УДК 577.117.2+577.151.63+577.112;004.942

ФЛУОРЕСЦЕИНА ДИГЕКСАНОАТ КАК ПРОФЛУОРЕСЦЕНТНЫЙ МАРКЕР ДЛЯ МОНИТОРИНГА ГИДРОЛИТИЧЕСКОЙ ДЕСТРУКЦИИ ПОЛИЛАКТИДНЫХ МАТЕРИАЛОВ

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Полилактид является одним из перспективных полимеров для получения биодеградируемых и биосовместимых материалов. Оценка скорости гидролитической деструкции материалов на основе полилактида имеет важное значение как в период планирования их применения, так и во время их эксплуатации. Рассматриваются синтез флуоресцеина дигексаноата и возможность его использования как профлуоресцентного маркера гидролитической деструкции материалов на основе двух типов полилактидов. Отмечается, что увеличение флуоресценции коррелировало с закономерно более быстрой деструкцией поли-D,L-лактида, а также меньшей стабильностью полилактидов в отношении щелочного гидролиза. Показывается применимость флуоресцеина дигексаноата для оценки гидролитической деструкции материалов на основе полилактида по флуоресценции практически в режиме реального времени.

Ключевые слова: полилактид; поли-L-лактид; поли-D,L-лактид; флуоресцеина дигексаноат; флуоресценция; гидролиз.

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FLUORESCEIN DIHEXANOATE AS A PROFLUORESCENT MARKER FOR MONITORING OF HYDROLYTIC DESCRUCTION OF POLYLACTIDE-BASED MATERIALS

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Polylactide (PLA) is one of the most promising biodegradable and biocompatible polymer materials. Estimation of hydrolytic destruction of PLA is of high importance for their applications both at planning and at real-time exploitation. This paper reports on synthesis and applicability of fluorescein-O-dihexanoate as a pro-fluorescent marker of hydrolytic destruction of two types of PLA-based materials. Fluorescence enhancement correlated with adequately more fast destruction of poly-D,L-lactide as well as less stability to alkaline hydrolysis. Thus, applicability of fluorescein-O-dihexanoate for fluorescence-based estimation of hydrolytic destruction of PLA-based materials is shown in quite real time mode.

Keywords: poly-L-lactide; poly-D,L-lactide; fluorescein-O-dihexanoate; fluorescence; hydrolysis.

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Introduction

Biodegradable materials are designed to degrade under environmental conditions or to be recycled in biological waste treatment plants. This degradation can follow the path of composting and microbiological biogasification of waste. For the medical biological and pharmaceutical industries, special requirements are imposed on the destruction process to control the biotransformation processes of the used polymeric materials. It defines the need for the development and standardisation of appropriate test methods for determining the biodegradation of polymers. Polylactide (PLA) has become a popular polymer to create materials for various biomedical applications because its biocompatibility and ability to hydrolytic decomposition [1–10].

Conversely, fluorescent polymers are widely used as fluorescent coatings, fluorescent probes, sensors, solid-state dye lasers and for the preparation of materials for luminescent conversion [11–17]. Notably that there are neither fluorescent no fluorogenic groups in common PLA. So, to use this phenomenon to analyse the state of PLA-based materials, the task to made PLA-based fluorescent of pro-fluorescent (fluorogenic) material has to be solved. One of such solutions can be a usage of a compound compatible with PLA matrices and live cells as well as able to change its fluorescence properties in accordance with polymer destruction. To do this, one of the technologically-convenient methods is to add a low molecular weight pro-fluorescent marker into a polymer solution followed by solvent removal. Despite the simplicity of this method of pro-fluorescent composite manufacturing, compatibility of a certain polymer with a certain marker must be taken into consideration.

Fluorescein is known to be one of the most abundant xanthene fluorophores that have found wide biomedical application, however, it is polar compound and not compatible with hydrophobic aliphatic polyester like PLA. Moreover, the compound's fluorescence does not dependent too much with respect to polarity and hydrophobicity of microenvironment. Conversely, non-fluorescent fluorescein esters like fluorescein-O-dihexanoate (FDHex) are hydrophobic and able to recover fluorescein and, thus, fluorescence during hydrolytic destruction [17–23]. Therefore, the aim of the study was to show compatibility of FDHex with PLA matrix and demonstrate correlation of hydrolytic destruction of FDHex and PLA matrix for the corresponding composite materials.

