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Removal of chloroform from biodegradable therapeutic microspheres by radiolysis

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Abstract

Radioactive holmium-166 loaded poly(L-lactic acid) microspheres are promising systems for the treatment of liver malignancies. These microspheres are loaded with holmium acetylacetonate (HoAcAc) and prepared by a solvent evaporation method using chloroform. After preparation the microspheres (Ho-PLLA-MS) are activated by neutron irradiation in a nuclear reactor. It was observed that relatively large amounts of residual chloroform (1000–6000 ppm) remained in the microspheres before neutron irradiation. Since it is known that chloroform is susceptible for high-energy radiation, we investigated whether neutron and gamma irradiation could result in the removal of residual chloroform in HoAcAc-loaded and placebo PLLA-MS by radiolysis. To investigate this, microspheres with relatively high and low amounts of residual chloroform were subjected to irradiation. The effect of irradiation on the residual chloroform levels as well as other microsphere characteristics (morphology, size, crystallinity, molecular weight of PLLA and degradation products) were evaluated.

No chloroform in the microspheres could be detected after neutron irradiation. This was also seen for gamma irradiation at a dose of 200 kGy phosgene, which can be formed as the result of radiolysis of chloroform, was not detected with gas chromatography–mass spectrometry (GC–MS). A precipitation titration showed that radiolysis of chloroform resulted in the formation of chloride. Gel permeation chromatography and differential scanning calorimetry showed a decrease in molecular weight of PLLA and crystallinity, respectively. However, no differences were observed between irradiated microsphere samples with high and low initial amounts of chloroform.

In conclusion, this study demonstrates that neutron and gamma irradiation results in the removal of residual chloroform in PLLA-microspheres.

Keywords: Holmium; Microspheres; PLLA; Irradiation; Residual solvents; Chloroform

1. Introduction

Radionuclide loaded microspheres are attractive and promising systems for the treatment of liver malignancies. When microspheres with a size between 20 and 50 μm are administered into the hepatic artery of patients suffering from liver malignancies, they will preferentially lodge in and around the tumour and subsequently irradiate the surrounding tissue (Nijsen et al., 2002b). Regarding its physical properties, holmium-166 is the ideal radionuclide for such therapies because it is the only element which can be neutron-activated to a beta- and gamma-emitter with a logistically favourable half-life, and which can also be visualized by MRI (Nijsen et al., 2002b, 2004). Using a solvent evaporation technique, non-radioactive holmium-165 can be incorporated into poly(L-lactic acid) (PLLA) microspheres as its acetylacetonate complex (HoAcAc). In a subsequent step the microspheres (Ho-PLLA-MS) can be rendered radioactive by neutron irradiation (Nijsen et al., 1999).

Organic solvents such as chloroform are widely used for the preparation of PLLA-microspheres (O'Donnel and McGinity, 1997; Chung et al., 2001), and it is also the solvent of choice for the preparation of Ho-PLLA-MS (Nijsen et al., 1999). However, these solvents are difficult to remove quantitatively and consequently traces hereof remain in the microspheres (Benoit et al.,

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1986; Mumper and Jay, 1992; Koegler et al., 2002). The ICH-guidelines (The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) prescribe a very low limit of 60 ppm for chloroform in pharmaceuticals (B'Hymer, 2003). Methods currently applied to reduce the organic solvent levels in polymeric microparticles are drying at elevated temperatures and reduced pressure (Freitas et al., 2005) or extraction using super critical carbon dioxide (Koegler et al., 2002; Herberger et al., 2003).

It is known that chloroform is highly susceptible for decomposition with high-energy radiation (Taghipour and Evans, 1997). Microspheres receive a very high radiation dose in a nuclear reactor (Nijsen et al., 2002a), and thus it is possible that residual chloroform decomposes during neutron activation resulting in the reduction or complete removal of the solvent. However, it should be realized that previous studies concerning the effect of radiation on residual solvents were done in a different setting. In particular, the removal of chlorinated hydrocarbons from wastewater using UV and gamma irradiation has been demonstrated. However, radiolysis of chlorinated solvents in polymer matrices has not been investigated before.

Provided that removal of residual solvent in microspheres can be achieved by irradiation, it is furthermore extremely important that the reaction products, which are the result of radiolysis, are not harmful for patients. The papers which have been published about the effect of UV and gamma irradiation on chlorinated hydrocarbons report on the formation of chloride as an end product of radiolysis (Dowideit et al., 1996; Taghipour and Evans, 1997; Hatashita et al., 2001; Mucka et al., 2003). However, another reaction radiolysis product that could be formed is the very toxic phosgene (Keeler et al., 1990; Dowideit et al., 1996; Chen et al., 2002). It is thus important to get a clear insight into the radiolytic pathway(s) of residual chlorinated solvents in polymeric microparticles.

In this study, we investigated whether neutron and gamma irradiation will result in the reduction/removal of residual chloroform in HoAcAc-loaded and placebo PLLA-microspheres. Microspheres prepared with a solvent evaporation process were dried at 50 or 70 °C under vacuum in order to obtain samples with high and low levels of residual chloroform and subsequently neutron irradiated or gamma irradiated with various dosages.

The microspheres were studied for their morphology, residual solvent levels, degradation products and for the molecular weight and crystallinity of PLLA.

