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Characterization of ciclosporin A loaded poly (D,L lactide-*co*-glycolide) microspheres using modulated temperature differential scanning calorimetry

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Abstract

The aim of this study was to investigate the physical structure of poly (D,L lactide-*co*-glycolide) (PLGA) microspheres loaded with ciclosporin A in terms of the amorphous properties of the individual components and the phase separation characteristics of the binary systems. Microspheres were prepared using a standard oil-in-water emulsion technique. The thermal properties of the PLGA, ciclosporin A and loaded spheres were investigated using modulated temperature differential scanning calorimetry (MTDSC) using a TA Instruments MTDSC 2920, with scanning electron microscopy (SEM), X-ray powder diffraction (XRD) and high-performance liquid chromatography used as supportive techniques. MTDSC indicated a glass transition for ciclosporin A in the reversing heat flow signal at 107°C, supported by temperature cycling studies, while XRD showed clear evidence for diffraction peaks, thereby indicating that the material as received is semi-crystalline. The unloaded PLGA spheres showed a glass transition (T_g) at 43°C, with no reduction in T_g being observed on loading the peptide up to 50%, w/w. Similarly, no evidence for diffraction peaks were seen for the drug-loaded systems, although the glass transition corresponding to the peptide was observed for the loaded microspheres, suggesting that the drug is present as a separate amorphous phase. Similarly, SEM studies showed the appearance of distinct ''islands'' on the surface of the spheres that are suggested to correspond to the drug phase, with the size of the islands increasing with drug loading. Evidence is therefore presented that ciclosporin A may exist in a range of solid states, with the degree of crystallinity being altered by processing. In addition, there appears to be little or no miscibility between the drug and PLGA using the manufacturing protocol employed here. These findings may have implications for the choice of manufacturing protocol, the release of peptide drugs from PLGA microspheres and the chemical and physical stability of such drugs.

Introduction

Ciclosporin A is a non-polar cyclic undecapeptide, used as a potent immunosuppresant to prevent tissue rejection in organ transplantation (Ptachcinski et al 1986; Sheng-Tanner et al 2000). However, the drug has several drawbacks including marked nephrotoxicity and variable oral bioavailability that are still pertinent considerations with regard to the commercially available dosage forms. In recent years, alternative formulation strategies have been examined, including liposomes (Al-Angary et al 1995), microemulsions (Mueller et al 1994), nanospheres (Barichello et al 1999) and microspheres. The possibility of using poly (D,L-lactic acid) (PLA) or poly (D,L lactide-*co*-glycolide) (PLGA) microspheres has attracted attention given the possibility of achieving sustained release over a therapeutically relevant period via parenteral administration, thereby avoiding the difficulties associated with oral absorption and repeat dosing. In particular, Sanchez et al (1993) prepared ciclosporin A loaded polylactide microparticles using the solvent evaporation method, and reported high encapsulation efficiency and a biphasic in-vitro release profile. These authors later investigated the in-vivo release properties of ciclosporin A loaded microspheres and nanospheres on subcutaneous injection using a mouse model, noting a burst followed by a sustained release effect for up to 3 weeks, depending on the sphere size

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(Sanchez & Alonso 1995). Similarly, Chacon et al (1996) analysed the effects of formulation conditions (polymer amount and stirring rate) on the yield, size, morphology and encapsulation efficiency in ciclosporin A loaded PLGA microspheres. More recently, Urata et al (1999) have studied modification of ciclosporin A release from PLGA microspheres by adding fatty acid esters as additives, noting that incorporation of ethyl myristate enhanced the release of the peptide in-vitro and in-vivo, thereby allowing optimization and tailoring of the release profile.

