Флуоресцеина диацинонат как профлуоресцентный маркер для мониторинга гидролитической деструкции полилактидных материалов

Д. А. БЕЛОВ1, Я. В. ФАЛЕТРОВ1, 2, П. С. ЯКОВЕЦ1, В. М. ШКУМАТОВ1, 2

1) Белорусский государственный университет, пр. Независимости, 4, 220030, г. Минск, Беларусь
2) Научно-исследовательский институт физико-химических проблем БГУ, ул. Ленинградская, 14, 220006, г. Минск, Беларусь

Полилактид является одним из перспективных полимеров для получения биодеградируемых и биосовместимых материалов. 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 диациноната 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 диацинонат; флуоресценция; гидролиз.

Благодарность. Работа выполнена при поддержке государственной программы научных исследований «Химические процессы, реагенты и технологии, биорегуляторы и биооргхимия» (№ гос. регистрации 20210560).
FLUORESCEIN DIHEXANOATE AS A PROFLUORESCENT MARKER FOR MONITORING OF HYDROLYTIC DESCRUCTION OF POLYLACTIDE-BASED MATERIALS

D. A. BELOV¹, Y. V. FALETROV¹, b, P. S. YAKOVETS¹, V. M. SHKUMATOVA¹, b

¹Belarusian State University, 4 Niezalieźnasci Avenue, Minsk 220030, Belarus
²Research Institute for Physical Chemical Problems, Belarusian State University, 14 Lieninhradskaja Street, Minsk 220006, Belarus

Corresponding author: D. A. Belov (beldis@tut.by)

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.

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 nor fluorogenic groups in common PLA. So, to use this phenomenon to analyse the state of PLA-based materials, the task to make 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 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 depend 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).
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.

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 elution.

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 NETZSCH 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

\[
\chi_{\text{DSC}} = \frac{\Delta H_m}{\Delta H_m^{100}},
\]

where \(\Delta H_m\) – enthalpy of crystalline fusion; \(\Delta H_m^{100}\) – fusion enthalpy of the 100 % crystalline material (93.1 J/g for poly-L-lactide and poly-D,L-lactide); \(\chi_{\text{DSC}}\) – 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 alkali 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 amorphous-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).

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.
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 in poly-D,L- and poly-L-lactide (50:50) 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.

Библиографические ссылки

1. Ikada Y, Tsuji H. Biodegradable polyesters for medical and ecological applications. Macromolecular Rapid Communications. 2000;21(3):117–132. DOI: 10.1002/(SICI)1521-3927(20000201)21:3<117::AID-MARC117>3.0.CO;2-X.
2. Vichaibun V, Chulavatnatol MA. A new assay for the enzymatic degradation of poly(lactic acid). Science Asia. 2003;29:297–300. DOI: 10.2306/scienceasia1513-1874.2003.29.297.
3. Alwattar A, Haddad A, Zhou Q, Nascimento T, Greenhalgh R, Medeiros E, et al. Synthesis and characterisation of fluorescent pyrene-end-capped polylactide fibres. Polymer International. 2018;68(3):360–368. DOI: 10.1002/pi.5712.
4. Fukuzaki H, Yoshida M, Asano M, Kumakura M. Synthesis of copoly(D,L-lactic acid) with relatively low molecular weight and in vitro degradation. European Polymer Journal. 1989;25(10):1019–1026. DOI: 10.1016/0014-3057(89)90131-6.
5. Richert A, Olewnik-Kruszkowska E. Enzymatic degradation of biostatic materials based on polylactide. Ecological Questions. 2018;29(2):91–97. DOI: 10.12775/EQ.2018.014.
6. Patel RB, Carlson AN, Solorio L, Exner AA. Characterization of formulation parameters affecting low molecular weight drug release from in situ forming drug delivery systems. Journal of Biomedical Materials Research. Part A. 2010;94(2):476–484. DOI: 10.1002/jbm.a.32724.
7. Emadian SM, Onay TT, Demirel B. Biodegradation of bioplastics in natural environments. Waste Management. 2017;59:526–536. DOI: 10.1016/j.wasman.2016.10.006.
8. Yew GH, Mohd Yusof AM, Mohd Ishak ZA, Ishiaku US. Water adsorption and enzymatic degradation of poly(lactic)/rice starch composites. Polymer Degradation and Stability. 2005;90(3):488–500. DOI: 10.1016/j.polymdegradstab.2005.04.006.
9. Tsuji H, Echizen Y, Nishimura Y. Enzymatic degradation of poly(L-lactic acid): effects of UV irradiation. Journal of Polymers and the Environment. 2006;14(3):239–248. DOI: 10.1007/s10924-006-0023-6.
10. Peng H, Ling J, Liu J, Zhu N, Ni X, Shen Z. Controlled enzymatic degradation of poly(ε-caprolactone)-based copolymers in the presence of porcine pancreatic lipase. Polymer Degradation and Stability. 2010;95(4):643–650. DOI: 10.1016/j.polymdegradstab.2009.12.005.
11. Li S, Liu L, Garreau H, Vert M. Lipase-catalyzed biodegradation of poly(ε-caprolactone) blended with various polylactide-based polymers. Biomacromolecules. 2003;4(2):372–377. DOI: 10.1021/bm025748j.
References

