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Gamma irradiation effects and EPR investigation on poly(lactide-co-glycolide) microspheres containing bupivacaine

L. Montanari [a,*], F. Cilurzo [a], B. Conti [b], I. Genta [b], A. Groppo [b], L. Valvo [c], A. Faucitano [d], A. Buttafava [d]

[a] *Istituto di Chimica Farmaceutica e Tossicologica, Università degli Studi di Milano, V. le Abruzzi 42, 20131 Milan, Italy*
[b] *Dipartimento di Chimica Farmaceutica, Università degli Studi di Pavia, V. le Taramelli 12, 27100 Pavia, Italy*
[c] *Laboratorio di Chimica del Farmaco, Istituto Superiore di Sanità, V. le Regina Elena 299, 00161 Rome, Italy*
[d] *Dipartimento di Chimica Generale, Università degli Studi di Pavia, V. le Taramelli 12, 27100 Pavia, Italy*

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Abstract

The effects of γ radiation on the stability of microspheres made of a polylactide-co-glycolide 50:50 copolymer (PLGA) and loaded with 40% bupivacaine (BU) were studied. The radiolysis mechanisms of BU and BU-loaded microspheres were investigated by using electronic paramagnetic resonance (EPR) analysis. Microspheres were prepared by means of a spray drying method. γ Irradiation was carried out in the open, at the dose of 25 kGy, by using a ^{60}Co source. The stability of BU-loaded microspheres was evaluated over a 1-year period on the basis of drug content and dissolution profile. Non-irradiated microspheres were stable over the whole period under consideration. Immediately after irradiation the amount of BU released after 24 h from irradiated microspheres increased from 17 to 25%; in the following 3 months of storage it increased to about 35%, and then it kept constant for 1 year. Radicals generated by BU irradiation were identified by EPR analysis; the sensitivity to γ radiation of BU was about four times lower than that of PLGA. Furthermore, the EPR spectra of loaded microspheres showed that the relative abundance of BU radicals plus PLGA radicals was proportionate to the electronic fractions of the components; this implies that no spin transfer BU/PLGA had occurred during γ irradiation. © 2002 Published by Éditions scientifiques et médicales Elsevier SAS.

Keywords: Gamma irradiation; Bupivacaine; Microspheres; PLGA; EPR

1. Introduction

Biodegradable polyesters based on glycolic acid (PGA) and lactic acid (PLA) and their copolymers poly-(D,L-lactide-co-glycolide) (PLGA) are often used as drug carriers for the development of parenteral drug delivery systems. As they are susceptible to heat sterilisation, ionising radiations are frequently used for final sterilisation or for sanitisation after aseptic preparation.

The investigations on the radiolytic pattern of these drug-delivery systems are useful in the evaluation of their safety.

The predominant effect due to γ radiations on PGA is thought to be chain scission within a radical mechanism where cage effects play a major role, and leads to a concentration of the radiation damage in amorphous regions [1]. Chain degradation leads to a faster loss of tensile strength and to an enhanced hydrolysis rate. A radiolysis mechanism mainly based on radical recombination, disproportionation and H abstraction reactions was postulated [1]. A similar behaviour was observed for PLA and PLGA with the exception of the enhanced sensitivity to radiation degradation, which is attributed to the presence of a tertiary carbon centre [1,2]. The relative importance of chain scissions and cross linking and their effects on average molecular weights and polydispersity index were investigated by several authors adopting thermal, viscometric and chromatographic methods [3–5]. Conflicting conclusions were reached as regards the relative importance of random chain scissions versus end chain scission.

* Corresponding author.
E-mail address: luisa.montanari@unimi.it (L. Montanari).

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The radiation chemistry investigations were also extended to the polymer/drug interactions with interesting results pertaining to the nature and role of polymer interaction. Lalla et al. [6] described a decreased release of piroxicam from γ irradiated PLA microspheres. Volland et al. [7] showed the same pattern from γ irradiated PLA microspheres containing captopril. On the contrary, Yoshoka et al. [5] described an increased progesterone release from irradiated PLA microspheres in relation to the irradiation dose. Mohr et al. [8] showed accelerated kinetics of estradiol release by increasing irradiation doses, due to dose-dependent polymer degradation.

The influence of drug loading on polymer degradation is also discussed. PLA degradation was independent from methadone loading [9], but it was higher when prometazine loading increased [10]. Bittner et al. [11] showed that PLGA degradation rate slowed down following the incorporation of tetracycline in the microspheres.

