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Effect of preparation temperature on the characteristics and release profiles of PLGA microspheres containing protein fabricated by double-emulsion solvent extraction/evaporation method

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Abstract

This study describes the influence of preparation temperature on the various characteristics and release profiles of poly(DL-lactide-co-glycolide) (PLGA) microspheres. The bovine serum albumin (BSA)-loaded microspheres were prepared using the water-in-oil-in-water (w/o/w) technique with poly(vinyl alcohol) as surfactant in the external aqueous phase. We have varied the preparation temperature to observe its effect on microsphere characteristics such as the microsphere shrinking rate during formation, particle size, density, surface and internal morphology, BSA encapsulation efficiency, BSA initial release, microsphere degradation and BSA in vitro release behaviour. During fabrication, a low preparation temperature of 5°C gives the fastest initial but the slowest overall shrinking rate. Microspheres formed at high temperatures of 38°C and 42°C on the other hand have the lowest initial yet the highest overall shrinking rate. Subsequently, microsphere mean size increases and the particle size distribution widens with increase in the preparation temperature. Although all the microspheres have a porous surface as well as internal structure, microspheres fabricated at high temperatures have a uniform internal pore distribution and a very thin dense skin layer, while microspheres fabricated at lower temperatures have a thicker but porous skin layer and bigger pores in the middle of the sphere. Microspheres formed at 33°C are found to give the highest initial burst release. In terms of in vitro release, microspheres fabricated at low temperatures (5°C, 15°C and 22°C) exhibit similar, steady rates. Microspheres formed at higher temperatures however give very low release rates after their initial release. The results obtained suggest that preparation temperature significantly affects microsphere formation, resulting in their structural and protein release profile differences. These differences ultimately work together to affect the initial release and overall release patterns of the microspheres.

Keywords: Microspheres; Poly(DL-lactic-co-glycolic acid); Preparation temperature; Solvent removal; Morphology; In vitro release profiles

1. Introduction

Although the concept of controlled release is quite old, it was not used for the controlled delivery of

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proteins from biodegradable microspheres until around the 1980s. Proteins possess small half-lives and are incapable of diffusing through biological membranes. Their instability in the stomach and intestines furthermore makes oral delivery of these drugs difficult. Alternative administration by frequent injections is also tedious and expensive. Indeed the development of biodegradable polymeric microspheres has been seen as a promising way to overcome the administering problems of these macromolecules. However, despite this great potential, there are still considerable difficulties that have limited the use of this system in the commercial market. One of the major problems lies in the controlling of the release rate of the system.

From the current number of studies being carried out by researchers all over the world, it is clear that release rates of microspheres depend on many possible factors. Besides the type of polymers used, processing conditions employed during preparation very much determine the properties of the microspheres formed. Properties such as the size and density, the matrix structure, which includes the surface and internal areas, the drug encapsulation efficiency and drug distribution all influence the release of drug from the system. Researchers have therefore been exploring the various preparation conditions that could influence the above properties.

Giunchedi et al. [1] and Witschi and Doelker [2] both experimented with the different methods of encapsulation in their preparation of the microparticles. Differences were found in terms of degradation rates, encapsulation efficiencies and microsphere yields. Crotts and Park [3] varied the volume of the inner aqueous solution to find that higher inner aqueous volume produced more surface porous microspheres. Jeffery et al. [4] studied the effect of antigen:polymer ratio, volume of external aqueous phase and concentration of emulsion stabiliser on the size of the microspheres and antigen entrapment.

The effect of solvent removal technique on poly-(DL-lactide-co-glycolide) (PLGA) microspheres has been studied in a series of publications. Mehta et al. [5] reported that the method and rate of solvent removal, namely the temperature gradient (Tmp) and the dilution (Dil) of the continuous phase methods, influenced the internal structure of the microspheres. Jeyanthi et al. [6] then related this effect to the differences in the density, specific surface area and protein loading of the microspheres formed. Li and co-workers [7,8] modelled the microsphere formation, incorporating solvent concentration and solvent removal profiles. Instead of using a temperature gradient, they noted that having different fixed preparation temperatures also affected the rate of solvent removal. Yang et al. [9] went further to investigate the effect of the preparation temperature on morphology and release profiles of microspheres at 22°C. Our objective in this study is to systematically investigate the fundamental influence of preparation temperature on microsphere formation and its shrinking rate during the early stage of formation, particle size, density, morphology, bovine serum albumin (BSA) encapsulation efficiency, initial burst and release behaviour of BSA-loaded PLGA microspheres at 37°C. This work is essential for scientists to understand microsphere formation mechanisms and the relationship between preparation temperature and properties of PLGA microspheres.

2. Materials and methods

2.1. Materials

PLGA with a copolymer ratio of 65:35 and a molecular mass (M_r) range of 40 000–75 000 (lot 98H1368) was obtained from Aldrich. 88% hydrolysed poly(vinyl alcohol) (PVA) with a M_r range of 31 000–50 000 was purchased from Fluka. BSA (fraction V, M_r 60 000) was obtained from Sigma. Methylene chloride of LC-grade was purchased from Merck. All other regents were of reagent grade.

2.2. Preparation of microspheres

BSA-loaded microspheres were fabricated using a water-in-oil-in-water (w/o/w) double-emulsion solvent extraction/evaporation technique as previously described by Yang et al. [9]. Briefly, 20 mg of BSA was dissolved in 0.5 ml phosphate-buffered saline (PBS) (pH 7.4) solution containing 0.05% (w/v) PVA as an emulsifier and mixed with 12 ml methylene chloride containing 400 mg of PLGA. The

emulsification was carried out by sonication for 15 s using a VC 50T sonicator (Sonics & Materials, CT, USA). The resulting emulsion was further injected into a 250 ml PBS (pH 7.4) solution containing 0.05% (w/v) PVA as an emulsifier to produce a double w/o/w emulsion. The dispersion was then stirred at a constant temperature for 30 min using a mixer (Cole-Parmer Instrument, IL, USA). In order to extract methylene chloride from the first emulsion into the external phase, a 640 ml PBS (pH 7.4) buffer solution containing 0.05% (w/v) PVA was added continuously at a rate of 3 ml/min using a Dosimat pump (Model 725, Metrohm, Swiss). The temperature of the second emulsion throughout the solvent extraction/evaporation stage was maintained constant using a low-temperature circulator (LTD6G, Grant Instruments, Cambridge, UK). The resulting BSA-loaded microspheres were collected by vacuum filtration and washed three times with PBS. The microspheres were then vacuum-dried overnight and stored at 4°C.

2.3. Optical observation of microsphere shrinkage

The rates of reduction in the microsphere diameter during the formation process were tracked with a Nikon polarising microscope (Optiphot2-pol, Japan).

