A Polysorbate-Based Non-Ionic Surfactant Can Modulate Loading and Release of β-Lactoglobulin Entrapped in Multiphase Poly(DL-Lactide-co-glycolide) Microspheres

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Purpose. The goal of the present paper was to investigate the role of a surfactant, Tween 20, in the modulation of the entrapment and release of β-lactoglobulin (BLG) from poly (DL-lactide-co-glycolide) microspheres.

Methods. Poly(DL-lactide-co-glycolide) microspheres containing BLG were prepared by a water-in-oil-in-water emulsion solvent procedure. Tween 20 was used as a surfactant in the internal aqueous phase of the primary emulsion. BLG entrapment efficiency and burst release were determined. Displacement of BLG from microsphere surface was followed by confocal microscopy observations and zeta potential measurements, whereas morphological changes were observed by freeze-fracture electron microscopy.

Results. Tween 20 was shown to increase 2.8 fold the encapsulation efficiency of BLG without any modification of the stability of the first emulsion and the viscosity of the internal aqueous phase. In fact, Tween 20 was shown to be responsible for removing the BLG molecules that were adsorbed on the particle surface or very close to the surface as shown by confocal microscopy and zeta potential measurements. Tween 20 reduced the number of aqueous channels between the internal aqueous droplets as well as those communications with the external medium. Thus, the more dense structure of BLG microspheres could explain the decrease of the burst release.

Conclusions. These results constitute a step forward in the improvement of existing technology in controlling protein encapsulation and delivery from microspheres prepared by the multiple emulsion solvent evaporation method.

KEY WORDS: biodegradable microspheres; poly(DL-lactide-co-glycolide); β-lactoglobulin; Tween 20; encapsulation; protein aggregation.

INTRODUCTION

Poly(DL-lactide-co-glycolide) (PLGA) microspheres have been studied extensively as proteins/peptides delivery systems. The biodegradable and biocompatible nature of PLGA (1) makes this polymer a suitable candidate, among other applications, for the systemic and oral administration of proteins. Various studies have demonstrated an enhanced serum and/or secretory antibody response following parenteral or oral administration of antigens entrapped into microspheres (2–4).

One of the most successful methods for the encapsulation of proteins and peptides is the double emulsion/solvent evaporation technique (5). Microspheres prepared by this technique have been proposed by many authors for loading proteins and peptides (6,7). Because of their high water solubility, these molecules are introduced in the internal aqueous phase of the multiple emulsion. This procedure results in an increased encapsulation efficiency of the microspheres as compared to particles produced by the single emulsion/solvent evaporation method (7). Formulation parameters considered in the development of such a procedure are protein and polymer concentrations (8), nature of the solvents used (9), ratio of solvent to aqueous solution, homogenization conditions of the primary and multiple emulsion (10–12,) and pH of the external aqueous phase (13).

During the preparation of microspheres by the multiple emulsion solvent evaporation method, proteins display amphiphatic properties and accumulate at the interface of the droplets of both the first and the second emulsion. This particular location of proteins may induce a stabilizing effect of the two emulsions that, in turn, contributes to a successful stabilization of the multiple emulsion (14–16). However, in the case of hydrophilic proteins, the partitioning into the external aqueous phase may lead to a low entrapment efficiency (<10%) (17). This transfer could occur during the formation of the multiple emulsion and may be due to shearing forces that induce coalescence of internal globules. Therefore, the loss of the protein in the external aqueous phase during the preparation of the microspheres is a major drawback during the process of preparation. In addition, because of their particular location in microspheres, proteins are generally released very quickly in the external media displaying the so-called burst release effect.

In this study, we have investigated the effect of a non-ionic surfactant that is able to compete with a protein on its accumulation at the interfaces. To address this question, the model protein, β-lactoglobulin (BLG), known to adsorb at oil/water interfaces (18) has been used. BLG was previously shown to compete at this interface with non-ionic surfactants such as Tween 20 (19). Therefore, Tween 20 was used with the purpose of increasing entrapment efficiency and reducing the burst release effect of BLG from PLGA microspheres.

MATERIALS AND METHODS

Materials

Poly(DL-lactide-co-glycolide) (copolymer composition 75:25; MW 19,500) was supplied by Birmingham Polymers (USA). Bovine β-lactoglobulin (BLG) (MW 18,400), three times crystallized and freeze-dried, Tris (hydroxymethyl) aminomethane (TRIS), Pluronic® F68 (MW 8,500) and Tween 20 (polyoxyethylene (20) sorbitan monolaurate) (MW 1,230) were obtained from Sigma (France). Methylene chloride (MC) from Prolabo (France) was used without further purification. Poly(vinyl alcohol) (PVA, Mowiol® 40–88, mean MW 127 000) was purchased from Aldrich Chemical (France).

