Influence of radiation sterilization on poly(ester urethanes) designed for medical applications

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Abstract Preparation of scaffolds for tissue regeneration requires elucidation of chemical and physical processes induced by ionizing radiation during radiation sterilization of polymeric materials used for these purposes. Such treatment induces many unintended and desired reactions that lead either to degradation or cross-linking of macromolecules. It was found that in poly(ester urethane) the ester segments are responsible predominantly for radiation induced phenomena. Some radicals generated following irradiation were identified under cryogenic conditions. Upon exposure to sterilizing dose cross-linking was confirmed on the basis of rheological changes in melted polymers. The results obtained by EPR spectroscopy and DRS revealed a relatively high susceptibility of poly(ester urethanes) on oxidation.

Key words polyurethane • radiation sterilization • EPR • cross-linking • degradation

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Introduction

Polyurethane based materials have recently received increasing attention as multi-component systems widely applied for medical devices [9]. They usually consist of two separated phases what provides a unique opportunity to control chemical and mechanical properties of the resulting material. The type and proportions between components determine the character of polymer and consequently their industrial utilization. They are considered as the resins of growing application in medicine, particularly in tissue engineering. Most of the polymeric materials are composed of two segments of different rate of biodegradation and various mechanical properties.

Resins contained urethanes and ester components were found unsuitable for long-term implants because of fast hydrolysis of the ester soft segments. On the other hand, such properties make them valuable, gradually degradable biomaterial that might be used as scaffolds for tissue engineering [3, 5, 10]. One of the most important aspects concerning fabrication of such materials is elimination of bioburdens. The application of ionizing radiation for the sterilization purposes has prompted studies on the response of poly(ether urethane) materials on irradiation. The process could induce undesired consequences, e.g. degradation, oxidation, release of harmful gaseous products, etc. Simultaneously, cross-linking of macromolecules might proceed together with formation of additional polar groups that seems to increase hydrophilicity and eventually adhesion of cells to polymeric matrix.

The elastomeric polyurethanes are known to be radiation stable materials for sterilizing doses [3, 6, 10].

Nevertheless, if additional components appear in the system, e.g. segments of oligoesters, then the influence of irradiation is poorly recognized. Contrary to polyurethanes, polyesters are characterized by high oxidizability, thus in their copolymers the oxidative processes are supposed to be at least partly limited.

Gorna and Gogolewski, while investigating molecular stability of polycaprolactone (PCL)-based polyurethanes confirmed that for weight-average molecular weight of 130.8 kDa gamma irradiation induces (i) a decrease in molecular weight by 30%, (ii) no changes in the polydispersity and (iii) a slight increase in contact angle vs. water, depending on the length of PCL segment [4]. If the copolymer contains third, hydrophilic component, e.g. polyethylene oxide, the effect is much more significant and mechanical properties deteriorate considerably.

It was also confirmed that in poly(ester urethane) UV light initiates free radical degradation and oxidation followed by the release of hydrogen at the α position to nitrogen atom (for $\lambda = 300$ nm) and additionally, for a shorter wavelength ($\lambda = 254$ nm), scission of C-N or C-O bonds [11]. Guan *et al.* found that poly(ester urethane)ureas based upon two lengths of PCL soft segments shows different phase separation and changes in morphology [5]. The other factor that determines the mechanisms of radical processes is the presence or absence of oxygen during irradiation [8].

The purpose of the presented study was to evaluate the effect of ionizing radiation on poly(ester urethanes) (PEU) that occurs during radiation sterilization. Thus, we have studied free radicals generated in polymers synthesized from 4,4'-methylene bis (*p*-cyclohexyl isocyanate), poly(ε-caprolactone)diol and ethylene glycol using the EPR method for their detection. We prepared two types of polymeric materials of various lengths of PCL units since the domain size is a factor that often influences the extent of phases compatibility.

In the studied polymers, various substituents and reactive groups are able to stabilize unpaired electrons. In order to identify primary and secondary paramagnetic species, the substrates of polymer matrix were examined and their spectra were compared with experimental signals of the resulting resins. Taking into account that free radical processes are responsible for the changes in physical properties, we also estimated the extent of cross-linking induced by ionizing radiation and contact angle for water, a parameter that indirectly characterize adhesion of the cells cultured on the surface of PEU.

In order to evaluate the influence of radiation on biocompatibility of poly(ester urethanes), we examined the viability of human bone derived cells (HBDCs) and their osteogenic activity *in vitro*.