2. Materials and methods

2.1. Materials

All chemicals were commercially available and used as obtained. Acetylacetone, 2,4-pentanedione (AcAc; >99%), chloroform ($CHCl_3$; HPLC-grade), poly(vinyl alcohol) (PVA; average M_w 30,000–70,000, 88% hydrolyzed), ammonium hydroxide (NH_4OH; 29.3% in water) were supplied by Sigma–Aldrich (Steinheim, Germany). Sodium hydroxide (NaOH; 99.9%) was purchased from Riedel-de Haën (Seelze, Germany). Holmium(III) chloride hexahydrate ($HoCl_3{\cdot}6H_2O$; 99.9%) and dichloromethane (CH_2Cl_2; HPLC-grade) were obtained from Phase Separations BV (Waddinxveen, The Netherlands). Poly(L-lactic acid) (PLLA; intrinsic viscosity 0.09 dl/g in chloroform at 25 °C) was purchased from Purac Biochem (Gorinchem, The Netherlands). Hydrochloric acid (HCl; 37%), nitric acid (HNO_3; 65.0%), silver nitrate ($AgNO_3$; 99.9%) and ethyl acetate (99.9%) were purchased from Merck (Darmstadt, Germany).

2.2. Preparation of HoAcAc and microspheres

HoAcAc was prepared as described previously (Nijsen et al., 1999). In brief: acetylacetone (180 g) was dissolved in water (1080 g). The pH of this solution was brought to 8.50 with an aqueous solution of ammonium hydroxide. Holmium chloride (10 g dissolved in 30 ml water) was added to this solution. After 15 h incubation at room temperature, the formed HoAcAc crystals were collected by centrifugation and washed with water. HoAcAc (10 g) and PLLA (6 g) were dissolved in 186 g chloroform. The resulting homogeneous solution was added to 1 l of an aqueous solution of PVA (2%). The mixture was stirred (500 rpm) for 40 h at room temperature and the formed microspheres were collected by centrifugation. The microspheres were washed three times with water, three times with 0.1 M HCl and finally three times with water. The washed microspheres were fractionated according to size using stainless steel sieves with a pore size of 20 and 50 μm, with a wet sieving system consisting of an Electromagnetic Sieve Vibrator (EMS 755) combined with an Ultrasonic Processor (UDS 751) (both from Topas GmbH, Dresden, Germany). The collected microsphere fraction (about 4 g, size between 20 and 50 μm) was divided into two equal portions of 2 g and dried at 50 °C for 48 h at normal pressure or at 70 °C under vacuum for 5 h using a rotating Glass Oven (B-580 GKR, Buchi). Placebo PLLA-microspheres without HoAcAc loading were prepared in the same way. All microsphere batches were prepared in duplicate; ~500 mg per batch was packed in polyethylene vials.

2.3. Neutron and gamma irradiation

Routinely, microspheres were neutron-activated in a reactor facility in Delft or gamma irradiated with various dosages. Since the reactor facility of Petten was used in previous studies of our group (Nijsen et al., 1999, 2002a), some microsphere batches were also neutron irradiated at this facility. Neutron irradiations were performed in the pneumatic rabbit system (PRS) in the reactor facilities in Delft and Petten. The thermal neutron flux in the Delft facility was $5 \times 10^{12}\ cm^{-2}\ s^{-1}$, while the thermal neutron flux in Petten was $30 \times 10^{12}\ cm^{-2}\ s^{-1}$. The irradiation times were 6 and 1 h, respectively, to ensure equal doses of microsphere-associated radioactivity (~14 GBq, immediately after irradiation).

Gamma sterilization of the samples with a dose of 25.0 kGy was performed using a cobalt-60 source (Isotron, Ede, The Netherlands). A Gammacell 200 cobalt-60 high dose rate research irradiator (Nordion, Canada) was used for irradiation

of the samples with higher dosages from 100 up to 1000 kGy. Also, this irradiator was used to study the effect of temperature during irradiation because differences in temperature in the Petten and Delft reactor facilities were expected due to differences in their thermal neutron fluxes (Mumper et al., 1991). To ensure low levels of microsphere-associated radioactivity, analyses of the neutron irradiated samples were performed after 1 month storage at room temperature in closed vials.

2.4. Determination of holmium and water content in microspheres

The holmium content in microspheres was determined by a complexometric titration as described before (Zielhuis et al., 2005). The water content in the microspheres was determined with the Karl–Fisher method. Therefore, 50 mg microspheres were dissolved in 1 ml of Hydranal Coulomat A (Riedel-de Haën) and the water concentration was determined using a Mitsubishi moisture meter model CA-05 (Tokyo, Japan) from which the residual water content of the microspheres was calculated.

2.5. Determination of particle size distribution and evaluation of the surface morphology of the microspheres

The particle size distribution of radiated and non-radiated microspheres was determined using a Coulter counter (Multisizer 3, Beckman Coulter, The Netherlands) equipped with a 100-μm orifice.

The surface morphology of the Ho-PLLA-microspheres was investigated by scanning electron microscopy (SEM) using a Philips XL30 FEGSEM. A voltage of 5 or 10 kV was applied. Samples of the different microsphere batches were mounted on aluminium stubs and sputter-coated with a Pt/Pd layer of about 10 nm.

