Research paper

Sterilization of gentamicin containing collagen/PLGA microparticle composites

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Abstract

In order to achieve implants which provide sustained release of gentamicin, microparticles based on a 50/50 Resomer® 503/Resomer® 502H blend were combined with collagen in order to achieve their fixation and to utilize the favorable effect of collagen on wound healing. Ethylene oxide treatment as well as β- and γ-irradiation were tested for sterilization of the collagen/PLGA-microparticle composite. All methods resulted in a decrease of molecular weight and glass transition temperature of polymer raw material and microparticles. In addition, ethylene oxide treatment yielded aggregation of microparticles leading to a substantial increase in the initially liberated gentamicin dose. Furthermore, chemical changes of gentamicin after ethylene oxide sterilization could be identified using NMR spectroscopy. Despite a decrease in the molecular weight and glass transition temperature after irradiation, neither morphological changes of the composites nor changes regarding the gentamicin release profile from β- and γ-sterilized material were observed. Free radicals, which could only be detected in gentamicin drug substance and at marginal level in gentamicin-loaded MPs, disappeared within 4 weeks. Additional microbiological testing verified the microbiological activity of gentamicin liberated from β-sterilized composites. Storage of β-sterilized composite at 4 °C/35% r.h. for 3 months did not influence morphology, molecular weight, glass transition temperature, and release profiles of microparticles and composites. However, at 25 °C/60% r.h. and 40 °C/75% r.h. a marked decrease in molecular weight and glass transition temperature resulted. This effect was due to a higher humidity, water uptake into polymers, and subsequent hydrolysis of polymers and microparticles, which was more pronounced for RG 502H because of its hydrophilicity. Upon storage at 25 °C/60% r.h. and 40 °C/75% r.h. particles collapsed resulting in an increased gentamicin liberation. Thus, all sterilization techniques have their pros and cons, but based on drug release profile and chemical changes of gentamicin irradiation treatment appears to be more suitable for collagen/gentamicin-loaded PLGA microparticle composites.

1. Introduction

Local parenteral application of antibiotics is indicated to achieve high drug levels at an infection site and to minimize systemic side effects. Different delivery systems, e.g., based on collagen or polymethylmethacrylate are available [1]. A new implantable, biodegradable drug delivery system based on a collagen sponge providing an initial antibiotic dose followed by a sustained delivery of gentamicin (GM) from embedded polylactide-co-glycolide (PLGA) microparticles within 1 week had been developed [2–4]. According to pharmacopoeial requirements these implants have to be sterile. The European Agency for the Evaluation of Medicinal Products considers terminal sterilization in the final container with dry heat (160 °C, 120 min) as the method of choice for non-aqueous systems [5]. For both collagen and PLGA sterilization with dry heat cannot be applied due to thermal instability – taking various efficient

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conditions into account. Therefore, following the EMEA decision tree for the selection of sterilization methods, ionizing radiation has to be selected with a sterility assurance level of 10^{-6}. Sterilization by irradiation is based on the fact that atoms are ionized. The free electrons which are created interact with DNA and kill microorganisms. In recent years, γ-irradiation at doses $\geqslant 25$ kGy was the method of choice for ionizing radiation of pharmaceutical products and medical devices [6–9]. γ-rays are electromagnetic waves of high energy created, e.g., by ^{60}Co sources. They show a high depth of penetration into matter but the degree of interaction is rather low. Sterilization time ranges from a few hours to several days depending on size, quantity, and type of sterilized good. As an alternative, β-irradiation by electron beam can be used. The focused, accelerated electrons exhibit a 10 times lower depth of penetration as compared to γ-rays, however, the electrons react more intensively with matter. Consequently, reaction time with the electron beam for sterilization is only a few seconds [10]. Therefore, the use of e-beam as compared to γ-irradiation has cost benefits. But the lower depth of penetration limits the application, since adequate sterility assurance for large or dense goods may be difficult to achieve [11].

In view of the known potential of ethylene oxide (EO) for genotoxic carcinogenicity, it is recommended that use of EO is acceptable only when pharmaceutically absolutely necessary [12]. In that case residual EO in the product should not exceed a limit of 1 ppm and ethylene chlorohydrin should be less than 50 ppm [13]. Despite the risks, EO continues to be used for increasing volumes of medical devices due to improvements of the equipment and the cycle efficiency as well as more accurate process monitoring [14]. Specifically in case of collagen products, EO is utilized for sterilization of commercially available suture material and wound dressings. A major advantage is the low temperature burden on the sterilization good.

The goal of this study was to evaluate the feasibility of γ- and β-irradiation as well as EO for sterilization of the GM-loaded collagen/PLGA-microparticles composites. γ-irradiation has frequently been described for sterilization of poly-α-hydroxyacid polymers with the potential of radical formation, chemical modification, polymer chain scission, and changes in the drug release profile depending on the dose [7,15–18]. Little is known about the effects of both electron beam and EO on these materials. EO treatment presents a viable method to obtain sterile collagen products. However, EO irreversibly alkylates amino acid residues, affecting various characteristics such as the number of free amino groups and the degradation rate [19,20]. γ-irradiation is another established sterilization method for collagenous devices and has been extensively used for the preparation of human bone and tendon grafts [21–23]. Several studies have reported two contrary effects on collagen: on the one hand, fragmentation of protein chains occurs, and on the other hand additional cross-links are formed during the process [24]. The prevalence of chain scission of cross-link formation depends on the moisture content

[25]. Dry samples were found to be more compromised in their mechanical strength [20,23,26] and more susceptible to enzymatic degradation [20,27]. Since both the biodegradability and the mechanical strength of collagen should not impact the antibiotic effect of the composite implant, collagen was only analyzed for formation and persistence of radicals which could influence the drug or the microparticles as well as microscopical changes in appearance. The PLGA polymers and microparticles were characterized with respect to changes in the chemical and physicochemical properties by size-exclusion chromatography, differential scanning calorimetry, and NMR spectroscopy. In addition, the GM release profile had to be tested including analysis of the biological activity assay of the antibiotic liberated. Furthermore, a short-term stability test was performed on β-irradiated microparticles and composites.

2. Materials and methods

2.1. Materials

Gentamicin-loaded PLGA microparticles were prepared based on a W/O/W-double emulsion technique as previously described [2]. A 50/50 blend of Resomer® RG 502H (RG 502H) (PLGA 50:50; uncapped) and RG 503 (RG 503) (PLGA 50:50; end-capped) was used. Insoluble fibrous bovine tendon collagen (p$I \sim 7.0$) (IC) was provided by Innocoll GmbH, Saal, Germany. Gentamicin sulfate was obtained from Innocoll GmbH and throughout this paper GM describes the sulfate form. Collagen sponge/PLGA composites were prepared by lyophilization of a 1% IC-dispersion, pH 4.5, containing 5 mg GM microparticles per milligram IC (reflecting 2 mg GM per 2.8 mg IC) as well as additional 2 mg GM per 2.8 mg IC dissolved in the IC dispersion for an initial GM bolus [4]. Suspensions were cast in PP dishes of 38 mm diameter and 4 mm height at 22 °C. The molds were placed on the lyophilizer shelves at 22 °C and freeze-dried (cooling to −40 °C at 20 °C/h and hold for 4 h, heating to −20 °C within 1 h at 0.12 mbar and hold for 20 h, and heating to 20 °C within 6 h at 0.12 mbar and hold for 6 h using a Christ Model Delta 1-24 KD, Osterode, Germany).

