Biological Activity of Lysozyme After Entrapment in Poly (d,l-lactide-co-glycolide)-Microspheres

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Received April 1, 1997; accepted August 5, 1997

Purpose. The purpose of this study was to investigate the process of preparing microspheres for maximising entrapment efficiency (EE) and retained biological activity (RBA) of peptides and proteins.

Methods. A controlled-release formulation based on poly(d,l-lactide-co-glycolide) was designed and produced using a small-scale double emulsion method. These PLG microspheres contained a model peptide, lysozyme. The retained bioactivity of the incorporated lysozyme was determined by bacterial assay. The size distributions and the morphology of the microspheres were characterized.

Results. The RBA and EE improved when the PLG concentration in the organic phase of the emulsion was increased. A high lysozyme concentration in the inner water phase of the emulsion resulted in decreased EE and an increase in the proportion of fragmented particles. The RBA of lysozyme in the microspheres varied between 30 and 80% with changes to the process.

Conclusions. The study shows that the RBA of lysozyme in PLG microspheres is strongly dependent on the experimental conditions for preparing the microspheres. Measurement of the EE alone, without the RBA is insufficient to evaluate the efficacy of the designed delivery system.

KEY WORDS: poly (d,l-lactide-co-glycolide); biodegradable microspheres; lysozyme; entrapment; enzyme; activity.

INTRODUCTION

In recent years, a large number of studies have investigated biodegradable polymer material as a drug carrier in various drug delivery systems. An increasing number of biological (peptides, proteins, antigens, etc.) and nonbiological drugs have been considered for incorporation into biodegradable microspheres.

There were several reasons for choosing the biodegradable PLG-polymer microspheres for this investigation. Firstly the material is biocompatible and degrades in vivo by forming the non-toxic monomers, lactic acid and glycolic acid. Secondly, drug carriers made from this material protect sensitive drugs or peptides against damaging environmental conditions which could cause pH or enzymatic degradation, especially after oral intake of the drug. Furthermore the release rate of the entrapped peptide can be controlled by varying the molecular weight of the carrier and the copolymer ratio (1–3).

Since a large number of recombinant proteins and synthetic peptides have been developed as potential therapeutic agents, it is of great interest to estimate the effect of the incorporation processes on the biological activity of the entrapped substance during the process of entrapment in the drug carrier (4,5).

The three-dimensional structure of proteins and peptides is an essential component of their biological activity. The tertiary structure of a protein is particularly sensitive. A disturbance in the linkages holding the tertiary structure in place could lead to deformation and loss of the biological activity of the protein.

The preparation of biodegradable microspheres requires the use of a volatile organic solvent such as dichloromethane (CH_2Cl_2; MC) (3). This solvent appears to interact with the hydrophobic parts of polypeptide chains, thereby reducing the biological activity of the peptide or protein during incorporation into the microspheres. A total loss of activity has been reported in some cases (6–8).

An alternative incorporation process for sensitive and water soluble drugs uses the double emulsion method in order to decrease contact between the organic phase and the active substance and increase the entrapment efficiency (EE) of water soluble drugs. This preparation method could also be used for the incorporation of unusual or one-off substances into microspheres since it may be performed on a small scale (3,9).

Briefly, the drug (peptide) is initially dissolved in a small aqueous aliquot, which is subsequently emulsified with the organic PLG solution to form a water-in-oil (w/o) emulsion. This emulsion is added to an aqueous solution and a second emulsification then takes place, resulting in a water-in-oil-in-water (w/o/w) double emulsion. The organic solvent is removed to leave an aqueous suspension of microspheres containing the drug.

The PLG microspheres used in this study were designed to a controlled-release formulation based on poly (d,l-lactide-co-glycolide).

Lysozyme was chosen as the model peptide because enzymes are highly dependent on their tertiary structure for keeping their biological activity, the enzyme activity of lysozyme is relatively simple to measure and lysozyme is a well known and characterised enzyme that has been used in other microsphere incorporation studies (5).

