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Gamma irradiation for terminal sterilization of 17β-estradiol loaded poly-(D,L-lactide-co-glycolide) microparticles

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Abstract

17β-Estradiol-loaded microparticles using poly-(D,L-lactide-co-glycolide) polymer (PLG) were prepared by a modified spray-drying method and the effects of γ-irradiation on drug substance, polymer and microparticles were investigated. Irradiation doses ranging from 5.1 to 26.6 kGy were applied using a ^{60}Co-radiation source. 17β-Estradiol drug substance showed excellent stability against γ-irradiation in the investigated dose range, whereas microencapsulated estradiol seems to be converted to conjugation products with PLG, and to a lesser extent to the degradation product 9,11-dehydroestradiol. The weight-average molecular weight of the PLG polymers decreased with increasing irradiation dose while polydispersity indices (M_w/M_n) remained nearly unchanged, compatible with a random chain scission mechanism in lactide/glycolide-copolymer degradation. In vitro drug release studies showed accelerated kinetics with increasing irradiation doses due to dose dependent polymer degradation. Microbiological process monitoring showed decreasing bioburden with increasing spraying time, which was successfully further reduced by applying irradiation sterilization. Microencapsulated test spore suspensions of *Bacillus pumilus* ATCC 27142, the official test specimen for the γ-sterilization process, revealed effective reduction of bioburden, confirming its published D_{10} value. In conclusion, our studies demonstrated efficacy of γ-irradiation as terminal sterilization method for poly-(D,L-lactide-co-glycolide) polymer-based drug delivery systems. The sterilization conditions need to be carefully adjusted for the final dosage form. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Parenteral drug delivery systems based on poly-(D,L-lactide-co-glycolide) polymers (PLGs), were intensively investigated in the last decades covering a wide variety of different drug substances.

While the modulation of release properties was achieved by selection of appropriate polymers and microencapsulation techniques, suitable sterilization methods remain a challenge for these parenteral depot systems. Since PLG-based drug delivery systems are very sensitive to dry or moist heat, and ethylene oxide is not applicable due to its toxic residues, γ-irradiation currently remains the only accepted method for terminal sterilization [1]. The potential of new techniques, e.g., low-temperature plasma sterilization is still under evaluation [2].

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Furthermore, terminal sterilization commonly leads to a higher sterility assurance level compared to aseptic processing which is of substantial interest for parenteral dosage form design [3,4].

The effects of γ-irradiation on the physico–chemical properties of biodegradable drug delivery systems are described controversially in the literature. It is now generally accepted that γ-irradiation causes a radiation dose dependent decrease in polymer molecular mass by radiolytic chain scission [5–12], and consequently an accelerated onset of polymer mass loss [5,8,9]. The question whether this polymer chain cleavage occurs randomly or preferentially at end-groups remains controversial, although results from EPR, electron paramagnetic resonance spectroscopy experiments clearly suggest a random chain cleavage only slightly affecting polymer dispersity [9].

These radiation effects on the biodegradable polyesters, namely the formation of reactive radicals, may also compromise the drug substance incorporated into the device. The drug stability under irradiation conditions and changes in drug release properties need to be carefully evaluated. The distribution of the drug in the biodegradable device can influence the radiolytic stability [5]. The drug release kinetics from these biodegradable drug delivery systems showed positive [5,9–13] or negative [5,14,15] deviations from unirradiated controls, depending on matrix type and irradiation dose.

We report here the effects of γ-irradiation on 17β-estradiol (E2), a fairly stable steroid hormone [16,17], after microencapsulation into a PLG-50/50 copolymer, typically forming a solid solution matrix. Microparticle formation was achieved using a pilot production plant spray dryer. The irradiation dose related changes in polymer molecular mass, polymer dispersity, drug stability and drug release properties were investigated. Since there are only scarce data available in the literature with respect to changes in bioburden due to processing conditions and sterilization method, we monitored bioburden of starting materials and spray-dried products before and after γ-irradiation, to assess the need of limiting bioburden of starting materials and equipment. Finally, the D_{10} value [3,18] of *Bacillus pumilus*, the official test microorganism for irradiation sterilization, microencapsulated into a PLG copolymer, was determined.

2. Materials and methods

2.1. Materials

Poly-(D,L-lactide-co-glycolide)-50/50, Resomer RG 503, molecular mass M_w (weight average) 34 000, and poly-(D,L-lactide-co-glycolide)-75/25, Resomer RG 752, M_w 17 000 were purchased from Boehringer Ingelheim (Ingelheim, Germany). The active principle 17β-estradiol-semihydrate (E2) was obtained from Diosynth (Oss, The Netherlands). Degradation products used as standards for examination by high-performance liquid chromatography (HPLC) were purchased from the following sources: 17-estradiol, Aldrich (Steinheim, Germany), Eetrone (E1), Diosynth, 9,11-dehydroestradiol (DHE), Schwarz Pharma (Monheim, Germany). Ethanolic test spore suspensions of *Bacillus pumilus* ATCC 27142, bioburden: 10^9, 10^7 and 10^5 CFU (colony forming units)/ml were provided by BioChem (Karlsruhe, Germany). Polyvinylalcohol Mowiol 18-88 used for spore encapsulation was from Hoechst (Frankfurt/Main, Germany). Gel permeation chromatographic calibration was performed using narrow distributed (ND)-polystyrene standards in a molecular mass range from M_w 5900 to 96 400, Waters/Tosoh (Eschborn, Germany).

All other reagents and solvents used were of analytical purity.

2.2. Preparation of the E2 microparticles

Preparation of the E2 microparticles was carried out using a spray-drying technique (NIRO Minor Atomizer, rotary atomizer, NIRO AS, Copenhagen, Denmark). In a typical experiment polymer and E2 were dissolved separately, polymer in 11.4% (w/v) dichloromethane (DCM), and estradiol in acetone according to the desired drug loading. The clear solutions were combined forming a mixture of DCM–acetone (7:3), and a final polymer concentration of 8% (w/v), respectively. The process parameters were set as follows: rotary nozzle speed: 35 000 rpm/min (pressure: 5 bar), aspirator setting: ST1/M, product feed rate: 20 ml/min, inlet temperature: 60°C, outlet temperature: 40°C. Batch size was ca. 100 g. The microparticles were collected from the

spray-drier cyclone, dried under vacuum (20 mbar) at room temperature for 48 h, and stored at 4–8°C over a desiccant.

2.3. Preparation of test spore microparticles

In order to reduce the contamination risk, microencapsulation of the test specimen was performed using a simple O/W-solvent–evaporation process under aseptic-conditions (LAF). All glass and plastic containers were sterilized prior to use and thereafter, using steam at 121°C, 2 bar, for 15 min. Bioburdens of the final microparticles were adjusted to 10^8, 10^6 and 10^4 CFU per gram, respectively. Therefore, to 5 g of PLG polymer (RG503) dissolved in 30 ml DCM a volume of 0.5 ml of the respective ethanolic spore suspension (10^9, 10^7 and 10^5 CFU/ml) was added and homogenized in an ultrasonic water bath for 2 min. The resulting suspensions were then added to 300 ml of a 0.5% (w/v) water-based poly(vinyl alcohol) (PVA) solution that was stirred (500 rpm/min) on a magnetic stirrer from Janke and Kunkel (Staufen, Germany).

The microspheres were stirred overnight to complete solvent evaporation, then collected on a nylon gauze sieve, pore size 400 mesh (37 μm) Spex (Ottobrunn, Germany) and washed three times with 50 ml sterile water. The microparticles were dried in vacuo (20 mbar) at room temperature for 48 h. Each batch was placed separately into vials which were sealed under argon atmosphere and stored at 4–8°C until use.

