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Research paper

A simple method for the preparation of monodisperse protein-loaded microspheres with high encapsulation efficiencies

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ABSTRACT

A simple method for the preparation of monodisperse protein-loaded polymer microspheres is presented in this paper. The method is based on the co-extrusion of an internal phase of an aqueous protein solution and an external phase of an organic polymer solution through a 200-micron-sized hole. Controlled in the correct flow region, this process produces a core–shell-structured laminar liquid jet, which breaks to form monodisperse compound liquid droplets. Stabilized in a dilute aqueous polyvinyl alcohol (PVA) solution, the droplets are converted into solid protein-loaded polymer microspheres through evaporation of the organic solvent. Results show that preparation parameters such as polymer concentration, total flow rate, flow rate ratio of the aqueous to organic phase have significant effects on the mean particle size, particle morphology and protein encapsulation efficiency (EE). The results of biodegradation and the protein release characteristics of the polymer microspheres are also presented.

1. Introduction

In recent years, the fabrication and synthesis of protein-loaded polymer particles have attracted extensive attention due to the protection function and controlled release properties of the particles [1–7]. These advantages are widely used in a variety of fields including biotechnology, pharmaceutics, medicine, ecology, catalysis and medicinal therapy. In most cases, the shell materials are biocompatible and biodegradable, allowing the protein-loaded particles to be swallowed or injected to human body [8–14]. The proteins can either diffuse from the pores of the shell layer or release from the degradation of the shell layers. As such, it is expected that the release pattern of the proteins can be achieved through the manipulation of shell polymer material, particle size and morphology, which can be controlled by the production conditions. It is shown that controlled release of drugs can improve the therapeutic efficiency and lower the dose of drugs required, which makes encapsulation even more attractive.

Many methods have been developed for the encapsulation of proteins, drugs and flavors. The most often employed methods are the double emulsion method (including water-in-oil-in-water, water-in-oil-in-oil, solid-in-oil-in-water, etc.) [12,15–18], post-fabrication encapsulation [19] and layer-by-layer (LbL) methods [20]. Among these methods, the double emulsion technology is studied and utilized most extensively.

A major drawback of the conventional double emulsion method is the broad size distribution of the resultant particles from this method. Due to the close relationship of particle size and its distribution with the performance of the particles in almost all applications, some new methods have been developed aimed at producing monodispersed or near monodispersed particles [21–26]. These include membrane emulsification [27–31], micro-channel emulsification [32], and two-dimensional [33,34] and three-dimensional micro-fluidic systems [35–37].

Encapsulation efficiency and payload are two key parameters for the encapsulation systems. They are influenced by not only the encapsulation processes but also the chemical nature of both the content and the encapsulating polymers (composition and molecular weight). In the case of protein encapsulation by conventional multiple emulsion methods, a large proportion of proteins can be lost from the merger of the internal aqueous phase with the external aqueous phase, resulting in a low encapsulation efficiency [15]. While the recently developed methods have been demonstrated to be able to produce very narrowly distributed particles with precisely controlled size and structures, low encapsulation efficiencies and payload remain to be a major problem when applied to the encapsulation of proteins with encapsulation efficiencies still being in the 40–60% range [16,18].

In our previous research, we developed a method that was capable of producing monodisperse polymer particles encapsulating an internal aqueous phase. The method is based on the breakup of a flow-focused emulsion jet [22] and a core–shell-structured laminar jet [38]. In both cases, however, encapsulation of the inner aqueous phase was confirmed only visually by the colour of the particles encapsulating a water-soluble colour agent. The objective of the

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current study is to apply the method that we developed to encapsulate a model protein to uniformly sized polymer particles. This article presents quantitative information on particle size, encapsulation efficiency and release kinetics of lysozyme in polycaprolactone (PCL) particles produced under different conditions using this method.