Lysozyme is a relatively small enzyme (14.6 kd). It preferentially hydrolyses the β-1,4 glucosidic linkages between N-acetylemuramic acid and N-acetylglucosamine which occur in the mucopeptide cell wall structure of certain micro-organisms. The rate of lysis of this specific linkage in the cell walls of micro-organisms can be measured and is known as the specific biological activity of lysozyme (10,11).

In this paper we have tried to evaluate how the process of microencapsulation can be optimised to achieve the maximum degree of retained biological activity (RBA) while increasing EE during the preparation process.

We have also shown how the particle size distribution and the morphology of the microspheres varies with changes in experimental parameters.

MATERIALS AND METHODS

Materials

Poly (d,l-lactide-co-glycolide): copolymer composition 50:50 (PLG), Resomer RG 502 H (inherent viscosity approximately 0.2) was supplied by Boehringer Ingelheim, Germany.

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Poly vinyl alcohol (13–23 K Da, 87–89 hydrolysed)(PVA) was obtained from Aldrich, USA. Dichloromethane (MC), acetone and dimethylsulfoxide (DMSO) of analytical grade were supplied by Merck, Germany. The NanoOrange protein quantitation kit (N-6666) was obtained from Molecular probes, Netherlands. Lysozyme from chicken egg white and *Micrococcus lysodeikticus* cells were supplied by Sigma, USA. All chemicals were used without further purification.

Microsphere Preparation

A solution of 100 μl lysozyme (1.2, 6, 20 and 30% w/w) in Milli-Q water (internal aqueous phase) was emulsified with a 1 ml solution of PLG (4.5 and 37% w/w) in dichloromethane (organic phase) using a polytron homogenizer (Kinematica AG PT 300) at high speed (approximately 15000 rpm for 10 seconds).

The resulting w/o emulsion was then dispersed in 1 ml of 10% w/v PVA solution (external aqueous phase) using a vortex mixer (Whirlmixer) for 5 seconds to produce a w/o/w emulsion.

The solvents in the organic phase containing PLG concentrations of 4.5 and 37% consisted of acetone and MC at different ratios (0:1, 1:1, 2:1). These preparations were used to study the effects of increasing the rate of solvent removal on microsphere characteristics.

The double emulsion was poured into 5 ml of 10% PVA aqueous solution, and stirred magnetically for 15–18 hrs at room temperature to allow solvent (MC) evaporation and microsphere formation. The microspheres were isolated by centrifugation (Eppendorf Centrifuge 5403) at 3000 rpm for 10 min., washed in water either three or five times depending on the amount of microspheres, and freeze-dried (Super Modulyo, Edwards). The final dried microspheres were stored in a desiccator at room temperature.

Size Distribution

The freeze-dried microspheres were dispersed in filtered (0.22 μm filter) 0.9% w/w NaCl. Volume size distributions were obtained using a multisizer (Coulter, Multisizer II). The results are presented as mean values with standard deviations from measurements of samples from at least three different batches.

Morphology of Microspheres

The external and internal morphology of the preparations were analysed using scanning electron microscopy (SEM). Carefully dried microspheres were cross-sectioned with a razor blade. Surfaces and cross-sections of microspheres were coated with gold—palladium (metallization) under an argon atmosphere at room temperature. The external and internal morphologies were then examined (Jeol—JSM—T 330 Scanning microscope).

Entrapment Efficiency

PLG microspheres (10 mg) were dissolved in 0.8 ml DMSO. A volume of 2.4 ml 0.001 M HCl was added to the solution. The fluorescence intensity of the solution was measured using a fluormeter analyser (Bio-Tek Instruments, FL).

This was compared with the fluorescence intensity obtained in a previously made standard curve for lysozyme. The amount of lysozyme trapped in the microspheres was then calculated. The limits of this protein assay method range from 10 ng to 10 μg protein/ml, which is a suitable range for detection of small amounts of proteins. The entrapment efficiency (%) was calculated by:

$$\% \text{ EE} = \frac{\text{measured amount of lysozyme per batch microspheres}}{\text{amount of lysozyme initially added to the internal aqueous phase}} \times 100$$

Biological Activity Assay

The rate of lysis of *Micrococcus lysodeikticus* cells by lysozyme was used to estimate the of biological activity of the enzyme.

