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Physical ageing and thermal analysis of PLGA microspheres encapsulating protein or DNA

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Abstract

PLGA microspheres undergo physical ageing but their ageing kinetics have not been reported, nor the effect of encapsulated protein or plasmid DNA on any associated changes to the glass transition. Differential scanning calorimetry (DSC) was used to measure the rate of ageing of various PLGA microsphere formulations, with temperature-modulated DSC used to accurately measure the associated glass transition. The Cowie–Ferguson model was applied to determine the parameters describing the enthalpy relaxation kinetics. We show that encapsulated proteins had no significant effect on the glass transition of the microspheres, whereas DNA and PVA were mild antiplasticising agents, particularly with high Mw PLGA. Physical ageing occurred through a range of enthalpy relaxation times (or modes) and was independent of both encapsulated protein and surfactant used during microsphere preparation. Analysis of accelerated ageing at 35 °C gave calculated enthalpy relaxation times to thermal equilibrium of 280–400 h. No ageing was observed ≤10 °C and at 25 °C estimated relaxation times were at least one order of magnitude greater than at 35 °C. Ageing of PLGA microspheres therefore occurs at temperatures >10 °C, but relaxation will be far from equilibrium unless storage times and/or temperatures are prolonged or nearing the glass transition, respectively.

Keywords: Poly(lactic-co-glycolic acid); Glass transition; Emulsification; Enthalpy relaxation; Physical ageing

1. Introduction

Physical ageing, or structural relaxation, of amorphous materials has been widely investigated and characterised for a large number of polymers (Hodge, 1995). Insight into the ageing process at the molecular level has also been gained by use of mobility-sensitive fluorescent probes which have enabled characterisation of ageing for individual layers within a polymeric film (Priestley et al., 2005). Despite this, reports describing physical ageing for biodegradable polyesters have been limited, particularly the change in thermomechanical properties for polyesters used in the context of drug delivery vehicles or medical implants (Rosilio et al., 1998). These include poly(lactic acid-co-glycolic acid) (PLGA) microspheres, which have demonstrated potential as controlled delivery vehicles for therapeutic proteins, vaccines and gene delivery (Nikou et al., 2005; Ennett et al., 2006). The ageing process for polyesters was first described for poly(lactic acid) (PLA) (Celli and Scandola, 1992) and also for non-medically relevant polyesters (Cortes and Montserrat, 1998). This study for the first time describes the ageing process for the medically relevant polyester PLGA fabricated into microspheres encapsulating model protein.

Thermal characterisation of PLGA microspheres by differential scanning calorimetry (DSC) has shown that physical ageing occurs on a time scale equivalent to the freeze-drying process (Bouissou et al., 2006; Mohamed and van der Walle, 2006); observed as an enthalpy relaxation peak which is absent following quench cool from temperatures greater than the glass transition temperature (T_g). Physical ageing, as distinct from polymorphism and degradation, is observed by superposition of the first heating curve with the curve following quench cooling. The difference between the curves is seen as an endothermic peak (which must not be confused with a melting endotherm), often termed the 'overshoot' or 'overheating' peak, the area of which is the enthalpy relaxation (Chartoff, 1997). The enthalpy relaxation can indicate how storage conditions have affected the material. PLGA, like other amorphous materials, 'relaxes' through molecular rearrangements at temperatures below the

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T_g; the molecular motions therefore involving side chains (β-relaxations) rather than long-range polymer backbone motion (α-relaxation). The β-relaxations represents energy minimisation of the non-equilibrium nature of the glassy state. This loss of enthalpy involves increased stability via a reduction in the free volume fraction and thereby an increase in density, which may be associated with the material becoming brittle and hard (Wang and Mano, 2006). Investigation of PLGA ageing will therefore be of importance to optimising freeze-drying parameters and storage variables for microspheres encapsulating a protein or DNA.

The temperature corresponding to the T_g of PLGA microspheres is influenced by the formulation parameters of the particles as well as the history of the sample. Previous work in this area has shown that residual water has a plasticising activity on PLGA (Passerini and Craig, 2001; Blasi et al., 2005). Similarly, use of Triton X-100 (polyethylene glycol *tert*-octylphenyl ether) during emulsification has a strongly plasticising effect, probably on account of the hydrated PEG chain which is known to interact with PLGA (Bouissou et al., 2006). Proteins have been widely encapsulated in PLGA but suffer denaturation, dependent on the protein and formulation (Bouissou et al., 2004; Duncan et al., 2005). Since certain proteins have strong emulsifying activity, and are indeed occasionally used in place of PVA as the primary emulsifier (Corrigan and Heelan, 2001), it is reasonable to ask what consequence encapsulation of protein has on the thermomechanical properties of PLGA. The same is true for polyester microspheres encapsulating plasmid DNA (pDNA), which have been proposed for gene delivery and DNA vaccines (Sahoo et al., 2002; Mohamed and van der Walle, 2006).

