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Effect of physical ageing on the performance of dexamethasone loaded PLGA microspheres

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ABSTRACT

The phenomenon of physical ageing or structural relaxation and its effect on the performance of dexamethasone loaded poly(lactide-co-glycolide) (PLGA) microspheres was evaluated. Microspheres were incubated at temperatures (−20 (control), 4 and 25 °C) below their glass transition temperature for 12 months. Physical ageing occurred in microspheres incubated at 25 °C due to structural relaxation of the polymer chains which occurs to achieve a lower equilibrium energy state. Significant physical ageing was not observed in microspheres incubated at 4 °C due to the lower molecular mobility of PLGA. The rate of structural relaxation (at 25 °C) was a function of free volume which decreased with time. The microspheres incubated at 25 °C for 12 months resulted in a slower release profile after day 25 when compared to the control microspheres. This was speculated to be due to a reduction in free volume upon physical ageing which in turn may reduce water absorption and retention of acidic degradation products in the PLGA matrix, hence reducing the degradation rate of PLGA. Therefore, exposure to ambient temperature during storage, shipping or handling may cause physical ageing in PLGA microspheres and hence, their performance may be affected. Storage temperatures of 4 °C or lower may be considered appropriate for PLGA microspheres.

1. Introduction

Physical ageing or structural relaxation is a well known phenomenon in polymer science and engineering. Physical ageing commonly occurs when polymers are rapidly quenched from an elevated temperature in processes such as polymer molding (White, 2006). Rapid cooling below the glass transition temperature (T_g) of the polymer results in a glassy state with high structural energy compared to the equilibrium state (Allison, 2008; White, 2006). When such a rapidly quenched polymer is stored at temperatures up to 30–40 °C below its T_g, the polymer chains gradually relax (due to molecular mobility) to attain their thermodynamic equilibrium state (Bailey et al., 2002; Liu et al., 2009; White, 2006). This phenomenon is referred to as physical ageing or structural relaxation. Physical ageing is associated with a decrease in the free volume of the polymer and hence, increase in its density (White, 2006). Physical ageing causes polymers to become brittle and have a greater tendency to break even at small elongations.

Physical ageing or structural relaxation has also been investigated for pharmaceutical solids (Liu et al., 2002). Amorphous solids are preferred due to their higher solubility and hence, high bioavailability compared to the corresponding crystalline forms. However, amorphous solids have a tendency to relax towards the equilibrium state due to molecular mobility. This structural relaxation can result in crystallization and hence, can affect product performance. Therefore, the main challenge in the development of amorphous pharmaceutical materials is to ensure stability throughout their shelf life (Miller and Lechuga-Ballesteros, 2006).

Poly(lactide-co-glycolide) (PLGA) microspheres are under extensive investigation for controlled delivery of drugs. A widely used method of preparation of PLGA microspheres is emulsion solvent evaporation (Burgess and Hickey, 2005; Yeo and Park, 2004). In this method, the polymer is dissolved in an organic solvent and the drug is either dissolved or dispersed in this organic phase, which is then emulsified in an aqueous solution. The organic solvent is evaporated or extracted into the external aqueous phase resulting in solidification of polymer particles. PLGA microsphere solidification by solvent removal (solvent quenching) is similar to the thermal quenching of an amorphous polymer from the molten state (Allison, 2008). Fast solvent quenching may result in polymeric matrices with high structural energy and lower density. The PLGA microsphere system would tend to relax towards the lower equilibrium

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energy state over time due to molecular mobility within the system. As mentioned earlier, physical ageing is associated with changes in the density and free volume of the PLGA matrix. Such changes can affect the performance of the microspheres.

The glass transition temperature (T_g) of various PLGA polymers used for microsphere preparation is usually in the range of 40–50 °C (Allison, 2008). Therefore, microspheres are in the glassy state at temperatures encountered during processing, storage and use. Physical changes in the PLGA microspheres can occur under these conditions and this may affect microspheres performance. The studies reported in literature addressing the physical ageing of PLGA microspheres are limited and focused only on the thermal and mechanical properties of PLGA microspheres (Rosilio et al., 1998; Rouse et al., 2007; Wang and Mano, 2006). To the best of our knowledge, the effect of physical ageing on the performance of the PLGA microspheres has not been studied before.