Experimental

Materials

The polyurethane based materials were prepared from 4,4'-methylene bis (*p*-cyclohexyl isocyanate) (HMDI), poly(ε-caprolactone)diol (PCL) and ethylene glycol (EG) used as a chain-extending agent. The synthesis was performed with the prepolymer method, Scheme 1. The substrates were obtained from Aldrich
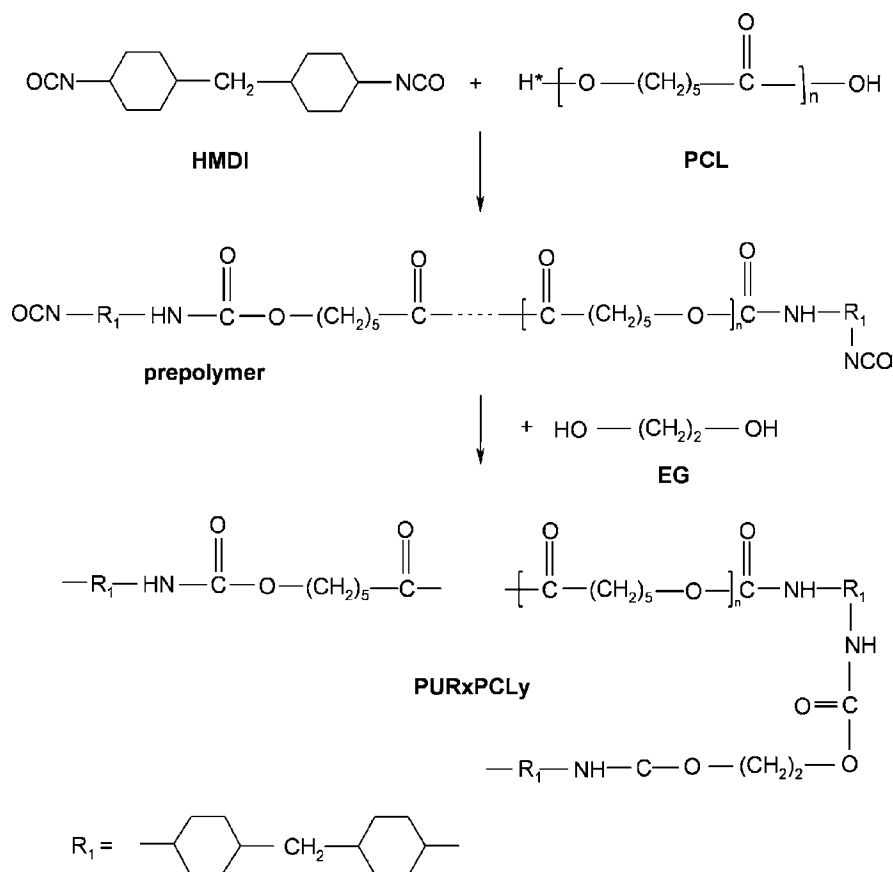
Scheme 1.

Chemical Co., Germany. The resulting elastomers contain components of the following molar ratios HMDI:PCL:EG = 4:1:3 for PEU1 and 2:1:1 for PEU2. The PCL oligomers have various molecular weights – 1250 Da in PEU1 and 530 Da in PEU2.

Samples investigated by EPR were irradiated to a dose of 6 kGy in a ^{60}Co gamma source (Issledovatel) under cryogenic conditions, i.e. at 77 K, since free radicals appeared to be unstable at ambient temperature. Other measurements were carried out upon exposure of the materials to a dose of 25 kGy in an electron beam accelerator LAE 13/9 at room temperature. In order to irradiate the samples with higher doses, the process was repeated until the expected total dose was achieved, e.g. a dose of 75 kGy was supplied during three times repeated irradiation with doses of 25 kGy. In this way the materials avoided overheating.

Methods

EPR measurements were performed on an X band Bruker ESP300 spectrometer. Spectra were recorded directly upon irradiation of the samples and after their annealing to the temperatures indicated in figures. The typical EPR parameters for spectra acquisition were as follow: sweep width 30.0 mT, microwave power 2 mW, modulation amplitude 0.02 mT, time constant 10 ms. Number of scans was adjusted to intensity of the experimental spectra. All signals were detected at 77 K. Annealing temperature was controlled by a thermocouple inserted in the centre of the sample. The signals were analyzed using Apollo software [1]. Radical concentration was determined by comparison of the spectral areas obtained by double integration. Simulation was carried out using SimFonia software (Bruker).

Dynamic contact angle was measured using a Tensiometer K100C (Krüss) supplied with a thermostattable sample vessel at 23°C. Applying the Wilhelmy method, six measurements were performed for each sample; a confidence interval was calculated for a confidence level equal to 0.90. The gel content was determined by solvent extraction in boiling cyclohexanone for 8 h and by measuring the content of solid fraction. The gel residue was dried to a constant weight by vacuum-pumping system for 24 h. The measurements were repeated three times.

Apparent viscosity was measured with a CAP 2000+H Brookfield viscometer at the temperature indicated in figures. The process was computer controlled via Brookfield CALPCALC software.

DRS (diffuse reflection spectrophotometry) measurements were carried out on a Perkin Elmer Lambda-9 apparatus.

In a preliminary biological study, PEU2 samples were irradiated with doses of 25 kGy and 75 kGy and then placed into the wells of tissue culture polystyrene (TCPS) plate.