In addressing the affect of protein emulsifying activity on the resultant material properties it is important to note that proteins are inherently surface seeking dependent on conformation and primary structure (Wilde et al., 2004). While albumins and caseins are known to be strong protein emulsifiers their conformations differ greatly (Caessens et al., 1999; Burnett et al., 2002). It would therefore be difficult to distinguish between changes to the microsphere's thermomechanical properties due to a protein's emulsifying activity or structure. However, we have recently designed a series of proteins based on a fibronectin type III domain pair which differ by only one amino acid but have either poor (FIII9-10 wt) or good (FIII9′-10) emulsifying activity (Annan et al., 2006). Here, we characterise the thermomechanical properties of PLGA microspheres encapsulating proteins of varying emulsifying activity and pDNA, comparing the shift in glass transition against commonly used polyvinyl alcohol and non-ionic surfactants. Characterising these properties alongside the physical ageing process for the same PLGA microspheres will be important if we are to fully understand microsphere behaviour during lyophilisation and storage.

2. Materials and methods

2.1. Microsphere fabrication

Bovine serum albumin (Fraction V) (BSA) and β-casein were purchased from Sigma–Aldrich (UK). The type III fibronectin domain pair (FIII9-10 wt) and its conformationally stable mutant (FIII9′-10) were produced by recombinant methods using *E. coli* and purified as previously described (Annan et al., 2006). Plasmid DNA (pGL3-control, Novagen, UK) was amplified in *E. coli* strain DH5α and purified to an A_{260}:A_{280} ratio of 1.8 using a modified method of Sambrook and Russell (2001). A water-in-oil-in-water (w/o/w) double emulsion-evaporation technique was employed. Briefly, 100 μl of protein (10 mg/ml in phosphate buffered saline, PBS) or plasmid DNA (5 mg/ml in Tris–EDTA) or emulsifier (2.5%, w/v), was injected into 1 ml of oil phase: dichloromethane (DCM) containing 5% (w/v) low or high Mw PLGA (0.18 dl/g or 0.83 dl/g, respectively; Purac, The Netherlands) and homogenised at 20,000 rpm (Turrax T18, IKA). This primary emulsion was transferred into 50 ml of water containing 0.5% (w/v) polyvinyl alcohol (PVA, Mw 22,000, 88% hydrolysed) and stirred at 500 rpm for 2 h at room temperature (r.t.). Microspheres were then harvested by centrifugation, washed three times in distilled water, snap-frozen in liquid nitrogen and lyophilised: frozen to −80 °C over 2 h, placed under a vacuum of 30 mTorr at −40 °C for 8 h, then progressively brought to room temperature and held under vacuum for 16 h (MicroModulyo, ThermoSavant). Lyophilised microspheres were kept at 4 °C in a sealed container with silica gel. Microspheres referred to as 'blank' used PBS as the inner aqueous phase.

2.2. Encapsulation efficiencies and microsphere morphology

Protein extraction and protein encapsulation efficiency, were determined as previously described (Bouissou et al., 2004). Briefly, lyophilised microspheres were added to 0.1 N NaOH, 2% (w/v) SDS and shaken overnight at r.t. After centrifugation the supernatant was added to bicinchoninic acid (BCA) working solution in a 96-well plate format. The absorption was read at 550 nm in triplicate and subtracted from background readings for supernatant of blank microspheres of a corresponding batch.

Scanning electron microscopy (SEM), size analysis and DNA encapsulation efficiency were determined as previously described (Mohamed and van der Walle, 2006). Briefly, for SEM, lyophilised microspheres were sprinkled onto a carbon adhesive disk mounted onto an aluminium stub. Samples were coated with a thin layer of gold and imaged on a Jeol JSM-6400 operating at 10 kV, 20 °C and 10^{-5} Torr. Particle size for non-lyophilised microspheres was measured by laser diffraction using a Malvern Mastersizer 2000 (Malvern Instruments, UK) with a laser obscurity between 12 and 25%. The mode diameter and standard deviation of the microspheres are reported. For microspheres encapsulating plasmid DNA, DNA was extracted into Tris–EDTA buffer for microspheres solubilised into chloroform (Mohamed and van der Walle, 2006). The samples were centrifuged and the aqueous supernatant (upper layer) was analysed by absorbance at 260 nm. DNA extracted per mg of PLGA was quantified by the Beer–Lambert law (with an absorptivity coefficient of 50 μg/ml at $A_{260\,nm} = 1$).

2.3. Thermal analysis

DSC plots were obtained using dynamic DSC (DSC 822e, Mettler Toledo, UK). Samples were prepared by carefully weighing ~2–3 mg of microspheres into a 40 μl aluminium pan, which was then hermetically sealed with a pinhole in the lid. An empty pin-holed 40 μl aluminium pan was used as a reference. Both the reference pan and the sample pan were allowed to equilibrate isothermally for 5 min at 0 °C. The pans were then heated at a rate of 10 °C/min from 0 to 85 °C (well above the T_g), quench cooled to −20 °C (to erase thermal history) and then heated again to 85 °C at 10 °C/min. The temperature and heat flow of the DSC instrument was calibrated with indium and zinc and each microsphere batch was subjected to two independent DSC scans under a nitrogen atmosphere. The results were analysed using Mettler STAR software, and onset of transition was reported as the corresponding glass transition temperature. The accuracy of the DSC sensor is 0.1 °C. Evaluations were undertaken consistently for measurement of the onset temperature by extrapolating a straight baseline in the glassy state to where this meets the steepest part of the curve, and extrapolating a straight baseline in the rubbery state to where this meets the curve, respectively.

