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Poly(lactide-co-glycolide) microspheres containing bupivacaine: comparison between gamma and beta irradiation effects

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Abstract

The β- and γ-irradiation effects on stability of microspheres made of poly(lactide-co-glycolide) 50:50 copolymer (PLGA) containing bupivacaine (BU) were studied. Microspheres containing 10, 25, and 40% w/w, respectively, of BU were prepared by spray drying and irradiated in air with β- and γ-irradiation at a dose of 25 kGy. Morphology (atomic force microscopy, particle-size analysis), physico–chemical characteristics (DSC and FT-IR spectroscopy), drug content and in vitro dissolution profile of microspheres were all determined; the stability of irradiated microspheres was evaluated over a 9-month period. The decrease of BU content in γ-irradiated microspheres was almost always constant independent of the amount of BU per sample, therefore it was in inverse proportion to drug loading (range between 5 and 15%). BU release rate increased immediately after irradiation and increased slightly until 90 days of storage. As far as β-irradiated microspheres are concerned, BU content decreased in a significant way (≈3%) only in microspheres containing 10% w/w of BU. Immediately after irradiation, drug release rate in β-irradiated microspheres increased less than in the corresponding γ-irradiated microspheres, and it did not change further over the following storage period. BU-loaded microspheres have been shown to be more stable against β- than γ-irradiation. AFM revealed that the surface roughness of the irradiated microspheres increases depending on irradiation. As such, if a parameter is quantifiable, it is proposed as a marker of degradation due to ionizing radiation.

Keywords: Gamma-irradiation; Beta-irradiation; AFM; Bupivacaine; PLGA

1. Introduction

Ionizing radiation is mainly used for the sterilization of heat sensitive materials and products. Nevertheless, irradiation could cause some alterations in many medicinal products, and treatment is approved

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only when it has been experimentally documented that no harmful side effects occur [1,2].

Raw materials or final products are typically sterilized by using γ-rays emitted from a nuclear source (^{60}Co or ^{137}Ce). However, electron beam irradiation is faster, easier and cheaper from an operational point of view, and it does not incur the environmental risk of nuclear irradiation; therefore, it is more likely to be used in the near future.

Sterilization with ionizing radiation is mainly due to the beam of secondary electrons that produce free radicals. Ionization events activate numerous chemical reactions, many of which, in the presence of oxygen, lead to oxidative degradation [3]. Although the main interaction with matter is basically the same for γ-rays and high energy electrons, minor differences between the two modes remain. In fact, γ-rays are a form of electromagnetic radiation characterized by high penetration into matter but at a very low dose rate (kGy/h). On the contrary, β-rays are a form of corpuscular radiation characterized by low penetration into matter, but at a very high dose rate (kGy/s) [4]. These peculiarities of β- and γ-rays can modify the performance of irradiated drug delivery systems. For the same administered dose, β-ray treatment may cause an overheating of the material while γ-ray treatment could prolong the peroxidative radiolitic mechanism due to the exposure time [3].

In the pharmaceutical field, one of the applications of ionizing radiation is the final sterilization of biodegradable poly(lactide-co-glycolide) microspheres intended for parenteral use [4]. The effects of γ-irradiation on poly(lactide-co-glycolide) and loaded microspheres were debated by a number of papers, and controversial results are reported, depending on the active ingredient used [5–9]. However, no information is available at the moment on the effects of β-irradiated microparticulate systems made up of such copolymers.

The aim of this work was to compare the effects of γ- and β-irradiation on the stability of microspheres made up of poly(lactide-co-glycolide) 50:50 copolymer containing bupivacaine.

BU was selected because it is a local anesthetic drug, usually administered by the parenteral route for the regional control of major pain and regional anesthesia for decreasing systemic administration of narcotic drugs [10]. In both regional control of major pain and regional anesthesia, the development of prolonged drug delivery systems seems interesting in order to avoid repeated administration or infusion via indwelling catheters. Le Corre and co-workers investigated the use of PLGA microspheres for the controlled spinal delivery of BU; promising results were obtained in the biopharmaceutical and pharmacodynamic evaluation [11,12].

Three formulations containing BU in the range 10–40% w/w were designed in order to verify the effect of the amount of drug loaded on the performance of the microparticulate systems. Placebo and BU loaded microspheres were prepared by means of a spray drying method.