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Sodium dodecyl sulfate (SDS) and Bio-Rad DC (Detergent Compatible) Protein Assay were supplied by Bio-Rad (France). 1,2 dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) was obtained from Avanti Polar-Lipids (USA). Fluorescein labeling kit was supplied by Boehringer Mannheim Biochemica (Germany) and MicroSpin™ G-25 Columns by Pharmacia Biotech (USA). Acrytol mounting medium was obtained from Surgipath Canada Inc (Canada). Aquamount mounting medium from BDH Chemicals Ltd. (England). 1,4-diazabicyclo-[2.2.2]octane (DABCO) was purchased from Sigma (France).

Microspheres Preparation

PLGA microspheres containing BLG were prepared by a W/O/W solvent evaporation technique as previously described (13). BLG was dissolved in 0.4 ml of TRIS buffer (20 mM, pH 7.5) at 50 mg/ml. When Tween 20 was introduced in the inner emulsion, its concentration ranged from 0 to 26 mg/ml. Others surfactants were used in different batches of microspheres as Pluronic® F68 (30 mg/ml) in the inner aqueous phase or 200 µl of DOPE in the organic phase (4 mg/ml). This aqueous solution was emulsified with 2.5 ml of MC (O) containing 250 mg of PLGA using an Ultraturrax® at 13,500 rpm for 2 min to form a primary W/O emulsion. This emulsion was then poured into 20 ml of a PVA/PBS solution (2% w/v, pH 6.5) and emulsified during 2 min at 13,500 rpm. The multiple W/O/W emulsion was stirred at 1,000 rpm for 3 hours at room temperature to allow solvent removal and microsphere formation. Microspheres were collected after centrifugation (4,000 rpm for 10 min), washed three times with distilled water, freeze-dried, and stored at 4°C.

Determination of Microsphere BLG Content

Protein content in microspheres was determined according to the method described by Hora *et al.* (20). Freeze-dried microspheres were dispersed in a 0.1 M NaOH solution containing SDS (1% w/v) to give a final concentration of 5.0 mg of particles/ml. The resulting suspension was kept under stirring at room temperature for 24 h. Samples were centrifuged (4,000 rpm for 5 min), and BLG concentration was measured in the supernatant by a Bio-Rad DC microassay. The encapsulation efficiency was expressed by the ratio between the actual and the theoretical BLG loading × 100.

Determination of Particle Size and Zeta Potential

Microsphere diameter and size distribution were measured using a Coulter Multisizer® II (Coultronics, France). For microsphere surface charge measurements, freeze-dried microspheres were introduced into a 10 ml glass vial and dispersed in distilled water. The zeta potential of the suspensions was determined using a Zetasizer 4 (Malvern, France), and the measurements were carried out at 25°C.

Stability of the Primary Emulsion

The stability of the primary emulsion was determined using the method described by Blanco *et al.* (15). In short, the primary emulsion, prepared as reported above, was stored in a 15 ml screw-capped tube fitted with a rubber septum. The time required for initial macroscopic phase separation (phase separation time) was determined at room temperature. The globule diameter of the inner aqueous phase was achieved using light microscopy immediately after the second emulsification (Olympus BH-2, France).

Rheological Analysis

The rheological analysis was performed at 20°C ± 1°C using a controlled stress rheometer Hacke® RS 100 (Rheo Champlan, France). The shear geometry was performed using a 3.5 cm diameter and a cone angle of 1 °C. The viscosity was measured for different solutions of BLG with and without surfactant (50 mg/ml of BLG and 0–26 mg/ml of Tween 20).

Fluorescent BLG-Loaded Microspheres

BLG was labeled using 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) by modifying aliphatic amines and forming stable carboxamide couplings. Non-reacted FLUOS was separated on a MicroSpin™ G-25 column. This procedure led to a molar ratio of FLUOS/BLG to about 0.6:1. Fluorescent BLG-loaded microspheres, whether or not containing Tween 20 (at a molar ratio 1:8) were prepared as described above.

