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Research article

Internal morphology of poly(D,L-lactide-co-glycolide) BCNU-loaded microspheres. Influence on drug stability

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Abstract

The solvent extraction/evaporation process has been used to form poly(D,L-lactide-co-glycolide) (PLAGA) BCNU-loaded microspheres designed for use as intracranial controlled-release implants. Their actual payload could reach 25% with a 20–50 μm size distribution. Scanning electron microscopy showed that such carriers had a smooth surface and a spherical geometry. Differential scanning calorimetry analyses carried out on drug-loaded microspheres established that the PLAGA T_g was markedly shifted towards the low temperatures along with the disappearance of the BCNU melting endotherm. Annealing experiments performed at room temperature did not induce any change of the loaded microsphere DSC profiles. These features indicated that the BCNU acted as a plasticizer for the coating material and formed with it a solid solution. Similarly, stability of encapsulated BCNU was assessed in different conditions of storage. It appeared that drug degradation increased with temperature increase: 5.4, 8.8, 32.4 and 51.2% of decomposition after 3 month storage at −18, 4, room temperature (RT) and 37°C respectively. Since the free drug was stable at 4°C and experienced only 10.6% decomposition at RT during the same storage time, the state of solid solution involving the intimate mixing of the drug and the polyester in the matrix favors a progressive decomposition of BCNU. However, keeping the microspheres 6 months at −18°C or 3 months at 4°C prevents a loss of drug superior to 10%. © 1998 Elsevier Science B.V.

Keywords: BCNU; Poly(D,L-lactide-co-glycolide); Microencapsulation; Solvent extraction/evaporation process; Solid solution; Differential scanning calorimetry; Drug stability

1. Introduction

A major obstacle in the treatment of many neurological disorders is the inability to deliver drugs into the brain, particularly within discrete regions, at a controlled rate. Recent reports have shown that polymeric devices implanted into the brain can release locally neuroactive substances for extended periods of time [1,2]. In this manner, the brain implantation of polymeric devices has been achieved in man for the treatment of malignant cerebral tumors [3]. We have demonstrated in a previous study the biocompatibility and biodegradability of blank poly(D,L-lactide-co-glycolide) 50:50 (PLAGA) microspheres implanted in the brain tissue [4]. Consequently, these microspheres could be of interest to carry BCNU (1,3-bis(-2-chloroethyl)-1-nitrosourea), one of the most effective antineoplastic agents for chemotherapy of malignant glial tumor, but displaying a short half-life and important adverse effects such as bone marrow suppression and pulmonary toxicity.

The solvent extraction/evaporation process was retained to produce the microspheres. However, it is

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known that a variety of physicochemical interactions between the polymeric carrier and drug can occur [5–7]. These interactions likely influence the therapeutic performance and stability of the final pharmaceutical form, especially when the drug is unstable like BCNU [8–10]. Accordingly, the objective of this paper was (i) to prepare BCNU-loaded microspheres with the smallest possible amount of organic solvent in order to avoid any interference of solvent traces on the microspheres morphology, (ii) to evaluate the thermal properties of BCNU-loaded microspheres stored in different conditions, in order to define their internal morphology and (iii) to follow drug stability in microspheres, that might be affected by the nature of the drug dispersion in the matrix.

2. Materials and methods

2.1. Chemicals

The poly (D,L-lactide-co-glycolide) 50:50 (PLAGA) copolymer was obtained from BI Chimie (Resomer® RG 506, Paris, France). Its $\bar{M}_w$ and $\bar{M}_n$ were respectively 75 320 and 22 470 with a polydispersity index of 3.3. 1,3-bis(-2-chloroethyl)-1-nitrosourea (BCNU) was supplied as a lyophilized powder by Laboratoires Bristol (Paris, France). Polyvinyl alcohol (PVA) (Rhodoviol® 4/125, 88% hydrolyzed) was used as the emulsifying agent. The methylene chloride (CH_2Cl_2) was used without further purification (Rectapur®, Prolabo, Paris, France).

2.2. Microsphere preparation

A previously described formulation based on the solvent extraction/evaporation method [11] was used to encapsulate BCNU. Microspheres were formed according to the following procedure: PLAGA (0.25 g) was dissolved at room temperature in a known volume of CH_2Cl_2 and cooled at 4°C. A definite amount of BCNU (5–300 mg) was then dissolved (completely shielded from light) in the resulting solution. This obtained organic phase was added under an agitation of 1600 rpm for 5 min, to 100 ml of a cooled aqueous phase (4°C) containing 2% (w/v) PVA. The extraction was carried out under agitation (1000 rpm) in water (500 ml) for 10 min and the formed microspheres were washed twice with 100 ml of deionized water, recovered by filtration and then freeze-dried.

Two main process parameters were investigated: the polymer/solvent ratio (w/v %) (4.16, 5, 6.25, 6.94, 7.14, 8.33, 12.5) and the stirring rate of the primary emulsion (1000, 1400, 1600, 2000 rpm).