The microspheres were irradiated in air either with β- or γ-irradiation at the dose of 25 kGy. A minimum absorbed dose of 25 kGy is considered adequate for the purpose of sterilizing pharmaceutical products without providing any biological validation [1].

Microsphere morphology was analyzed by atomic force microscopy (AFM).

The physico–chemical properties of non-irradiated and irradiated microspheres were evaluated by using DSC and FT-IR spectroscopy. Drug content and in vitro BU release profile were evaluated over 9 months of storage.

2. Materials and methods

2.1. Materials

Poly(lactide-co-glycolide) 50:50 (PLGA), Resomer® RG 503, inherent viscosity 0.39 dl/g, and M_w of 34,000 (Boehringer Ingelheim KG, Ingelheim am Rheim, G). Bupivacaine base (BU) was obtained by adding an aqueous solution of NH_4OH 30% w/w to an aqueous solution of bupivacaine hydrochloride (S.I.M.S., Florence, Italy) with stirring until complete precipitation. The precipitate was filtered, washed with distilled water and dried in a desiccator under vacuum with silica gel at room temperature until a constant loss on drying was obtained.

All solvents, unless specified, were of analytical grade.

2.2. Preparation of BU microspheres

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

The microspheres were obtained by spraying 2% w/v feed made of PLGA and BU in methylene chloride through a standard nozzle with inside diameter of 1 mm. Three microparticulate systems were designed with the following BU/PLGA ratio: 10:90 w/w (formulation 1); 25:75 w/w (formulation 2); and 40:60 w/w (formulation 3). The process parameters were set as follows: inlet temperature 50 °C; outlet temperature 34–36 °C; and flow rate 15 ml/min. Placebo microspheres were also prepared by spraying a 2% w/v PLGA solution at the conditions described above. After preparation, the microspheres were stored at 4±1 °C until use.

2.3. β- and γ-irradiation of Bu and placebo and loaded microspheres

2.3.1. γ-Irradiation

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 a dose of 25 kGy, applied at a 516 Gy/h dose rate and irradiation temperature of 25 °C.

2.3.2. β-Irradiation

BU, placebo and BU loaded microspheres were irradiated by using an electron beam accelerator (Bioster, Italy). Irradiation was performed in the presence of air, calorimetry dose 25.1 kGy, energy 10 MeV, and irradiation temperature of 25 °C.

2.4. Size distribution

Particle-size analysis was performed by a light diffraction method with a Coulter apparatus, model LS 230 (Coulter Corporation, Florida, US); this instrument works on laser diffraction optics and on another system based on polarized light of three wavelengths termed P.I.D.S. The size range of the LS230 version was from 0.04 to 2000 μm. The samples of microspheres were suspended in filtered water, sonicated for 30 s and subsequently analyzed.

2.5. Atomic force microscopy (AFM)

Morphological and topographical modifications of microsphere surface were investigated by AFM with an AutoProbe CP Research scanning probe microscope (ThermoMicroscope, Sunnyvale, CA, US) in air and under constant applied force conditions (non-contact mode) with a cantilever resonant frequency of approximately 90 kHz and a theoretical spring constant k=3.2 N/m. Microspheres were nebulized at a distance of approximately 15 cm on a carbon conductive adhesive tape (Ted Pella Inc., Redding, CA, US), which had been glued onto a steel disc to enable magnetic fixation under the scanning tip.

Images were processed and analyzed using the Image Processing Data Analysis 2.0 software provided by ThermoMicroscope. Microsphere surface roughness is given as root-mean-square (rms) data, that is the standard deviation over all height values within an area of 1 μm^2. The results are the average of rms surface roughness values obtained from 10 AFM images taken from 10 different microspheres.

2.6. FT-IR spectroscopy

FT-IR spectra were recorded with an FT-IR spectrometer Paragon 1000 PC (Perkin Elmer, US). Sixteen scans were collected for each sample at a resolution of 2 cm^{-1} over the wave number region 4500–500 cm^{-1}. Samples were prepared in KBr dies by compaction (compaction force: 10 tons; holding time: 10 min). The weight ratio of KBr to powder was about 100.

2.7. Thermal analysis

DSC thermograms were recorded by using a DSC 2010 TA (TA Instruments, US). The samples of 5 mg exactly weighted (±0.01 mg) were sealed in aluminum pans and heated in an inert atmosphere (70 ml/min of N_2). The reference was an empty pan. The equipment was calibrated with an indium sample.