2.2. Methods

2.2.1. Sterilization

For EO treatment an EO sterilizer Type 15009 VD (DMB, D-Wiesbaden) with 1500 l chamber volume was used. The chamber was filled with pressurized air at 4 bar, a vacuum of 0.8 bar was applied, and EO was introduced to 4 bar, 32 °C, 6 h using a 15:85 (m:m) EO:CO_2 mix. EO was desorbed over 12 h in 30 min cycles between +0.6 and −0.8 bar. β-sterilization with 25.3 kGy was performed at Ristron, D-Saal, using a Rhodotron TT 300 (IBA, B-Louvain-la-Neuve) electron beam accelerator. γ-irradiation with 28.9 kGy was performed at Gammaster®, D-München, with a Co-60 source.

2.2.2. Microscopical investigation

A stereological microscope (Zeiss, D-Oberkochen) was used for sample characterization. For SEM, samples were sputtered with gold in a sputter chamber (Hummer JR Technics, Alexandria, VA, USA) and investigated with a scanning electron microscope (Amray Model 1810 T, Amray Inc., Bedford, MA, USA).

2.2.3. Atomic force microscopy

For atomic force microscopy (AFM) a 0.1% IC-dispersion, pH 4.5, was spread on a glass coverslip of 15 mm diameter, washed three times with water, and air-dried. Microscopy was performed with a NanoScope® 3 (Digital Instruments, D-Mannheim) in contact mode.

2.2.4. Release testing

Composites corresponding to 100 mg GM containing PLGA microparticles (reflecting 14 mg GM) plus additional 14 mg non-encapsulated GM (1.6 cm^3 composite of mg) were incubated in 5 ml PBS, pH 7.4 (5.2 g/L K$_2$HPO$_4$/1.415 g/L KH$_2$PO$_4$), containing 0.2% sodium azide (Merck, D-Darmstadt) at 37 °C in a shaking bath at 50 strokes per minute ($n = 3$). At designated time points 3 ml of the release medium was exchanged and the GM concentration was determined [3].

2.2.5. Molecular weight analysis

Resomer® molecular weight (molecular weight weight-average (m_w), molecular weight number-average (m_n), and polydispersity index (pi)) was determined as described previously using size-exclusion HPLC [2].

2.2.6. Differential scanning calorimetry

The measurements were carried out on a DSC machine with a low temperature feature (Polymer Laboratories Inc., Loughborough, UK). Samples of 10 ± 5 mg were sealed in 40 μl aluminum containers and heated at 5 °C/min starting at 0 °C. Studies were performed in triplicate.

2.2.7. Electron spin resonance spectroscopy

The ESR-Spectra at x-band were analyzed with an ECS D40 XG Microwave bridge X/ECS 106 (Bruker, D-Rheinstetten) at 9.5 GHz.

2.2.8. ^{1}H NMR and ^{13}C NMR measurements

Polymers were dissolved in CDCl$_3$ and GM in D$_2$O. Samples were analyzed with a Bruker AC 250 and a Bruker AM 360 spectrometer (Finnigan MAT GmbH, D-Bremen) respectively (internal standard: tetramethylsilane).

2.2.9. Microbiological assay of gentamicin

The GM released from e-beam-sterilized samples was tested for activity according to the Ph. Eur. Suppl. 2000 method 2.7.2 by the diffusion method using *Staphylococcus aureus* as test organism. Samples were transferred into Petri dishes of 100 mm diameter and after 18 h the inhibition zone was measured.

2.2.10. Stability testing

For stability testing samples were stored at 4 °C/35% r.h., 25 °C/60% r.h., and 40 °C/75% r.h. for 4 and 12 weeks, and analyzed for molecular weight, glass transition temperature, morphology, and GM release. The water content was determined via gravimetric analysis of the loss on drying at 70 °C.

3. Results and discussion

3.1. Sterilization with EO

3.1.1. Glass transition temperature and molecular weight of PLGA

According to Athanasiou et al. [18] there is little literature available on the influence of EO on preparations of PLGA polymers. Therefore, not only the effect of EO on the composite but also that on PLGA bulk material, GM bulk, and GM containing microparticles was investigated. EO-treatment caused a marked reduction in the molecular weight of PLGA – polydispersity only slightly changed – (Table 1) and in the glass transition temperature (T_g) by approximately 5 °C. On the one hand, ester bonds in the polymer chain could be cut by EO-sterilization or transesterification could occur and on the other hand EO could react with free carboxylic groups of the polymer chain [28]. For the GM containing microparticles the decrease in m_n and m_w by 5–6% is less than that for RG 502H powder with 14% and 19%, respectively, and RG 503 powder with 24% and 15%, respectively. This could be due a lower specific surface area and reduced EtO concentrations in the inner of the MPs.

3.1.2. ^{1}H NMR and ^{13}C NMR spectroscopy of PLGA and GM

In order to analyze the chemical changes of PLGA and GM by EO-treatment in more detail and to detect possible

Table 1
Molecular weight weight-average (m_w), molecular weight number-average (m_n), polydispersity index (pi), and glass transition temperature (T_g) of RG 503 and RG 502H bulk material as well as 50/50 RG 503/RG 502H MPs before sterilization and after EtO, e-beam or γ treatment ($n = 3$)

	m_w (Da)	m_n (Da)	pi	T_g (°C)
RG 503				
Non-sterilized	36,200	16,300	2.2	48
EtO	30,900	12,400	2.5	44
e-beam	29,000	12,000	2.4	43
γ	30,900	10,000	3.1	43
RG 502H				
Non-sterilized	13,500	6,700	2.0	46
EtO	11,000	5,800	1.9	40
e-beam	10,300	5,500	1.9	42
γ	10,600	5,300	2.0	44
MPs				
Non-sterilized	21,700	7,700	2.8	47
EtO	20,700	7,200	2.8	41
e-beam	18,400	7,000	2.8	41
γ	19,000	6,200	3.1	43

attachment of EO, ^{1}H NMR spectroscopy of the polymers and both ^{1}H NMR and ^{13}C NMR spectroscopy of GM were performed. All PLGA spectra showed a peak at ≈1.5 ppm which could be attributed to the methyl-group of lactic acid (Fig. 1a). At 4.8 ppm a peak of the methylene-group of glycolic acid and an additional characteristic peak of lactic acid (methine-group) at 5.2 ppm are found [38–40]. Integration of the peaks gives the ratio of lactide/glycolide as 1:1. Significant differences between the ^{1}H NMR spectra of RG 503 and RG 502H prior and after EO-sterilization were not found and the lactide/glycolide ratio is not changed. Small differences in peak height and width are due to the viscosity of the polymer solutions. Thus, a chemical change in the polymer chain composition could not be detected in ^{1}H NMR spectroscopy.