2.3. Microsphere characterization

2.3.1. Size distribution analysis

Microsphere size distribution was determined using a Coulter® counter (Multisizer, Coultronics, Margency, France). Twenty milligrams of the sample were suspended in an aqueous solution of Tween 80®, diluted in saline (Isoton II®, Coultronics, Margency, France) and analyzed.

2.3.2. Morphology studies

Microsphere morphology was determined by optical microscopy (Olympus® BH-2, OSI, Paris, France) and scanning electron microscopy (Jeol JSMR 3 JC, Jeol, Paris, France). In the latter case, a layer of gold was deposited by evaporation on the sample (Ion Sputter JFC 1100, Jeol, Paris, France).

2.3.3. Determination of BCNU content and stability study

According to a procedure previously described [12], a weighed amount of BCNU-loaded microspheres (10 mg) was dissolved in 0.8 ml methylene chloride. The sample volume was adjusted to 10 ml with ethanol to precipitate the polymer. The resulting suspension was centrifuged at 18 000 rpm for 20 min (4°C). The supernatant was assayed according to the Bratton–Marshall colorimetric method, adapted by LOO and DION for BCNU determination [13].

A 10 μl aliquot of the clear supernatant was added to 0.5 ml of 0.5% sulfanilamide in 2 N HCl (Prolabo, Paris, France) and 0.99 ml of deionized water. After agitation, the sample was heated at 50°C for 45 min. Afterwards, the medium was rapidly chilled in an ice bath and 0.1 ml of *N*-(1-naphthyl) ethylenediamine dihydrochloride (Bratton–Marshall reagent) in deionized water (3 mg/ml) was added (Aldrich, Saint-Quentin Fallavier, France). The color was allowed to develop for 10 min and the optical density was read at 540 nm by spectrophotometry (Uvikon 940®, Kontron, St Quentin-en-Yvelines, France). The assays were performed in triplicate on freshly made microspheres and on particles stored in containers at different temperatures: −18, 4, RT and 37°C.

The theoretical (T.E.R) and actual (A.E.R) encapsulation ratios were respectively defined by the following expressions:

$$\text{T.E.R } (\%) = \frac{\text{Drug weight}}{\text{Drug weight} + \text{polymer weight}}$$

$$\text{A.E.R } (\%) = \frac{\text{Drug weight}}{\text{Microsphere weight}}$$

The encapsulation yield (%) was given by the A.E.R/T.E.R ratio.

2.3.4. Thermal analysis

Thermal gravimetric (TGA) and differential scanning calorimetry (DSC) analyses were achieved on free BCNU, blank and BCNU-loaded microspheres. Analyses were carried out with a differential scanning calorimeter (DSC TC II, Mettler Toledo, Viroflay, France). DSC analysis was made by heating samples at 10°C/min, in a sealed pan, over a temperature range −100–200°C. Reported glass transition temperatures (T_g) are midpoint values. Studies were performed on freshly-prepared and aged microspheres stored under previously described conditions. TGA analysis was carried out on free BCNU, blank and BCNU-loaded microsphere samples heated at 10°C/min, in an opened pan, over a temperature range 30–300°C.

3. Results and discussion

3.1. Microsphere preparation

A previous study was performed in our group to determine the process variables allowing the formation of BCNU-loaded PLAGA microspheres [11]. However, by initially setting the theoretical drug content at 16.67%, the actual BCNU content barely went above 10%.

The objective of the present study was to obtain more highly loaded-BCNU microspheres using the lowest volume of organic solvent as the dispersed phase. This approach should minimize the amount of entrapped methylene chloride in the microspheres and, thus, avoid plasticization of the polymer. This condition must be met to have a clear interpretation of the drug polymer interactions, without interferences due to entrapped solvent.

The solvent extraction/evaporation process was selected versus the classical evaporation procedure because it was believed that the extraction step improved the elimination of methylene chloride without altering the BCNU content. The smallest volume of dispersed phase investigated in the study of Torres et al. [11] was 6 ml. Fig. 1 shows the effect of methylene chloride volume on the microsphere size, for amounts below 6 ml, the stirring rate being set at 1000 rpm. It was noticeable that the size of the microspheres was influenced by the polymer concentration in the dispersed phase during preparation. An increase in the polymer concentration resulted in a larger particle diameter. This might be explained by a greater probability of fusion of semi-formed particles when they ran into each other in the medium. In addition, increasing the concentration of dissolved polymer also increased the viscosity of the organic phase, which might prevent an optimal shearing of the emulsion when agitated. Similar results have been previously reported [14,15].

