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European Journal of Pharmaceutics and Biopharmaceutics 59 (2005) 139–146
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Effects of gamma-irradiation on trehalose–hydroxyethylcellulose microspheres loaded with vancomycin

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Received 1 December 2003; accepted in revised form 21 June 2004
Available online 6 October 2004

Abstract

Ionizing radiation can be used as a drug sterilization technique, provided that the drug itself is not modified and that no toxic products are produced; moreover, if the irradiated product is a drug delivery system, the drug release characteristics must not be significantly altered by radiation. The aim of this work was to study the effects of sterilization by ionizing radiation on hydroxyethylcellulose/trehalose spherical micromatrices, containing the antibiotic vancomycin. Our experimental results showed that gamma-rays did not alter the chromophore groups of vancomycin (UV measurements), and did not modify the kinetic behavior of drug release from microspheres. Moreover, no significant changes in the shape and in the size distribution of microspheres were found after irradiation. The electron spin resonance (ESR) spectroscopy was proven to be a valid identification method of the executed radiation treatment, even after 5 years. The experimental results showed that the therapeutic application of the pharmacological system investigated was not compromised by irradiation, and that ESR spectroscopy can be used to distinguish irradiated from non-irradiated products.

Keywords: Drug sterilization; ESR; Quality control; Ionizing radiation; Drug delivery system; Microspheres; Gamma-irradiation

1. Introduction

In the last few years, ionizing radiation has gained much interest in sanitization/sterilization processes for food, drugs, pharmaceutical systems and medical supplies as an alternative method to heat, gas exposure and other sterilization techniques.

Ionizing radiation, such as gamma-irradiation generated from a ^{60}Co source, is able to kill the pathogens that could contaminate therapeutic products.

The main advantage of using radiosterilization are the high penetration power and the isothermal character of the gamma-rays that allows a suitable treatment for heat-sensitive materials. Moreover, gamma-irradiation assures homogeneous sterilization and is useful for packaged products, thus avoiding further risk of microbe contamination.

On the basis of the European Committee for Standardization requirements, the sterilization procedures must guarantee a sterility assurance level (SAL) of at least 10^{-6} in the final product [1]. Considering the number and the resistance of typical naturally occurring contaminating microorganisms in the raw materials, the dose required to achieve this goal is assumed to be 25 kGy [2–4].

Nevertheless, as for the other sterilization methods, radiosterilization has some drawbacks. The energy transfer could induce fragmentations of covalent bonds and production of free radicals that, in turn, are responsible for the majority of the damage that occurs to irradiated materials as a consequence of chemical attack, e.g. radiation could cause alteration of the physico-chemical properties,

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doi:10.1016/j.ejpb.2004.06.011

decrease of the amount of active ingredient by partial decomposition, or create molecular fragments that may result in a toxicological hazard.

Moreover, as a general rule, substantial modifications may occur during the irradiation of a polymer. Consequently, for pharmaceuticals that contain polymers as formulation components, ionizing irradiation could generate degradations that adversely affect the behavior of the polymer [5,6].

The Commission of the European Communities has given some guidelines about the use of ionizing radiation in the manufacture of pharmaceuticals. The knowledge of the irradiation effects on the stability of materials and formation of degradation products are required [7]. For this reason, the use of ionizing radiation as a sterilization method for medicinal products requires accurate analyses, to check that the active ingredient has not been modified and no toxic products have been produced. Moreover, if the drug is dispersed in a solid drug delivery system, the components of formulation may be altered by radiation as a consequence of radiolysis, thus affecting the drug release from the dosage form and hence, safety [8,9]. In the present work we describe the effects of sterilization by ionizing radiation on the previously reported hydroxyethylcellulose/trehalose spherical micromatrices containing the antibiotic vancomycin [10].