2. Experimental

2.1. Materials

Polycaprolactone (PCL), polyvinyl alcohol (PVA, MW 13000, 86.7–88.7% hydrolysis), lysozyme (90% protein), dichloromethane (DCM, 99.5% ACS reagent) and ethyl acetate (EA, 99.5% ACS reagent) were all purchased from Sigma–Aldrich and used as received without further treatment. Deionized water was used throughout the experiment.

2.2. Preparation of lysozyme-loaded PCL particles

To prepare lysozyme-loaded PCL particles, a flow cell designed in our laboratory was used. Fig. 1 shows the schematic diagram of the experimental set-up. Two syringe pumps (Harvard Apparatus, PHD 2000) were used to pump an aqueous lysozyme solution (1.0 mg/ml, internal phase) and a PCL in DCM-EA mixture solution (0.8–5.0/100 ml, external phase) to flow concurrently through the 200-micron-sized hole in the bottom plate of the flow cell. The DCM-to-EA-volume ratio in the DCM–EA mixture is 2:1 so that the density of the jet is slightly heavier than that of the bulk PVA solution in the collection chamber, ensuring the resultant droplet from the jet breakup would sink. During the jetting period, the liquid droplets were stabilized by the PVA (0.3 wt.%) in the bulk aqueous phase against coalescence. After the jetting completed, the PVA-stabilized droplets were stirred gently for a further 2 h and to evaporate the organic solvent, resulting in the formation of lysozyme-loaded PCL solid particles. The as-prepared polymer particles were separated from the PVA solution by filtration, and each sample was washed with deionized water for four times, then collected and dried in an oven at 40 °C for about 3 h for further use.

2.3. Characterization

2.3.1. Particle morphology

The morphology of the prepared particles was examined by both optical microscope (OM) and scanning electron microscope (SEM). OM observation was conducted by using an optical microscope (Olympus CH.2, Japan), with the particles dispersed in distilled water on a glass slide. SEM studies were conducted with a JEOL JSM-5410LV scanning electron microscope operating at 10 keV. The particles were scattered onto a conductive tape and sputter-coated with gold prior to SEM examinations.

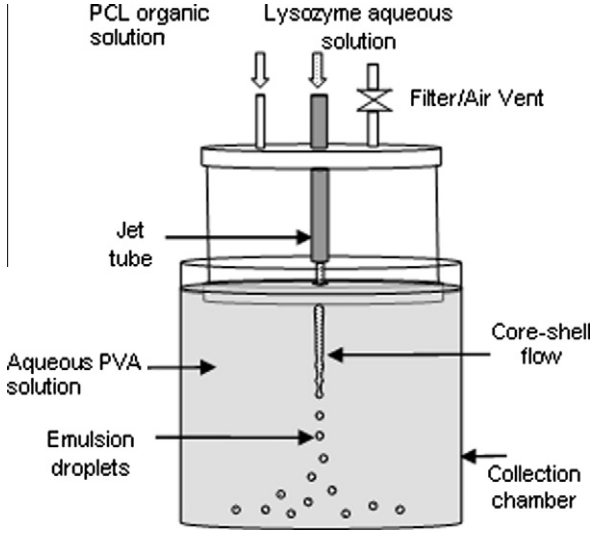

Fig. 1. Schematic image of the flow-focused jetting cell and particles' formation process.

2.3.2. Particle size

The size and size distribution of prepared lysozyme-loaded PCL particles were determined by the laser-light-scattering method on a Mastersizer 2000 instrument (Malverm Instruments, UK). Each sample was tested for three times, and the values of mean particle size and size distribution are the average values of the three measurements.

2.3.3. Encapsulation efficiency (EE)

Polymer microspheres (20 mg) were dissolved in 1.0 ml of DCM, and 1.0 ml of distilled water was added to extract the proteins. Subsequently, the aqueous phase and organic phase were separated to obtain the aqueous protein solution. The extraction of proteins was repeated for three times. The proteins were collected, and their quantity was determined by a micro-protein assay method, the bicinchoninic acid (BCA) method. The protein content was compared with the content of protein used in the preparation of protein-loaded polymer spheres to calculate the encapsulation efficiency using the following equation:

$$\mathrm{EE} = \frac{M_{\mathrm{en}}}{C \cdot V} \tag{1}$$

where M_{en} is amount of protein encapsulated as measured by the BCA method; C is the protein concentration in the aqueous phase; and V is the volume of the protein solution used as measured by the syringe pumps.