2.6. Determination of chloride and chlorine content

Gas chromatographic analyses were performed on a Shimadzu type 14 B GC equipped with a flame ionization detector, employing an OV-17 on Chromosorb W at 175 °C. The injection port and the detector temperature were 200 °C. Microsphere samples (50 mg) were dissolved in 2 ml of dichloromethane for the analysis of chloroform. Standards were prepared by adding varying amounts of chloroform to dichloromethane solutions containing PLLA (25 mg/ml) or both PLLA (12.5 mg/ml) and HoAcAc (12.5 mg/ml). The detection limit is defined as a signal-to-noise ratio of three (Miller and Miller, 2005). The concentration of chloroform in the microspheres (ppm) was converted to concentrations of chlorine (ppm) (1000 ppm chloroform corresponds with 892 ppm chlorine). To verify the results of the GC analyses, the total chlorine content of microspheres was also determined by neutron activation (NRG, Petten, The Netherlands) (Franca et al., 1999). Some selected samples with a residual solvent level below the detection limit of conventional GC were also subjected to headspace GC. These analyses were performed using European Pharmacopoeia method 2.4.24, 'Identification and Control of Residual Solvents for Water Insoluble Substances', and were carried out by Farmalyse B.V., Zaandam, The Netherlands.

The concentration of chloride in the different microspheres was determined using a precipitation titration. Therefore, microspheres (100 mg) were heated for 1 h at 100 °C to evaporate residual chloroform. GC analyses showed that the chloroform levels were indeed below detection limit. Thereafter, the microspheres were dissolved in 2 ml of 2 M NaOH at 100 °C and the resulting solutions were neutralized with 2 M HNO_3. The solutions were subsequently titrated with 0.005 M $AgNO_3$ and the endpoint was detected potentiometrically.

2.7. Gas chromatography–mass spectrometry (GC–MS)

GC–MS for the detection of phosgene after derivatisation with *N*,*N*-dibutylamine (DBA), according to the method of Schoene et al. (1993), was performed at The Netherlands Organisation for Applied Scientific Research (TNO, Prins Maurits Laboratory, Delft, The Netherlands) (in the results named as method 1). Neutron irradiated microspheres samples (∼50 mg), which were dried at 50 °C, were extracted with 1 ml hexane. Next, 20 μl of DBA was added and this solution was subsequently analysed. The detection limit of phosgene was determined by analysing solutions of phosgene in hexane, after the addition of 20 μl of DBA.

Identification of organic acids, including lactate, lactyl lactate and longer oligomers of lactic acid, was carried out by GC–MS on a Hewlett-Packard 5890 series II gas chromatograph linked to a HP 5989B MS-Engine mass spectrometer (in the results named as method 2). Prior to this GC–MS analysis, the organic acids were trimethylsilylated with *N*,*N*-bis(trimethylsilyl)trifluoroacetamide/pyridine/trimethylchlorosilane (5:1:0.05, v/v/v) at 60 °C for 30 min. The gas chromatographic separation was performed on a 25 m × 0.25 mm capillary CP Sil 19CB column (film thickness 0.19 mm) from Varian/Chrompack, Middelburg, The Netherlands.

2.8. Gel permeation chromatography (GPC)

The weight average molecular weight (M_w) and number average molecular weight (M_n) of PLLA were determined by GPC with two thermostated (35 °C) columns in series (PL gel Mixed-B, Polymer Laboratories) equipped with a refractive index detector (type 410, Waters, Milford, USA). Samples of approximately 5 mg were dissolved in 5 ml chloroform and filtered through 0.45 μm HPLC-filters (Waters). Elution was performed with chloroform and the flow-rate was 1 ml/min. The columns were calibrated using poly(styrene) standards of known molecular weights (Polymer Laboratories, Shodex and Tosoh, Amherst, USA). Analyses were performed in duplicate.

2.9. Differential scanning calorimetry (DSC)

Modulated DSC (MDSC) analysis was performed with a DSC Q1000 (TA Instruments, USA). Samples of approximately 5 mg were transferred into aluminium pans. Scans were recorded under 'heating only' conditions, with a heating rate of 1 °C/min

and cooling rate of 2 °C/min. The settings were periods of 30 s and a temperature modulation amplitude of 0.5 °C was applied. Samples were heated from 20 to 200 °C. The Universal Analysis 2000 software (Version 3.9A) was used for evaluation. Analyses were performed in duplicate.

3. Results and discussion

3.1. Preparation of microspheres

Ho-PLLA-MS with 17.0 ± 0.5% (w/w) of holmium, corresponding with a loading of the HoAcAc complex of ∼50% (w/w), were prepared using a solvent evaporation method with chloroform as organic solvent (Nijsen et al., 1999).

The water content of Ho-PLLA-MS and PLLA-MS batches dried at 50 °C for 48 h at normal pressure or at 70 °C under vacuum for 5 h was 2 ± 0.5% (w/w). Previous work from our group demonstrated that the amount of water in Ho-PLLA-MS had an influence on the microsphere characteristics after neutron irradiation in terms of the morphology and size distribution (Nijsen et al., 1999). Since the used drying method resulted in equal water contents, possible differences in the microsphere characteristics after irradiation are not caused by different amounts of water in the microspheres.

3.2. Particle size distribution and SEM analysis of microspheres

After sieving, more than 97% (volume-based) of the microspheres had a size between 20 and 50 μm. No differences in size distributions were observed after gamma irradiation (25 kGy), whereas after neutron irradiation more than 94% of the microspheres had a size between 20 and 50 μm.