Human bone derived cells (HBDC) used in this experiment were harvested from bone chips. Prior to the tests, cells isolated from tissue were cultured in an incubator under the standard conditions (37°C, 90% humidity, 5% CO_2). After reaching confluence, HBDCs were detached by trypsinisation (0.05% trypsin), counted, and seeded on a PEU2 matrix.

The experiment was performed in a 96-well TCPS plate. HBDC suspension (1.5×10^3 cells/sample) was slowly dispersed over the surface of the PEU. Cells seeded just on the bottom of the TCPS plate wells served as a positive control. After 7 days, the viability of HBDCs was estimated with an XTT assay. It is a colorimetric test routinely used in toxicology *in vitro*. The final product of the reaction is measured spectrophometrically in an ELISA reader at 450 nm. The results for PEU2 were compared with the data obtained for cells cultured directly on TCPS.

Simultaneously, ALP (alkaline phosphatase) activity was measured by means of an ALP SIGMA Diagnostic Kit. The amount of the final product was determined by absorbance at 405 nm using the ELISA reader. The results for the examined material were related to the ALP activity of cells seeded on TCPS, and are expressed as percent of positive control.

In order to determine changes in biocompatibility of PEU with elapse of time, the same experimental procedure was performed 7 and 42 days after irradiation.

Discussion

In the paper, we present development of EPR spectra under cryogenic conditions since radicals induced by ionizing radiation in aliphatic poly(ester urethanes) are thermally unstable at RT. In order to interpret properly the signals obtained for irradiated polymers we compared them with the spectra recorded for irradiated substrates used for their synthesis.

The experimental spectrum of EG at 77 K consists of a dominant, asymmetric singlet and two peaks of low intensity at both the sides of the central signal, Fig. 1a. Upon gradual warming to 160–180 K, external lines grow and a sharp peak between them is revealed. We suggest that alkoxyl radical formed in EG is a precursor of α-hydroxyl carbon centered radical that exhibits usually slightly smaller hydrogen coupling than typical alkyl radicals. Therefore, the detected triplet of $A(2H)_{iso}$ = 1.93 mT was attributed to the OHCH$_2^{\bullet}$ radical. The intermediate subsequently undergoes oxidation since the newly-formed peak at g = 2.0345, characteristic of peroxyl radicals, appears at elevated temperatures. The presence of other possible carbon centered radical, OHCH$_2$CH$^{\bullet}$OH, was not confirmed since the overall width of spectrum, 4.25 mT, is too small to cover hyperfine splitting (hfs) of one α- and two β-protons.

Free radicals generated in the irradiated HMDI gave the spectra collected in Fig. 1b. Unresolved peak of ΔH_{pp} = 1.19 mT seems to correspond to the radical formed upon the abstraction of hydrogen at carbon atom bonded to the isocyanate group. The strong influence of the substituent can eliminate the interaction of unpaired spin with β-protons. Other possible carbon centered radicals would show hyperfine splitting typical for alkyl radicals. Moreover, in the range of temperatures 140–170 K, the product converts to peroxyl radical, typical product of oxidation. Taking into account above
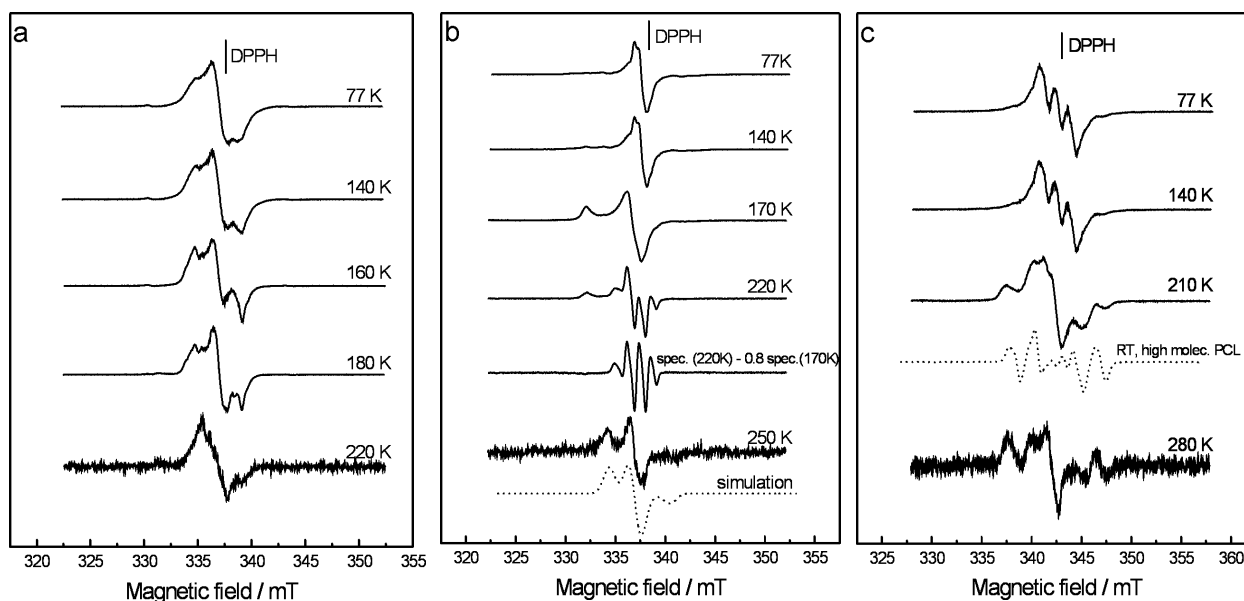
Fig. 1. EPR spectra of PEU substrates irradiated at 77 K to a dose of 6 kGy upon annealing to indicated temperatures (a) EG, (b) HMDI and (c) PCL (dotted line – see text).