Temperature-modulated DSC (TM-DSC) was used to determine the mid point of the glass transition, observed without superposition of the enthalpy relaxation, circumventing the need to undergo a quench-cool step to remove thermal history for the material (Royall et al., 2001). Specifically, the T_g is represented by the reversing signal which describes a kinetic event, whereas the relaxation endotherm is represented by the non-reversing heat flow.

2.4. Physical ageing and non-linear regression analysis

Physical ageing was measured for the high Mw PLGA (0.83 dl/g) only. Each sample was subjected to the same (non-TM) DSC scans with the exception of the temperature and time following the quench-cool step. To measure ageing as a function of time and temperature, following quench-cool an isothermal annealing step was included wherein the sample was held *in situ* for 30 min to 12 h at constant temperature (0, 10, 25 and 35 °C). The enthalpy relaxation was calculated by integration of the overheating peak, extending the baseline that followed from the glass transition. Prolonged ageing at 35 °C, required to bring the microspheres towards thermal equilibrium (to estimate ΔH_∞), involved incubation of the microspheres at 35 °C *ex situ* for 24 and 48 h, and then measuring the overheating peak in the first DSC heating scan.

The ageing data were fitted to the Cowie–Ferguson model (see Section 3 for equations and notations used) in GraphPad Prism ver. 4.1, GraphPad Software, CA, USA, with the function $Y = Y_{max}(1 - (\exp((-1)(X/\tau)^{\beta})))$ and substituting ΔH_∞ (Y_{max}) for the experimentally observed enthalpy relaxation for microspheres approaching equilibrium. Statistical analysis of the parameters β and τ were performed using a one-way ANOVA with comparison of all values using Bonferroni's post-test. A significance level of $P < 0.05$ was used to denote significance in all cases.

3. Results

3.1. Encapsulation efficiencies and microsphere morphology

A porous microsphere surface morphology was observed by SEM for the microspheres produced by the w/o/w emulsion-evaporation method (Fig. 1). The microsphere size distributions corresponded to values previously obtained in our laboratories, as may be expected for the equivalent addition of PVA to the secondary emulsion, the volume ratio of primary:secondary emulsion, and the homogenisation speed (Table 1). pDNA encapsulation efficiency was equivalent to that for primary emul-

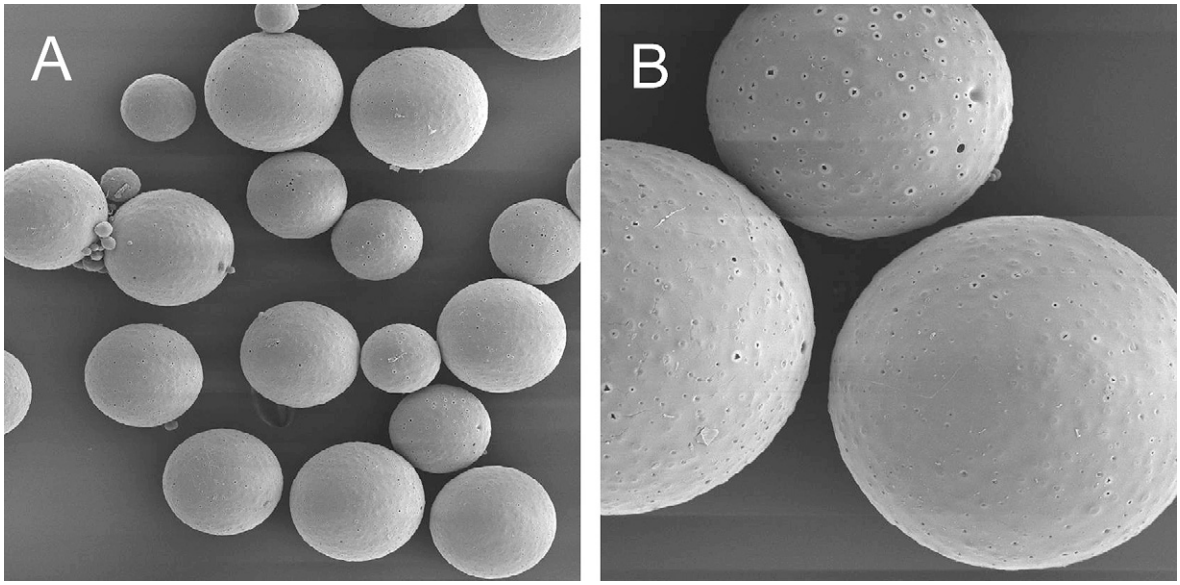

Fig. 1. Representative SEM micrographs for microspheres encapsulating FIII9′-10 and including PVA in the secondary emulsion step. (A) microspheres at 100× magnification and (B) microspheres at 500× magnification.