A volume of 2.9 ml of *Micrococcus lysodeikticus* cells in suspension was pipetted into a cuvette and incubated for 4–5 minutes to establish the baseline and achieve temperature equilibrium. An aliquot of 0.1 ml of lysozyme solution from the entrapment efficiency test was added to the cuvette and the change in A_{450} per minute from the initial linear portion of the curve was recorded over 5 minutes. Enzyme activity was deduced from the slope of the curve. Since the concentration of lysozyme was known, the percentage retained biological activity of lysozyme was calculated by:

$$\% \text{ RBA} = \frac{(DA_{450}/\text{minute}) \text{ apparent}}{(DA_{450}/\text{minute}) \text{ theoretical}} \times 100$$

The theoretical value was taken as the enzymatic activity of 0.1 ml of a freshly prepared lysozyme solution (1.2, 6, 20 or 30% w/w). During our study we observed that DMSO had no effect on the biological activity of lysozyme.

RESULTS AND DISCUSSION

Morphology of Microspheres

Representative SEM pictures of the external surfaces of PLG microspheres are shown in Figure 1. Examination of the external morphology showed that all prepared microspheres were spherical in shape with very few pores on the external surfaces. The number of pores on the external surfaces of the microspheres was increased with higher concentrations of lysozyme in the inner water phase while the PLG concentration was kept constant (Figure 2). The number of fragmented particles increased when acetone was used in the organic phase (Figure 3).

Particle Size Distribution

Table I shows how the particle size distributions were affected with different concentrations of PLG in the organic phase and lysozyme in the internal water phase. The particle size increased with increasing polymer concentration. The results are in agreement with earlier findings using a similar method of preparation (9,12). The median diameter of microspheres prepared at a PLG concentration of 37% was about twice that of microspheres prepared with 4.5% PLG. This was also theoretically expected since the volume of a sphere is