Although the effects of ionising radiation on PLA and PLGA molecular weight and drug release were discussed by a number of papers, very few works were focused on the ageing of irradiated microparticulate systems [12,13].

In this work, the research is focused (a) on the evaluation of the stability of PLGA microspheres loaded with bupivacaine (BU) and (b) on the elucidation of radical components production (release), of the radiolytic mechanisms and of their modifications as a consequence of drug–polymer molecular interactions, by matrix EPR spectroscopy.

Placebo and BU-loaded microspheres were prepared by means of a spray drying method.

Microspheres were irradiated by a ^{60}Co source in the open, at the dose of 25 kGy. A minimum absorbed dose of 25 kGy is regarded as adequate for the purpose of sterilising pharmaceutical products without providing any biological validation [14].

The stability of non-irradiated and irradiated microspheres loaded with BU was evaluated for a period of more than 1 year, on the basis of their drug content and dissolution profile.

BU was selected because it is a local anaesthetic drug, usually administered by parenteral route for the regional control of major pain and regional anaesthesia, together with a consequent decreased systemic administration of narcotic drugs. In both regional control of major pain and regional anaesthesia, the development of prolonged drug delivery systems appears interesting in order to avoid repeated administration or infusion via indwelling catheters. LeCorre et al. [14] investigated the use of PLA microspheres for the controlled spinal delivery of BU; promising results were obtained in the biopharmaceutical and pharmacodynamic evaluation of the anaesthetic action in rabbit.

2. Materials and methods

2.1. Materials

Polylactide-co-glycolide 50:50, (PLGA) Resomer® RG 503, inherent viscosity 0.39 dl/g, 34 000 Mw (Boehringer Ingelheim KG, Ingelheim am Rheim, G).

Bupivacaine hydrochloride (Sigma, St. Louis, MO, USA); bupivacaine base (BU) was obtained from bupivacaine hydrochloride following the method of LeCorre et al. [14].

L-Alanine; 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Fluka, Milan, I).

Paraffin wax (Aldrich, Milan, I).

Unless specified, all other compounds were of analytical grade.

2.2. Preparation of microspheres

Microsphere preparation was performed by using the spray-dryer Lab-Plant model SD04 (Lab-Plant LTD, West Yorkshire, UK).

Placebo microspheres were obtained by spraying 1.8% w/w solution of PLGA in methylene chloride through a standard nozzle (inner diameter: 1 mm). The process parameters were set as follows: inlet temperature, 48 °C; outlet temperature, 32–33 °C; flow rate, 10 ml/min.

BU-loaded microspheres were prepared by spraying—under the same conditions as above—1.8% w/w solution of PLGA in methylene chloride in which BU had been previously solubilised in the following ratio: PLGA:BU 60:40 w/w. After preparation, both placebo and BU microspheres were lyophilised by using a Modulyo 4K Freeze Dryer (Edwards, England), and stored in an airtight container at 4 °C until use.

2.3. γ Irradiation of bupivacaine-loaded microspheres

BU, placebo and BU-loaded microspheres were irradiated by using ^{60}Co as irradiation source (Gammacell, Nordion Inc., Canada). Irradiation was performed in the presence of air at 25 kGy dose, applied at a 516 Gy/h dose rate; irradiation temperature: 25 °C.

2.4. Microsphere characterisation

2.4.1. Morphology

Microparticle size and morphology before and after irradiation were evaluated. The microsphere shape and surface were analysed using SEM (JSM-T 800, Jeol Italia S.p.A., Pieve Emanuele, I). The samples were sputtered with an Au/Pd coating in an argon atmosphere.

The microsphere size was determined by light blockage method and an HIAC/ROYCO apparatus, model

3000, equipped with an HC60 sensor. Samples of microspheres were suspended in filtered and bi-distilled water, and analysed while gently stirring. The results are the average of five determinations.

2.4.2. Drug content

BU content in the microspheres was determined by dissolving 40 mg of BU-loaded microspheres in CH_2Cl_2 (1 ml); the drug was extracted with 5 ml of H_2SO_4 1 N. After 2 min stirring and centrifugation for 15 min at 1000 rpm, 50 μl of aqueous phase were diluted in 5 ml of mobile phase. The samples were assayed by HPLC method modified from LeCorre et al. [14]. The HPLC system was HP1100 Chemstation (Hewlett Packard, USA). Chromatographic conditions: column: Bondclone C18, 10 μm, 300 × 3.9 mm I.D. (Phenomenex, USA); mobile phase: KH_2PO_4 (pH 4.0; 0.01 M)/acetonitrile (70/30 v/v); flow rate: 1 ml/min; temperature: 30 °C; wavelength set: 205 nm; injection volume: 5 μl. The drug concentrations were determined from standard curves (2–50 μg/ml) and the method gave 98.5% recovery of theoretical value (C.V. < 1%).