Table 1
Microsphere size and protein/pDNA encapsulation efficiencies

Formulation[a]	High Mw PLGA (0.83 dl/g)		Low Mw PLGA (0.18 dl/g)	
	Diameter ± S.D. (μm)[b]	EE (%)[c]	Diameter ± S.D. (μm)[b]	EE (%)[c]
PVA	110 ± 15	n/a	96 ± 12	n/a
Triton X-100	125 ± 17	n/a	107 ± 13	n/a
Tween 20	102 ± 14	n/a	92 ± 8	n/a
BSA	95 ± 9	35	101 ± 11	26
β-Casein	115 ± 14	25	95 ± 10	20
FIII9-10 wt	123 ± 15	18	89 ± 7	14
FIII9′-10	103 ± 10	23	92 ± 7	31
pDNA	119 ± 16	17	117 ± 13	10

n/a: no protein or pDNA encapsulated.
[a] Protein, pDNA or emulsifier added to the primary emulsion, concentrations as described in Section 2.
[b] Median diameter ± standard deviation.
[c] Encapsulation efficiency expressed as the percentage of the theoretical load (1 mg protein, or 0.5 mg pDNA, per 50 mg PLGA).

sions stabilised with Pluronic™ copolymers but around half compared with PVA (Table 1) (Mohamed and van der Walle, 2006). FIII9-10 wt and FIII9′-10 encapsulation efficiencies were equivalent to that primary emulsions stabilised with PVA but around half compared with Triton X-100 (Bouissou et al., 2004); the encapsulation of BSA was around half compared with PVA-stabilised primary emulsions (Duncan et al., 2005).

3.2. Determination of the glass transition

The glass transition of the high and low Mw PLGA microspheres was observed during the DSC scans as a change in the heat capacity slope, the first heating scan showing the enthalpy relaxation peak superposed on the T_g (Fig. 2). Although T_g can be determined from the second heating scan for quench-cooled material, this would expose the encapsulated protein (and to a lesser extent, pDNA) to high temperatures (85 °C) and so cause thermal denaturation and, concomitantly, the material properties of the PLGA microsphere. However, estimation of the T_g from the first scan is complicated by the overheating peak, and this favours measurement of the onset T_g value (Fig. 2). Since T_g is an important parameter we investigated TM-DSC in order to facilitate measurement of the T_g in the first heating scan at the midpoint of the associated heat capacity change. The midpoint was preferred estimate of the T_g since it has been shown that this method is more reliable than measurement of the onset temperature (Wunderlich et al., 1997).

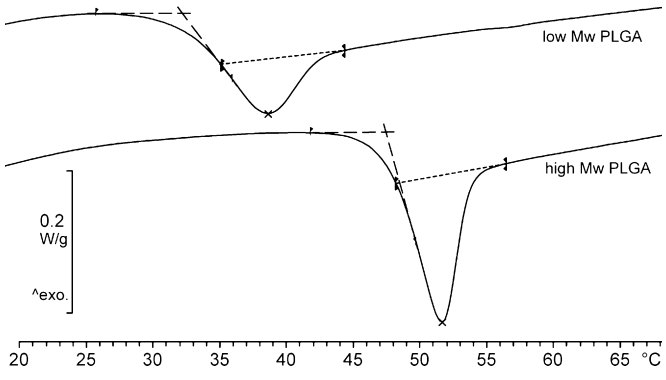

Fig. 2. Representative total heat flow DSC heating thermograms for high and low Mw PLGA microspheres in the glass transition region (labels next to curves). Dotted lines extrapolate a baseline to delineate the overheating peak area, integrated to give the enthalpy relaxation. Dashed lines extrapolate the baseline and steepest point on the curve to measure the onset T_g (see Section 2).

Analysing the PLGA microspheres using TM-DSC, we were able to isolate the T_g from the enthalpy relaxation, as previously observed (Passerini and Craig, 2002). The onset and midpoint T_g values for the blank PLGA microspheres were typical for the high and low Mw PLGA used (Table 2). Onset T_g values measured by DSC were lower than the midpoint T_g values measured by TM-DSC. The midpoint T_g for high and low Mw PLGAs varied between 46.0 to 48.9 and 34.1 and 37.3 °C, respectively (Table 2). The reason that the difference between the onset and midpoint T_g values were less for the high Mw PLGA microspheres than the low Mw PLGA microspheres was the steeper slope of the glass transition for high Mw PLGA (Fig. 3). The origin of the "ripples" on the thermograms acquired by TM-DSC is a consequence of the Fourier transformation of the modulated heat flux signals; they do not represent noise on the signal (Jiang et al., 1998).

Table 2
Microsphere glass transitions measured as the onset T_g and the midpoint T_g[a]

Formulation[b]	High Mw PLGA (0.83 dl/g)		Low Mw PLGA (0.18 dl/g)	
	Onset T_g (°C)	Midpoint T_g (°C)	Onset T_g (°C)	Midpoint T_g (°C)
No additive	47.3	47.7	32.1	36.1
PVA	47.6	48.9	32.9	37.3
Triton X-100	44.5	46.0	28.5	34.1
Tween 20	47.9	47.4	31.3	36.3
BSA	48.1	48.0	33.2	37.0
β-Casein	48.0	48.5	33.1	36.4
FIII9-10 wt	47.7	48.3	32.8	37.1
FIII9′-10	47.5	48.2	32.9	36.9
pDNA	48.4	48.7	33.0	37.0

[a] Average of two independent DSC scans: the difference between the two values ranged from 0.02 to 0.50 °C (the error in instrument sensitivity is 0.1 °C).
[b] Protein, pDNA or emulsifier added to the primary emulsion, concentrations as described in Section 2.