1. Ikada Y, Tsuji H. Biodegradable polylesters for medical and ecological applications. Macromolecular Rapid Communications. 2000;21(3):117–132. DOI: 10.1002/1521-3927(20000221)21:3<117::AID-MARC117>3.0.CO;2-X.

2. Vichasun V, Chulavattmol MA. A new assay for the enzymatic degradation of polylactic acid. Science Asia. 2003;29:297–300. DOI: 10.2306/sciencia1513-18742003.29.29.297.

3. Alwattar A, Haddad A, Zhou Q, Nascimento T, Greenhalgh R, Medeiros E, et al. Synthesis and characterisation of fluorescent pyrene-end-capped polylactide fibres. Polymer International. 2018;68(3):360–368. DOI: 10.1002/pi.35712.

4. Fukuzaki H, Yoshida M, Asano M, Kumakura M. Synthesis of copoly(D,L-lactic acid) with relatively low molecular weight and in vitro degradation. European Polymer Journal. 1989;25(10):1019–1026. DOI: 10.1016/0014-3057(89)90131-6.

5. Richert A, Olejnik-Kruszewska E. Enzymatic degradation of biostatic materials based on polylactide. Ecological Questions. 2016;29(2):91–97. DOI: 10.12775/EQ.2018.014.

6. Patel RB, Carlson AN, Solorio L, Exner AA. Characterization of formulation parameters affecting low molecular weight drug release from in situ forming drug delivery systems. Journal of Biomedical Materials Research. Part A. 2010;94(2):476–484. DOI: 10.1002/jbma.32724.

7. Emadian SM, Onay TT, Demirel B. Biodegradation of bioplastics in natural environments. Waste Management. 2017;59:526–536. DOI: 10.1016/j.wasman.2016.10.006.

8. Yew GH, Mohd Yusof AM, Mohd Ishak ZA, Ishiak US. Water adsorption and enzymatic degradation of polylactic/rice starch composites. Polymer Degradation and Stability. 2005;90(3):488–500. DOI: 10.1016/j.polymdegradstab.2005.04.006.

9. Tsuji H, Echizen Y, Nishimura Y. Enzymatic degradation of poly(L-lactic acid): effects of UV irradiation. Journal of Polymers and the Environment. 2006;14(3):239–248. DOI: 10.1007/s10924-006-0023-6.

10. Peng H, Ling J, Liu J, Zhu N, Ni X, Shen Z. Controlled enzymatic degradation of poly-(ε-caprolactone)-based copolymers in the presence of porcine pancreatic lipase. Polymer Degradation and Stability. 2010;95(4):643–650. DOI: 10.1016/j.polymdegradstab.2009.12.005.

11. Li S, Liu L, Garreau H, Vert M. Lipase-catalyzed biodegradation of poly(ε-caprolactone) blended with various polylactide-based polymers. Biomacromolecules. 2003;4(2):372–377. DOI: 10.1021/bm025748j.

12. Göpferich A. Mechanisms of polymer degradation and erosion. Biomaterials. 1996;17(2):103–114. DOI: 10.1016/0142-9612(96)85755-3.