implications we suggest that the most probable candidate for primary radical is $> C^{\bullet}(NCO)$. Quartet of the proportion 1:2:2:1 and hfs = 1.4 mT selected at 220 K, corresponds probably to $> CHNH^{\bullet}$ radical. The spectrum measured upon annealing to 250 K, showing two weak asymmetric external lines, must arise from the interaction between nitrogen and unpaired spin. Character of the spectrum is comparable to a simulated pattern of nitroxyl radical that was computed using the following parameters: $A(N)_{||} = 3.0$ mT; $A(N)_{\perp} = 0.5$ mT and $\Delta g = 0.005$.

The spectrum detected at 77 K directly upon irradiation of PCL is a superposition of at least two components – a triplet and a wide signal comprising weak peaks on both the sides of main absorption band. The spectral range of spectrum dominant at 77 K is comparable to that found in EG and assigned to $OHCH_2^{\bullet}$ radical. Therefore, we interpret the triplet as the spectrum corresponding to species formed upon scission of C-C bond in the main chain. Hyperfine splitting, smaller than in typical alkyl radicals, indicates that the functional groups containing oxygen are involved, thus the triplet might be assigned either to $OHCH_2^{\bullet}$ or $-OCH_2^{\bullet}$. The unambiguous interpretation of the singlet is not possible on the basis of obtained results, but we suggest that the peak represents radicals formed upon scission of the main chain at heteroatoms. They might be precursors of radicals situated in α position to ether groups $-CH_2CH^{\bullet}OC(O)-$, a fraction of which is already formed during irradiation at 77 K. Such result is in accordance with conclusions presented by Ohrlander *et al.* [7] who, studying the effect of electron beam on PCL, found that an alkylether radical is formed predominantly. Similar intermediates are formed in other polyesters [2]. To confirm unambiguously above interpretation the spectrum of irradiated high molecular weight PCL (80 kDa) was recorded at ambient temperature, Fig. 1c. (doted line). The signal corresponds to the radical characterized by the following EPR

parameters: $A(H_{\alpha})_{av} = 2.07$ mT; $A(H_{\beta 1})_{av} = 3.54$ mT; $A(H_{\beta 2})_{av} = 2.60$ mT.

Free radical processes induced by ionizing irradiation in PEU polymers synthesized from HMDI, PCL and EG might proceed in the entirely different way than in their substrates due to totally different conditions for the dissipation of energy absorbed in polymer chains, for transfer of excitations and charges along macromolecules, and for localization of unpaired spin that might proceed at newly formed functional groups, i.e. urethane linkages. The unambiguous interpretation of PEUs spectra presented in Fig. 2 is difficult as the primary species already above 170–180 K convert to peroxyl radicals of typical large, nearly axial γ-anisotropy. At lower temperatures two components could be distinguished. Except the wide singlet, the spectra detected directly upon irradiation and after annealing to temperatures below 160 K, consist of 6 lines. The spectral distances among lines are consistent with hfs of alkyl radical whose unpaired electron interacts with 5 equivalent hydrogen atoms $A(H_{\alpha}) = A(4H_{\beta}) = 2.23$ mT. The intensity of two central peaks is too high for 1:5:10:10:5:1 ratio, thus a sextet has to be overlapped by a doublet of comparable hfs. Hence, contrary to radicals identified in components used for PEUs synthesis, radical centers in polymeric materials are localized also at the inner sequences of hydrocarbon chain, not only at α position to heteroatom. Their contents is smaller in PEU1 than in PEU2.