One of the main indication of vancomycin is the treatment of several wound infections, caused by methicillin resistant staphylococci, that could occur in patients with extensive skin barrier disruption (burns, abrasions). Systemic administration of vancomycin may be associated with several adverse effects that could be lowered by topical applications. Since skin is seriously damaged and no barrier opposes drug transfer into biological tissues, drug penetration may be regulated by the topical application of an adequate drug delivery system [10]. This is a good approach for obtaining a therapeutic effect in a predetermined period, meanwhile minimizing side effects. Nevertheless, the drug delivery system before application needs to meet the pharmacopoeia requirements of sterility.

Since vancomycin decomposition by melting was observed at 90 °C [10], dry or wet heat is not applicable for sterilization of microspheres. On the other hand, ethylene oxide and the other sterilizing gases are not advisable due to the risk of reaction with the components of the matrix, modifying the mechanism of drug release and/or producing toxic residues. Moreover, the system preparation under aseptic conditions is a very complex and expensive process. Gamma-irradiation currently remains the only alternative method for terminal sterilization of the microspheres containing vancomycin.

Poor data are reported in literature on the radiosensitivity of vancomycin, hydroxyethylcellulose and trehalose [11,12]. Nevertheless, it is known that ionizing radiation induces some structural modifications of polysaccharides such as cellulose, starch, chitin/chitosan and their water-soluble derivatives in aqueous solution. For instance, such irradiated polymers can be to a certain extent cross-linked in highly concentrated solutions and can adopt more ordered and more stable conformations. It has been proved that the cross-linking reaction was remarkably affected by polysaccharide concentration. It was assumed that the reactions were mainly due to the mobility of side chains of these polysaccharides derivatives. Side-chain radicals were formed mostly via indirect effects, by the abstraction of H atoms by the intermediate products of water radiolysis [13,14].

After controlled exposure to gamma-rays, solid dosage forms containing polysaccharides may also undergo alterations as a result of possible cross-linking reactions. Therefore, it is necessary to establish that modification of formulation components affecting deviations in drug release performance had not occurred.

Moreover, before irradiation of pharmaceuticals, it is desirable to ascertain experimental methods of discrimination between irradiated and non-irradiated products, even after long time from treatment.

Electron spin resonance (ESR) spectroscopy appears to be useful to determine and quantify long-living free radicals in complex media. It is already a well-established method used for the identification of some kind of irradiated food [15,16]. For the same reason, ESR spectroscopy has been used for identification of some irradiated drugs [17–19] and irradiated parenteral microparticulate drug delivery systems [20,21].

The first aim of this work was the assessment of potential alterations of formulation components of hydroxyethylcellulose/trehalose spherical micromatrices loaded with vancomycin and deviations in drug release performance after exposure to gamma-rays. The effects of gamma-irradiations were evaluated by observing the main differences in the system before and after treatment, using various techniques.

We also present and discuss the detection of free radicals induced by gamma-radiation using the ESR spectroscopy data. This technique allows the evaluation of free radicals even after long time from irradiation.

2. Materials and methods

2.1. Materials

- Vancomycin hydrochloride (Eli Lilly Indianapolis, IN) and Veloderm were kindly supplied by ARNAS Ospedale Civico (Palermo, Italy), and Bioskin Trade Company (Ancona, Italy), respectively.
- Trehalose (dihydrate) was purchased from Hayashibara Shoij, Inc. (Okayama, Japan).
- Hydroxyethylcellulose (Natrosol®) and liquid paraffin were purchased from Galeno (Florence, Italy).

- SPAN® 60 was purchased from Fluka (Buchs, Switzerland).

All chemicals and solvents were used without further purification.

2.2. Preparation of microspheres

Trehalose/hydroxyethylcellulose microspheres loaded with vancomycin were prepared as previously reported [10]. The system composition was: Natrosol® 10.0%, trehalose dihydrate 80.0% and vancomycin hydrochloride 10.0%. Microspheres were produced by the solvent (water) evaporation method using liquid paraffin as external phase and SPAN® 60 as dispersing agent. Each batch was prepared using an overall amount of 1.0 g of material. Reproducible, spherical particles were obtained with a vancomycin content of 9.99%, determined by UV spectrophotometric quantitative analysis, as described in Section 2.4. Reproducibility was evaluated on six different batches.