Experimental section

Poly-L-lactide 4042D (M_w = 125 kDa) and poly-D,L-lactide Ingeo 2002D (M_w = 150 kDa) (*Nature Works*, USA) (fig. 1) was used. Its molecular weight characteristics were confirmed by gel-permeation chromatography (chromatographic system HPLC (*Agilent Technologies*, USA) refractometric detector, eluent trichloromethane, 30 °C; NUCLEOGEL GPC 50-5 column; pores size is 50 Å). The number-average molecular weight was estimated at 75 000 (poly-L-lactide), 79 000 (poly-D,L-lactide); degree of polydispersity – at 1.62 (poly-L-lactide) and 1.9 (poly-D,L-lactide).

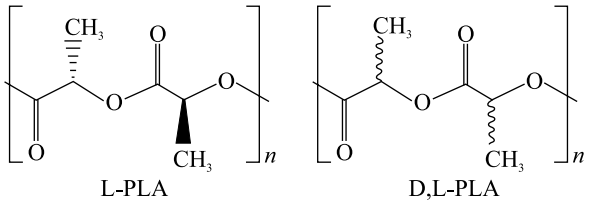

Fig. 1. A structural formula of poly-L-lactide and poly-D,L-lactide

Poly-L-lactide, poly-D,L-lactide and blend films (50 : 50) with 0.1 % FDHex were produced by casting the solution of the polymer in chloroform with PLA concentration of 1 g/L onto silicon and glass substrates, as well as by the centrifugation technique [3]. The thermal history of the film was erased by additional heat treatment at 180 °C.

Synthesis of FDHex are schematically shown on fig. 2.

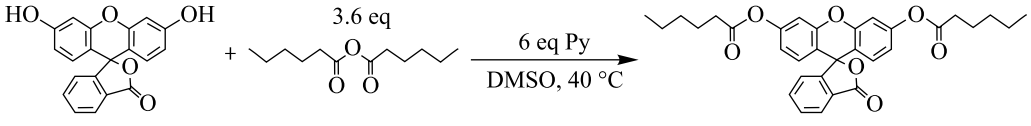

Fig. 2. Scheme for FDHex

Briefly, fluorescein disodium salt (*Sigma*, Germany) has been added to the penicillin vial as 50 mg and dissolved in 6 mL of dimethyl sulfoxide (DMSO; *Fartechnologia*, Belarus). To the resulting solution 66 μL of pyridine (*Lenin Nizhny Tagil Iron and Steel Works*, USSR) followed by 111 μL of hexanoic acid anhydride (*Fluka*, Germany) were added dropwise. The penicillin bottle was equipped with a magnet. To the reaction mixture by hexanoic acid anhydride (111 μL), dissolved in 1 mL of DMSO with constant stirring. The resulting mixture was stirred 4 h at temperature 40 °C until clarification of the solution. The progress of the reaction was monitored using method of thin layer chromatography. At the end of the reaction, the mixture filtered through cotton wool using a glass funnel. The product was purified using silica gel (*Applichem*, Germany) column chromatography (4 × 10 cm) using benzene – acetone (*Ekos*, Russia) gradient eluation.

The solvent as well as hexanoic acid and pyridine (byproducts) was removed using freeze-drying resulting in FDHex as yellowish solid with the yield about 70 %.

Liquid chromatography-mass spectrometry (LC-MS) measurements were performed using the LCMS-2020 (*Shimadzu*, Japan) system equipped with a photodiode array detector and a mass-spectrometer. The latter was operated in the LC-MS mode at a detector voltage of 1.2 kV, heat block temperature of 400 °C and desolvation line temperature of 250 °C. Both negative and positive ions were monitored simultaneously. Nitrogen was used as a drying and nebulizer gas at flow rates of 1.5 and 15.0 L/min respectively. Synthesised compounds were directly injected as methanol solutions.