In the present work, the changes in the glass transition temperature and enthalpy of relaxation associated with long term physical ageing of PLGA microspheres was determined. The effect of physical ageing on the *in vitro* release profile of dexamethasone loaded PLGA microspheres was also investigated. This work was performed with the objective of selecting appropriate storage conditions for PLGA microspheres to ensure shelf life stability.

2. Materials and methods

2.1. Materials

Poly(D,L-lactic-co-glycolic acid) (PLGA) polymer, PLGA Resomer® RG503H 50:50 (MW: 25 kDa) was a gift from Boehringer-Ingelheim. Methylene chloride, tetrahydrofuran (optima grade) and acetonitrile (HPLC grade) were purchased from Fisher Scientific (Pittsburg, PA). Dexamethasone and poly(vinyl alcohol) (PVA) (MW: 30–70 kDa) were obtained from Sigma–Aldrich (St. Louis, MO). Nanopure™ quality water (Barnstead, Dubuque, IA) was used for all studies.

2.2. Methods

2.2.1. Preparation of microspheres

Dexamethasone loaded PLGA microspheres were prepared by oil-in-water (o/w) emulsion solvent extraction/evaporation technique (Zolnik et al., 2005). 2 g of PLGA was dissolved in 8 ml methylene chloride. 200 mg dexamethasone was dispersed in the PLGA solution using a homogenizer (PowerGen 700 D, Fisher Scientific) at 10,000 rpm for 30 s. This organic phase was then added slowly to 40 ml of a 1% (w/v) aqueous poly(vinyl alcohol) (PVA) and homogenized at 10,000 rpm for 2 min. This emulsion was added to 500 ml of a 0.1% (w/v) aqueous PVA solution and stirred at 600 rpm under vacuum for 6 h at 25 °C. The resulting microspheres were filtered (Durapore® membrane filter, 0.45 μm, Fisher Scientific), washed three times with de-ionized water and vacuum dried for 24 h.

2.2.2. Microspheres incubation conditions

The dexamethasone loaded PLGA microspheres were placed in tightly closed scintillation vials in vacuum desiccators and incubated at −20, 4 and 25 °C. Three batches of dexamethasone loaded PLGA microspheres were incubated at each temperature. Approximately 300 mg of microspheres were sampled from each of three batches incubated at 4 and 25 °C at every time point *i.e.* day 4, 1 month, 3 months, 6 months, 9 months and 12 months. The microspheres stored at −20 °C were used as a control and were sampled initially (day 0) and at the end of 12 months.

2.2.3. Characterization of microspheres

2.2.3.1. High performance liquid chromatography (HPLC). The concentration of dexamethasone was determined using a Perkin Elmer HPLC system (series 200) with a UV absorbance detector (Perkin Elmer, Shelton, CT) set at 242 nm. The mobile phase was acetonitrile:water:phosphoric acid (30:70:0.5%, v/v/v). A Zorbax® C18 (4.6 mm × 15 cm) analytical column was used with the flow rate set at 1 ml/min. The chromatographs were analyzed using PeakSimple™ Chromatography System (SRI instruments, Torrace, CA). This method is a stability indicating HPLC assay.

2.2.3.2. Drug loading. 5 mg of dexamethasone encapsulating PLGA microspheres were dissolved in 10 ml tetrahydrofuran (THF). The solution was filtered (Millex® HV, PVDF 0.45 μm syringe filter) and the dexamethasone concentration was determined *via* HPLC as described before using an injection volume of 2.5 μl. Drug loading was determined as: percent drug loading = (weight of drug entrapped/weight of microspheres used) × 100. All measurements were conducted in triplicate and the results are reported as the mean ± SD.

2.2.3.3. Particle size analysis. An AccuSizer 780A autodiluter particle sizing system (Santa Barbara, CA) was used to determine the mean particle diameter. About 50 mg of microspheres were dispersed in 2 ml of 0.1% (w/v) PVA solution. 200 μl of the dispersion was used for particle size analysis. All measurements were conducted in triplicate and the results are reported as the mean ± SD.