2.3. Gamma-irradiation of microspheres

Irradiations of the microspheres (as pellets for EPR studies, and as powder for the other studies) were carried out at room temperature with a ^{60}Co panoramic irradiator IGS-3 at dose values of 5, 10, 15, 25 and 40 kGy. The dose of 25 kGy was chosen since it is the recommended dose for sterilization treatment of drugs. Lower doses were used to study the dependence of ESR signal intensity on dose. The 40 kGy irradiation was performed to study the effects caused by doses higher than recommended.

The dose rate (measured with the Fricke dosimeter) was 2.00 kGy/h. Uncertainty in dose value was less than 2%. Non-irradiated samples were kept as reference.

2.4. UV spectrophotometric determinations

Spectrophotometric analyses were performed using an UV/VIS spectrophotometer (Shimadzu, model 1601).

To evaluate drug content in microspheres before and after exposure to gamma-radiation, the amount of vancomycin was measured by spectrophotometric quantitative determination. For this purpose, aliquots of 10 ± 0.5 mg of microspheres were transferred into 50 ml flasks and dissolved, by sonification, in an isotonic pH 7.4 phosphate buffer solution prepared according to Italian Pharmacopoeia [22], thus releasing the encapsulated vancomycin. Drug content was detected at $\lambda = 280$ nm, using the appropriate blank and calibration curve ($E_{1\%} = 0.0436$ in phosphate buffer solution). At 280 nm the peak was highly reproducible and linearly related to concentration over a range 0.001–0.3 mg/ml. The sensitivity was better than 0.001 mg/ml. Intraday and interday variations, observed during collection of experimental data, were below sensitivity.

Analyses were performed on six different batches of non-irradiated and irradiated microspheres. The average drug content in microspheres ($9.99 \pm 0.01\%$) was unchanged before and after irradiation at each dose under test.

2.5. Surface morphology of microspheres

The microspheres were characterized, before and after gamma-irradiation, for shape and surface characteristics with a scanning electron microscope Philips XL30 ESEM. For this observation, microspheres were mounted on metal stubs with conductive silver paint and then sputtered with a 15 nm thick layer of gold.

2.6. Size distribution of microspheres

Particle size distribution measurements were carried out using an image processing and analysis system (Leica Quantimet Q500), equipped with a Leica Wild 3D stereomicroscope. This image processor calculates the particle area and converts it to an equivalent circle diameter. Size distribution of microspheres before and after gamma-irradiation was performed on six different batches of microspheres.

2.7. Differential scanning calorimetry (DSC)

Thermal analyses were carried out with a Perkin–Elmer DSC7 (power compensation) differential scanning calorimeter connected to a computer via a TAC7/DX thermal analysis instrument controller. The thermal unit was thermostated by means of an external thermocryostat in which the coolant was kept at -30 °C; a nitrogen flux was used as a purging gas for the furnace. Calibration was performed using indium and zinc at the same scan rates used in the experiments. DSC scans were run before and after irradiation on samples of drug-loaded microspheres weighing 10 ± 0.5 mg and sealed in pierced aluminium Perkin–Elmer DSC pans. Analyses were conducted in triplicate, and were performed at a constant heating rate of 5 °C/min, in the range of 40–230 °C.

2.8. Electron spin resonance studies

Solid state, cylindrically shaped pellets were realized and irradiated for ESR analysis. Aliquots of 30 mg were inserted in each housing of a stainless steel mould; the pellets were obtained by pressing the chosen quantities of microspheres with stainless steel pistons of appropriate length, by means of a hand-tabletting press; a pressure of about 5×10^6 Pa was applied, to obtain pellets with expected thickness of 1.5 mm and diameter of 4.8 mm.

