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Localization of bovine serum albumin in double-walled microspheres

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Abstract

Phase separation of binary blends of various combinations of poly (L-lactide) (PLA), and poly (D,L-lactide-co-glycolide) (PLGA), was investigated using differential scanning calorimetry (DSC). Based on this phase separation phenomenon, double-walled microspheres were fabricated. A model agent, bovine serum albumin (BSA) labeled with fluorescein isothiocyanate (FITC-BSA) was localized in each layer. Scanning electron microscopy (SEM) and fluorescence microscopy (FM) were used to assess the formation of double-walled microspheres and the localization of the drug, respectively. When a 1:1 polymer ratio was used, the FITC-BSA was localized in the outer layer. When the relative ratio of PLGA to PLA was increased to 3:1 using the same overall polymer concentration, the FITC-BSA was localized in the inner core. Release studies were carried out to evaluate the advantage of double-walled microspheres compared to single walled microspheres. Microspheres made with FITC-BSA localized in the inner core exhibited a significantly lower initial release rate compared to microspheres where the drug was located in the outer layer, or compared to microspheres made from PLA only. Hence microspheres with a double-walled morphology have the potential for therapeutic use where a high burst might be detrimental.

Keywords: Double-walled; Phase separation; Blend; Enthalpic relaxation; DSC; Microencapsulation

1. Introduction

The limitation of microspheres made of a single polymer encapsulating drugs includes an initial burst caused by the release of the drug trapped on the surface during the encapsulation process and a progressively slower release rate. Therefore, microspheres made with a two-layered structure may have certain advantages over their counterparts made from single polymers. In some applications (e.g. Lupron Depot®), where the therapeutic range of the drug is wide or the drug is nontoxic, this burst is not detrimental. However, for molecules with narrow therapeutic ranges or high toxicity, this initial burst of drug can be a problem for the patient. In an attempt to better control the release kinetics, the formation of double-walled microspheres with the drug loaded in the inner core could provide release kinetics with a lower burst effect than polymeric microspheres made from a single polymer. There are several methods of

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making microspheres with a two-layered structure from polymer blends. One method is to simply encapsulate a therapeutic agent in microspheres using a conventional microencapsulation technique and then to coat the microspheres with a second polymer. This coating would reduce the burst effect since no protein or drug would be encapsulated on the surface. A second method entails polymer–polymer phase separation of a binary blend of polymer solutions, which results in the formation of microspheres that have a two-layered structure [1–3].

Despite the potential advantages of double-walled microspheres, encapsulation techniques are quite crude and not appropriate for small sized microspheres. Dip or pan coating processes yield microspheres with uneven or incomplete polymer coatings and each coating adds an additional step to the manufacturing process and further decreases the yield [4]. Air suspension coating methods generally produce a more evenly coated product, but are limited by the size of the particles that can be suspended in a fluidized bed; particles less than 100 μm are difficult to coat by this method [4].

The solvent evaporation method has been modified in our laboratory to prepare double-walled microspheres. The usual process of microencapsulation by solvent evaporation entails the formation of an "oil-in-water" emulsion of a polymer solution in an aqueous nonsolvent. This emulsion creates the spherical droplets, which then harden as the solvent evaporates, creating solid polymer microspheres. To form microspheres from a single polymer, the polymer is dissolved in a volatile organic solvent, such as methylene chloride, and mixed with the substance to be encapsulated (i.e. drug or protein), before adding to an aqueous nonsolvent bath. The solvent evaporation method has been used extensively to prepare microspheres from PLA and PLGA [5–13].

In the modified solvent evaporation process used to form double-walled microspheres, two polymer solutions are briefly mixed before adding to the aqueous nonsolvent bath. As the solvent is slowly lost, the droplets of the polymer–polymer solution become more concentrated and the polymers begin to phase-separate. A homogeneous polymer solution undergoes phase separation into one phase rich in one polymer, and a second phase rich in the second polymer.

The polymers PLA, a polyester, and poly(1,3-bis(*p*-carboxyphenoxy)propane-co-(sebacic anhydride)), 20:80, (P(CPP:SA) 20:80), a polyanhydride, have been used previously to fabricate double-walled microspheres. These microspheres had a core of P(CPP:SA) 20:80 and an external coat of poly (L-lactic acid) (PLLA) [2,3,14–16]. The fabrication of these double-walled microspheres was optimized to determine manufacturing conditions, which affect yield and reproducibility, and characterized to understand their chemical and physical properties. Five process variables were studied: polymer solution concentration, polymer weight ratio, polymer solution volume ratios, encapsulation temperature, and airflow rate across the top of the encapsulation vessel [16].

In addition, Heller et al. prepared double-walled microspheres using a poly(ortho ester) (POE) and 50:50 PLGA. The morphology of the microspheres varied depending on the POE content. When the POE content was 50, 60 or 70 wt.%, double-walled microspheres with a dense core of POE and a porous shell of PLGA was formed [17]. Further work used POE–PLGA (50:50) double-walled microspheres to encapsulate hydrophilic BSA and hydrophobic cyclosporin A (CyA). Release studies indicated that nearly 100% BSA and >95% CyA were released from the double-walled POE/PLGA microspheres in a sustained manner. It was concluded that the distinct structure of double-walled POE/PLGA microspheres would make an interesting delivery system for therapeutic agents [18].

