New Biocomposites Based on Bioplastic Flax Fibers and Biodegradable Polymers

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A new generation of entirely biodegradable and bioactive composites with polylactic acid (PLA) or poly-ε-caprolactone (PCL) as the matrix and bioplastic flax fibers as reinforcement were analyzed. Bioplastic fibers contain polyhydroxybutyrate and were obtained from transgenic flax. Biochemical analysis of fibers revealed presence of several antioxidative compounds of hydrophilic (phenolics) and hydrophobic [cannabidiol (CBD), lutein] nature, indicating their high antioxidant potential. The presence of CBD and lutein in flax fibers is reported for the first time. FTIR analysis showed intermolecular hydrogen bonds between the constituents in composite PLA+flax fibers which were not detected in PCL-based composite. Mechanical analysis of prepared composites revealed improved stiffness and a decrease in tensile strength. The viability of human dermal fibroblasts on the surface of composites made of PLA and transgenic flax fibers was the same as for cells cultured without composites and only slightly lower (to 9%) for PCL-based composites. The amount of platelets and Escherichia coli *cells aggregated on the surface of the PLA based composites was significantly lower than for pure polymer. Thus, composites made of PLA and transgenic flax fibers seem to have bacteriostatic, platelet anti-aggregated, and non-cytotoxic effect.*
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Introduction

Flax (*Linum usitatissimum* L.) is best known as a plant cultivated for industrial purposes. It is a source of oil as the basal component or an additive for various paints or polymers, and a source of fibers that can be used in the textile and paper industries. However, there is a growing interest in flax fibers as a component of composites. Composites are materials consisting of a matrix that is reinforced with fibers. Their application as medical devices is favorable because they are more biocompatible to the human body than those from a single material (polymers, metals, or ceramics).[1] Single-material devices are often too flexible or too weak or too

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stiff to host tissues, and some may also be sensitive to corrosion or cause allergic reactions (e.g., nickel and chromium). Therefore, the present research is focused on developing biocompatible and bioactive composites reinforced with flax fibers derived from transgenic flax-producing PHB polymer that can be used primarily as tissue engineering scaffolds.

Composites containing biodegradable polymers as the matrix can be divided into two groups. One has a natural polymer (starch, alginate, silk, collagen, and fibrin gel) matrix reinforced with natural fibers (lignocellulose or cellulose polymers), and the other has a synthetic polymer [polylactic acid (PLA), polyglycolic acid, poly-ε-caprolactone (PCL)] matrix in combination with hydroxyapatite, glass and glass-ceramic fillers.[2] This last element has recently been replaced by natural fibers embedded in a biodegradable polymer matrix.[3] The composites containing biodegradable polymers reinforced with natural fibers have been used in several branches of industry (i.e., automobile) and in medicine, with special focus on artificial tissue scaffolds, drug-release systems, cardiovascular patches, and nerve cuffs.[4,5]

PLA is synthesized by the condensation of lactic acid or the ring-opening polymerization of lactide, and being di-ester of lactic acid. Lactic acid is produced by the fermentation of dextrose from natural sources. PLA can be used for biomedical purposes because it is biodegradable in contact with biological tissues.[6] The degradation process depends on characteristics like structure, molecular weight and mass, and it usually takes days to years. It was shown that the biodegradation time for PLA is from 12 to over 24 months depending on the stereoizomeric form of PLA.[2]

PCL is another biodegradable, hydrophobic polymer used in composites, where it increases strength and stiffness and causes the composite to absorb less water.[7] The biodegradation time of PCL takes over 24 months.[2]

Unfortunately, the synthetic polymers (PLA, PGA, PCL) on their own are useless for biomedical purposes because they lack bioactivity and the desired mechanical properties. Therefore, they are combined with natural fibers to yield biological activity and improved strength. Such a natural fibers could derived from flax, the crop plant cultivated in a temperate climate, which is now a source of fibers with great agricultural significance.

The aim of this study was to prepare and analyze a new generation of entirely biodegradable composites enriched with bioactive transgenic flax fibers. Two kinds of matrix were used in the preparation of the composites: PLA and PCL. These polymers were reinforced with transgenic flax (M50 transgenic line) fibers which contain poly-β-hydroxybutyrate (PHB).[8,9] For comparison, the polymers were also combined with control, unmodified flax fibers. Trangenic flax fibers were used because of their two features: first of all they are more compatible to polymer matrix which resulted in improved mechanical properties and secondly because of their chemical composition they add bioactive properties to the composites. The wide spectrum of secondary metabolites [phenolics, cannabidiol (CBD), and lutein] in transgenic fibers exhibit antioxidant properties and remain very important to potential tissue engineering purposes.

In a recent study, transgenic fibers enriched with PHB (also called bioplastic fibers) were combined with non-biodegradable polypropylene (PP) to yield improved stiffness of the composite.[10] PHB (poly-β-hydroxybutyrate) is a natural, biodegradable polymer produced by many bacteria, where it acts as a source of carbon and energy. Previous studies showed that PHB synthesis in fibers does not affect their major constituents in comparison to control fibers, while their mechanical properties were better than those for control fibers. Thus, bioplastic fibers were combined with entirely biodegradable polymers (PLA and PCL) and the mechanical and biological properties of the prepared composites were determined.

It was noticed that the adhesion rate for natural fibers and hydrophobic polymers usually used as a matrix in composites determines the mechanical properties of composites. As the PLA used in this study has a hydrophilic nature, it should have a better degree of adhesion to the flax fibers. It should be also pointed out that PLA and PHB have similar chemical structures. For this reason also, better adhesion of the composite constituents was expected. In the case of high compatibility between the fibers and the matrix in a composite, the mechanical stress is transferred from the matrix to the fibers, which should yield improved mechanical properties of the composite.

For this study, four types of composite were prepared, containing flax fibers from transgenic or control plants combined with a PLA or PCL matrix, and their mechanical properties were measured. All of the composites showed a higher degree of stiffness (higher Young modulus), and the highest was for the PLA composites. However, the strength of all of the composites was lower than for pure matrix polymers. FTIR analysis revealed that in PLA composites strong intermolecular hydrogen bonds between the components were formed. Different results were obtained for PCL composites, for which hydrogen bonds were weaker than in pure PCL. Thus, the hydrogen bond formation between flax cellulose polymers and matrix polymers is indicated to not significantly contribute to composite mechanical properties.