2.2.3.4. Molecular weight determination. The molecular weight of the microspheres was determined by gel permeation chromatography (GPC; Waters) with an evaporative light scattering detector (ELSD). The mobile phase was THF with a flow rate of 2 ml/min at 40 °C. 10 mg microspheres were dissolved in 10 ml tetrahydrofuran (THF) and filtered through 0.45 μm filters for GPC analysis. The data collection and analysis were performed using Waters Millenium software. Polystyrene standards (2000, 900, 824, 400, 200, 110, 43, 18.80, 17.60, 6.93, 2.61, 0.98 kDa) were used for calibration and weight average molecular weights (MW) were calculated. All measurements were conducted in triplicate and the results are reported as the mean ± SD.

2.2.3.5. Glass transition temperature and enthalpy of relaxation. The glass transition temperature and enthalpy of relaxation of the microspheres were analyzed using a TA instrument Q100 differential scanning calorimeter (New Castle, DE). Samples were heated from −40 °C to 100 °C, cooled to −40 °C and heated again to 100 °C at a rate of 20 °C/min. The first cycle of the thermograms was used to determine the glass transition temperature (T_g) and the enthalpy of relaxation (peak area) of the microspheres using Universal Analysis software (TA Instruments). All measurements were conducted in triplicate and the results are reported as the mean ± SD.

2.2.3.6. In vitro release. A modified USP apparatus 4 (Sotax CE7 smart with CY 7 piston pump, Sotax, Horsham, PA) was used for *in vitro* release testing (Zolnik et al., 2005). Flow through cells (12 mm diameter) packed with glass beads (1 mm diameter) were used in a closed system. The system was temperature controlled at 37 °C. Approximately 40 mg of microspheres were dispersed in the flow through cells (fitted with regenerated cellulose filter 0.45 μm) and 250 ml of 0.05 M phosphate buffer saline pH 7.4 with 0.1% sodium azide was circulated at a flow rate of 8 ml/min. 1 ml samples were withdrawn and replaced with fresh media at suitable time intervals. The samples were analyzed using HPLC as described before using an injection volume of 20 μl. Release media was replaced with fresh media at appropriate time intervals to maintain sink conditions. The media replacement during the

Table 1
Drug loading, particle size and molecular weight of dexamethasone PLGA microspheres incubated at −20, 4 and 25 °C.

Temperature	−20 °C (control)		4 °C		25 °C	
Parameter/time	Day 0	12 months	Day 0	12 months	Day 0	12 months
Drug loading (%w/w)	7.41 ± 0.10	7.59 ± 0.35	7.41 ± 0.10	7.49 ± 0.23	7.41 ± 0.10	7.69 ± 0.22
Particle size (μm)	6.94 ± 0.50	7.29 ± 0.23	6.94 ± 0.50	7.37 ± 0.25	6.94 ± 0.50	7.43 ± 0.35
Molecular weight (kDa)	26.29 ± 0.68	25.58 ± 0.81	26.29 ± 0.68	25.51 ± 1.01	26.29 ± 0.68	24.83 ± 0.93

Table 2
Glass transition temperature of dexamethasone PLGA microspheres incubated at −20, 4 and 25 °C.

Time	−20 °C (control)	4 °C	25 °C
Day 0	39.70 ± 0.38	39.70 ± 0.38	39.70 ± 0.38
Day 4	–	40.87 ± 0.15	42.49 ± 2.05
1 month	–	41.47 ± 0.22	52.39 ± 2.80
3 months	–	43.48 ± 0.42	55.20 ± 0.72
6 months	–	42.11 ± 0.31	54.93 ± 0.22
12 months	40.32 ± 0.43	42.28 ± 0.87	57.46 ± 0.57

release study was taken into account in the calculation of cumulative percentage release.