Double-walled fabrication based on a phase separation phenomenon of PLA and PLGA has not been shown even though they are the most widely used biodegradable polymers. Their commercial availability with different lactide to glycolide ratios, molecular weight, stereochemistry and co-monomer sequence makes them an interesting class of polymers to study from a phase separation perspective. Recently, Wang et al. demonstrated the fabrication of composite double-walled microspheres with biodegradable PLA shells and PLGA cores and highly water-soluble etanidazole entrapped within the core as solid crystals. However, the phase separation phenomenon was not reported for that system [19]. The objective of this paper was to determine the correct conditions [20,21] for phase separation of several polyesters and to demonstrate for the first time the formation and use

of double-walled microspheres using PLA and PLGA. FITC-BSA, was used as a model drug to examine its localization both in the inner and outer layer of these microspheres. DSC was used to study the phase separation phenomenon along with enthalpic relaxation of 24 kDa PLA and 50 kDa PLGA. The formation of double-walled microspheres and the localization of the FITC-BSA were investigated using SEM and FM, respectively. Release studies were also carried out to evaluate the sustained release potential of the double walled microspheres.

2. Materials and methods

2.1. Materials

PLA, poly(L-lactide), 24 kDa and 90 kDa and PLGA 50/50, 50 kDa were purchased from Birmingham polymers (BPI). PLA (2 kDa), and additional 50/50 PLGA polymers including Resomers® RG 502 (Mw 18 000), 502H (Mw 10 000), 503 (Mw 30 000), 503H (Mw 19 000) were purchased from Boehringer Ingelheim. Poly(vinyl alcohol), 30–70 kDa, was purchased from Sigma Chemical Company. BSA (fraction V) and FITC-BSA were purchased from Sigma. All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Film casting

Films were cast from polymers dissolved in methylene chloride. A 1:1 w/w blend of PLA, 24 kDa, and PLGA RG503 was made by dissolving, 0.1 ± 0.005 g PLA and 0.1 ± 0.005 g of PLGA in 1 ml of methylene chloride (20% w/v). The solution was poured onto a petri dish to give a thin film that was analyzed by DSC. Similarly films were made with PLA 24 kDa and PLGA RG503H, PLA 24 kDa and PLGA RG502, PLA 24 kDa and PLGA RG502H, PLA 24 kDa and PLGA 50 kDa, PLA 2 kDa and PLGA 50 kDa, PLA 90 kDa and PLGA 90 kDa.

2.2.2. Differential scanning calorimetry

DSC was used to determine the existence of glass transition temperature. A Perkin–Elmer DSC-7 with an intracooler attachment was used, and data were collected on a PC. The instrument was calibrated for enthalpy and temperature using the melt of ultra pure indium (28.42 J/g). A standard glass of the sample was formed by heating above the T_g to remove thermal history and then quenching at a standard cooling rate of 100 °C/min. Subsequent samples were held for 1 min at 180 °C, cooled from 180 to 28.17 °C at 100 °C/min so that a standard glass was formed, held for 60 min, then cooled from 28.17 to −20 °C at 100 °C/min. The samples were subsequently heated from −20 to 180 °C at 10 °C/min, cooled from 180 to −20 °C at 100 °C/min and then again heated from −20 to 180 °C at 10 °C/min.

The extent of relaxation was determined by heating through the T_g at 10 °C/min, quenching to form the standard glass at 100 °C/min and then reheating through the T_g at 10 °C/min. The quenched polymer trace was subtracted from the aged polymer and the appearance of single or multiple peaks enabled determination of distinct glass transition points.

2.2.3. Liquid–liquid phase separation of PLA 24 kDa and PLGA 50 kDa

To find the point of phase separation of a 1:1 PLA, 24 kDa, and PLGA, 50 kDa, solution, several concentrations of each of the polymers were made in methylene chloride between 5% and 40% (w/v) in increments of 5% in 7 ml scintillation vials. As an example, to prepare the 20% w/v mixture, 0.4 g of each polymer was dissolved in 2 ml of methylene chloride. The vials were mixed for 2–4 h in a rotomixer (Roto-Rack Mixer, Fisher Scientific). Each of the PLA solutions was then mixed with the equivalent PLGA solution. The vials were allowed to sit undisturbed for 24-h. Appearance of two distinct phases was monitored by a difference in color between the polymers. The total height of the solvent in the vials was measured before and after phase separation until a constant height was obtained. This was measured using digital calipers (ABS Digimatic Solar, CD-S6"C, Mitutoyo Corp.). The concentration at which two immiscible phases was seen was noted. The points of phase separation at other ratios of PLA, 24 kDa and PLGA, 50 kDa were similarly determined.

2.2.4. Fabrication of blank double-walled microspheres from1:1 PLA PLGA

The modified solvent evaporation technique utilized a nonsolvent bath containing 200 ml of deion-

ized water with 0.5% w/v polyvinyl alcohol solution as a surfactant. 0.2 g of the polymers, PLLA, 24 kDa and PLGA 50:50 were each dissolved in 1 ml of methylene chloride, at an overall polymer concentration of 20% w/v. The PLGA solution was mixed in a rotomixer (Fisher Roto-Rack) for 45 min. The 20% w/v PLA solution was added to the 20% w/v PLGA solution (ratio of 1:1) and the mixture was injected into the nonsolvent bath using a 10 cc syringe immediately above the agitator. Stirring was continued for 4–5 h at 500–800 rpm before the microspheres were washed with 500 ml of distilled water and then lyophilized.

2.2.5. Preparation of FITC-BSA particles

BSA (30 g) was added to FITC-BSA(1 g) in water (total of 600 ml). The solution was then injected into a spray dryer (Labplant SD-04). Variables that were controlled for particle size included air pressure and solution flow rate set at 10 ml/min, with an inlet temperature set at 110 °C. The particle size of the dried product was about 5 μm. Powder losses to the cyclone attachment in the spray drier were washed with deionized water and spray dried a second time. Yields of ~45% were obtained. An electrophoresis gel of FITC-BSA after spray drying and after release did not show any change in the product [22].

