Gamma Sterilization of a Semi-Solid Poly(ortho ester) Designed for Controlled Drug Delivery—Validation and Radiation Effects

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Radiation sterilization is becoming increasingly popular for the sterilization of many pharmaceutical products. Although this technique is not limited to the sterilization of polymers, it is probably the most suitable method for such materials. This method however suffers several drawbacks. The sterilization of a product must lead to a safety level of 10^{-6}, i.e. one chance in a million to find a contaminated sample. In many cases, this assurance of sterility can be achieved by using a uniform treatment dose of 2.5 Mrad, recommended by the pharmacopeia. We investigated the possibility of using doses of radiation inferior to 2.5 Mrad to sterilize a semi-solid poly(ortho ester) (POE) developed for use as carrier in controlled drug delivery. After determination of the initial bioburden, the polymer was intentionally contaminated with the bioindicator *Bacillus pumilus E 601*. Following exposure to gamma irradiation, the D_{10} value of the radio resistant bioindicator was determined. Using the initial contamination value, the reduction factor D_{10} and the safety level, it is possible to calculate an optimal sterilizing dose for POE. All polymers are affected by ionizing radiation and the amount of radiation which produces a significant change in properties may vary from one polymer to the other. A molecular weight and dynamic viscosity decrease resulting from backbone cleavage was observed for this POE at a dose lower than 2.0 Mrad. Evaluation of the structure using ^{1}H-NMR, ^{13}C-NMR and IR analysis shows that for doses higher than 2.0 Mrad, another degradation process takes place. Formation of two isomeric esters of the triol used for the synthesis was identified by these methods. Cleavage of the monomer cycle is believed to be the main cause of the degradation observed. A radiation dose of not less than 7 times the D_{10} value but less than 2.0 Mrad was used for this semi-solid biodegradable poly(ortho ester) in order to ensure its sterility and avoid an excessive formation of degradation products.

KEY WORDS: poly(ortho ester); drug carrier; gamma radiation sterilization; *Bacillus pumilus* E 601; degradation; polymer characterization.

INTRODUCTION

Controlled delivery of pharmacologically active compounds from biomaterials is important for improving drug administration in the treatment of many diseases. The use of bioerodible polymers for the release of active compounds is well-established. The desirability of developing bioerodible drug delivery devices where the erosion process is confined to the polymer-water interface was at the origin of the development of the poly(ortho esters) (POE). Recently, a new semi-solid hydrophobic POE has been described (1,2). The possibility of incorporating a therapeutic agent into the polymer by a simple mixing at room temperature and without the use of solvent is of particular interest for the controlled release of peptides and thermolabile drugs. An additional advantage of the semi-solid bioerodible controlled release system is that it can be directly injected at the targeted site using a hypodermic syringe. This semi-solid polymer is currently under investigation regarding its biocompatibility (3). Controlled peptide delivery (4,5) and ophthalmic implant injections, releasing the antimetabolite 5-fluorouracil (6), have been reported recently.

These studies were carried out with sterile preparations. The only terminal sterilization method which is practicable in the case of this semi-solid POE was exposure to radiations. Conventional methods such as dry heat or autoclaving (steam sterilization) were discarded as an option due to the moisture sensitivity of the polymer and its susceptibility to degradation at elevated temperatures. Ethylene oxide sterilization also was discarded because this method may leave undesirable residues in the product despite extensive outgassing. Mutagenic and possibly carcinogenic properties due to its strong alkylating properties have also been reported (7). The two types of ionizing radiations in use are beta radiations generated through the use of high energy electron beam or gamma radiations generated from a radioisotope source. In our study, gamma radiation from a cobalt-60 source was used as a means to terminally sterilize the semi-solid POE. A minimum irradiation dose of 2.5 Mrad (25 kGy) is generally accepted as being satisfactory for sterilizing pharmaceutical products, in accordance with good manufacturing practices (8).

It is however possible to validate the use of doses lower than the usual value of 2.5 Mrad by evaluating the initial bioburden and its resistance to irradiation, and by determining the sterility assurance level (SAL). A product is generally considered sterile when not more than one surviving microorganism can be found in one million sterilized units (9).