2.3.1. Initial shrinkage

The first sample was drawn 5 min after injection of the first emulsion into the external aqueous phase. For the next 3 min, photomicrographs of the sample were captured continuously at 15-s intervals. This was taken as the initial rate of reduction in diameter of the microspheres.

2.3.2. Continuous shrinkage

Samples were continuously drawn at 5-min intervals for the first 30 min after the first sampling. Following this, samples were still regularly collected throughout the whole dosing period. Photomicrographs were taken for all the samples. The arithmetic mean diameters of microspheres at the various times were determined by averaging the diameters of over 100 microspheres.

2.4. In vitro BSA release study

In vitro release tests were carried out in triplicate at 37°C. A 30-mg amount of dried microspheres was suspended in a 1.5-ml microfuge tube containing 1 ml of PBS and incubated at 37°C. The tubes were periodically removed and centrifuged for 4 min at 10 000 rpm using an Eppendorf Centrifuge (Model 5415C). The supernatant was then collected and its BSA content was analysed using a high-performance liquid chromatography (HPLC) system (HP1050, Waters). The tubes were returned to the incubator after fresh buffer solution was added. The amount of BSA released in the first 24 h was defined as the initial BSA burst in this study.

2.5. Determination of BSA encapsulation efficiency

The BSA amount per unit weight of microspheres was determined by an extraction method. A 10-mg amount of dried microspheres was dissolved in 1 ml of ethyl acetate for 15 min and 10 ml of PBS (pH 7.4) was then added. The mixture was vigorously shaken for 2 min in order to extract the BSA from the organic solution into the PBS. After clarifying for 1 h, the aqueous solution was filtered and the BSA content of the solution was analysed by HPLC. The encapsulation efficiency is calculated as the ratio of actual and theoretical BSA content.

2.6. Particle size distribution

The particles were sized using a Coulter LS 230 particle size analyser. Samples were prepared by re-dispersion of about 10 mg microspheres in Ultrapure Milli-Q water. The results were reported as a volume size distribution.

2.7. Scanning electron microscopy (SEM)

The surface and internal morphology of microspheres, fabricated under different preparation conditions or after a certain period of release, was observed by SEM (Model JSM-5310, JEOL, Tokyo, Japan). The microspheres were mounted on brass

stubs using double-sided adhesive tape and vacuum-coated with a thin layer (100–150 Å) of gold.

2.8. Atomic force microscopy (AFM)

A Multimode Atomic Force Microscope from Digital Instruments was used to study the surface morphology of the microspheres. The samples were mounted on metal slabs using double-sided adhesive tapes and observed under microscope that was maintained in a constant-temperature and vibration-free environment. A scan size of 10 μm was used.

2.9. Determination of polymer molecular masses

Molecular masses of the PLGA microspheres before and during the in vitro release were determined by gel permeation chromatography (Waters 2690) with a differential refractometer detector (Waters 410). Two columns, Styragel@HR 5E, lot No. T81081, 300×7.8 mm and Styragel@HR 4E, lot No. 780751, 300×7.8 mm, with the effective molecular mass ranges of 2000–4 000 000 and 50–100 000, respectively (MA, USA) were connected in series. The mobile phase was tetrahydrofuran (THF) with a flow-rate of 1 ml/min. The dried microspheres were dissolved in THF and filtered. The polymer solution injection volume was 100 μl. The data collection and analysis were done using Waters Millennium[32] software. Weight and number average molecular masses were calculated from a calibration curve using a series of polystyrene standards (Polymer Labs., MA, USA) with molecular masses of 1350, 4920, 9790, 28 400, 71 550, 150 500, 417 500, 1 254 000 and 3 502 000). The total analysis time for every sample was 50 min.

2.10. Bulk density

The microspheres fabricated were weighed and transferred to a 10-ml glass graduated cylinder. The cylinder was tapped using an autotrap (Quantachrome, FL, USA) until the microsphere bed volume stabilised. The bulk density was estimated by the ratio of microsphere weight to the final volume of the tapped microsphere bed.

3. Results and discussion

3.1. Effect of preparation temperature on net solvent removal rate and skin formation

In the double emulsion solvent extraction/evaporation process used, after the first emulsion is injected into the external aqueous phase, dichloromethane dissolves into the external aqueous phase and evaporates at the air–liquid interface. The extent and speed of solvent extraction from the dispersed phase into the external aqueous phase depends on the solubility as well as the rate of solvent mass transfer. Solvent evaporation on the other hand depends mostly on temperature and the concentration of the solvent in the air.

Crotts and Park [3] and Li et al. [8] concentrated their work on residual solvents in the microspheres or the amount of solvents present in the external aqueous phase. Here, our research emphasised another aspect, the net solvent removal rate from the microsphere and microsphere shell formation during the entire fabrication process. Since solvents diffused out through the nascent microsphere skin, the microsphere diameter decreased. The net solvent removal rates could then be estimated by the volume shrinkage of a nascent microsphere. We determine the volume shrinkage by two means; namely (i) an initial shrinkage rate at time $t=0$ (taken to be 5 min after injection of first emulsion) and (ii) an overall shrinkage rate for the entire period of fabrication.

3.1.1. Initial shrinkage rate

The analysis here is based on the shrinking rate of a single microsphere from a drop taken from the external aqueous phase 5 min after injection of the first emulsion into the external aqueous phase ($t=0$). Two assumptions are made to enable the analysis namely: (a) the temperature of the aqueous droplet does not vary too much from that in the actual fabrication emulsion and (b) the droplet's small volume does not seriously affect the extent of contribution of the two mechanisms involved in solvent removal.

The samples were observed under the optical microscope. The reduction in diameter of the microsphere formed at 5°C is shown in Fig. 1. Interestingly, the rate of diameter reduction is constant during

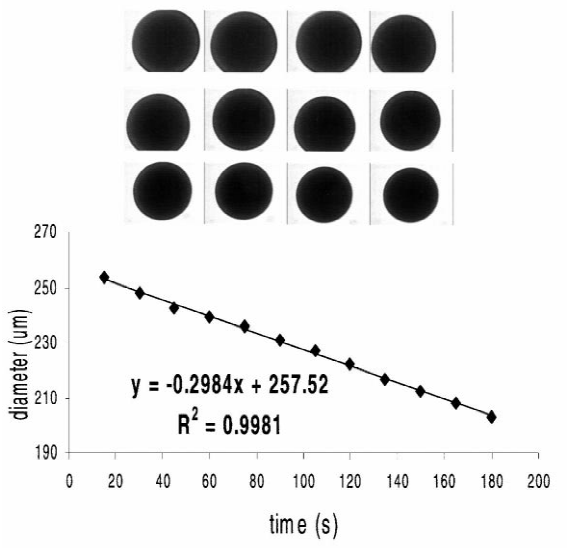

Fig. 1. Top: Photo-micrographs for microspheres at onset of fabrication at 5°C and time interval between photos is 15 s. Bottom: The corresponding diameter reduction of microspheres.