One area that arguably merits further exploration not only for these specific systems, but also for peptide-loaded microspheres in a broader sense, is the nature of the distribution of the drug within the spheres. The study of Sanchez et al (1993) involved the use of scanning electron microscopy (SEM), differential scanning calorimetry (DSC) and X-ray powder diffraction (XRD) as means of studying the nature of the interaction between the peptide and carrier; the authors suggested that the former was present as a molecular dispersion in the latter. In this investigation, we examine the use of modulated temperature DSC (MTDSC) as a further means of studying equivalent systems, particularly in terms of examining the physical properties of ciclosporin itself. MTDSC involves the application of a sinusoidal signal to a sample and the deconvolution of the response into reversing (heat capacity) and non-reversing (kinetic) signals according to:

$$dQ/dt = C_p(b + Bx\cos(x\,t)) + f'(t, T) + C\sin x\,t \qquad (1)$$

where dQ/dt is the total heat flow, C_p is the heat capacity, b is the underlying heating rate, B is the amplitude of the non-kinetic (heat capacity) modulation, x is the frequency, $f'(t,T)$ is the underlying kinetic response excluding the effect of the modulation and C is the amplitude of the kinetic response to the sine wave modulation (usually assumed to be negligible) (Coleman & Craig 1996). This technique not only enables the operator to separate the glass transition from the accompanying endothermic relaxation, but also facilitates measurement of the heat capacity from the magnitude of the response to the perturbation via:

$$C_p = K_{Cp}(Q_{amp}/T_{amp})(1/x) \qquad (2)$$

where K_{Cp} is a heat capacity calibration constant, Q_{amp} is the modulated heat flow amplitude and T_{amp} is the modulated temperature amplitude. In addition, the baseline sensitivity of the reversing signal is such that subtle glass transitions may be seen using this approach that would be indistinguishable using the conventional technique. The ability to measure and identify glass transitions with greater clarity has several implications for the study of PLA/PLGA microspheres, both in terms of understanding release behaviour and characterizing the physical form of the drug within the spheres. In terms of the latter, previous studies on PLA/PLGA microspheres using MTDSC have indicated that incorporated progesterone may exist as a molecular dispersion, as a separate amorphous phase or as a separate crystalline phase within PLGA spheres depending on the loading (Hill et al 1998). Later studies using microthermal analysis presented evidence for these separate phases being at least partially present on the surface of the spheres, with concomitant implications for release (Royall et al 2001). However, although several groups have studied the macroscopic phase separation of proteins and peptides within microspheres (Sanchez et al 1993; van de Weert 2000), the issue of the physical structure of the drug itself remains largely unexplored. The objective of this study was therefore to investigate the effect of drug loading on the physical properties of ciclosporin A loaded microspheres, using a combination of thermal spectroscopic and microscopic techniques, with a view to exploring the utility of MTDSC as a means of examining the physical properties of pharmaceutically relevant peptides and peptide-loaded spheres.

Materials and Methods

Materials

Ciclosporin A (MW 1202.6) was used as received. The weight loss, determined by thermogravimetric analysis and assumed to correspond to water loss, was $1.15 \pm 0.22\%$ (see below). PLGA 50:50 (MW 40 000–70 000) was obtained from Sigma. Dichloromethane and polyvinylalcohol (PVA; 98% hydrolysed, MW 22 000) were supplied by BDH. Cyclohexane (99.9%) and *n*-octodecane (99.9%) were supplied by Reidel-de-Haen (Germany), and indium (99.999%) and aluminium oxide (99.9%) were supplied by Aldrich.

Preparation of the microparticles

The microparticles were prepared according to the solvent evaporation technique (O'Donnell & McGinity 1997). PLGA and ciclosporin A (total mass 500 mg) were dissolved in 10 mL dichloromethane; ciclosporin A contents of 0, 10, 20, 30 and 50% (w/w) were used. The organic phase was emulsified in 125 mL of a 2% (w/v) aqueous solution and stirred at 750 rev min^{-1} for 5 min. The stirring was then reduced to 250 rev min^{-1} and maintained at this speed overnight to produce spheres in the 50–150-1 m diameter range. The microspheres were then collected by vacuum filtration, washed 3 times with water to remove the PVA and dried by freeze-drying. The freeze-drying protocol was as follows: 20 min at -50°C, then 3 h at -20°C and 100 mbar, 2 h at 0°C and 200 mbar, 4 h at 30°C and 500 mbar and, finally, 4 h at 30°C and 1000 mbar. The water content of the drug and the microspheres were determined using a TGA 2950 (TA Instruments) using a dry nitrogen purge. Indium was used to calibrate the temperature, and the mass measurement was calibrated according to the manufacturer's instructions. A heating rate of 10°C min^{-1} and open pans were used to determine the mass loss from ambient temperature to 130°C. The water content of the formed spheres after drying