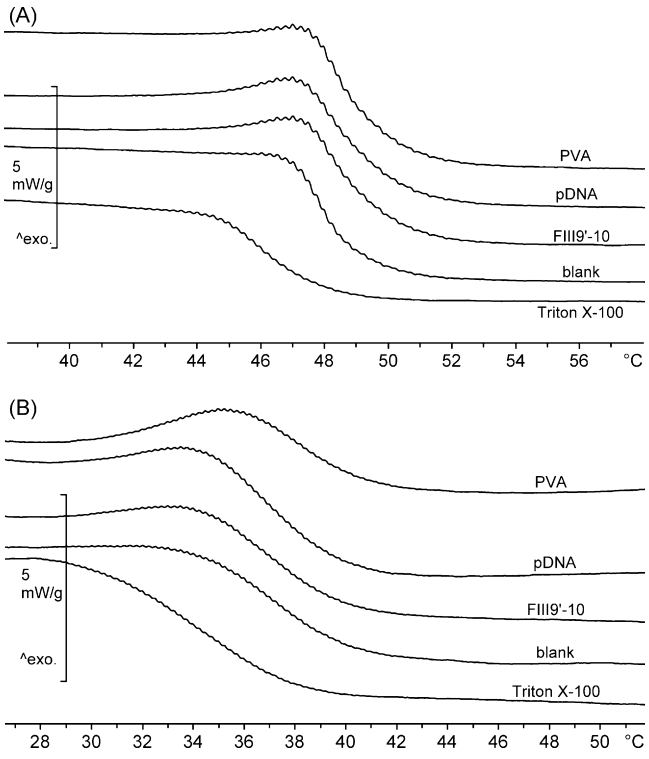

Fig. 3. Representative reversing heat flow TM-DSC heating thermograms for high (A) and low (B) Mw PLGA microspheres encapsulating PVA, pDNA and FIII9′-10, compared with Triton X-100 and blank formulations (labels next to curves).

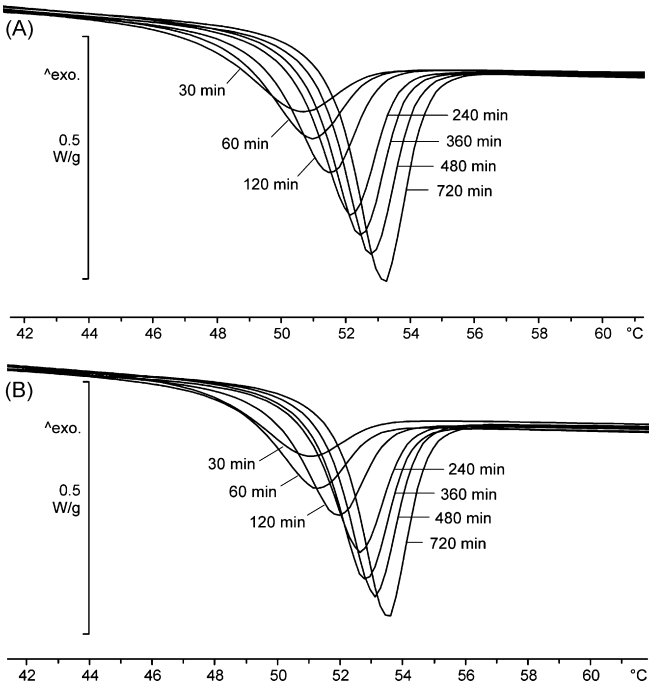

Fig. 4. Representative total heat flow DSC heating thermograms for high Mw PLGA microspheres fabricated with PVA (A) and BSA (B) in the glass transition region following various ageing times (indicated) at 35 °C. Overlaid curves superpose above and below the glass transition.

The T_g values did not vary significantly upon the encapsulation of proteins, irrespective of the proteins' emulsifying activities. (On the basis of the curve reproducibility and curve analysis method, a difference of 1 °C was taken as a significant difference.) For the encapsulation of FIII9′-10 at least, this is in contrast to previous data showing a 2 °C increase upon FIII9′-10 encapsulation into low Mw PLGA microspheres (Bouissou et al., 2006). The data acquired here for FIII9′-10 show a 0.8 °C increase in the onset T_g for low Mw PLGA (Table 2 and Fig. 3B); the difference between the two studies is therefore small and may be accounted for by the relative inaccuracy in using the onset T_g from DSC thermograms compared with TM-DSC reversing heat flow thermograms (Wunderlich et al., 1997). The known plasticising effect of Triton X-100 is consistent with the large fall in the T_g for both high and low Mw PLGA microspheres. In contrast, PVA and pDNA were observed to be mildly antiplasticising to high Mw PLGA and, for PVA alone, low Mw PLGA microspheres, when measured by the midpoint T_g using TM-DSC.