2.3. Fabrication of microspheres containing 5% FITC-BSA using different polymer ratios

A suspension of FITC-BSA (0.02 g) in a 1% w/v solution of PLA (0.05 g) in methylene chloride(5 ml) was sonicated for 5 min. Following infiltration at 4 °C overnight, the methylene chloride was evaporated using compressed air and the dry protein and polymer were reconstituted by adding PLA(0.15 g) and methylene chloride(1 ml), to form a 20% w/v polymeric solution. An additional 20% PLGA solution (0.2 g in 1 ml of methylene chloride) was added to the protein–PLA solution to prevent loss of the protein during transfer from one vial to another. The solution was mixed for 15–30 s and then injected into aqueous 0.5% polyvinyl alcohol (250 ml) using a 10 cc syringe immediately above the agitator. The mixture was stirred for 3.5 h with the agitator speed at 500–800 rpm. Similarly microspheres were made using PLA and PLGA in the ratios of 1:2 and 1:3, with 5% w/w protein loading.

2.3.1. Morphology of microspheres studied using SEM

Microspheres were mounted on metal stubs and cross sectioned using a razor blade to view the internal structure. The samples were sputter coated with a 50–100 Å layer of gold–palladium (Polaron instrument E5100) and observed using a Hitachi S-2700 scanning electron microscope at an accelerating voltage of 10 kV.

2.3.2. Morphology of FITC-BSA particle loaded microspheres studied by fluorescence and optical microscopy

All microspheres were embedded in Leica Historesin (#7022–18 500, Leica Instruments GmbH) using a technique developed to maximize the infiltration of the embedding solution and preserve the core of the DW microspheres. Small samples (~15 mg) of each sphere batch were placed in 1.5-ml microcentrifuge tubes. Leica infiltration solution was prepared according to directions. Half of this solution was placed in a separate vial and diluted with an equal volume of 100% ethanol. One milliliter of this 50% infiltration solution was added and the sample tubes were placed on their side and the microspheres were soaked for 12 h at 4 °C. The microspheres were re-suspended in activated Leica Historesin and placed in the sample troughs of an embedding tray. Sections (8 μm) were cut from the embedded microspheres using a microtome with a glass knife (Reichert-Jung 2050, Cambridge Instruments GmbH).

Histosections were analyzed on a light microscope (Olympus IX70) under both white light and fluorescence (488 nm for FITC). The embedded cross sections were also analyzed for birefringence under an optical microscope (Zeiss Model IM 35). The extent of birefringence was qualitatively observed for the cross sections when the polarizer and analyzer were set in crossed positions (0° and 90°, respectively). This showed whether the outer or the inner layer was mostly birefringent, indicating the polymer it is rich in.

2.3.3. In vitro release studies

In vitro studies were carried out using FITC-BSA loaded microspheres. The amount of protein encap-

sulated was calculated based on losses to the non-solvent and the wash. In vitro studies involved taking 10 mg of samples in triplicate, adding PBS buffer (1 ml) and incubating at 37 °C. At predetermined time intervals, the supernatant was removed, stored at 4 °C for later analysis, and the microcentrifuge tube replenished with fresh PBS buffer till the next time point. The supernatant was analyzed using a micro BCA assay.

3. Results and discussion

The polylactides and polylactide-co-glycolides were used to study the phase separation phenomenon because of their commercial availability and the ease in controlling degradation times depending on their molecular weight, lactide to glycolide ratio and crystallinity [23–25].

3.1. Determination of solubility parameters

In order to form double walled microspheres with PLA and PLGA it was first important to study their phase separation properties. In general most polymer blends are immiscible in a range of temperatures and concentrations, and the specific conditions under which they are immiscible must be determined. Phase separation of binary blends of PLA and PLGA is not as likely as with many other polymer combinations due to their similarity in chemical structure. Basic thermodynamics teaches that polymers will be miscible whenever the free energy of mixing as given by Gibb's equation Eq. (1) is negative:

$$\Delta G_{\mathrm{mix}} = \Delta H_{\mathrm{mix}} - T\Delta S_{\mathrm{mix}} \quad (1)$$

Here ΔH_{mix} is the enthalpy of mixing, ΔS_{mix} is the entropy of mixing, ΔG_{mix} is the free energy of mixing and T is the temperature. For polymers, ΔS_{mix}, which is a function of molecular sizes, approaches zero. Therefore, polymer–polymer mixing is dependent on ΔH_{mix}, which can be understood in terms of a difference in the pure component solubility parameter [21]. The greater the difference in solubility parameters, the more likely that polymers are immiscible.

The value of the solubility parameter can be calculated from the group molar attraction constants, G, for each chemical group,

$$\delta = \frac{\rho \sum G}{M} \quad (2)$$

where δ is the solubility parameter, ρ the density of the polymers, and M is the molecular weight of the monomer unit [26]. Group molar attraction constants, G, have been calculated by Small and Hoy [26] for individual chemical groups, some of which are shown in Table 1. The solubility parameters calculated by this method were 10.21 $(\mathrm{J/cm^3})^{0.5}$ for RG 502, 503, 502H and 503H, 10.94 $(\mathrm{J/cm^3})^{0.5}$ for PLGA 50 kDa, 10.7 $(\mathrm{J/cm^3})^{0.5}$ for PLA 2 kDa and 24 kDa and 11.56 $(\mathrm{J/cm^3})^{0.5}$ for PLA 90 kDa. These solubility parameters are very similar, the difference being less than 0.65 $(\mathrm{J/cm^3})^{0.5}$. For miscibility over the entire composition range, the difference in solubility parameters of the two polymers cannot exceed 0.7 [27], thus suggesting that binary blends of PLA's and PLGA's are probably miscible over most compositions. Therefore, an experimental analysis of phase separation between PLA and PLGA's of various molecular weights was undertaken.