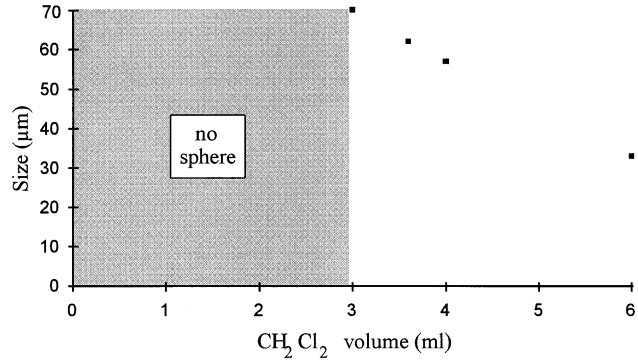

Fig. 1. Influence of the methylene chloride volume on blank microspheres size (stirring rate 1000 rpm).

However, below a 3 ml volume, the formation of microspheres did not occur and between 3 and 3.6 ml, the microscopic observation revealed the presence of polymeric fibers along with microspheres. The smallest volume of methylene chloride leading to regular microspheres without any debris, in these conditions, was 3.6 ml. But their mean size was 62 μm, incompatible with the planned surgical application. A stereotaxic injection on rats required a size distribution in between 20 and 50 μm.

Taking into account the adjustment of the methylene chloride volume to 3.6 ml, the effect of the stirring rate of the emulsion on the microsphere size was evaluated by electronic count (Table 1). A stirring speed of 1600 rpm allowed the formation of 34 μm microspheres. The adjustment of the previous process conditions was done without the presence of BCNU.

In a last series of experiments, BCNU was introduced in increasing amounts in the organic phase.Table 2 reports the encapsulation data obtained along with the mean sizes of the particles. The microsphere size was not affected by the presence of the drug.

In terms of A.E.R, the best results were obtained for T.E.R (28.6%) since the drug content reached the value of 22.2% equivalent to an encapsulation yield of 77.8%. Interestingly, the encapsulation yield dropped to 52.1 and 45.5% for higher T.E.R (44.4 and 54.5%), the A.E.R leveling off at 23–25%. Since BCNU is a very fragile molecule, the validity of the analytical method used in the study could be questioned. It must be noted that the drug content was determined by a colorimetric assay that allowed the quantification of the intact nitro-

Table 1
Influence of stirring rate of the emulsion on the particle size

Stirring rate (rpm)	Microsphere size (μm)
1000	62.3 ± 22.6
1400	43.5 ± 18.4
1600	34.2 ± 14.1
2000	32.7 ± 12.8

Table 2
Influence of the initial BCNU amount introduced in CH_2Cl_2 (3.6 ml) on drug content, encapsulation yield and microsphere size (stirring rate: 1600 rpm)

BCNU (mg)	BCNU encapsulation ratio (%)		Encapsulation yield (%)	Particle size (μm) $\pm$ S.D.
	Theoretical	Actual		
5	1.96	1.35 ± 0.24	68.8	29.2 ± 10.9
20	7.41	5.47 ± 0.45	73.8	34.2 ± 14.3
50	16.66	11.64 ± 0.74	69.9	31.2 ± 12.7
100	28.57	22.24 ± 0.89	77.8	30.2 ± 13.5
200	44.44	23.14 ± 0.51	52.1	34.1 ± 12.8
300	54.54	24.82	45.5	35.8 ± 14.6

sourea molecule [13]. The first step involves the spontaneous decomposition of BCNU. The subsequent denitrosation generated nitrous acid that was the chemical entity assayed. Therefore, none of the decomposition products of BCNU interfered with the reaction.

SEM observations showed that microspheres were spherical and exhibited a very smooth surface (Fig. 2). There was no evidence of pore formation. Unlike CCNU-loaded PLA microspheres [12], free BCNU crystals were never observed on the microsphere surface, or in the aqueous phase. That could be explained by the fact that BCNU is 80 times more soluble in water (4 mg/ml) than CCNU (< 0.05 mg/ml) [16]. This difference in behavior could also explain why the maximal BCNU encapsulation yield in PLAGA microspheres (78%) was lower than that concerning CCNU in PLA particles (90–100%), although prepared according to very similar conditions.

3.2. Internal microsphere morphology

Fig. 3 contains five DSC scans that illustrate the thermic behavior of free BCNU, PLAGA, a physical mixture of these compounds, blank microspheres and BCNU-loaded microspheres. Curve A shows a narrow melting endotherm at 30.4°C (123 J/g) and an exotherm starting at 90°C supporting a degradation phenomenon of the drug. Indeed, when free BCNU samples were heated at a rate of 10°C/min using an oil bath, an important degradation occurred after 80°C. In this temperature range, Loo et al described a cyclization of BCNU [8]. Moreover at this temperature range, the TGA weight losses that the BCNU sample experienced were close to 100%. They could be attributed to volatilization of BCNU degradation products.