2.4.3. In vitro dissolution test

In vitro dissolution test was performed by the USP 24 paddle dissolution method (Erweka DT6, Erweka, G).

Each sample of 60 mg microspheres was treated with 1 ml wetting aqueous solution containing 2.5% w/w mannitol. The samples were stirred for 10 min, then suspended in 500 ml dissolution medium made of 0.01 N HCl aqueous solution (pH 2.0) containing NaCl 0.2% w/w; the suspension was maintained at 37 °C under stirring at 100 rpm for 24 h. The acidic medium was used to improve the BU solubility.

The amounts of BU released from the microspheres were spectrophotometrically determined at 205 nm wavelength. The test was performed in triplicate.

The test was performed soon after irradiation (time 0), and repeated after 15, 30, 60, 90, 120, 150, 180 and 365 days.

The release rate constant was calculated according to Higuchi's equation as follows: $M_t/M_\infty = kt^{0.5}$ where M_t is the amount of drug released at time t, M_∞ is the drug loaded in the matrix and k is the release rate constant expressed as $h^{-0.5}$.

2.5. EPR analysis

Irradiation was performed at the Applied Nuclear Energy Laboratory (L.E.N.A., Pavia University, I) by using a ^{60}Co gamma source calibrated against Alanine and Fricke dosimeters, under the following conditions:

- samples sealed under high vacuum in EPR quartz tubes, dose rate 1.3 kGy/h, total dose 25 kGy; irradiation temperature $T = 77$ K (liquid nitrogen);
- irradiation temperature $T = 298$ K, samples sealed under high vacuum in EPR quartz tubes, dose rate 1.3 kGy/h, total dose 25 kGy; irradiation temperature $T = 298$ K;
- sample containers open to air during irradiation; dose rate 1.3 kGy/h, total dose 25 kGy; irradiation temperature $T = 298$ K.

After irradiation, part of the sample tubes was flamed by using the sliding technique in order to eliminate the radiation-induced quartz paramagnetic centres. During this operation, the sample temperature was not allowed to rise above 77 K for samples irradiated at 77 K.

The radiolytic radical yields were determined through the comparison of the EPR signals areas using alanine standards with a known number of spins. The alanine standards were prepared by extrusion of alanine powder/wax mixture (ca 20% wax) in the form of cylinders having the same geometry of the samples. Stable alanine radicals were generated by irradiation and their concentration determined by comparison with a standard solution of DPPH.

EPR analysis was performed by using a Varian E-109 spectrophotometer (Palo Alto, California, USA) equipped with a data acquisition system and a temperature control apparatus. The EPR spectra were analysed by computer simulation by using the Hamiltonian:

$$H = -\beta S\tilde{g}H + \Sigma_i S_i\tilde{A}_i I_i - \Sigma_i I_i H$$

where: H is the spin Hamiltonian, β is the Bohr magneton, $\tilde{g}$ is the g tensor, H is the external magnetic field, S is the electron spin operator, $\tilde{A}$ is the hyperfine tensor, and I is the nuclear spin operator.

3. Results and discussion

3.1. Morphology

Scanning photomicrographs of the drug-loaded microspheres before irradiation and after irradiation at 25 kGy are shown in Fig. 1a and b, respectively. BU-loaded microspheres had spherical shape and porous surface. Microsphere size, as determined by granulometric analysis, resulted in a range between 2 and 5 μm (Fig. 2). The γ irradiation did not seem to cause morphological changes.

3.1.1. Drug content

Purity of BU loaded in the microspheres resulted to be equivalent to that of bupivacaine hydrochloride, demonstrating that it was not affected by microsphere preparation process.

BU content in the microspheres used for stability studies resulted to be 40.1 ± 0.2% w/w before irradiation and 38.3 ± 0.6% w/w immediately after irradiation.