3.2. Miscibility behavior using the DSC

In general, a miscible blend of two polymers will have properties between those of the two unblended polymers. The existence of two glass transitions, each representative of the original polymers, is evidence of a phase-separated blend. The existence of a single glass transition(T_g), between the T_g's of the original polymers, indicates a miscible blend [28–30]. The difficulty in evaluating a blend of polylactides and

Table 1
Group molar attraction constants, G (according to small; derived from measurement of heat of evaporation)

Group	G
$-CH_3$	214
$-CH_2$	133
$-CH$	28
$-CO$	275
$-COO$	310
$-O$	70

polyglycolides is that the T_g's of the polymers are similar. For example PLA, 24 kDa has a T_g of 48.6 °C and PLGA 50 kDa has a T_g of 38.2 °C. As such, a broad single T_g cannot be distinguished from two single glass transitions.

Therefore, the films prepared from binary polymer solutions were physically aged and held for a certain period of time at a temperature below the T_g. When a polymer chain is quenched below the T_g, the polymer chains are not in an equilibrium conformation and in time gradually relax towards equilibrium. This process is thermodynamically driven and is known as enthalpic relaxation. Enthalpic relaxation in polymers results in changes in mechanical properties and this process is known as physical aging [31]. The polymers in the blend relax at different rates towards the T_g and when heated, the distinction between two existing T_g is more pronounced.

Films were made from a combination of the following polymers by solvent casting-PLA 2 kDa/PLGA 50 kDa, PLA 90 kDa/PLGA 50 kDa, PLA 24 kDa/PLGA 50 kDa, RG502 /PLA 24 kDa, RG502H /PLA 24 kDa, RG503 /PLA 24 kDa, RG503H /PLA 24 kDa. All the films were physically aged for a period of 1 h at a temperature of 28.17 °C, 5–10 °C below the T_g of PLGA. Fig. 1 showed that certain blends of PLA and PLGA were miscible by the appearance of a single peak (RG503/PLA 24 kDa and PLA 2 kDa/PLGA 50 kDa), tabulated in Table 2, whereas others formed immiscible blends as shown by the appearance of two peaks (RG502/PLA 24 kDa, RG502H/PLA 24 kDa and PLA 24 kDa/PLGA 50 kDa. RG503H/PLA 24 kDa showed a small second peak, and therefore, could be classified as immiscible. PLA 90 kDa/PLGA 50 kDa also had a small second peak indicating some immiscibility. Increasing the aging time will increase the area underneath the peaks but will not affect the number of peaks appearing [28–30]. Therefore, phase separation was observed in certain combinations of polymer such as RG502/PLA 24 kDa, RG502H/PLA 24 kDa, PLA 24 kDa/PLGA 50 kDa, RG503H/PLA 24 kDa and PLA 90 kDa/PLGA 50 kDa whereas two other combinations did not show any phase separation. Of the polymer combinations that showed phase separation, PLA 24 kDa/PLGA 50 kDa was studied further for the development of double-walled microspheres.

3.3. Miscibility of polymer solutions

The next objective was to study the behavior of these polymers in solution. Methylene chloride was

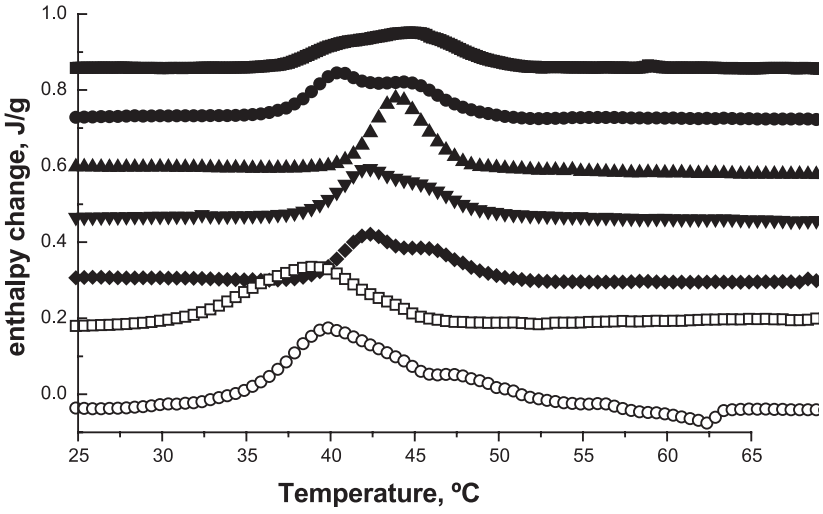

Fig. 1. Enthalpic relaxation curves of different solvent cast blends of PLA and PLGA in the ratio of 1:1 with (■) representing RG502 and PLA 24 kDa, (●) representing RG502H and PLA 24 kDa, (▲) representing RG503 and PLA 24 kDa, (▼) representing RG503H and PLA 24 kDa, (♦) representing PLGA 50 kDa and PLA 24 kDa, (□) representing PLGA 50 kDa and PLA 2 kDa and (○) representing PLGA 50 kDa and PLA 90 kDa.

Table 2
Miscibility of different solvent cast blends of PLA and PLGA in the ratio of 1:1

PLA (kDa)	PLGA	Miscibility
24	RG502	Immiscible
24	RG502H	Immiscible
24	RG503	Miscible
24	RG503H	Immiscible
24	50 kDa	Immiscible
2	50 kDa	Miscible
90	50 kDa	Immiscible

the solvent of choice. Each of the polymers, PLA 24 kDa and PLGA 50 kDa, ranging in concentration from 5% w/v to 40% w/v in increments of 5% was dissolved in methylene chloride, then combined to make up solutions of PLA 24 kDa and PLGA 50 kDa in the ratio of 1 to 1 so the final concentrations ranged from 5% to 40% in increments of 5%. These solutions were monitored over 24 h for the appearance of two distinct phases that could be distinguished due to difference in color between the PLGA and the PLA. The cloud point, the concentration at which they phase separated to form two immiscible phases, was determined to be 30% w/v. This implies that at concentrations above 30% w/v, a 1:1 combination of PLA, 24 kDa and PLGA 50 kDa will phase separate to form two immiscible phases and when utilized in the solvent evaporation process will potentially form double-walled microspheres. Phase separation in solution at 30% w/v was seen to be rapid with two distinct phases forming within 5 min, with the volumes of the two phases remaining constant even at 24 h, indicating that equilibrium was probably reached early on in the process. The upper phase was a clear solution whereas the bottom phase was an amber colored solution. For PLA and PLGA ratios of 1:2 and 1:3, similar cloud points were obtained at 30% w/v. This suggests that the polymer ratio has no effect on the phase separation point and the same starting polymer concentration can be used with different polymer ratios in the solvent evaporation process.