Radiation sterilization is increasingly used for the sterilization of many pharmaceutical products. Although this technique is not limited to the sterilization of polymers, it is probably the most suitable method for such materials. The advantages of sterilization by irradiation include low chemical reactivity, low measurable residues, and the fact that there are fewer variables to control. In fact, radiation sterilization is unique in that the basis of control is essentially that of the absorbed radiation dose, which can be precisely measured. The possibility to sterilize the polymer directly in its final container, in our case a hypodermic syringe, is an important advantage of this method. However, this method is not always without problems. The interaction of high energy radiations with polymers can produce crosslinking, backbone scission, hydrogen evolution, which can all influence chemical and physical properties such as crystallinity vs. amorphous state, mechanical strength or stability (10). Polymers vary greatly in their interaction with ionizing radiation and thus the dose necessary to produce similar effects in two

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different polymers may vary from values as low as about 0.04 Mrad for polytetrafluoroethylene to 5'000 Mrad for polystyrene or polyimide (11). In our work, we used incremental doses of cobalt 60 radiation as a means to determine an optimal radiation sterilization dose and evaluate the effect of these doses on the physico-chemical properties of semi-solid POE.

MATERIALS AND METHODS

Polymer Synthesis

The synthesis of the semi-solid POE was carried out under anhydrous conditions by a transesterification reaction between trimethyl orthoacetate and 1,2,6-hexanetriol (Aldrich® Chemie, Steinheim, Germany) (12). After heating for 18 hrs in cyclohexane (Fluka® Chemie AG, Buchs, Switzerland) and subsequent cooling to room temperature, the solvent was decanted and the polymer placed in a vacuum oven to remove residual solvent. The polymer was then purified by precipitation to eliminate residual monomers and oligomers. By using two different ratios between the two precursors, two semi-solid POEs with an average molecular weight of 17'400 (17.4 kDa) and 33'300 (33.3 kDa) were obtained.

POE Bioburden Determination

Using a laminar air-flow cabinet and immediately after synthesis, each one of the two polymers were introduced into respective sealed glass bottles containing 20.0 ml of 0.85% sodium chloride solution (Fluka® Chemie AG, Buchs, Switzerland) with 0.1% peptone (Sigma® Chemie AG, Buchs, Switzerland). The 1.0 g POE samples were dissolved by agitation using a horizontal shaker (GFL®, Burgwedel, Germany) at 4°C for 4 days. After complete dissolution, 1.0 ml aliquots of each bottle were plated in triplicate without further dilution on Tripticase Soy Agar (Sigma® Chemie AG, Buchs, Switzerland), Sheep Blood Gelose 5% and Chocolate Gelose 10% (Bio-Life®, Milan, Italy) as nutrient media. The Petri dishes were then incubated for 48 hrs at 37.0°C. After incubation, the number of colony-forming units (CFU) per g of sample was calculated. The standard plate count method was used to enumerate the different organisms.

Sterility Testing with Contaminated POE

The efficacy of the radiation-sterilization process was assessed by the sterility testing of 1.0 g samples of the two POEs of different molecular weights deliberately contaminated before the irradiation with a spore suspension of the radiation resistant *Bacillus pumilus* E 601 (ATCC 27142, La 3687) purchased from the Institute of Microbiology, CHUV, Lausanne, Switzerland. After a heat shock treatment to ensure that the suspension was only composed of spores and not of vegetative cells, 100 µl containing 5.35×10^6 *Bacillus pumilus* spores per ml were placed into each bottle containing the POE samples. The inoculated polymer was then dried overnight under vacuum at 50°C. Before sterilizing the samples, the bottles were sealed with rubber stoppers and aluminium caps, and placed in a box containing dry ice.

The different sterilized polymer aliquots were then dissolved, under aseptic conditions, in 20.0 ml of the peptone salt solution. A positive control was carried out on unsterilized contaminated aliquots. After dissolution in a horizontal shaker, the resulting solution was plated directly or plated after further appropriate dilutions with the peptone salt solution, in Pepticase Soy Agar.