2.2.3.7. Statistical analysis. Statistical analysis to evaluate significant differences between different microspheres formulations was performed using JMPIN® software. Results were analyzed using one-way analysis of variance (ANOVA). Any significant difference was further analyzed by Tukey–Kramer HSD (Honestly Significantly Different) post hoc test, a multiple range test to determine significant difference between more than two groups. The level of significance was accepted at $p < 0.05$.

3. Results

Incubation temperatures below the T_g of the microspheres (40 °C) were selected. 4 °C was selected since microspheres are usually stored under refrigerated conditions. The microspheres were incubated at 25 °C to simulate ambient conditions that may be encountered during shipping and handling where humidity is controlled using desiccant in the finished packages. The microspheres incubated at −20 °C were used as a control since polymer chains do not have mobility at approximately 50 °C below T_g (Aklonis and MacKnight, 1983).

The drug loading and particle size of the microspheres were approximately 7% (w/w) and 7 μm, respectively. The molecular weight of PLGA used for microsphere preparation was approximately 26 kDa. As shown in Table 1, the drug loading, particle size and polymer molecular weight did not change significantly after incubation at −20, 4 and 25 °C for 12 months.

The effect of microsphere incubation at different temperatures on the glass transition temperature (T_g) and enthalpy of relaxation is shown in Tables 2 and 3. The glass transition temperature and enthalpy of relaxation of the prepared microspheres (day 0) were approximately 40 °C and 5 J/g, respectively. The T_g and enthalpy of relaxation of the microspheres stored at −20 and 4 °C for up to 12 months did not differ significantly from their initial values. At 25 °C, no significant increase in the T_g and the enthalpy of relaxation was observed after storage for 4 days. However, the T_g increased significantly by approximately 12 and 17 °C following incubation for 1 and 12 months, respectively ($p < 0.05$) (Table 2). The enthalpy of relaxation increased by 6 J/g after incubation at 25 °C for 1 month. The enthalpy of relaxation measured after 12 months at 25 °C was not significantly different from that observed after 1 month (Table 3). An initial increase in the enthalpy of relaxation followed by a plateau after 1 month incubation was observed at 25 °C as shown in Fig. 1. Fig. 2 shows DSC thermograms where the enthalpy of relaxation peak is superimposed on the glass transition temperature. The T_g and the enthalpy of relaxation peaks were observed to shift towards higher temperature with increase in ageing times at 25 °C (Fig. 2).

Table 3
Enthalpy relaxation of dexamethasone PLGA microspheres incubated at −20, 4 and 25 °C.

Time	−20 °C (control) (J/g)	4 °C (J/g)	25 °C (J/g)
Day 0	5.48 ± 1.14	5.48 ± 1.14	5.48 ± 1.14
Day 4	–	6.05 ± 0.17	7.14 ± 1.28
1 month	–	6.74 ± 0.46	11.69 ± 0.70
3 months	–	7.22 ± 0.16	13.38 ± 0.52
6 months	–	6.82 ± 0.37	12.20 ± 0.11
12 months	5.13 ± 0.61	7.03 ± 0.79	12.50 ± 0.29

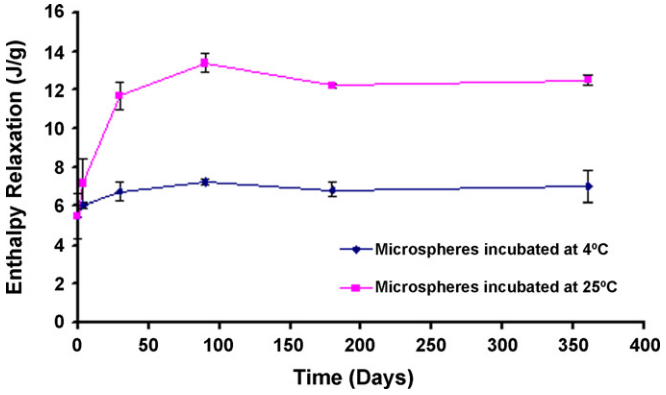

Fig. 1. Enthalpy relaxation of the microspheres aged at 4 and 25 °C.