2.3.4. Polymer degradation and in vitro lysozyme release

To observe the degradation of the polymer shells and the release of proteins, 20 mg protein-loaded polymer microspheres were placed into Eppendorf tubes and incubated in phosphate buffer saline (PBS, pH 7.4) solution at 37 °C under agitation, and the samples were centrifuged at certain intervals. The supernatant was withdrawn for BCA assay to obtain the amount of proteins released from the polymer particles. The polymer microspheres were incubated in fresh PBS solution medium for the next release test. For biodegradation observation, one batch of protein-loaded PCL microspheres was divided into four groups. They were then dispersed in phosphate buffer saline solution (PBS, pH 7.4) in four separate Eppendorf tubes and kept in an incubator at 37 °C. At pre-determined time intervals, the particles in one tube would be taken out of the solution and their morphologies were analyzed by SEM observation for structure and/or morphological changes.

3. Results and discussion

To make the encapsulating process readily visible, the lysozyme was dissolved in 100 ppm aqueous bromophenol blue (BB) solution, using BB as an indicator. Attainment of blue polymer microspheres suggested that the encapsulation of protein solution was successful. Typical SEM and OM images of the resultant microspheres are shown in Fig. 2. It can be seen that, in both images, the particles are reasonably uniform in size. Quantitative measures in our previous work on the uniformity of particle size showed that the coefficient of variance (defined as the number-based standard deviation divided by the mean particle size) reached as low as 3.1% [38]. The SEM image also shows that the surfaces of the particles are generally smooth. However, there are many small specks in

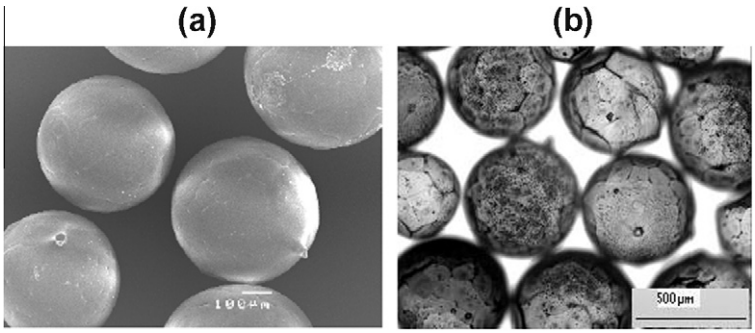

Fig. 2. Typical SEM image and OM images of the lysozyme-loaded PCL particles.

the OM images, suggesting that lysozyme was successfully encapsulated inside the PCL shell.

In our previous work, it was found that total jetting rate, jetting ratio of the organic phase to aqueous phase (O/A) and PCL concentration in the organic phase all have notable effects on the mean size and size distribution of the resultant PCL particles [22]. In this work, the effects of these factors on particle morphology, protein encapsulation efficiency, biodegradation behavior and protein release from the polymer spheres will be discussed in detail in the following paragraphs.

3.1. Particle size and morphology

As expected, when all other conditions are kept unchanged, PCL concentration in the organic solution has a significant effect on the particle size: the higher the PCL concentration, the larger the mean particle size. In this study, when 0.8 g/100 mL, 1.5 g/100 mL, 3 g/100 mL and 5 g/100 mL PCL organic solutions were used, the mean particle size increased from about 115 μm to550 μm, as shown in Table 1.