1.0 ml aliquots were used for the standard plate counts of the surviving *Bacillus pumilus* spores after exposure to cobalt 60 gamma radiation. All aliquots were heated at 65°C for 30 min to destroy any vegetative cells present. A 20.0 ml sample of the peptone salt solution served as a negative control. CFU determinations were performed in triplicate after 48 hrs incubation at 37°C.

Gamma Irradiation

Irradiations were performed using a 22'000 Ci activity ^{60}Co source of the Federal Research Institute, Wädenswil, Switzerland. The POE samples were gamma-irradiated at −78°C at a dose rate of 0.093 Mrad/hr. Low temperature was selected to avoid a possible degradation of the polymer during the sterilization process by a temperature increase produced by the irradiation. At this temperature the semi-solid polymer is at a glassy state (2). The doses of gamma radiation ranging from 0.1 Mrad to 4.0 Mrad were measured using a red Perspex dosimeter type 4034 BM (Harwell® Laboratory, UK). The change in absorbance of the dosimeters after exposure to gamma radiation was measured at 640 nm using a Hitachi® U 2000 spectrophotometer. All absorbance measurements were corrected for thickness variation of the dosimeters and calibrated using the Fricke dosimeters (Ferro sulphate dosimetry, Fe^{2+} . . . . . Fe^{3+}).

Molecular Weight Determination

The average molecular weights of the semi-solid POE were determined by gel permeation chromatography (GPC) using a Waters® 150 CV apparatus with three Ultrastyragel® (Waters®, Volketswil, Switzerland) columns 100, 500, 10^3 Å pore size placed in series and tetrahydrofurane (THF) (Romil® Chemicals, Leics, England) as eluent. The flow rate was set at 1.0 ml/min and the column temperature was maintained at 40°C. A viscosimeter coupled with a differential refractometer was used as detector. Samples were prepared by dissolution of the polymer in THF at a concentration of 2.0 mg/ml and 200 µl were injected each time. Polystyrene monodisperse standards were used for the calibration.

Polymer Characterization

The structure of POEs was examined by ^1H and ^{13}C-NMR. The 200 MHz ^1H and 50 MHz ^{13}C spectra of POE in a 20% (w/v) solution in $CDCl_3$ (Dr. Glaser AG, Basel, Switzerland) at room temperature was obtained with a NMR Bruker® AC-F 200 Spectrometer (Spectrospin® AG, Fällanden, Switzerland). A pulse sequence termed APT (Attach Proton Test) was used with parameters chosen so that differences between CH, CH_2 and CH_3 appear in the ^{13}C spectra.

The molecular composition determination of the polymer by analyzing the characteristic vibrations of functional groups was carried out with a 1600 series FT-IR spectrom-

eter (Perkin Elmer® AG, Küsnacht, Switzerland). Sample preparation of the semi-solid POE was achieved by coating the viscous paste directly on the surface of a sodium chloride disc.

Viscosity Determination

A Bohlin® Controlled Stress Rheometer with a cone-plate CP 4/40 or a parallel plate PU 20 device (Bohlin® Rheology GmbH, Mühlacker, West Germany) was used for measuring the viscosity. A stress viscometry test was applied to the samples which were placed on the stationary lower plate. The temperature was controlled during the test with a Bohlin® Extended Temperature Option (ETO) and the controlled torque was applied on the rotatable upper geometry using a drag-cup motor principle. Shear stresses ranging from 100 to 4500 Pa were used for the determinations. For all samples, the strain delay time was 20 s, the integration time 20 s and the measurement interval 10 s.