BU samples were scanned at 10 °C/min from 30 to 130 °C.

The microsphere samples and the corresponding physical mixtures were heated at 10 °C/min from 30 to 60 °C in order to avoid BU melting, then cooled

down from 60 to 0 °C at 20 °C/min and re-heated up to 130 °C at 10 °C/min. All the determinations were performed in triplicate.

2.8. Drug content assay

Forty milligrams of BU loaded microsphere was dissolved in methylene chloride (1 ml); the drug was extracted with 5 ml of 1 N H_2SO_4. After 2 min of stirring and centrifugation for 15 min at 1000 rpm, 50 μl of aqueous phase was diluted in an appropriate amount of mobile phase. The samples were tested by the HPLC method described in Section 2.10.

2.9. Dissolution test

In vitro dissolution tests were carried out in bottles closed by screwed stoppers and stirred in a shaker incubator (100 strokes/min) at 37 °C. The microspheres were suspended in 400 ml of dissolution medium (pH 7.4 phosphate buffer containing 0.02% w/v of SDS) and the amount of BU released at fixed time intervals was tested by the HPLC method described in Section 2.10.

Samples were exactly weighed in order to get 5.00 mg of BU.

The test was performed soon after irradiation (time 0), and repeated before and after 90 and 270 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 amount of drug loaded in the matrix and k is the release rate constant expressed as h^{-1}.

2.10. HPLC analysis

The HPLC method followed for the analysis of the amount of drug loaded in the microspheres (Section 2.6) and of the drug released during dissolution test (Section 2.7) was as follows. The HPLC system was a HP1100 Chemstation (Hewlett Packard, US). Chromatographic conditions were as follows: column, Bondclone C_{18}, 10 mm, 300×3.9 mm I.D. (Phenomenex, US); mobile phase: KH_2PO_4 (pH 4.0; 0.01 M)/acetonitrile (70:30 v/v); flow rate, 1.5 ml/min; temperature, 25 °C; wavelength set, 205 nm; and injection volume, 10 μl. The drug concentrations were determined from standard curves in the range 1–50 mg/ml [13].

2.11. Statistical analysis

Tests for significant differences between means were performed by Student t-test or one-way ANOVA, by using the software SPSS 11 (Spss Inc., US). In order to identify the parameters that affected the in vitro BU drug release, a multilinear regression analysis was also performed with the same software. Differences were considered significant at the $P < 0.05$ level.

3. Result and discussion

3.1. Morphological and topographical characterization of microspheres

The particle-size distributions of the BU microspheres are summarized in Table 1. As expected, the particle-size distribution of the different types of microspheres overlapped. Moreover, irradiation did not influence the size distribution.

Sterilization of BU loaded microspheres by γ- and β-irradiation induced modifications of surface morphology, which were easily detected by visual inspection of AFM images. As shown in Fig. 1, the surface of non-irradiated microspheres (Fig. 1A) is relatively smooth in comparison to treated samples: AFM images of β- (Fig. 1B) and γ- (Fig. 1C) irradiated samples reveals the appearance of bumpy features on the surface of the microspheres. The

Table 1
Microsphere particle-size distribution (data are expressed as a percentage in number)

Form. no.	Radiation	d_{10} (μm)	d_{50} (μm)	d_{90} (μm)
1	–	0.42	0.59	1.06
	β	0.42	0.60	1.08
	γ	0.42	0.60	1.08
2	–	0.42	0.59	1.02
	β	0.42	0.59	1.02
	γ	0.42	0.59	1.04
3	–	0.42	0.58	0.99
	β	0.42	0.58	0.99
	γ	0.42	0.59	1.00