2.4. γ-Irradiation

E2 drug substance, E2/PLG microparticles and *Bacillus pumilus* microparticles were placed in vials, sealed under an argon atmosphere as described above and were irradiated using a ^{60}Co-radiation source, Fa. Willi Rüsch (Waiblingen, Germany). The actual radiation doses were 5.1 kGy, 15.2 kGy and 26.6 kGy, respectively (26.0 kGy and 34.1 kGy in the case of RG752). To prevent thermal effects, the γ-irradiation was carried out at a defined temperature using dry ice (−78.5°C).

2.5. Analytical methods

To quantitate E2 and its degradation products we used a reversed-phase (RP) HLPC method on a Millipore–Waters system (Eschborn, Germany), equipped with the following components: a WISP 717plus autosampler, double piston pump Model 510 operated at a flow-rate of 2 ml/min, column oven CHM with temperature controller TCM. E2 content was determined using a Waters UV detector Model 481. The degradation products were detected by a Model LC-240 fluorescence detector, Perkin-Elmer (Langen, Germany) operating in-line with the UV detector. Data collection and evaluation were performed on a MicroVax-3100 data-system with Waters Expert Ease 860/V2.3 software. A Merck LiChrospher 100 RP18 column (5 μm, L=250 mm) was used for separation, thermostatted at 35°C. The mobile phase consisted of a mixture of acetonitrile–water (55:45, v/v). The limit of detection for estradiol was 0.025 μg/ml.

2.6. E2 content in biodegradable microparticles

To determine the content of E2 and E2 degradation products 10–20 mg microparticles, accurately weighed, were dissolved in 1 or 2 ml acetonitrile and then subsequently diluted to 100.0 ml or 200.0 ml, respectively, using a mixture of methanol–water (1:1, v/v). Suspensions were shaken for 1 h at 200 rpm/min. The precipitated polymer was separated by filtration through a 0.45-μm Millex-HV filter. The clear filtrate was assayed spectroscopically for E2 at 280 nm (UV), and for degradation products with the fluorescence detector.

Evaluation of estradiol/polymer conjugates was carried out in triplicate using a hydrolytic degradation method. For comparison an unirradiated control of the same batch was used, and a E2-spiked placebo polymer sample treated the same way. To an amount of ca. 20 mg microparticles or polymer 80 ml of 1 M methanolic potassium hydroxide was added. The suspension was shaken (10 rpm/min) for 72 h in a water bath thermostatted at 37°C. An aliquot of the clear hydrolysate was neutralized with 25% hydrochloric acid and diluted to 200.0 ml with

a mixture of methanol–water (1:1), and filtered in HPLC vials using a 0.45-μm Millex-HV filter. UV detection of E2 was at 280 nm.

2.7. In vitro drug release studies

In vitro drug release studies were conducted in a rotating bottle apparatus (NF XIII) consisting of 80-ml screw capped glass bottles rotated at 10 rpm in a water bath at 37°C. Ten or 20 mg microparticles ($n=3$ for each batch) depending on drug loading were investigated. As release medium, phosphate-buffered saline (PBS), pH 7.4 was used, containing 0.05% (w/v) sodium azide to prevent microbiological contamination and 0.05% (w/v) Pluronic F68 as a wetting agent. The buffer was completely replaced three times on the first day to provide sink conditions and daily thereafter. The E2 content was determined directly in the samples using the RP-HPLC method described above.

2.8. Gel permeation chromatography (GPC)

Polymer molecular masses and molecular mass distribution were determined on a Waters GPC system (Eschborn, Germany) equipped with: a WISP 712 autosampler, double piston pump Model 590 operated at a flow-rate of 1 ml/min (tetrahydrofuran, THF), column oven CHM, refractive-index (RI) detector Model 410 with integrated temperature controller TCM maintained at 40°C. Data collection and evaluation were performed on a MicroVax 3100 data system with Waters Expert Ease 860/V2.3, GPC module software. For molecular mass determination three Waters Ultrastyrogel columns (pore sizes: 500 Å, 10^3 Å and 10^4 Å) were used in-line at 35°C. A universal calibration was carried out based on ND (narrow distributed)-polystyrene standards of the following molecular masses, M_w: 5900; 9100; 18 100; 37 900 and 96 400. The mobile phase was THF. Polymer and microparticle samples were dissolved in an appropriate solvent (i.e., THF, $CHCl_3$) to form a clear solution (1%, w/v). Chromatography was carried out after sample dilution with THF (1:1), and filtration through a 0.45-μm Millex-HV filter.

2.9. Differential scanning calorimetry (DSC)

Glass transition temperatures (T_g) of polymers and microparticles were measured with a DSC-7 differential scanning calorimeter, Perkin-Elmer. Samples (5–10 mg) were sealed in aluminum pans and heated under nitrogen atmosphere two times at 10 K/min from 0°C to 200°C. All reported thermograms were analyzed using the second measurement. Data evaluation was performed with the proprietary Perkin-Elmer Thermal Analysis Software, Version 2.0. Calibration was carried out using indium (T_m: 156.6°C) and zinc (T_m: 419.6°C).

2.10. Scanning electron microscopy (SEM)

Microparticle size and morphology were investigated after sputter coating with gold in an argon atmosphere (2.4 kV, 20 mA, 180 s, Polaron-Sputter-Coater, Polaron Instruments, Watford, UK). The samples were analyzed using a scanning electron microscope, type: SEM 505, Philipps (Eindhoven, The Netherlands).

2.11. Particle size measurement of microspheres

Particle size and size distribution of microparticles were determined by laser diffractometry using a Malvern Master Sizer MS20, Malvern (Herrsching, Germany), equipped with an MS15 dispersion sample unit. Microparticle samples were dispersed in water containing 0.5% Tween 80 using an ultrasonic bath (5 min). The measurements were carried out in triplicate with a 45-mm lens covering a size range of 0.1 to 80 μm. Results are expressed as mean volume diameter D (v, 0.5).

2.12. Determination of bioburden in microspheres

One g of either γ-irradiated or non-irradiated microparticle samples was transferred under aseptic conditions to 9 ml of sterile peptone solution, pH 7.0 and homogenized for 30 min by shaking (200 rpm/min). From these homogenates further dilutions in a ratio 1:10 were prepared using sterile isotonic sodium chloride solution. To detect aerobic bacteria,

yeasts and molds, 0.1 ml of these suspensions were transferred to soya peptone agar containing 6% defibrillated sheep blood and to sabouraud agar for yeasts and moulds, respectively. Blood agar plates were incubated at 30–35°C for a maximum of five days, sabouraud agar plates at 20–25°C for the same period. Colonies grown after incubation were counted, and total bioburden was determined using the corresponding dilution factor. The limit of detection was 10^2 CFU/g.

The rest of the above mentioned 10% homogenates were transferred to 200 ml casein soya peptone bouillon and incubated for a further 48 h at 30–35°C. From these bouillons, subcultures were prepared on casein soya peptone agar containing 6% defibrillated sheep blood which were incubated for another 48 h at 30–35°C.

Colonies grown either on subculture agar plates or in bouillons were isolated, counted and identified with commercially available Api identification kits, BioMèrieux (Nürtingen, Germany). The limit of detection was 10^1 CFU/g.

To determine germicide property of dichloromethane, a spore suspension of *Bacillus pumilus* ATCC 27142 in dichloromethane was prepared with an initial bioburden of $1.0 \cdot 10^5$ CFU/ml. The suspension was incubated for 24 h at 25°C. After 1 h, 6 h and 24 h samples (1 ml) were withdrawn to perform a determination of bioburden as described above.