Chromatography was performed on a Discovery C8 column (150 × 4 mm, 5 μm; *Phenomenex*, USA) using 100 % methanol as the eluent, elution rate 0.4 mL/min.

On the chromatogram is shown one distinct peak of the target product (fig. 3); $m/z = 529.23$ for the $[M + H]^+$ ion confirms the molecular weight of the FDHex (empirical formula $C_{32}H_{32}O_7$).

Fluorescence. Spectrophotometry and fluorimetry were conducted using a spectrofluorimeter SM2203 (*Solar*, Belarus). The slits were set at 5 nm for all measurements.

Differential scanning calorimetry. The study of crystallisation and melting of polylactide crystallites and their mixtures was carried out by differential scanning calorimetry (DSC). DSC curves were recorded and processed on a NETZCSH STA 449C thermal analyser (Netherlands). For each test, between 5 and 7 mg of samples were weighted and then sealed in an aluminium sample pan. When using the DSC, all samples were subjected to a heating rate of 10 °C/min from 20 to 200 °C in a nitrogen atmosphere (flow rate of 20 mL/min) to determine the melting temperature and percentage crystallinity of the sample. The value of the enthalpy of crystalline fusion is possible to estimate the crystallinity of the material using equation

$$\chi^{\mathrm{DSC}} = \frac{\Delta H_m}{\Delta H_m^{100}},$$

where ΔH_m – enthalpy of crystalline fusion; ΔH_m^{100} – fusion enthalpy of the 100 % crystalline material (93.1 J/g for poly-L-lactide and poly-D,L-lactide); χ^{DSC} – crystallinity, %.