ESR spectra were recorded at room temperature using a Bruker ECS106 spectrometer (operating in the X-band), equipped with a TE_{102} rectangular cavity operating at approximately 9.7 GHz. Samples were inserted in standard

quartz tubes of 5 mm internal diameter. Since the shape of the ESR spectrum did not show any dependence on dose, the peak-to-peak height of the central line of the ESR spectrum (in the following 'signal intensity') could be used as the dose dependent parameter, as usual in ESR dosimetry [23].

The following ESR recording parameters were appropriately chosen to obtain the highest signal to noise ratio: central field: 349 mT; sweep width: 15 mT; microwave power: 1.2 mW; modulation amplitude: 0.11 mT; time constant: 328 ms; receiver gain: 2.8×10^2; number of cumulated scans: 3.

2.9. In vitro drug release tests

To evaluate any possible release alteration induced by radiation, in vitro kinetic studies were carried out before and after exposure to gamma-rays.

Aliquots of 150 ± 5 mg of microspheres were placed in the cell-cap (donor chamber) of a flow-through Franz type diffusion cell of 45 ml. As previously described [10], drug transfer was tested using a freely permeable membrane (Veloderm®), and a receptor chamber consisting of an isotonic phosphate buffer solution (pH 7.4) maintained at a constant temperature of 37 ± 0.5 °C (Polimix EH2 thermostatic bath, Kinematica, Switzerland) simulating biological medium. The exposed surface area was 10.7 cm^2. A magnetic stirrer rotating at 600 rpm (Stirrer RECO® S5, Kinematica, Switzerland) assured homogeneous and uniform concentration in the receptor phase. One-millilitre aliquots of the receptor phase were taken at predetermined time intervals of 10 min, and replaced by an equal volume of fresh buffer solution, to maintain 'sink' conditions and avoid saturation phenomena.

The released vancomycin was determined by UV spectrophotometric quantitative analysis of the receptor phase at $\lambda=280$ nm.

Experiments on drug release were conducted in triplicate.

3. Results and discussion

3.1. UV spectrophotometry measurements

By comparing the shape of the UV spectra of vancomycin released from irradiated and non-irradiated microspheres (recorded in the range 200–400 nm), it was possible to verify that gamma-rays did not alter the chromophore groups of drug molecule. The second order derivative UV spectra of irradiated microspheres showed peaks and valleys that overlap with those of non-irradiated microspheres. Moreover, peaks and valleys were identical to those of a pure sample of vancomycin at the same concentration. By measuring the absorbance of vancomycin at $\lambda=280$ nm, no reduction of drug content was found. Moreover, full recovery of the vancomycin entrapped in the microspheres was observed following irradiation both at 25 and 40 kGy.

Moreover, biological essays showed that the in vitro antibacterial activity of vancomycin was not reduced after irradiation (data not reported).

These results confirmed that ionizing radiation caused neither qualitative nor quantitative alteration of vancomycin entrapped in microspheres.

3.2. Kinetics of vancomycin release from microparticulate systems

Drug discharge from microspheres was monitored by analysis of the cumulative amount of vancomycin reaching the receptor phase. Fig. 1 shows kinetics of drug release from non-irradiated microspheres, and microspheres irradiated at 25 and 40 kGy. The 'lag-time' at the beginning was ascribed to the time needed for establishing interactions with water, the following swelling process, and the formation of a hydrophilic and viscous gel that allows drug diffusion. After the 'lag-time', the percentage of released vancomycin increases gradually.

We fitted the most common models, used in dissolution analysis, to our experimental results (bilog, cube root, exponential, Higuchi, Hill and Weibull [10,24]). The method of least squares showed that the Weibull model was the most appropriate for describing the kinetics of drug release: for non-irradiated microspheres the correlation coefficient was 0.999, and the χ^2 test gave a goodness of fit better than 98%; analogous results were observed for irradiated samples both at 25 and 40 kGy.