There are two factors that contribute to the increase in mean particle size with increasing PCL concentration in the organic phase. At a higher PCL concentration, more PCL is available from each liquid drop to precipitate resulting in a larger solid PCL particle. A more significant factor is, however, the increase in the viscosity of the organic phase with increasing PCL concentration, which causes the formation of larger liquid drops from the breakup of the jet [38].

Change in mean particle or liquid drop size with liquid flow rates is more complex. To produce monodisperse liquid droplets as a template for monodisperse solid particles using our method, the total liquid flow rate (the sum of both aqueous and organic phase flow rates) has to be controlled within what is defined as the laminar jet region. Within this region, the mean particle size decreases first, reaches a minimum and then increases with increasing liquid flow rate. In this work, when the organic phase flow rate increased from 180 to 240 ml/h, the mean particle diameter decreased from 317 μm to 300 μm because the flow condition was in the early part of the laminar jet breakup region. Interested readers are referred to our earlier publications for more details on particle size change with flow conditions [38].

The particle size distribution can be expressed by the coefficient of variation C_v, which is defined as the standard deviation divided by the mean diameter [22]. The C_vs of the particles obtained under different preparation conditions are shown in Table 1. Compared with those produced by conventional multiple emulsion methods where C_vs as high as 40% are not uncommon [39,40], particles produced by this method are much more uniform in size with all sample particle C_vs being below 10%.

A further observation of the resultant particles was that the particle surfaces became rougher with increasing PCL concentrations. This phenomenon is shown in Fig. 3. The effects of PCL concentration on surface roughness may be explained as follows. When high concentration solutions were used, during solvent evaporation, a relatively large amount of PCL solid would precipitate out at the same time. This meant that the PCL shell was formed in a relatively short time, resulting in the rough surfaces of the final particles. In comparison, when a dilute PCL solution was used to encapsulate the protein, with the evaporation of the solvents, the PCL shell formed more gradually, allowing the PCL to precipitate out in a more orderly manner and form a smoother surface.

Table 1
Particle sizes with the change in preparation conditions.

Run	PCL concentration (g/100 ml)	Organic phase jetting rate (mL/h)	Aqueous phase jetting rate (mL/h)	Particle size (μm)	C_v (%)
1	5.0	180	30	557	3.8
2	5.0	180	60	317	7.5
3	5.0	240	60	300	4.6
4	3.0	180	30	327	7.9
5	1.5	180	30	126	6.4
6	0.8	180	30	113	8.3

3.2. Encapsulation efficiency (EE)

While there has been much progress in the generation of structured monodisperse liquid droplets of which some have been converted into structured monodisperse solid particles, encapsulation of proteins remains a practical challenge. It is not uncommon to see encapsulation efficiencies of proteins in the 40–50% range [2,16,18].

In a two-step encapsulation process such as the one used in this paper, the protein may be lost in either the emulsification/droplet formation step or the solvent evaporation/extraction step. In this work, it is found that the encapsulation efficiency is strongly influenced by the process conditions including PCL concentration in the organic phase and the flow rate ratio of the aqueous phase to the organic phase. The results are summarized in Table 2.

From the table, it can be seen that when the flow rates of both the aqueous and organic phases were kept constant but PCL concentration in the organic phase was increased from 0.8 (w/v)% through 3.0 (w/v)% to 4.0 (w/v)%, the encapsulation efficiency decreased from 79% to 77% and 42% respectively. In other words, the higher the PCL concentration, the lower the encapsulation efficiency. Given the possible errors introduced in the micro-protein assay test for protein content, there is practically no difference in the encapsulation efficiency between the microparticles prepared at PCL concentration of 0.8 (w/v)% and 3.0 (w/v)%. However, when PCL concentration was increased to 4.0 (w/v)%, the encapsulation efficiency decreased dramatically to 42%. This is likely to be caused