Probably, the decreased BU recovery after irradiation with respect to irradiated samples was the consequence of BU sensitivity to γ irradiation. As a matter of fact, BU irradiated at the same dose as the microspheres (25 kGy) led to a recovery of 97.56 ± 0.04% that was quite similar to the amount of BU degraded in the microparticles.

After 1 year of storage BU content did not significantly change both in non-irradiated and irradiated microspheres.

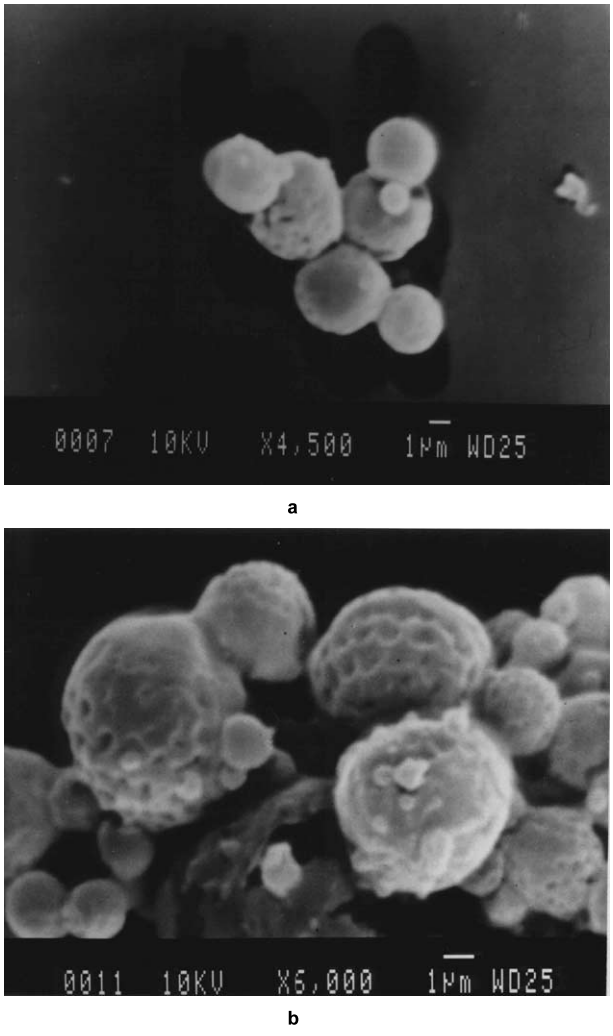

Fig. 1. Photomicrographs of BU-loaded microspheres (a) before irradiation, (b) after irradiation at 25 kGy.

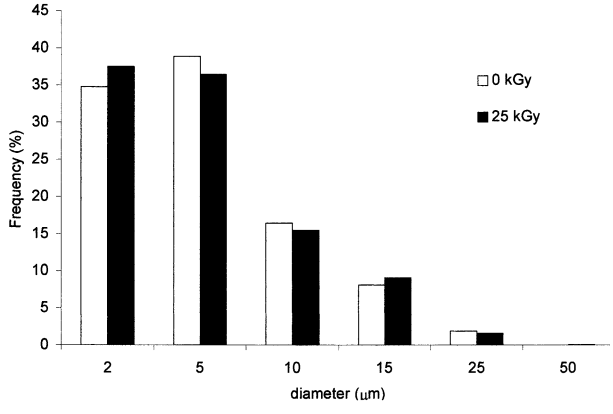

Fig. 2. Particle size distribution of BU-loaded microspheres before irradiation, and after irradiation at 25 kGy.

3.1.2. In vitro release profile

BU dissolution data obtained from both non-irradiated and irradiated microspheres over a period of 1 year are reported in Tables 1 and 2. The BU release was controlled by the diffusion of the drug from a monolithic matrix and followed the square root of time relationship throughout the test period according to the Higuchi model (Tables 1 and 2).

The amount of BU released from non-irradiated microspheres, immediately after the preparation, was about 17% in 24 h, and it did not significantly change further over the considered period of time (1 year) (Table 3).

Immediately after irradiation the amount of BU released from the microspheres was 25% and increased to about 35% after 3 months of storage. In the following period of time the amounts of BU released in 24 h did not change significantly; this fact suggesting a stabilisation of microsphere structure.

The increased BU release in the first 3 months of storage after irradiation could be due to the decay of the molecular weight of irradiated PLGA [2].