3. Results and discussion

3.1. Characterization of polymers and estradiol (E2) microparticles

The characteristics of E2 microparticles prepared by a modified spray-drying technique, a technology commonly used for microencapsulation [1,5,9,19–22], are summarized in Table 1 together with the respective polymer properties. For our investigations we used two different PLGs with different monomer composition and molecular masses. Moreover, the microparticles were prepared with different E2 loading levels. Detailed DSC studies conducted with the PLG polymers and E2 as drug substance revealed that up to 20% (w/w) drug loading the microspheres form a solid solution morphology (data not shown). The glass transition temperature T_g of PLG polymers and E2 microspheres are shown in Table 1, supporting the molecular dispersion of E2 in the PLG matrix up to 18.4% loading. The high product yield of more than 90% is a result of the pilot plant spray-dryer design and demonstrates impressively the advantage of the pilot plant equipment in comparison to laboratory-scale spray-dryers [5,9,19,22]. The high microencapsulation efficiency of ca. 90% is the consequence the one-step process [20,21] with little risk of potential drug loss. The drug substance 17β-estradiol was found to be relatively stable in terms of

Table 1
Polymer and 17β-estradiol (E2) microparticle characteristics

Batch	Polymer	Physical form	Actual drug load (%)	Loading efficiency (%)	Yield (%)	Particle size[a] (μm)	M_w [b] (Da)	M_n [b] (Da)	M_w/M_n	T_g [c] (°C)
211794 (BI)	RG 503	Starting material	–	–	–	–	33.700	16.500	2.03	49.2
NI-Y-00	RG 503	Placebo – MP	0	–	91.5	26±8	33.300	16.500	2.01	42.8
NI-Y-01	RG 503	MP	7.92	87.2	89.3	31±5	34.800	18.600	1.87	32.1
NI-Y-02	RG 503	MP	18.40	89.9	93.6	28±9	35.200	20.100	1.75	27.7
10705 (BI)	RG 752	Starting material	–	–	–	–	17.300	12.200	1.42	47.5
NI-Y-03	RG 752	MP	18.41	92.1	91.7	22±10	17.900	12.900	1.39	31.5
NI-Y-04	RG 752	MP	44.42	88.8	92.4	18±9	17.600	12.600	1.40	28.2

[a] By laser diffraction.
[b] Molecular mass by GPC.
[c] Glass transition temperature by DSC.
BI = Boehringer Ingelheim = polymer supplier.
MP = Microparticles.

detectable degradation products during microencapsulation.

Fig. 1a and c shows the typical morphology of a spray-dried product, yielding regular shaped, discrete spheres with a smooth surface texture. The particle sizes derived from laser diffraction measurements given in Table 1 are in same order of magnitude as the corresponding SEM micrographs, and demonstrate the influence of polymer molecular mass on solution viscosity and resulting particle size, since polymer concentrations in the sprayed solutions were the same for both polymers despite all other process parameters.

We noted slight influence of the spray-drying process on the polymer molecular mass as shown in Table 1. In particular, number-average molecular weight was affected in terms of a slight increase whereas weight-average molecular weight remains nearly unchanged. As observed in former studies [5] this seems to be due to the extraction of low-molecular-mass components during processing.

3.2. Effect of γ-irradiation on PLG polymer

Since standard conditions for γ-sterilization have not been generally accepted and previous studies [5,23–26] had shown a certain influence of humidity and oxygen, environmental conditions during irradiation had to be controlled carefully to reduce the radiolytic effects on the drug delivery systems. Therefore, we carried out the γ-irradiation of polymers and microparticles on dry ice (−78.5°C), after sealing the vials in an argon atmosphere. Irradiation doses ranging from 5.1 kGy up to 26.6 kGy were

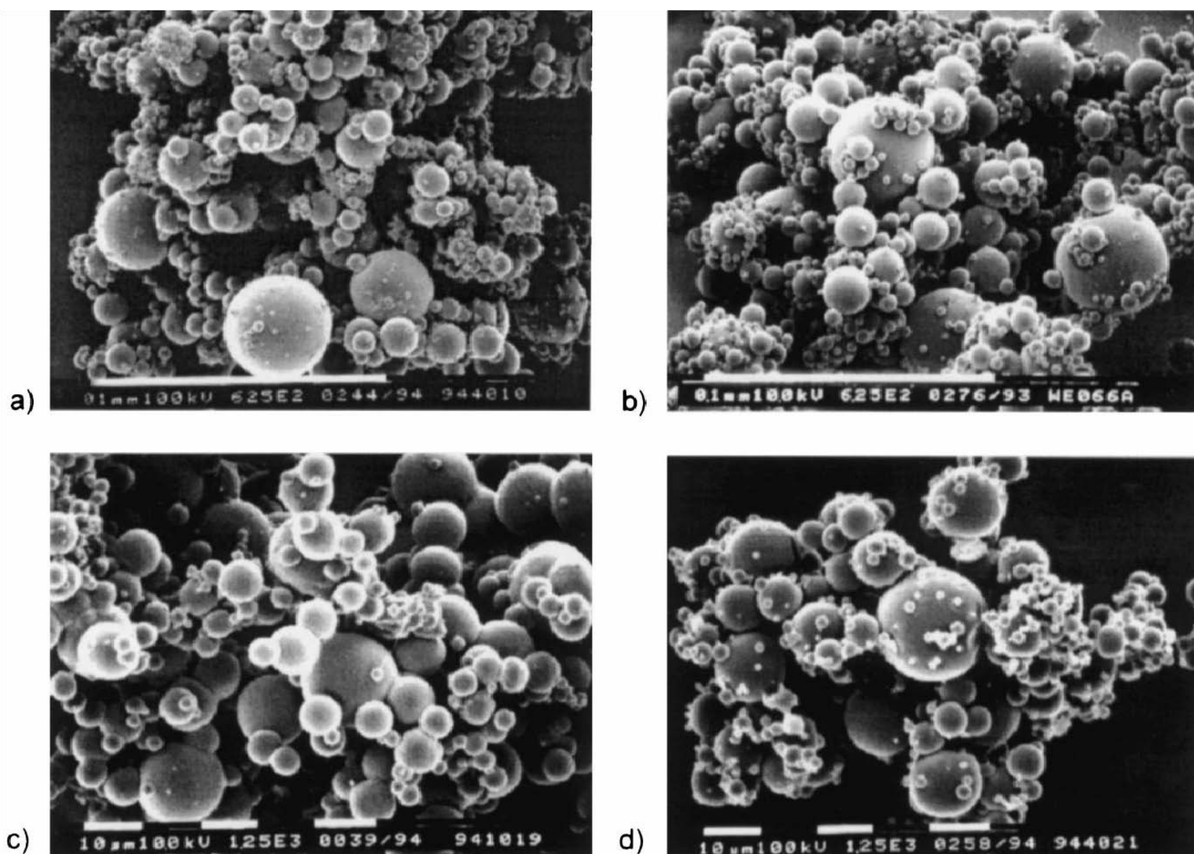

Fig. 1. SEM micrographs of 17β-estradiol/PLG microparticles before and after γ-irradiation. (a) NI-Y-02 RG503/E_2 18% – 0 kGy, (b) ditto 26.6 kGy (size bar = 100 μm), (c) NI-Y-04 RG752/E_2 44% – 0 kGy, (d) ditto 26.6 kGy (size bar = 10 μm).

applied using a ^{60}Co-source, covering the defined overkill standard dose of 25 kGy [3,4,18]. Fig. 1b and d show two representative E2 microparticle samples after γ-irradiation. Obviously, the surface morphology of microspheres remains unchanged by γ-irradiation, as already described by other investigators [5,8–10].