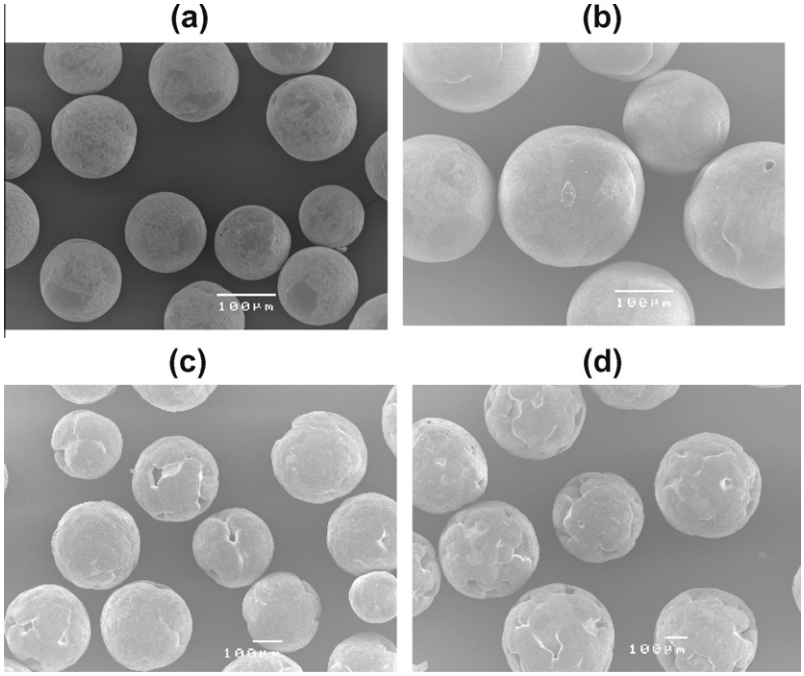

Fig. 3. SEM of lysozyme-loaded PCL particles prepared at the concentration of PCL at (a) 0.8 g/100 ml, (b) 2 g/100 mL, (c) 3 g/100 mL and (d) 5 g/100 mL respectively.

Table 2
Effect of PCL concentration and jetting rate ratio on protein encapsulation efficiency.

Run	PCL concentration (g/100 ml)	Organic phase jetting rate (mL/h)	Aqueous phase jetting rate (mL/h)	EE (%)
1	0.8	150	30	79
2	3.0	150	30	77
3	4.0	150	30	42
4	0.8	150	50	62

by the difference in the breakup behavior of the jet. Our previous results showed that the viscosity of the organic phase increased exponentially with the PCL concentration [22]. At a higher viscosity, the jet breakup becomes more difficult and incomplete, resulting in an imperfect encapsulation process and loss of the internal aqueous protein solution to the bulk PVA solution.

Comparing the encapsulation efficiency of Run Number 1 with that of Run Number 4 in Table 2, the effect of the total flow rate, in particular, the flow rate ratio of aqueous to organic phases is obtained. In these two runs, the PCL concentration was kept at 0.8 (w/v)%, and the outer organic phase flow rate at 150 ml/h. As the aqueous phase flow rate increased from 30 ml/h to 50 ml/h, the encapsulation rate decreased from 79% to 62%. This is to be expected. With a given amount of shell material represented by the constant PCL concentration and external organic phase flow rate, it would become increasingly difficult to encapsulate the inner phase as its amount (in the form of flow rate) increases.

It is worth noting that even the worst encapsulation efficiency in our runs is over 40% with the highest reaching almost 80%. With some further process optimization, the protein encapsulation efficiency is expected to reach beyond 80%, which is a significant improvement in protein encapsulation compared with other protein encapsulation methods reported in the open literature.

3.3. Polymer degradation and protein release

There are two broad mechanisms for the encapsulated proteins to release from the capsules to their surrounding environment, namely, bulk diffusion through the shell and burst release from bulk erosion or degradation of the shell material. Irrespective of the release mechanism, the release characteristics of the proteins is intimately related to the degradation of the polymer shells.

Fig. 4 shows the morphological and topological changes in the particles. It can be seen that, within the first 100 h, the only change occurred to the shells of the particles visible at this magnification is that their surface became rougher. After 2 weeks, some holes were clearly visible on the surface of the particles. After 4 weeks, most particles were damaged and collapsed.