Radicals combine inducing cross-linking or, upon oxidation, are precursors of the polar groups in polymeric material. It was found that more than 70% of radicals in PEU2 detected directly upon irradiation convert to the peroxyl radical at elevated temperatures, Fig. 3. The oxidation opens other termination channel, $2ROO^{\bullet} \rightarrow ROOR + O_2$. High concentration of peroxyl radicals indicates that cross-linking between two carbon atoms is less possible than the formation of relatively unstable peroxides.
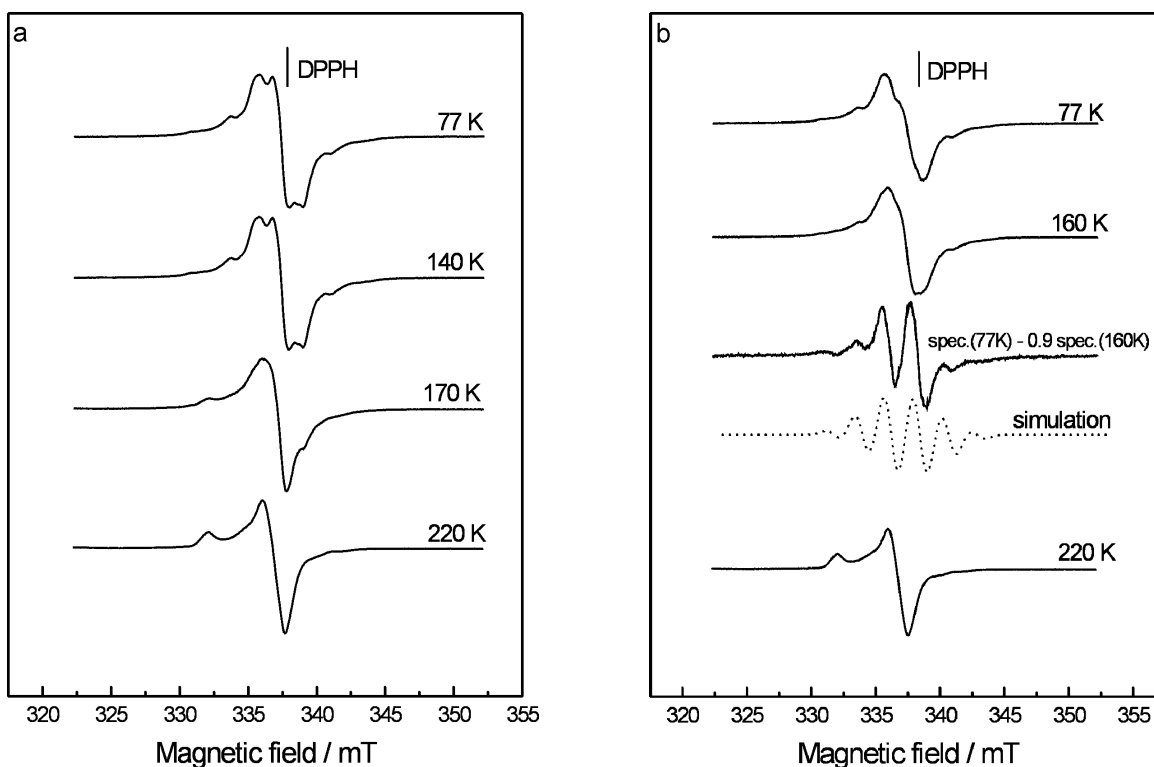
Fig. 2. EPR spectra of PEU1 (a) and PEU2 (b) irradiated at 77 K to a dose of 6 kGy upon annealing to indicated temperatures.

The interpretation of the single center line observed at low temperatures, Fig. 2, is not possible, however on the basis of results obtained by Wilhelm *et al.* [11] we suppose that such a peak might be assigned to heteroatom centered radicals formed upon homolytic cleavage of the bond between carbon and nitrogen or oxygen atoms as the C(O)-OR and C(O)-NHR bond scissions are expected to be prevalent over the C-C(O)OR breakage.

We estimated the degree of cross-linking of PEUs upon exposure to doses of 25 and 100 kGy determining a gel fraction after 8 h lasting dissolution in cyclohexanone. The results presented in Table 1 indicate that for a dose of 25 kGy the yield of cross-linking is insig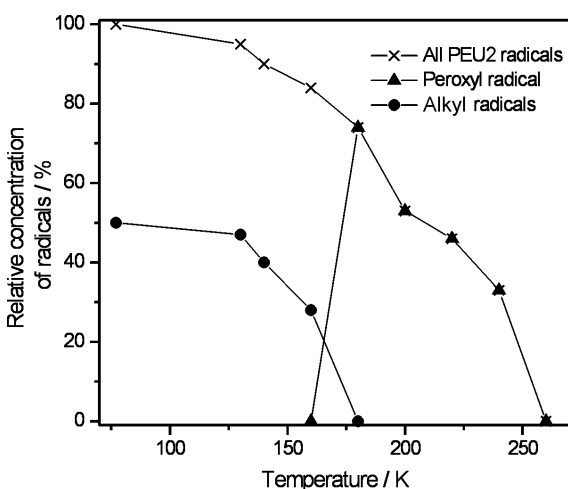nificant. After exposure to a dose of 100 kGy, 10% of gel fraction was found gravimetrically. Consequently, formation of bonding system among macromolecules in PEUs upon sterilizing dose is very limited.