was 1.12±0.09%, 1.28±0.11%, 1.63±0.07% and 1.51±0.06%, respectively, for 0, 10, 30 and 50% loading (determined by TGA, previous studies having established that dichloromethane levels are negligible using this protocol; Passerini & Craig 2001). The ciclosporin A content in microspheres was determined by reversed-phase high-performance liquid chromatography (RP-HPLC), using the method previously reported by Sanchez et al (1993). The microspheres were dissolved in acetonitrile and diluted adequately before injection onto the HPLC system (Perkin-Elmer, Norwalk, USA). The HPLC system consisted of a LC 410 pump and a LC235 DAD (diode array detector). Separation was achieved by using a reversed-phase column (Spherisorb ODS1 5S 4.6×250 mm; Waters, USA) thermostated at 70°C. The mobile phase was acetonitrile/water (65:35), the flow rate was 1.0 mL min^{-1} and the ciclosporin A was monitored at 210 nm. The entrapment efficiencies were 95.0, 97.0 and 95.8% for the 10, 30 and 50% systems, respectively.

Physical characterization of microspheres

The morphology of the microparticles was examined using a SEM XL20 (Philips, Eindhoven, Netherlands). Conventional DSC and MTDSC experiments were performed using a MDSC 2920 (TA Instruments) with a refrigerated cooling system (TA Instruments). The instrument was calibrated for temperature using cyclohexane, *n*-octane and indium, and for the enthalpy using indium. The heat capacity constant was determined at 45°C using aluminium oxide. Both conventional DSC and MTDSC analysis were performed in aluminium hermetic pans with a sample mass of approximately 4 mg. DSC studies were conducted at 5°C min^{-1}. For MTDSC experiments, the parameters used were: 1°C min^{-1} heating rate, ±0.5°C modulation amplitude and 40-s modulation period. For the temperature cycling experiments, the following method was used: an initial 10-min isothermal period at 20.0°C was used to allow the equilibration of the sample, followed by a heating scan at 1°C min^{-1} to 130°C. This in turn was followed by a cooling scan at 1°C min^{-1} to 25°C and a second heating scan at 1°C min^{-1} to 130°C. The experiments were repeated at least three times. The glass transition was calculated from the midpoint of the transition using Universal Analysis TA Instruments software. XRD analysis on ciclosporin A and on ciclosporin A loaded PLGA microspheres were obtained using a Siemens D500 X-ray diffractometer with CuK radiation (40 mA). The step size was 0.02° and the step time was 20 s.

Results

Ciclosporin A

The physical state of the drug as received was evaluated in order to detect possible modifications of the physical structure when incorporated into the microspheres. Conventional DSC (Figure 1) showed a discontinuity in the heat flow commencing at 100.2±1.6°C; this could be owing to a glass transition of the drug associated with a relaxation endotherm, suggesting that the drug is in a wholly or partially amorphous state. In order to establish whether the observed event was indeed a glass transition, cycling MTDSC experiments were performed. This provides two mechanisms of verification. In the first instance, a glass transition may be expected to be observed in the reversing rather than the non-reversing signal in which the accompanying relaxation endotherm may be observed. Second, temperature cycling indicates whether the transition is reversible, as would be expected for a glass transition. The results of these experiments are shown in Figures 2 and 3. Figure 2 shows the first heating scan, indicating the total heat flow, the reversing and the non-reversing signals. The transition is clearly seen in the reversing signal, while the relaxation endotherm is seen in the non-reversing signal, supporting the hypothesis that the transition represents a T_g. It should be noted, however, that this is the T_g of the partially hydrated rather than the dry material. Furthermore, there is an apparent discrepancy between the T_g measured in the total and reversing

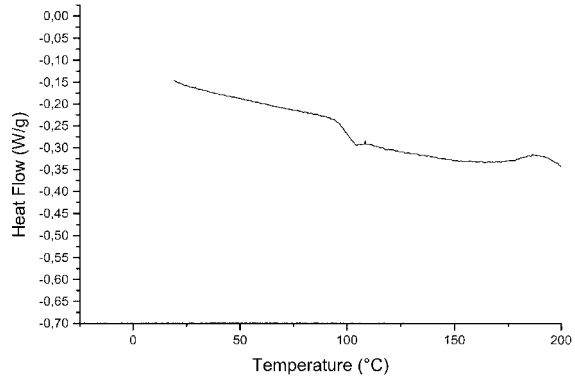

Figure 1 Conventional DSC for ciclosporin A.

