

EPR Diagnostics of D,L-Polylactide Porous Matrices Formed in Supercritical CO₂

N. A. Chumakova^{a, *}, E. N. Golubeva^a, T. A. Ivanova^a, N. N. Vorobieva^b,
P. S. Timashev^{b, c}, and V. N. Bagratashvili^b

^aMoscow State University, Moscow, Russia

^bInstitute of Photonic Technologies, Crystallography and Photonics Research Center,
Russian Academy of Sciences, Moscow, Russia

^cInstitute for Regenerative Medicine, Sechenov University, Moscow, Russia

*e-mail: harmonic2011@yandex.ru

Received February 16, 2018

Abstract—The regularities of formation of D,L-polylactide porous matrices in supercritical CO₂ environment with simultaneous impregnation with paramagnetic biologically active 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) was studied using spin probe electron paramagnetic resonance (EPR) and optical microscopy. The dependence of the average and local concentration of a dopant on impregnation conditions was assessed. The resulting matrices meet important requirements for porous materials for tissue engineering. Considering that impregnation of a polymer with a paramagnetic compound makes it possible to study the uniform distribution of a dopant in a sample at macroscopic and microscopic levels and to study a structure of a polymer matrix, EPR spectroscopy is a promising method for studying porous polymeric materials formed under supercritical conditions.

Keywords: impregnation, supercritical carbon dioxide, EPR, spin probe method, matrices for tissue engineering

DOI: 10.1134/S1990793118080031

INTRODUCTION

Three-dimensional porous biodegradable structures based on foamed polylactides and polylactoglycolides are promising materials for reconstructive surgery as carriers of progenitor and specialized cells. A matrix is replaced by newly formed tissues in an organism, which gradually grow through the pores of a polymer in parallel with to material degradation both via hydrolysis of ester bonds of polymers and cellular response. One of the technologies for the production of porous polymer matrices is foaming in a supercritical (SC) fluid environment, primarily carbon dioxide [1–4]. The main advantages of these technologies are the rejection of hard to remove organic solvents and the possibility of direct production of matrices with a certain structure by varying the parameters of the foaming process [5]. The pore system of a material must meet some requirements: it should be open and uniform and should include pores larger than 100 μm for the growth of blood vessels and smaller pores for germination of cells; the total pore volume should be at least 80% of the volume of a material.

Up to 100% of the pores in the three-dimensional structures should be linked together, so that nutrients and oxygen can penetrate into cells and the products of the vital activities of the cells may be removed [1].

The development of SC foaming technology to create materials with mutually bound pores of a given size is therefore an urgent task of medical materials science. Many methods, including quantitative ones, have been developed to study the permeability of pores via measurement of the pressure jump during passage of gases, primarily air [6] and nitrogen [7], and various liquids (water, methanol, and acetone [8]), and measurement of flow rate through a matrix.

Biologically active substances are added to a polymer matrix to prevent inflammation and stimulation of tissue growth. Introduction of biologically active dopants during SC fluid polymer modification is the most promising method [1, 9, 10]. One of the factors that constrain the widespread use of polymer impregnation in the SC-CO₂ environment with biologically active substances is the regulation and control of a uniform distribution of dopant throughout a sample. In fact, the processes taking place during swelling of polymers in the SC-CO₂ and subsequent release of pressure are non-equilibrium. As a result, the formation of a dopant microphase in the polymer bulk and the concentration of doping molecules in the near-surface layer, etc., are possible. Moreover, when the pressure is released, a significant amount of active substance can be precipitated both on the outer sur-

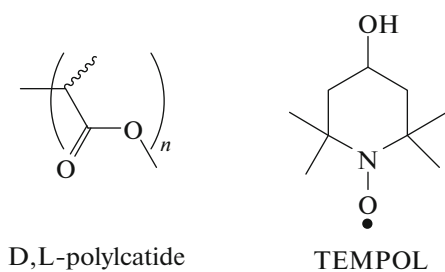


Fig. 1. The structural formulas of the polymer and spin probe.