the first 3 min, indicating a constant flux (mass transfer per unit surface area) during this period of time if the density is assumed to be almost invariant because

$$\frac{dM}{dt} = \frac{d(V\rho)}{dt} = \frac{d\left(\frac{4}{3}\pi r^3 \rho\right)}{dt} \cong 4\pi r^2 \rho \cdot \frac{dr}{dt}$$

$$\frac{1}{4\pi r^2} \cdot \frac{dM}{dt} \cong \rho \cdot \frac{dr}{dt}$$

where M is the mass of solvents within a microsphere, t is the time and ρ is the density. These data suggest that the structures of the nascent skin and the mass transfer resistance across the skin at this temperature do not vary much in the early stage (first 3 min) of formation.

Fig. 2 shows the initial rates of reduction in diameter of microspheres fabricated at various temperatures. It is observed that the initial rate of reduction in the diameter of the microspheres is the highest for a low temperature of 5°C. The rate drops for 15°C but climbs up slightly at 22°C and 33°C. The rate falls again at 38°C before becoming negligible at 42°C. A high rate of reduction in diameter means that dichloromethane is leaving the sphere more rapidly. Thus it can be deduced that initially

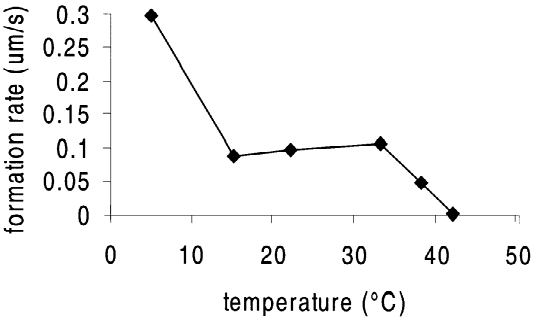

Fig. 2. Initial rate of reduction in diameter of microspheres as a function of fabrication temperature.

solvent is leaving the sphere faster at 5°C than at 15°C or 22°C. From solubility data [10], it is known that the solubility of dichloromethane in water decreases with increase in temperature. Thus at the beginning, the slightly higher solubility (less than 0.5 wt.% difference) of dichloromethane at 5°C as compared to 15°C and 22°C enables a faster removal of the solvent out of the sphere.

However besides solubility, the diffusion coefficient and the concentration gradient of the solvent, both significant factors in mass transfer, are also dependent on temperature. The diffusion coefficient increases with temperature. Higher temperatures will also provide greater driving force for evaporation at the air/liquid interface thus reducing the solvent concentration in the sink. The concentration gradient between the two liquid phases will then increase, favouring mass transfer. Thus it could be argued that at 5°C, since mass transfer is slow, the concentration of the solvent in the external aqueous phase would still be low at the beginning compared to the other temperatures. The concentration gradient is probably still steep, providing the driving force for diffusion of the solvent out into the external phase. This coupled with a higher solubility, results in the high initial shrinking rate of the microsphere at 5°C.

On the other hand, at temperatures 15°C and above, faster mass transfer results in faster saturation of the external aqueous phase. At 15°C and above, less than 5 g of dichloromethane (as compared to about 16 g used in experiment) will saturate the 250 ml of water used. Evaporation is then the predominant process.

The drop in rate of reduction in diameter at 38°C

and 42°C to an almost negligible rate probably means that the shrinking rate slows down and even levels off. Since at such temperatures close to or exceeding the normal boiling point of dichloromethane (39.8°C [11]), the diffusion and evaporation of solvent would be so rapid and effectively over-ride the small difference in solubility. Even within the first 5 min of formation, most of the solvent already escapes from the microspheres, resulting in rapid precipitation of the microspheres and the apparent low initial shrinkage rate.

3.1.2. Overall formation rate

Although the solubility of methylene chloride in water is relatively higher at lower temperatures, it is on the whole not large. Saturation of methylene chloride in water can thus be rapidly achieved. As a result, without fast diffusion and evaporation, the impact of this thermodynamic property at low temperatures can become insignificant during the overall formation. When we track the whole formation process of the microspheres, it is observed that the overall reduction in diameters of the microspheres is rapid at 5°C, gradual at mid-range temperatures like 15°C and 22°C and slow at high temperatures of 38°C and 42°C. Fig. 3 shows the change in diameter of the microspheres fabricated at the various temperatures.

From the graphs in Fig. 3, we observe that except for microspheres fabricated at 5°C, the other microspheres already shrink substantially within the first few minutes. Two possible reasons could result in this large difference in size right at the beginning. First, the reduced viscosity of both phases at higher temperatures result in the spheres being easily broken up by the vigorous stirring (500 rpm) during the first 5 min before the first sample is taken. Second, as mentioned in previous paragraphs, higher temperatures result in a higher mass transfer coefficient for extraction and rapid evaporation. The microspheres thus harden at the very beginning for temperatures 33°C and above due to most of the solvent being already removed. Consequently, microspheres fabricated at 33°C and above have low overall shrinkage rates.

At high temperatures such as 38°C, equilibrium in the system is not reached since the evaporation of solvent is very rapid, removing almost all the solvent through extraction and then evaporation within the first few minutes. This is reflected in the almost negligible initial formation rate (Fig. 2) and the microsphere rapidly reaches the final diameter (Fig. 3c).

3.2. Effect of preparation temperature on size, density and morphology

Since temperature affects the way and the rate at which microspheres formed, it is conceivable to expect the physical characteristics of the microspheres to be affected by preparation temperature as well. The physical characteristics investigated including size, density and morphology are reported below.

3.2.1. Effect on particle size distribution and density

The size of microparticles can be easily affected by a variety of processing factors. Jeffery et al. [4] reported in their study that parameters such as volume of inner and external aqueous phase and the viscosity of inner and external aqueous phase influenced the size of the microspheres. Increase in the molecular mass of polymer also leads to an increase in the size of microspheres [12]. The control of the size of microspheres is important since size affects degradation rate [5], loading and initial burst release [13] of microspheres. The utility of the microspheres is also very much governed by the suitability of the size [4,14].

The size distribution curves for all samples prepared at seven temperatures are as shown in Fig. 4. Due to the different methods used, the sizes of the microspheres measured here using light scattering technique are larger than that observed in Section 3.1.2 using microscopy. With microscopy, the equivalent diameter is obtained from the projected area. In the Coulter particle size analyser, the equivalent diameter used is the volume diameter, which emphasises large particles.