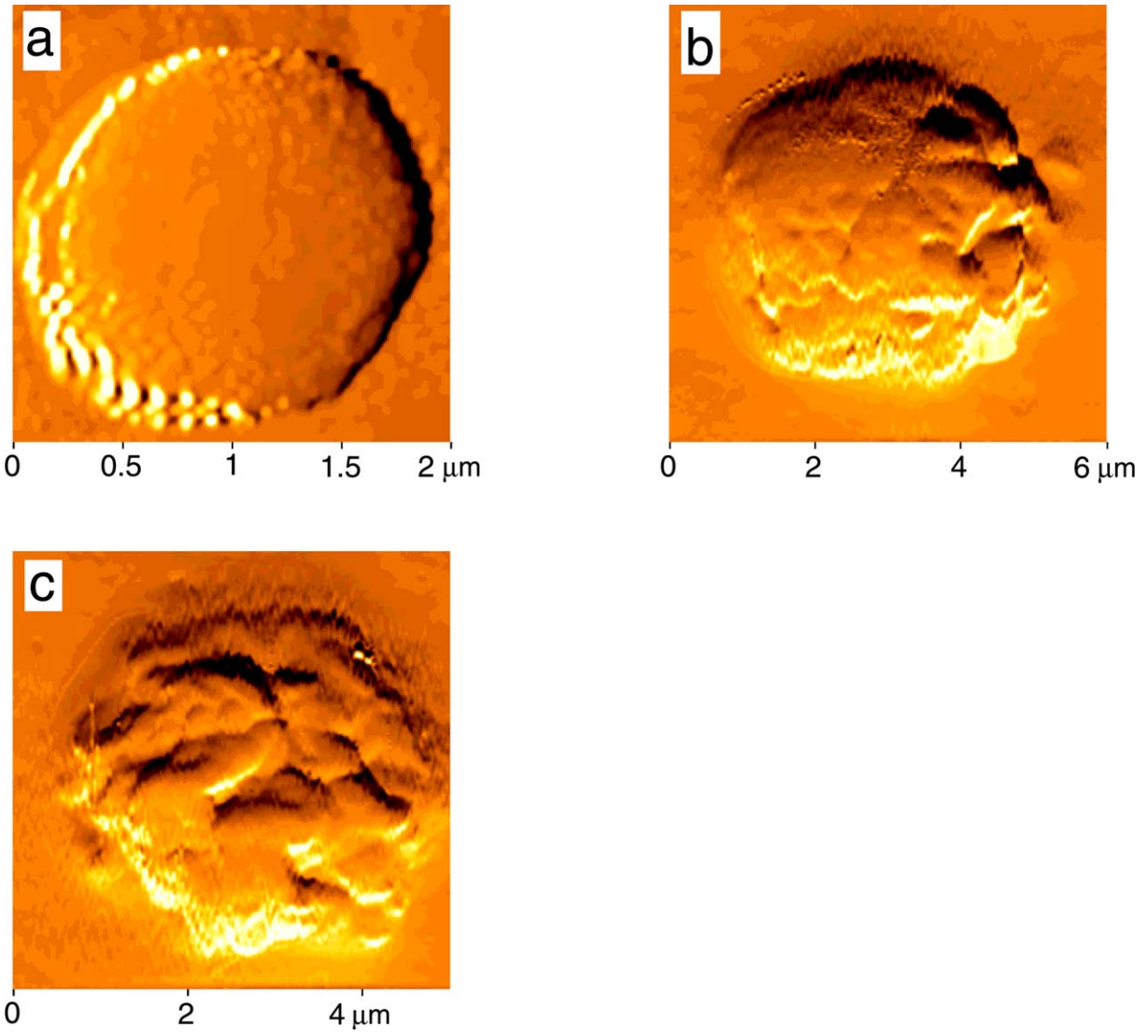

Fig. 1. Error signal AFM image of formulation 1 non-irradiated (a), β-irradiated (b) and γ-irradiated (c). Scan size is 2×2 μm^2 (a), 6×6 μm^2 (b) and 5×5 μm^2 (c).

same surface modifications were highlighted in all microsphere formulations.

The quantification of the surface roughness through the calculation of rms values confirms the effects of irradiation treatment on the microsphere surfaces. As reported in Table 2, the analysis of rms surface roughness points out highly significant differences among non-irradiated and irradiated samples, which were independent from the BU content of microspheres (one-way ANOVA, $P<0.05$). Furthermore, based on rms analysis, the surface of microspheres was slightly more affected by γ- than β-irradiation treatment (Table 2).

The increase in surface roughness did not cause any modification in particle size distribution of irradiated microspheres. Such a phenomenon could be due at least to two events. During the dose administration the microspheres swelled as a conse-

Table 2
Microsphere rms roughness values (mean±S.D., $n=10$)

Form. no.	Radiation	rms (nm)
Placebo	–	32.0±3.7
	β	70.6±8.2*
	γ	80.3±9.8*
1	–	32.2±4.8
	β	71.7±17.3*
	γ	87.4±19.7*
2	–	37.7±3.8
	β	74.5±17.2*
	γ	83.5±13.2*
3	–	39.9±3.5
	β	73.5±12.9*
	γ	84.6±8.7*

*$P<0.05$.

quence of the interaction either with γ- or β-rays. As the process ended, the microsphere structure collapsed. Nevertheless, investigations about this mechanism should require more specific work.