Confocal Laser Scanning Microscopy

Specimens were mounted in Aquamount containing 100 mg/ml of DABCO (an antifading agent) and fixed with Acrytol. Confocal microscope analysis was carried out using the TSC 4D confocal imaging system (Leica instrument, Heidelberg, Germany), equipped with a 63× objective (plan apo; NA = 1.4). For the fluorescein derivative, an Argon-Krypton ion laser adjusted at 488 nm was used. The signal was treated with line averaging to integrate the signal collected over 8 lines in order to reduce noise. The confocal pinhole was adjusted to allow a field depth of about 0.5 µm corresponding to the increment between two adjacent sections. Focal series of up 8 sections were collected for each specimen. Micrographs were sent via Ethernet to a computer for processing with Adobe Photoshop® 4.0. Images were assembled and printed directly from the computer on dye sublimation printer (Colorease Kodak).

Freeze-Fracture Electron Microscopy

For freeze-fracture electron microscopy, a small drop of a pelleted aqueous suspension of microspheres was deposited on a thin copper planchette and rapidily frozen in liquid propane. Fracturing and replication were conducted with a Balzers BAF 301 freeze-etching unit with platinium-carbon shadowing. The replicas, after the digestion of organic material with methylene chloride and washing with alcohol, were observed using a Philips 410 electron microscope.

In Vitro Burst Release Study

For the burst release studies, freeze-dried microspheres corresponding to 0.7 mg of BLG were accurately weighed in test tubes and dispersed using a vortex in 4 ml of TRIS buffer (20 mM, pH 7.5) at 37°C. At 2 minutes after dispersion, the suspension was centrifuged (4,000 rpm for 5 min) and the BLG

Table 1. Effect of Tween 20 on Particle Size, Loading, and Encapsulation Efficiency

Protein:Tween 20 (molar ratio)	Average size (μm)	Loading efficiency (mg BLG/100 mg MS)	Encapsulation efficiency (%)
1:0	6.6	2.4	32
1:0.01	6.8	2.5	33
1:0.018	6.7	3.4	46
1:0.1	7.0	3.4	46
1:0.5	6.9	3.7	50
1:1	7.0	4.1	55
1:4	7.2	5.9	80
1:8	6.2	6.7	91

Note: Theoretical loading was 7.4 mg BLG/100 mg MS, (n = 3).

concentration was determined in the supernatant by a Bio-Rad DC protein microassay.

Adsorption of BLG onto Unloaded Microspheres

25 mg of unloaded freeze-dried microspheres prepared with or without Tween 20 (26 mg/ml of inner aqueous phase) were placed into test tubes in 2 ml of 2% PVA/PBS (w/v, pH 6.5 or pH 5.6) containing 8 mg of BLG. The suspension was magnetically stirred for 4 hours at room temperature. At selected intervals (0.25, 0.5, 1, 2, 3, and 4 h), one tube was removed and the microspheres were collected by centrifugation, washed three times with double-distilled water and freeze-dried. The amount of BLG absorbed onto the microspheres was determined as described above.

RESULTS AND DISCUSSION

BLG-loaded microparticles were prepared at concentration of 50 mg of BLG /ml and various concentrations of Tween 20 (molar ratio: protein/surfactant of 0 to 8). Average particle size, protein loading, and entrapment efficiency were determined (Table 1). All batches of BLG microspheres displayed a mean diameter from 6.2 to 7.2 μm. The Tween 20 did not modify microsphere size characteristics. However, the amount of surfactant affected both loading and encapsulation efficiency. The introduction of Tween 20 in the inner aqueous phase improved the encapsulation of BLG. Nevertheless, the protein entrapment efficiency was improved only from a molar ratio of protein/Tween 20 of 1:0.018. Under this ratio, the surfactant is below its critical micellar concentration (CMC: 0.06 mg/ml). In addition, the aqueous solubility of the BLG in the presence of Tween 20 (at 25°C and at a molar ratio protein/Tween 20 of 1:8) increased by 25%.

The solubilization of BLG by micelles formed within the inner aqueous phase may be responsible for reducing the protein to protein or protein to polymer interactions. Consequently, this process could increase the amount of protein located within the aqueous globules. Moreover, the formation of micelles makes possible an homogeneous distribution of BLG into microspheres.