Fig. 3 shows the *in vitro* release profiles of: (1) control microspheres (−20 °C); (2) microspheres incubated at 4 °C for 12 months; and (3) microspheres incubated at 25 °C for 12 months. Dexam-

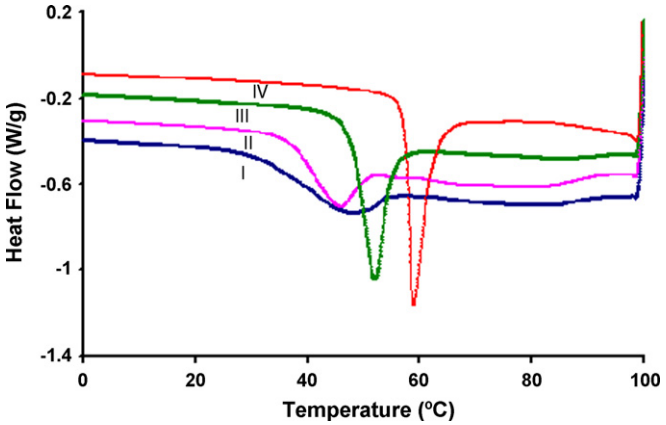

Fig. 2. DSC thermograms showing increase in glass transition temperature and enthalpy relaxation with time following incubation of dexamethasone loaded PLGA microspheres at 25 °C. The figure shows the first heating scan (DSC) where the enthalpy relaxation peak is superposed on glass transition temperature. I: day 0; II: day 4; III: 1 month; IV: 12 months.

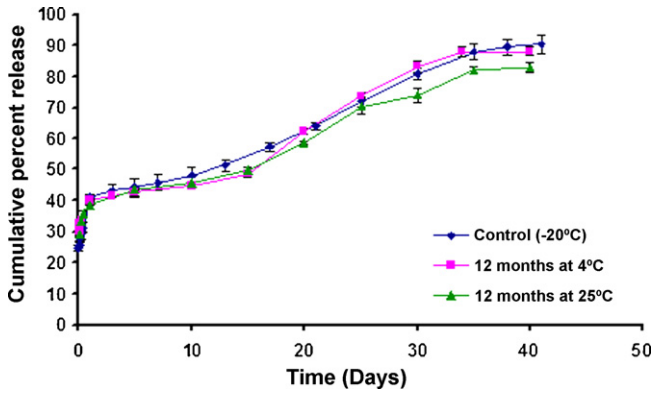

Fig. 3. *In vitro* release profile of dexamethasone loaded PLGA microspheres: (1) control microspheres; (2) microspheres incubated at 4 °C for 12 months and (3) microspheres incubated at 25 °C for 12 months.

ethasone loaded PLGA microspheres exhibited a typical triphasic release profile. There was an initial burst release (24 h release) of approximately 40% followed by a lag phase of about 10 days. A secondary zero order release phase was observed after the lag phase. As shown in Fig. 3, the *in vitro* release profiles were similar for the control microspheres (−20 °C) and those incubated at 4 °C for 12 months. The microspheres incubated at 25 °C for 12 months showed an *in vitro* release pattern similar to the control group for up to 25 days. There was a decrease in the cumulative percent release by approximately 6% after day 25 for the microspheres incubated at 25 °C for 12 months.

4. Discussion

The lack of change in microsphere drug loading following incubation at 4 and 25 °C for 12 months indicates that there was no drug degradation or diffusion of drug from the polymeric matrix (Table 1). Since the incubation temperatures were well below the T_g, no particle size increase due to microsphere aggregation was observed (Table 1). Ionizing radiations, presence of moisture and high temperature can cause PLGA degradation. These studies indicate that PLGA microspheres are not susceptible to degradation in conditions of ambient temperature and low humidity, indicating that moisture is necessary for polymer chain degradation (Table 1). This also indicates that the changes in the T_g and enthalpy of relaxation observed were only due to the physical ageing of the PLGA microspheres.