RESULTS AND DISCUSSION

The initial bioburden of the non-sterilized POE samples was determined using two polymers of different molecular weight. Polymers having a molecular weight up to 40 kDa can be produced by using different ratios of precursors in the synthesis (12). These polymers exhibit a very broad range of viscosities. In order to determine the influence of the physico-chemical properties and hence of the molecular weight on the microbial burden, two polymers having a molecular weight of respectively 17.4 kDa and 33.3 kDa were used. Table 1 gives the results of this bioburden. Each value represents the mean of 3 determinations. The results show that the initial number of CFUs per gram of POE is low. Different reasons can explain these results: the viscous texture of the synthesized POE is not favourable for microbial growth, and the use of high temperature (~120°C) during the synthesis is an additional factor reducing the initial contamination of the two precursors used for the synthesis. At this point of the study and in order to determine if we were in presence of radio resistant or radio sensitive organisms, it was important to identify the contaminants. The microorganisms isolated were identified as belonging to the species *Staphylococcus epidermidis* and *Streptococcus α-hemoliticus*. These organisms are likely to have originated from the manufacturing environment and are not considered to be radio resistant (13).

The amount of POE injected for the biocompatibility studies was 250 mg (200 μl) and would contain on the average 10 microorganisms since we had found an average of 40 per gram of POE.

Because of the low bioburden of the POE, the reduction factor appears to be difficult to determine. Therefore, the reduction factor was determined using samples contaminated with the radiation resistant *Bacillus pumilus*. *Bacillus pumilus* spores are a generally recognized biological indicator for monitoring an ionizing radiation sterilization process (14).

Table 2 shows that the number of survivors after irradiation decreases with the dose. Schematically, the decrease of a population of a given species (in our case *Bacillus pumilus*) after irradiation is a linear relation in a semi-logarithmic scale (fig. 1). The reduction factors D_{10} of the spores inoculated on the POE samples were extrapolated from the slopes of the curves. The D_{10} is defined as the dose producing a tenfold reduction of viable spores (i.e. a 90% destruction).

The semi-logarithmic plots of viable spores per gram of POE versus radiation dose give a slope of 0.26 Mrad/log CFU for the 17.4 kDa polymer and 0.23 Mrad/log CFU for the 33.3 kDa polymer. These results are in good agreement with the D_{10} values found in the literature (15). For the 33.3 kDa POE, a D_{10} value lower than the 17.4 kDa POE was obtained. It appears that low viscosity POE (17.4 kDa) provided a better protection of the *Bacillus pumilus* from the radiation. Whitby and Gelda (16) reported that the radiation resistance of organisms can vary and was dependent on the experimental conditions employed. It is well established that microorganisms are more resistant to the lethal effects of radiation in a dry state (17).

The same difference between the two polymers was found regarding the bioburden prior to sterilization. Differences in the texture of these two POEs were probably the

Table 1. Determination of the Initial Bioburden

Sample [kDa]	Nutrient media	No of organisms found per gram	Type of organism
POE 17.4	TSA[a]	17	Staph. epi.
POE 33.3	TSA[a]	13	Staph. epi.
POE 17.4	Chocolate Gelose	27	Staph. epi.
			Strep. α-hemo.
POE 33.3	Chocolate Gelose	20	Staph. epi.
POE 17.4	Sheep Blood Gelose	17	Staph. epi.
			Strep. α-hemo.
POE 33.3	Sheep Blood Gelose	10	Staph. epi.

[a] Tripticase Soy Agar.

Table 2. Radiation Resistance of *Bacillus pumilus* Spores Inoculated on Two Different POE Molecular Weights

Radiation dose [Mrad]	Sample [kDa]	Colony-forming units [CFU/g]	Log_{10} CFU	Percentage survival
0.00	33.3	3.90×10^5	5.59	72.90
	17.4	5.30×10^5	5.72	99.07
0.10	33.3	1.44×10^5	5.16	26.92
	17.4	2.24×10^5	5.35	41.87
0.25	33.3	2.30×10^4	4.36	4.30
	17.4	4.90×10^4	4.69	9.16
0.50	33.3	2.80×10^3	3.45	0.52
	17.4	7.20×10^3	3.86	1.35
0.75	33.3	3.20×10^2	2.51	0.06
	17.4	6.20×10^2	2.79	0.12
1.00	33.3	2.00×10^1	1.30	0.003
	17.4	8.00×10^1	1.90	0.01
1.50	33.3	0	0	0
	17.4	0	0	0
2.00	33.3	0	0	0
	17.4	0	0	0