From Fig. 4, one can observe a general increase in the size of the microspheres as temperatures increase. The average size of microspheres was the smallest for 5°C (about 88 μm) and largest for high temperatures 38°C (about 96 μm) and 42°C (about 130 μm). For the rest, particle size ranged from 107

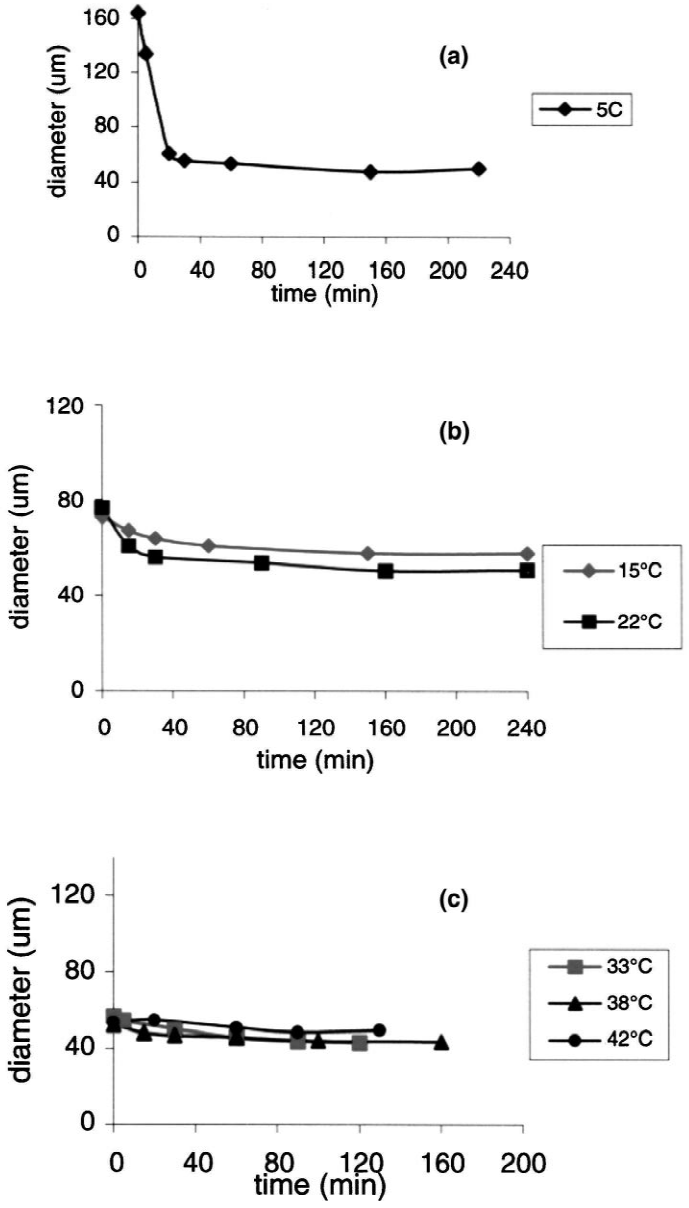

Fig. 3. Change in diameter of microspheres during fabrication at (a) 5°C, (b) 15°C and 22°C, (c) 33°C, 38°C and 42°C.

to 115 μm. Microspheres made at lower temperatures of 5°C, 15°C and 22°C possess the same mode (curve pattern) of 87.9 μm. Similarly microspheres made at higher temperatures of 28°C, 33°C and 38°C have the same size of 96.5 μm.

It is further observed from Fig. 4 that the distribution curves are broader with lower peaks at high temperatures of 38°C and 42°C. Microspheres fabricated at lower temperatures have narrow size distribution curves with higher peaks. Microspheres fabricated at high temperatures start from low viscosity solutions, but spheres harden much rapidly. This probably causes the spheres to have a broader size distribution because shearing forces induced by

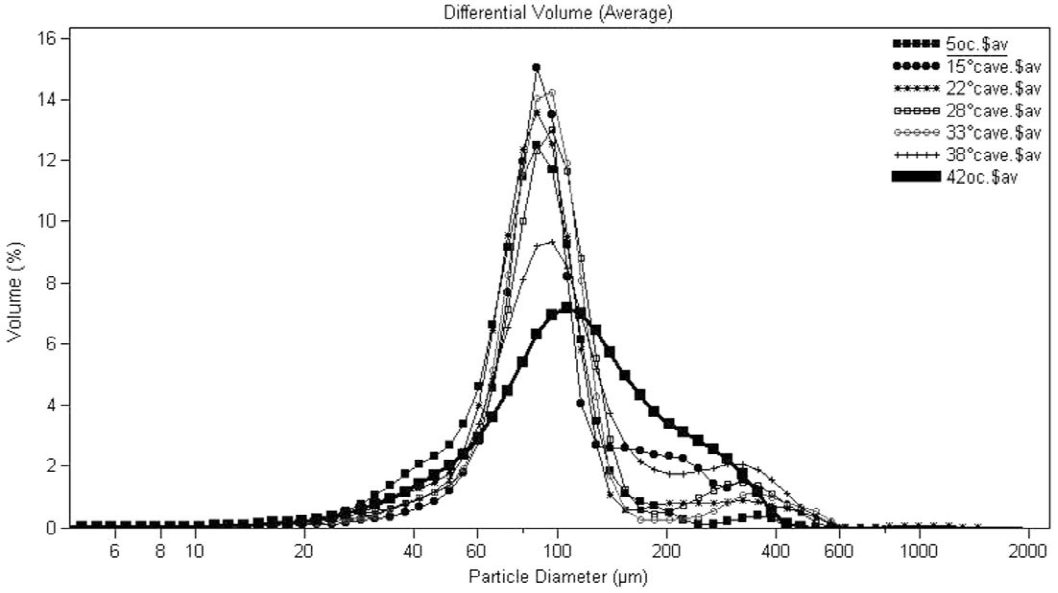

Fig. 4. Size distribution of microspheres as measured with a Coulter particle size analyser.

the stirrer have limited effect on them once the initial spheres are formed. For all the temperatures, a small secondary peak after the major peak is also observed. In addition to previous explanation for broadening size distribution, this characteristic could also be attributed to particle agglomeration in the suspension during the analysis of particle size or the presence of broken spheres and polymer bits.