The drug release rate from irradiated micromatrices was negligibly faster than from the non-irradiated ones. For instance, at 20 min after the beginning of the experiment, the percentage of vancomycin released from non-irradiated microspheres, and microspheres irradiated at 25 and 40 kGy, was 2.7, 2.9 and 5.1%, respectively. The differences among the extrapolated steady state release rate

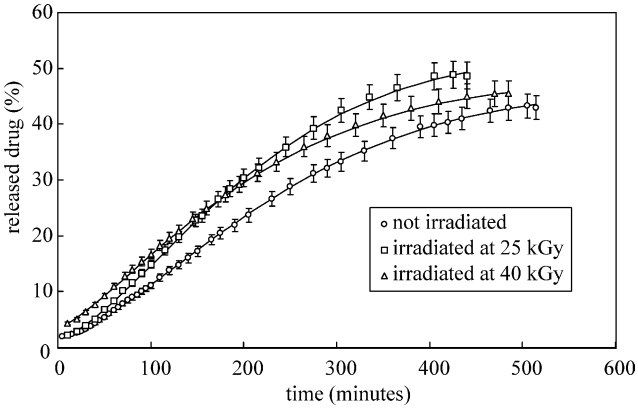

Fig. 1. Percentage of vancomycin released from microspheres before (○), and after irradiation at 25 kGy (□), and at 40 kGy (△); the continuous lines represent the results of the fit with the Weibull model.

(45% for non-irradiated, 52% for irradiated at 25 kGy and 47% for irradiated at 40 kGy) are not relevant, considering the uncertainties associated with the experimental method (±5%). As each experiment was conducted in triplicate, no statistical test was applied to quantify the significance of differences.

These results show that not only the microspheres irradiated at 25 kGy, but also those irradiated at 40 kGy have the same kinetic behavior of drug release as the non-irradiated microspheres. Since no effects due to gamma-irradiation were observed at 25 and 40 kGy, experiments were not repeated at lower doses. It is therefore possible to conclude that gamma-rays, even at a dose higher than the one recommended for sterilization, do not determine cross-linking or modification reactions in the matrix components, and that the delivery system under test is stable and the safe use of irradiated microspheres is not endangered.

Since a stable ESR signal was observed in the irradiated matrix, the long term effects of the radicals on the drug release profile was evaluated. Six months after irradiation, the release pattern perfectly overlaps that observed 24 h after irradiation.

3.3. Microsphere characterization

Fig. 2 shows the scanning photomicrographs of non-irradiated microspheres and after irradiation at 25 and 40 kGy: the microspheres irradiated were of good morphological characteristics, spherical shape and smooth surface, as the non-irradiated ones; this result shows that the irradiation, also at dose value higher than sterilization standard dose, does not cause any morphological alteration.

The histograms in Fig. 3 show dimensional distribution of non-irradiated microspheres, and those irradiated at 25 kGy; in both cases, about 85% of microspheres were in the same size range between 5 and 20 μm. Moreover, the *t*-test did not show any significant difference for each interval, between percentage content of irradiated and non-irradiated microspheres, even in the interval 10–15 μm ($P < 0.10$), where the highest difference was observed (41 and 30%, respectively). Similar results were obtained after irradiation at 40 kGy.

3.4. Thermal behavior

The non-irradiated microspheres, and those irradiated at 25 and 40 kGy, were treated in a heating–cooling cycle at 5 °C/min heating rate in the range 40–230 °C. In a previous paper [10] we described the stabilizing effects of trehalose on vancomycin in microspheres by DSC analysis and drug recovery after heating the system. In particular, we observed that the thermogram of the physical mixture of vancomycin natrosol and trehalose, in the ratio 1:1:8, resulted as a combination of the signals observed for the corresponding pure substances. The thermogram of the drug loaded