The release of lysozyme from the PCL particles was determined by BCA assay. Three groups of PCL microspheres with high encapsulation efficiencies were chosen to investigate their release characteristics. The cumulative release versus time is shown in Fig. 5. During the preparation of all the PCL microspheres, the lysozyme concentration in the inner aqueous phase was maintained constant at 1.0 mg/ml. The flow conditions and the PCL concentration in the organic phase for these PCL microparticles are different and are described in the caption of Fig. 5.

From Fig. 5, it can be seen that the cumulative release of lysozyme increased for all three groups of PCL particles tested. The release appears to occur in two stages: a fast release period followed by a more gradual release in the second stage with the majority of the content (up to almost 80%) released in the first stage. The fast release ended in the first week of the *in vitro* environment. With reference to the morphological changes in the particles shown in Fig. 4, visible holes at the level of magnification used only appeared on the surface of the particle after 2 weeks. It is therefore reasonable to suggest that the dominant release mechanism for all three groups of particles tested in this study is bulk diffusion of free proteins inside the capsules through the micropores in the PCL wall of the particles not visible in Fig. 4. In comparison, the slow release of protein in the second stage is postulated to be a result of desorption of the lysozyme from the PCL walls.

On the basis of percentages of content release, there is little difference between Curves a and b, considering the accuracy of the BCA assay. This suggests that, within the range tested here, the jetting rate ratios of the organic to aqueous solutions did not have

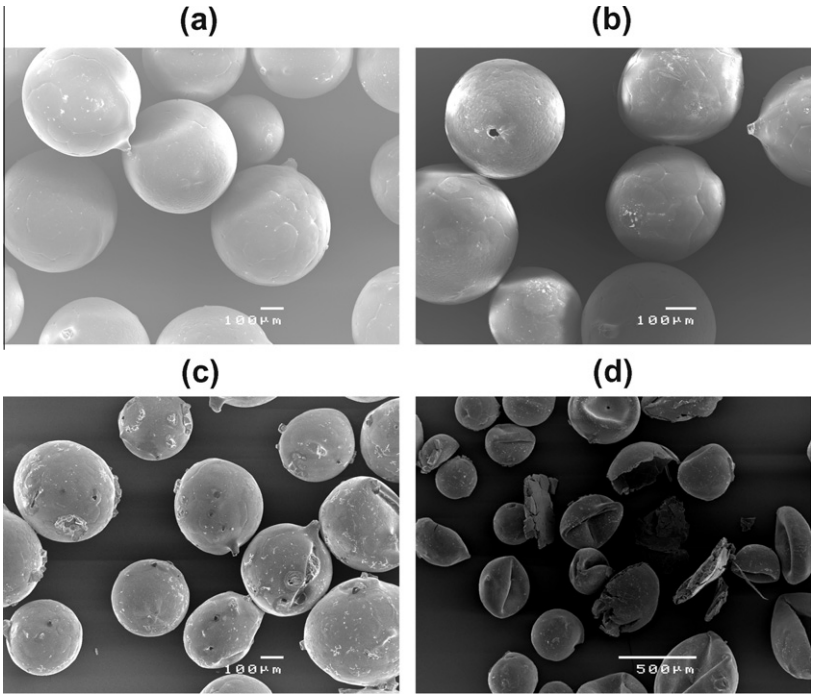

Fig. 4. SEM images of the PCL particles degraded with time in PBS solution. (a) 48 h, (b) 100 h, (c) 2 weeks and (d) 4 weeks.