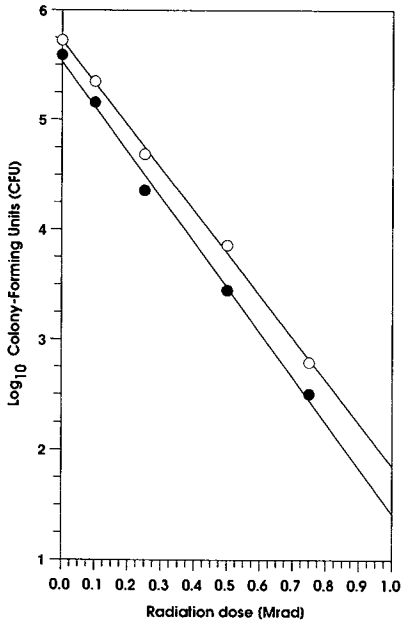

Figure 1. Semi-logarithmic plot of colony-forming units for *Bacillus pumilus* spore versus dose of radiation in megarad (Mrad). ●: POE 33.3 kDa, ○: POE 17.4 kDa.

reason why the reduction factor was not the same and why the initial contamination was more important for the 17.4 kDa POE.

A study from the Association for the Advancement of Medical Instrumentation (AAMI) shows that common microorganisms (*Staphylococcus epidermidis, Streptococcus α-hemoliticus* etc . . .) exhibit a low radio resistance, characterized by a D_{10} lower or equal to 0.10 Mrad (1.0 kGy) for 65.48% and lower or equal to 0.28 Mrad (2.8 kGy) for 98.65% of them (18). Therefore, by using the bioindicator reduction factor (D_{10}) and by knowing the POE bioburden (No) it is possible to calculate the sterilizing dose (D.S.) with the following formula (9):

$$D.S. = D_{10} (\text{Log No} - \text{Log SAL})$$

In order to ensure a sufficient margin of safety, a SAL of 10^{-6} was used. The D.S. calculated were of 1.82 for the 17.4 kDa polymer and 1.61 for the 33.3 kDa polymer respectively. For the two determinations, an initial bioburden of No = 10 was used.

These results show that the usually recommended 2.5 Mrad dose was totally satisfactory for the sterilization of this injectable POE. The linearity of the relationship between CFU and gamma radiation doses for the contaminated POEs over as many as 4 log units ensures the reliability of extrapolations to values of CFU beyond the measurable range. Extrapolation indicates that a dose of 2.5 Mrad achieves a probability of occurrence of a non-sterile sample in the population of 10^{-9} (SAL = 10^{-9}), a clear case of over-kill when considering the pharmacopeial criterion of sterility (19).

To study the influence of gamma-irradiation upon the physico-chemical properties of the POEs, a broad range of irradiation doses was employed. The doses used ranged from 0.5 to 4.0 Mrad. A dose-dependent decrease in the average

Table 3. Effect of Gamma-Sterilization on the Molecular Weight (M_W) and the Dynamic Viscosity of Two Different Molecular Weight POEs

	POE 33.3 kDa		POE 17.4 kDa	
Dose [Mrad]	M_w [kDa]	Viscosity [Pa.s]	M_w [kDa]	Viscosity [Pa.s]
0.5	21.6	7082.52	10.3	804.47
1.0	18.6	2212.78	9.5	579.43
1.5	15.3	919.59	8.5	445.92
2.0	11.9	508.98	8.4	321.25
2.5	11.0	294.50	7.3	146.44
3.0	7.9	254.19	5.7	94.82
3.5	6.6	294.41	6.2	313.26
4.0	6.3	251.44	5.9	170.49

molecular weight and in the dynamic viscosity of the polymer was observed. These results (Table 3) can be explained by the fact that irradiation of polymers can produce a complex cascade of events such as electron ejection, excited state formation and finally C – C scission (20). For polymers with a linear backbone, which is scissioned at random, the final number average molecular weight (M_n) per gram can be determined using the modified formula (10):