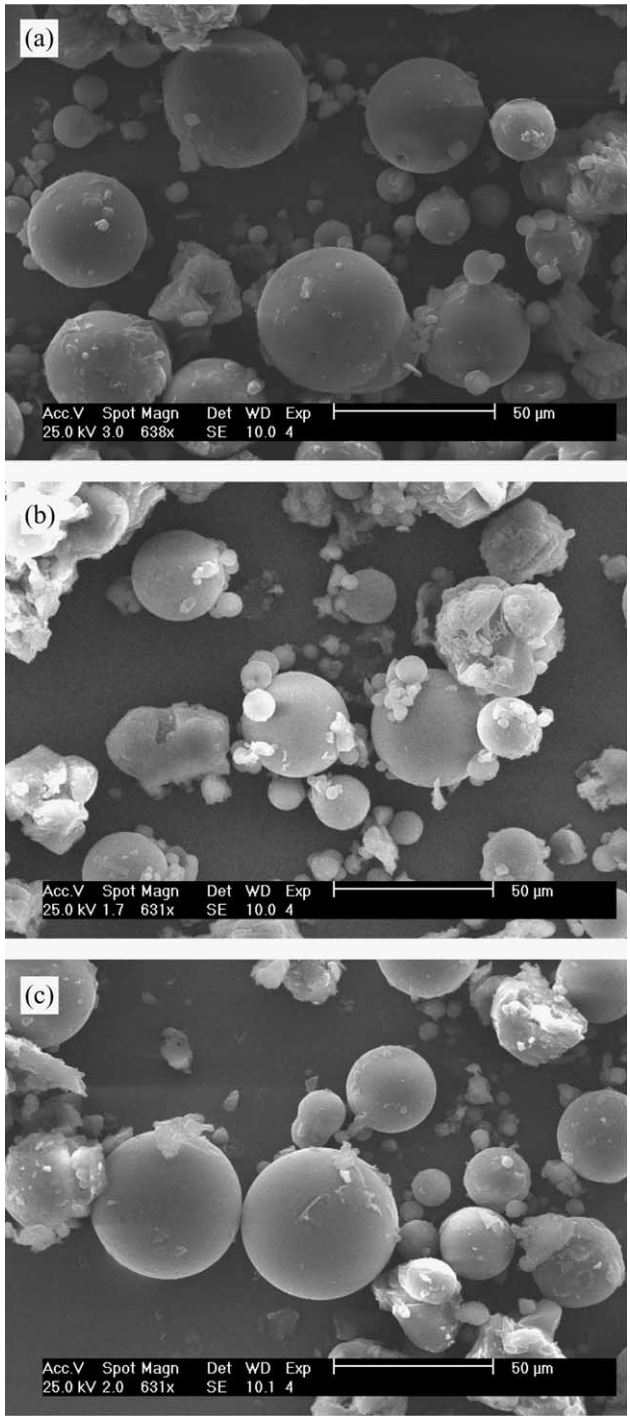

Fig. 2. Photomicrographs of microspheres containing vancomycin: (a) before irradiation; (b) after irradiation at 25 kGy; (c) after irradiation at 40 kGy.

microspheres showed only a sharp endotherm peak, centered at 90 °C, attributed to the loss of the residual water entrapped in the cellulose polymer network during the microencapsulation process. Moreover, the thermogram showed the complete amorphization of the system.

The same behavior was observed after irradiation up to 40 kGy. No peaks indicative of other transitions or other

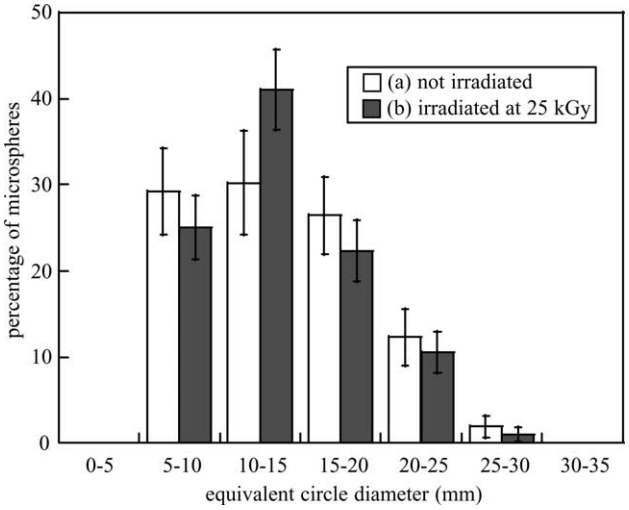

Fig. 3. Dimensional distribution of microspheres before irradiation (a), and after irradiation at 25 kGy (b).

substances unrelated to formulation components were observed in irradiated microspheres.