Fibroblasts were cultured on the composite surfaces and grew with good spreading. The viability of fibroblasts was between 86 and 95%, while in a medium without composite, the cells viability was 95%. Determining the level of platelet aggregations on the surface of the prepared composites and the level of their colonization by bacteria [*Escherichia coli* (*E. coli*)] showed that the composites had antiaggregation and bacteriostatic properties, which indicated their potential for medical applications. These last features might have resulted from the chemical constituents of flax fibers, which contain antioxidative compounds like phenolics, CBD and lutein. Those compounds are perhaps the reason for the high antioxidant potential and bioactivity of flax fibers.

Materials and Methods

Preparation of composites

Samples with a 20% content of fibers (wt and M50) and PLA or PLC as a matrix were used for composite preparation. The PLA and PCL were obtained from Biomer (Germany). Finally, pulverized combed flax fibers were mixed with PLA or PCL granules at 170°C. The mixture was mechanically pressed into sheets. These were cut into smaller pieces and heat-pressed between Teflon sheets for 30–60 s at 175°C (for the PLA composite) or 100°C (for the PCL composite) to produce final composite sheets that were 0.2-mm thick.

Mechanical testing of the composite sheets

The Young's modulus and maximum tensile strength (R max) of the composite sheets were tested using a

computer-driven Instron 5542 system (High Wycombe, UK) as described in a previous paper.[10] The composite sheet of 25-mm long sections cut into "dog bone" shape were fixed between clamps at the initial distance of 5 mm. The samples were stretched at a rate of 20 mm per min. The load-extension ratio was recorded and used to construct the stress–strain diagram and to calculate the mechanical parameters. All of the calculations were done automatically by the onboard Instron Merlin software package. Sheets of pure PLA and PCL were used as a reference.

Scanning electron microscopy

To determine the degree of adhesion between the fibers and the matrix, the surface morphology of fractured composite sheets was examined using a TESLA BS 340 scanning electron microscope. The samples were coated with graphite before analysis.

FT-IR and FT-Raman measurements

Infrared spectra (IR) in the 4000–400 cm^{-1} range were measured on the BIORAD 575 Fourier transform spectrophotometer. The transmission spectra were recorded using the samples in the form of KBr pellets. Because of strong intensity of spectral patterns in the regions 1000–1500 and 500–800 cm^{-1}, the spectra of the thick plates were measured at near-normal incidence using a specula reflectance accessory. The IR reflection spectra were analyzed using four-parameters model as described in Gervais and Echegut.[11] The Kramers–König analysis was performed to fit the wavenumber dependence of the absorption coefficient and the imaginary part of the inverse dielectric function.

The Raman spectra (RS) were measured in the 4,000–80 cm^{-1} range using a BRUKER 110/S spectrometer with the Nd:YAG (1,064 μm) excitation. The IR and RS were recorded with 2 cm^{-1} resolution. IR spectroscopy is a commonly used method for composites analysis and other details and applications can be found elsewhere.[12–20]

Extraction of phenolic components

One gram of fibers were ground in a Retsch mill to a fine powder and extracted three times with methanol. The extracts were pooled, evaporated under a vacuum, resuspended in 1 mL methanol and used for the analysis of the free phenolic components. The remaining material was hydrolyzed in 2 N NaOH at room temperature for 24 h to release ester-bound phenolics. The supernatants were adjusted to pH 3, and extracted three times with ethyl acetate to separate the phenolic components. The ethyl fraction was dried under a vacuum, resuspended in 1 mL of methanol, and used for the analysis of the ester-bound phenolic components.[22]

Total phenolic estimation

The content of total phenolic compounds in methanol (free phenolics) and in NaOH hydrolyzed extracts (ester-bound phenolics) was determined using the Folin–Ciocalteu method.[23] Diluted Folin–Ciocalteu reagent was added to an aliquot of the extract. After the subsequent addition of saturated sodium carbonate and water, the absorbance at 725 nm against a reagent blank was measured using a Carry UV–vis spectrophotometer. The amounts were expressed as gallic acid equivalents.

Ultra-performance liquid chromatography analysis of phenolics

The content of phenolic components in the methanol and NaOH extracts were assessed using the Acquity ultra-performance liquid chromatography (UPLC) system (Waters). A 2-μL sample was applied to an Acquity UPLC BEH C18 column (2.1 $\times$ 100 mm^2, 1.7 μm). The mobile phase was passed through the column at a flow rate of 0.4 mL/min. The mobile phase consisted of the following components, A: 0.1% formic acid and B: 100% acetonitrile. For the first minute, isocratic elution was performed using 97% of A in B. From 1 to 6 min, a linear gradient was applied using 97–30% A in B. From 6 to 7 min, a linear gradient was applied using 30–0% A in B. In the final minute, the concentration of A was returned to 97%. The column was kept at 25°C. A photodiode array (PDA) was used to detect the absorption between 210 and 500 nm.

Extraction of hydrophobic components

For the UPLC analysis, 100 mg of fibers were extracted three times with chloroform, dried, and redissolved in 100 μL of methanol. The extracts were filtered through a 0.25-μm Acrodisc and 10 μL was injected onto an Acquity UPLC BEH C18 column (2.1 $\times$ 150 mm^2, 1.7-μm particles).

UPLC analysis of hydrophobic components

The analysis was performed with a Waters Acquity ultra-performance liquid chromatograph equipped with a PDA detector. The mobile phase was an acetonitrile-water gradient from 70 to 30% for 1 min followed by a gradient from 70 to 100% acetonitrile for 5 min, 100–0% for 10 min, then a 1-min return to 30 to 70% for a further 1 min (flow 0.4 mL min^{-1}). 0.05% trifluoroacetic acid was added to both solvents to eliminate tailing. The column was kept at 40°C and the PDA detector was used to register absorption between 210 and 500 nm. The detection and integration of the peaks was performed for CBD at 230 nm and for lutein at 445 nm.

TBARS determination

The level of thiobarbituric acid-reactive substances (TBARS) in the samples was determined.[24] Oil samples (4 μL) were oxidized at 140°C for up to 45 min with or without a 4-μL flax fiber extract in 0.9% NaCl. Two milliliter of reagent A (15% trichloroacetic acid and 0.37% thiobarbituric acid in 0.25 M HCl) was added and the mixture was thoroughly blended. Test tubes containing the samples were stopped with glass marbles, heated at 100°C for 15 min, cooled under running tap water, and centrifuged for 10 min at 2,000 g. The absorbance was measured at 535 nm using a spectrophotometer (Cecil CE-2020). The reference blank contained the TBA reagent.