face of a matrix and on the inner surface of the pores [10]. A biologically active compound will then unevenly release during biodegradation of a temporary prosthesis. This distribution is necessary to be controllable without destroying the material to develop an impregnation technology of polymeric materials in the SC fluid environment, which leads to this uniform distribution of the doping substance. One of the promising methods for the analysis of the distribution of molecules in a matrix is spin probe electron paramagnetic resonance (EPR) spectroscopy. In fact, polylactides and polylactide glycolides do not possess paramagnetic properties and when a stable paramagnetic dopant (spin probe) is introduced into the polymer its distribution in the matrix may be controlled with EPR spectroscopy. EPR makes it possible to find the number of paramagnetic molecules in a sample within 10^{14} – 10^{15} particles (10^9 – 10^{10} mol) with an accuracy of 10–20% [11]. In addition, the width of EPR lines is sensitive to the distance between molecules, on which the interaction of the magnetic dipoles of spin probes depends [12].

Currently, the spin probes that are most widely used are stable nitroxyl radicals [13]. EPR spectra of nitroxyls are sensitive to mobility and to orientational and translational distribution of paramagnetic molecules due to high spatial anisotropy of magnetic parameters. These substances are widely used for the study of the structure and dynamics of polymeric materials [14]. A nitroxyl group is sufficiently resistant to chemical modification, such that it may be introduced into many molecules, including biologically active substances [15]. Their localization, orientation, mobility, and other properties may be in vitro and in vivo observable with EPR spectra [12, 16]. In addition, nitroxyl radicals possess their own biological activity, which has a positive effect on many processes in the body [17–19]. Nitroxyl radicals, as average members of a nitrosonium ion–nitroxyl radical–nitroxylamine redox triad, can neutralize the active forms of oxygen and, therefore, perform a protective antioxidant function in the body [16]. The toxicity of nitroxyl radicals is low and is 1.5–2.0 mmol/kg, when 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) is intravenously administered into mice [16]. When nitroxyl

fragment is introduced into a biologically active compound, its biological effect can be strengthened, so that synergism takes place [13, 20]. The high level of chemical and thermal stability of nitroxyl radicals makes it possible to use them in an SC-CO₂ environment [21, 22]. The introduction of a stable nitroxyl radical into a polymer during its supercritical fluid treatment, therefore, makes it possible to control the distribution of a dopant throughout a sample at the microscopic and macroscopic levels, to study the mobility of an active substance in a polymer matrix, and to control the release of the dopant from the polymer. The aim of this work was to develop methods for the formation and diagnostics of D,L-poly(lactide) porous materials formed in an SC-CO₂ environment, which contain a stable nitroxyl radical TEMPOL, which is the most commonly used spin probe.

MATERIALS AND METHODS

PURASORB PDL 04 D,L-poly(lactide) (Purac Biochem), with $M_w = 45$ kg/mol and a glass transition temperature of 325 K, and TEMPOL stable nitroxide radical (Sigma-Aldrich) were used without further purification. Figure 1 shows the structural formulas of the polymer and spin probe. Chemically pure carbon dioxide, toluene, and acetonitrile were also used without further purification.

The polylactide was foamed and simultaneously impregnated with a spin probe using a SCF minilaboratory universal instrument [23]. A cylindrical foil shape containing a probe sample (~2.5 mg) was placed into 18 mL reactor. Polymer pellets (~0.4 g) were ground to a size of 100–200 μm and placed into Teflon cups with a diameter of 10 or 18 mm and a height of 20 mm, and the entire construction was loaded into the reactor. The reactor was filled with liquid CO₂ at room temperature and then heated to 313 K for 20–30 min. The thermostating accuracy was ± 1 K and the accuracy of maintaining the pressure was 0.1–0.2 MPa. Samples were held in a SC-CO₂ environment for a certain time, the pressure was then decreased, and temperature was lowered to room temperature. The pressure was manually decreased with a fine adjustment valve. The holding time and the pressure relief mode are thoroughly discussed in the next section. A polymer foam containing a paramagnetic dopant was obtained at the end of the process. The vessel containing TEMPOL was empty at the end of the process, which indicates redistribution of the radical throughout the reactor.