Fig. 5 shows a graphical representation of the mean size and the density of the microspheres. From the graph we observe that microspheres of a larger mean size (at 38°C and 42°C) have a lower density. The variation in density for the other microspheres is less significant. The density relationship with process temperature is consistent with the report conducted by Jeyanthi et al. [15]. In short, at high temperatures, solvent removal is rapid and microspheres harden almost instantaneously. Thus, microspheres fabri-

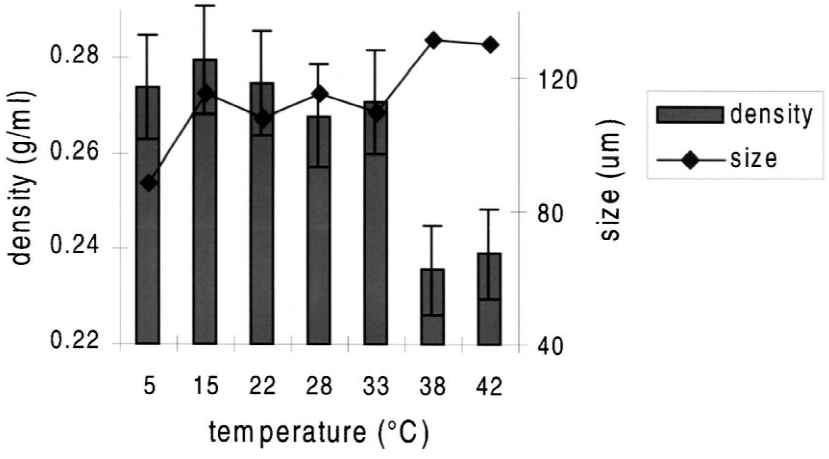

Fig. 5. Plot of density and size of microspheres versus formation temperature.

cated at high temperatures are characterised by a lower density, fully porous internal structure, larger mean size and broader size distribution. While for microspheres fabricated at low temperatures, solvent removal is slow and microspheres take a longer time to solidify, thus permitting a greater shrinkage and densification. The resultant spheres are smaller and denser. However the observed differences in density could also be due to the way measurements are taken. The fact that smaller spheres can pack more tightly than larger spheres when tapped using an autotrap will also contribute to the density differences obtained.

3.2.2. Effect on morphology

Both surface and internal morphologies of the microspheres are examined using SEM or AFM.

3.2.2.1. Surface and internal morphology with SEM. From SEM scans taken (not shown), the microspheres are observed to be all surface-porous with no sharp visible differences amongst the different temperatures. From the cross-sectional images of the microspheres as shown in Fig. 6, it is clear that although internally the microspheres are all highly porous, there are no distinct hollow cores. Porosity of microspheres is known to vary with numerous factors such as polymer molecular mass, co-solvent concentration, dispersed phase to continuous phase (DP/CP) ratio, peptide concentration, solvent removal rate as well as method [5,15,16]. In our case, the internal porosity is generally high for the microspheres since the polymer concentration is quite low. Low polymer concentration means a high distribution of solvent and internal water in the polymer. During precipitation, more water from the CP could diffuse in to create more water pockets [7]. Once the microspheres are dried, the water pockets, which include the internal water, and the water diffused from CP will become the holes. It was reported by Mehta et al. [5] that rapid removal of solvent using the temperature gradient technique resulted in hollow core formation. Here, although solvent removal is rapid at high temperatures, the microsphere interior is more uniformly porous instead.

The absence of core formation could be due to the presence of emulsifier in the inner aqueous phase, which prevents coalescence of the internal water droplets. Moreover, at higher temperatures like 33°C and 38°C, solvent removal is rapid right from the beginning. A dense and tight skin layer of the microspheres form quickly, hindering the influx of water. In fact the whole sphere probably solidifies so quickly without allowing many water droplets to interact within the matrix. With less water entering, there are no large water droplets diffusing into the inner matrix. Rapid solidification really gives little time for instability to set in on the water droplets. They are probably still fairly uniformly distributed within the dispersed phase when the polymer solidifies giving the even pore distribution throughout the matrix as observed in Fig. 6D and E.

For the slower forming microspheres at low temperatures, skin formation is slower and the interior remains soft for a longer period of time. External aqueous phase could diffuse in, creating more water pockets. The inner water droplets, given the time, could move within the soft interior of the matrix, coalescing to form bigger droplets. As the polymer solidifies from the peripheral towards the centre, the water droplets would also be pushed gradually towards the centre. All these eventually give the bigger holes observed in the centres of the microspheres.

From Fig. 6, it can also be seen that the walls of the microspheres fabricated at low temperatures are slightly porous and thicker (A, B) than the higher temperatures (D, E). The microsphere skin layer formation is often likened to the phase separation and precipitation of asymmetric membranes. Nucleation and growth tends to be the dominant mechanism at low temperature precipitation, which yields a slightly porous, but thicker skin layer structure [17]. High-temperature solvent removal results in less defective membrane skin layer. Fig. 7 shows a simplified ternary phase diagram for a solvent/coagulant/polymer system often used to illustrate the precipitation characteristics for such a system. For microspheres made at high temperatures, the dispersed phase will most likely take the coagulation path (a) during formation. Since solvent removal is rapid, out-flow of solvent will be higher than in-flow of water. Polymer concentration within the dispersed phase increases quickly, solidifying into a dense layer. Whereas for microspheres made at low temperatures, polymer precipitation will probably follow