AFM was revealed to be a very useful technique to quantify the basal surface roughness of PLGA microspheres, as well as their different morphologic modifications induced by γ- and β-irradiation.

The AFM technique shows great advantage with respect to SEM: first of all it does not require any treatment in the sample preparation (vacuum and metalization), and in addition it avoids any possible damage to the sample due to the β-beam of the SEM.

3.2. Physico–chemical characterization of microspheres

The FT-IR spectra of the BU microspheres exhibited a transmission pattern that appeared as the sum of the two components according to their relative abundance, indicating that no specific interactions between BU and PLGA occurred (Fig. 2).

The placebo microspheres exhibited a glass transition temperature (T_g) at 46.8±0.2 °C, which overlapped with that of the PLGA raw material (T_g= 46.9±0.4 °C), revealing that the production process did not affect the copolymer structure.

The DSC thermograms of BU/PLGA physical mixtures showed that the thermal pattern of the two components was substantially the same of the two single substances, and no interaction could be attributed to the heating processes (Fig. 3). As far as BU microspheres are concerned, by decreasing the BU loading, the onset temperature of the melting peak shifted slightly toward a lower value than that of pure BU due to an increasing of the peak width. The T_g of microspheres was lower than that of PLGA, but it increased upon increasing of the drug loading (Table 3). The reason for this pattern has not been fully understood yet. It could be attributed to the heating process, or it could be related to a plasticizing effect of the non-crystallized BU fraction that decreased upon increasing of the drug loading.

As shown in Table 4, irradiation caused a small reduction in the T_g value that followed the rank order: T_g of non-irradiated microspheres>T_g of β-irradiated microspheres>T_g of γ-irradiated microspheres. The T_g reduction of the irradiated microspheres was statistically different from those of the non-irradiated microspheres except in the case of form. no. 1 (Table 4). This phenomenon may be due to the different exposure time during the sterilization process. In the case of γ treatment, the longer exposure time could prolong the peroxidative radiolitic degradation discussed in a previous work, which led to PLGA chain scission [7]. Moreover, the T_g slight reduction of irradiated microspheres did not seem to be influenced by the BU/PLGA ratio (Table 4). This result is in agreement with the EPR study reported in a previous work [13]. No spin transfer BU/PLGA, and consequently no interaction occurred during irradiation, as the relative abundance of BU radicals and polymer radicals was proportional to the electronic fractions of the components.

3.3. Drug content and drug release

The drug content of non-irradiated and irradiated microspheres is reported in Table 5.

The decrease of BU content in γ-irradiated microspheres was always almost constant independent of the amount of BU per sample, therefore it was in inverse relation to drug loading (in a range between 5 and 15%); after β-irradiation, the BU content decreased in a significant way (≈3%) only in those microspheres containing 10% w/w of BU (Table 5).

This pattern is in agreement with the results regarding the sensitivity of BU to irradiation. BU is much more sensitive to γ-rays (loss of about 2%