$$1/M_n = 1/M_{(n)o} + 1.04 \cdot 10^{-6} G \cdot \tau$$

where $M_{(n)o}$ is the initial number average molecular weight, G the average number of reactions (scissions) per eV of absorbed energy and τ the dose in Mrad. Plots of $1/M_n$ versus τ give a straight line with a slope of $1.04 \cdot 10^{-6}$ G, so that the yield of scissions per unit dose can be easily calculated (fig. 2). The G values were respectively of 73 with a correlation coefficient of r = 0.988 for the 33.3 kDa POE and 48 with r = 0.941 for the 17.4 kDa POE. These results give a good indication of the sensitivity of this semi-solid polymer to gamma radiation.

Figure 3 shows that the high POE molecular weight is more sensitive to gamma radiations. An important decrease is already observed for small doses such as 0.5 Mrad. The molecular weight decreases from 33.3 kDa to 21.6 kDa for the 33.3 kDa POE. For the same dose, a decrease of 7 kDa is observed for the 17.4 kDa POE. These results are in good agreement with the literature (10).

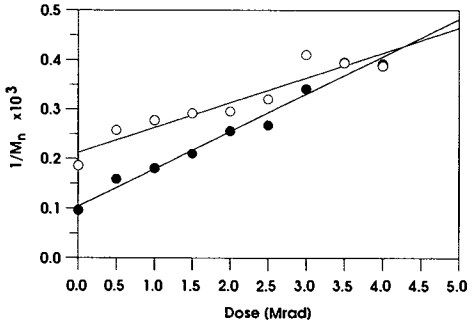

Figure 2. Reduction of the number average molecular weight (M_n) in function of the radiation dose in megarad (Mrad). ●: POE 33.3 kDa, ○: POE 17.4 kDa.

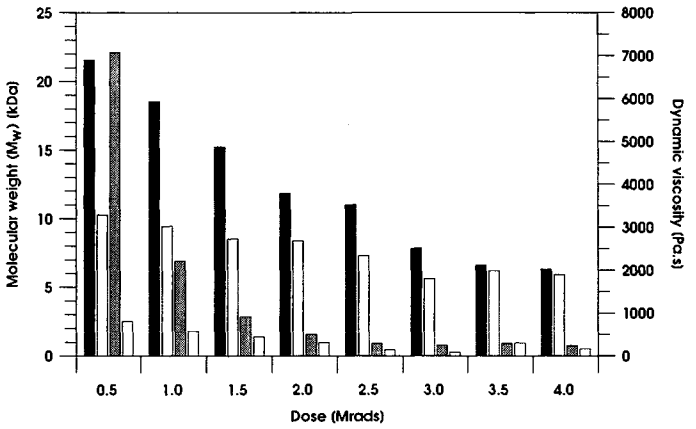

Figure 3. Influence of the gamma-radiation on the average molecular weight (M_w) and the dynamic viscosity of two different molecular weight POE. ■: molecular weight of the 33.3 kDa POE, □: molecular weight of the 17.4 kDa POE, ▨: dynamic viscosity of the 33.3 kDa POE, □: dynamic viscosity of the 17.4 kDa POE.

The decrease of the POE viscosity resulting from the backbone scission is not to be underestimated in the case of a polymer used as a drug release system. In a previous work (12) we have described the influence of the change in POE viscosity on the 5-FU release. It was therefore important to take in consideration these changes before synthesizing a POE which is to release a therapeutic agent over a determined period of time.

A structural change of the polymer, produced by the use of an incremental radiation dose, can be observed on the FT-IR spectra (fig. 4). The characteristic broad vibration of the stretching O – H (3200–3650 cm^{-1}) of the hydroxyl group increases as a function of the dose used. This phenomenon can be explained by the fact that the proportion of hydroxyl groups of the terminal chain increases proportionally, after rearrangement, with the number of scissions. At the value of 2.0 Mrad, we can note an increase of the peak at 1750 cm^{-1}. This wave number is characteristic of the IR absorption band corresponding to the carbonyl group of an ester. The hydrolysis of the POE was previously described as the formation during the first step of two isomeric esters of the triol followed by a slower reaction leading to the formation of the original hexanetriol and a carboxylic acid (5). Despite the