The oil from the flax seeds used for the reaction was prepared in accordance with the method described.[25] Briefly, 1 g of seeds was ground in a mortar with 1 mL of water. The homogenate was suspended in 2 mL of methanol and 4 mL of chloroform. 3.5 mL of 0.9% NaCl was then added. The mixture was gently agitated and left to settle for 24 h at room temperature. The lower chloroform phase was collected and the extraction was repeated. The chloroform was evaporated using a rotary vacuum evaporator. The lipids were resuspended in chloroform/methanol (1:2, v/v) and stored at −20°C.

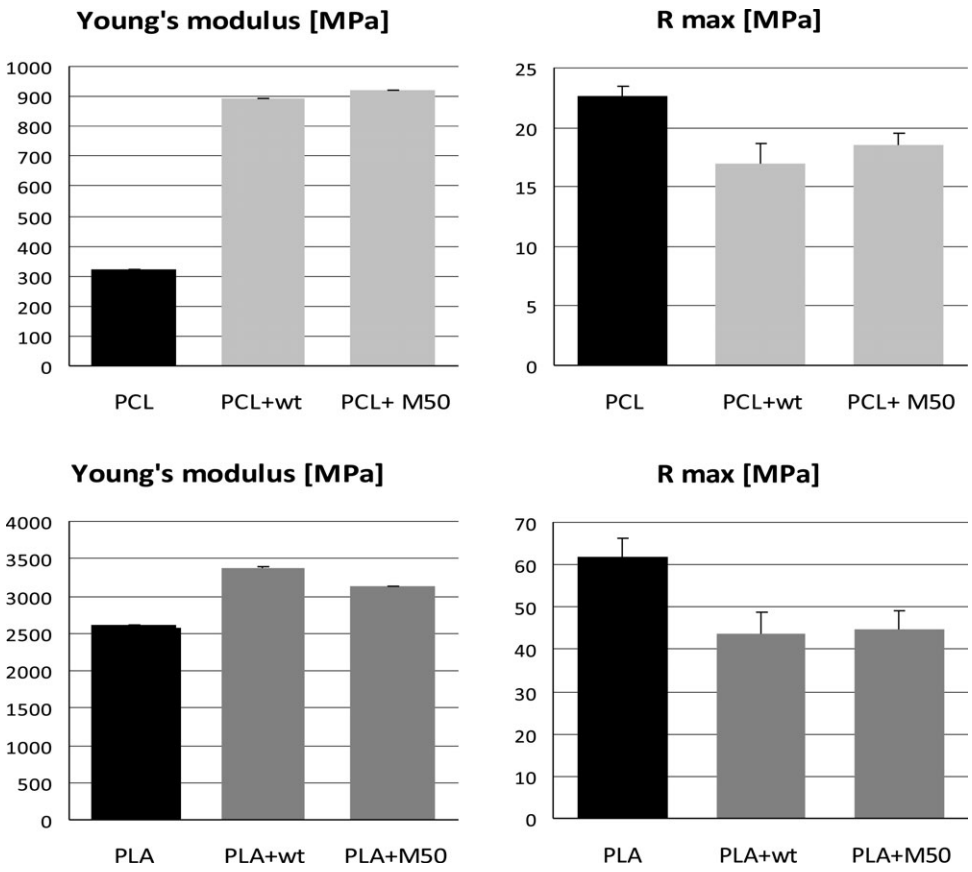

Figure 1. The Young's modulus and strength of the composites containing fibers from the transgenic (M50) and control plants.
Sheets of pure PLA or PCL were used as a reference. The measurements were conducted as described in the Materials and Methods section.

Cell culture growth on the composites, assessed via hoechst staining and viability assay

Normal human dermal fibroblasts (NHDF) from the Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences in Wrocław were cultivated at 37°C and 5% (v/v) CO_2 in alpha-MEM medium (derived from Institute of Immunology and Experimental Therapy, Wrocław, Poland) containing 10% (v/v) fetal calf bovine serum (Abo, Poland) and antibiotics (100 U/mL penicillin, 100 U/mL streptomycin) and 0.29 mg/mL of glutamine (HyClone). The cells for the experiments were used between passage 13 and 17. After preculturing, the cells were transferred to 24-well plates containing the composites: PCL, PCL+wt fibers, PCL+M50 fibers, PLA, PLA+wt, and PLA+M50 fibers. After 24 h, the fibroblasts were harvested with trypsin treatment and counted in the Bürker chamber. The experiment was performed twice and the result is presented as the average number of cells per milliliter of medium. In a simultaneous experiment, fibroblasts cultured for 24 h were stained with Hoechst (Invitrogen), and their nuclei were analyzed under a fluorescence microscope at 200× magnification. The fibroblast viability was also measured using Trypan Blue exclusion. Viable and dead cells were counted using standard light microscopy.

Aggregation of platelets on the surface of the composite sheets

The aggregation of platelets on the composite sheets was examined using scanning electron microscopy (SEM) as described previously.[26] Sheets of pure PP were used as a reference.

Venous blood was collected from healthy volunteers in tubes containing 3.8% sodium citrate. The tubes were centrifuged for 10 min at room temperature at 1,000*g*, after which the plasma was drawn off. Samples of composite were incubated in the plasma for twelve hours at 4°C, after which the samples were rinsed with buffered phospho-saline. The samples were then incubated with citrated whole blood for 1 h with gentle agitation, after which they were washed with buffered phospho-saline.

The samples were then fixed for 1 h at 4°C with 3% glutaraldehyde in buffered phospho-saline. The fixed samples were serially dehydrated in ethanol, air-dried, and coated with a 20-nm thick layer of metallic gold in a Jeol JEE-4X sputtering apparatus. Platelets that adhered to the surface of the composite sheets were visualized using a Hitachi S-3000N scanning electron microscope.