SEM analysis of microspheres showed drying-related differences in their morphology. Ho-PLLA-MS that were dried at 50 °C had a smooth surface (Fig. 1a), whereas small fragments were released from the surface and the surfaces showed more roughness after neutron irradiation (Fig. 1b). Importantly, the microspheres retained their spherical character. Ho-PLLA-MS that were dried at 70 °C showed a surface that was slightly wrinkled (Fig. 1c). After neutron irradiation, their surfaces showed more roughness and also small fragments were formed (Fig. 1d). The formation of these fragments is very probably the cause of the small changes in the particle size distribution (97% versus 94%). After gamma irradiation no surface changes were observed using SEM analysis (not shown). Placebo PLLA-MS (before and after neutron irradiation) had the same morphology as Ho-PLLA-MS (SEM pictures not shown).

3.3. Determination of chlorine and chloride levels

The chloride and chlorine levels in the different microspheres dried at 50 °C before and after neutron irradiation and gamma irradiation are shown in Table 1. The (Ho)-PLLA-MS had a chloroform content, as determined by GC, varying from 1100 to 6700 ppm, which corresponds with 1000–6000 ppm chlorine. Table 1 also shows that the chlorine contents in non-irradiated microspheres as determined with neutron activation analysis and GC were similar, indicating that no residual chloride (from hydrochloric acid, that was used during the washing procedure) remained in the microspheres.

After the standard gamma sterilization dose of 25 kGy, no significant changes were seen for the chlorine concentration, and no chloride could be detected in (Ho)-PLLA-MS (see Table 1). The initial chlorine content of the microsphere batch was 2000 ppm and after irradition with a dose of 100 kGy, the chloride and

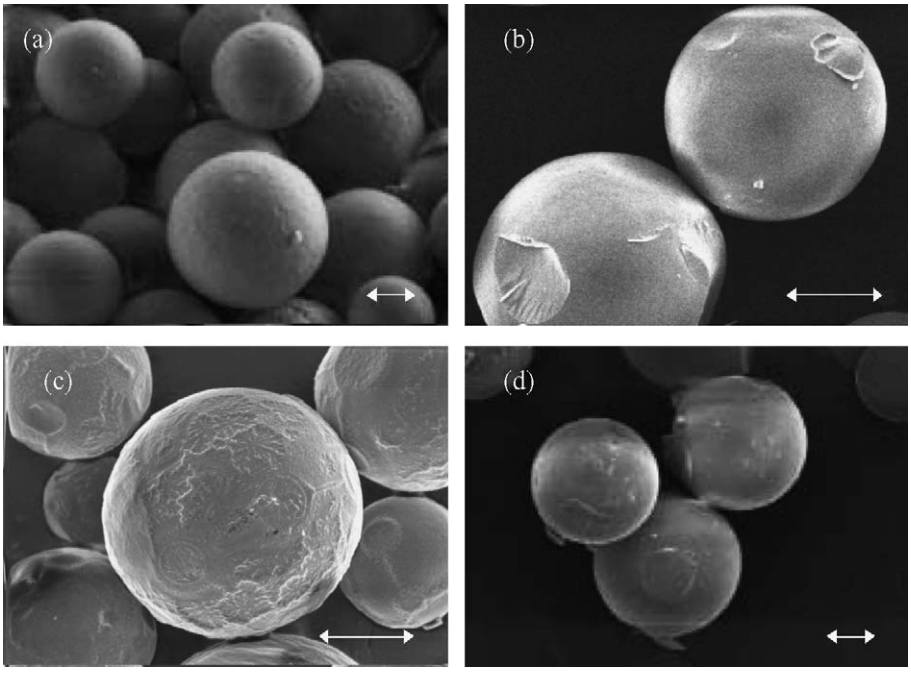

Fig. 1. SEM pictures of Ho-PLLA-MS dried at 50 °C before (a) and after (b) neutron irradiation and Ho-PLLA-MS dried at 70 °C before (c) and after (d) neutron irradiation. Bars represent 10 μm.

Table 1
Chlorine (Cl) and chloride (Cl^-) contents (in ppm ± 10%) in PLLA-MS and Ho-PLLA-MS

Irradiation[a]	Batch[b]	Cl before irradiation (GC)	Cl before irradiation (neutron activation analysis)	Cl after neutron irradiation (GC)	Cl^- after neutron irradiation (titration)
Neutron Delft	A	1000	1000	nd[c]	1000
Neutron Delft	B	1500	1500	nd	1500
γ 25 kGy	A	1000	1000	1000	nd
γ 25 kGy	B	1500	1500	1500	nd
Neutron Delft	C	4400	4400	nd	1000
Neutron Delft	D	1400	1400	nd	1500
γ 25 kGy	C	4400	4400	4400	nd
γ 25 kGy	D	1400	1400	1400	nd
Neutron Petten	E	6000	6000	nd	2000
Neutron Petten	F	5000	5000	nd	1700
Neutron Petten	G	5000	5000	nd	1700
Neutron Petten	H	1800	1800	nd	600
γ 100 kGy	I	2000	2000	1400	600
γ 200 kGy	I	2000	2000	nd	2000
γ 400 kGy	I	2000	2000	nd	2000
γ 500 kGy	I	2000	2000	nd	2000
γ 1000 kGy	I	2000	2000	nd	2000

Samples were dried at 50 °C and subsequently irradiated in different ways.
[a] Neutron irradiated in the Delft or Petten facility or gamma irradiated with various dosages.
[b] A, B, E and F are PLLA-MS batches and C, D, G, H and I are Ho-PLLA-MS batches.
[c] nd: not detectable.

chlorine levels were 600 and 1400 ppm. Chlorine was quantitatively converted into chloride after an irradiation dose of 200 kGy. This demonstrates that at higher doses of gamma irradiation radiolysis of $CHCl_3$ occurred. Table 1 shows that after neutron irradiation, no chloroform could be detected with GC in both HoAcAc-loaded and placebo PLLA-MS dried at 50 °C with high initial levels of chloroform. As for gamma irradiated samples, chloride was detected in these microspheres implying that radiolysis also had occurred after neutron irradiation.