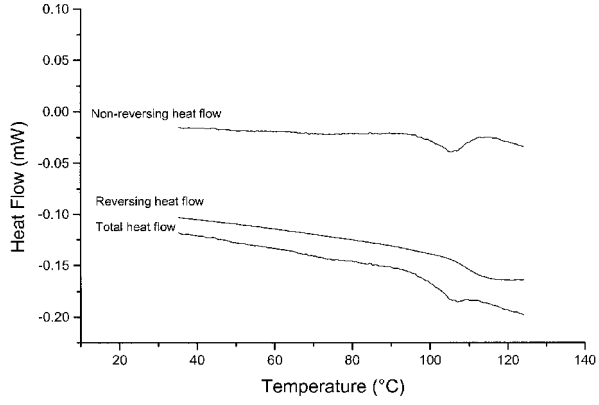

Figure 2 MTDSC signals for ciclosporin A, showing the total reversing and non-reversing heat flow.

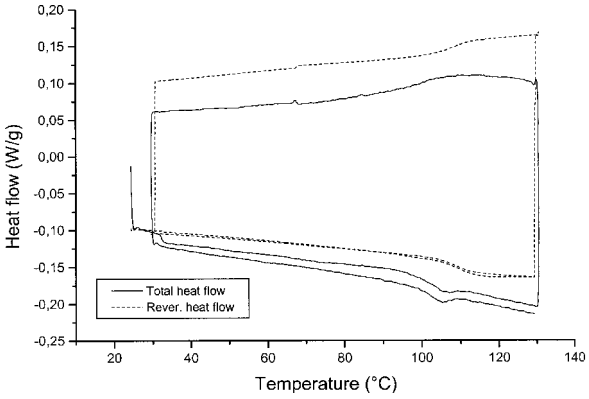

Figure 3 MTDSC temperature cycling experiments for ciclosporin A.

signals. From Figure 2, the total heat flow indicated that the transition occurs over a temperature range of 98 to 109°C. It is not possible to accurately assign a specific value for the T_g midpoint owing to the obscuration of the response by the relaxation endotherm, although previous studies have utilized the fictive temperature to obtain a more reliable single value (Craig et al 2000). The midpoint T_g observed in the reversing heat flow is 107 ± 3°C; clearly this value may be obtained directly from the data and illustrates one of the advantages of the modulated technique. Even in the absence of an accurate midpoint value for the total heat flow, it is clear that the reversing heat flow value is higher. This is further illustrated by observing the total heat flow during the cycling studies (Figure 2) whereby, on cooling, the relaxation endotherm in the total heat flow is absent (or strictly speaking very much reduced in size; Craig et al 2000; Van den Mooter et al 2001). In this signal, the total heat flow value is around 100°C ± 3°C in contrast to the reversing heat flow values of 106 ± 3°C and 107 ± 3°C for the first heat and cool, respectively. Such differences have been previously noted (Royall et al 1998; Lammert et al 1999) and have been ascribed to the transition being measured as a response to the modulated signal in the reversing signal, but to the average underlying signal in the total heat flow. This "T_g shift" effect reflects the fact that the glass transition is dependent on the rate of application of the heating signal being different with respect to the oscillation and the underlying (linear) programmes. Both values are accurate in as much as the T_g is not a fixed value for a particular material, but varies according to the measuring conditions (an exception being the fictive temperature, which is independent of heating rate).