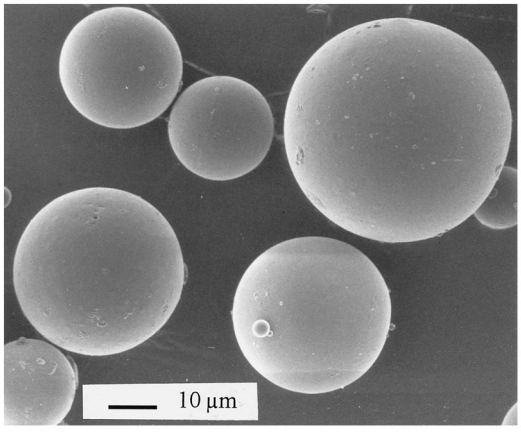

Fig. 2. Scanning electron micrograph of 22.2% BCNU-loaded PLAGA microspheres.

Curve B is the DSC profile of the starting PLAGA with a glass transition at 49.4°C.

Curve C corresponds to the DSC chart obtained from a physical mixture of BCNU and PLAGA (20% w/w). It shows all the thermal events previously described: the PLAGA glass transition at 48.3°C, the melting endotherm at 33°C and the degradation exotherm both typical of BCNU. Curve D is a DSC profile given by blank microspheres with a polymer glass transition located at 48.3°C. Curve E illustrates the thermic behavior of freshly made BCNU loaded-microspheres with a 22.2% actual drug content. Three characteristic features could be brought about. First, the PLAGA T_g fell at 12.9°C. Secondly, no BCNU melting endotherm was detectable. Thirdly, the exothermic event remained located at the same temperatures. The absence of detectable crystalline domains in the PLAGA microspheres along with the presence of BCNU degradation signal, demonstrated that intact drug is molecularly dispersed in the matrix. The type of molecular dispersions, stable or metastable, could be defined by examining how the drug affects the T_g of the coating material [5–7]. By comparing the DSC charts of the starting PLAGA (curve B), the physical mixture (curve C) and the blank microspheres (curve D), it must be noted that the T_g values do not vary significantly (48.3–49.4°C). Interestingly, methylene chloride was known to have a high affinity for PLAGA and to plasticize it [5]. However, in this case, the residual traces entrapped were too low to affect the PLAGA T_g, partly because the microspheres

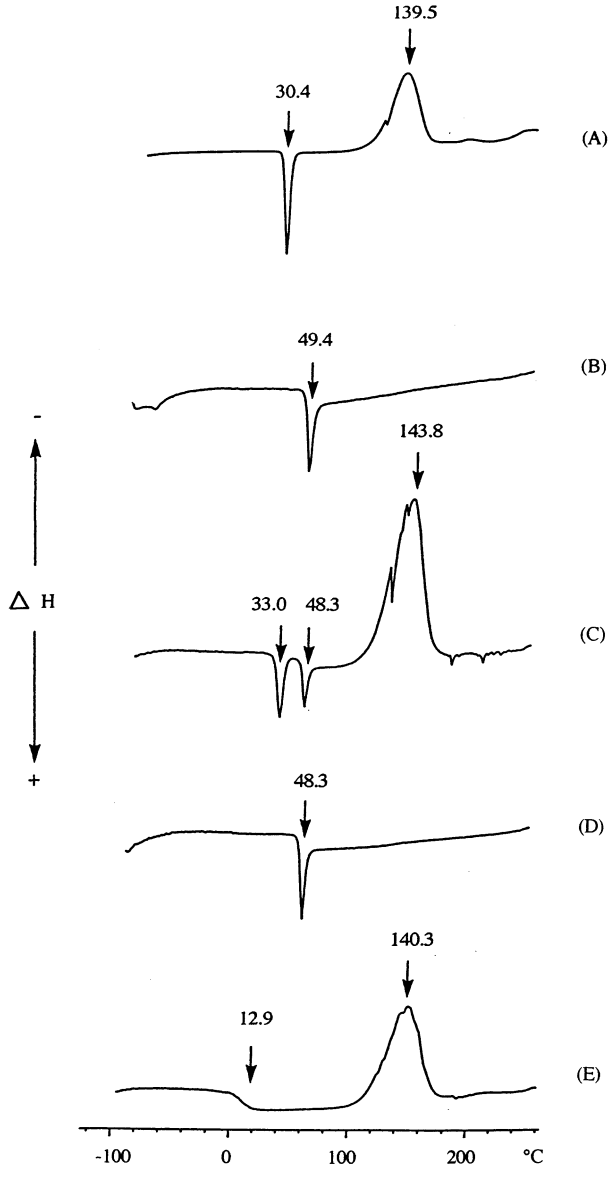

Fig. 3. DSC scans obtained with (a) BCNU, (b) PLAGA, (c) 20% BCNU–PLAGA mixture, (d) blank microspheres and (e) 22.2% BCNU-loaded microspheres.

were processed with the lowest amount of CH_2Cl_2 allowing their formation. This was supported by the absence of weight loss during the TGA scan performed on blank and drug-loaded microspheres, between 30 and 80°C. Therefore, the T_g shift recorded on curve E was solely due to the presence of BCNU and supported the fact that the drug acted as a strong plasticizer for the polymer. In other words, the drug had strong miscibility with PLAGA.