Tween 20 also induced the formation of more homogeneous internal aqueous globules than those obtained in the absence of surfactant (Table 2 and Fig. 2). On the other hand, confocal microscopy examinations (Fig. 1) showed clearly that in the presence of Tween 20 (at a molar ratio protein/surfactant of 1:8), BLG was located inside the internal globules in a very homogeneous fashion as compared to microspheres that do not contain the surfactant. In addition, freeze-fracture micrographs revealed coalescence and connections of internal aqueous globules as well as the existence of aqueous connections with the external aqueous medium (Fig. 2A). Such coalescence and connections were not observed for samples prepared using high Tween 20:protein ratio (8:1) (Fig. 2B).

In order to investigate other parameters involved in the improvement of encapsulation by Tween 20, the stability and viscosity of the emulsion were characterized. Stability of the inner emulsion was shown by several authors to be a fundamental prerequisite for the successful entrapment of hydrophilic compounds in microspheres prepared by the double emulsion technique (16,21,22). Thus, the stability of inner emulsions, whether or not they contained Tween 20, was measured by determination of the phase separation time of the emulsion. The role of different types of surfactants was investigated. Pluronic® F68 induced a weaker stability of the inner emulsion. Other surfactants tested (Tween 20 and DOPE) did not seem to improve the stability of the emulsion, suggesting in our experimental conditions, there was no correlation between the stability of the inner emulsion and the encapsulation efficiency (Table 2). In addition, at all molar ratios (BLG/Tween 20) used in this study, the viscosity of the primary emulsion was not significantly changed by the presence of Tween 20 (1.44 ± 0.06 mPa.sec).

Since Tween 20 has an HLB (Hydrophilic-Lipophilic Balance) of 15, it has a strong tendency to stabilize O/W emulsions. Therefore, during the process of microsphere preparation, a part of the surfactant could migrate to the interface between oil and the outer aqueous phase modifying the surface properties of the microspheres. *In vitro* release experiments with different molar ratios of BLG/Tween 20 showed that microspheres prepared in the absence of Tween 20 displayed the highest burst effect (23%). As the amount of Tween 20 was increased the burst release was reduced: at a molar ratio of 1:4, the burst effect was 16% and at 1:8, only 1% (Fig. 3). The large burst effect observed for the microspheres without Tween 20 could be explained by the presence of a high amount of BLG onto microsphere surface (Fig. 1A) and also from its release through pores and channels identified within microspheres (Fig. 2A).

Table 2. Stability of the Inner Emulsion Containing or Not Surfactants, Globule Diameter of the Inner Aqueous Phase and the Resulting Encapsulation Efficiency

Surfactant (mg/ml)	Phase separation time (minutes)	Globule diameter (μm)	Encapsulation efficiency (%)
None	180	1–10 (100% < 10)	32
Tween 20 (26 mg/ml)	180	1–10 (90% < 5)	91
Pluronic F68 (30 mg/ml)	120	1–10 (100% < 10)	24
DOPE (4 mg/ml)	180	1–10 (100% < 10)	31

Note: BLG concentration was 50 mg/ml (lower concentration of DOPE was used because of its solubility). Theoretical loading was 7.4 mg BLG/100 mg MS, (n = 3).