3.4. Double-walled microsphere fabrication using PLA and PLGA

A starting 1:1 PLA:PLGA solution of 20% w/v, less than the 30% w/v cloud point, was used to obtain a homogeneous miscible polymer solution. This miscible polymer solution was injected into a nonsolvent bath containing water and polyvinyl alcohol, at which point the methylene chloride was lost by evaporation and extraction, resulting in the concentration of the polymer solution. Phase separation ensued at the cloud point (30% w/v), causing microspheres to form. The process itself is dynamic, and therefore, phase separation

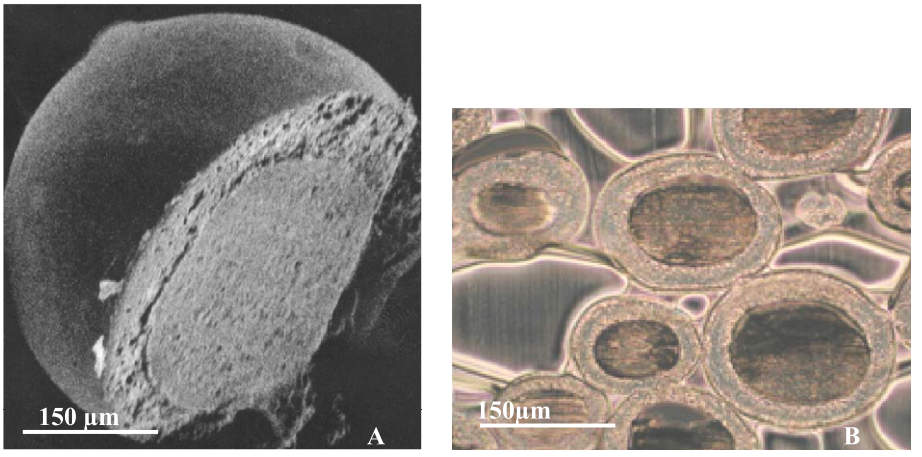

Fig. 2. (A) Scanning electron micrograph of double-walled microsphere prepared using the solvent evaporation method. The bar represents 150-µm. (B) Optical microscopy of cross sections of double-walled microspheres with two distinct layers.

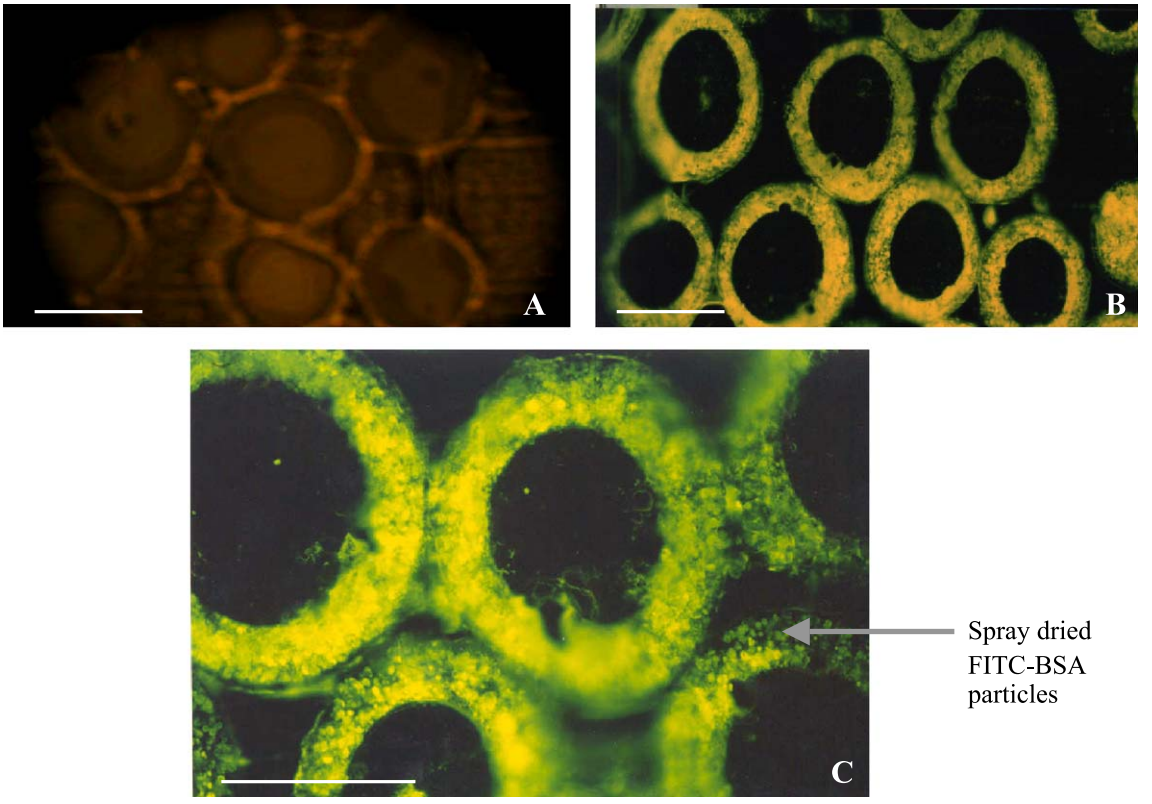

Fig. 3. (A) Cross sections of blank double-walled microspheres under fluorescence microscope. (B) Cross sections of double-walled microspheres using FM (10×) made from PLA:PLGA 1:1 with FITC-BSA localized in the outer layer (C) represents a 20× magnification where the spray dried FITC-BSA particles can be seen embedded in the outer layer. The white bar represents 150 μm.

as a result of thermodynamics was balanced with the kinetic factors of the solvent evaporation technique, to create the final morphology of the microspheres.