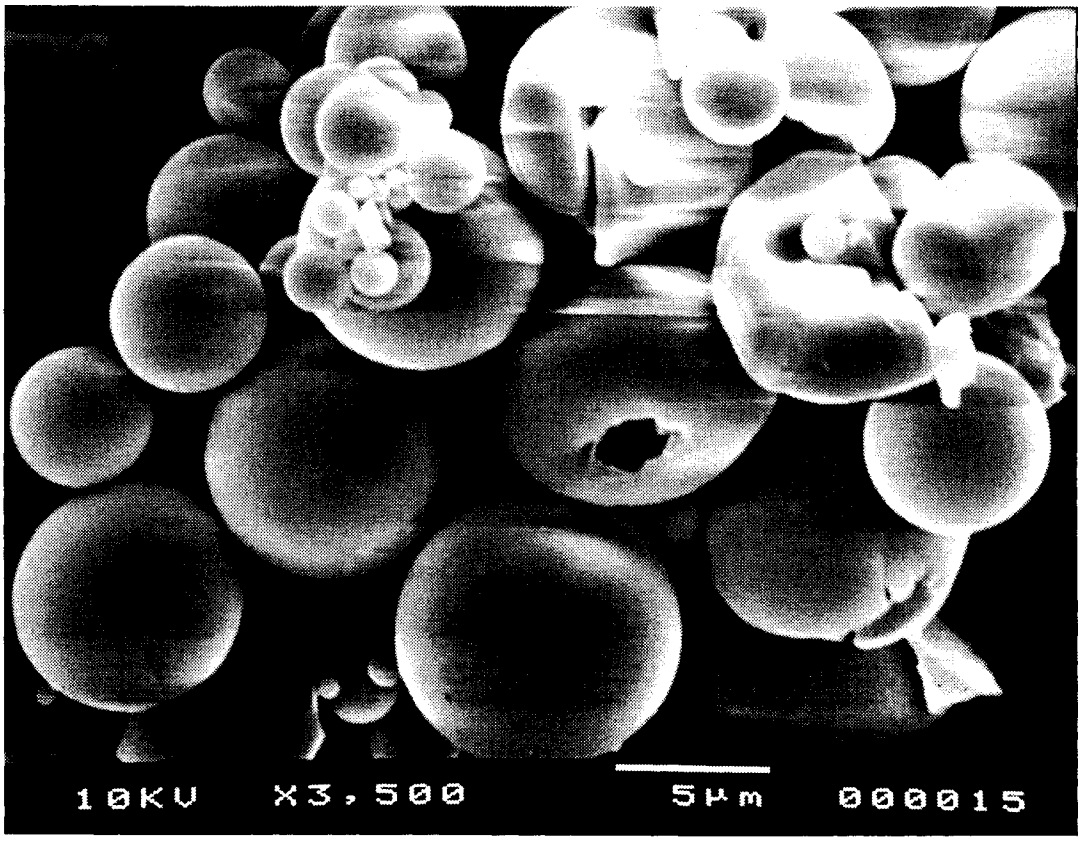

Fig. 1. Representative SEM picture of the external surface of PLG microspheres prepared using 4.5% PLG in the organic phase and 1.2% lysozyme in the internal aqueous phase.

proportional to the third power of its diameter. Changing the concentration of lysozyme in the internal aqueous phases had no effect on the size distributions of the microspheres prepared with 4.5% PLG. However increasing the lysozyme concentration from 1.2 to 6.0% for microspheres prepared with 37% PLG increased the mean diameter by almost 30%. Furthermore the external porosity of the microspheres increased with increasing concentrations of lysozyme.

The mean size of porous particles will be underestimated when coulter multisizer equipment is used. The pores in the particles will be occupied by the measuring medium (electrolyte solution), hence reducing the signal obtained from the electrical resistance of the solid content of the particles. Examination of the SEM pictures taken of the porous microspheres prepared with 37% PLG at lysozyme concentrations above 6% revealed that the actual sphere size was around 20 μm.

In general, a lysozyme concentration higher than 6% causes increases in the proportion of fragmented microspheres. This fragmentation may be the result of crystallisation of lysozyme either during the solidification process or during freeze drying.

Table II shows that the mean particle size was unaffected by the addition of higher proportions of acetone in the organic phase. The effect of the addition of acetone was predominately an increase in the solidification rate of the microspheres caused by the rapid extraction of acetone into the external aqueous phase.

Entrapment Efficiency (EE)

Increasing the PLG concentration in the organic phase led to increases in the entrapment efficiency of lysozyme. It was also found that the EE decreased with increasing concentrations of lysozyme in the inner aqueous phase. The results are shown in Figure 4.

As the polymer concentration in the organic phase was increased, the EE increased more than tenfold. This is in agreement with earlier studies on the entrapment of mannitol in PLG microspheres (9).

The SEM pictures showed an increase in the proportion of fragmented particles and microspheres when the lysozyme concentration was increased. This may explain the decrease in EE at higher lysozyme concentrations.

Most of the free unentrapped lysozyme was recovered from the external aqueous phase of the double emulsion. Thus the dominating route for loss of lysozyme is assumed to be the result of transport of droplets from the internal aqueous phase to the external aqueous phase. As the double emulsion is formed, progressive loss of the organic solvent eventually leads to solidification of microspheres and the cessation of this route of droplet transport. With an increase in the PLG concentration in the organic phase, the time to reach solidification will be decreased. The increased viscosity of the organic phase caused by the increased PLG concentration will also decrease the loss of lysozyme by this route and contribute to the enhanced entrapment efficiency. The time to reach solidification of the micro-