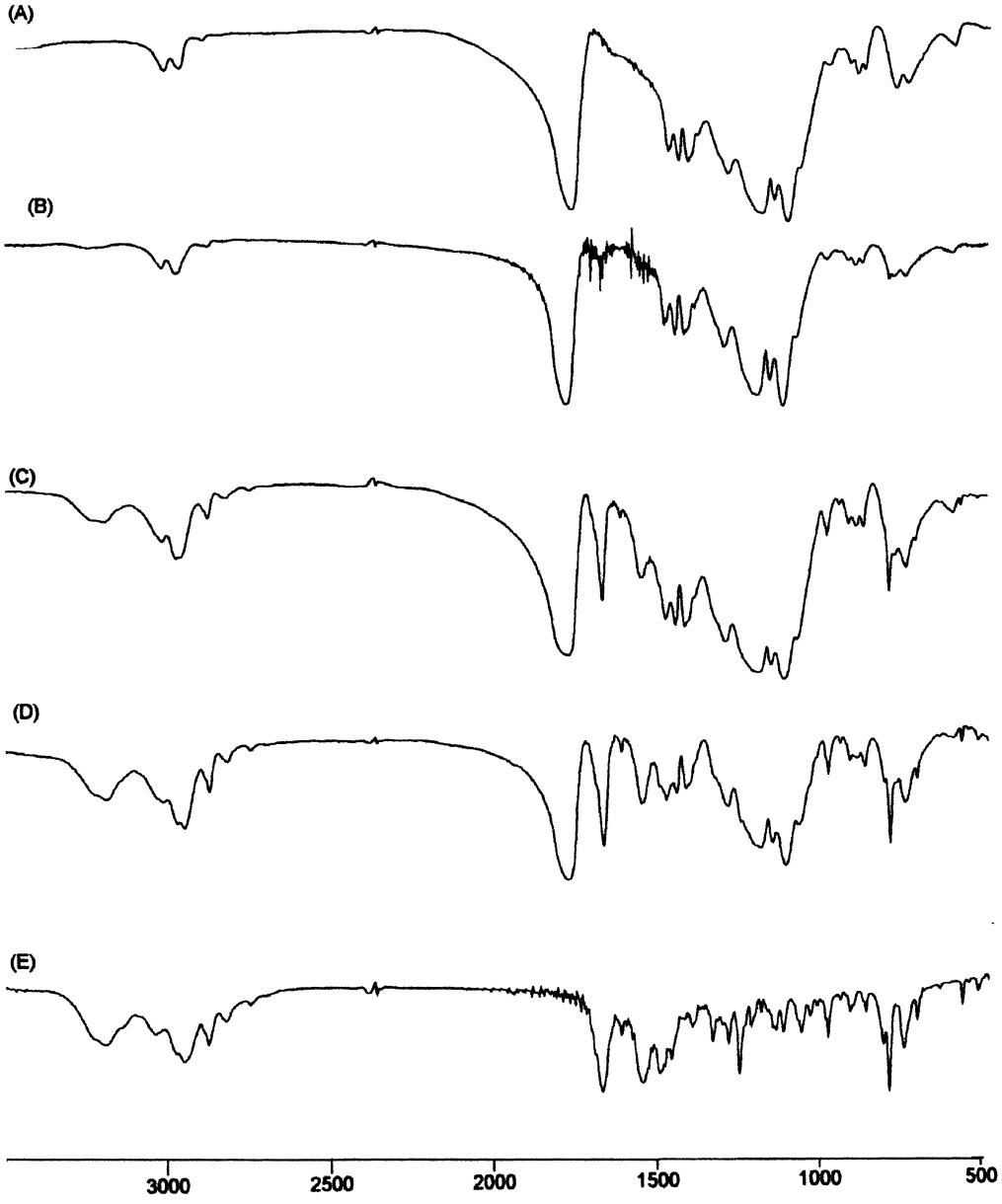

Fig. 2. FT-IR spectra of PLGA (A), formulation 1 (B), formulation 2 (C), formulation 3 (D) and BU (E).

w/w) than β-rays. In the case of β-irradiation, the BU recovery was not significantly different from that of non-irradiated drug.

In vitro release profiles of BU from non-irradiated microspheres are shown in Fig. 4. The drug release was dependent on the relative BU/PLGA ratio and increased upon decreasing the drug loading according to the percentage of crystallinity and T_g of the systems (Table 3). The Higuchi model only agrees with the experimental data over a period of 6 days ($r^2 > 0.995$), indicating that during the early stage of BU release, the process was controlled purely by diffusion, and the effect of PLGA degradation was not yet evident.