Fig. 4 shows the part of thermogram in which the endothermic signal appears, between 30 and 120 °C, of a sample before irradiation and after irradiation at 25 kGy. After irradiation even the amorphous status of the system was maintained. Analogous behavior was observed in the microspheres irradiated at 40 kGy.

Our results demonstrate that radiation sterilization at the standard dose of 25 kGy, and even at the dose of 40 kGy, alter neither the matrix composition in which the vancomycin is dispersed, nor the kinetics of release of vancomycin, and so do not compromise its possible therapeutic application.

3.5. Electron spin resonance studies

The ESR spectrum of microspheres containing vancomycin irradiated at 25 kGy is shown in Fig. 5; the spectrum

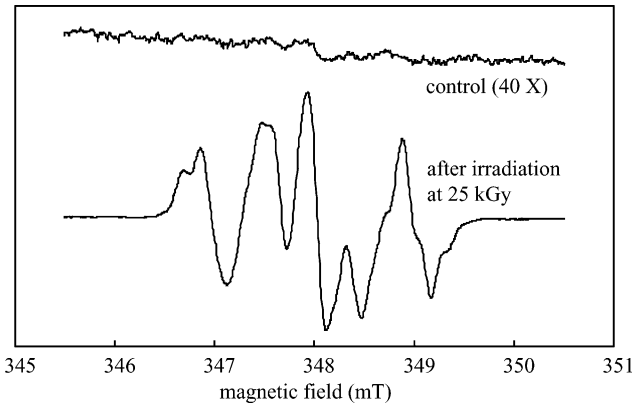

Fig. 5. ESR spectrum of microspheres before irradiation (control, magnified 40 times), and after irradiation at 25 kGy.

of non-irradiated sample (control), recorded with a receiver gain 40 times higher, is also shown for comparison.

The ESR spectrum observed in irradiated microspheres consists of several lines, indicating the presence of more than one type of free radical species induced by gamma-radiation. The same type of ESR spectrum was obtained in irradiated unloaded trehalose/hydroxyethylcellulose microspheres. This result suggests that the signal is due to more free radicals produced in matrix components; however, the precise assignment of these radicals was beyond the goal of the present research.

The signal intensity in the control sample (even though magnified 40 times) is negligible with respect to the irradiated one, the ratio between the two signal intensities being actually about 400. This result strengthens the hypothesis that ESR spectroscopy can be used as a powerful method to distinguish irradiated microspheres from non-irradiated ones. A more reliable and final confirmation can be obtained from the time stability of the ESR signal in the irradiated sample that should be at least comparable with the shelf life of the pharmaceutical product. Fig. 6 shows the signal intensity (H) as a function of storage time (t) at room temperature (15–25 °C). Best fit of the experimental results ($r^2 = 0.999$) was obtained using the function

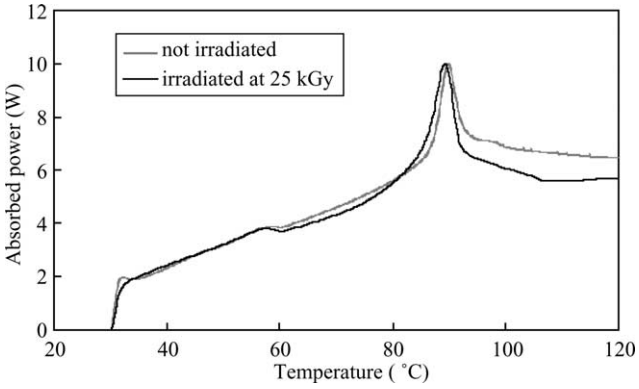

Fig. 4. Heating thermogram of microspheres before irradiation and after irradiation at 25 kGy.