The microspheres were determined to have a size range of 150–200 μ by SEM and, when sliced in half, two discontinuous distinct walls were seen (Fig. 2A). To obtain an estimate of the percentage of microspheres that had double-walled morphology, cross sections (5 μm thick) of embedded microspheres were prepared. The cross sections delineated a two-layered structure shown in Fig. 2B. The efficiency of the formation of double-walled microspheres was quantified by counting the microspheres that had a two-layered structure as seen from five 1 cm^2 sections of the embedded microspheres, and was estimated to be at ~100%.

Thus it was possible to fabricate double-walled microspheres with high efficiency from two polymers

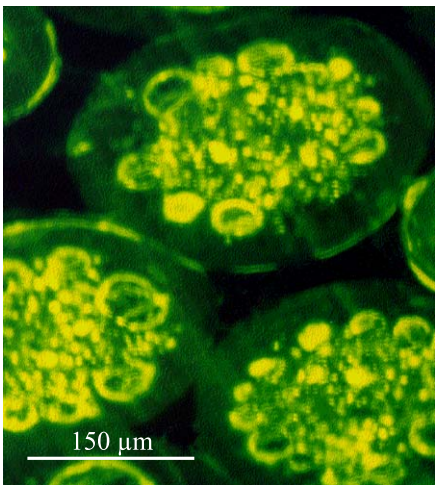

Fig. 4. Cross sections of microspheres under FM made from PLA:PLGA 1:3 with FITC-BSA localized in the inner core under 20× magnification.

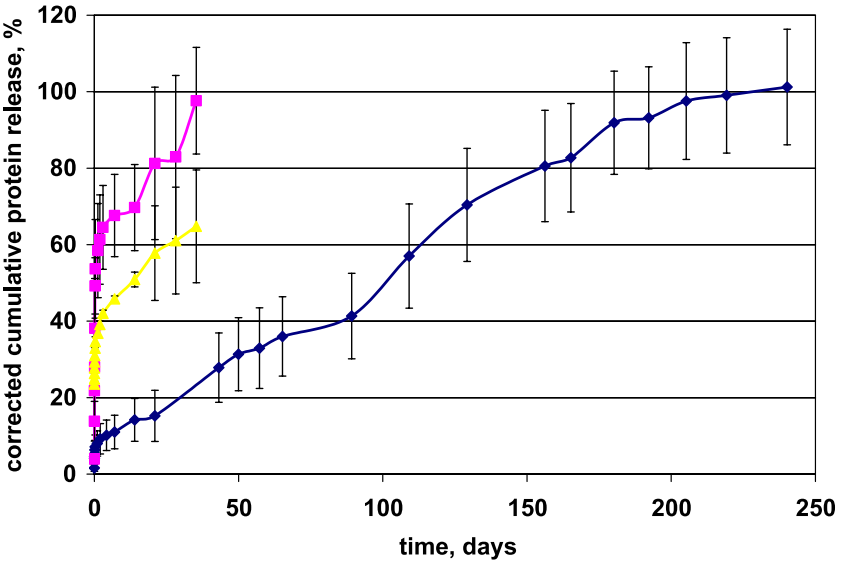

Fig. 5. This figure represents the corrected cumulative protein release showing the effects of the relative polymer ratio and localization of the protein within the microspheres where (■) represents double-walled sphere made from PLA:PLGA 1:1 with the protein localized in the outer core and (▲) represents single walled microspheres made from PLA and (◆) represents double-walled microspheres made from PLA:PLGA 1:3 where the protein is localized in the inner core.

that are chemically very similar. At this point a drug could be placed in either the PLA or the PLGA polymer solution during the pre-encapsulation steps. FITC-BSA, 5% w/w polymer loading, was placed in the PLA solution and, after microencapsulation, its distribution was investigated by preparing cross sections of