Another way to make the distinction between the two types of molecular dispersions was to determine what happened to drug-loaded PLAGA microspheres stored for prolonged periods at temperatures above the T_g of PLAGA, but below the melting temperature of the drug [5–7]. In this situation, drug trapped as a metastable molecular dispersion in the PLAGA glass will have its diffusivity greatly increased due to the large increase in PLAGA chain mobility that occurs above T_g. Under such conditions, molecularly dispersed drug molecules immiscible with PLAGA could diffuse together to nucleate and grow finite crystal domains. If the drug was miscible with PLAGA, it would remain molecularly dispersed during and after heat treatment. Room temperature was selected because it was above the polymer T_g of the loaded microspheres and below the drug melting point. No drug melting event was observed over 6 months. This feature strongly supported the fact that BCNU formed a stable solid solution with the polymer. Another point strengthened the proposed structure of the matrix. When BCNU alone was in its amorphous state (obtained by quench-cooling its liquid form), its conversion to crystalline domains occurred after 7 months of storage at +4°C. No such transformations took place in the microspheres kept at room temperature indicating that the matrix internal structure was thermodynamically stable. This particular interaction between a drug and its homing polymer was already suspected for PLA microspheres carrying another nitrosourea, CCNU [6]. Finally, it was previously shown that the encapsulated drug content leveled off at approximately 22–25%, irrespective of the initial amount introduced in the organic phase. Fig. 4 shows how the actual encapsulation ratio affects the PLAGA T_g. It fell from 48.3 down to 29.2°C when small amounts of drug (5 mg) were introduced to the medium. When the A.E.R increased from 1.4 to 22.2%, the PLAGA T_g decreased linearily. It was clear that the extent of polymer plasticization depended on the amount of entrapped drug until a certain limit was reached. When microspheres were made with initial amounts of BCNU ranging from 100 to 300 mg, the PLAGA T_g did not significantly change (from 12.9 to 10.4°C). This must be related to the fact that the optimal encapsulation ratio was almost obtained with only 100 mg of BCNU. The use of higher initial amounts of drug did not bring about improvement. The value of 24.8% leading to a T_g of 10.4°C was believed to be close to the solubility limit of the drug in the polymer. Beyond this limit, the drug was not able to stay entrapped in the matrix, even under the state of crystalline dispersion. The encapsulation of BCNU in PLAGA (50:50) via the solvent extraction/evaporation process (according

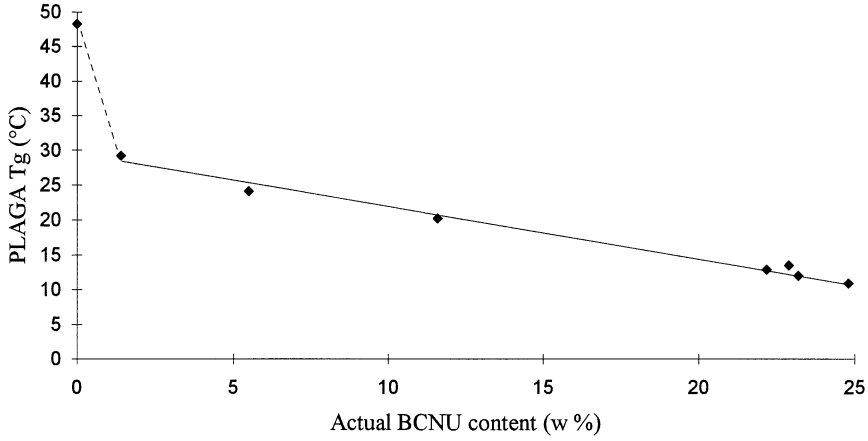

Fig. 4. Effect of BCNU content in the microspheres on the PLAGA T_g.

to the process variables previously described) occurred through the establishment of a solid solution. When the solubility limit was attained in the polymer, the remaining fraction of drug was rejected in the aqueous phase.