Radiolysis of chloroform results in the formation of chloride, as was previously described by Hatashita et al. (2001) and Taghipour and Evans (1997):

Step 1. H_2O + gamma ray → $H_2O^+ + e^-$ (with a yield of 0.28 μmol per absorbed Joule). Taghipour and Evans (1997) furthermore reported the formation of $^\bullet H$, $^\bullet OH$, H_2O_2, H_2, OH^- and OH^+.

Step 2. $CHCl_3 + e^- \rightarrow {}^\bullet CHCl_2 + Cl^-$.

Step 3. Decomposition of $^\bullet CHCl_2$ by H_2O and O_2 to $2Cl^-$, CO, CO_2 and H_2O.

It is important to note that the above given radio lysis of chloroform occurs in water. In contrast, the water content in our microspheres is rather low (2%). It is, therefore, likely that other free radicals or 'lost electrons' are the initiators of the radiolysis of chloroform in PLLA-microspheres. Indeed, Montanari et al. (1998) described the radiolysis of PLGA and the formation of radicals by electron loss. Table 1 shows that chlorine was quantitatively converted into chloride for samples which were irradiated in the Delft facility (Table 1). In contrast, samples which were neutron irradiated in the Petten facility showed a chloride content which was about one third of the initial chlorine amount. The differences in chloride content between the two reactor facilities can be caused by a temperature difference during irradiation. However, varying the temperature in the facilities of either Delft or Petten to study the effect of temperature differences is impossible. Therefore, the effect of temperature during irradiation was studied at a fixed gamma dose of 200 kGy, since at this dose chlorine was also converted into chloride (Table 1). The temperature was varied between 30 and 70 °C and the results are given in Fig. 2. This graph shows after irradiation that the Cl^- concentration in the microspheres slightly decreased from 30 to 50 °C. Above 50 °C a strong decrease in Cl^- concentration was observed. DSC analysis (shown in Fig. 3) showed that the onset of the glass transition temperature of the microspheres started at 50 °C. Therefore, it is likely, that with increasing temperature chloroform evaporated particularly above the T_g of the PLLA matrices during irradiation, which resulted in lower Cl^- levels. Ho-PLLA-MS and PLLA-MS dried at 70 °C for 5 h had a chlorine content below the GC detection limit (~300 ppm chloroform). However, neutron activation analysis of these samples showed that their chlorine content varied from 50 to 100 ppm, correspond-

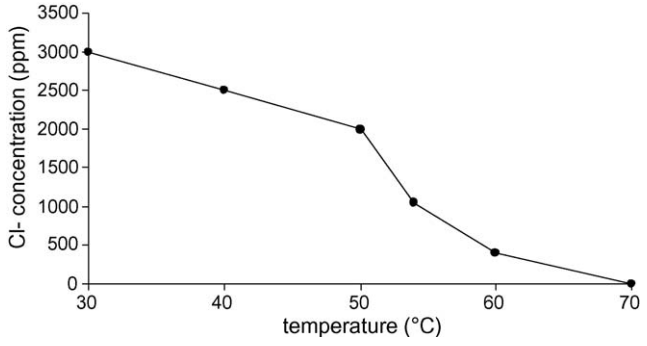

Fig. 2. Cl^- concentrations (ppm) in Ho-PLLA-MS dried at 50 °C after gamma irradiation with 200 kGy at varying temperatures.

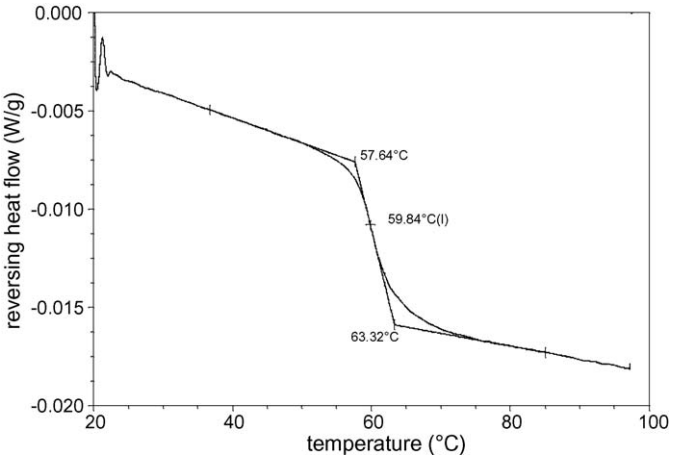

Fig. 3. Enlargement of the MDSC thermogram (reversing heat flow) of Ho-PLLA-MS irradiated with 200 kGy. The glass transition starts at 50 °C and the T_g was determined at 60 °C.

ing with 60–110 ppm chloroform. These levels are just above the earlier mentioned chloroform limit (from the ICH-guidelines) of 60 ppm. If these samples were subsequently neutron irradiated no chloroform could be detected using headspace GC, making Ho-PLLA-MS suitable for clinical application considering their residual solvent levels.