A D,L-poly(lactide) sample, in which the TEMPOL radical was introduced from an acetonitrile solution, was prepared for comparison. For this, acetonitrile containing the dissolved polymer and radical was poured onto a smooth cellophane surface (GOST 7730-89, 30–45 g/m²). A film with a thickness of ~200 μm was obtained after drying of the solvent. The

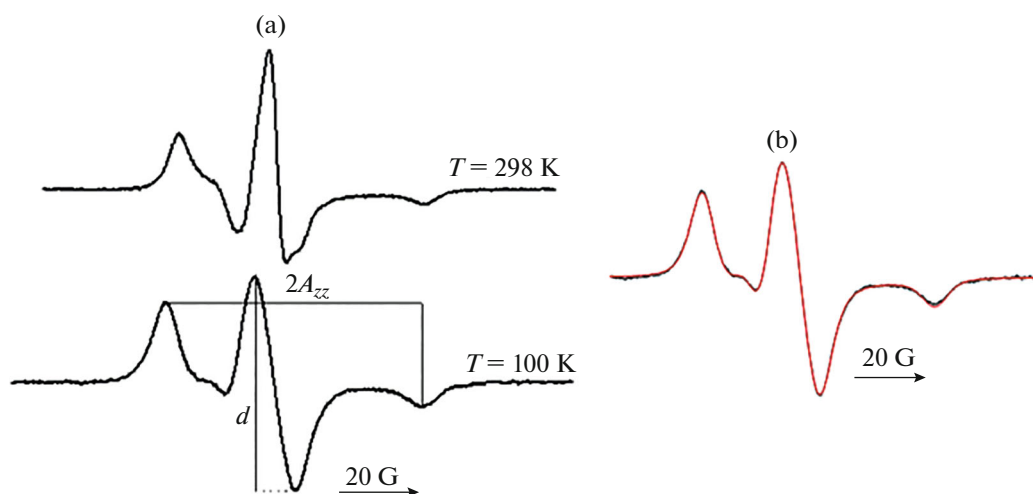


Fig. 2. (a) The EPR spectra of foamed poly lactide samples containing a spin probe and (b) computer simulation result of spectrum recorded at 100 K. The black line denotes the experimental spectrum; the red line indicates the simulation result.

solvent residues were removed by holding the film at 5×10^{-6} MPa for 5 days.

EPR spectra were acquired on a Bruker EMX-500 X-band radio spectrometer at 100 and 298 K. When the spectra were recorded at low temperature, the sample was in the resonator of the spectrometer in a Dewar tube, which was purged with nitrogen. The gas temperature was maintained with an accuracy of ± 1 K on special Bruker equipment. The microwave radiation power was 0.8 mW. The fact that there was no saturation of TEMPOL signals in the poly lactide at this radiation power within this temperature range was established after special experiments.

The foamed polymer samples were cut into $4 \times 2 \times 2$ -mm fragments to determine the amount of paramagnetic substance. The amount of paramagnetic molecules in each fragment was found via double integration of its EPR spectrum. The average concentration of radicals in different samples calculated as the total number of paramagnetic molecules in all fragments relative to the mass of the sample was $(2-8) \times 10^{18}$ particles per gram. With this concentration, the average distance between the radicals was ~ 50 Å, and the dipole-dipole broadening of EPR spectra was no more than 0.4 G. The local concentration of spin probes was assessed according to the method from an empirical parameter, which characterizes the shape of an EPR spectrum recorded without rotational mobility of paramagnetic molecules (in the hard limit) [13].

In this work, to determine the permeability of the pores of the foamed poly lactide, the samples were immersed into an aqueous dye solution (ink for a MOORIM inkjet printer) for 1 day, after which they were dried in air for 2 days and after that were cut. The sample stain visible at the cut was used as a characteristic of permeability of polymer pores. Micrographs of

the samples were obtained on a BLM-500 optical microscope.