The initial enthalpy of relaxation (approximately 5 J/g, day 0) is considered to be due to vacuum drying of microspheres for 24 h at room temperature following preparation (Table 3). The enthalpy of relaxation peak that appears in the DSC thermograms (Fig. 2) upon ageing is due to recovery of the heat lost during the ageing process. The significant increase in the T_g and enthalpy of relaxation at 25 °C with ageing/incubation (Tables 2 and 3; Figs. 1 and 2) was a result of structural relaxation of the polymer chains ($p < 0.05$). Structural relaxations in polymers below their T_g are secondary (β) relaxations that arise due to localized vibrations and re-orientation of small polymer chains or side groups. Translational and rotational motions of large polymer chains are restricted in the glassy state (Aklonis and MacKnight, 1983; Liu et al., 2009).

The change in the rate of structural relaxation at 25 °C (faster change in enthalpy of relaxation in the beginning followed by a gradual change (Fig. 1)) can be explained by the fact that the rate of structural relaxation of a polymer is a function of free volume which decreases with time (Aklonis and MacKnight, 1983; Allison, 2008). The time dependent volume recovery of the polymers is given by the equation: $d\delta/dt = \delta/\tau$ where 'δ' is the normalized volume departure from the equilibrium;'t' is the ageing time and 'τ' relaxation time. Therefore, the rate of structural relaxation towards equilibrium '$d\delta/dt$' is proportional to δ itself (Aklonis and MacKnight, 1983).

The low PLGA chain mobility at 4 °C, which is approximately 35 °C below the microsphere T_g, may be responsible for the lack of any significant change in microsphere thermal properties following 12 months incubation at this temperature (Tables 2 and 3; Fig. 1). It is well documented that the relaxation time of polymers increases as the annealing temperature is lowered below the T_g due to slower molecular motions (Aklonis and MacKnight, 1983; Rouse et al., 2007). Therefore, it may take several years for structural relaxation or physical ageing to occur in PLGA microspheres at 4 °C. Accordingly, the *in vitro* release profile did not change following 12 months shelf life stability testing at 4 °C (Fig. 3).

The slower release profile observed in the microspheres incubated at 25 °C for 12 months is speculated to be due to physical ageing. The reduced free volume of the microspheres incubated at 25 °C is likely to reduce water absorption and subsequent hydrolytic degradation of PLGA (Allison, 2008). Reduced free volume may also result in lower retention of the acidic degradation products within the PLGA matrix. This would also result in slower PLGA degradation in these physically aged microspheres compared to the control. Therefore, the decreased hydrolytic degradation of the polymer may be responsible for the slow release of dexamethasone from the microspheres after day 25.

It appears that the physical ageing or structural relaxation of the PLGA microspheres below their T_g may affect their performance. Accordingly, the physical ageing tests along with the stability tests specified in ICH guidelines should be considered for the evaluation of PLGA microsphere stability and determination of appropriate storage conditions. Further work needs to be performed in this area for confirmation of the effect of physical ageing. In particular, evaluation of any change in molecular weight of the aged PLGA microspheres during release in comparison with control freshly prepared microspheres should be conducted.

The extent to which the performance of PLGA microspheres is affected by physical ageing is likely to depend on its processing conditions as well as the T_g and the storage temperature. Therefore, the physical ageing test conditions and results may vary for different PLGA microspheres.

5. Conclusions

The effect of physical ageing on the *in vitro* release of dexamethasone loaded PLGA microspheres was evaluated and appropriate storage condition to ensure their shelf life stability was determined. Physical ageing occurred in the microspheres at 25 °C as a result of structural relaxation of the polymer chain to achieve lower equilibrium energy state and was evident from the increase in T_g and enthalpy of relaxation with time. Physical ageing also resulted in a slower cumulative percent drug release from the microspheres. Therefore, evaluation of the effect of physical ageing is important to determine the impact of inadvertent excursion of formulations outside their recommended storage conditions (ambient temperatures) during shipping or handling. It is recommended that PLGA microsphere formulations should be stored under refrigerated conditions for shelf life stability of these products. The results emphasize the need of evaluation of the effect of physical ageing of PLGA microspheres as part of stability testing for determining PLGA microsphere shelf life and appropriate storage conditions. On the basis of the current work, storage temperatures of 4 °C or below may be considered appropriate for PLGA microspheres. However, the physical ageing test conditions and results may vary depending upon the PLGA microspheres.

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