3.4. Gas chromatography–mass spectrometry

No phosgene could be detected in neutron irradiated microspheres using GC–MS (method 1), which means that the level was below detection limit (20 ppb). However, it cannot be excluded that phosgene was formed in microspheres during radiation. However, phosgene might have reacted with the water present in the microspheres (2%, Section 3.2). GC–MS (method 2) of PLLA-MS and Ho-PLLA-MS dried at 50 and 70 °C before irradiation showed that trace amounts of lactic acid were present. After neutron irradiation the amount of lactic acid increased substantially. Moreover, lactic acid oligomers like the lactyl lactate dimer, trimer and tetramer were also detected. We previously showed that chain scission of PLLA occurred during neutron irradiation of (Ho)-PLLA-MS (Nijsen et al., 2002a,b). The detection of lactic acid oligomers confirms these findings.

3.5. Molecular weight determinations

The molecular weights of PLLA in (Ho)-PLLA-MS before and after irradiation (gamma and neutron) are given in Table 2. GPC analysis of Ho-PLLA-MS showed that the M_w and M_n of PLLA were lower than the molecular weights in PLLA-MS. As reported before, this decrease in molecular weight is not caused by Ho-induced degradation of PLLA (Nijsen et al., 2001, 2002a), but is due to interactions between PLLA and Ho-AcAc which results in a decrease in the hydrodynamic volume of PLLA and thus in an apparent lower molecular weight. After gamma irradiation (25 kGy) the M_w and M_n of PLLA in PLLA-MS and

Table 2
Weight average and number average molecular weights of PLLA in Ho-PLLA-MS, PLLA-MS and PLLA references which were dried and subsequently irradiated in different ways

Sample	Drying procedure	Irradiation	M_w	M_n
PLLA-MS	50 °C	Non	85000	40000
PLLA-MS	50 °C	γ 25 kGy	45000	18000
PLLA-MS	50 °C	Neutron[a]	2200	1900
PLLA-MS	70 °C, vacuum	Non	84000	40000
PLLA-MS	70 °C, vacuum	γ 25 kGy	43000	17000
PLLA-MS	70 °C, vacuum	Neutron[a]	2200	2000
Ho-PLLA-MS	50 °C	Non	66000	48000
Ho-PLLA-MS	50 °C	γ 25 kGy	55000	31000
Ho-PLLA-MS	50 °C	Neutron[a]	1500	1200
Ho-PLLA-MS	70 °C, vacuum	Non	64000	48000
Ho-PLLA-MS	70 °C, vacuum	γ 25 kGy	53000	30000
Ho-PLLA-MS	70 °C, vacuum	Neutron[a]	1700	1200
Ho-PLLA-MS	50 °C	γ 100 kGy	9000	6000
Ho-PLLA-MS	50 °C	γ 200 kGy	6100	4700
Ho-PLLA-MS	50 °C	γ 400 kGy	4600	3300
Ho-PLLA-MS	50 °C	γ 500 kGy	3200	2500
Ho-PLLA-MS	50 °C	γ 1000 kGy	1600	1400
PLLA (control)	–	Non	88000	41000
PLLA (control)	–	γ 25 kGy	49000	21000
PLLA (control)	–	Neutron[a]	2400	2100

[a] Neutron irradiated in the Delft facility.

Ho-PLLA-MS decreased with ∼45 and ∼20%, respectively. This decrease in molecular weights is caused by chain scission induced by gamma irradiation (Montanari et al., 1998), and was independent of the applied drying procedure of the samples. Higher dosages of gamma irradiation (from 100 up to 1000 kGy) resulted in a further decrease of the molecular weight (M_w from 9000 to 1600 and M_n from 6000 to 1400 g/mol; Table 2).

In agreement with previous findings (Nijsen et al., 2002a) neutron irradiation of (Ho)-PLLA-MS caused a substantial decrease (∼95%) in the M_w and M_n of PLLA. Again, the changes in molecular weight due to neutron irradiation were independent of the residual chloroform levels of the microspheres.

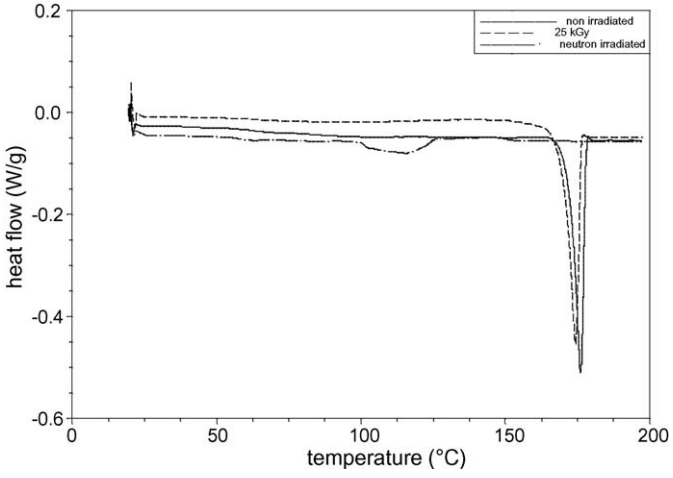

Fig. 4. MDSC thermograms (heat flow) of PLLA-MS before and after gamma irradiation (25 kGy) and neutron irradiation.