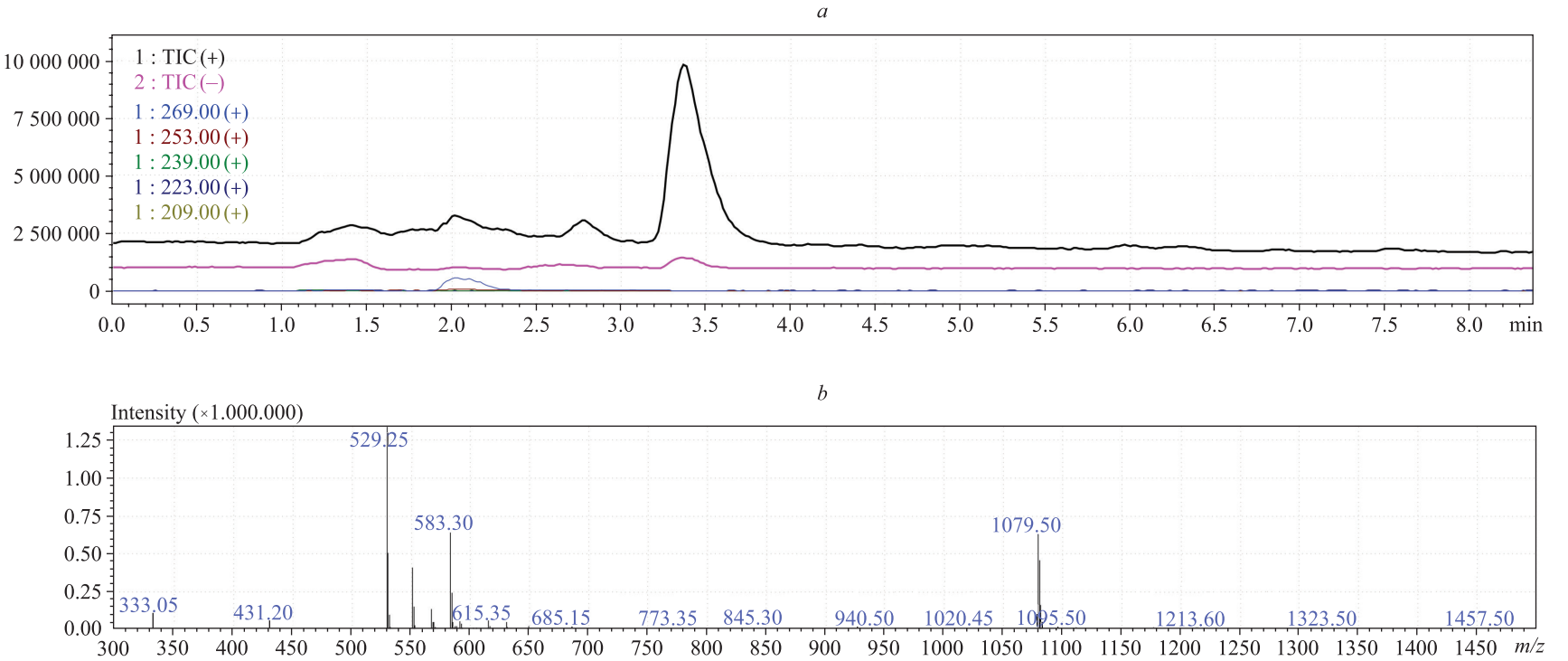

Fig. 3. LC-MS chromatogram (*a*) and mass-spectrum (*b*) of FDHex

Results and discussion

The PLA matrix is widely used in biomedical applications as a material for orthopedic and tissue engineering structures, in this case it requires high strength and retention of properties for a significant period of time, however, the usefulness of PLA is not limited to these areas, since it can serve as a component of the controlled release of biologically active substances. For these purposes, it is necessary to use PLA with special properties (easy processability, relatively short degradation period) and provide the ability to control the process of destruction of the carrier matrix. Tactical poly-D,L-lactide in the form of film samples containing a compatible hydrophobic fluorescent agent was chosen as such a material.

A fluorescent compound is necessary for studying the destruction process, since it is quite difficult to evaluate objectively and comprehensively due to the many reactions that occur. The release of a substance with fluorescent properties allows us to correlate the destruction of the polymer and changes in the physical properties of the liquid medium. To optimise the study time, solutions of sodium hydroxide and hydrochloric acid were used to accelerate destruction.

In this work, the intensity of fluorescence was recorded as a function of the residence time of the image in a 0.1 mol/L solution of sodium hydroxide. As shown earlier [24–26], the hydrolytic destruction of PLA in saline (*in vitro*) and in living organisms (*in vivo*) depends to an important degree on the relaxation and phase state of polymers, and takes place over a period from several months to a year.

Thereby, the use of alkaline solution makes it possible to simulate the process of hydrolytic destruction in a short time. At the same time, a correlation is observed between the physical destruction of the polymer body – the loss of mass by the sample – and the fluorescence intensity, due to the release of FDHex into the solution.

As known, an important role of the processes of hydrolytic destruction of PLA is played by its phase state, highly ordered regions of amorphous-crystalline poly-L-lactide (degree of crystallinity is 37 %), give much greater resistance to destruction of relatively incapable of crystallisation atactic poly-D,L-lactide (degree of crystallinity 0 %). However, in the case where destruction is a factor responsible for the release of any substances from the polymer matrix, it is necessary to be able to vary this parameter. The simplest way to achieve this seems to be the use of mixtures of amorphous- and amphoric-crystalline PLA (the degree of crystallinity of the mixed sample is 16 %). As follows from the graphs, the presence of ordered regions in PLA leads to a slowdown in hydrolytic degradation, the shape of the mixture curve is near poly-L-lactide (fig. 4).