The second means of verification, other than the observation of a heat capacity shift in the reversing heat flow signal, is the reproducibility of the response on cycling using identical heating and cooling rates. Indeed, this is a highly effective means of establishing whether a response is indeed a glass transition unless the sample undergoes a further event, such as decomposition, during the cycle. The cycling studies therefore lend further support to the suggestion of the event being a glass transition, indicating that

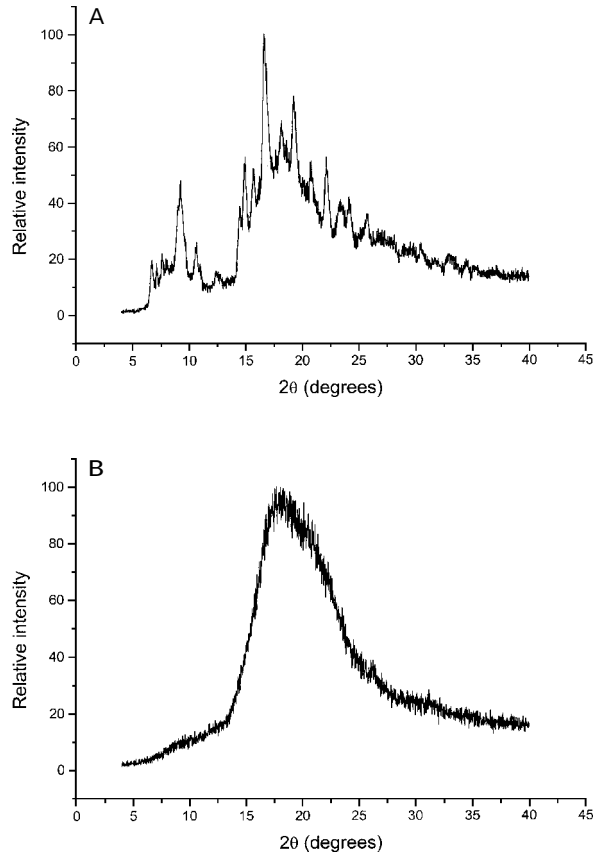

Figure 4 Powder X-ray diffraction for (A) ciclosporin A as received and (B) 50% w/w ciclosporin A loaded microspheres.

the material is partially or wholly amorphous. However, it was interesting to note that XRD studies (Figure 4A) clearly indicate that the material has a crystalline content. These studies therefore indicate that the material as received is semi-crystalline in nature. Studies are ongoing regarding the melting/decomposition behaviour of this material.

Ciclosporin A loaded microspheres

Low magnification SEM studies indicated that the spheres were approximately 100–150 1 m in diameter. To evaluate the surface characteristics, analysis at higher magnification on empty and drug-loaded (10, 30 and 50% ciclosporin A) microspheres was performed. The surface of empty microparticles (Figure 5) was completely smooth and homogeneous, whereas the surface of ciclosporin A loaded microspheres showed "islands" surrounded by a continuous matrix. The dimensions of these islands increased with increasing drug loading, leading to the tentative suggestion that the circular region is the drug and the surrounding area is the polymeric matrix, thereby suggesting phase separation between the two components.

In order to further evaluate the physical form of the drug in the spheres, MTDSC studies were conducted on the