Colonization of the composites by E. coli

The composites were sterilized by treatment with 70% EtOH, and washed in sterile YPG medium. Then, the composites (10×10 mm^2) and stainless steel were placed into separate flasks and immersed in a medium containing NaCl (1%), bactopeptone (1%), and yeast extract (0.5%), pH 7.0.[27] The medium was supplemented with a small number of *E. coli* cells (2×10^3) and cultured for 48 h at 28°C with gentle shaking. The *E. coli* cells were analyzed via fluorescence microscopy with the use of bis-benzidine. The composite samples

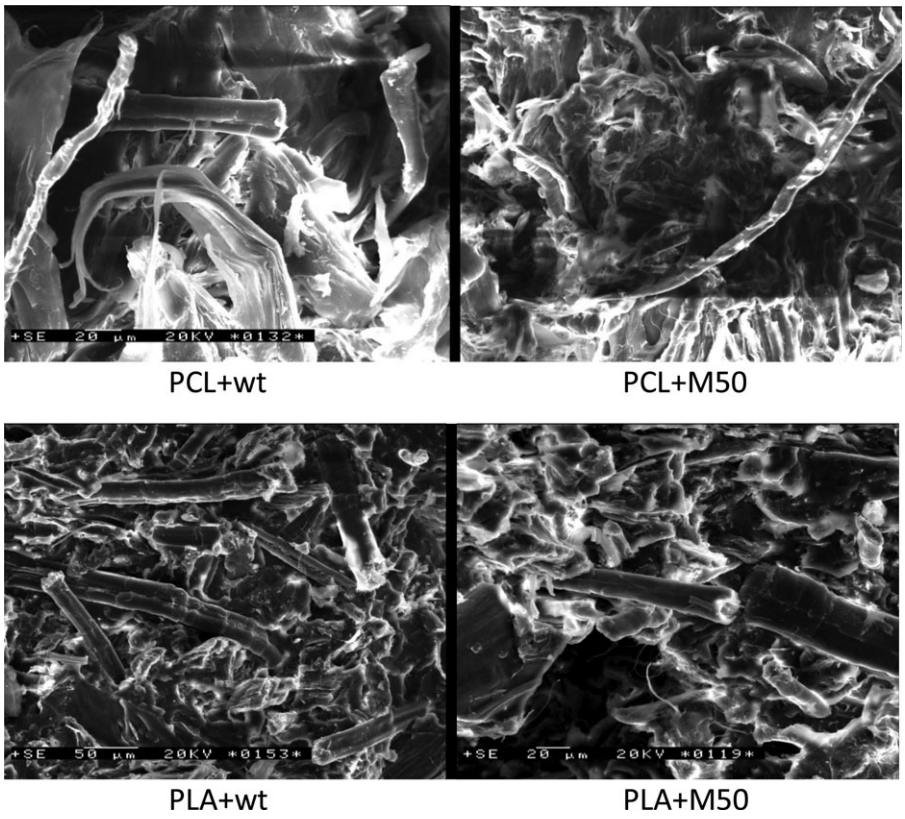

Figure 2. SEM of fractured composite sheets containing fibers from the transgenic (M50) and control plants (wt).
The microscopy was performed as described in the Materials and Methods section.

were removed from the growth medium and washed with water. After that, the samples were fluorescently stained with bis-benzidine, which binds to dsDNA (20 μL of a stock 100 μg/mL solution was added to each sample). The process was carried out at 28°C in the dark for 10 min. The bacteria were detected using a fluorescence microscope (Nikon TE 200).

Results and Discussion

The biomechanical properties of the composites sheets

The interfacial adhesion of the composite components appeared to be an important factor which determines its quality. The adhesion between the hydrophilic cellulose polymer and the usually used hydrophobic matrix was insufficient. Therefore, coupling agents were generally used to improve the interfacial association of composite components.[28]

Recently, a different strategy has been used to improve adhesion. Our recent study showed that the cellulose polymer in fibers from a polyhydroxybutyrate-producing transgenic plant (M50) was more structured than the control fibers, and that PHB was strongly bound to the cellulose polymer by covalent and hydrogen bonds, which resulted in sufficient adhesion to the PP matrix.[10]

For this reason in this study, we used fibers with PHB instead of coupling agents for composite preparation. PLA and PCL was used as the matrix components. Mechanical parameters of the prepared composite sheets and the SEM of the composite fractures were analyzed to characterize the adhesion of the components and the stiffness of the composites. The biomechanical properties were measured as described in the Materials and Methods section, and the data is presented in Figure 1. The composites with a PCL matrix and flax fibers (from M50 or untransformed, wild-type plants) showed about a three-fold higher Young's modulus than the pure PCL used as a reference. An increase in the Young's modulus was also detected when the composites with a PLA matrix were analyzed, but it was not as high as for the PCL composites. An almost 20% increase in the Young's modulus was observed for the PLA+M50 composite and an almost 30% increase for the PLA+wt. The data shows that the stiffness of all of the composites was improved compared to that for pure synthetic polymers.

However, the maximum tensile strength (R max) decreased for all the tested composites compared to that for pure PLA and PCL. Almost the same result was recently reported for a composite with a PLA matrix and fibers from Holstein-type flax.[3] The reason for this is as yet unknown. Tensile strength strongly depends on whether the fiber type is bundle or single filament. Also, important for tensile strength value is the adhesion of the fibers to the matrix polymer. Poor adhesion, or insufficient or incomplete surrounding of the fibers by the matrix might also affect the tensile strength. To analyze this, SEM of the fractured composite was conducted.

SEM of the composite sheets

SEM images of fracture surfaces of all of the types of composite are shown in Figure 2. The microscopy revealed good adhesion between the matrix and the flax fibers: the transgenic or control fibers were well embedded in the PLA and PCL matrix; no gaps were detected in the fracture

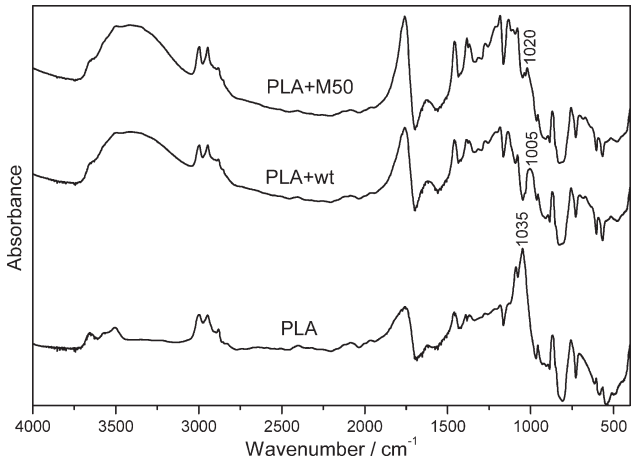

Figure 3. Transmittance FTIR spectra of composites PLA+wt, PLA+M50.

Sheets of pure PLA were taken as a reference. The measurements were conducted as described in the Materials and Methods section.