The characteristic peaks of the ^{1}H NMR spectrum of GM have been listed by Rosenkrantz et al. [29]. The spectrum shows clear changes in the peak pattern in the 3.8–4.0 ppm range after EO-treatment (Figs. 1b and c). This region represents the coupling of protons in proximity to

oxygen and could be an indicator for EO-attachment to GM during sterilization. ^{13}C NMR spectra showed an additional peak at 62.9 ppm after EO-treatment (Figs. 1d and e) which is characteristic for the –CH_2O– group found in EO-adducts. As described by Schönfeldt, EO is highly reactive and reacts with alcohol and amino groups which are found in the GM structure [28]. Despite the obvious attachment of EO to GM, the antibiotic is only slightly reduced in its microbiological activity and the activity of EO-sterilized material is still sufficient after 2-years of storage [30].

3.1.3. AFM of collagen

In addition to the effects of EO-treatment on PLGA and GM, the question of the impact of the gassing on collagen needs to be answered. Olde Damink found that EO-sterilization did not lead to a change in the mechanical properties of collagen [20]. However, collagen does not stay completely unaffected by EO. The amino groups react with EO which leads to an increase in pH after resuspension in
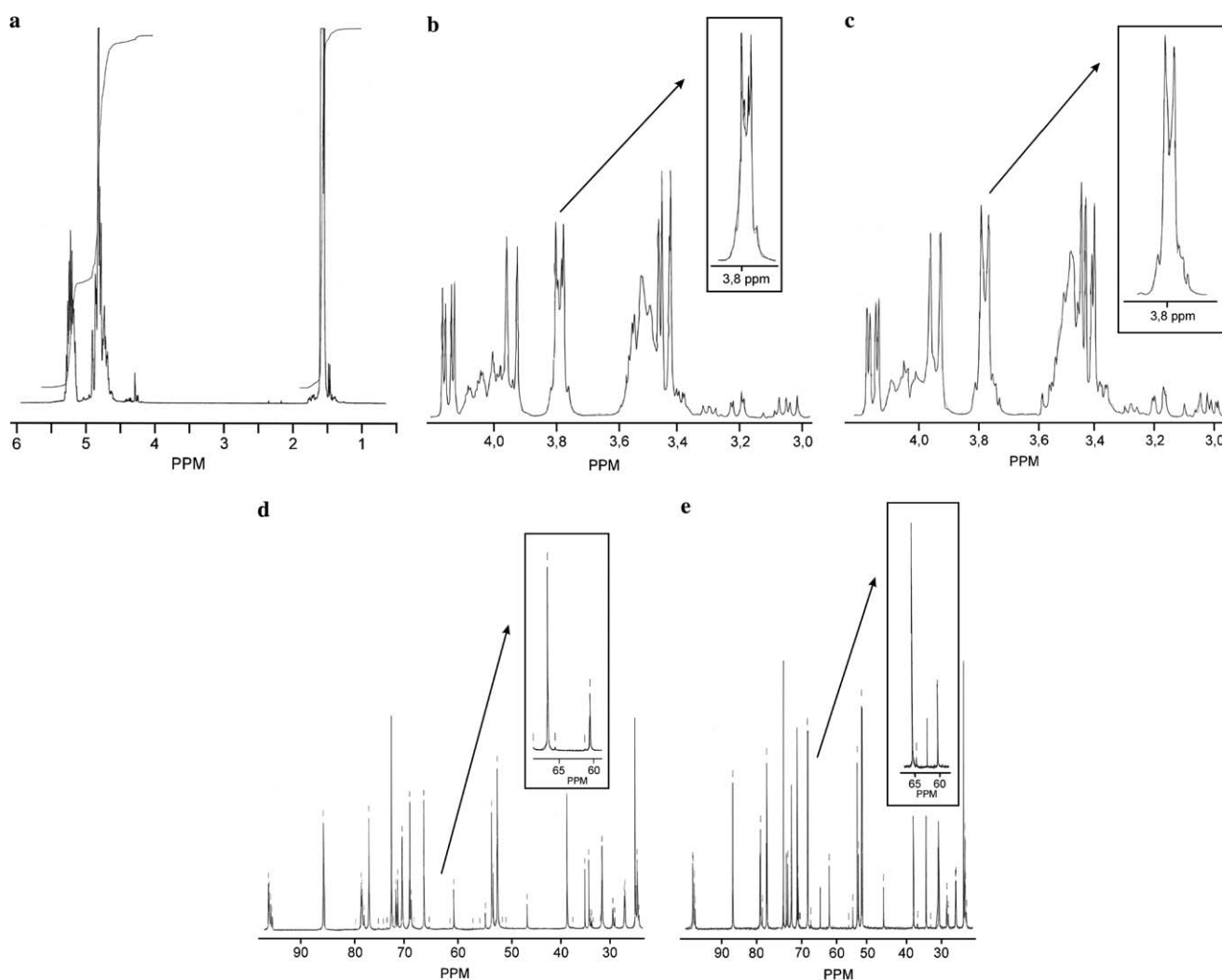

Fig. 1. ^{1}H NMR spectra of EO-treated RG 502H (a), non-sterilized gentamicin (b), EO-treated gentamicin (c) and ^{13}C NMR spectra of non-sterilized gentamicin (d), and EO-treated gentamicin (e).

water, an increase in the isoelectrical point (pi), and a decrease in helical stability indicated by a lower melting temperature [6,21]. In different studies, EO-treatment of collagen material led to either slightly increased or decreased collagenolytic degradation rates [19,20]. Overall EO-gassing only slightly influences the biological effects of collagen materials like enhanced wound healing and cell penetration [30]. In order to gain insight into EO-treated collagen fibers, AFM was performed. In contrast to non-treated collagen fibers (Fig. 2a), EO-sterilized collagen fibers demonstrate irregular structures and the characteristic periodic pattern can hardly be detected (Fig. 2b). The decrease in helix stability described by Gorham [21] due to attachment of collagen could be related to the changes in collagen structure as demonstrated microscopically.