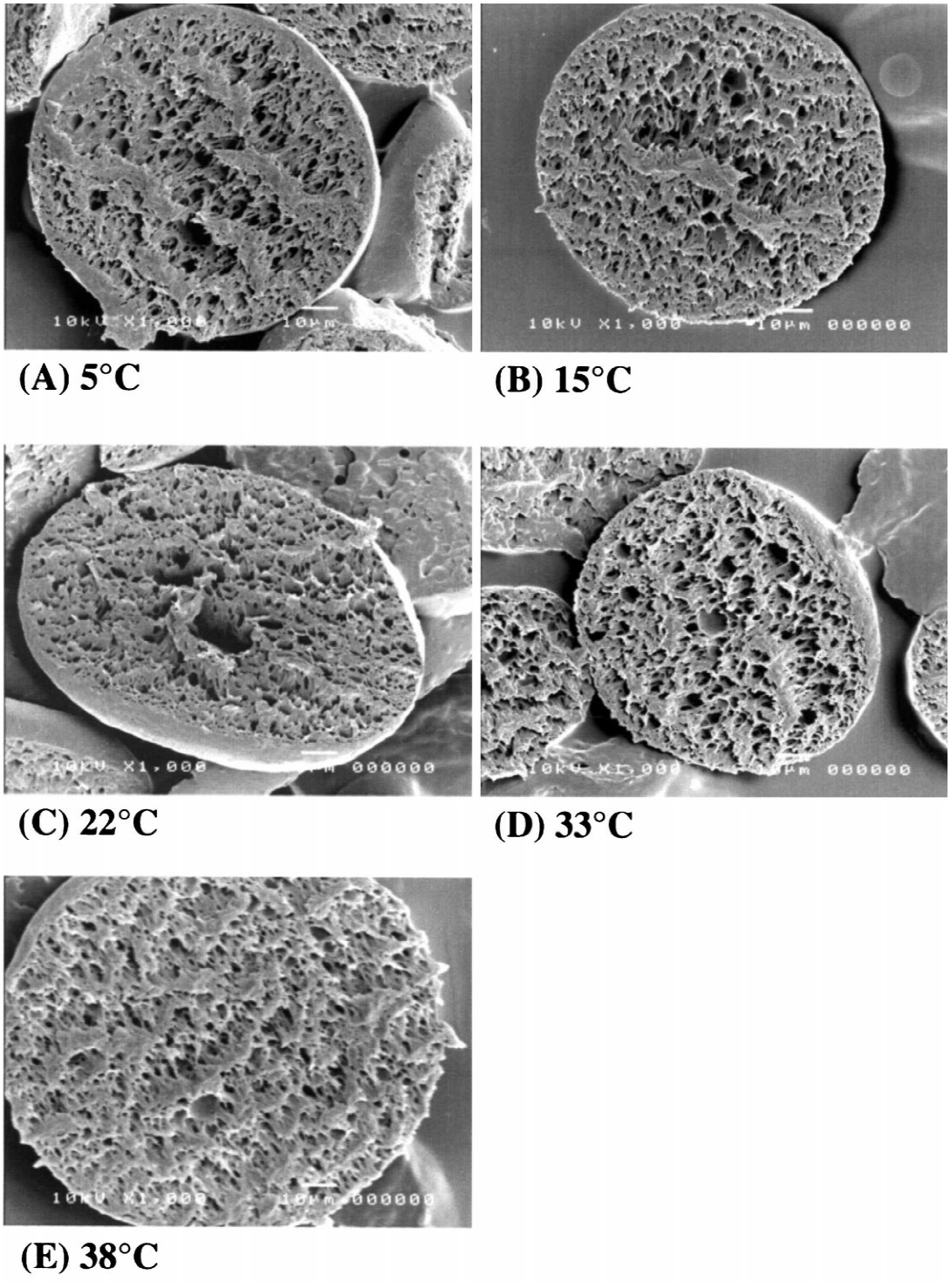

Fig. 6. Cross-sectional SEM scans for (A) 5°C, (B) 15°C, (C) 22°C, (D) 33°C and (E) 38°C.

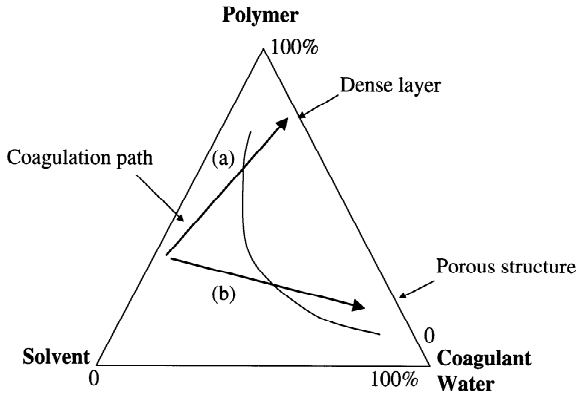

Fig. 7. Ternary phase diagram for the microspheres forming in the external aqueous phase.

path (b). Here solvent out-flow is slower allowing increased in-flow of coagulant water. A more porous, spongy microsphere wall is thus formed.

3.2.2.2. External morphology with AFM. Fig. 8 shows the external morphology of microspheres as observed using AFM. Comparison of the degree of porosity may be difficult here due to limited scan size. However we can observe that the microspheres made at higher temperatures (38°C and 42°C) appear to have a much rougher surface. This coarse skin is probably due to rapid evaporation and phase separation during the shrinking and hardening stage.

The microspheres made at lower temperatures on the other hand have a relatively ''slightly smoother'' surface. Since the spheres fabricated at lower temperatures undergo slower but more extensive shrinking during their formation, it may result in this smoother sphere surface. Since the surface roughness of microspheres was suggested to affect their adhesion onto the intestine's Payer's patch [18], it is thus a meaningful characteristic to consider.

3.3. Effect of preparation temperature on BSA encapsulation efficiency

Table 1 shows the BSA encapsulation efficiencies of various microspheres prepared at different temperatures and the overall average is about 58±5%. This encapsulation efficiency value may be different from those previously obtained by other researchers mainly because we have different research objectives. The difference in encapsulation efficiency is also due to the differences in sample preparation conditions [4], microsphere sizes, targeted loadings, polymer concentrations, polymer molecular masses, copolymer compositions and characterisation techniques [12].

It has been frequently reported that the rapid solidification of polymer giving rise to denser skin layer increases loading efficiency [7,12]. The fast solidification of the polymer can be a result of many factors such as a higher solvent removal rate [7], higher polymer concentration or a lower dispersed phase to continuous phase DP/CP ratio [12]. Yet in our case, although the rate of solvent removal and thickness of the microsphere skins are different, these are not reflected in the encapsulation efficiency among the different microspheres.

Instinctively, since microspheres made at high temperatures (such as at 38°C and 42°C) solidify rapidly, forming a dense thin skin, drug encapsulation efficiency should be high. Naturally a dense skin will provide impedance, making it difficult for proteins to diffuse through. However, preparation temperature affects not only the mass transfer and evaporation rate of the solvent but also the mass transfer and solubility of the protein. Although faster skin formation may reduce protein loss, the increase in solubility of protein at higher temperature and the faster mass transfer may also increase the amount of BSA leaving the dispersed phase during formation. Furthermore protein denaturation at high temperature may also result in less active proteins encapsulated.

By the same token, although the microspheres fabricated at lower temperatures solidify slower, the lower solubility and mass transfer of BSA at those temperatures probably compensate for the relative ease of diffusing through the softer, less dense skin. The activity of proteins also remains fairly intact at these lower temperatures. The resultant encapsulation efficiencies for microspheres fabricated at higher or lower temperatures are thus similar.