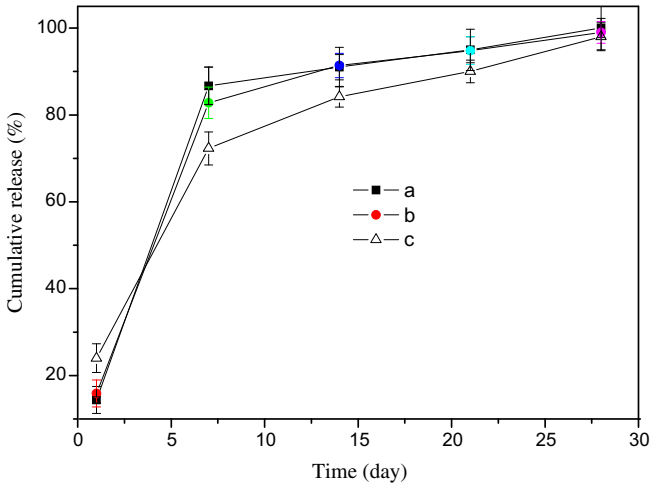

Fig. 5. Cumulative release of lysozyme from PCL particles. (a) Protein-loaded particles prepared under 3 g/100 ml PCL solution and 150 ml/h: 30 ml/h (organic solution to aqueous solution, the same below); (b) 3 g/100 ml PCL solution and 150 ml/h: 50 ml/h; (c) 2 g/100 ml PCL solution and 150 ml/h: 30 ml/h. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

a remarkable effect on the release of the resultant particles. In contrast, particles prepared under exactly the same flow conditions but at a lower PCL concentration have a lower release rate in the first stage of release, i.e. release by bulk diffusion, as seen in Curve c. A possible explanation for this phenomenon is that particles prepared at lower PCL concentrations have a smoother surface as shown in Fig. 3. As the particles are formed by solute precipitation, it is highly likely that particles with a smooth surface also have a much less porous wall, inhibiting the bulk diffusion of lysozyme through it.

Similarity between the curves can further be tested by the similarity factor, f_2, adopted by FDA, which can be calculated using the following equation:

$$F_2 = 50 \times log\left\{\left[1 + \frac{1}{n}\sum_{t=1}^{n}(R_t - T_t)^2\right]^{-0.5} \times 100\right\} \quad (2)$$

where n is the number of time points, R_t is the dissolution value of a reference product and T_t is the dissolution value for the test product at time t.

If f_2 is between 50 and 100, the two profiles are considered to be similar and bioequivalent [41,42]. The f_2 between Curves a and b is 79.9, and that between Curves a and c is 49.1. From this, we can conclude that the dissolution profiles of the two samples made under different flow conditions (Samples a and b) can be considered similar, while the two samples made at different PCL concentrations but the same flow condition different.

The release data can also be analyzed using the Korsmeyer equation to determine the release mechanism:

$$\frac{M_t}{M_\infty} = Kt^n \quad (3)$$

where M_t/M_∞ is the protein fraction released at time t [43], K is the Korsmeyer constant and n is a parameter that defines the release mechanism. The n values for Curves a, b and c, obtained by linear regression analysis, are 0.59, 0.56 and 0.53, respectively. Judged by the n values (between 0.5 and 1.0), the release mechanism of the proteins from the polymer microspheres is non-Fickian diffusion.

4. Conclusion

In this study, a simple method developed in our laboratory has been employed successfully to encapsulate lysozyme inside polycaprolactone (PCL) particles. Particles prepared by this method are shown to be monodisperse in size, which is more desirable for controlled release. The mean particle size can be tuned by either the flow conditions or the PCL concentration of in the organic solution. As well as the mean particle size, PCL concentration also influences the wall porosity and surface morphology of the polymer particles and, consequently, the release behavior of the content. The higher

the PCL concentration, the rougher the surface of the particles obtained and faster release. The method is also shown to be capable of efficiently encapsulate proteins, as high as 79% of encapsulation efficiency has been achieved. Results on the biodegradation of the polymer and the release of the protein from the polymer particles suggest that the predominant release mechanism for the protein release is bulk diffusion through the micropores of the polymer walls. Initial trials also show that the method presented in this paper can be applied to encapsulate solid particles and gas bubbles. These are currently under way.

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