RESULTS AND DISCUSSION

Figure 2a shows the EPR spectra of the foamed D,L-poly lactide samples containing a spin probe recorded at 100 and 298 K. The shape of the spectrum at 100 K is typical for a nitroxyl radical without rotational mobility of paramagnetic molecules (in the hard limit) and is due to anisotropy of the spin-Hamiltonian parameters [13]. In particular, the distance between the extreme components in the spectrum is the doubled hyperfine coupling constant $2A_{zz}$ of an unpaired electron with a nitrogen nucleus [12]. The following spin-Hamiltonian parameters were found for TEMPOL radical in poly lactide matrix via computer simulation of the hard-limit spectrum: $g_{xx} = 2.0093 \pm 0.0002$, $g_{yy} = 2.0060 \pm 0.0002$, $g_{zz} = 2.0020 \pm 0.0002$, $A_{xx} = 7.3 \pm 0.1$ G, $A_{yy} = 5.3 \pm 0.1$ G, and $A_{zz} = 34.16 \pm 0.05$ G. The simulation was performed according to the methodology described in [13]. Figure 2b shows the simulation result. The values obtained are close to the spin Hamiltonian parameters for TEMPOL in nonpolar solvents [24], which indicates the low polarity of the local environment of the probe in poly lactide matrix.

The shape of the EPR spectra of nitroxyl radicals is sensitive to the rotational mobility of paramagnetic molecules. One of the main characteristics of a spectrum, reflecting the rotational mobility of radicals, is the distance between the extreme components. This parameter for the spectrum recorded at 298 K is 65.5 G, which corresponds to a rotational correlation time of paramagnetic molecules in a polymer matrix of no more than 1×10^{-8} s [25, 26]. In fact, D,L-poly lactide

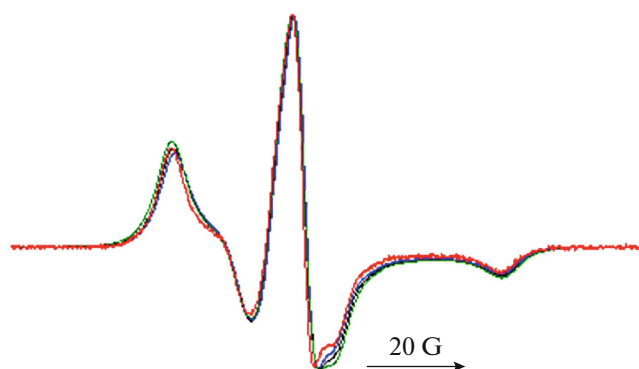


Fig. 3. The EPR spectra of the TEMPOL radical in polylactide samples obtained via codissolution of polymer and a paramagnetic substance in acetonitrile with subsequent evaporation of solvent (red line) and impregnated under supercritical conditions: (black line) 15 MPa and pressure release time of 7 min, (blue line) 12 MPa and pressure release time of 3 min, and (green line) 16 MPa and a pressure release time of 48 min.

is glassy at 298 K, which leads to low mobility of the doping molecules.

Figure 3 shows the EPR spectra of TEMPOL in several polylactide samples impregnated in SC-CO₂ and in a sample obtained via coprecipitation of polymer and TEMPOL radical from acetonitrile for comparison. It is seen that the shape of the EPR spectra for the paramagnetic substance in all samples is similar. The local environment of paramagnetic molecules in D,L-polylactide, therefore, is almost independent on impregnation method of polymer, because the shape of the EPR spectra of nitroxyl radicals in polymer matrices is sensitive to the local environment of the probes [26].

A significant factor that determines the quality of matrices obtained via impregnation of polymers in SC fluid is the uniformity of distribution of dopant throughout a sample. In fact, the polymer swelling and foaming of a polymer matrix during a pressure release are non-equilibrium. In this case, unevenly impregnated samples may be obtained with great probability.