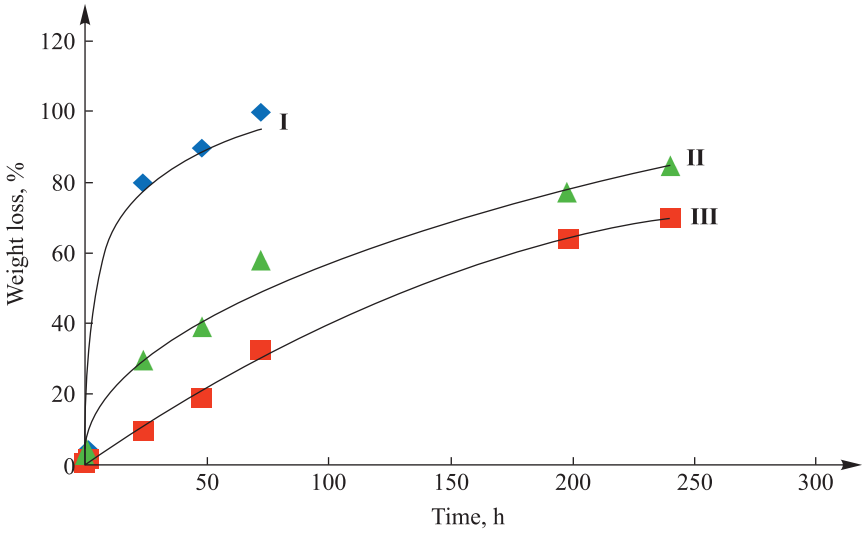

Fig. 4. Weight loss during exposure poly-D,L-lactide (**I**), poly-D,L- and poly-L-lactide (50 : 50) (**II**), poly-L-lactide (**III**) polymer – FDHex composite in 0.1 mol/L NaOH solution

By method fluorescence, the similarity of the curves of mixtures of PLA and poly-D,L-lactide is assessed. The crystalline regions in the mixed sample, even with their small amount (16 %), prevent the destruction of the amorphous regions, however, the smaller size and defectiveness of the supramolecular formations in the PLA mixture leads to an intensive washout of the fluorescent marker from the polymer sample (fig. 5).

So, it was revealed that the dependence of the fluorescence intensity of the solution is influenced not only by the rate of destruction, but also by the phase state of the polymer: the presence of crystalline regions extends the time of hydrolytic decomposition, but to a lesser degree prevents the elution of the fluorescent marker from the polymer sample.

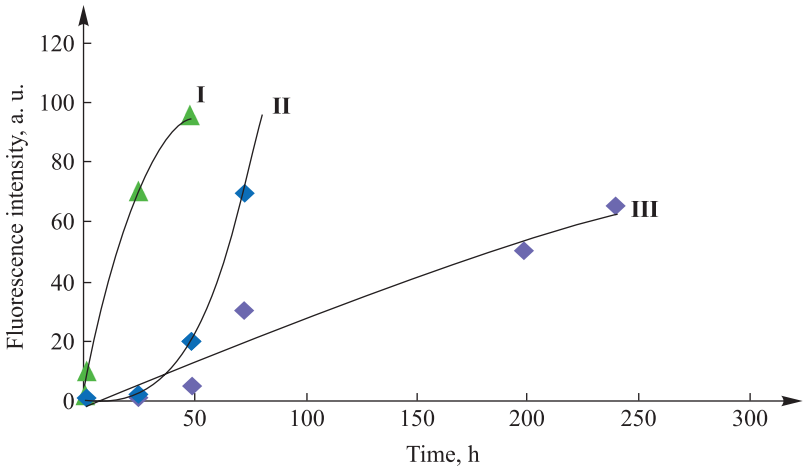

Fig. 5. Effect of exposure poly-D,L-lactide (**I**), poly-D,L- and poly-L-lactide (50 : 50) (**II**), poly-L-lactide (**III**) polymer – FDHex composite in 0.1 mol/L NaOH solution

So, it was found that the dependence of the fluorescence intensity of the solution is influenced not only by the hydrolysing agent, but also by the phase state of the polymer: the presence of crystalline regions increases the time of hydrolytic decomposition and weight loss. At the same time, the small size, defectiveness of crystallites and, as a consequence, the low degree of crystallinity in the poly-D,L- and poly-L-lactide (50 : 50) mixed sample leads to comparable elution of the fluorescent marker from the polymer matrix to amorphous poly-D,L-lactide.

Conclusions

As a result of the work, a fluorescent compound FDHex was synthesised, which has an affinity for the organic phase of an aliphatic biodegradable polyester, PLA. The introduction of FDHex into the matrix made it possible to evaluate the hydrolytic destruction of PLA, to establish the regularities of the effect of low molecular weight components on the release of fluorophore and weight loss by the polymer. The formation of PLA films containing fluorescent can give structural insights of the structure of the polymer itself or make it possible to create biodegradable materials, products from which will find application in the biomedical field.

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