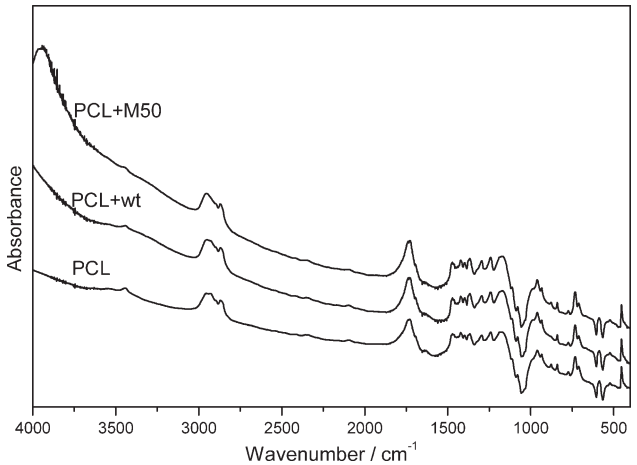

Figure 4. Transmittance FTIR spectra of composites PCL+wt, PCL+M50.

Sheets of pure PCL were taken as a reference. The measurements were conducted as described in the Materials and Methods section.

surface, and fibers were rather broken than pulled out of the composites. A similar effect was seen when composites with PP as the matrix was tested.[10] Thus, the data from the SEM analysis proved good adhesion of the composite components.

IR and RS analysis of composites

IR spectroscopy has been widely used in the studies of biocomposites prepared in the form of blend, foil or matrices.[12–20] However, different conclusions have been drawn from these studies. Ciardelli et al.[15] stated that no chemical interaction exists between blend components (PCL and polysaccharides) when analyzed by FTIR-ATR spectra. Neves et al.[14] also maintained that no significant frequency shifts of the characteristic functional groups occur when pure polymers spectra are compared to those of blends, that corroborates previous conclusions about the interactions between two polymers in blends.[17,18]

On the other hand, Sawpan et al. suggest that the interactions between the OH groups of cellulose of hemp fibers and the carbonyl (C=O) and carboxylic (COOH) groups of PLA occur.[11] Similar bonding interactions have been described by Huda et al for silane grafted kenaf and pineapple l-(ε-caprolactone) of fiber reinforced PLA composites.[19,20] Kumar et al. also explained the changes seen in their IR spectra measurements as a result of the interactions between the hydrophilic units (COOH, NH_2) and the hydroxyl groups of the fibers.[15]

It should be noted that the conclusions described above have been derived from the studies that differ in the experimental technique used. Some of them have been obtained from the transmission or absorption methods but others from the reflectance studies and thus might affect the result obtained. For example in the reflectance IR, the penetration depth of the light is of the order a few microns, and thus only the components on the surface layer are excited i.e. PLA and PCL polymers in case of this work.

In this work the intermolecular hydrogen bonds in composite materials is suggested upon IR absorption spectra analysis (Figure 3). First, new strong bands contour appeared in the ranges 3,100–3,700, 1,550–1,700, and 950–1,050 cm^{-1} in the IR spectra of PLA+wt and PLA+M50 composites. These new, not detected in pure PLA, broad contours correspond to the in-plane stretching ν(O-H···O), in-plane bending δ(O-H···O) and out-of-plane bending γ(O-H···O) vibrations of the intermolecular hydrogen bonds, respectively. Second, It should be pointed out that the strong band at 1,035 cm^{-1} seen in the spectrum of PLA shifts to 1,020 cm^{-1} for PLA+M50 composite and to 1,005 cm^{-1} for PLA+wt composite. This band corresponds to the ν(C-O) stretching vibration of the carboxyl group of the PLA that participate in the formation of the C-H···O bond. The clear difference in the position of this band for the PLA+wt and PLA+M50 composites suggests that the strength of the hydrogen bond between the PLA and cellulose is higher in the PLA+wt composite.

The same analysis was conducted for the IR and RS of the PCL, PCL+wt and PCL+M50 samples. In opposite to PLA composite those with PCL do not shown shift in bands wavenumber or intensity (Figure 4) characteristic for newly generated hydrogen bonds. Since there were no changes in the absorption spectra, the lack of strong molecular interactions between the PCL and cellulose is concluded. This agrees with the conclusion reported by Ciardelli et al.[16] for the blends composed of PCL and polysaccharides.

The reason for opposite behaviors of the studied PLA and PCL blends is as yet unknown, it is speculated that it might arise from the differences in the length of their repeated units and steric hindrance of the methyl group in the lactic units.

In conclusion, polymers of the PLA matrix clearly interact with cellulose polymers of flax fibers in a composite material via hydrogen bonds and this is not the case for PCL composite. Thus, observing differences in mechanical properties of PLA and PCL composites with flax fibers was expected. This was, however, not the case. The maximum tensile strength decreased for both PLA and PCL composites as compared to pure matrices. Conversely, the stiffness of all composites was improved according to pure synthetic polymers. Also, the microscopy study showed high compatibility of composite components. Although the reason for this discrepancy is as yet unknown, we speculate that addition of

Table 1. The Content of Antioxidative/Anti-inflamatory Components in the Control and M50 Fibers

	wt	M 50
Total phenolics (μg/mg DW)	1 ± 0.1	1.1 ± 0.08
Including:		
Ferulic acid	0.29	0.35
Vanilin	0.46	0.55
4-Hydrobenzoic acid	0.30	0.16
Acetovanillone	0.07	0.08
Cannabidiol (ng/mg DW)	4.88 ± 0.4	5.03 ± 0.28
Lutein (ng/mg DW)	1 ± 0.06	4.01 ± 0.66
Antioxidant capacity (mmol/g)	0.40 ± 0.05	0.28 ± 0.05

Phenolic components were extracted as described in the Materials and Method section and measured in UPLC. Antioxidant capacity was measured as TBARS formation in Linola flax oil in the presence of the flax fibre extract. Crude oil extracted from Linola seeds alone or supplemented with an extract from transgenic or control fibres was heated for 40 min at 140°C. The data is the mean TBARS levels measured spectrophotometrically at 535 nm.

hydrophilic cellulose polymers to the hydrophobic matrix might modify intramolecular and intermolecular hydrophobic interaction in matrix polymers and hydrogen bonds between components of composite is not significant.

Biochemical analysis of the flax fibers

Our earlier study showed that the levels of the major components (cellulose, lignin, and pectin) of flax fibers in the M50 transgenic plant were unchanged compared to the levels in the control fibers.[21] However, the levels of the other components that might affect the biomedical application of flax fiber composites were not analyzed.

Therefore, the biochemical composition of the flax fibers was studied in detail, and compounds that exhibited antioxidative properties were found (Table 1). The phenolic components, CBD, and lutein were identified in extracts from transgenic and control fibers and their quantity measured via UPLC, as described in the Materials and Methods section. This is the first time that CBD and lutein were detected in flax fibers.