3.3. Physical ageing

Total heat flow DSC thermograms are shown for one set of scans for high Mw PLGA microspheres fabricated with PVA or BSA and aged over 12 h at 35 °C prior to measurement (Fig. 4). Similar thermograms were observed for PLGA microspheres fabricated with Triton X-100 and repeated scans. For the shortest ageing times of 30 min at 25 °C, a minimal endothermic overheating peak was observed superposed on the glass transition region for all the microspheres. At higher temperatures and longer ageing times the respective endothermic peaks were seen to increase correspondingly. This endothermic peak represents the recovery of heat loss during the ageing process (the enthalpy of relaxation). With increasing ageing times the T_g and the temperature at the apex of the overheating peak were observed to shift to higher temperatures. The linear plot for ΔH versus log(ageing time) is consistent with the Bauwenscrowet and Bauwens model of physical ageing as a consequence of decreasing molecular mobility (Fig. 5) (Bauwenscrowet and Bauwens, 1986).

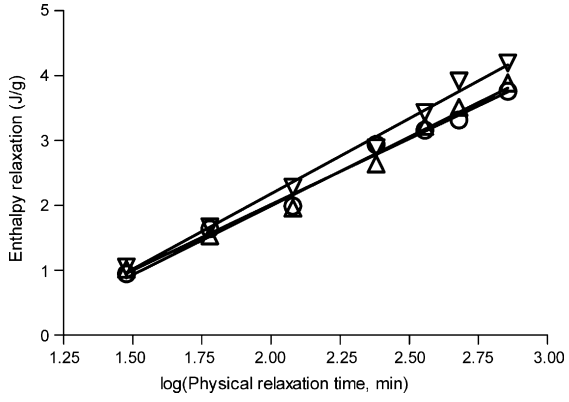

Fig. 5. Linear regression of the logarithm of the physical ageing time for the PLGA microsphere formulations and the enthalpy relaxation at 35 °C (r^2-values are 0.99 for each curve). Emulsifiers used in the primary emulsion were PVA (up triangles), BSA (down triangles) and Triton X-100 (circles).

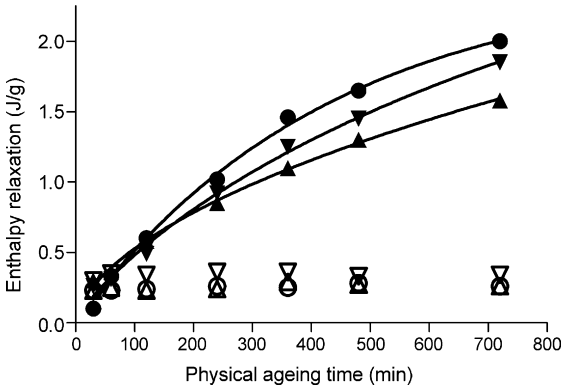

Fig. 6. Effect of high Mw PLGA microsphere formulation on the enthalpy relaxation kinetics at 25 °C (filled symbols) and 10 °C (open symbols). Emulsifiers used in the primary emulsion were PVA (up triangles), BSA (down triangles), Triton X-100 (circles). Curves represent the data fitted to the Cowie–Ferguson model (fitting was inappropriate for data acquired at 10 °C).

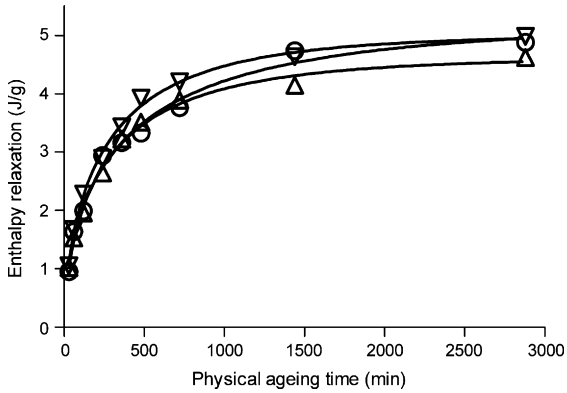

Fig. 7. Effect of high Mw PLGA microsphere formulation on the enthalpy relaxation kinetics at 35 °C. Emulsifiers used in the primary emulsion were PVA (up triangles), BSA (down triangles), Triton X-100 (circles). Curves represent the data fitted to the Cowie–Ferguson model.

We further investigated the effect of the primary emulsifier and temperature of storage on the physical ageing process of the PLGA microspheres. ΔH for the microspheres aged for up to 12 h at 0 and 10 °C did not show any corresponding increase (Fig. 6; data not shown for 0 °C). For microspheres aged at 25 °C, ΔH increased slowly with increasing ageing times and after 12 h remained far from the ΔH measured for microspheres subject to prolonged ageing at 35 °C (around 5 J/g, Table 3); the enthalpy taken to approximate ΔH_∞. In contrast, ΔH calculated for all microsphere formulations aged for up to 12 h at 35 °C did near the value taken as ΔH_∞ and the curves showed evidence of reaching a plateau (Fig. 7). Therefore, while the microspheres clearly aged at room temperature, the ageing process was much more rapid as the temperature of the isothermal annealing step approached T_g. It was also evident on inspection of the curves in Figs. 6 and 7 that ΔH did not vary much between the three-microsphere formulations tested.