Table 3
DSC data of Ho-PLLA-MS, PLLA-MS and PLLA references which were dried and subsequently irradiated in different ways

Sample	Drying procedure	Irradiation	T_g (°C)	T_m, maximum melting enthalpy	
				°C	J/g[a]
PLLA-MS	50 °C	Non	70	176	55
PLLA-MS	50 °C	γ 25 kGy	68	174	49
PLLA-MS	50 °C	Neutron[b]	nd	116	12
PLLA-MS	70 °C, vacuum	Non	69	177	57
PLLA-MS	70 °C, vacuum	γ 25 kGy	60	173	52
PLLA-MS	70 °C, vacuum	Neutron[b]	nd	114	13
Ho-PLLA-MS	50 °C	Non	60	141	35
Ho-PLLA-MS	50 °C	γ 25 kGy	60	139	32
Ho-PLLA-MS	50 °C	Neutron	50	nd	nd
Ho-PLLA-MS	70 °C, vacuum	Non	62	135	40
Ho-PLLA-MS	70 °C, vacuum	γ 25 kGy	66	141	8
Ho-PLLA-MS	70 °C, vacuum	Neutron	50	nd	nd
Ho-PLLA-MS	50 °C	γ 100 kGy	63	143	23
Ho-PLLA-MS	50 °C	γ 200 kGy	60	129	15
Ho-PLLA-MS	50 °C	γ 400 kGy	59	nd	nd
Ho-PLLA-MS	50 °C	γ 500 kGy	58	nd	nd
Ho-PLLA-MS	50 °C	γ 1000 kGy	49	nd	nd
PLLA (control)	–	Non	66	176	54
PLLA (control)	–	γ 25 kGy	nd	176	49
PLLA (control)	–	Neutron	nd	119	15

[a] Corrected for HoAcAc loading.
[b] Neutron irradiated in the Delft facility.

3.6. Differential scanning calorimetry

Table 3 summarizes the results of the DSC analysis of the different microspheres and some representative thermograms are given in Figs. 4 and 5. No differences in the DSC pattern were observed between PLLA-MS which were dried at 50 °C for 48 h or at 70 °C under vacuum, for 5 h. Gamma irradiation (25 kGy) of PLLA-MS did not result in major changes in the DSC pattern (Fig. 4). However, after neutron irradiation both the melting temperature (T_m) and melting enthalpy decreased tremendously whereas no glass transition temperature (T_g) was detected (Fig. 4). Comparable DSC data were previously obtained with samples neutron irradiated in the Petten reactor facility (Nijsen et al., 2002a). Before irradiation, Ho-PLLA-MS had a lower T_g, T_m and melting enthalpy than non-loaded PLLA-MS (Table 3). Gamma irradiation (25 kGy) of Ho-PLLA-MS did not result in major changes in the DSC pattern (Fig. 5). However, with increased dose the T_g as well as the T_m and melting enthalpy decreased significantly. These results are in agreement with the GPC data of Table 2, which show that polymer degradation had occurred. With doses of 400 kGy or higher and also after neutron irradiation of Ho-PLLA-MS lowering of the T_g was observed, whereas no T_m was detected (Fig. 5). This means that neutron and high dose gamma irradiation result in a substantial decrease in PLLA molecular weight by which crystallization was prevented. It is however important to note that the structural integrity of the Ho-PLLA-MS was preserved.

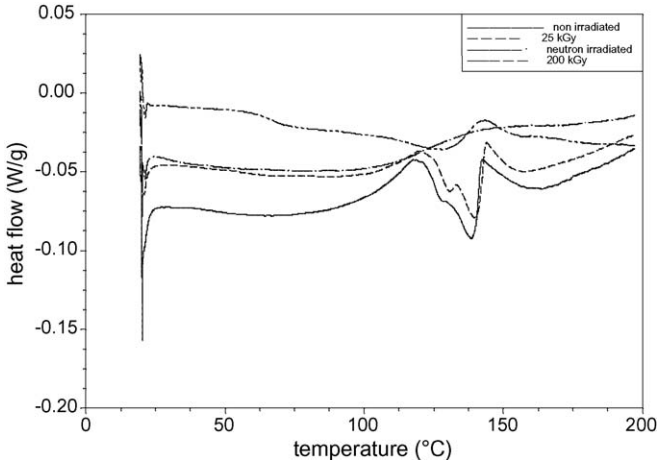

Fig. 5. MDSC thermograms (heat flow) of Ho-PLLA-MS before and after gamma irradiation (25 and 200 kGy) and neutron irradiation.

4. Conclusion

This study shows that residual chloroform in Ho-PLLA-MS can be effectively removed by neutron irradiation or a gamma irradiation at a dose of 200 kGy. As a result of radiolysis chloroform was converted into chloride and no harmful phosgene could be detected in Ho-PLLA-MS. Although the microspheres were affected by these high-energy radiations in terms of their molecular weight and crystallinity, the particles retained their integrity and desired size between 20 and 50 μm, which is the main requirement for their clinical application.

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References

B'Hymer, C., 2003. Residual solvent testing: a review of gas-chromatographic and alternative techniques. Pharm. Res. 20, 337–344.

Benoit, J., Courteille, F., Thies, C., 1986. A physicochemical study of the morphology of progesterone-loaded poly(-lactide) microspheres. Int. J. Pharm. 29, 95–102.

Chen, F.Y., Pehkonen, S.O., Ray, M.B., 2002. Kinetics and mechanisms of UV-photodegradation of chlorinated organics in the gas phase. Water Res. 36, 4203–4214.

Chung, T.W., Huang, Y.Y., Liu, Y.Z., 2001. Effects of the rate of solvent evaporation on the characteristics of drug loaded PLLA and PDLLA microspheres. Int. J. Pharm. 212, 161–169.