3.3. Stability studies

Identification of the matrix structure might have some important implications in terms of drug stability. The necessity of such an investigation was reinforced by the peculiar instability of BCNU. Several microsphere samples were stored for 13 months under different conditions, −18, 4, RT, 37°C, and assayed for their drug content parallel to the determination of their thermal behavior (Table 3). It appeared that some degradation occurred irrespective of the experimental conditions and its extent was more marked when the storage temperature increased: 5.4, 8.8, 32.4 and 51.2% of decomposition after a 3 month storage at −18, 4, RT and 37°C respectively. Since free BCNU was stable at 4°C and experienced only 10.6% decomposition at RT after a 3 month storage, it was believed that the intimate mixing of the drug and the polyester under the form of a solid solution favored the slow but progressive decomposition of the encapsulated nitrosourea. No stabilization effect was obtained through encapsulation in PLAGA except at 37°C where encapsulated BCNU degraded much more slowly than the free form (Fig. 5). In this case, the matrix system partly protected BCNU against heat decomposition. Similarly, it is worth noting that the degradation kinetics of BCNU in the microspheres follow a first order law (Fig. 5). This kinetic pattern was previously reported when the drug was dissolved in petroleum ether [8]. This feature is consistent with the formation of a solid solution when BCNU is encapsulated in PLAGA. Last but not least, it must be noted that a rather good correlation exists between the BCNU contents assessed either by the Bratton–Marshall method or by DSC through the energy emitted during the BCNU degradation process (Fig. 6). In this case, the thermal analysis could supply a reliable follow-up of the BCNU stability in the microspheres. In addition, it was noted that the remaining amount of intact encapsulated BCNU did not crystallize at room temperature, irrespective of the storage time. Interestingly, the PLAGA T_g value slightly increased by a few degrees during storage, especially at 37°C, confirming loss of pure BCNU although impossible to quantify. Both observations were consistent with the existence of a solid solution between the drug and its homing polymer.

4. Conclusion

The encapsulation of BCNU in PLAGA (50:50) by the solvent extraction/evaporation process proceeds through the formation of a solid solution. The peculiar structure of the matrix involving an intimate mixing of the drug and the polyester, was shown to favor a progressive decomposition of the encapsulated nitrosourea during storage at a temperature equal to or below RT. A thorough study of stability allowed the selection of conditions where less than 10% of drug were degraded in the microspheres: 6 months at −18°C or 3 months at +4°C. These storage conditions were particularly important to define in order to start biological studies and to make sure that BCNU doses used would

Table 3
Effects of storage conditions on the thermal events exhibited by BCNU-loaded microspheres (22.2% loaded)

Month	−18°C			+4°C			Ambient temperature			+37°C		
	PLAGA T_g (°C)	Exotherm event (J/g)	BCNU into MS (%)	PLAGA T_g (°C)	Exotherm event (J/g)	BCNU into MS (%)	PLAGA T_g (°C)	Exotherm event (J/g)	BCNU into MS (%)	PLAGA T_g (°C)	Exotherm event (J/g)	BCNU into MS (%)
0	11.9	200.4	100	11.9	200.4	100	11.9	200.4	100	11.9	200.4	100
1	8.9	190.4	97.6 ± 1.8	10.5	173.2	94.4 ± 1.8	12.6	178.1	83.2 ± 5.7	16.4	164.2	70.9 ± 4.3
2	13.7	188.9	94.2 ± 1.3	11.2	182.9	89.4 ± 2.5	13.5	183.0	75.4 ± 1.9	19.4	105.5	54.7 ± 2.3
3	13.0	183.3	94.6 ± 3.5	13.7	176.9	91.2 ± 3.6	13.2	173.5	67.6 ± 5.3	22.6	85.5	48.8 ± 4.1
4	12.8	178.8	91.5 ± 2.1	13.8	186.3	86.3 ± 5.1	12.5	166.7	62.3 ± 3.5	20.3	67.4	33.1 ± 6.8
5	13.4	183.6	89.5 ± 5.8	14.9	165.8	84.6 ± 5.6	14.6	138.0	56.5 ± 6.02	22.5	?[a]	23.7 ± 5.9
6	13.2	200.1	90.3 ± 3.2	14.1	—	81.5 ± 4.4	14.2	168.1	52.4 ± 5.1	18.3	?	17.2 ± 3.2
7	11.6	191.1	—	14.8	184.9	—	13.9	148.8	—	19.2	?	—
8	12.9	193.6	87.1 ± 5.2	15.9	165.4	76.3 ± 4.6	13.8	131.7	43.3 ± 7.0	24.9	?	7.8 ± 5.4
13	14.0	174.8	80.3 ± 6.4	15.5	170.6	65.1 ± 5.4	13.9	96.1	29.6 ± 8.1	17.1	?	<1

[a] The question mark (?) designates cases where the event could not be characterized reliably because it was too diffuse.