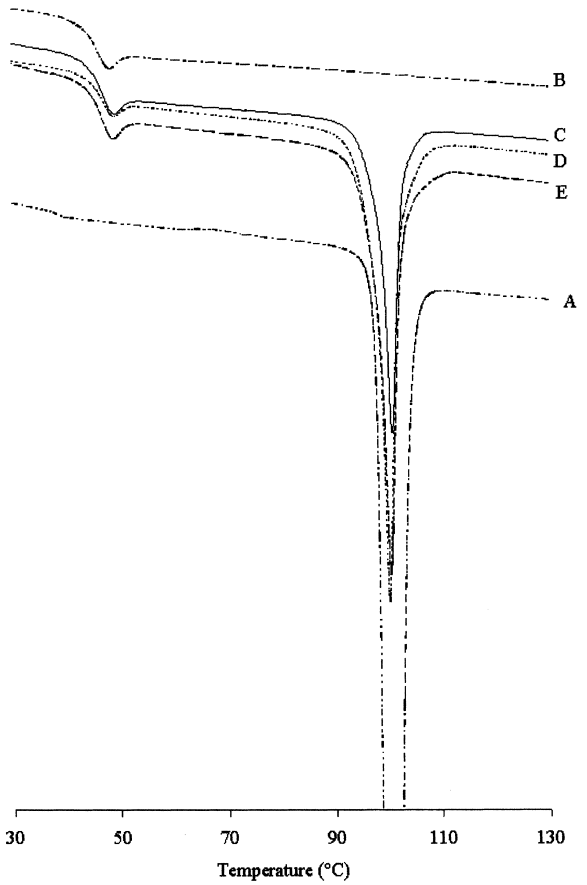

Fig. 3. DSC traces of: (A) BU ($T_{m,BU}$ = 97.6 °C, ΔH = 71.3 J/g); (B) re-heated PLGA; (C) BU/PLGA 10:90 physical mixture ($T_{m,BU}$ = 98.0 °C, ΔH = 7.3 J/g); (D) BU/PLGA 25:75 physical mixture ($T_{m,BU}$ = 97.6 °C, ΔH = 18.3 J/g); (E) BU/PLGA 40:60 physical mixture ($T_{m,BU}$ = 97.6 °C, ΔH = 26.4 J/g).

Both γ- and β-irradiation affected the drug release rate. PLGA decomposition and the decrease in the average molecular weight [7,13] increased both the extent and the rate of BU released from PLGA microspheres (Table 6).

Table 3
DSC data of non-irradiated BU microspheres (mean±S.D., n = 3)

Form. no.	T_g (°C)	$T_{m,BU}$ (°C)	Crystalline BU[a] (%)
1	42.8±0.5	94.8±1.1	12.8
2	44.7±0.3	96.0±1.9	33.5
3	45.4±0.3	97.4±2.4	77.1

[a] Calculated on the basis of ΔH of BU (ΔH = 71.0±1.0 J/g).

In γ-irradiated microspheres, the initial BU release rate increased by irradiation, compared to the non-irradiated controls, independently of the drug content (Table 6). The rate remained relatively constant for the following storage periods beyond 90 days (Table 6). The detrimental effects of γ-irradiation on PLGA lasted until 90 days of storage, independent of the BU/PLGA ratio. This pattern confirmed the results reported in a previous work [13].

As far as the β-irradiated microspheres are concerned, the drug release rate constant that was determined immediately after the treatment increased less than that in the corresponding γ-irradiated microspheres in all cases. Moreover, it did not change further in the following 90 days of storage (Table 6) and therefore the test at 270 days was not performed.

The potential predictors of the release rate constant k (i.e. drug content, DC; microsphere surface roughness, rms; and glass transition, T_g) were analyzed by multiple regression analysis. At a first run, it was evident that there was a significant collinearity between predictors that prevented the correct estimate of possible non-significant contributors. The analysis was re-run using the Z-score transformation of the predictors. This procedure solved the problem of collinearity and yielded T_g as the sole non-significant predictor (P = 0.170). Performing again the multiple regression analysis on the original data after removal of T_g, the following equation was obtained:

$$k = -0.053\pm0.008\,\mathrm{DC} + 0.045\pm0.005\,\mathrm{rms} + 3.661\pm0.387$$

(n = 9; corrected r-square: 0.940; F = 64.215; P < 0.001) in which all included terms are statistically significant ($P \leq 0.001$).

This result suggests that, in relation to the BU release, the small changes of T_g (Table 4) were irrelevant in comparison with rms and DC.

4. Conclusion

An interesting conclusion can be drawn from the comparison of the effects obtained by β- and γ-irradiation performed on BU loaded PLGA microsphere.

Table 4
T_g of non-irradiated and irradiated placebo and BU microspheres (mean±S.D., $n=3$)

	$T_{g,PLGA}$ (°C)			
	Placebo	Form. 1	Form. 2	Form. 3
Non-irradiated	46.8± 0.2	42.8±0.5	44.7±0.3	45.4±0.3
β-Irradiated	45.1± 0.2*	42.3±0.1	42.1± 0.0*	43.6±0.7*
γ-Irradiated	43.7± 0.5*	41.6±0.4	40.6±0.2*	41.8±0.5*

*t-test, $P<0.05$.