In Fig. 2 the relative changes in molecular mass and polydispersity for the PLG-50/50 polymer are shown as a function of γ-irradiation dose. The polydispersity of PLG, placebo MP and E2 MP remain unchanged (hollow symbols). Independent from drug loading we detected a linear decrease in weight-average molecular weight with increasing irradiation dose without significant changes in polydispersity. In the range of the standard overkill dose of 25 kGy a relative loss in M_w for all drug loadings of about 15% was found. Volland et al. [5] reported similar results with ca. 8% reduction for the same polymer. The slight difference in molecular mass decrease was attributed to the greater stability of 17β-estradiol upon irradiation (less energy absorption), resulting in a higher energy exposition for the polymer matrix.

Fig. 3 shows the corresponding results for the PLG-75/25 polymer. Again, a linear decrease in weight-average molecular weight was found (8% at 25 kGy), while polydispersity remained nearly unchanged. This lower degree in molecular mass decrease for PLG-75/25 could be explained with the cage effect postulated by Chu and Campbell [7], taking into consideration the better opportunities of stabilization for primary formed radicals from higher-molecular-mass polymers as seen with PLG-50/50 in contrast to a low-molecular-mass polymer, which tend to recombination as the preferred reaction pathway.

The effects of γ-irradiation on the PLG polymer are affected both by molecular mass and the distribution of the drug in the polymeric matrix. The chain cleavage mechanism of lactide-glycolide polymers in our study are in agreement with a random

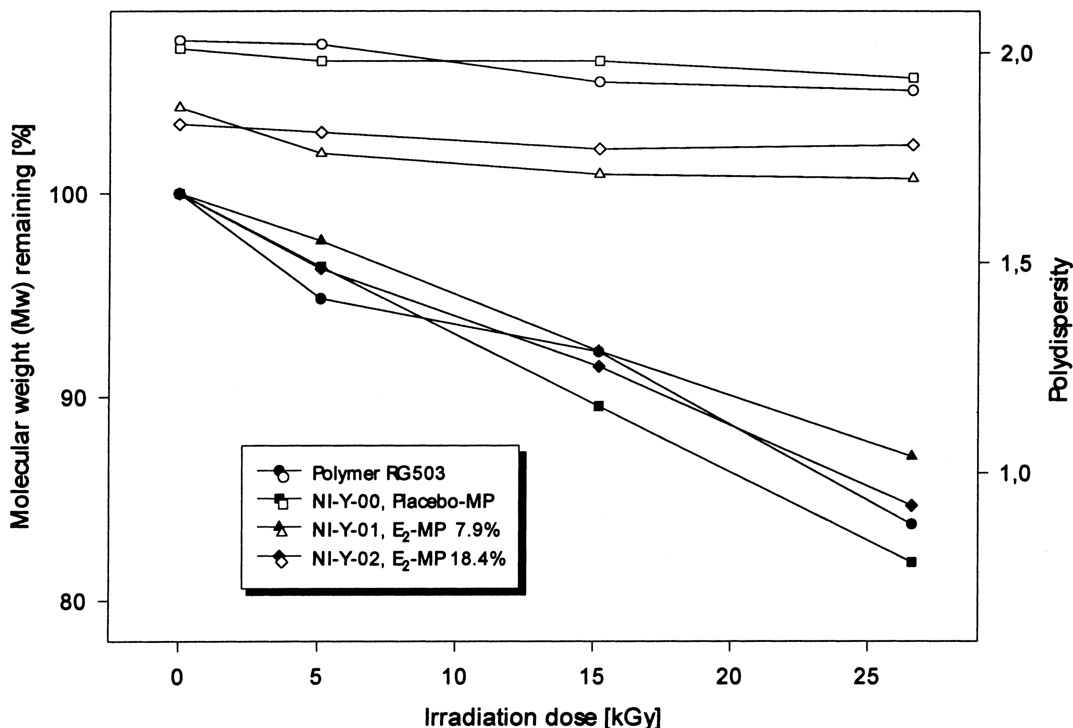

Fig. 2. Changes in polymer molecular mass (filled symbols) and polydispersity (hollow symbols) as a function of irradiation dose. Polymer: PLG-50/50 (Resomer RG503).

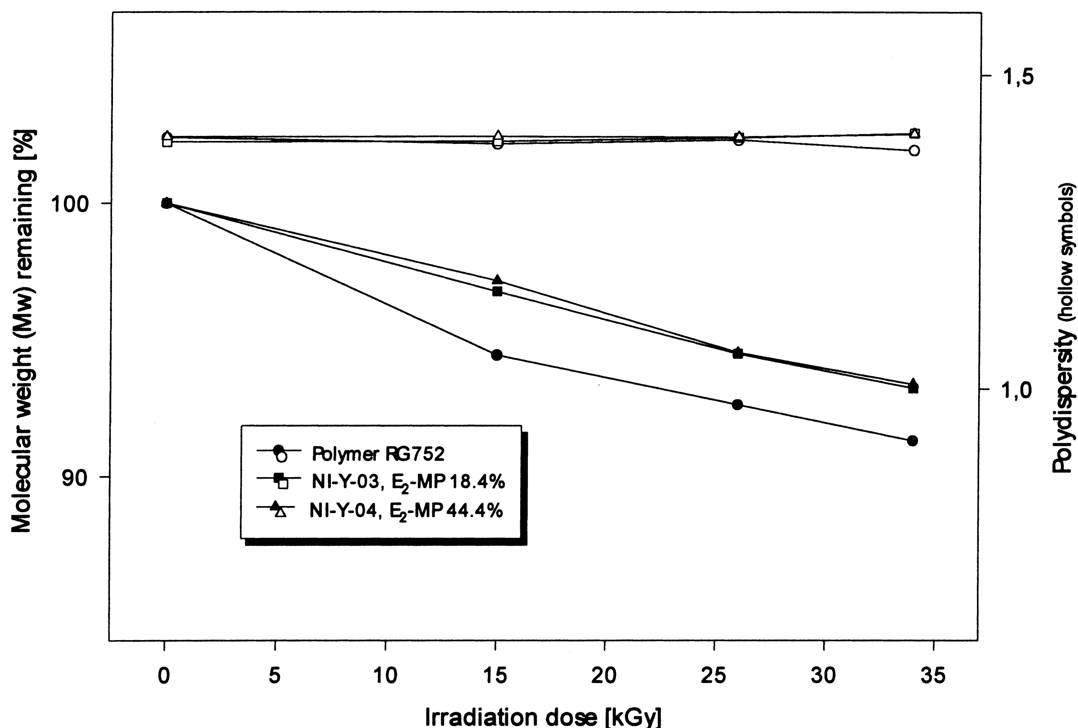

Fig. 3. Changes in polymer molecular mass and polydispersity (hollow symbol) as a function of irradiation dose. Polymer: PLG-75/25 (Resomer RG752).

radiolytic cleavage process, as indicated by the unchanged polydispersity under γ-irradiation, confirming the results of other investigations [5,8,9].

3.3. Effects of γ-irradiation on E2 stability

As mentioned above γ-irradiation was performed on dry ice (−78.5°C) to avoid thermal decomposition. Fig. 4 summarizes potential radiolytic cleavage sites in 17β-estradiol. The main radiolytic degradation product under standard storage conditions was 9,11-dehydroestradiol (9,11-DHE). In Table 2 the results of the observed γ-irradiation effects on either microencapsulated (PLG-50/50) E2 or the drug substance complied. E2, in unencapsulated form was resistant to radiolytic effect up to 26.6 kGy and no impurities could be detected by our analytical RP-HPLC method. By contrast, microencapsulated E2 showed a γ-irradiation dose loss of drug substance up to 5%. Fig. 5 summarizes the effects of the γ-irradiation dose on drug loading and formation of by-products. The loss of E2 in microspheres reaches a plateau at an irradiation dose of 15 kGy independent from drug loading. Furthermore, there seems to be an influence of drug loading on the niveau of that plateau [5], with a higher drug load

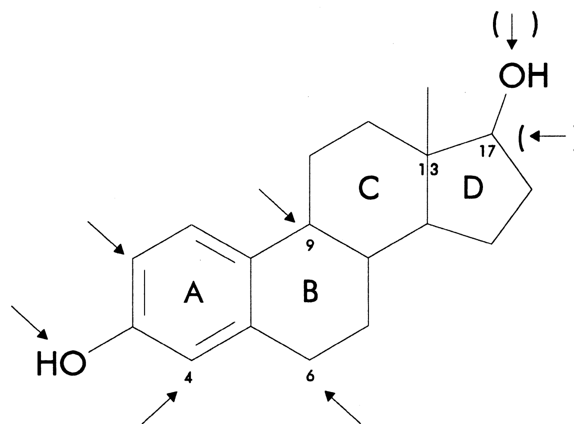

Fig. 4. 17β-Estradiol (E2), activated molecule sites for γ-irradiation induced degradation.