3.1.4. GM release after EO-gassing

The changes in PLGA m_w and T_g, in collagen fiber structure as well as the addition of EO to GM indicate that the EO-sterilization process could lead to significant changes of the collagen/GM containing PLGA microparticle composite. Especially, conservation of the GM release profile from sterilized composites is an important criterion. The in vitro experiments demonstrated an increase in the amount of GM released from PLGA microparticles initial-

ly within the first 4 h from 11% to 55% for EO-sterilized material (Fig. 3). Subsequently, the antibiotic release is completed within 1 week. Release of GM from sterilized composites reaches 83% at day 1 and is significantly higher than the 60% GM released from non-sterilized composites within the same time. The increase corresponds to the amount which is additionally liberated from the MPs.
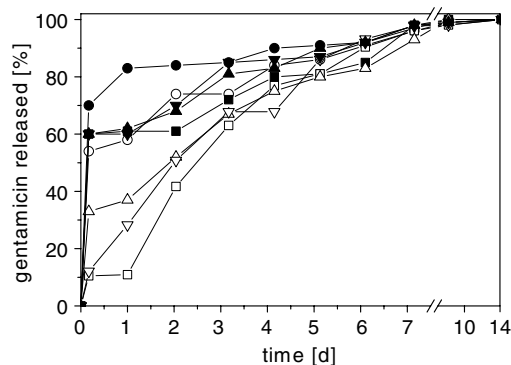
Fig. 3. Gentamicin release from 50/50 RG 503/RG 502H microparticles (open symbols) and collagen/PLGA-microparticle composites (full symbols) non-sterilized (■□) EtO treated (●○), e-beam treated (▼▽), and γ treated (▲△).
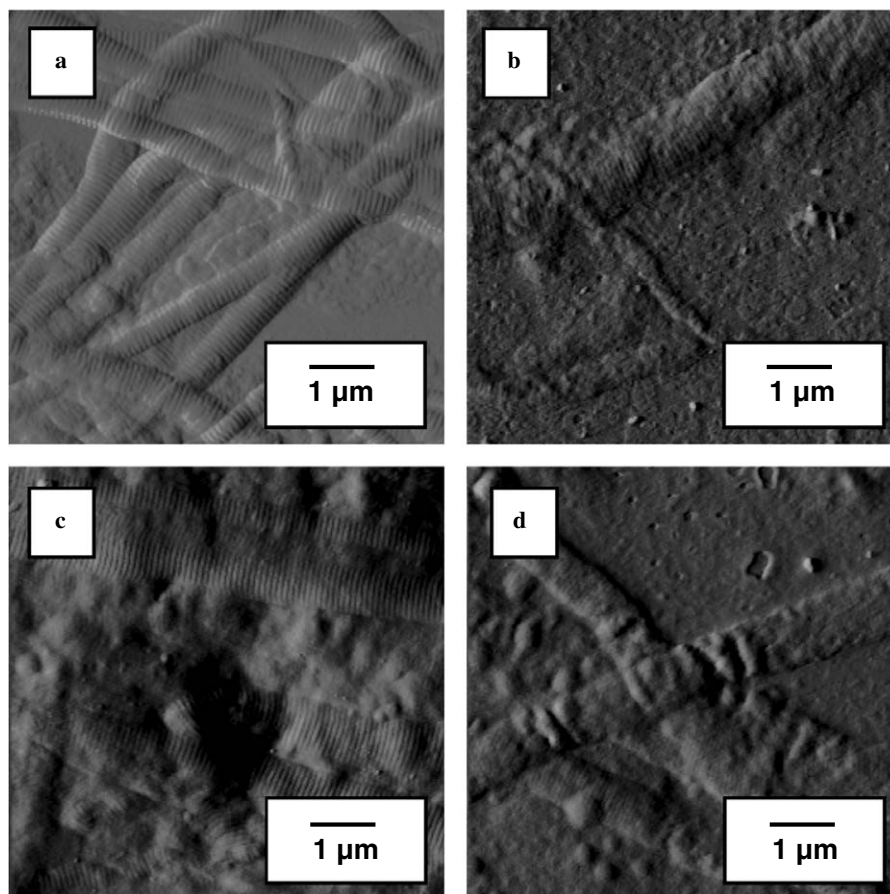
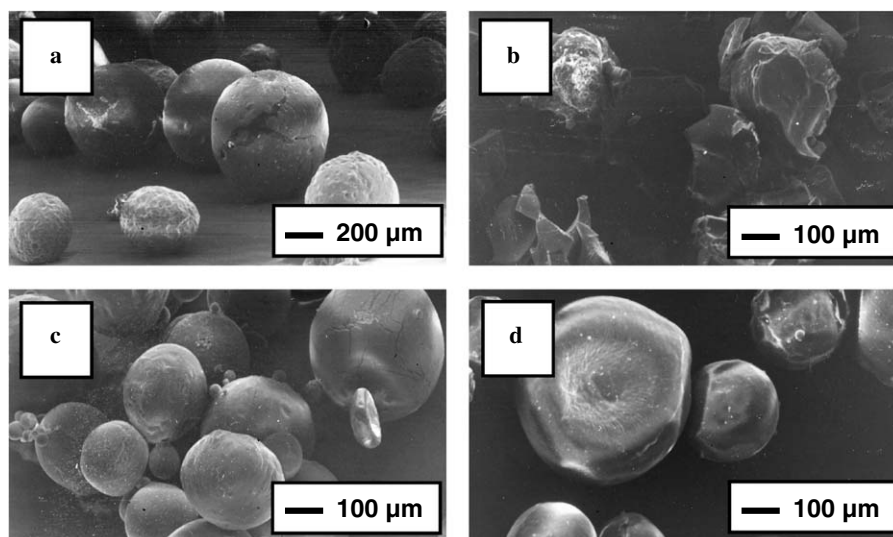Fig. 2. AFM of redispersed collagen fibers: non-sterilized (a), EtO treated (b), e-beam treated (c), and γ treated (d).

Fig. 4. SEM of microparticles: non-sterilized (a), EtO treated (b), e-beam treated (c), and γ treated (d).