The phenolic components are known antioxidants that retard microorganism growth. Lutein is one of the carotenoids: it is oxygen-containing xanthophyll with antioxidative functions, and since it has less hydrophobic properties than others carotenoids, it acts in the hydrophilic side of the membrane.[29] Lutein, β-carotene, and zeaxanthin, is also retained in human tissues (liver and retina), where it acts as an antioxidant. Lutein prevents age-related macular degeneration and atherosclerosis, and stimulates the immune response. CBD with its ability to reduce oxidative stress is also a known antioxidant.[30,31]

The level of phenolic compounds (i.e., ferulic acid, vanillin, and acetovanillone) was higher in the transgenic fibers than in the control fibers, but the difference was not significant. The level of lutein and CBD was also higher in the transgenic fibers, with the highest difference observed for lutein, which was at a fourfold higher level than in the control. The presence of several antioxidative compounds in the fibers might affect their antioxidative potential, and thus, the bacteriostatic and platelet anti-aggregated properties of the composite.

Antioxidative potential of the flax fiber extract

As the compounds (phenolic acids, lutein, and CBD) detected in the fibers are known antioxidants, an increase in the antioxidative status of the fibers was expected. Therefore, extracts of the fibers were used to measure the antioxidant capacity in terms of their ability to prevent the peroxidation of polyunsaturated fatty acids from flax seed oil. Lipid peroxidation was evaluated by measuring the level of TBARS, which are the index of lipid peroxidation and oxidative stress. For the extracts from fibers of the transgenic M50 plants, the level of TBARS was about 28% lower than that from the control fibers (Table 1). Therefore, the antioxidant capacity was higher for the extract from the transgenic fibers than for that from the control fibers. Because reactive species of oxygen are known to appear in first stage of bacterial infection, fibers with antioxidant properties should have protective function. This was analyzed in further experiments, in which we examined platelet aggregation, bacterial colonization and fibroblast growth on the surface of flax fiber-reinforced PLA and PCL composites.

Fibroblast growth on flax biocomposites

The adhesion and proliferation of cells on scaffold material is crucial for tissue engineering, thus human fibroblasts were grown on the surface of tested composites. We examined the morphology of NHDF (NHDFs) after 24 h of in vitro culturing on the surface of the composites. The results are presented in Figure 5. The fibroblasts exhibited normal cell morphology. They were spread well on the surface of the analyzed composites, except composites based on PCL matrix, which exhibited significantly lower NHDF amounts in comparison to control (Figure 5A). The fibroblasts were stained with Hoechst dye and analyzed under a fluorescence microscope (Figure 5C). The analysis revealed no differences in the morphology of the nuclei of tested fibroblasts cultured on analyzed composites. The fibroblasts were also stained with Trypan blue to distinguish between living and dead cells (Figure 5B). The amount of dead fibroblast cells was on the level observed for fibroblasts cultured without composites (control); only in the case of PCL and PCL+wt the amount of dead cells was slightly higher (14 and 13%, respectively), but it was still lower than the amount of dead L929 mice fibroblasts on the same composites observed in a prior study.[32]

In another in vivo study, a flax fiber -reinforced PLA composite was implanted into rat skeletal muscles (Musculus latissimus dorsi). After four weeks, it was resected and the effect on muscle function and gene expression was evaluated.[33] It was shown that the implanted composites did not cause any inflammation response and that the composite was surrounded and even overgrown by blood vessels and connective tissue. The composite used in that study showed better biocompatibility than pure PLA implants, and did not have negative effect on the gene expression of many growth factors, such as vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF) and growth differentiation factor 8 (GDF8). Thus, the data from that in vivo study[33] and present data made on human fibroblasts demonstrate that prepared composites, especially based on a PLA matrix are not cytotoxic and can be considered for implants used in medical applications.

Platelet aggregation on the composites

Platelet aggregation can lead to the formation of a thrombus, and because of that to severe diseases like thrombosis and atherosclerosis. Because of the potential of composites used in biomedical devices to produce thrombus when contact