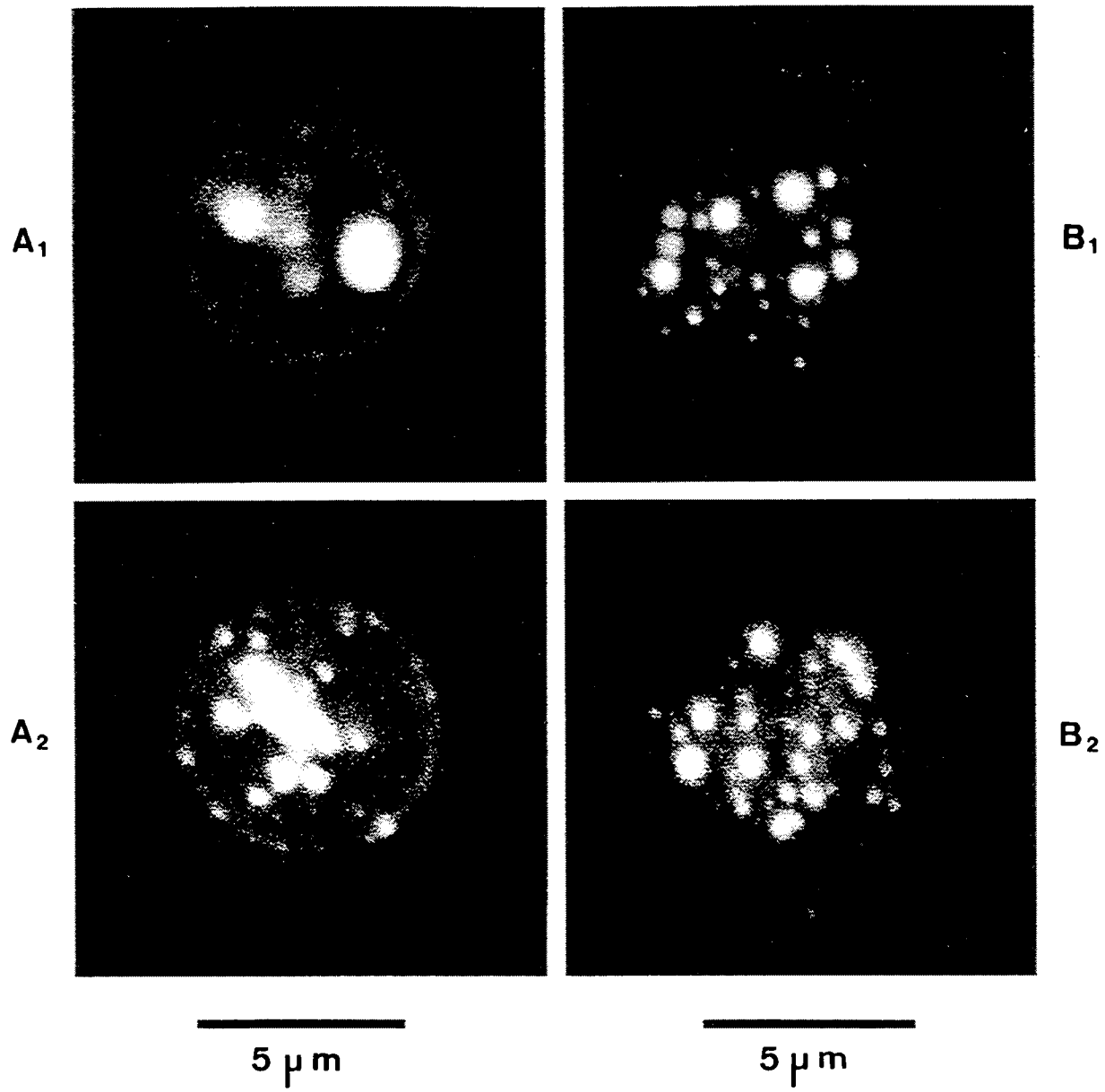

Fig. 1. Confocal micrographs of BLG microspheres formulated (A_1, A_2): without Tween 20. (B_1, B_2): with Tween 20, molar ratio 1:8.

On the contrary, as Tween 20 was able to migrate from the first emulsion to the outer surface of the microspheres, it made possible a reduction of the amount of BLG adsorbed onto the microsphere surface. On the other hand, DOPE and Pluronic® F68 did not seem to be able to reduce the burst effect from BLG microspheres, they displayed respectively, 27% and 35% of protein immediately released.

Protein adsorption onto particle surfaces or onto regions very close to particle surfaces is an important drawback of protein encapsulation into polymeric particles. During adsorption, proteins undergo conformational changes that increase their surface affinity (23). When BLG adsorbs onto microspheres, the protein partially unfolds and its internal free sulfhydryl group becomes available for polymerization at the interface of polymer/water. In addition, it is noteworthy that sulfhydryl residues are mainly responsible for the formation of strong protein-protein interactions (24). Confocal microscopy revealed the presence of protein aggregates; these could be due to the existence of protein-protein or protein-polymer interactions in microspheres prepared without surfactant (Fig. 1 A_1, A_2).

On the contrary, in the presence of Tween 20, BLG was located inside the internal globules in a very homogeneous fashion (Fig. 1, B_1 and B_2) without localization at the periphery of the microspheres. This could be due to surface displacement of BLG by Tween 20. In effect, Courthaudon *et al.* (18) found, at the oil-water interface for a molar ratio BLG/Tween 20 of 1:8, there was a complete protein displacement from the emulsion droplet surface. A high concentration of Tween 20 was shown to be able to induce a low equilibrium tension at the oil/water interface and a more efficiently packed monolayer. This is probably the main thermodynamic basis for the competitive displacement of BLG from the interface by Tween 20 (19).