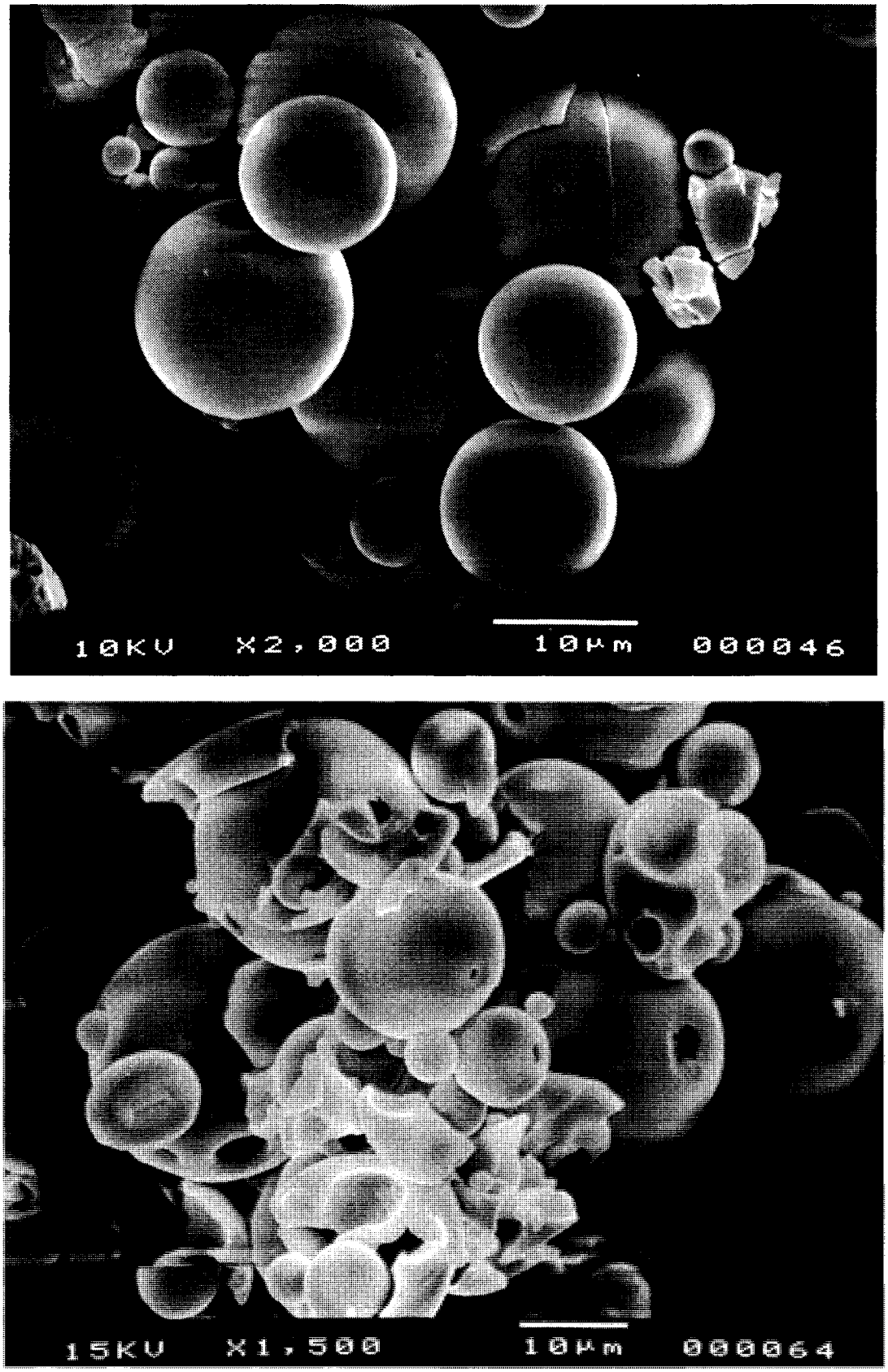

Fig. 2. SEM pictures of PLG microspheres prepared using 37% PLG in the organic phase and (A) 1.2%; (B) 30% lysozyme in the internal aqueous phase.