3.4. Effect of preparation temperature on polymer degradation

All microspheres show a small decrease in molecular mass after release. There is no significant

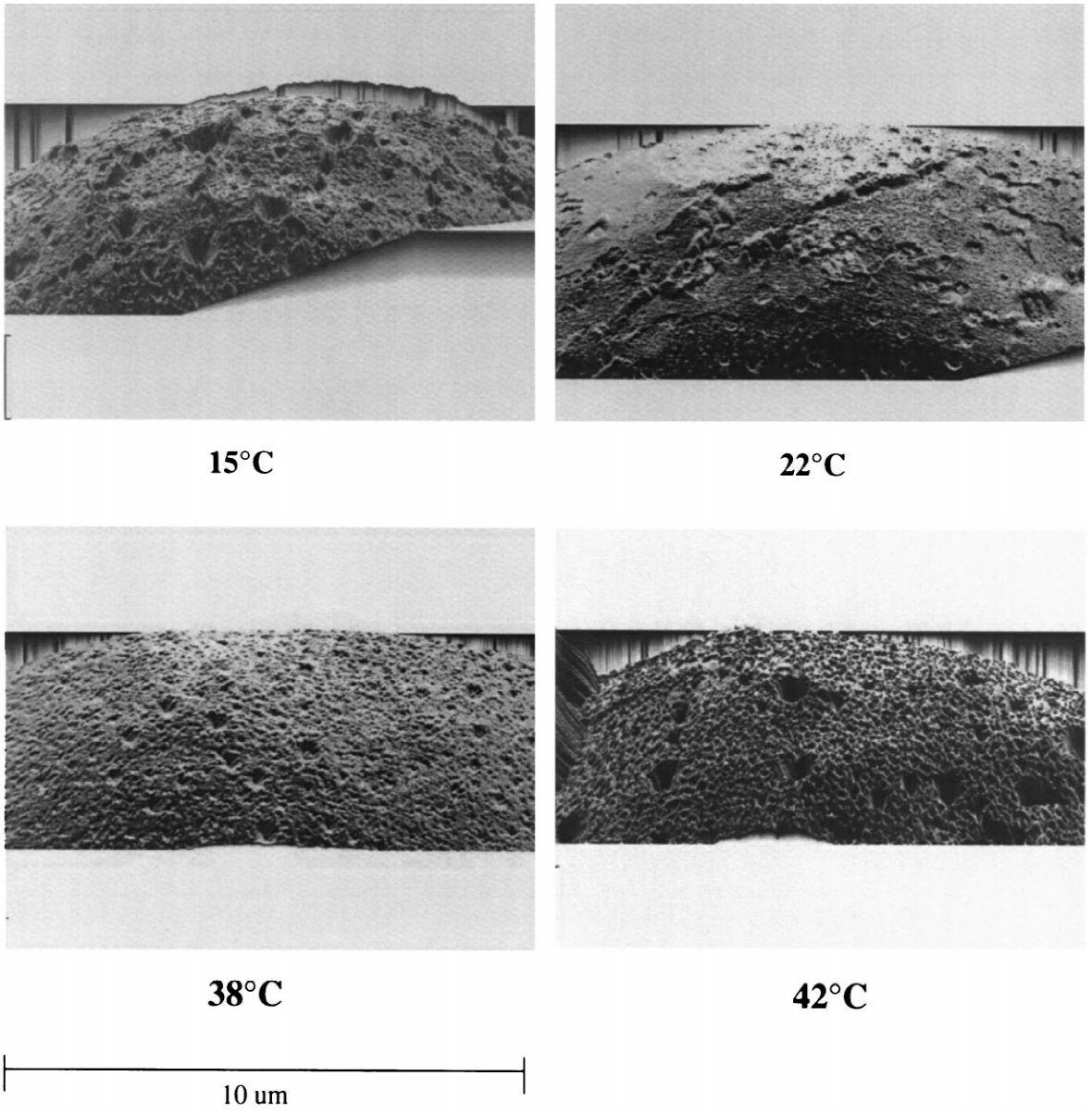

Fig. 8. AFM images for microspheres fabricated at various temperatures.

Table 1
Encapsulation efficiency of the microspheres

	Temperature (°C)						
	5	15	22	28	33	38	42
Encapsulation efficiency (%)	59.6	59.6	52.7	56.6	62.5	54.1	57.8

difference in the decrease, which averaged about 17% after 28 days.

Fig. 9 shows the typical morphology of the degraded microspheres. From the SEM images, it is observed that all the microspheres go through similar rates of degradation regardless of their fabrication temperature. After about 14 days of incubation at 37°C close to human body temperature, the surfaces of the spheres become wrinkled and the internal pores can be seen.

3.5. Effect of preparation temperature on initial BSA release

The variation in the initial burst with preparation temperature is shown in Fig. 10. It is a very interesting phenomenon that lower and higher temperatures yield the lowest initial BSA burst while the highest initial burst peaks at a preparation temperature of 33°C. The initial release basically increases gradually from about 20% to about 61% when temperature is increased from 5°C to 33°C. Subsequently, initial release dips back to a low of about 20% at 42°C.

As was described by O'Hagan et al. [19], the initial burst release observed in microspheres is probably the result of poorly entrapped protein and protein loosely attached to the internal and outer surface. Initial release thus depends on the ability of the polymer matrix to encapsulate the protein, making diffusion difficult [12]. Increase in polymer concentration [20] or polymer molecular mass [12] both have the same effect of increasing the speed at which the polymer solidified. Faster solidification of the polymer means a better entrapment of the protein thus reducing initial release, which is expected. Increase in protein loading on the other hand has the effect of increasing the surface embedded proteins and thus increasing burst.

Based on the results we have obtained, it is likely that preparation temperature affects both the entrapment efficiency as well as the amount of surface proteins available for initial release. Temperature again has a dual effect on initial release, like in the case of the encapsulation efficiency. Specifically, high temperature speeds up solidification of polymer thus improving entrapment. Once the outer skin is formed, this dense layer may serve to prevent easy