Dowideit, P., Mertens, R., von Sonntag, C., 1996. Non-hydrolytic decay of formyl chloride into CO and HCl in aqueous solution. J. Am. Chem. Soc. 118, 11288–11292.

Franca, Y.V., Leitao, F., Shihomatsu, H.M., Scapin, W.S., de Moraes, N.M.P., Salvador, V.L., Figueiredo, A.M.G., Muccillo, E.N.S., Muccillo, R., 1999. Determination of yttrium and lanthanum in zirconium dioxide by HPLC, X-ray fluorescence and neutron activation analyses. Chromatographia 49, 91–94.

Freitas, S., Merkle, H.P., Gander, B., 2005. Microencapsulation by solvent extraction/evaporation: reviewing the state of the art of microsphere preparation process technology. J. Control. Release 102, 313–332.

Hatashita, M., Yamamoto, T., Wu, X.Z., 2001. Chromatographic study of gamma-ray irradiated degradation of chlorinated hydrocarbon in water. Anal. Sci., 623–625.

Herberger, J., Murphy, K., Munyakazi, L., Cordia, J., Westhaus, E., 2003. Carbon dioxide extraction of residual solvents in poly(lactide-*co*-glycolide) microparticles. J. Control. Release 90, 181–195.

Keeler, J.R., Hurt, H.H., Nold, J.B., Lennox, W.J., 1990. Estimation of the Lct50 of phosgene in sheep. Drug Chem. Toxicol. 13, 229–239.

Koegler, W.S., Patrick, C., Cima, M.J., Griffith, L.G., 2002. Carbon dioxide extraction of residual chloroform from biodegradable polymers. J. Biomed. Mater. Res. 63, 567–576.

Miller, J.C., Miller, J.N., 2005. Statistics for Analytical Chemistry, third ed. Ellis Horwood Limited, Chichester.

Montanari, L., Costantini, M., Signoretti, E.C., Valvo, L., Santucci, M., Bartolomei, M., Fattibene, P., Onori, S., Faucitano, A., Conti, B., Genta, I., 1998. Gamma irradiation effects on poly(DL-lactictide-*co*-glycolide) microspheres. J. Control. Release 56, 219–229.

Mucka, V., Polakova, D., Pospisil, M., Silber, R., 2003. Dechlorination of chloroform in aqueous solutions influenced by nitrate ions and hydrocarbonate ions. Radiat. Phys. Chem. 68, 787–791.

Mumper, R.J., Jay, M., 1992. Poly(L-lactic acid) microspheres containing neutron-activatable holmium-165: a study of the physical characteristics of microspheres before and after irradiation in a nuclear reactor. Pharm. Res. 9, 149–154.

Mumper, R.J., Ryo, U.Y., Jay, M., 1991. Neutron-activated holmium-166-poly(L-lactic acid) microspheres: a potential agent for the internal radiation therapy of hepatic tumors. J. Nucl. Med. 32, 2139–2143.

Nijsen, J.F.W., Seppenwoolde, J.H., Havenith, T., Bos, C., Bakker, C.J.G., van het Schip, A.D., 2004. Liver tumors: MR imaging of radioactive holmium microspheres—phantom and rabbit study. Radiology 231, 491–499.

Nijsen, J.F.W., van het Schip, A.D., van Steenbergen, M.J., Zielhuis, S.W., Kroon-Batenburg, L.M., van de Weert, M., van Rijk, P.P., Hennink, W.E., 2002a. Influence of neutron irradiation on holmium acetylacetonate loaded poly(L-lactic acid) microspheres. Biomaterials 23, 1831–1839.

Nijsen, J.F.W., van het Schip, A.D., Hennink, W.E., Rook, D.W., van Rijk, P.P., de Klerk, J.M.H., 2002b. Advances in nuclear oncology: microspheres for internal radionuclide therapy of liver metastases. Curr. Med. Chem. 9, 73–82.

Nijsen, J.F.W., van Steenbergen, M.J., Kooijman, H., Talsma, H., Kroon-Batenburg, L.M., van de Weert, M., van Rijk, P.P., De Witte, A., van het Schip, A.D., Hennink, W.E., 2001. Characterization of poly(L-lactic acid) microspheres loaded with holmium acetylacetonate. Biomaterials 22, 3073–3081.

Nijsen, J.F.W., Zonnenberg, B.A., Woittiez, J.R., Rook, D.W., Swildens-van Woudenberg, I.A., van Rijk, P.P., van het Schip, A.D., 1999. Holmium-166 poly lactic acid microspheres applicable for intra-arterial radionuclide therapy of hepatic malignancies: effects of preparation and neutron activation techniques. Eur. J. Nucl. Med. 26, 699–704.

O'Donnel, P.B., McGinity, J.W., 1997. Preparation of microspheres by solvent evaporation technique. Adv. Drug Del. Rev. 28, 25–42.

Schoene, K., Bruckert, H.J., Steinhanses, J., 1993. Derivatization of acylating gases and vapors on the sorbent tube and gas-chromatographic analysis of the products by atomic emission and mass-spectrometric detection. Fresenius J. Anal. Chem. 345, 688–694.

Taghipour, F., Evans, G.J., 1997. Radiolytic dechlorination of chlorinated organics. Radiat. Phys. Chem. 49, 257–264.

Zielhuis, S.W., Nijsen, J.F.W., Figueiredo, R., Feddes, B., Vredenberg, A.M., van het Schip, A.D., Hennink, W.E., 2005. Surface characteristics of holmium-loaded poly(L-lactic acid) microspheres. Biomaterials 26, 925–932.