Polar groups produced as the stable products of oxidative degradation, i.e. hydroxyl, carbonyl and carboxyl groups, suppose to decrease contact angle vs. water. Surprisingly, its value slightly increases upon irradiation, Table 1. Such phenomenon was earlier observed by Gorna and Gogolewski [4]. They claimed that after irradiation contact angle values of hydrophilic materials are reduced, whereas in hydrophobic poly(ester urethanes) the tendency is opposite. It seems that in such materials ionizing radiation induces reorganization of the domains, leading to migration of soft segments towards the surface and to growth of hydrophobic properties. In spite of observed tendency towards the increase of contact angle upon irradiation, production of oxidizing groups was confirmed unambiguously by EPR and DRS methods. If, instead of electron beam, gamma irradiation is used, as in the case of Gorna and Gogolewski's studies, then the extend of oxidation has to be even larger. In order to achieve a dose of 25 kGy, the material must be usually irradiated for hours, depending on a dose rate of the gamma source. Gradually formed radicals have contact with oxygen, limited only by its diffusion rate. Contrary to this situation, radicals generated almost simultaneously by electron beam might undergo competitive reactions as the distance between primary products is shorter and locally depletion of oxygen could be expected.

Fig. 3. Relationship between annealing temperatures and relative concentrations of radicals in PEU2 estimated on the basis of EPR spectra.

The apparent viscosity of polymers in the melted state is a sensitive indicator of changes induced by ionizing radiation, Fig. 4. At 215 K, viscosity of the non-irradiated materials is in the range 150–200 Pa·s and enhances by ca. 1/4 upon exposure to a dose of 25 kGy

Table 1. Gel contents and contact angles of PEUs

Measured values	Non-irradiated	Upon irradiation	
		25 kGy	100 kGy
Gel content (average results for PEU1 and PEU2)	0	traces	10%
Contact angle PEU1/water	82.0 ± 2.5 deg	94.7 ± 3.0 deg	–
Contact angle PEU2/water	87.5 ± 2.7 deg	91.7 ± 2.4 deg	–
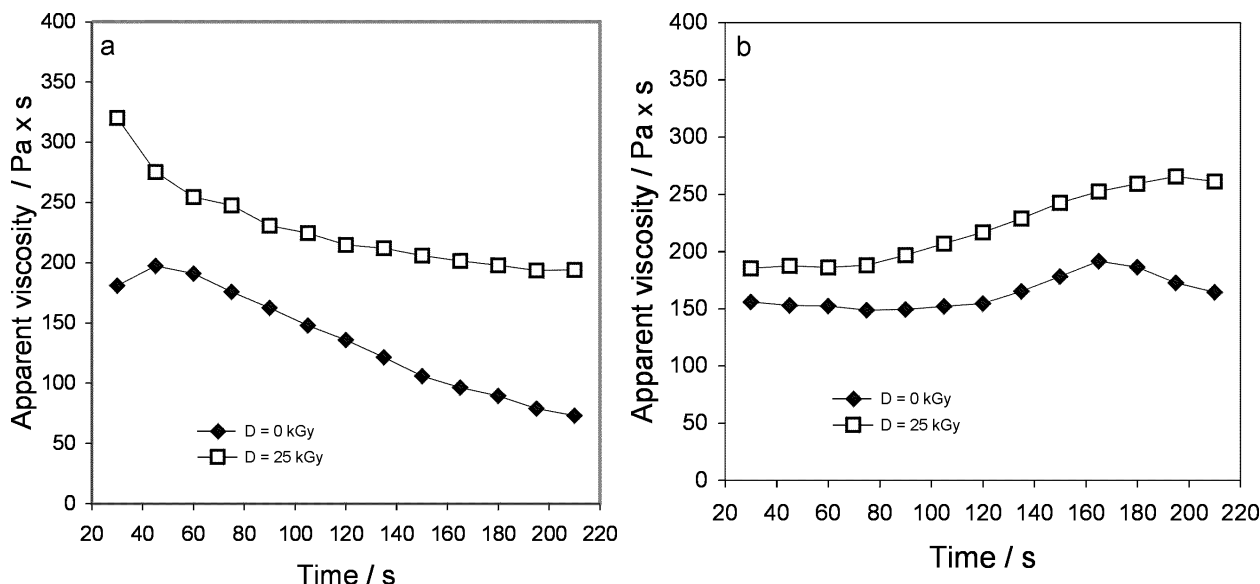
Fig. 4. Apparent viscosity of melted polymers, PEU1 (a) and PEU2 (b), in function of time of shearing, before and after irradiation with a dose of 25 kGy; $t = 215°C$, shear rate $20\ s^{-1}$.

both in PEU1 and PEU2. The effect is small, but noticeable and confirms limited cross-linking, in accordance with the gravimetric evaluation of gel fraction. The yield of cross-linking for a dose of 100 kGy turned out to be too high to measure apparent viscosity under the same conditions. It was also found that PEU1 undergoes destruction resulting from thermodegradation and long lasting shearing. The sample is less thermodynamically stable than PEU2 whose viscosity even slightly increases, probably due to further cross-linking. Such an effect might also results from more substantial phase separation in PEU1 due to longer

PCL segments than in PEU2. Presented results were calculated as the average value taken from three independent measurements.