3.2. EPR analysis

3.2.1. Bupivacaine

The 77 K EPR spectrum of BU obtained following vacuum irradiation at the same temperature has the appearance of a large partially resolved structure with an overall width of ca. 11 mT (Fig. 3). On warming at 298 K the larger component decays leaving a triplet pattern of 4.5 mT which in turn, and by prolonged storing at the same temperature, changes progressively into a doublet with the same overall width and a 2.4 mT peak to peak splitting. The doublet was computer simulated by considering the interaction of two inequivalent protons with couplings 1.2 and 2.0 mT and a nitrogen with coupling of 0.5 mT: such hyperfine features are reckoned with the α-amido radical formally arising from loss of a hydrogen at the site adjacent to the amido group in the piperidine moiety (radical **C** in Fig. 3). The inequivalence of the β hydrogens is accounted for in terms of a chair ring conformation giving one C–H bond closer to the symmetry axis of the u.s. orbital and the other closer to the nodal plane. By subtracting the doublet from the Fig. 3b spectrum a triplet pattern with a nitrogen splitting of ca. 2.0 mT was obtained which was assigned to the anilido radical

Table 1
In vitro release data of non-irradiated BU-loaded microspheres

Storage time (days)	BU amount released after 24 h (%)	BU release rate ($h^{-0.5}$)	r^2
0	17.2 ± 0.5	2.82×10^{-02}	0.9988
30	17.4 ± 0.2	3.35×10^{-02}	0.9891
180	17.4 ± 0.1	2.45×10^{-02}	0.9933
360	17.3 ± 0.5	2.51×10^{-02}	0.9942

Table 2
In vitro release data of irradiated BU-loaded microspheres

Storage time (days)	BU amount released after 24 h (%)	BU release rate ($h^{-0.5}$)	r^2
0	24.9 ± 0.9	3.69×10^{-02}	0.9960
15	27.3 ± 0.3	4.20×10^{-02}	0.9984
30	28.1 ± 1.0	3.70×10^{-02}	0.9924
60	30.8 ± 1.5	4.43×10^{-02}	0.9890
90	35.7 ± 1.7	5.03×10^{-02}	0.9909
120	35.4 ± 0.8	5.07×10^{-02}	0.9883
180	34.4 ± 1.4	5.03×10^{-02}	0.9856
360	33.7 ± 1.9	5.70×10^{-02}	0.9899

B (Fig. 3). Finally by subtracting the Fig. 3b spectrum from that of Fig. 3a, the larger hyperfine component was isolated: it consisted of a sextet with an average splitting of 2.2 mT. This latter pattern was assigned by computer simulation to the species, the **A1** and **A2** formally arising, respectively, from the rupture of methylene C–H bonds in the piperidine and *n*-butane moieties

$$-CH_2-C\overset{\bullet}{H}-N< \quad \textbf{(A1)} \qquad -CH_2-C\overset{\bullet}{H}-CH_2- \quad \textbf{(A2)}$$

The hyperfine coupling used were:
Radical **A1**: a(N) = 0.4 mT; a(H) = 1.4 mT; 2a(H) = 2.4 mT
Radical **A2**: 2a(H) = 1.9 mT; 2a(H) = 4.4 mT; a(H) = 2.3 mT

According to the above assignments, the EPR changes observed by increasing the temperature up to 298 K are interpreted in terms of hydrogen abstraction reactions by the species **A1**, **A2** and **B** from the most favoured site in the piperidine ring leading to the species **C** which is stable for months at room temperature under vacuum (only 13% decay observed after 21 days) but reacts readily with oxygen on admission of air.

The hydrogen abstraction leading to the species C (Fig. 3) is expected to be activated by a captodative effect stemming from the amino and amido substituents.

3.2.2. Irradiation of BU-loaded microspheres at 77 K

The EPR spectra recorded at room temperature following the irradiation under vacuum at 77 K show the hyperfine components due to the radical species arising from the polymer radiolysis as a major constituent (Fig. 4). Both chain scission and H abstraction radicals are presented; the latter becoming the dominant species on warming to room temperature as a consequence of H abstraction reaction at the favoured tertiary and secondary C–H sites in the polymer chain. The H abstraction at the secondary sites seems to be activated by a nearly parallel orientation of the C–H bond with respect to the unpaired spin orbital which partially overcomes the unfavourable bond breaking energy with respect to the tertiary C–H bonds (stereo electronic effects). Two different types of $-C^{\bullet}(CH_3)-$ radicals are detected after prolonged storing at room temperature, showing significantly different H coupling. The $-C^{\bullet}(CH_3)-$ species developing on prolonged storing at room temperature is thought to be associated with terminal units of the formula: $-C(=O)OC^{\bullet}(CH_3)-OH$.

