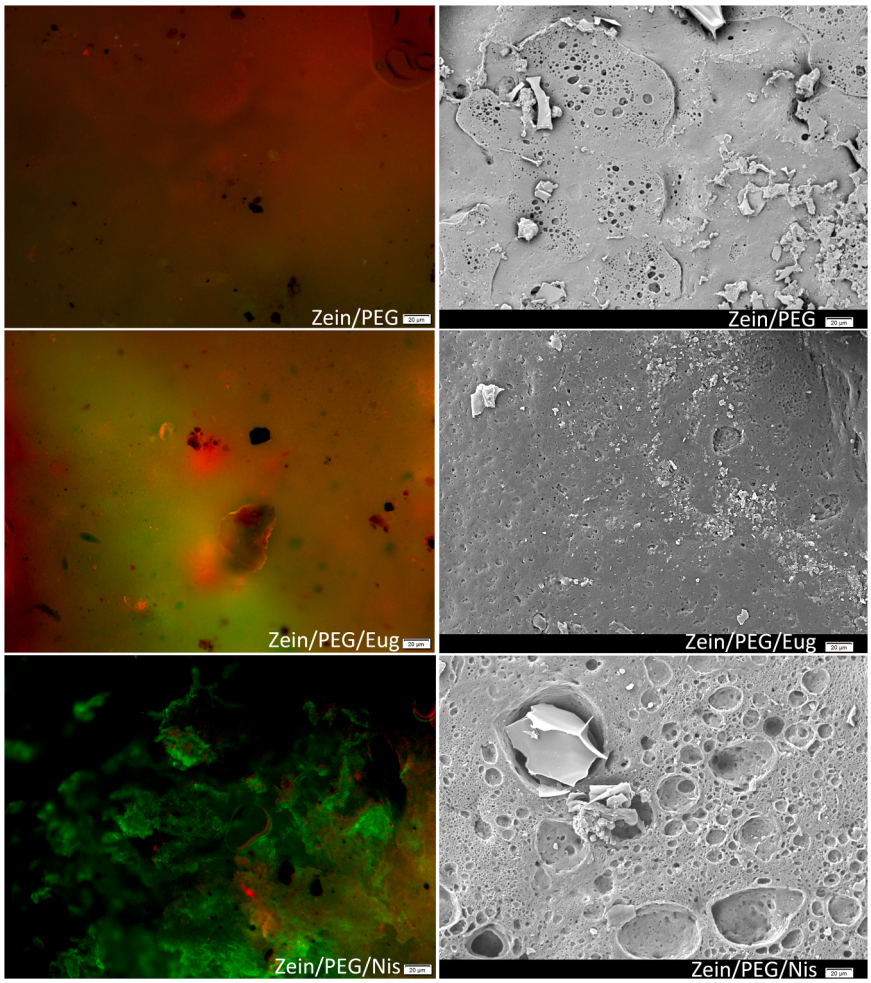

Fig. 7. Fluorescence and scanning electron microscopy of zein/PEG samples (zein/PEG, zein/PEG/Eug, Zein/PEG/Nis) after 7 days' degradation process.

pronounced antibiofilm activity against Gram-positive and Gram-negative bacteria was achieved. It could be concluded that the developed zein/PEG membranes encapsulated with natural antimicrobials can be used as functional sustainable packaging to enhance the quality of food products and combat the biofilm formation of foodborne pathogens.

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CRediT authorship contribution statement

Sedlaříková Jana: Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Krejčí Ondřej:** Methodology, Investigation. **Šišková Alena Opálková:** Writing – review & editing, Validation, Methodology, Investigation. **Matošková Lucie:** Writing – review & editing, Funding acquisition. **Bartošová Lucie:** Writing – original draft, Methodology, Investigation. **Pleva Pavel:** Writing – review & editing, Visualization, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Polášková Martina:** Writing – review & editing, Validation, Methodology, Investigation, Funding acquisition. **Janalíková Magda:** Writing – review & editing, Validation, Methodology, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. None

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2025.03.005.

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