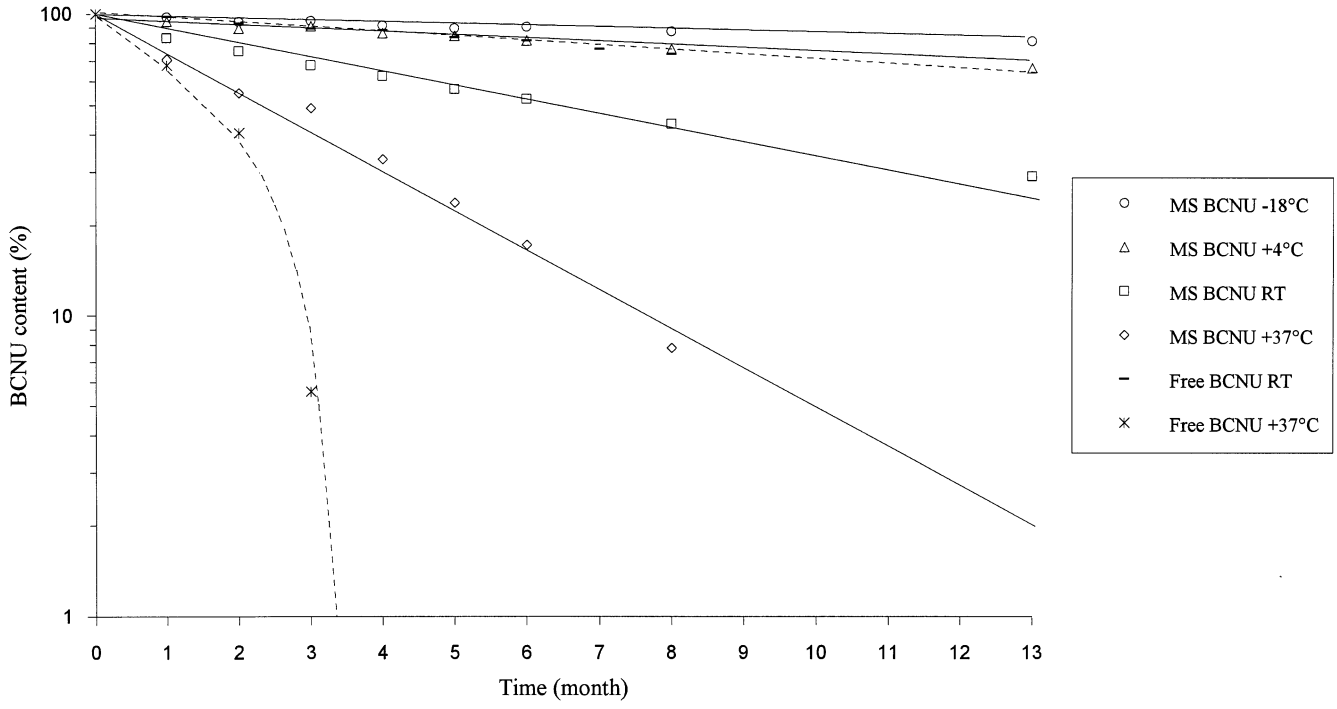

Fig. 5. Degradation kinetics of BCNU (free and encapsulated, actual content: 22.2%) during storage (MS, microsphere; RT, room temperature).