Together with polymer radicals, BU radicals are also contributing to the overall spectrum with relative abundance corresponding to the electron fraction additivity law (Table 3). This leads to the conclusion that spin transfer reactions between the polymer matrix and the active ingredient are of minor importance.

Table 3
Radical radiolytic yields in γ irradiated neat BU, placebo microspheres and BU-loaded microspheres submitted to γ irradiation under vacuum at 77 K

Sample	Radiolytic yield (μmol/J)
BU	0.07 ± 0.01
Placebo microspheres	0.26 ± 0.05
BU-loaded microspheres	0.20 ± 0.04
Calculated [a]	0.20 ± 0.04

[a] Linear combination of the neat compounds radiolytic yields weighted by the electron fractions.

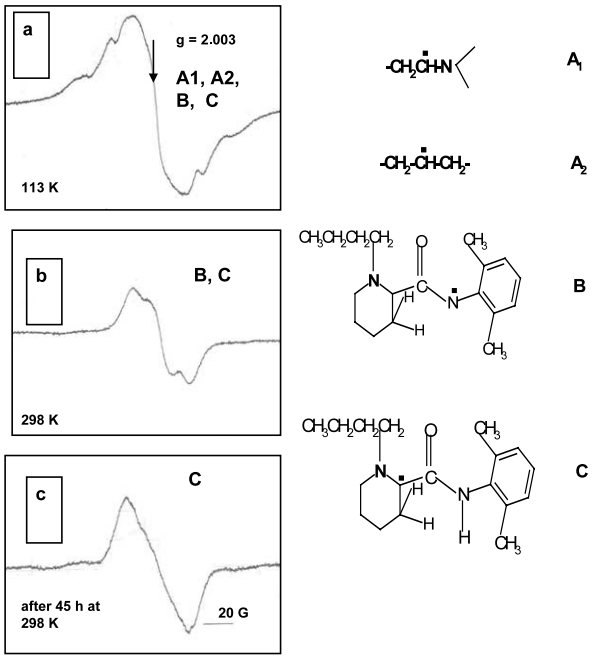

Fig. 3. EPR spectra of BU after under vacuum γ irradiation at 77 K, its attributed radicals and effect of the annealing temperature.

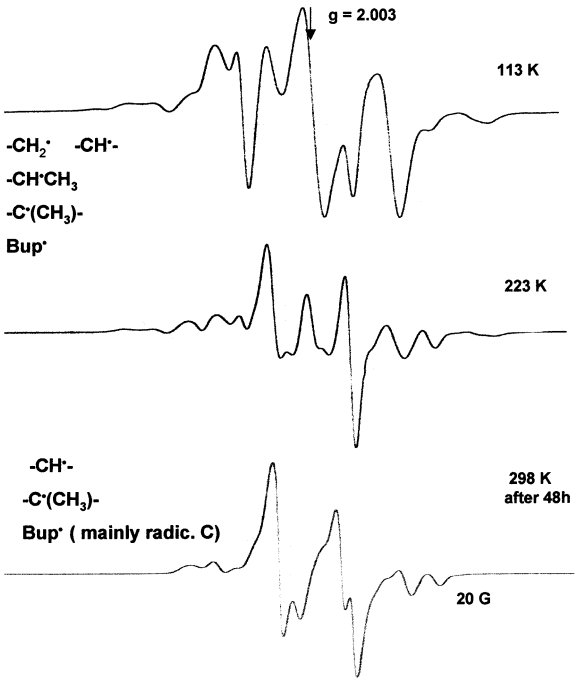

Fig. 4. Effect of the temperature on EPR spectra of BU-loaded microspheres after under vacuum γ irradiation at 77 K. Bup• stands for BU radicals A_1, A_2, B, C (Fig. 3); the other species are PLGA radicals which are the major contributors to the spectra.