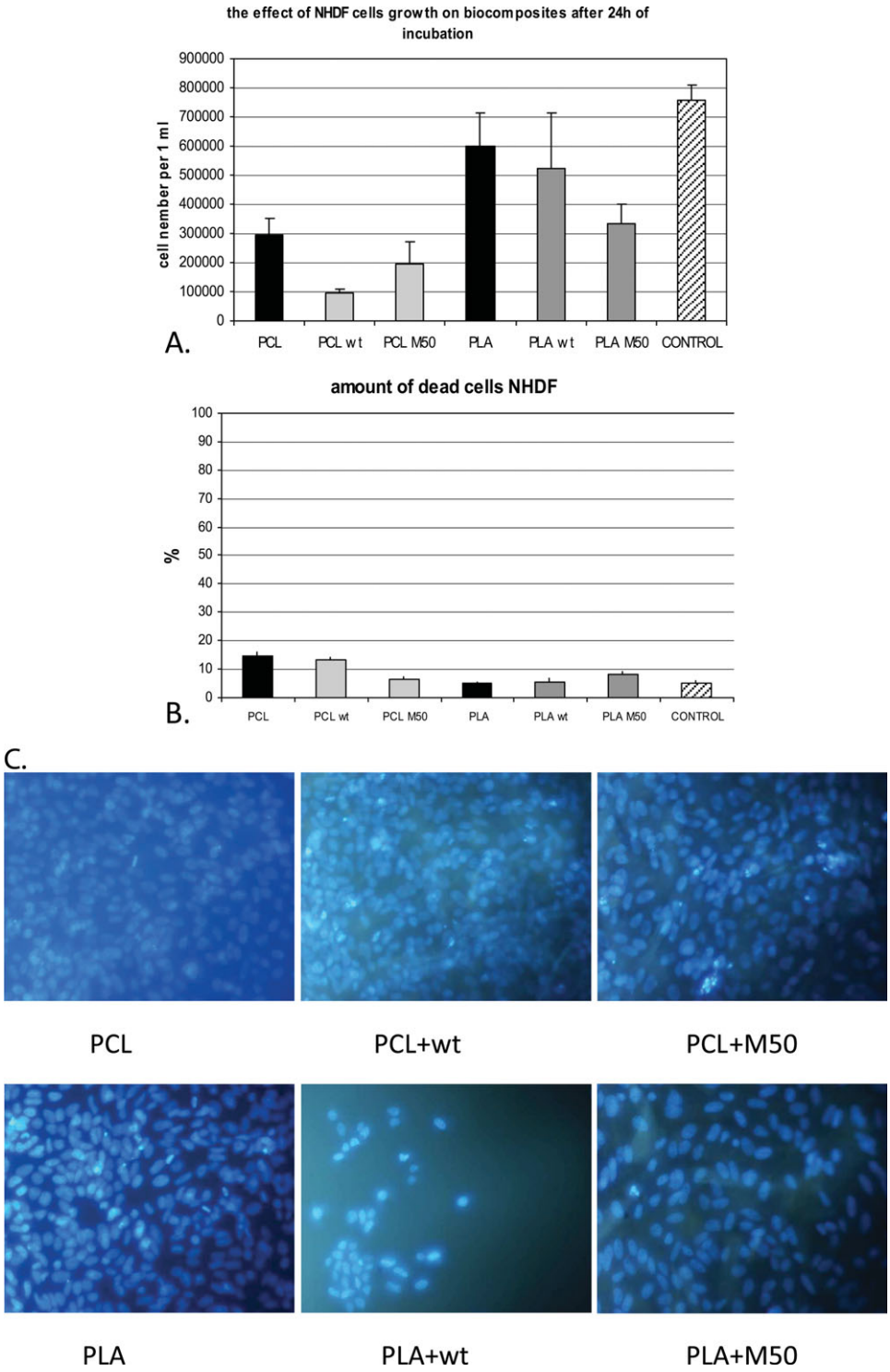

Figure 5. NHDFs growth on the composite surface after 24 h culture.

Fibroblasts were grown on the composites as described in the Materials and Method section. The amount of fibroblast was counted and is presented (panel A). The amount of dead fibroblast cells was examined after Trypan blue staining (panel B). The fibroblasts were also stained with Hoechst dye, and the nuclei of those cells were analyzed under a fluorescent microscope (panel C).

with blood occurs, the adhesion of platelets to all the composites was tested. SEM images of the surfaces of each composite incubated with blood platelets are presented in Figure 6. Platelet aggregation was higher on the composites with PCL as the matrix and flax fibers as fillers than on pure PCL or surgical stainless steel (316L), where the latter is commonly used in medicine (Figure 6; Table 2). For unknown reasons, the addition of flax fibers to the PCL matrix caused an about 2.5-fold increase in the amounts of platelets adhered to the composites. However, the area of composite occupied by the platelets was almost fourfold smaller than on surgical steel (316L). Thus, it can be deduced that on PCL-based composites platelets form thrombus.

A different phenomenon was observed for the composite with PLA as the matrix. On those composites, the amount of aggregated platelets was significantly (threefold to fivefold)

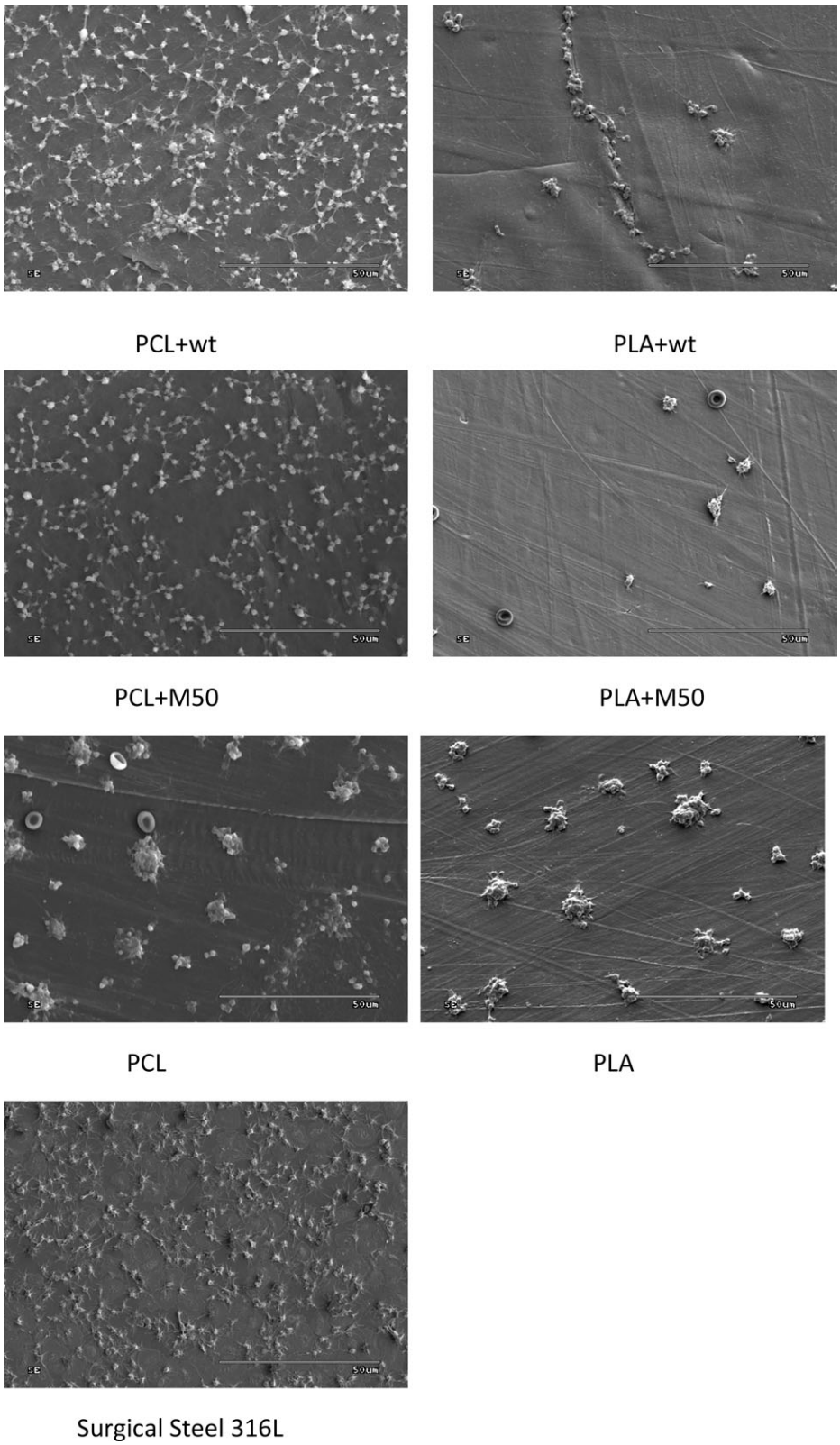

Figure 6. Platelet aggregation on composites containing fibers from transgenic plants (M50) and control plants (wt).