The DRS absorption of poly(ester urethanes) in the range 290–400 nm grows after irradiation with doses of 25 and 50 kGy, whereas upon exposure to a dose of 100 kGy, the samples become nontransparent for waves shorter than 320 nm, Fig. 5. The absorption increase is attributed to radiation-induced oxidative processes whose earlier stages were observed by EPR spectroscopy. Functional polar groups formed upon oxidation of alkyl radicals result in the shift of absorption threshold
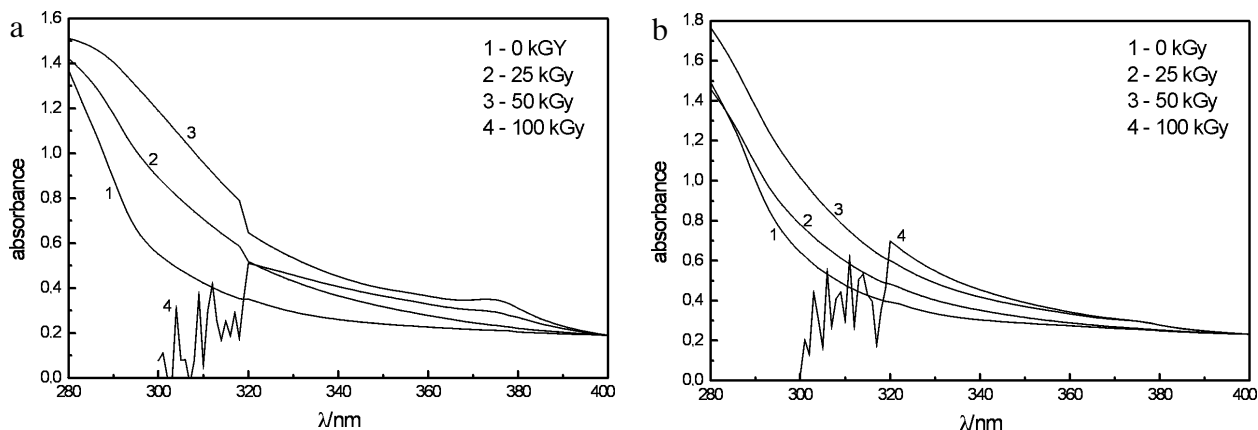
Fig. 5. DRS spectra of PEU1 and PEU2 before (a) and after irradiation (b) with doses of 25, 50 and 100 kGy.
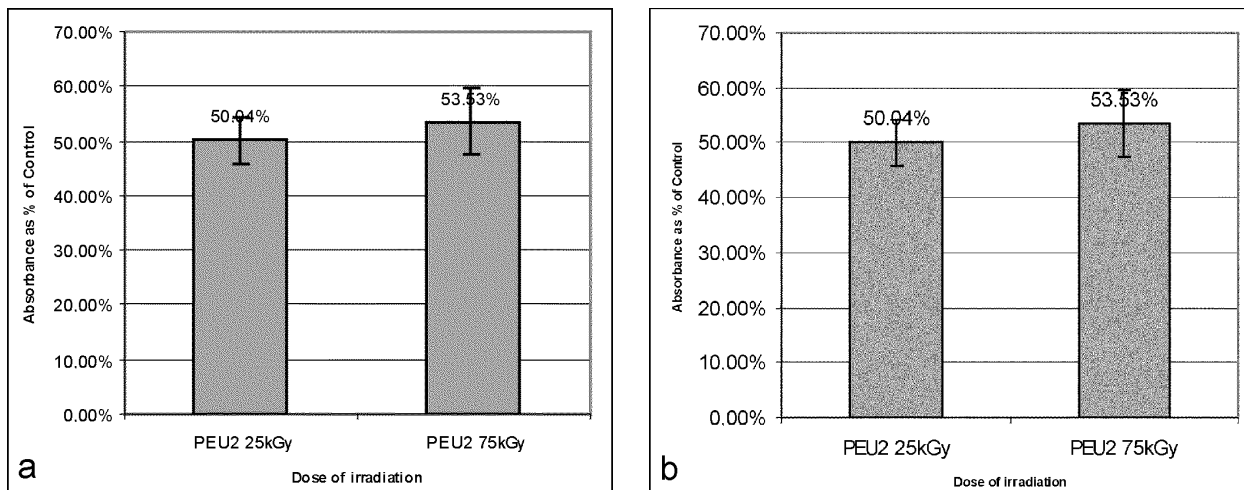
Fig. 6. Viability of HBDC in contact with PEU2 estimated on the basis of XTT assays (a) 7 days and (b) 42 days upon irradiation. Data are presented in percent of positive control.

towards longer wavelength. An accumulation of oxidizing products in the samples irradiated with the dose of 100 kGy results in a significant increase of absorption below 320 nm, what eventually reduces the intensity of reflected light to the undetectable level. The DRS method and the basis of interpretation of DRS spectra measured for irradiated polymers are explained in [13].