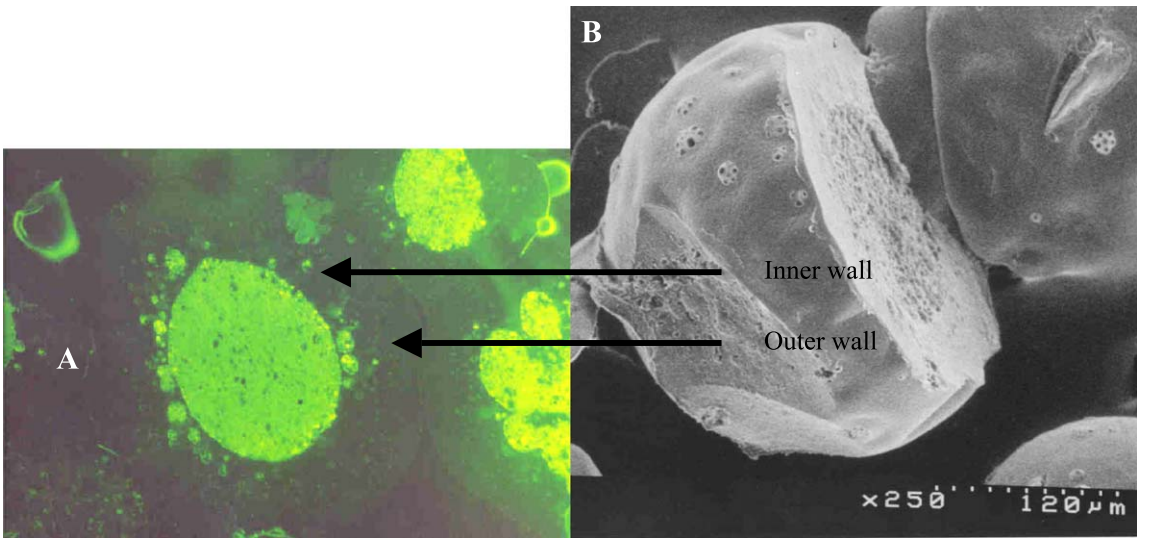

Fig. 6. Cross sections of double-walled microspheres made from PLA:PLGA 1:2 with FITC-BSA at 20% loading (A) under FM 20× (B) microspheres cut in half under SEM.

the resulting microspheres. Fig. 3A,B depict the results. FITC-BSA was consistently observed to be in the outer layer of the cross sections using fluorescence microscopy (FM), as shown in Fig. 3B. The outer layer was of same thickness as the blank microspheres. Cross sections of blank double-walled microspheres made in the same way are shown in Fig. 3A and it can be seen that auto fluorescence does not confound the localization of the FITC-BSA in the outer layer. Fig. 3C shows a 20 × magnification where the FITC-BSA spray-dried particles can be individually seen embedded within the outer layer. When the FITC-BSA was placed in the PLGA polymer solution it again appeared in the outer layer, yielding a similar distribution of the FITC-BSA as seen when placed in the PLA solution. This suggests that the FITC-BSA has a higher affinity towards the outer layer, which is PLA rich, or that the layers have reversed when the FITC-BSA is placed in the PLGA during pre-encapsulation. Preliminary data suggests that the layers do not reverse and that the PLA layer is the outer layer. The FITC-BSA appears in the exterior layer due to the combination of the hydrophilic nature of the nonsolvent and, to a certain extent, its affinity for the PLA. However, the exact mechanism is not known yet.

The FITC-BSA could not be localized in the inner core by placing the drug in either the PLA or the PLGA polymer solution during the pre-encapsulation

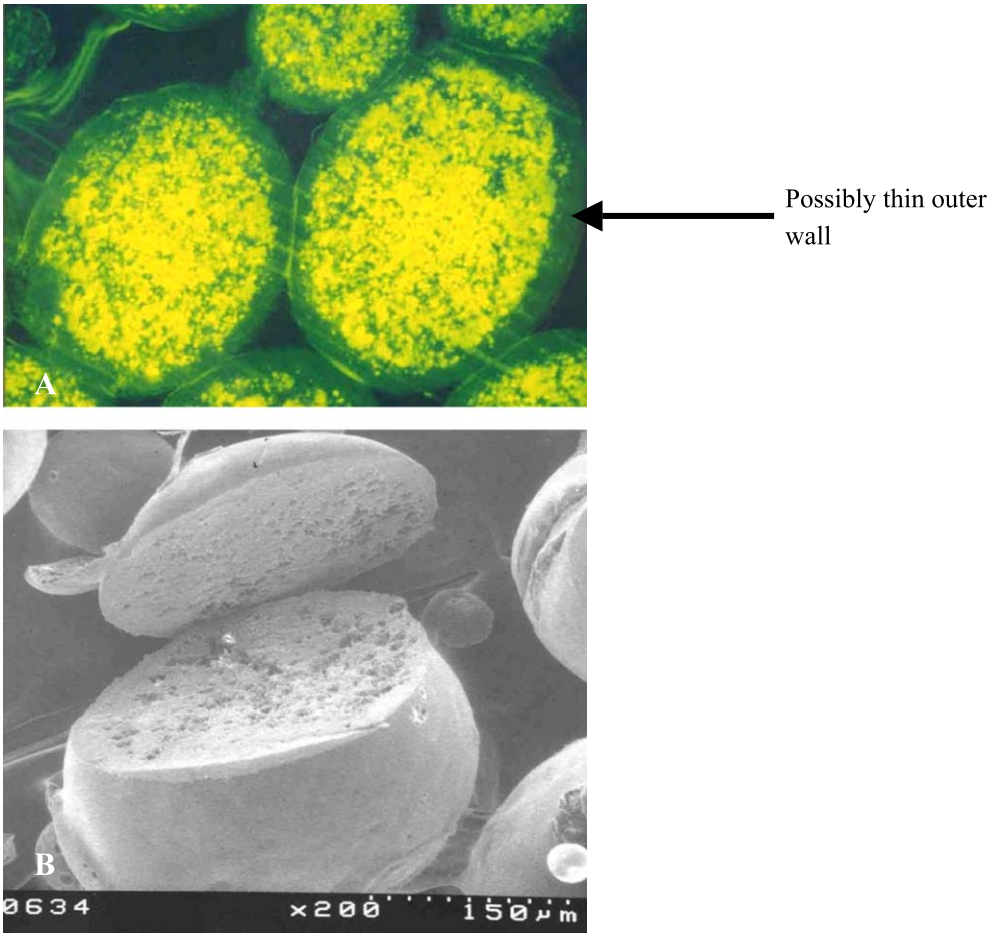

Fig. 7. Cross sections of double-walled microspheres made from PLA:PLGA 1:3 with FITC-BSA at 20% loading (A) under FM 20× (B) microspheres cut in half under SEM illustrating the thin outer shell in these double-walled microspheres.

steps. Therefore, other ways to localize the drug in the inner core had to be investigated so that it was possible to make use the outer shell to reduce the burst effect. Increasing the relative polymer concentration of the PLGA to the PLA from 1:1 to 3:1 and placing 5% w/w FITC-BSA in the PLGA solution at 5% w/w FITC-BSA caused the drug to localize in the inner layer, as shown in Fig. 4. The drug was encapsulated in small domains in the inner core.