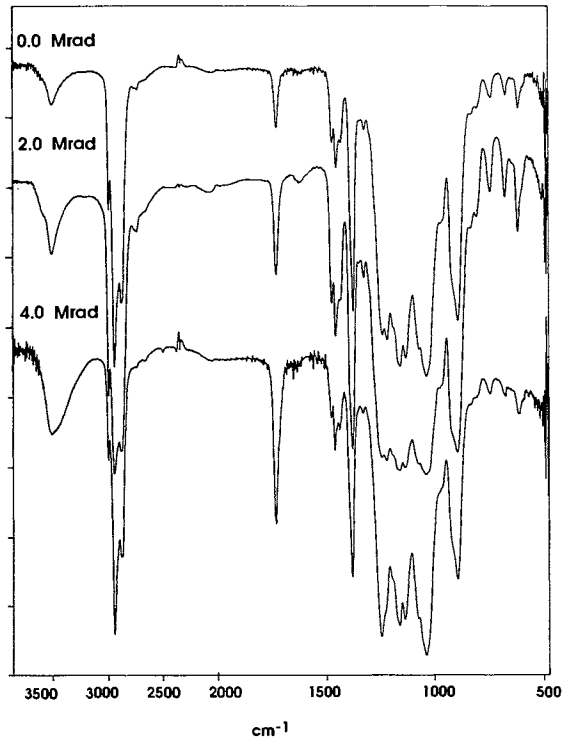

Figure 4. IR spectra of the 33.3 kDa POE non-irradiated and irradiated at 2.0 and 4.0 megarad (Mrad).

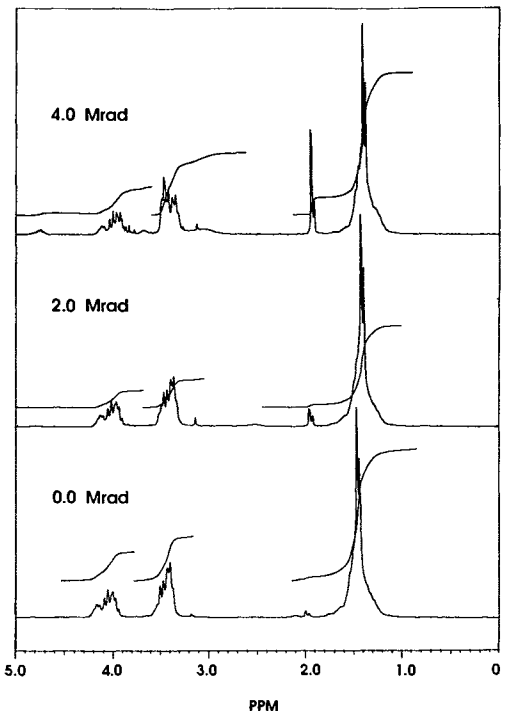

Figure 5. Proton NMR spectra of the 33.3 kDa POE non-irradiated and irradiated at 2.0 and 4.0 megarad (Mrad).