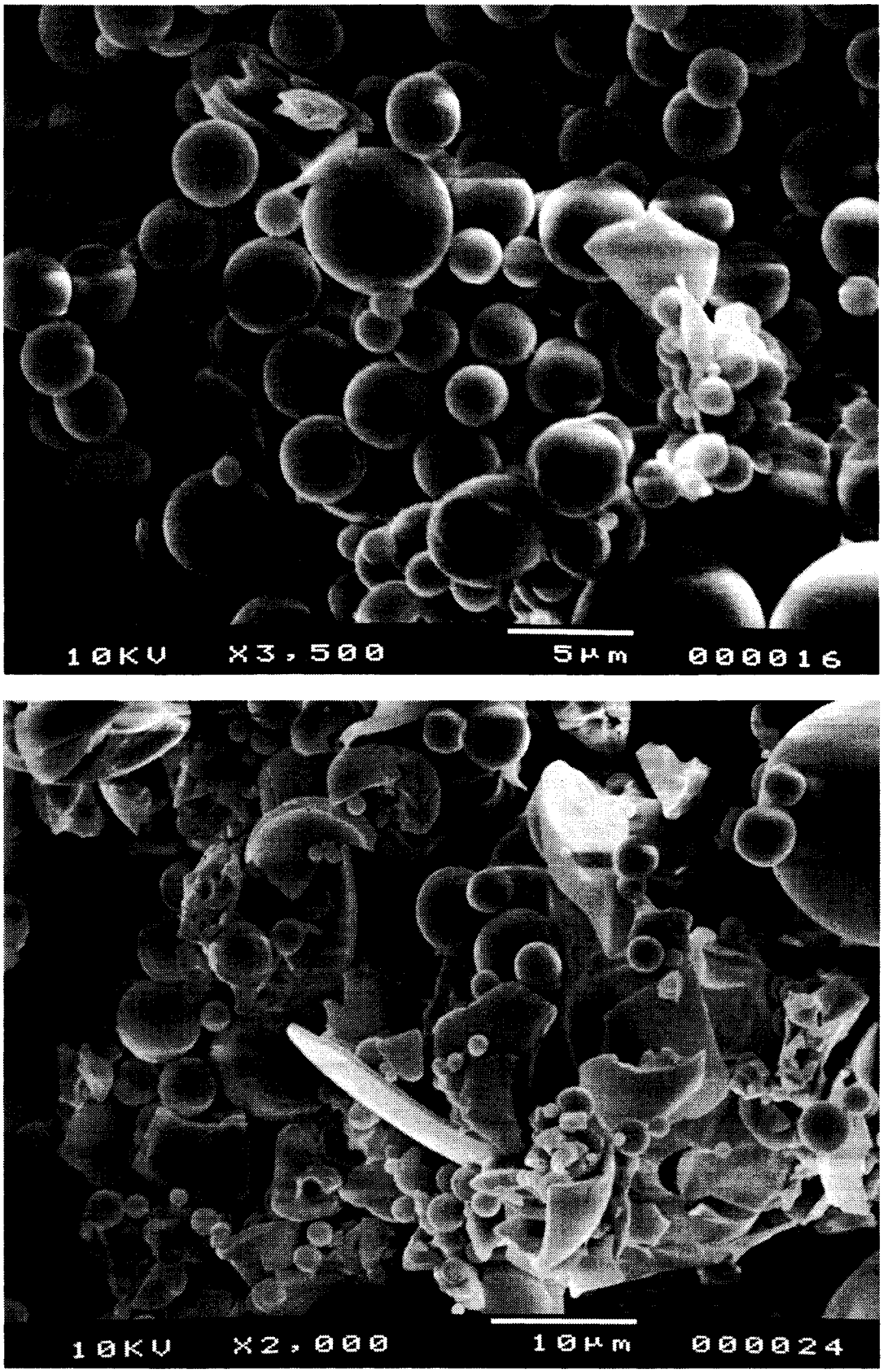

Fig. 3. SEM pictures of lysozyme-loaded microspheres prepared under the same processing conditions (4.5% PLG in the organic phase and 1.2% lysozyme in the internal aqueous phase) but using different proportions of acetone : dichloromethane (A) 1:1; (B) 2:1 in the organic phase.