Table 2
Changes in 17β-estradiol content and degradation product profile of PLG microparticles under γ-irradiation

Batch	Irradiation dose (kGy)	17β-Estradiol		9,11-Dehydroestradiol[a]		E_2/PLG conjugates[b]	
		(%, w/w)	(Σ, %)	(%, w/w)	(Σ, %)	(%, w/w)	(Σ, %)
NI-Y-01	0	7.92	99.94	0.005	0.06	0.0	0.0
NI-Y-01	5.1	7.59	95.77	0.01	0.13	0.33	4.1
NI-Y-01	15.2	7.50	94.64	0.016	0.20	0.41	5.16
NI-Y-01	26.6	7.50	94.64	0.04	0.51	0.38	4.85
NI-Y-02	0	18.40	99.99	0.001	0.01	0.0	0.0
NI-Y-02	5.1	18.04	98.04	0.02	0.11	0.34	1.85
NI-Y-02	15.2	17.81	96.79	0.026	0.14	0.57	3.07
NI-Y-02	26.6	17.76	96.52	0.036	0.20	0.60	3.28
17β-Estradiol, lot 786	0	100.0	ditto	n.d.	n.d.	–	–
17β-Estradiol, lot 786	5.1	99.8	ditto	n.d.	n.d.	–	–
17β-Estradiol, lot 786	15.2	99.9	ditto	n.d.	n.d.	–	–
17β-Estradiol, lot 786	26.6	99.6	ditto	n.d.	n.d.	–	–

[a] Besides 9,11-DHE no other known degradation products were detectable.
[b] Unknown, not detectable E_2/PLG conjugates.
n.d. = Not detectable.

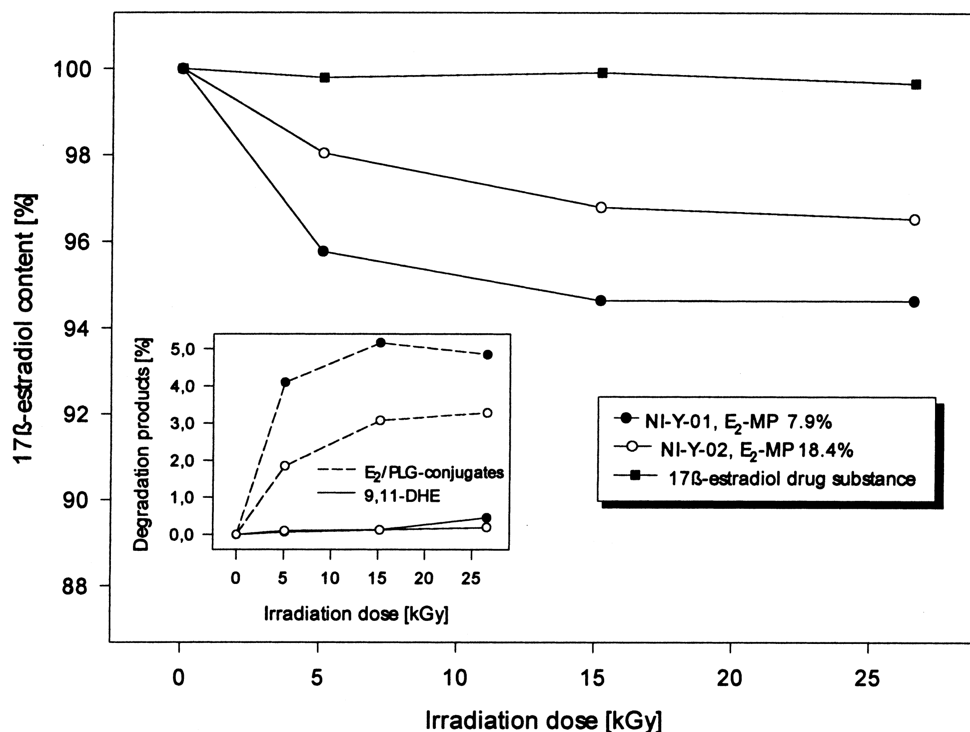

Fig. 5. Changes in E2-content and degradation product profile under γ-irradiation (polymer: PLG-50/50, Resomer RG503).

obviously having a positive effect on overall stability. Analyzing the type of degradation products, the surprising result obtained from a mass balance study was, that predominantly (>96%) not directly leachable radiolytic reaction products were formed under γ-irradiation. These products are presumably conjugation products of polymeric primary radicals with the drug substance. This effect is possibly due to the chemical structure of main degradation product 9,11-DHE, which can react with radicals generated in the polymeric matrix.

To further characterize the conjugation products we carried out some degradation experiments using alkaline hydrolysis for accelerated matrix degradation. In case of hydrolytically cleavable conjugates these should be detectable with standard RP-HPLC methods. As a result from these experiments it could be concluded, that no intact E2 was found after matrix hydrolysis, confirming our assumption that predominantly covalent recombinations of type –C–C– or –C–O–C– were formed upon irradiation.

The positive influence of a high drug load on the overall system stability is possibly due to a saturation effect, in terms of an irradiation dose dependent formation of a constant fraction of radicals, while a constant amount of drug substance acts as a radical scavenger, resulting in seemingly better stability at higher E2 loadings. Better mechanistic understanding of radical formation and quenching rates as well as the preferred recombination pathways are to be expected from studies using EPR spectroscopy [9].

Thermoanalytic examination of PLG-50/50 microspheres (Fig. 6) did not show strong dose dependent effects on the glass transition temperature. Compared to placebo microspheres a strong reduction in T_g is seen, compatible with residual solvents in the micro-