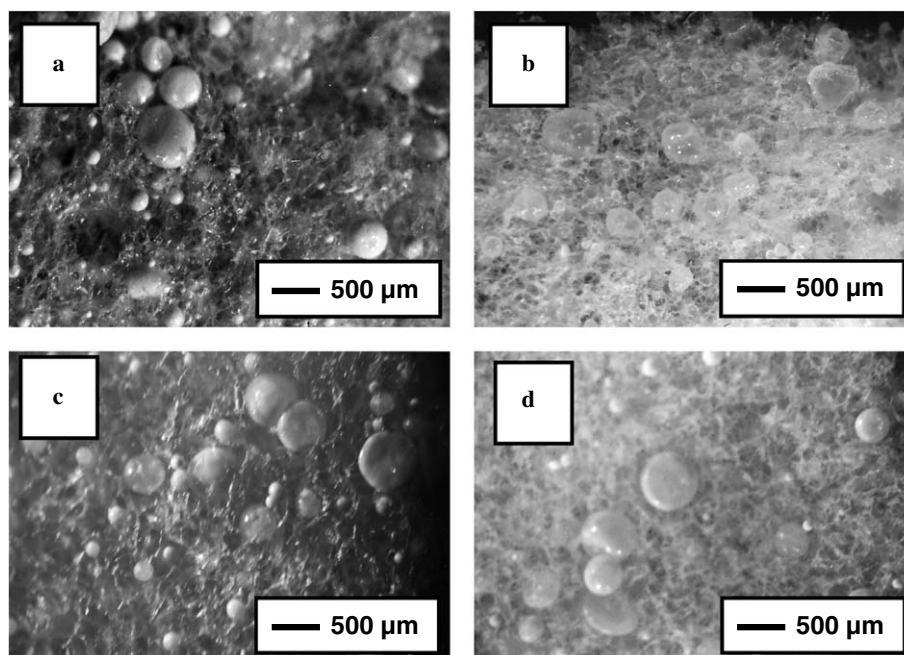

Fig. 5. Macroscopic appearance of collagen/PLGA-microparticle composites: non-sterilized (a), EtO treated (b), e-beam treated (c), and γ treated (d).

Microscopy of MPs and composites provides insight into the reason for the marked increase in initial GM release. Sterilization with EO resulted in a collapse and agglomeration of the MPs (Figs. 4a and b, and 5a and b). Whereas Choi et al. [31] observed a similar effect for poly-(L-lactide) microparticles, Jeong et al. [32] did not notice a morphological change in Resomer® 503 H microparticles – both sterilizing at 37 °C and reduced pressure. The particles embedded in composites appear transparent after EO-treatment and also present deformed structures. The sponge structure itself, however, remains unaffected by sterilization as found by Olde Damink et al. [20]. Despite the low sterilization temperature of 32 °C, which is below

the T_g, deformation of the microparticles occurs as a consequence of the high pressure of 4 bar. Thus, EO-gassing under the applied conditions (32 °C, 4 bar for 6 h) is unsuitable for the composite.

3.2. Sterilization by irradiation

3.2.1. m_w, T_g, and NMR spectroscopy of PLGA

The molecular weight and the glass transition temperature of RG 503 and RG 502H bulk powder as well as the 50/50 RG 503/RG 502H microparticles prior and after irradiation are summarized in Table 1. As described in the literature, γ-irradiation of PLA- and PLGA-materials

leads to a decrease in the molecular weight and the glass transition temperature of the polymers [7,15,16,33]. The m_w is reduced by 12–24% after irradiation and the m_n by 9–40%. The effect is more pronounced than with EO-treatment. Polydispersity is not changed significantly by irradiation. On the one hand, ester bonds are cleaved at random, while on the other hand irradiation induced cross-linking is discussed [34–36] and the decrease in molecular weight has been shown to be irradiation dose-dependent. In addition, the properties of the polymers influence the decrease in m_w and T_g [17,18], and the influence on m_w has been shown to be more significant for hydrophilic non-end-capped polymers like RG 502H due to the higher sensitivity of free carboxylic groups to irradiation as compared to end-capped polymers like RG 503 [7]. The T_g is reduced by 4–6 °C, similar as with EO-treatment. As shown for EO-treatment, additional ^{1}H NMR spectroscopy of the polymers after irradiation did not indicate changes in the chemical structure of the polymers (Fig. 6a shows the spectra of γ-irradiated RG 502H as an example). The lactide/glycolide ratio in the polymers after irradiation is still 1:1.

3.2.2. NMR spectroscopy of GM

The NMR spectra had demonstrated attachment of EO to GM after gas sterilization. Treatment of drugs with γ-irradiation has frequently been described as a possibility of sterilization without induction of chemical changes [34,35,8]. As shown in Figs. 6b and c (shown for γ-irradiated GM as an example), sterilization with β- and γ-irradiation did not induce changes in the NMR spectra, specifically in the 3.8–4 ppm region.

3.2.3. AFM of collagen

γ-Irradiation has been described to induce the cleavage of covalent bonds in collagen material leading to enhanced susceptibility to enzymatic degradation and reduced mechanical strength [20]. AFM of the material tested demonstrated, that the collagen fibers still present the characteristic banding pattern after β- and γ-irradiation (Figs. 2c and d) and a few spots without this striation. Deformed fibers can be detected after irradiation, however, to a lesser extent than after EO-gassing.

3.2.4. ESR spectroscopy

During irradiation, radicals are formed which could lead to chemical changes in drugs, excipients, and drug products both during sterilization itself as well as upon storage. Radicals sustain in the sterilized product as a function of irradiation dose and material properties [8,15]. The presence of free radicals was analyzed by ESR-spectroscopy. Tests were performed on GM, Resomer RG 502H and 503 powder, PLGA microparticles, insoluble collagen sponges, GM-loaded PLGA microparticles and composites prior to sterilization and 24 h, 7 and 28 days post-sterilization. As shown in Fig. 7a (after β-sterilization as an example) the spectra of the polymers prior and 24 h after irradiation do not show deflections of the baseline which could indicate the presence of free radicals showing spin resonance. Thus, radicals formed during sterilization could recombine due to the polymer chain mobility or participate in reactions leading to diamagnetic products [15]. Differences in the spectra between RG 503 and RG 502H due to esterified or free terminal acid group could not be detected and should not have an influence on the formation and stability of radicals [7].

The spectrum of the unsterilized collagen sponge already shows a slight deflection of the baseline (Fig. 7b). The deflection is caused by the porous sponge structure which changes resonance conditions and the pattern is not changed after β- or γ-irradiation (Fig. 7b after γ-sterilization as an example).
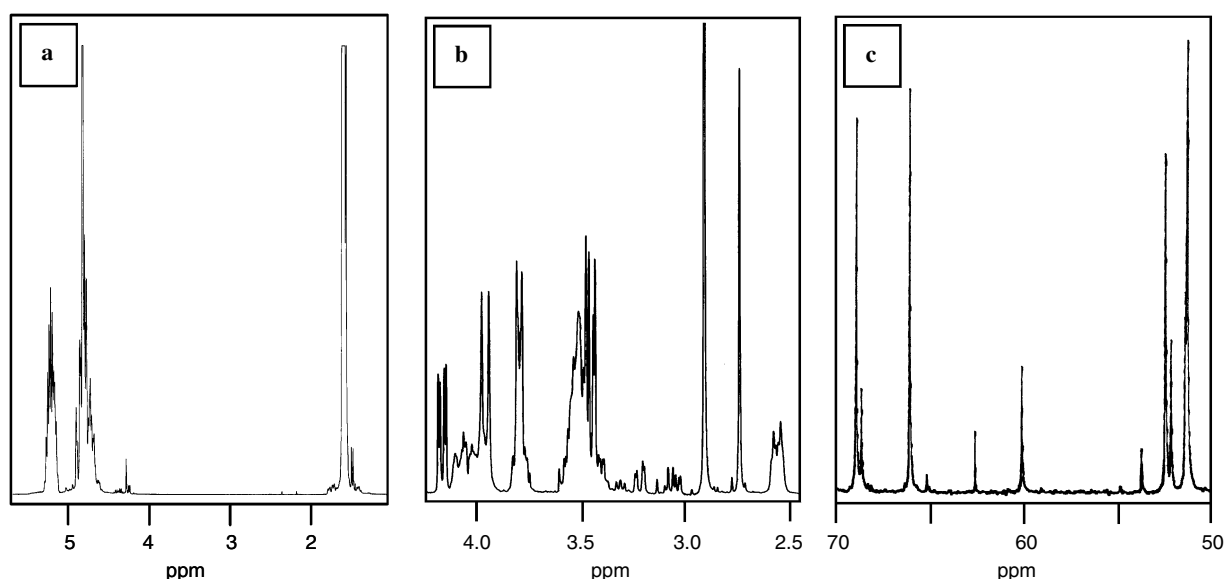