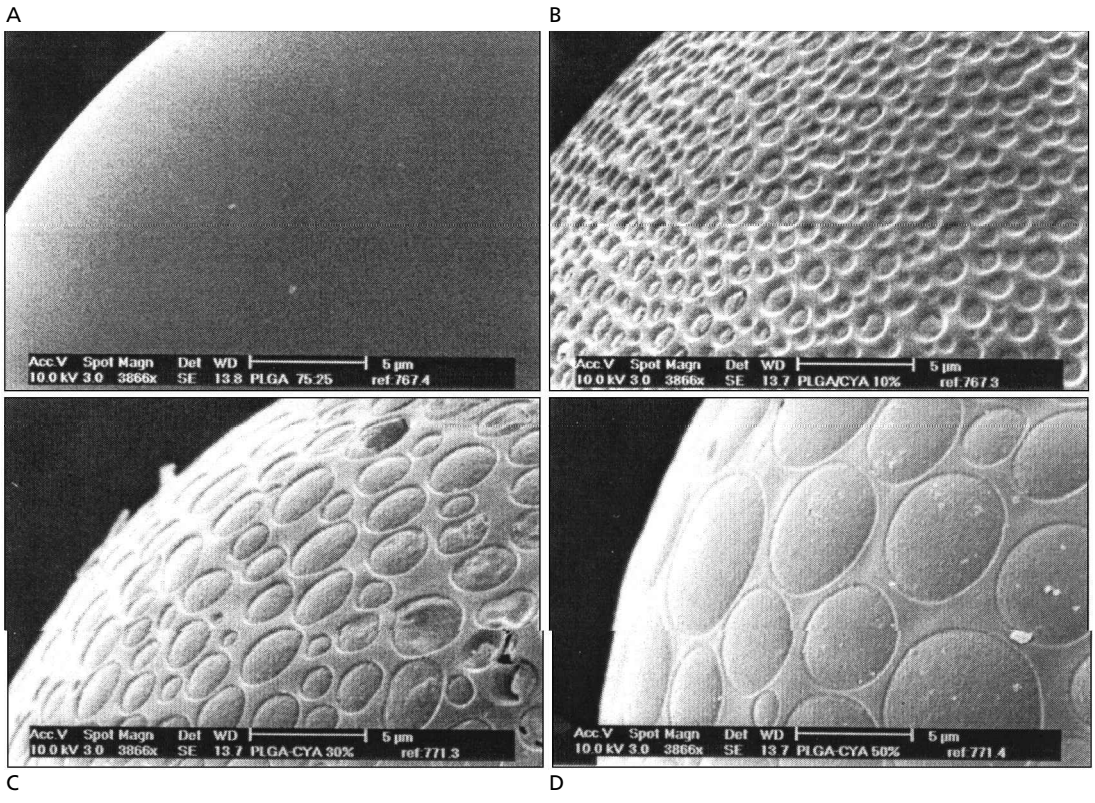

Figure 5 SEM images of ciclosporin A loaded microspheres containing (A) 0%, (B) 10%, (C) 30% and (D) 50% w/w drug loading.

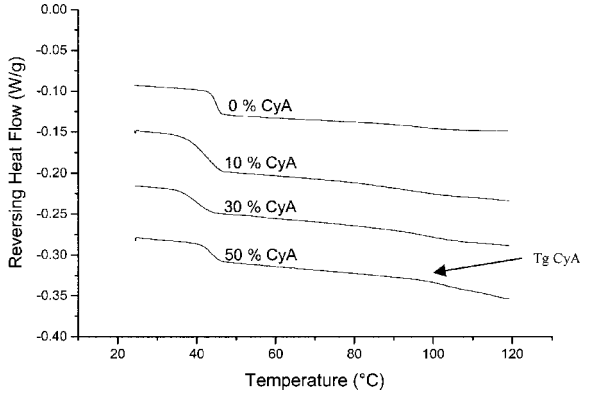

Figure 6 Reversing heat flow for 0, 10, 30 and 50% ciclosporin A loaded microspheres.

unloaded and loaded systems. Figure 6 shows the reversing heat flow of the 0, 10, 30 and 50% ciclosporin A loaded microparticles. The empty microspheres show a transition at 43 ± 2°C (calculated in the reversing heat flow), this being ascribed to the glass transition of the polymer. On incorporating the ciclosporin A into the spheres, the glass transition of PLGA did not change significantly, with T_g values for the 10, 30 and 50% loaded microspheres being measured as 42 ± 1°C, 41 ± 1°C and $43 \pm < 1$°C, respectively. This absence of any discernible plasticization effect suggests the absence of interactions between the polymer and the drug. Moreover, when detectable, the T_g of the drug itself was not modified when ciclosporin A was incorporated in microspheres. For example, at 50%, w/w, drug loading, a second glass transition at $108.0 \pm < 1$°C was observed, corresponding to the amorphous ciclosporin A, this value being very close to the T_g of pure ciclosporin A (107 ± 3°C in the first heating scan).

XRD analyses were performed to detect possible modifications in ciclosporin A physical state (originally semi-crystalline) following incorporation into the microspheres. As expected, empty PLGA microspheres show an XRD response typical of a complete amorphous system. However, this diffraction pattern was maintained for the 10 and 50% loaded microspheres (Figure 4B). The absence of any XRD peaks associated with the pure ciclosporin A suggest that, during the preparation process, the drug is transformed to a largely or completely amorphous state that is phase separated from the polymeric matrix. This is, we believe, of considerable potential interest, as although we have demonstrated that such effects may occur for low molecular weight drugs such as progesterone (Hill et al 1998), the possibility of amorphous phase separation occurring with peptide and proteinaceous drugs has not been previously considered.