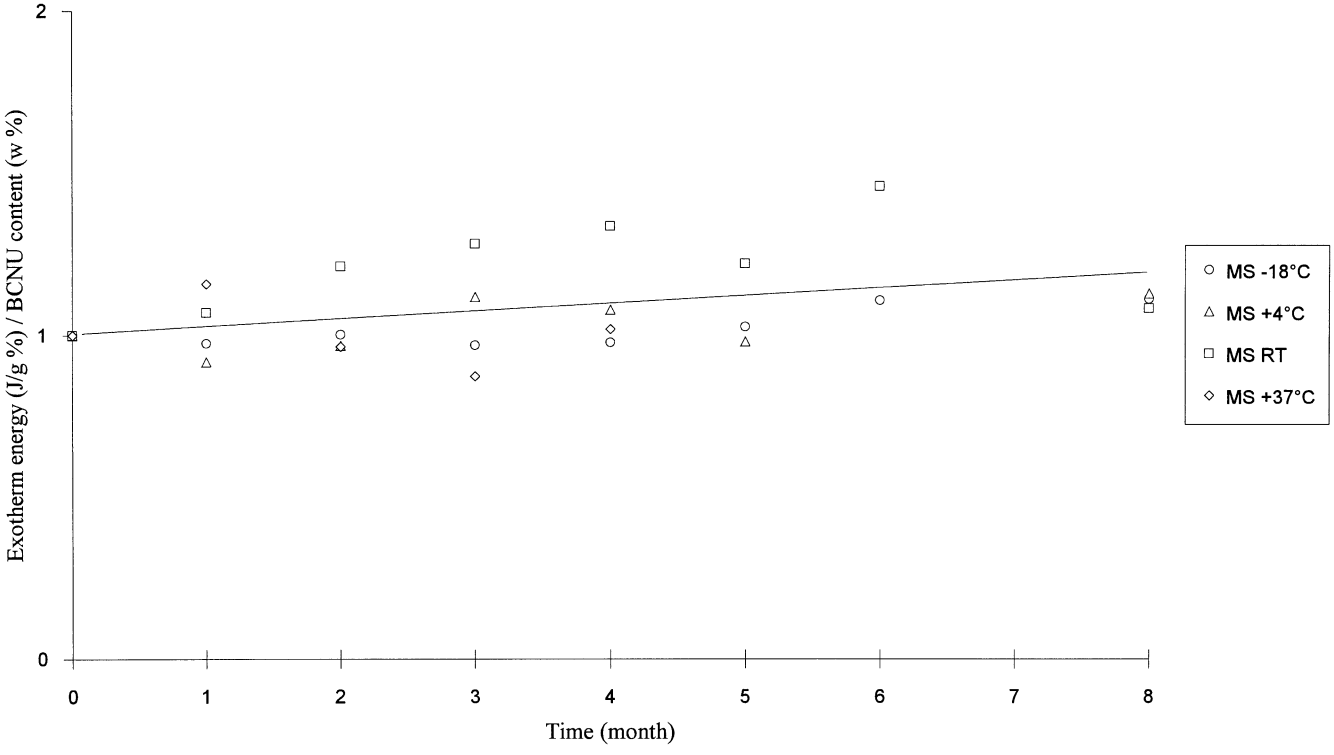

Fig. 6. Evolution of drug degradation energy (J/g %)/drug content (wt.% as assayed by spectrophotometry) ratio as a function of storage time (MS: microspheres; RT: room temperature).

not be overestimated. The release kinetics from the microspheres and the toxicological studies of drug-loaded microspheres intracranially implanted in rats are now ongoing.

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References

[1] P. Menei, M. Boisdron-Celle, A. Croué, G. Guy, J.P. Benoit, Effect of stereotactic implantation of biodegradable 5-fluorouracil-loaded microspheres in healthy and C6 glioma-bearing rats, Neurosurgery 39 (1) (1996) 117–124.

[2] P. Menei, M.C. Venier-Julienne, J.P. Benoit, Drug delivery into the brain using implantable polymeric systems. S.T.P. Pharma Sci. 7 (1) (1997) 53–61.

[3] H. Brem, S. Piantadosi, P.C. Burger, M. Walker, R. Selker, N.A. Vick, K. Blak, M. Sisti, S. Brem, G. Mohr, P. Muller, R. Morawetz, S.C. Schold, Placebo-controlled trial of safety and efficacy of intraoperative controlled delivery by biodegradable polymers of chemotherapy for recurrent gliomas, Lancet 345 (1995) 1008–1012.

[4] P. Menei, V. Daniel, C. Montero-Menei, M. Brouillard, A. Pouplard-Barthelaix, J.P. Benoit, Biodegradation and brain tissue reaction to poly(D,L-lactide-co-glycolide) microspheres, Biomaterials 14 (1993) 470–478.

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

[6] J.P. Benoit, G. Madelmont, F. Puisieux, C. Thies, The storage behavior of CCNU-loaded poly (D,L-lactide) microspheres, Polym. Prepr. 27 (1986) 27–28.

[7] F. Courteille, J.P. Benoit, C. Thies, The morphology of progesterone-loaded polystyrene microspheres, J. Control. Release 30 (1994) 17–26.

[8] T.L. Loo, R.L. Dion, R.L. Dixon, D.P. Rall, The antitumor agent, 1,3-bis(2-chloroethyl)-1-nitrosourea, J. Pharm. Sci. 55 (1966) 492–497.

[9] A.G. Bosanquet, Stability of solutions of antineoplastic agents during preparation and storage for in vitro assays, Cancer Chemother. Pharmacol. 14 (1985) 83–95.

[10] K. Fredriksson, P. Lundgren, Stability of carmustine-kinetics and compatibility during administration, Acta Pharm. Suec. 23 (1986) 115–124.

[11] A.I. Torres, M. Boisdron-Celle, J.P. Benoit, Formulation of BCNU-loaded microspheres: influence of drug stability and solubility on the design of the microencapsulation procedure, J. Microencapsulation 13 (1996) 41–51.

[12] S. Benita, J.P. Benoit, F. Puisieux, C. Thies, Characterization of drug-loaded poly(D,L-lactide) microspheres, J. Pharm. Sci. 73 (1984) 1721–1724.

[13] T.L. Loo, R.L. Dion, Colorimetric method for the determination of 1,3-bis(2-chloroethyl)-1-nitrosourea, J. Pharm. Sci. 54 (1965) 809–810.

[14] C. Sturesson, J. Carlfors, K. Edsman, M. Andersson, Preparation of biodegradable poly(lactic-co-glycolic) acid microspheres and their in vitro release of timolol maleate, Int. J. Pharm. 89 (1993) 235–244.

[15] H. Jeffery, S.S. Davis, D.T. O'Hagan, The preparation and characterization of poly(lactide-co-glycolide) microparticles 1: oil-in-water emulsion solvent evaporation, Int. J. Pharm. 77 (1991) 169–175.

[16] The Merck Index: an encyclopedia of chemicals, drugs and biologicals. 10th ed. Merck, Rahway, NJ, 1983.