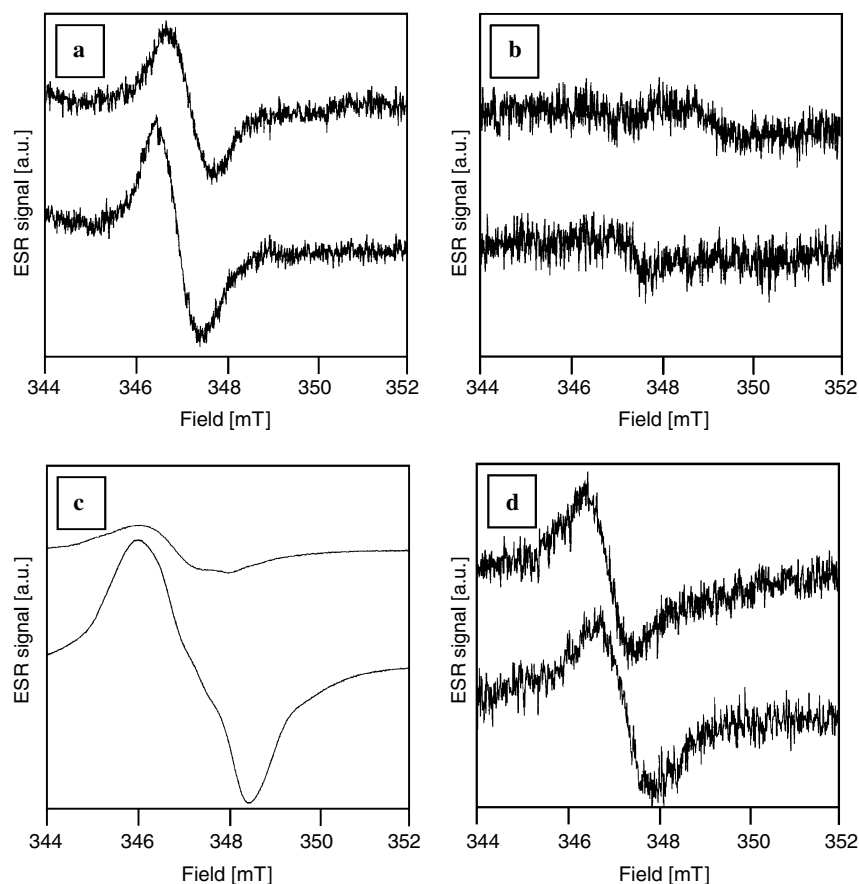Fig. 6. ^{1}H NMR spectrum of γ-treated RG 502H (a), γ-treated gentamicin (b), and ^{13}C NMR spectra of γ-treated gentamicin (c).

Fig. 7. ESR-signal non-sterilized (top) and 24 h after irradiation treatment (bottom) of RG 503 e-beam treated (a), collagen sponge γ treated (b), gentamicin e-beam treated (c), and collagen/PLGA-microparticle composite γ treated (d).

Significant resonance can be seen in the spectrum of irradiated GM bulk drug substance. Whereas the spectrum of non-sterilized GM does not show any deflection of the baseline, the spectra 24 h after β- or γ-sterilization show resonance phenomena (Fig. 7c after β-sterilization as an example). The signal intensity after 24 h was higher in β-sterilized material as compared to γ-irradiated. At day 7 after irradiation, the intensity of the signal was reduced to 20% and 30%, respectively of the intensity on day one for β- and γ-sterilized material. After 28 days, no radicals were detected and the spectra corresponded to those of non-irradiated samples. As shown by the ^{1}H NMR spectroscopic investigation, chemical changes in GM caused by the formation of radicals could not be detected. Since the radicals persist not longer than 4 weeks, long-term stability problems are unlikely to occur.

The resonance of PLGA microparticles depends on the GM loading. Spectra of non-loaded microparticles prior and after irradiation did not show a deflection of the baseline. In the spectra of GM-loaded microparticles, a deflection at ≈350 mT was observed prior to sterilization and an additional signal was found at 348 mT after irradiation. At day 28 after sterilization, the spectra were identical to the ones obtained for non-irradiated samples. Thus, GM radicals are less pronounced in GM-loaded microparticles than in GM bulk substance.

The spectra of non-irradiated collagen/GMPM-composites with additional free GM were similar as the ones for pure collagen sponges (Fig. 7d after γ-sterilization as an example). After irradiation, changes in shape or position of the deflection could not be detected, indicating either strongly reduced formation of much shorter duration of GM radicals formed in the composite.

3.2.5. GM release after irradiation

The GM release profile from irradiated microparticles and composites is shown in Fig. 3. The amount of GM liberated within the first 4 h was increased to ≈33% for γ-irradiated MPs as compared to 12% for β-irradiated and non-sterilized particles. After the first day, 28% were released from e-beam-sterilized samples and 37% from γ-sterilized particles whereas the release stayed at 12% for non-sterilized samples. The remaining amount of drug was released from both irradiated and non-sterilized MPs during the following week with similar profiles after day 3. For all composites, initial GM release within 4 h reached 60% followed by sustained release over 1 week. Thus, despite the decrease in m_w and T_g caused by γ- and β-sterilization, the GM release profile from both MPs and composites was not dramatically changed. Yoshioka et al. [17] describes a dose-dependent change in the release profile from PLA microparticles.

Additional microscopic images of microparticles and composites (Figs. 4c and d, and 5c and d) demonstrated their integrity after β- and γ-sterilization as compared to EO-treatment. In a study by Montanari et al. [7], the morphology of RG 503 microparticles was preserved after γ-irradiation. Even though irradiation caused a similar decrease in T_g as EO-gassing, structural collapse did not occur. This indicated that the higher pressure during EO-sterilization is the reason for microparticle deformation and agglomeration. Since the material might slightly warm up during irradiation, the temperature increase during EO-treatment to 32 °C is probably not the decisive factor for the microparticle collapse. Similar as after gas sterilization, the porous structure of the composites appeared to be unaffected by irradiation.