3.2.3. Irradiation of BU-loaded microspheres at room temperature and oxygen effect

The samples irradiation at room temperature under vacuum leads to the production of the same radical products observed when the irradiation is performed at 77 K and the samples are successively gradually warmed up to room temperature (Fig. 5a). The irradiation in the presence of air (Fig. 5b) leaves only traces of peroxyl radicals in the microsphere samples and a weak signal with the appearance of a singlet superimposed on a triplet, probably generated by nitroxyl and peroxyl species, in the case of BU and BU-loaded microspheres. The latter sample EPR signal is, indeed, at the limit of detection. Nitroxyls may arise from aminyl radicals formed by rupture of C–N bonds in the piperidine ring followed by oxygen reaction according to the scheme:

$$>N^{\bullet} + O_2 \rightarrow >NOO^{\bullet}$$

$$>NOO^{\bullet} \rightarrow >N\text{–}O^{\bullet}$$

An alternative mechanism commonly proposed for tertiary alkyl amines-based polymer stabilisers is the reaction sequence initiated by the oxygen addition to alkyl aminyl radicals:

$$>NCH^{\bullet}CH_2\text{–} + O_2 \rightarrow >NCH(OO^{\bullet})CH_2\text{–} \rightarrow \rightarrow >N\text{–}O^{\bullet}$$

Finally also the anilido radicals **B** (Fig. 3) are capable of reacting with oxygen giving acyl nitroxyls:

$$\text{–C(=O)–N}^{\bullet}\text{–ph} + O_2 \rightarrow \text{–C(=O)–N(–O}^{\bullet}\text{)–Ar}$$

Acyl nitroxyls are known to be characterised by small N hyperfine couplings (5–7 G) which in the solid state are likely not to be resolved, thus contributing to the observed singlet component of the EPR spectra.

Judging from the low intensity of the EPR signals detected, the formation of nitroxyls in the radiolysis under air of BU-loaded microspheres must anyway be considered of minor importance.

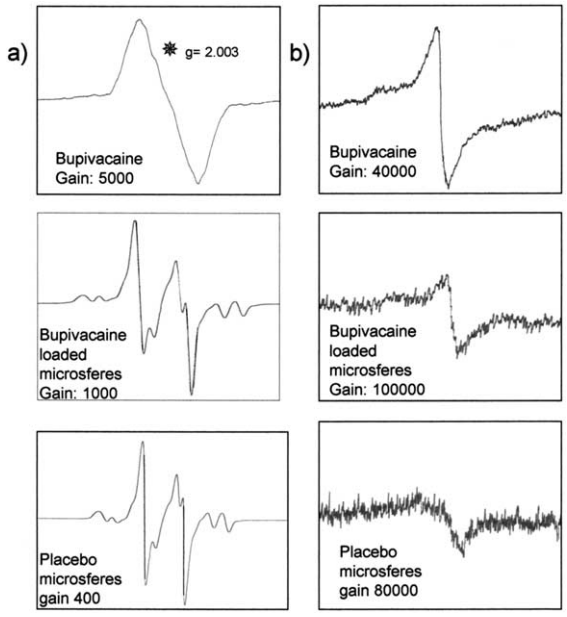

Fig. 5. EPR spectra obtained after room temperature γ irradiation: (a) under vacuum, (b) in the air.

3.2.4. Radical decay pattern

The decay rate for polymer radicals under vacuum is characterised by $t_{1/2}$ of about 5 days whilst in the same time only 5% decay is observed for crystalline BU. Both BU samples and the BU-loaded microspheres are permeable to oxygen. When BU-loaded microspheres are opened to air, the oxygen diffusion takes less than 1 min to convert the vacuum radical population into the corresponding peroxyl radicals. The peroxyl radicals are expected to carry on some oxidative degradation through propagation cycles based both on peroxyl and alkoxyl intermediates [2]. This post oxidation process is rapidly completed, the decay rate of the intermediate peroxyl radicals being 9.7 min at room temperature.

4. Conclusions

The non-irradiated BU-loaded microspheres stored at 4 °C were stable over the period of 1 year.

γ Irradiation at the dose of 25 kGy caused a decrease of BU content of about 4% due to radiolytic decomposition of the active ingredient.

Immediately after irradiation the in vitro drug release increased and further increased in the following 90 days of storage; then it kept constant for 1 year. This behaviour suggested that a stabilisation of microsphere structure occurred 90 days after irradiation.

EPR analysis showed that BU is sensitive to γ irradiation, possibly generating four radicals whose stability is higher for the piperidine ring radical. With respect to radical products, the sensitivity to γ radiation of BU was found to be about four times lower as compared to the polymer sensitivity.

The EPR spectra of BU-loaded microspheres showed the BU radicals plus the polymer radicals with relative abundance proportional with the electronic fractions of the components; this implies that no spin transfer BU/PLGA had occurred during irradiation.

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