The microscopy was performed as described in the Materials and Methods section. Sheets of pure PLA or PCL and medical steel were used as the reference. The bar represents 50 μm.

lower than for steel. PLA+M50 exhibited the lowest result: fivefold lower amount of aggregated fibroblasts than for conventionally used medical steel. Thus, this material with platelet anti-aggregation properties might serve as a source of biomedical devices exhibiting an anti-thrombosis effect. The reason for the observed lower platelet aggregation on the analyzed composites might derive from the chemical composition of flax fibers, that is, the presence of the antioxidant compounds and the improved antioxidant capacity.

Table 2. The Adhesion of Platelets to the Analyzed Composite Surfaces

Composite	Amounts of Platelets (for 100 μm)	Area of Composite Occupied by Platelets (%)
PCL	1.67 ± 0.51	19.4 ± 6.9
PCL+wt	4.21 ± 1.35	23.15 ± 4.92
PCL+M50	4.29 ± 0.63	19.45 ± 4.19
PLA	1.41 ± 0.33	12.6 ± 2.12
PLA+wt	0.6 ± 0.24	5.87 ± 2.36
PLA+M50	0.38 ± 0.32	1.8 ± 2.0
316L	1.91 ± 0.26	77.25 ± 6.34

The data was obtained ($n = 10$) after 1 h contact with the blood. The amounts of platelets (for 100 μm) and the area of composite occupied by the platelets (%) were measured and are presented. Sheets of pure PLA or PCL and surgical steel 316L were used as controls.

Colonization of the composites by E. coli

The bacterial infections that potentially occur during implantations of medical devices remain an important clinical problem, so, we tested the colonization of the surface of the prepared composites by the bacteria *E. coli*. The antimicrobial activity of composites and nanoparticles against *E. coli* as a model for Gram-negative bacteria are demonstrated in literature.[34,35] Data concerning study on composites effect against *E. coli* are presented in Figure 7. The addition of control and transgenic (M50) flax fibers to the PLA matrix caused decreases in the level of bacteria colonies on the composites. The lowest level of colonization was detected for the composite with the control fibers (PLA+wt): 40% lower than for surgical steel (316L). The growth of bacteria on PCL+wt fibers was also lower than on pure PCL, and almost 20% lower than for surgical steel. Colonization of the PLA+M50 composite was about 20% lower than that of the pure polymer (PLA). Thus, bacteriostatic properties were displayed by the PLA-containing composites, especially by the PLA composite reinforced with control fibers. The reason for this is perhaps the presence of compounds with antioxidative properties in the flax fibers. Both the transgenic and control fibers showed almost the same level of phenolic compounds and CBD, while only the level of lutein was higher (about fourfold) in the transgenic fibers than in control. This might suggest that too high concentrations of lutein may act in the opposite way and promote oxidation. Indeed, the dual action of antioxidants was shown to depend on their concentration: at the micromolar concentration they protect polyunsaturated fatty acids against oxidation, while milimolar concentrations promote their oxidation.[36]

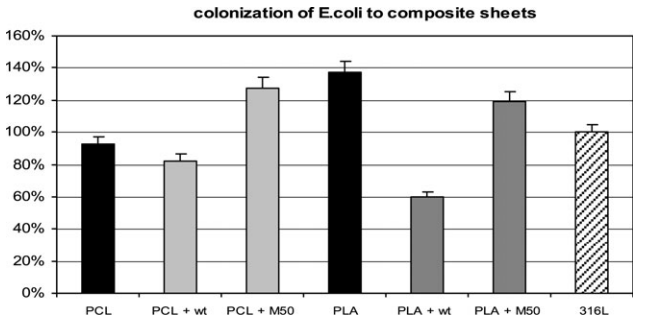

Figure 7. Colonization of the analyzed composites by *E. coli*.

Bacteria were incubated with the composites as described in the Materials and Methods section and then colonization was measured under a fluorescence microscope (Nikon TE 200). Sheets of pure PLA or PCL and surgical steel (316L) were used as controls.

Conclusions

Composites have gained popularity in biomedical products that need to be primarily biocompatible and bioactive. Biocompatible means hydrophilic (because the human body is approximately 70% water), non-inflammatory and non-thrombogenic. When used as a tissue engineering scaffold, bioactive means containing compounds that actively participate in the attachment, growth and proliferation of the new natural tissue. Aliphatic polyesters like PLA and PCL reinforced with natural flax fibers appear to be excellently biocompatible, nontoxic toward human tissues, and bioactive. Their bioactivity derives from compounds in the flax fibers with general antioxidative functions and anti-inflammatory (CBD and lutein) properties. Transgenic fibers derived from flax producing PHB also potentially have cell growth-promoting activity because PHB in contact with body fluid is degraded to 3-hydroxybutyrate, which upregulates cell proliferation.

Four types of composite were fabricated and analyzed: two different synthetic polymers for the matrix (PLA or PCL) and flax fibers from non-transformed or transgenic plants as reinforcement. All of the composites showed sufficient adhesion of the components and thus had improved stiffness. It was shown that the flax fibers provide several antioxidative compounds, and two of these (CBD and lutein) are reported on here for the first time. The composites based on PLA matrix and flax fibers showed platelet anti-aggregation activity and low colonization by bacteria, the highest Young's modulus and thus the highest stiffness. The number of human fibroblast cells attached to all of the composites was slightly lower than for pure polymers, but the number of dead fibroblasts was on the level observed for control (fibroblasts grown without composite). Thus, it is suggested that the composites are not cytotoxic.

FTIR analysis of composites revealed that polymers in PLA matrix clearly interacts with cellulose polymers of flax fibers in composite material via hydrogen bonds and this is not the case for PCL composite. Thus, the differences in mechanical properties of PLA and PCL composites with flax fibers were expected. Measured mechanical properties showed however decreased maximum tensile strength for both PLA and PCL based composites in comparison to pure matrices and improved stiffness of all type composites. The SEM study indicated high compatibility of composite components.

New composites with bacteriostatic and platelet anti-aggregated properties were investigated in this study, and they might serve as a new source of material for medical applications. They can potentially be used as implants/membranes to replace damaged tissues, and preliminary data on composite implanted into rat skeletal muscle revealed no negative effect on muscle function and gene expression (i.e., VEGF, IGF, and GDF8) [33]. Experiments aimed at determining the biodegradability of the composites in implanted tissues will be performed in the near future.

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