Table 1. The dependence of the maximum ratio of TEMPOL concentrations in different parts of a foamed sample on impregnation conditions

Holding time of sample in SC-CO ₂ , min	Pressure		
	16 MPa	18 MPa	22 MPa
20	—	82 ± 17	—
40	—	40 ± 8	—
120	1.8 ± 0.4	2.3 ± 0.5	4.0 ± 0.8
480	1.1 ± 0.2	—	—

In this work, both macroscopic and microscopic distributions of a paramagnetic substance in a polymer matrix were determined. The macroscopic distribution, as we assume, is the amount of a dopant per unit of polymer mass in different fragments of the sample. The microscopic distribution is the ratio of the mean and local concentrations of paramagnetic molecules.

The macroscopic distribution of a probe throughout a sample largely depends on the impregnation conditions: the amount of probe is at a maximum in its upper part, because polymer is impregnated in a Teflon cup with a probe solution in carbon dioxide precisely from the upper part of the sample. Table 1 shows the dependence of the maximum ratio of the amounts of paramagnetic substance per gram of the polymer in various fragments of the foam sample on impregnation conditions. It is seen that the difference in the amount of the dopant in different parts of the sample varies depending on its keeping time at 18 MPa from 82 (20 min) to 2.3 times (120 min). There was an inverse relationship between pressure and the uniformity of the distribution of paramagnetic substance throughout the sample at different pressures with the same reduction time. The most uniform distribution of the dopant throughout the sample occurred during impregnation at a CO₂ pressure of 16 kPa, a holding time of 480 min, and a pressure reduction time of 50 min.

The slower the pressure decreases, the more balanced the release process of carbon dioxide from the polymer matrix is. In this case, the doping substance is most evenly distributed in the foamed polymer. We may assume that the pressure release process in an automatic mode with a feedback device will allow obtaining samples with a uniform distribution of dopant in the sample. The uniform distribution of a probe depends on the pressure of SC-CO₂ to a lesser extent. In this work, we attempted to perform an experiment at a pressure below 16 MPa, but we found that unevenly foamed samples are formed in this case (Fig. 4, color insert).

The following phenomenon was also observed in the samples in which there was the greatest macroscopic inhomogeneity of the dopant distribution. The amount of paramagnetic substance determined from the EPR spectra of foamed samples is less than that of the same samples dissolved in toluene by from a few percent to a factor of two. There was no such difference is not observed for the samples obtained at a pressure reduction time of 480 min. The results apparently indicate that microphases of a paramagnetic substance are formed inside the polymer matrix. Such microphases are probably formed during the non-equilibrium release process of carbon dioxide from the polymer matrix with a decrease in pressure. The spectra of magnetically concentrated particles may be greatly broadened due to dipole–dipole and/or spin–spin coupling interactions, which leads to an actual merging of these spectra with the background. The pressure

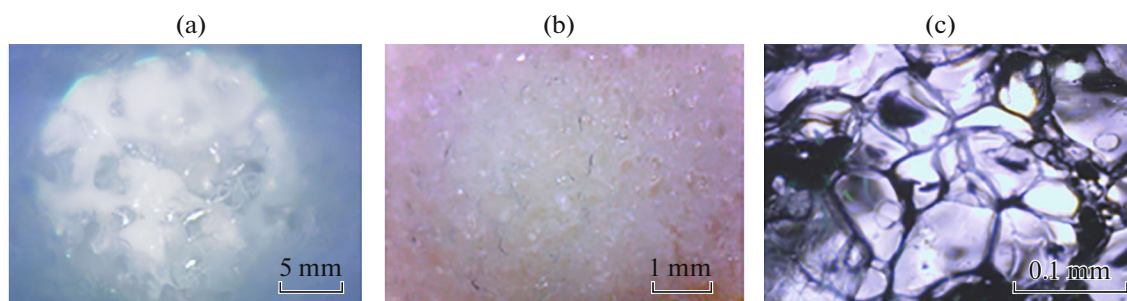


Fig. 4. Microphotographs for foamed polylactide samples obtained at: (a) 14 and (b) 16 MPa, and (c) a sample prepared in dye solution at 16 MPa.

reduction process of an SC fluid is therefore an important stage of impregnation, which determines the uniformity of distribution of a dopant in the polymer matrix.