As can be estimated on the basis of XTT assay, there is a post-irradiation effect in the HBDCs (Fig. 6). A few days after irradiation the cells are more viable than upon a few weeks. However, these differences are statistically significant only for the samples irradiated with a dose of 25 kGy (Kruskal-Wallis Test). This observation is in accordance with results obtained in our previous study on biocompatibility of PEU1 [12].

There was no unambiguous dependence between the viability of HBDCs and the dose of irradiation. Absorbance of samples seeded on a matrix irradiated with 75 kGy was lower than in the case of material sterilized with 25 kGy. However, after 42 days the relationship was inversed.

Osteogenic activity of HBDCs shows a weak dependence on the radiation dose, contrary to the viability, Fig. 7. The activity of cells seeded on polyurethane irradiated with a dose of 75 kGy was higher if the material was irradiated with 25 kGy, both after 7 and 42 days, although the absorbance was not significantly different (Kruskal-Wallis Test). This observation also corresponds with our previous studies of PEU1 [12].

Conclusions

Damages induced by gamma rays in two urethane based materials have been investigated by EPR spectroscopy. Results confirmed that the urethane segments are more resistant towards ionizing irradiation and the presence of ester units facilitated the generation of free radicals. It was also found that in PCL segments ionizing radiation induces radicals that are able to introduce cross-linking of macromolecules, and consequently
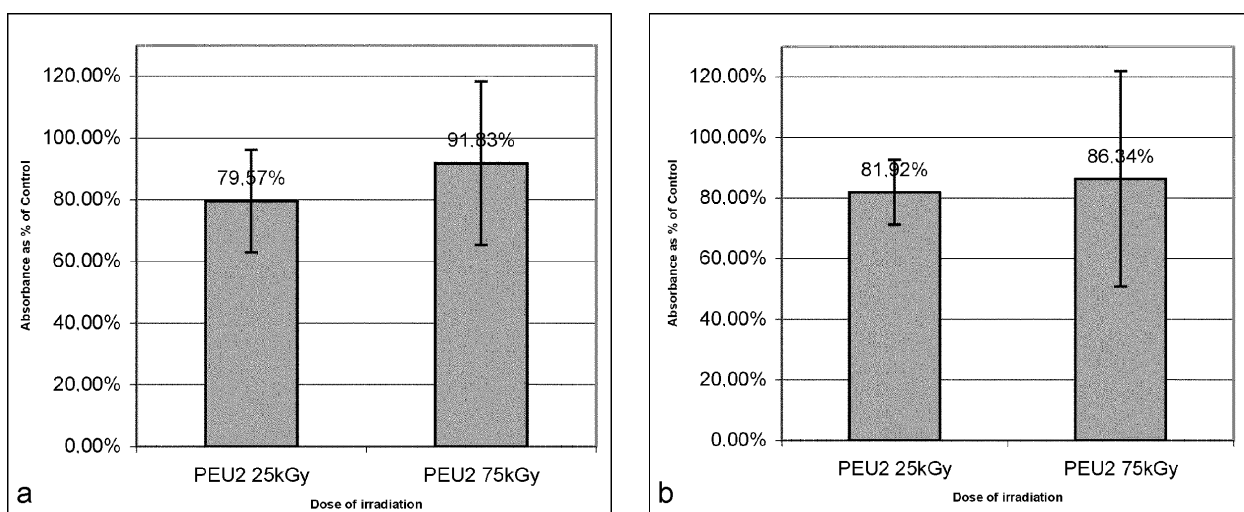

Fig. 7. Osteogenic activity of HBDC in contact with PEU2 estimated on the basis of ALP activity (a) 7 days and (b) 42 days upon irradiation. Data are presented in percent of positive control.

reduce the ability to biodegradation. Oxidation is a competitive reaction that refers to almost 70% of all initially generated radicals. The increase in water contact angle suggests that the surface of the irradiated materials might become more hydrophobic.

The dose used for poly(ester urethane) irradiation does not significantly influence viability and differentiation of HBDCs cultured on the surface of the examined material. The osteogenic activity of human bone derived cells estimated on the basis of ALP activity increases insignificantly after irradiation with high doses. There was a weak relationship between the post-irradiation-time and the viability of human bone derived cells (HBDC) cultured on the examined polyurethane samples.

Such a post-irradiation effect must result from slow physical evolution of polymeric materials, as at ambient temperature all generated radicals convert to diamagnetic species already during irradiation. However, oxidative degradation is still possible due to many unstable products presented in the material, e.g. peroxides. Thus, to limit such an effect, radiation sterilization requires rather irradiation under inert gas atmosphere.

Further biological investigations are necessary to confirm the reported preliminary study, as well as to explain the factors responsible for the observed phenomena.

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