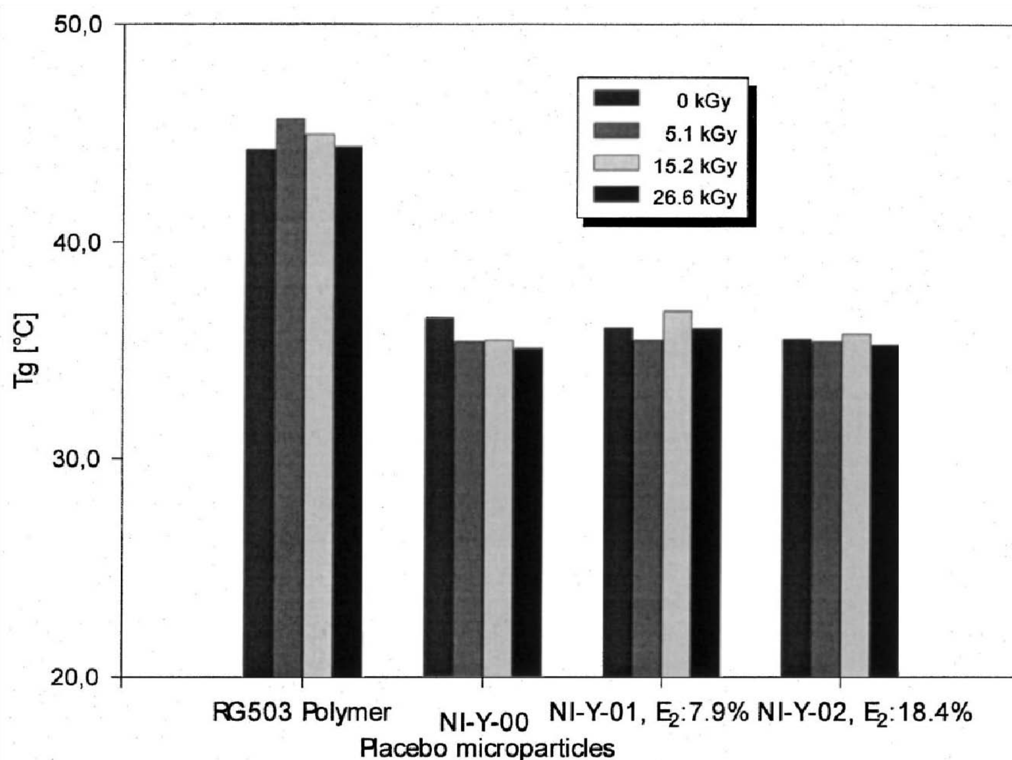

Fig. 6. Influence of irradiation dose on glass transition temperature (polymer: PLG-50/50, Resomer RG503).

spheres. This problem and the toxicity of the radiolytic cleavage products needs to be studied in more detail.

3.4. Influence of γ-irradiation on the in vitro drug release of 17β-estradiol

The in vitro drug release experiments were carried out in a rotating bottle apparatus (NF XIII) at 37°C using PBS, pH 7.4 as release medium. As illustrated in Figs. 7 and 8 we found a remarkable dose related influence of γ-irradiation on the in vitro release properties of E2 from PLG-50/50 microparticles. Independent from drug loading there was a significant acceleration of drug release with increasing γ-irradiation dose. Since E2 microparticles show a solid solution morphology, drug release kinetics are influenced by different mechanisms. Initial drug release occurs predominantly by matrix diffusion, followed by polymer erosion, which typically leads a to sigmoid release profile. The γ-irradiation leads to an acceleration of drug release as shown in Figs. 7 and 8, caused by the radiolytic degradation of polymer molecular mass. This effect becomes most prominent, when the onset of the erosion release phase is regarded. In the case of E2 microspheres with 7.9% drug loading (Fig. 7) we see a more or less parallel shift of the erosion controlled drug release phase with increasing irradiation. Nevertheless, there is a significant acceleration of drug release between 0 kGy and 26.6 kGy irradiated E2 microparticles, quantitated by time of 50% drug release, which is 28.5 days compared to 19 days (Student *t*-test, $p<0.05$). Higher drug loading of 18.4% (w/w) in PLG microspheres (Fig. 8) leads to flatter, more linear release profiles, but also to a more pronounced retardation, due to the lipophilic drug substance. Also in this case an acceleration of the in

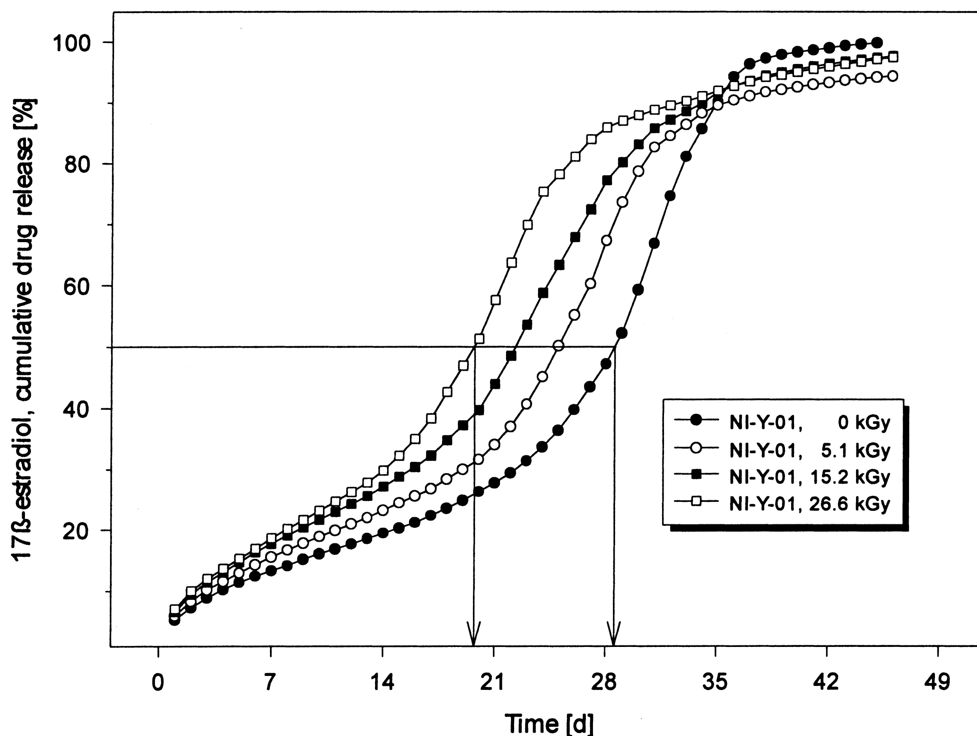

Fig. 7. Influence of γ-irradiation on in the vitro release kinetic of E2 microparticles (polymer: PLG-50/50, 7.9% actual E_2-drug load).

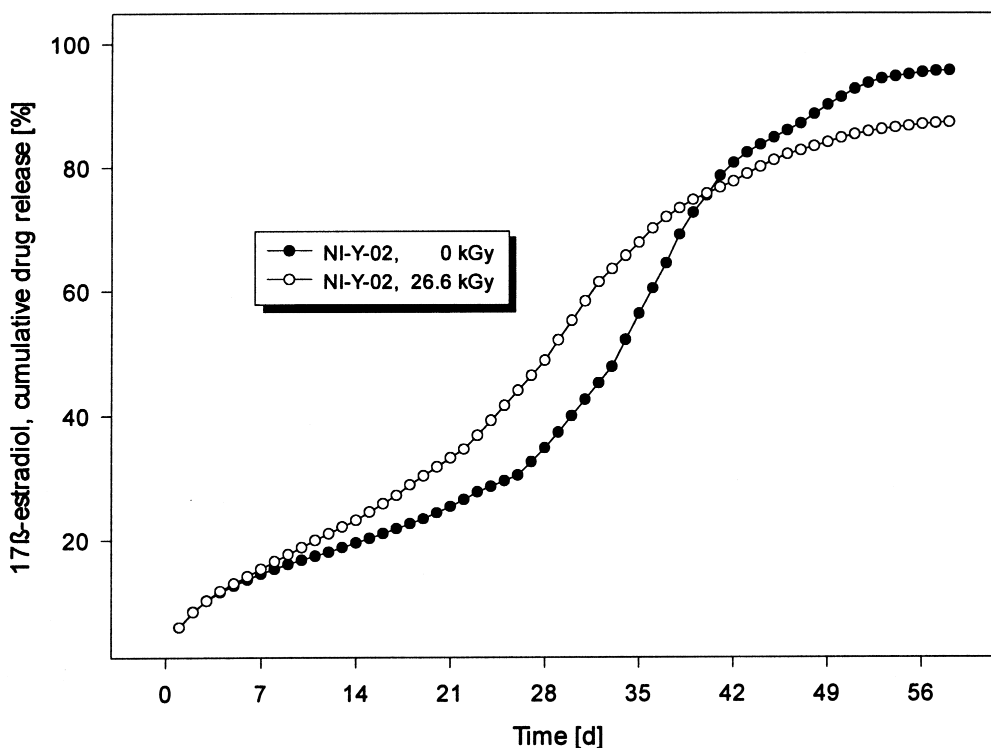

Fig. 8. Influence of γ-irradiation on the in vitro release kinetic of E2 microparticles (polymer: PLG-50/50, 18.4% actual E_2-drug load).

vitro release of E2 from PLG microspheres after γ-irradiation is observed.