In addition, the absence of diffraction peaks for the loaded drug presents an opportunity to characterize the degree of crystallinity via:

$$\Delta C_p = u \Delta C_p^0 \quad (3)$$

where ΔC_p and ΔC_p^0 is the heat capacity change for the semi-crystalline material and the wholly amorphous material, with the parameter u representing the degree of crystallinity. Unfortunately, it was not possible to obtain reliable ΔC_p values for the ciclosporin A within the spheres because of the absence of a clear step change. This was probably owing to the softening of the PLGA above the T_g (Royall et al 2001), resulting in the drug effectively being surrounded by liquid, which may alter the thermal response. However, with refinement, the approach may be developed as a novel means of assessing the degree of crystallinity of pharmaceuticals.

Discussion

This study has highlighted three issues: the identification of the glassy nature of ciclosporin A, the observation that the drug may be incorporated into microspheres as a separate amorphous phase, and the utility of MTDSC as a means of characterizing the glass transitional behaviour of both the peptide and the microspheres. In terms of the glassy nature of the peptide itself, it is perhaps surprising that little is known regarding amorphous peptides. Indeed, the topic of the glassy behaviour of proteinaceous materials is recognized as being an important issue for future study (Angell 1995a, b), although work in this area has been very limited to date, almost certainly owing to the difficulties involved in measuring the glass transitions of such molecules (Frauenfelder et al 1991; Green et al 1994). In particular, although it is possible to measure a discrete heat capacity step change for some proteins, many such materials show no discernible T_g using conventional DSC. It has been suggested that such difficulties are associated with the heterogeneity of the relaxation processes associated with proteins (Shamblin et al 1998), rendering the visualization of a clear step change in heat capacity extremely difficult. The difficulty is compounded by an absence of extensive study on the issue; most of the available published work has appeared in the food or carbohydrate literature (e.g. Castelli et al 2000). This is almost certainly because of the extensive use of proteinaceous material such as gluten in the solid state within these areas, whereas most studies involving pharmaceutical peptides and proteins are concerned with the molecular rather than the solid (or glassy) state properties of these systems. Nevertheless, the use of peptide/protein delivery systems and in particular freeze-dried systems may involve the drug forming some type of physical array, and hence we believe that more advanced knowledge regarding the solid state properties of these materials is essential. Interesting studies in this area are nevertheless ongoing, with a recent investigation (Breen et al 2001) having also utilized MTDSC for detecting the glass transition of a freeze-dried protein and reporting that conventional DSC was insufficiently sensitive to detect such small heat capacity changes. In addition, while studies on glassy pharmaceutical proteins are rare, studies on glassy pharmaceutical peptides are almost totally absent. In particular, the evidence that ciclosporin A may exist in a semi-crystalline form has important implications for both chemical and physical stability as well as release characteristics and, we believe, merits further investigation.

The observation that the drug appears to not only phase separate, but also to do so into separate amorphous phases is a key finding of this study, again with implications for stability and release. However, it should be emphasized that these results differ from those reported by Sanchez et al (1993), who reported no obvious surface deposition of drug and suggested that the ciclosporin A is molecularly dispersed throughout the spheres. These discrepancies may arise because of differences in manufacturing method or choice of polymer, both of which are known to influence the nature of the final product (Mehta et al 1996). The contrast between the two studies is in itself of interest as it clearly demonstrates how different manufacturing methods may result in markedly different physical structures.

Finally, this study has demonstrated that MTDSC may be of use not only in detecting subtle transitions (itself an important consideration for proteins), but also (potentially) for assessing the degree of crystallinity of semi-crystalline peptides. It is therefore likely that as this field develops, MTDSC will prove to be a pivotal tool in studying the glass transitions of proteins and peptides.

Conclusion

This study has demonstrated that ciclosporin A may exist in a semi-crystalline state, but may be converted to a wholly amorphous state on incorporation into PLGA microspheres, with the glassy materials existing as a separate phase within the polymer matrix. MTDSC has been shown to be capable of not only detecting, but also quantifying, the amorphous material present. These findings have implications not only for the development of rational formulation strategies for PLGA systems containing proteins and peptides, but also for understanding the solid state properties of these molecules in a broader sense.

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