Table I. Particle Size Distributions Obtained for Microspheres Prepared with 4.5 or 30% PLG in the Oil Phase and Varying Lysozyme Concentrations in the Internal Aqueous Phase

Lysozyme concentration % w/v	Diameter[a] (μm) of microspheres					
	4.5% w/w PLG			37% w/w PLG		
	10	50	90	10	50	90
1.2	16(2)	9.6(1)	4.9(0.8)	22(3)	15(2)	7.8(0.9)
6.0	17(3)	9.7(1)	4.9(0.4)	28(2)	20(0.8)	9.5(2)
20	19(0.4)	10(0.3)	5.0(0.4)	19(0.7)	11(0.6)	5.6(0.3)
30	17(1)	9.1(0.9)	4.6(0.5)	23(2)	14(1)	6.5(0.7)

Note: The table shows the percentage of the batch that was composed of microspheres with a diameter greater than that indicated.
[a] Standard deviation given in brackets.

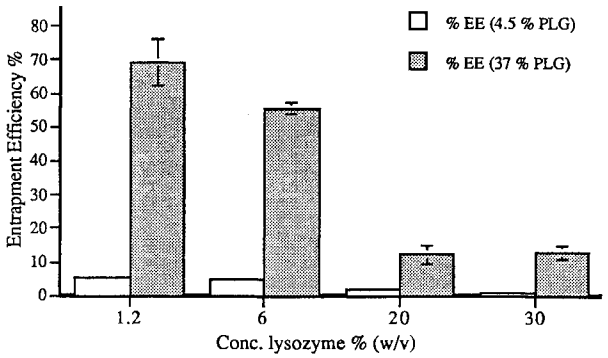

Fig. 4. The entrapment efficiency of lysozyme in microspheres prepared with 4.5 or 37% PLG at differing concentrations of lysozyme.

spheres is therefore critical for the entrapment efficiency of the double emulsion method. An increase in the solidification rate, by the addition of acetone to the organic phase, improved the EE in some cases. However, if the rate of solidification is too high, increased fragmentation of microspheres can result in decreased EE (see Table III).

Previous studies have also shown that a high rate of solvent removal may result in increased porosity of the microspheres.(13,14).

Retained Biological Activity (RBA)

When the PLG concentration was increased from 4.5 to 37% and a high concentration of lysozyme (30%) was used, the RBA of the entrapped lysozyme was increased from 59 to 83%. The improvement was probably caused by the higher rate of solidification of the microspheres as a result of the higher PLG concentration (see above).

Thus the encapsulated lysozyme would be protected from MC, which we found to have the greatest influence on the biological activity of lysozyme, in the organic phase. The longer the contact time between MC and lysozyme, the more enzyme activity was lost. In other words, a higher solidification rate would benefit the RBA of the enzyme provided it did not negatively affect the formation of the particles.

Table II. The Mean Particle Size Distribution of PLG Microspheres Prepared with Varying Proportions of the Organic Phase Components

Ratio[a] Acetone: CH_2Cl_2	% w/w PLG	Diameter[b] (μm) of microspheres		
		10	50	90
0:1	4.5	16(3)	9.6(2)	4.9(0.8)
1:1	4.5	14(0.3)	7.7(0.8)	3.4(0.1)
2:1	4.5	23(2)	12 (2)	5.2(0.7)
0:1	37	22(3)	15 (2)	7.8(0.9)
1:1	37	21(2)	12.7(1)	7.2(0.7)
2:1	37	22(3)	12.3(0.9)	6.9(0.3)

Note: The internal aqueous phase of the microspheres contained 1.2% lysozyme. The table shows the percentage of the batch that was composed of microspheres with a diameter greater than that indicated.
[a] Ratio of Acetone:CH_2Cl_2 within the organic phase.
[b] Standard deviation given in brackets.