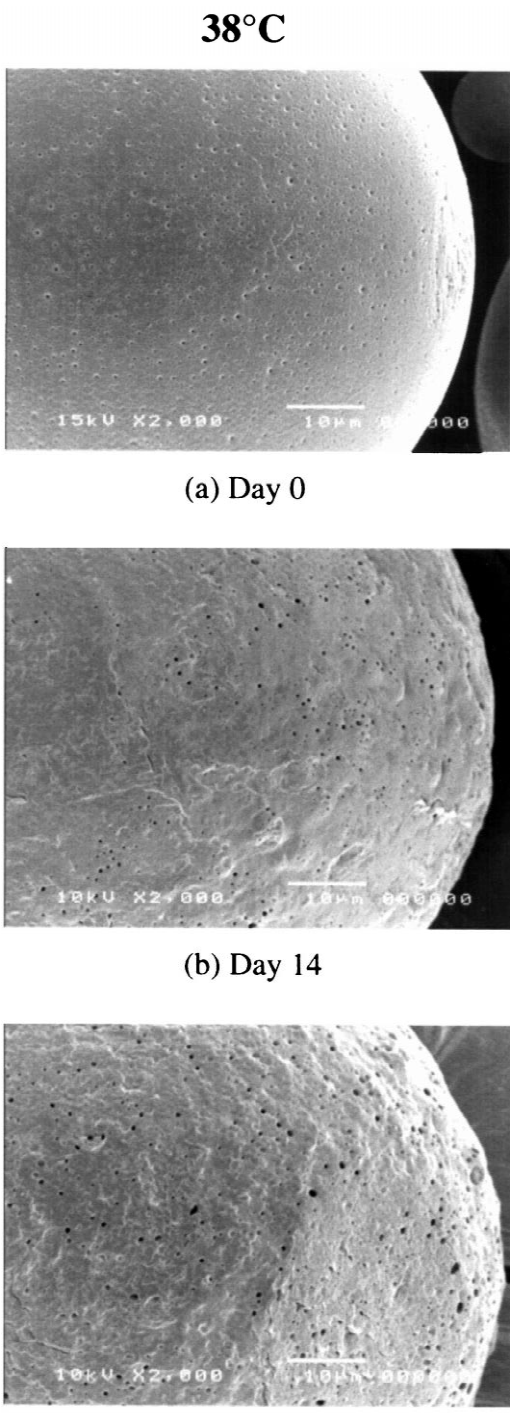

Fig. 9. SEM scans showing degradation of microspheres fabricated at 38°C at (a) day 0, (b) day 14 and (c) day 28.

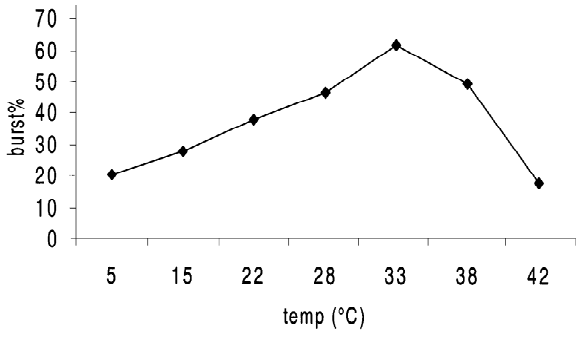

Fig. 10. Initial burst release as a function of preparation temperature.

release of the protein although the protein diffuses more quickly at high temperatures. As a result, microspheres made at 38°C and 42°C have decreasing burst. Similarly although microspheres made at lower temperatures have more porous skin, distribution of the protein near the surface is low as less protein can move to the surface. Thus initial release decreases as fabrication temperatures drop from 33°C to 5°C. It appears that at 33°C, the temperature is high enough for the speedy diffusion of protein to the surface and through sufficiently porous skin layer.

3.6. Effect of preparation temperature on BSA release profile

The release characteristics of the various microspheres are summarised in the % cumulative release profiles shown in Fig. 11. From the release profiles, there appear to be two distinct groups of microspheres with different patterns and rates of release. Specifically, microspheres fabricated at higher temperatures (from 28°C onwards) experience a high initial rate of release before levelling off very quickly after 2 to 6 days. Subsequent rate of release is then very slow as seen by the almost straight horizontal lines. For microspheres fabricated at lower temperatures of 5°C, 15°C and 22°C, there is still a relatively higher and steady rate of release after the initial phase of fast release.

Since there are no significant degradation rate differences observed, the variation in the release profile of the microspheres may very likely come primarily from the difference in the diffusion process. For the microspheres fabricated at high temperatures, the high initial release of about 2–6 days may be a release of the surface proteins available. However, due to the fact that these microspheres solidify mainly through an evaporation process, they have denser and tighter skin structures and the release of the deeply embedded protein becomes

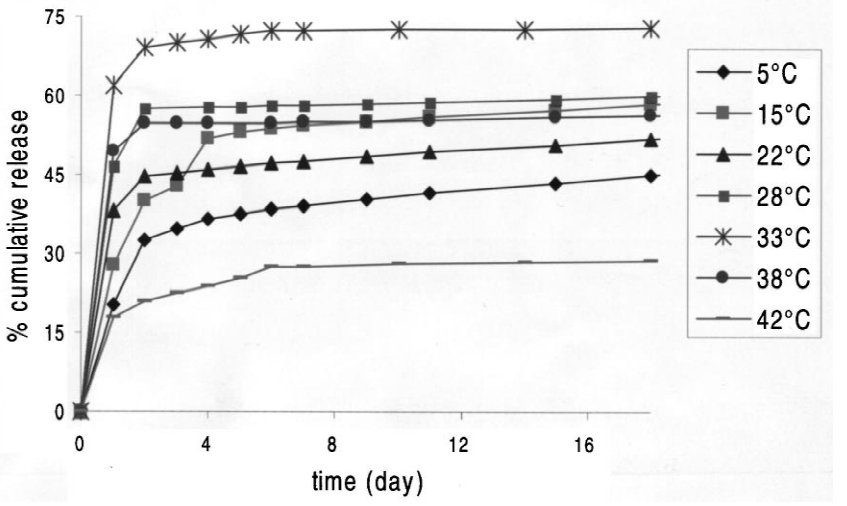

Fig. 11. In vitro release profiles of the microspheres.

difficult. Another possible reason is that the bigger microspheres produced at higher temperatures lead to a slower release profile due to their smaller surface area for protein diffusion.

Low-temperature microspheres, on the other hand, solidify less quickly giving a thicker but spongy skin layer. Although their initial burst is not exceptionally high, their resultant release rates are sustained at a rate higher than the microspheres fabricated at high temperatures. Interestingly, the microspheres produced at 5°C, 15°C and 22°C have almost the same release profile after initial bursts. It may come from the similar surface and internal morphology of these microspheres as presented above. The results show consistency with previous work [9] where in vitro release was carried out at room temperature (22°C). It means that even at 37°C, the microspheres still undergo a predominantly diffusion-based drug release during the first 20 days.

4. Conclusions

We have determined the effects of preparation temperature on the various characteristics and BSA release profiles of PLGA microspheres. The microspheres were prepared using the w/o/w technique with PVA as surfactant in the external aqueous phase. Very low preparation temperature (5°C) results in slow formation of microspheres. The resultant microspheres are the smallest in size, with porous surface and internal structure. They have a low initial burst and a characteristic steady release of BSA. Very high preparation temperature (38°C and 42°C) on the other hand gives large microspheres with a wide size distribution and rough surface because of rapid solvent removal.

During fabrication, a low preparation temperature of 5°C gives the slowest overall shrinking rate. Microspheres formed at high temperatures of 38°C and 42°C on the other hand have the highest overall shrinking rate. Subsequently, microsphere mean size increases and the particle size distribution widens with increase in the preparation temperature. Microspheres formed at 33°C is found to give the highest initial burst release. The results obtained suggest that preparation temperature significantly affects microsphere formation, resulting in their structural and protein release profile differences.

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