Volland et al. [5] found a decrease of the release kinetics with increasing irradiation doses for captopril microspheres using the same polymer, which could be attributed to the more hydrophilic nature of the captopril molecule that is released predominantly by pore diffusion. Decreased mechanical strength of polymers under irradiation due to polymer chain breakdown leads in conclusion to a disturbed pore network within the microspheres that decreases free pore diffusion.

3.5. Microbiological characteristics of γ-irradiated E2 drug delivery systems

γ-Irradiation has been used as a method for terminal sterilization for biodegradable delivery systems. Either the standard overkill dose procedure using 25 kGy regardless the nature of bioburden. Alternatively, a bioburden related procedure using lower irradiation doses was applied [3,4,18]. Unfortunately, the latter method requires substantial characterization of the microbiological contaminants and needs extensive validation efforts to demonstrate reliability of sterilization efficiency.

Application of the standard overkill procedure using 25 kGy for terminal γ-sterilization typically implies a six log sterility assurance level. With respect to *Bacillus pumilus*, the official test microorganism for γ-sterilization, 25 kGy is equivalent to about eight times its D_{10} value. Therefore, starting materials are required to have a maximum bioburden of 10^2 CFU/g. Furthermore, any secondary contamination by the manufacturing process itself has to be strictly avoided. Commercially available polymers such as PLG as well as synthetic drug substances commonly fulfill these microbiological requirements. To assess the influence of the manufacturing process on changes in bioburden we have monitored the bioburden of the microparticle batches. The microparticles were finally treated with increasing irradia-

Table 3
Determination of bioburden of starting materials and PLG microparticles before and after γ-irradiation[a]

Irradiation dose	Polymer (RG503) starting material	17β-Estradiol starting material	Placebo microparticles	E_2-MP NI-Y-01 (7.9% E_2)	E_2-MP NI-Y-02 (18.4% E_2)
Before irradiation	Bioburden $<10^2$ CFU/g	Bioburden $<10^2$ CFU/g	Bioburden, $1.6\cdot10^4$ CFU/g	Bioburden, $0.94\cdot10^4$ CFU/g	Bioburden $<10^2$ CFU/g
5.1 kGy	Bioburden <10 CFU/g	Bioburden <10 CFU/g	Bioburden, $8.0\cdot10^2$ CFU/g	Bioburden $<10^2$ CFU/g	Bioburden <10 CFU/g
15.2 kGy	Bioburden <10 CFU/g	Bioburden <10 CFU/g	Bioburden, $2.5\cdot10^2$ CFU/g	Bioburden $<10^2$ CFU/g	Bioburden <10 CFU/g
26.6 kGy	Bioburden <10 CFU/g	Bioburden <10 CFU/g	Bioburden $<10^2$ CFU/g	Bioburden <10 CFU/g	Bioburden <10 CFU/g

[a] Bioburden of yeasts and moulds: all irradiation doses below limit of detection, limit of detection was 10^2 CFU/g (CFU = colony forming units).

tion doses up to the overkill dose (Table 3), and monitored for contamination level by determination of number of CFU instead of using a sterility test, which is essentially only a qualitative test. The limit of detection for all contaminants including yeasts and moulds was 10^2 CFU per gram E2 microspheres. In the case of undetectable growth contamination was expressed as below 10^1 CFU/g.

Regarding the initial microbiological quality of polymer and drug substance (Table 3) both meet the bioburden specification $<10^2$ CFU/g. On the other hand placebo batches and E2 microspheres show additional microbiological contamination due to the encapsulation process. Although the microparticle batches listed in order of preparation (from left to right) indicate an increasing microbiological quality with increasing processing time, it is obvious that the required sterility assurance level of six log reduction cannot be achieved reliably by applying the standard overkill dose of 25 kGy. In case of limiting the initial bioburden to max. 10^2 CFU/g reliable sterility was already achieved with the lowest irradiation dose of 5.1 kGy.

Two conclusions could be drawn from these results: first, γ-irradiation is a suitable sterilization method for parenteral drug delivery systems based on PLGs with respect to sterility. Secondly, it is the important to limit bioburden of starting materials and to establish rigorous cleaning procedures for the equipment.

3.6. Result of the D_{10} value examination of microencapsulated *Bacillus pumilus*

The D_{10} value or decimal reduction number is defined as: the extent of the adjustable variable parameter characterizing a particular sterilization method needed for a reduction of the respective test specimen (bioindicator) by one log. As bioindicator for γ-sterilization usually *Bacillus pumilus* ATCC 27142 is used with a D_{10} value in the range of 2.2 to 3.0 kGy [3,4]. The question was if microencapsulated spores of *Bacillus pumilus* exhibit a different behavior under γ-irradiation and yield a different D_{10} value.

For this purpose we used a simple O/W-solvent–evaporation process for microencapsulation. To limit any risk of contamination all work was performed under aseptic conditions. Three different levels of spore-bioburden within the microspheres were adjusted: 10^4 CFU/g, 10^6 CFU/g and 10^8 CFU/g. As shown in Table 4 this target was not achieved.

Table 4
Changes in bioburden of *Bacillus pumilus* ATCC 27142 microencapsulated in PLG copolymer under terminal γ-irradiation[a]

Irradiation dose	Initial bioburden of *Bacillus pumilus*		
	10^8 CFU/g	10^6 CFU/g	10^4 CFU/g
Microparticle bioburden before irradiation	*Bacillus pumilus* $3.9\cdot10^4$ CFU/g	*Bacillus pumilus*, $12\cdot10^2$ CFU/g	Bioburden $<10^2$ CFU/g
5.1 kGy	*Bacillus pumilus* identified, $3.0\cdot10^2$ CFU/g	Bioburden $<10^2$ CFU/g	Bioburden $<10^2$ CFU/g
15.2 kGy	Bioburden $<10^2$ CFU/g	Bioburden $<10^2$ CFU/g	Bioburden $<10^2$ CFU/g
26.6 kGy	Bioburden $<10^2$ CFU/g	Bioburden $<10^2$ CFU/g	Bioburden $<10^2$ CFU/g

[a] Limit of detection was 10^2 CFU/g (CFU = colony forming units).

Ethanolic spore suspensions obviously showed a different behavior in the O/W-microencapsulation process leading to encapsulation efficiencies in the order of 10^4 CFU/g. To support these results a test was applied to investigate potential spore reducing properties of the solvent used, which was dichloromethane, but there was no significant spore reduction found after 24 h of incubation. Microencapsulated bioindicator batches were finally irradiated with increasing doses again up to the overkill dose of 25 kGy.

For the calculation of the D_{10} value several ways are possible. Lewis [27] suggested the following formula where log a is the bioburden before irradiation and log b the bioburden after irradiation and dose the corresponding dose used for irradiation.

$$D_{10} = \frac{\text{dose}}{\log a - \log b}$$

Calculations performed with our data set using the above mentioned formula revealed a D_{10} value of 2.4 kGy, which is in accordance with the reported literature data. This is not unexpected, since γ-irradiation is characterized by a very short wavelength, easily allowing for penetration of the materials used for microencapsulation.

4. Conclusions

We investigated the influence of terminal γ-irradiation on 17β-estradiol loaded PLG microparticles prepared by a spray-drying technique as a model controlled release system for a drug substance of substantial stability.