We have checked this hypothesis by employing a method of increasing the rate of solidification (i.e. addition of acetone to the organic phase). Since acetone has a higher solubility in water than MC, the solidification rate will be increased. We observed, however, that when a high fraction of acetone was added to the organic phase it no longer favored a high RBA. This may have been caused by disturbed particle formation since, from SEM observations, the proportion of fragmented microspheres was increased, suggesting decreased protection for the lysozyme from the organic solvent.

It should be kept in mind, however, that the RBA is not the same as the EE for biologically active substances. The fraction of enzyme bound to, or recovered from, the surface of the microspheres was biologically less active than the fraction entrapped inside the microspheres. The EE value relates to the whole amount of lysozyme entrapped in or bound onto the surfaces of the microspheres. In fact, the RBA we observed in the washing water of the PLG microspheres was almost the same (30%) as that seen in a lysozyme solution mixed with an adequate volume of MC. To obtain an accurate measure of the biological activity of prepared microspheres containing peptide/protein it is therefore recommended that the EE is multiplied

Table III. The Entrapment Efficiency (EE) and Retained Biological Activity (RBA) of Lysozyme Entrapped in PLG Microspheres Prepared by Different Methods

Concentration		% RBA[d]	% EE	% EE × RBA	% MF[e] × RBA
% PLG (w/w)	% Lysoz. (w/v)				
4.5[a]	1.2	59 (0.5)	5(1)	3.0 (0.7)	0.06(0.02)
4.5[b]	1.2	72 (12)	14(2)	10 (2.3)	0.21(0.05)
4.5[c]	1.2	32 (8.5)	4(0.1)	1.3 (0.4)	0.03(0.01)
4.5[a]	30	55 (5.0)		0.34(0.1)	0.17(0.07)
37[a]	1.2	63 (4.0)	61(8)	38 (2.4)	0.09(0.01)
37[b]	1.2	62.9(0.6)	27(5)	17 (3)	0.04(0.01)
37[c]	1.2	38.1(1.1)	6(1)	2.4 (1)	0.01(0.00)
37[a]	30	83 (10)	12(6)	11 (6.4)	0.65(0.39)

[a] 0:1 acetone:CH_2Cl_2.
[b] 1:1 acetone:CH_2Cl_2.
[c] 2:1 acetone:CH_2Cl_2.
[d] Standard deviation given in brackets.
[e] Mass Fraction (lysozyme/lysozyme + PLG).

by the RBA. This would result in a more reliable measure of the efficiency of the incorporation process.

From Table III it may be seen that when a high concentration of PLG was used in the organic phase while keeping the lysozyme concentration low in the internal aqueous phase of the emulsion, 38% bioactive lysozyme was recovered from the resulting microspheres. When a less favourable incorporation process was selected, only 0.3% bioactive lysozyme was recovered.

The mass fraction of bioactive lysozyme (MF × RBA), a measure of the potency of microspheres, is shown in Table III. These values are useful when determining the amount of microspheres necessary to achieve a desired dose of the bioactive peptide. From Table III it may be seen that employing a high PLG concentration in the organic phase and a high lysozyme concentration in the inner aqueous phase of the emulsion yields the preparation which requires the least amount of microspheres to achieve a high dose of bioactive lysozyme.

CONCLUSIONS

This study shows how important it is to optimise the process of manufacturing microspheres in order to give the peptide maximum protection against chemical degradation during the preparation process. It is not sufficient to ascertain the value of the EE alone for biological substances (peptides, proteins, antigens, etc.) entrapped in a controlled release system. Both the EE and the RBA should be determined for these systems. It was shown that between 0.3 and 38% of the initially loaded bioactive lysozyme was recovered from PLG microspheres depending on simple variations in the process parameters. A reliable evaluation of these drug delivery systems is gained by multiplying the RBA with the EE to obtain the biological entrapment efficiency (BEE).

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