In order to analyse the data in more detail, the enthalpy relaxation curves were fitted using a phenomenological multi-order parameter model described by Cowie and Ferguson (1989a,b) based on Eq. (1), wherein t is the ageing time, ΔH_∞ the enthalpy as t approaches ∞, and $\phi(t)$ the relaxation function describing the kinetics of the enthalpy relaxation:

$$\Delta H(t) = \Delta H_\infty[1 - \phi(t)] \quad (1)$$

$\phi(t)$ is commonly described quantitatively in terms of the following Kohlrausch–Williams–Watts type stretched exponential equation (Eq. (2)), wherein the stretched exponential function contains an overall relaxation time τ and a non-exponential parameter β:

$$\phi(t) = \exp\left[-\left(\frac{t}{\tau}\right)^{\beta}\right] \quad (2)$$

The parameter β is a measure of the degree of distribution of the relaxation time and is constrained to values $0 < \beta < 1$. Large values of β mean that there is a narrow range of relaxation times ($\beta = 1$ implies a single relaxation mode), with small values implying many coexisting relaxation modes and a broad range of relaxation times. β and τ are determined by fitting the ΔH versus t curve to the Cowie–Ferguson equation (Eq. (3)); substituting Eq. (2) into Eq. (1):

$$\Delta H(t) = \Delta H_\infty\left\{1 - \exp\left[-\left(\frac{t}{\tau}\right)^{\beta}\right]\right\} \quad (3)$$

Since the values for ΔH were observed to approach equilibrium for ageing at 35 °C, here, the value of ΔH_∞ was determined experimentally by conducting ageing experiments at 35 °C for periods of 24 and 48 h (1440–2880 min) to ensure that the microspheres had come close to equilibrium. Fitting of the data to the Cowie–Ferguson model was then possible as described in Section 2.

Table 3
Glass transition and enthalpy relaxation for high Mw PLGA microsphere formulations maintained at 35 °C

Formulation[a]	Onset T_g after 30, 240, 720 min ageing[b] (°C)	τ, relaxation time[c] ± S.E.[d] (min)	β, spectral width[c] ± S.E.[d]	Calculated ΔH_∞ (J/g)	Measured ΔH_∞[e] (J/g)
PVA	47.8, 50.1, 51.3	277 ± 21	0.62 ± 0.03	5.0 ± 0.1	4.98
BSA	48.1, 50.6, 51.6	288 ± 30	0.61 ± 0.04	4.62 ± 0.1	4.62
Triton X-100	44.2, 45.7, 46.1	405 ± 93	0.56 ± 0.06	5.2 ± 0.3	4.88

[a] Protein or emulsifier added to the primary emulsion.
[b] Measured using (non-TM) DSC.
[c] Determined by curve fitting to the Cowie–Ferguson model (Eq. (3)), substituting ΔH_∞ with the measured value.
[d] One-way ANOVA with a Bonferroni post-test did not show any significant difference between the groups ($P > 0.05$).
[e] For microspheres aged for a further 48 h, average of two DSC scans.

The effect of emulsifier used during microsphere fabrication on the kinetics of the enthalpy relaxation appeared to be minimal at best (Table 3). Although microspheres fabricated with Triton X-100 had relaxation times of 405 min, somewhat higher than the relaxation times for PVA and BSA, statistical analysis did not suggest this to be significant. Fitting the enthalpy relaxation curves acquired at 35 °C ageing to the Cowie–Ferguson model yielded calculated ΔH_∞ corresponding to the experimentally determined values (Table 3 and Fig. 7). No change to the enthalpy relaxation was observed for microspheres aged at 10 °C and these data were therefore not fitted to the model. For microspheres aged at 25 °C, values for ΔH following 12 h *in situ* isothermal annealing remained far from ΔH_∞ (Fig. 6). This introduced a degree of uncertainty in the fitting of the ageing data acquired at 25 °C to the Cowie–Ferguson model and the returned values for the relaxation times (2000–9000 h) should be treated as estimates. Clearly, prolonged ageing over several months at ambient temperatures (and possibly years at 10 °C) would be needed to fit the ageing data more accurately.

4. Discussion

Encapsulation efficiencies varied considerably between the proteins used. This is not unusual and appears to be dependent on the PLGA Mw, protein and co-emulsifier added to the primary emulsion (Blanco and Alonso, 1998). The absence of added surfactant to the primary emulsion tended to result in low encapsulation efficiencies of proteins and pDNA, though this depended upon the reported values used for comparison. The reason for the generally low encapsulation efficiencies was likely to have been due to poor emulsion stability, as previously observed for both encapsulation of proteins and pDNA (Rojas et al., 1999; Mohamed and van der Walle, 2006).