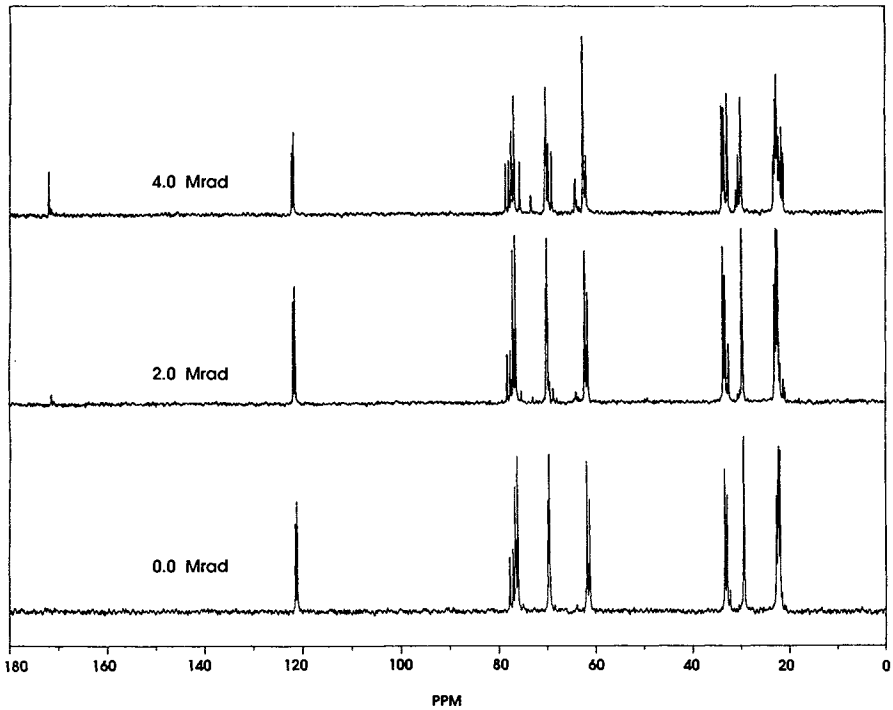

Figure 6. Carbon NMR spectra of the 33.3 kDa POE non-irradiated and irradiated at 2.0 and 4.0 megarad (Mrad).

absence of water during the gamma irradiation (the bottles containing 1.0 g of POE were sealed under anhydrous conditions using argon), we suppose that a similar degradation mechanism leading to the formation of these two isomeric esters takes place.

This assumption is confirmed by the proton and carbon nuclear magnetic resonance analysis of the irradiated POE. As shown in the ^{1}H-NMR spectra (fig. 5), we can see the appearance of a multiplet at 2.0 ppm which corresponds to the carboxylic ester chemical shift. The absence of the COO−H chemical shift at ~12 ppm of the carboxylic acid confirms that the degradation products are very likely the two intermediate isomeric esters of the triol and not the final carboxylic acid. A change in the multiplicity of peaks in the 3.5 ppm area is also noted and this is characteristic of the carboxylic ester chemical shift. Finally, confirmation of the presence of an isomeric ester of the triol resulting from the cycle opening is given by the ^{13}C-NMR spectra.

Figure 6 shows for doses higher or equal to 2.0 Mrad, the appearance of a little peak at 171 ppm corresponding to the carbonyl shift of the above described carboxylic ester. Changes in the 20 and 80 ppm area are also noted but the interpretation of these changes cannot be easily given due to the presence of a mixture of the polymer with its degradation products. Different chemical changes are produced by exposure to radiations, the nature of these changes is largely determined by the chemical structure of the polymer and the dose used. In our case, two different and additional mechanisms depending of the dose can be proposed. For doses lower than 2.0 Mrad the degradation mechanism is governed by the backbone scission of the polymeric chain. This mechanism leads to a fast average molecular weight decrease and consequently to a decrease of the dynamic viscosity. For doses higher than 2.0 Mrad, an additional degradation mechanism takes place. Cycle opening of the monomer by cleavage of the ortho ester bonds is certainly the governing additional degradation mechanism.

CONCLUSION

On the basis of these observations, we conclude that in the case of a low initial bioburden of the POE, the dose required to achieve a product SAL of 10^{-6} may be less than 2.5 Mrad. It was however important to know the D-values of each organism composing this bioburden. The results show that environmental microorganisms were the main source of contamination of POE, and that their radiation resistance and consequently their D-values were low. The use of *Bacillus pumilus* as a radiation resistant bioindicator gave excellent informations to validate a sterilization procedure.

The polymer tested is affected by ionizing radiations. The amount required to produce significant changes in properties may vary from doses as low as 2.0 Mrad to 4.0 Mrad. In our case, by using doses between 1.5 and 2.0 Mrad, and depending of the POE molecular weight, it was possible to obtain a sterile polymer and to limit the degradation of this semi-solid by reducing backbone scission. For these lower doses, the presumed formation of the two isomeric esters of the triol can be avoided. The principal changes are characterized by a decrease of the average molecular weight and consecutively a decrease of the dynamic viscosity.

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