Standard release tests were performed with UV-detection of GM after derivatization. Since ESR spectroscopy indicated significant radical formation, the biological activity of GM released was tested. Microbial assays are requested by the pharmacopoeias for antibiotics which are multicomponent mixtures like GM. Therefore, the amount of GM released from β-sterilized composites as well as commercially available GM-loaded collagen sponges sterilized with EO was analyzed by both UV-detection and microbiologically. Table 2 summarizes the amounts quantified by UV-detection and the microbiological activity of the GM released as the ratio of the GM concentration in the release medium quantified microbiologically to photometrically. In all cases, the deviation between microbiological test and standard UV-detection was at maximum 16%. This difference is within the error of the microbiological assay which is less precise, e.g., caused by the inaccuracy in the measurement of the diffusion radius [37]. Based on the GM concentrations obtained microbiologically the same overall GM release profile from collagen/PLGA-microparticle composites results in 50% release within the first day followed by sustained release of the other 50% over 1 week. Thus, the microbiological

assay demonstrated that β-irradiation of the composites did not lead to significant loss in GM activity. Stemberger et al. [30] had found only a small decrease in microbiological activity of the aminoglycoside antibiotics GM and netilmicin after EO-sterilization of collagen sponges.

3.3. Short-term stability testing

In order to analyze any short-term storage effects of irradiation on stability, β-sterilized GM containing PLGA microparticles as well as composites stored at 4 °C/35% r.h., 25 °C/60% r.h., and 40 °C/75% r.h. over 12 weeks was tested for polymer m_w and T_g as well as appearance and GM release.

3.3.1. m_w and T_g of PLGA-polymers and microparticles

Storage at 4 °C/35% r.h. did not lead to a decrease in m_w either for RG 503 and RG 502H powder for GM-loaded microparticles over 12 weeks (Table 3). Whereas the T_g remained constant for RG 503 and 50/50 RG 503/RG 502H microparticles, the glass transition temperature of RG 502H decreased by 7 °C from 42 to 35 °C over 3 months. At 25 °C/60% r.h. no marked changes in m_w during 4 weeks were observed. The T_g during that time stayed consistent for RG 503 and the GPMPs, however, decreases to 32 °C in case of RG 502H. After 12 weeks at 25 °C/60% r.h. the m_w of RG 503 and 50/50 RG 503/RG 502H microparticles decreased slightly whereas the T_g of RG 503 was unchanged and for microparticles it was reduced by 3 °C. RG 502H was melted after 12 weeks and showed a m_w of only 1500 Da. Storage at 40 °C/75% r.h. had a deleterious effect on the polymers and the microparticles. RG 502H and the microparticles liquefied already after 4 weeks with a dramatic decrease in m_w. RG 503 showed a T_g of only 33 °C and a m_w of 2400. After 12 weeks, m_w and T_g could not be analyzed in any sample. As found by Spenlehauer et al. [16] m_w of PLA and PLGA polymers significantly

Table 2
GM concentration in release medium quantified via UV-detection after derivatization and microbiological potency (c_{UV}/c_{mb} *100) of GM released from e-beam-treated collagen/PLGA-microparticle composites and EO sterilized gentamicin-loaded collagen sponge (* referring to photometric analysis) ($n = 3$)

Sample	Time point (d)	GM concentration (mg/ml)	Microbiological potency (%)
Composite	0.2	2.25 ± 0035	115 ± 4
	1	0.60 ± 0.050	84 ± 13
	2	0.73 ± 0.030	99 ± 5
	3	0.94 ± 0.161	104 ± 8
	4	0.577 ± 0.070	100 ± 8
	5	0.417 ± 0.020	90 ± 2
	6–11	0.090 ± 0.018	105 ± 16
GM containing IC sponge	0.2	1.42 ± 0.096	84 ± 4
	0.2–11	0.14 ± 0.030	83 ± 29

Table 3
Molecular weight weight-average (m_w) and glass transition temperature (T_g) of e-beam-treated RG 503 and RG 502H bulk material as well as 50/50 RG 503/RG 502H MPs upon storage

	4 °C/35% r.h.		25 °C/60% r.h.		40 °C/75% r.h.	
	m_w (Da)	T_g (°C)	m_w (Da)	T_g (°C)	m_w (Da)	T_g (°C)
RG 503						
$t = 0$	29,000 Da/43 °C					
4 weeks	28,000	44	28,000	44	2,400	33
12 weeks	28,000	44	25,200	44	n.d.	n.d.
RG 502H						
$t = 0$	10,300 Da/42 °C					
4 weeks	10,000	38	10,000	32	<400	n.d.
12 weeks	10,000	35	1,500	n.d.	n.d.	n.d.
MPs						
$t = 0$	18,400 Da/41 °C					
4 weeks	18,000	41	16,600	41	<400	n.d.
12 weeks	18,000	41	12,300	38	n.d.	n.d.

n.d., not detectable ($n = 3$).

decreased at storage above 4 °C. Wang et al. [38] observed a decrease in m_w and T_g for PLA/PLGA polymers and microparticles due to higher storage temperatures and humidities. Non-end-capped polymers like RG 502H are more hydrophilic due to the free carboxylic group and take up water faster and to a larger extent than polymers with a similar m_w but end-capped carboxylic function [3]. This manifested itself in faster hydrolysis and a more rapid decrease in T_g due to the plasticizer effect of water. Whereas the water content was below 2% over 12 weeks for RG 503 and the microparticles stored at 25 °C/60% r.h., it increased to approx. 5% for RG 502H after 4 weeks. The plasticizer effect of water could explain the initial decrease in T_g without strong decrease in m_w followed by an enhanced polymer hydrolysis. In addition, this effect of moisture on RG 502H resulted in significant hydrolysis and the decrease in T_g of the 50/50 RG 503/502H microparticles after 12 weeks at 25 °C/60% r.h.. At 40 °C/75% r.h., the water content of RG 502H increased dramatically to 5% after 2 week, 10% after 4 weeks to 67% after 12 weeks. As the content of highly hygroscopic and liquid D,L-lactic acid – also forming hydroxycarboxylic acid oligomers – as the end-product of polymer hydrolysis increases, the water content of the degraded polymer rises. At 40 °C/75% r.h. the water content of RG 503 only slightly increased to 3% after 12 weeks whereas the water content of the MPs reached 38%.