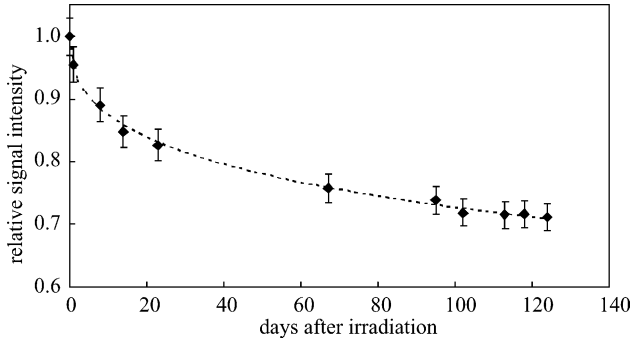

Fig. 6. ESR signal intensity of microspheres irradiated at 25 kGy as a function of time after irradiation.

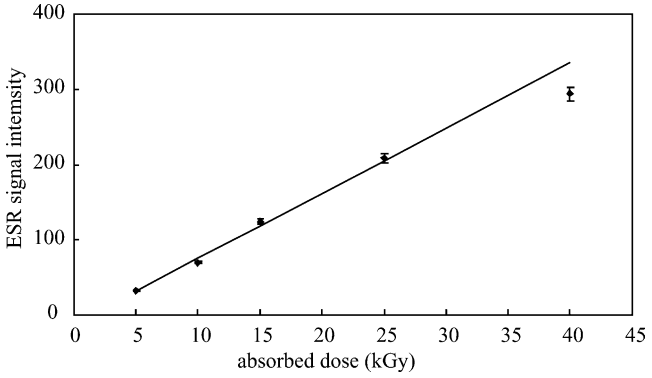

Fig. 7. Dose dependence of ESR signal intensity of irradiated microspheres.

$$H = \frac{1}{1 + at^b}$$

that describes a 'non-homogeneous' kinetics [25] (a and b are constants, optimized by the least squares fitting process). The intensity was normalized to the value measured soon after irradiation at 25 kGy.

Using the above function, the value of H was extrapolated for 5 years after irradiation, and still resulted in being about 100 times higher than in the control sample. This time interval is comparable to or even longer than the shelf life of the pharmacological product. This result is the final confirmation that ESR is a powerful technique for identification of microspheres realized with natrosol and trehalose studied in this research, and irradiated for sterilization purposes.

Similar results were obtained with samples stored at 4 °C; a faster decrease rate was instead observed in samples stored at 40 °C; nevertheless, even in this condition the signal intensity was estimated to remain well detectable 5 years after irradiation.

Samples of microspheres were irradiated at various dose values in the range of 5–40 kGy to observe the dependence of H on dose. The results (average of three samples for each dose) are shown in Fig. 7: H increases linearly with dose up to 25 kGy; for higher values, a saturation behavior appears, due to the reduced concentration of molecules, where a stable free radical has not yet been produced by radiation.

ESR spectroscopy turns out to be a good technique to detect free radicals induced by interactions with gamma-photons in the trehalose/hydroxyethylcellulose microspheres; it is consequently able to distinguish irradiated from non-irradiated products. Moreover, because of the long time stability of these radicals, ESR spectroscopy can be used to carry out controls on the sterilized micromatrix containing vancomycin, for long time intervals, even after the duration of its shelf life.

Acknowledgements

This work was financially supported by Istituto Nazionale di Fisica Nucleare and by Università di Palermo (Dottorato di Ricerca in Tecnologie delle Sostanze Biologicamente Attive). The authors are grateful to Dr P. Pierro for her technical assistance in size distribution measurements, to Dr D'Agostino for SEM analyses, and to Dr N. Tzankova for differential scanning calorimetry.

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