Table 5
Drug content of non-irradiated and β- and γ-irradiated BU microspheres (mean±S.D., $n=3$)

Form. no.	Non-irradiated (% w/w)	β-Irradiated (% w/w)	γ-Irradiated (% w/w)
1	10.55±0.38	10.00±0.12*	8.81±0.23*
2	25.47±0.48	25.57±0.37	23.55±0.64*
3	40.01±0.37	39.49±0.35	38.44±0.73*
BU			

*$P<0.05$.

β- and γ-rays modified the performance of the BU loaded microspheres in a significantly different way as regards surface morphology, drug content and drug release pattern. In particular, the microspheres were more sensitive to γ-irradiation, which caused an increase of in vitro drug release during the following 90 days of storage. Thus, although the effects on sterilization are basically the same for electromagnetic rays and high-energy electrons, the choice of irradiation type should be considered as a critical parameter to be carefully evaluated in the production of sterile dosage forms made of oxidable or thermosensitive materials.

In this work, AFM was used as an alternative to SEM analysis and was applied routinely to morphologic characterization of microspheres.

According to our results, AFM can be proposed as a technique to analyze the surface morphology of microspheres, and specifically to measure—in a quantitative way— their surface roughness, which could be considered as a marker of degradation due to ionizing radiations.

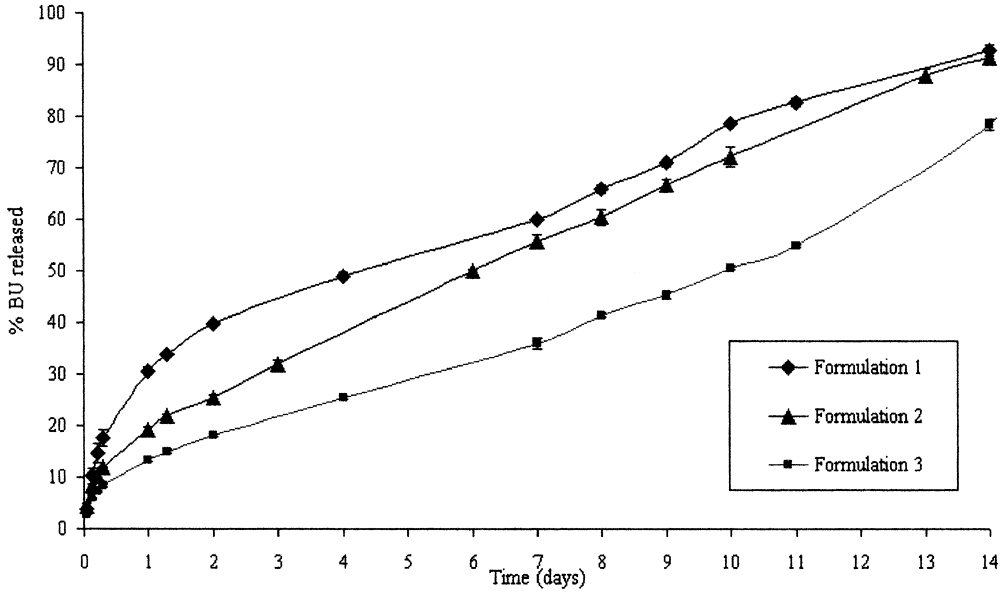

Fig. 4. BU release profiles from non-irradiated microspheres.

Table 6
BU release rate constant (K) calculated in the first 6 days of non-irradiated and irradiated microspheres soon after irradiation ($t=0$) and after 90 and 270 days of storage (mean±S.D., $n=3$)

Form. no.	Non-irradiated [K ($h^{-1}\times10^{-2}$)]			β-Irradiated [K ($h^{-1}\times10^{-2}$)]			γ-Irradiated [K ($h^{-1}\times10^{-2}$)]		
	$t=0$	$t=90$	$t=270$	$t=0$	$t=90$	$t=270$	$t=0$	$t=90$	$t=270$
1	4.5±0.1	4.3±0.1	–*	6.1±0.0	5.9±0.1	–*	6.9±0.1	7.5±0.6	7.2±0.1
2	4.2±0.1	4.1±0.0	–*	6.0±0.1	5.9±0.1	–*	6.5±0.2	7.2±0.2	7.4±0.0
3	3.2±0.0	3.2±0.0	–*	4.5±0.1	3.9±0.1	–*	5.4±0.0	5.8±0.3	5.6±0.1

*Not performed.

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