Proteins are surface active and during w/o/w emulsification are largely denatured or suffer subtle conformational changes (van de Weert et al., 2000; Duncan et al., 2005). The molecular basis for their surface activity has been characterised for albumins (Burnett et al., 2002) and β-casein (Caessens et al., 1999) and more recently for β-sandwich proteins like FIII9-10 wt (Annan et al., 2006). However, their influence on the emulsification efficiency of the primary emulsion and subsequent material properties of the microspheres have been largely ignored. This is in contrast to the known emulsification efficiencies of PVA, Triton X-100 and Tween 20. T_g measurements were therefore used to find evidence of plasticising or antiplasticising influence following encapsulation of the proteins or pDNA in the microspheres, compared with microspheres fabricated with PVA, Triton X-100 and Tween 20. We chose not to add co-emulsifiers to the primary emulsion during protein or pDNA encapsulation so that their effect on the material properties of the microspheres would not be masked.

The strong plasticising effect of Triton X-100 on PLGA (Table 2) has previously been observed and accounted to the miscibility of the polyethylene oxide (PEO) head group with the PLGA chains (Chung et al., 2002). The inclusion of Triton X-100 also increases the amount of residual water in the microspheres (Bouissou et al., 2006), which in turn increases PLGA chain mobility and thereby decreases T_g (Passerini and Craig, 2001). The mildly antiplasticising effect of PVA and pDNA on PLGA must therefore involve some restriction of PLGA chain mobility. Interestingly, a recent study wherein dendrimers were blended with polyesters showed that crosslinking between the two polymers had an antiplasticising effect on the polyester; this was suggested to be due to strong intermolecular interactions via hydrogen bonding restricting the polyester chain mobility (Xu et al., 2006). The hydroxyl groups of PVA and the sugar-phosphate backbone of DNA both have the potential to interact with PLGA via electrostatic interactions and this may explain their mildly antiplasticising effect. It should be noted that pDNA did not significantly increase the T_g of the low Mw PLGA microspheres; the reason for this is unclear but may be due to the lower encapsulation efficiency (Table 1). In contrast, none of the proteins had any significant effect on the material properties of PLGA. This was presumably either due to poor interaction between the protein and PLGA chains or phase separation of the protein and PLGA during emulsification–encapsulation. The complex chemical nature of proteins (considering the nature of the backbone and side chains and denaturation) makes further interpretation of these data difficult.

On the basis of the TM-DSC experiments, we chose to study the physical ageing process for microspheres fabricated with PVA, BSA and Triton X-100, since these were good examples of antiplasticising, neutral and plasticising agents, respectively. The physical ageing experiments were conducted at temperatures corresponding to those that would be encountered by the microspheres during the secondary drying stage of lyophilisation. That is, as the temperatures are increased from below 0 °C to room temperature (Tangpasuthadol et al., 1996). We also investigated temperatures around 10 °C below the T_g of the high Mw PLGA used in these experiments, in order to observe the accelerated ageing process. The increase in the T_g during physical ageing is well documented in the literature for various amorphous polymers and is due to the concomitant decrease in the molecular mobility of the polymer. Evidence that the decrease in molecular mobility is involved in the structural relaxation process can be seen by the linear relationship between ΔH (the enthalpy calculated by integration of the endothermic peak) and the time of ageing plotted on a logarithmic scale (Fig. 5). This relationship has previously been shown to imply that molecular mobility is a general feature of the structural relaxation of glassy polymers (Bauwenscrowet and Bauwens, 1986).

A commonly used mathematical model for analysis of the structural relaxation of glassy polymers is that of Cowie and Ferguson (1989a,b). Here, we found that the relaxation time (τ) was independent of encapsulation of protein or fabrication with antiplasticising and plasticising agents (although Triton X-100 did appear to increase τ, this was not statistically significant). This suggests that the mechanism of the structural relaxation involved only the PLGA chains and was limited by the mobility of the PLGA polymer backbone. This interpretation is further supported by analysis of the width of the relaxation times (β) for the three microsphere formulations, which were statistically indistinguishable; i.e. the relaxation modes were not that broad to suggest contributions from macromolecules other than PLGA.

Lowering the isothermal annealing temperature increased the relaxation time (Figs. 6 and 7), due to the slower molecular motions necessitating longer times for the structure to relax (Hancock et al., 1995; Bailey et al., 2002). Since these motions increase with temperature, it is likely that microsphere ageing during lyophilisation occurs mainly during the secondary stage of the lyophilisation process, as the temperature is slowly increased to ambient temperatures (Tangpasuthadol et al., 1996). However, given that the relaxation time is around 280–400 h at 35 °C it must be the case that, while the microspheres will age during lyophilisation, this process will be far from complete and practically suspended if the microspheres are subsequently stored dry in a fridge. The ageing process for PLA has further been described at the molecular level, wherein spectroscopic and thermal techniques were used to determine the preferred rotational isomeric states (ttt, ttg′, tg′, tg′g′) of the polymer backbone (Aou et al., 2005). Interestingly, a PLA sample with at least 60% amorphous content in a deformed structure was required before (fiber) shrinkage was observed during ageing around the glass transition.

Our observations have consequences for the manner in which PLGA microspheres are manufactured and deployed as drug delivery vehicles. First, the physical ageing process for PLGA microspheres appears to be independent of the emulsifier used or upon encapsulation of protein. Second, ageing will theoretically continue during cold chain storage and shipping, with changes in the microspheres' physicomechanical properties occurring at ambient temperatures, before administration (particularly, at high ambient temperatures).

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