13. Göpferich A, Langer R. Modeling of polymer erosion. Macromolecules. 1993;26(16):4105–4112. DOI: 10.1021/ma00068a006.

14. Freichels H, Danhier F, Prétat V, Lecomte P, Jérôme C. Fluorescent labeling of degradable poly(lactide-co-glycolide) for the cellular nanoparticles tracking in living cells. The International Journal of Artificial Organs. 2011;34(2):152–160. DOI: 10.5301/ijao.2011.6420.
15. Tosi G, Rivasi F, Gandolfi F, Costantino L, Vendelli MA, Forni F. Conjugated poly(D,L-lactide-co-glycolide) for the preparation of in vivo detectable nanoparticles. *Biomaterials*. 2005;26(19):4189–4195. DOI: 10.1016/j.biomaterials.2004.10.025.

16. Hu J, Guo J, Xie Z, Shan D, Gerhard E, Qian G, et al. Fluorescence imaging enabled poly(lactide-co-glycolide). *Acta Biomaterialia*. 2016;29:307–319. DOI: 10.1016/j.actbio.2015.10.010.

17. Robin MP, O’Reilly RK. Strategies for preparing fluorescently labelled polymer nanoparticles. *Polymer International*. 2014;64(2):174–182. DOI: 10.1002/pi.4842.

18. Hu J, Park H, Na Y, Kim S, Il Choi W, Lee JH, et al. Novel fluorescein polymer-based nanoparticles: facile and controllable one-pot synthesis, assembly, and immobilization of biomolecules for application in a highly sensitive biosensor. *RSC Advances*. 2020;10(5):2998–3004. DOI: 10.1039/C9RA09106H.

19. Jarzębski M, Peplińska B, Florczak P, Gapiński J, Flak D, Mała P, et al. Fluorescein ether-ester dyes for labeling of fluorinated methacrylate nanoparticles. *Journal of Photochemistry and Photobiology. Part A: Chemistry*. 2019;382:111956. DOI: 10.1016/j.jphotochem.2019.111956.

20. Demina TS, Grandišs Ch. Solid-state modified polylactides for processing of 3D materials with enhanced biocompatibility. *Materials Today: Proceedings*. 2019;12(1):93–96. DOI: 10.1016/J.MATPR.2019.03.072.

21. Ma H, Zhang A, Zhang X, Zhao H, Cui Z, Fu P, et al. Novel platform for visualization monitoring of hydrolytic degradation of biodegradable polymers based on aggregation-induced emission (AIE) technique. *Sensors and Actuators B: Chemical*. 2020;304:12734. DOI: 10.1016/j.snb.2019.127342.

22. Bardakova KN, Grebenik EA, Minaev NV, Churbanov SN, Moldagazyeva Z, Krupinov GE, et al. Tailoring the collagen film structural properties via direct laser crosslinking of star-shaped polylactide for robust scaffold formation. *Materials Science and Engineering: C*. 2020;107:110300. DOI: 10.1016/j.msec.2019.110300.

23. Agnieszka R, Dąbrowska GB. Enzymatic degradation and biofilm formation during biodegradation of polylactide and polycaprolactone polymers in various environments. *International Journal of Biological Macromolecules*. 2021;176:226–232. DOI: 10.1016/j.ijbiomac.2021.01.202.

24. Belov DA. Influence of the processes of radiation and hydrolytic destruction on the phase and relaxation transitions in poly-L- and poly-D,L-lactides. *Proceedings of the National Academy of Sciences of Belarus. Chemical Series*. 2010;1:40–43. Russian.

25. Krul LP, Belov DA, Butovskaya GV. Structure and physicochemical properties of biodegradable materials based on polylactides. *Vestnik BGU. Seriya 2. Khimiya. Biologiya. Geografiya*. 2011;3:5–11. Russian.

26. Krul LP, Belov DA, Butovskaya GV, Poloiko NA, Artushkevich AS. Poly-D,L-lactide destruction in vivo and in vitro. In: Kricheldorf HR, Schwarz G, Wutz C, editors. *Biomaterials. 29th Hamburger makromoleculares symposium; 2006 October 1–4; Hamburg, Germany*. Hamburg: Universitit Hamburg; 2006. p. 88.

Received 29.10.2021 / revised 29.10.2021 / accepted 09.12.2021.