The technique described in [13] was used for determination of the uniformity of distribution of the paramagnetic substance in the polymer matrix at the molecular level. The technique is based on an analysis of dipole–dipole broadening in EPR spectrum of a sample recorded in the absence of mobility of paramagnetic molecules. The spectra acquired at 100 K were analyzed in this work. Due to this approach, the local concentration of paramagnetic molecules may be found from the difference between the d_1/d value (Fig. 2a) for the EPR experimental spectrum and the spectrum without dipole–dipole broadening. The d_1/d value without broadening can be calculated from the formula: $d_1/d_0 = 1.73 - 0.035A_{zz}$. Analysis of the EPR spectra of all the samples showed that there was no dipole–dipole broadening, so that impregnation of polylactide under supercritical conditions does not lead to a local concentration of a paramagnetic substance in the polymer phase.

The system of connected pores is one of the most important parameters for three-dimensional matrices considered from the point of view of the formation of cell-engineering constructs. The degree of pore penetration for the dye solution depends on the rate of pressure reduction to a large extent (Fig. 5). If the pressure reduction time is 20 min or more, most of the pores are painted. Figure 4c shows the morphology of the dyed porous polymer with a large increase. It is seen that the polymer contains pores of various sizes (from 5 to $> 100 \mu\text{m}$), which are interconnected and permeable to the aqueous environment.

CONCLUSIONS

In summary, EPR spectroscopy is a promising method for studying porous polymeric materials, including those formed during treatment of an SC fluid environment. Impregnation of a polymer with a paramagnetic substance makes it possible to study the uniformity of distribution of dopant in a sample at the macroscopic and microscopic levels and to study the structure of the polymer matrix. EPR diagnosis of foamed samples made it possible to obtain and to characterize the foamed D,L-polylactide matrices, which meet the basic requirements for porous bio-compatible materials for tissue engineering.

ACKNOWLEDGMENTS

The authors are grateful to the Russian Science Foundation (project no. 14-33-00017-P) for financial support of preparation of porous matrixes and to the Russian Foundation for Basic Research (project nos. 16-03-00333 and 17-02-00445) for support of structure elucidation of matrixes with EPR spectroscopy. The authors are also grateful to Ph.D Gromov O.I. for his support and advice in preparation of matrixes, and to Prof. Vorobiev A.Kh., Prof. Kokorin A.I., and Prof. Dubinskii A.A. for their consultations in quantitative EPR measurements.

This work was performed on equipment acquired through the Development program of Moscow University.

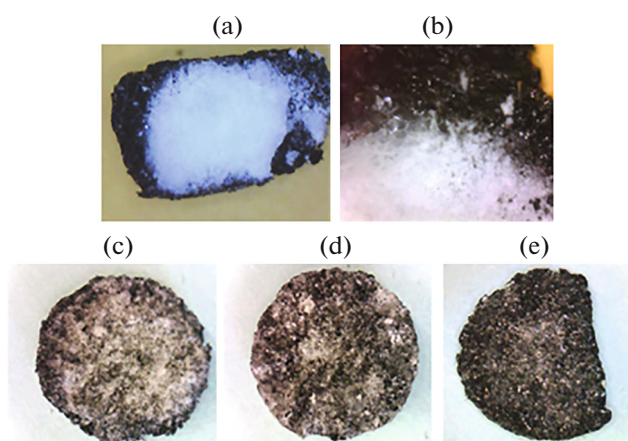


Fig. 5. Microphotographs for sections of samples prepared at 16 MPa and pressure reduction times: (a and b) 3, (c) 5, (d) 7, and (e) 20 min. The diameter of the samples is 10 mm.

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Translated by A. Tulyabaev