3.3.2. GM release from microparticles and composites

In addition to the physicochemical properties of the microparticles, the GM release was tested. Due to melting of the particles stored at 40 °C/75% r.h., only samples stored at 4 °C/35% r.h. and 25 °C/60% r.h. were investigated. Upon storage at 4 °C/35% r.h. for 4 and 12 weeks the release profile exhibited only a slight increase in the amount released at day 1 and 2, whereas the initial release within the first 4 h of 15% and the sustained release over 1 week remained unaffected (Fig. 8). After 4 weeks at 25 °C/60% r.h., the initial burst was increased by 8% and after 12 weeks by almost 30%. The GM release was completed after
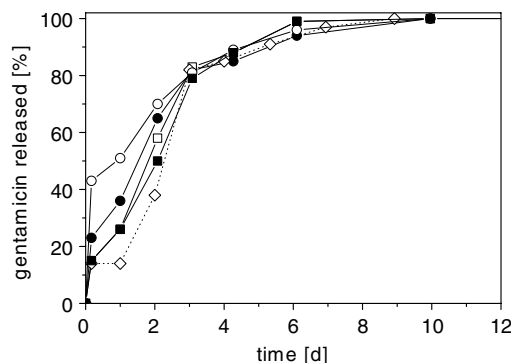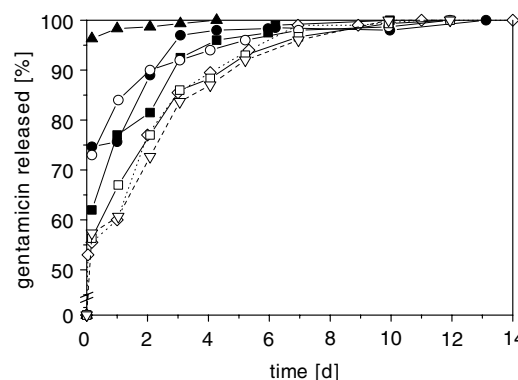
Fig. 9. Gentamicin release from collagen/PLGA-microparticle composites: $t = 0$ (◇) 4 weeks at 4 °C/35% r.h. (■) 12 weeks at 4 °C/35% r.h. (□), 4 weeks at 25 °C/60% r.h. (●) and 12 weeks at 25 °C/60% r.h. (○), 4 weeks at 40 °C/75% r.h. (▲), and 12 weeks at 22 °C/desiccator (▽).

1 week. As shown in Table 3 the m_w of the microparticles after 12 weeks of storage at 25 °C/60% r.h. was 12,300 which is below the critical m_w for particle collapse [3] and leads to faster GM liberation.

For composites the GM release profile did not change after 4 or 12 weeks storage at 4 °C/35% r.h. with an initial boost of 60% followed by sustained release for approximately 1 week (Fig. 9). For composites stored at 25 °C/60% r.h. the burst was increased corresponding to the results for GM-loaded microparticles. In contrast, liberation of GM from composites stored for 12 weeks at r.h. <10% and 25 °C was identical to the profile at $t = 0$. This demonstrated the importance of the relative humidity on polymer hydrolysis. Microscopic images of the composites stored at 4 °C demonstrated no changes in the structure and appearance of the composites (Figs. 10a and b). Macroscopically a fraction of translucent microparticles and some melted material could be identified in composites stored at 25 °C after 12 weeks (Fig. 10c) and a polymer layer originating from melted particles on the collagen sponge structure could be visualized. Corresponding to the partial collapse the initial release from the composites is increased after 12 weeks of storage at 25 °C/60% r.h. The GM release from composites stored for four weeks at 40 °C/75% r.h. reached 100% drug liberation within 4 h (Fig. 8). Due to the low m_w and T_g of the microparticles stored under these conditions (Table 3), and their melting GM release is accelerated dramatically. Upon storage at 40 °C/75% r.h. the MPs had melted in the composites and microscopically a spread polymer coat could be identified on the surface of the collagen structures (Fig. 10d).

In conclusion, EO-gassing, β- and γ-irradiation were tested for sterilization of GM-loaded PLGA microparticles/collagen composites. EO led to chemical modification of the drug GM and a slight reduction in PLGA T_g and m_w, and mechanical collapse of the microparticles. Irradiation treatment also caused a similar decrease in T_g and m_w of the polymers. Radicals occurred in pure GM bulk substance but could not be detected in the composite. Since

Fig. 8. Gentamicin release from 50/50 RG 503/RG 502H microparticles: $t = 0$ (◇) 4 weeks at 4 °C/35% r.h. (□) 12 weeks at 4 °C/35% r.h. (■), 4 weeks at 25 °C/60% r.h. (●) and 12 weeks at 25 °C/60% r.h. (○).
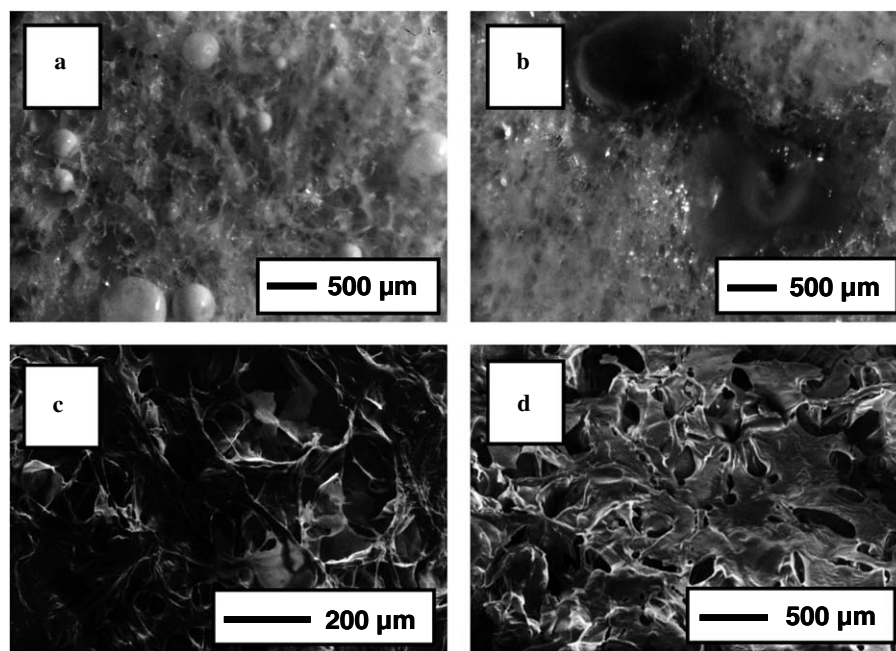
Fig. 10. Appearance of collagen/PLGA-microparticle composites: 4 weeks at 4 °C/35% r.h. (a), 12 weeks at 4 °C/35% r.h. (b), SEM after 12 weeks at 25 °C/60% r.h. (c), and after 4 weeks at 40 °C/75% r.h. (d).

the release profile is only marginally affected by irradiation, this sterilization procedure appears to be more appropriate for the implantable systems studied.

Acknowledgements

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