Pure E2 showed good stability in the investigated dose range up to the standard overkill dose of 25 kGy, whereas the microencapsulated drug substance that forms a solid solution matrix in the loading range studied here, showed some radiolytic degradation. Up to 5% degradation products were found depending on drug loading. With higher drug loadings a better stability was obtained due to a constant amount of degradation products formed upon irradiation. The investigation of the nature of these degradation products revealed surprisingly, that with more than 96%, there were covalent conjugation products formed with the matrix. For PLG polymer degradation under γ-irradiation a linear decrease in molecular mass was found independent from monomer composition and drug loading, while polydispersity remained nearly unchanged indicating random chain scission is the preferred chain cleavage mechanism.

The in vitro drug release kinetics showed remarkable irradiation dose related acceleration independent from investigated drug load. This was mainly caused by polymer degradation and therefore loss of mechanical strength and accelerated onset of polymer erosion, is leading to a synchronization of diffusion and erosion controlled drug release, thus, giving a more linear release profile.

The assessment of the bioburden before and during manufacturing of the microparticles demonstrated the contamination risk caused by the spray-drying process. It is, therefore, necessary to limit bioburden of starting materials and to establish and validate equipment cleaning procedure also from a microbiological perspective. For the sterilization of parenteral delivery systems γ-irradiation is an efficient procedure, provided the drug substance and the polymers show sufficient radiolytic stability.

The microencapsulation of the bioindicator *Bacillus pumilus* and subjection to γ-irradiation demonstrated that spores were not additionally protected by this process, but rather confirmed the literature data of the respective D_{10} value of 2.4 kGy.

From our results we conclude that γ-irradiation can be a suitable sterilization procedure for parenteral drug delivery systems, such as microspheres and implants based on biodegradable polyesters. The characterization and toxicological assessment of radiolytic degradation products remains a challenging task with respect to regulatory requirements, and should be addressed as early as possible within the development of a microparticulate controlled release system.

References

[1] T. Kissel, A. Rummelt, W. Schmutz, Technical aspects of microspheres for depot injection: Parlodel LAR, a one-a-month delivery system for bromocriptine, Triangle 29 (1990) 205–214.

[2] S. Gogolewski, P. Mainil-Varlet, J.G. Dillon, Sterility, me-

chanical properties and molecular stability of polylactide internal-fixation devices treated with low-temperature plasmas, J. Biomed. Mater. Res. 32 (1996) 227–235.
[3] K.H. Wallhäusser, in: 5th ed., Sterilisation durch Bestrahlung, Praxis der Sterilisation, Desinfektion-Konservierung, Vol. 4.4, Georg Thieme, Stuttgart, New York, 1995.
[4] B. Gorden et al., Sterilization of parenterals by γ-radiation, J. Parenteral Sci. Technol. 42 (1988) 1–9, Technical report No. 11.
[5] C. Volland, M. Wolff, T. Kissel, The influence of terminal γ-sterilization on captopril containing poly(D,L-lactide-co-glycolide) microspheres, J. Control. Rel. 31 (1994) 293–305.
[6] H.T. Wang, H. Palmer, J.R. Linhardt, D.R. Flanagan, E. Schmitt, Degradation of poly(ester) microspheres, Biomaterials 11 (1990) 679–685.
[7] C.C. Chu, N.D. Campbell, Scanning electron microscopic study of the hydrolytic degradation of poly(glycolic acid) suture, J. Biomed. Mater. Res. 16 (1982) 417–430.
[8] A.G. Hausberger, R.A. Kenley, P.P. De Luca, Gamma irradiation effects on molecular weight and in vitro degradation of poly(D,L-lactide-co-glycolide) microparticles, Pharm. Res. 12 (1995) 851–856.
[9] B. Bittner, K. Mäder, C. Kroll, H.-H. Borchert, T. Kissel, Tetracycline-HCl, glutathione and L-alanine loaded poly(DL-lactide-co-glycolide) microspheres prepared by a spray drying technique: Influence of γ-irradiation on radical formation and polymer degradation, J. Control. Rel. 59 (1999) 23–32.
[10] Y. Aso, S. Yoshioka, T. Otsuka, S. Kojima, Effects of γ-irradiation on the drug release characteristics of poly(D,L-lactide) microspheres, Proceed. Int. Symp. Control. Rel. Bioact. Mater. 22 (1995) 386–387.
[11] G. Spenlehauer, M. Vert, J.P. Benoit, A. Boddaert, In vitro and in vivo degradation of poly(D,L-lactide/glycolide) type microspheres made by solvent evaporation method, Biomaterials 10 (1989) 557–563.
[12] L.R. Beck, V.Z. Pope, T.R. Tice, R.M. Gilley, Long-acting injectable microsphere formulation for the parenteral administration of levonorgestrel, Adv. Contraception 1 (1985) 119–129.
[13] L.M. Sanders, J.S. Kent, G.I. McRae, B.H. Vickery, T.R. Tice, D.H. Lewis, Controlled release of a luteinizing hormone-releasing hormone analogue from poly(D,L-lactide-co-glycolide) microspheres, J. Pharm. Sci. 73 (1984) 1294–1297.
[14] L.R. Beck, V.Z. Pope, Controlled-release delivery systems for hormones. A review of their properties and current therapeutic use, Drugs 27 (1984) 528–547.
[15] J.K. Lalla, K. Sapna, Biodegradable microspheres of poly-(DL-lactic acid) containing piroxicam as a model drug for controlled release via the parenteral route, J. Microencapsul. 10 (1993) 449–460.
[16] E.G. Salole, in: K. Flowry (Ed.), Analytical Profiles of Drug Substances, Vol. 15, Academic Press, New York, 1986, pp. 283–318.
[17] Monograph Estradiol (1991) pp. 1–15, Arzneistoffprofile (1982–1994), Govi-Verlag, Frankfurt.
[18] The United States Pharmacopoeia, Sterilization <1211>, Rockville, MD, 23rd revision, 1995, p. 1978.
[19] F. Pavanetto, I. Genta, P. Giunchedi, B. Conti, Evaluation of spray drying as a method for polylactide and polylactide-co-glycolide microsphere preparation, J. Microencapsul. 10 (1993) 487–497.
[20] J. Broadhead, S.K. Edmond Rouan, C.T. Rhodes, The spray drying of pharmaceuticals, Drug Div. Ind. Pharm. 18 (1992) 1169–1206.
[21] K. Masters, Spray Drying Handbook, 5th ed., Longman Scientific and Technical, Harlow, 1991.
[22] R. Bodmeier, H.G. Chen, Preparation of biodegradable poly(±)lactide microparticles using a spray drying technique, J. Pharm. Pharmacol. 40 (1988) 754–757.
[23] S.R. Hartas, J.H. Collett, C. Booth, The influence of γ-irradiation on poly(lactide-co-glycolide) microspheres, J. Pharm. Pharmacol. 43 (Suppl.) (1992) 29P.
[24] J.H. Collett, L.Y. Lim, P.L. Gould, γ-Irradiation of biodegradable polyesters in controlled physical environments, Polym. Preprints, A. Chem. Soc., Div. Polym. Chem. 30 (1989) 468–469.
[25] L.Y. Lim, P. Gould, N.A. Dickinson, J.H. Collett, The influence of environmental conditions on the damage induced in L-polylactic acid during γ-irradiation, Proc. Int. Symp. Control. Rel. Bioact. Mater. 15 (1988) 111–112.
[26] S.D. Bruck, E.P. Mueller, Radiation sterilization of polymeric implant materials, J. Biomed. Mater. Res.: Appl. Biomat. 22 (1988) 133–144.
[27] D.H. Lewis, in: M. Chasin, R. Langer (Eds.), 1st ed., Biodegradable Polymers as Drug Delivery Systems, Vol. 45, Marcel Dekker, New York, 1990, pp. 1–41, Ch. 1.