3.5. Release kinetics of double-walled microspheres

The double-walled microspheres with the drug localized in the inner core were compared to double-walled microspheres with the drug in the outer layer and to control PLA microspheres. The release profile in Fig. 5 shows that the microspheres made from 1:3 PLA and PLGA, with the drug localized in the inner core, had a much lower burst compared to double-walled microspheres made from 1:1 PLA and PLGA with the drug localized in the outer layer. The release due to diffusion quantified at 6 h for the microspheres with the protein localized in the inner core was $7.1 \pm 3.1\%$ for double-walled microspheres; with protein in the outer core it was $32.9 \pm 1.3\%$; for single walled microspheres made from PLA it is $49.2 \pm 7.3\%$. The release profile for microspheres with the drug in the inner core was very close to a zero order release profile.

The advantages of the double-layered structure were more pronounced at higher loading where the burst effects tended to be larger. Fig. 6A shows fluorescent micrographs of double-walled microspheres made with 1:2 PLA and PLGA, 20% w/w FITC-BSA loading, with the drug in the inner core and a clear outer shell. Fig. 6B is a SEM showing the distinct phases within the microsphere. When the relative PLGA concentration was increased to a 1:3 ratio of PLA to PLGA, it was very difficult to observe the double-layered structure of the microspheres probably because the outer wall becomes considerably thinner. Fig. 7A and B are fluorescent and SEM micrographs of theses microspheres. The release profiles comparing the 20% loaded microspheres using the PLA to PLGA ratio of 1:2 and 1:3 is shown in Fig. 8. A higher initial release due to diffusion took place with the 1: 2 blend compared to the 1:3 blend even though the 1:2 blend had a thicker wall. This result could be due to a higher content of PLA with its semicrystalline properties that causes the FITC-BSA to be released much faster. The release profiles indicate that one can manipulate drug release in these microspheres fabricated from PLA and PLGA by controlling the relative polymer ratio of the polymers in the binary blend and the polymer into which the drug is placed during the pre-encapsulation steps of the process.

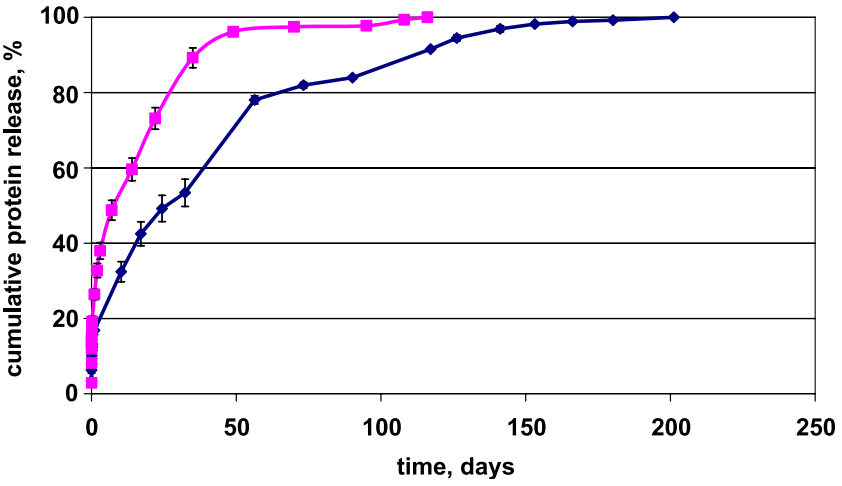

Fig. 8. Cumulative protein release showing the effects of the relative polymer ratio at higher loading of 20% where (■) represents double-walled microspheres made from PLA:PLGA 1:2 and (◆) represents double-walled microspheres made from PLA:PLGA 1:3.

4. Conclusions

The choice of a two-polymer system for the purposes of protein/drug encapsulation to be used for sustained in vivo delivery depends on whether the binary blend can provide release profiles that cannot be attained with the individual polymers by themselves. The polylactides and the polylactide-co-glycolides are chemically similar in structure and one would expect they would probably form miscible blends. The appearance of two distinct glass transitions using DSC showed that some of these blends were immiscible, whereas others were not. Immiscibility was demonstrated in the case of PLA, 24 kDa, and 1:1 PLGA, 50 kDa, in a common solvent, methylene chloride.

The immiscible blend, PLA, 24 kDa and 1:1 PLGA, 50 kDa, could be used for the fabrication of double-walled microspheres by the solvent evaporation procedure. Double-walled microspheres were produced with almost 100% efficiency using 1:1 and 1:2 polymer ratios. Using a PLA:PLGA ratio of 1:1, FITC-BSA was localized in the outer core, when the drug was placed in either the PLA or the PLGA solution. With 1:2 and 1:3 PLA to PLGA ratios, the model agent, FITC-BSA was localized in the inner core by placing it in the PLGA solution.

Using a 5% w/w FITC-BSA loading, almost zero-order release kinetics was obtained with a PLA:PLGA ratio of 1:3. These microspheres had a lower burst effect compared to microspheres made from 1:1 PLA and PLGA, where the FITC-BSA was localized in the outer core, and to microspheres made from PLA alone with 5% protein loading. At a higher loading of 20% FITC-BSA, the protein was still localized in the inner core, with the 1:2 blend having a thicker and more distinct outer wall than the 1:3 blend. Therefore, localization can be achieved even when the drug loading is increased from 5% to 20%. Thus these microspheres provide great versatility as drug delivery platforms, providing sustained delivery of therapeutic agents that might have a low therapeutic index.

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