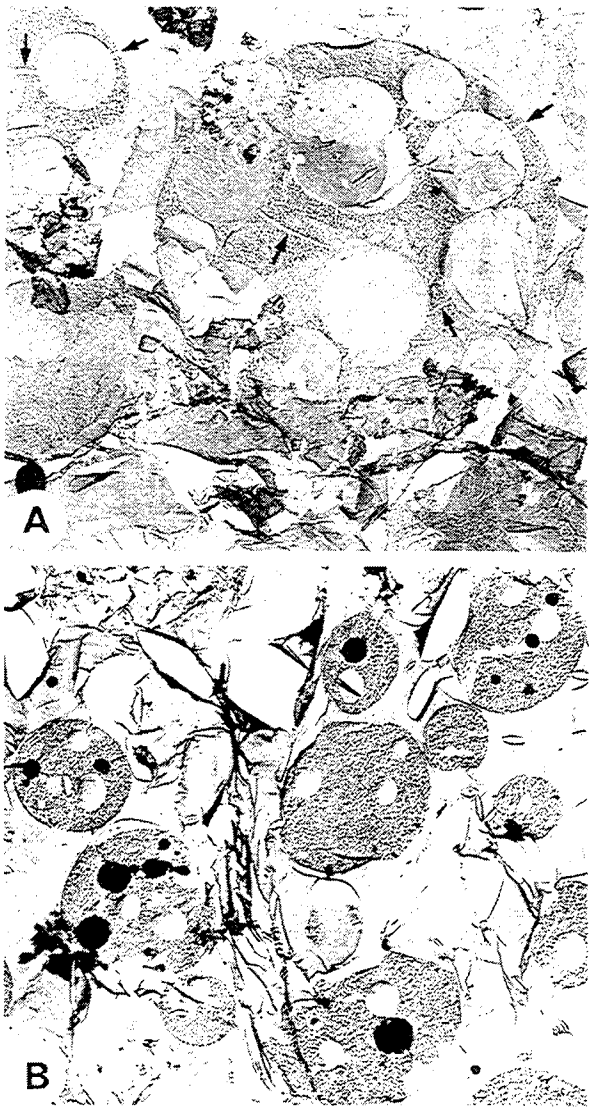

Fig. 2. Freeze-fracture electron microscopy of BLG microspheres formulated (A): without Tween 20. (B): with Tween 20, molar ratio 1:8. Magnification: × 5100; reduced to 90% for reproduction.

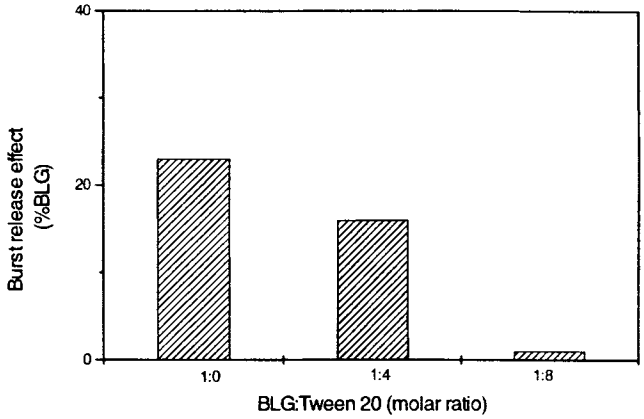

Fig. 3. Effect of Tween 20 on the burst release effect. BLG was introduced at a concentration of 50 mg/ml of inner aqueous phase (n = 3).

This poor localization at the surface was confirmed by competitive adsorption studies which enabled measurement the adsorption of BLG onto the surface of unloaded microspheres. After 4 h at pH 5.6, 2.9 mg of BLG were adsorbed, and at pH 6.5, 0.13 mg were adsorbed (Fig. 4A). A stronger adsorption at a lower pH was shown to be due to conformational changes of BLG occurring at values close to its isoelectric point (pI 5.2). At this pH, hydrophobic interactions between the protein and the polymer are more important (13). However, lower adsorption occurred when microspheres were prepared with Tween 20. After 4h at both pHs, only poor amounts of BLG were adsorbed (0.034 mg at pH 5.6 and 0.019 mg at pH 6.5) (Fig. 4B).

The influence of Tween 20 on microsphere surface was also evaluated by measuring particle zeta potential. Unloaded microspheres displayed a potential of −24.8 mV, and BLG microspheres prepared without Tween 20 displayed a potential of −8.9 mV (Table 3). However, as the amount of Tween 20 increased, microsphere zeta potential diminished until it reached a value that was very close to that of unloaded microspheres.

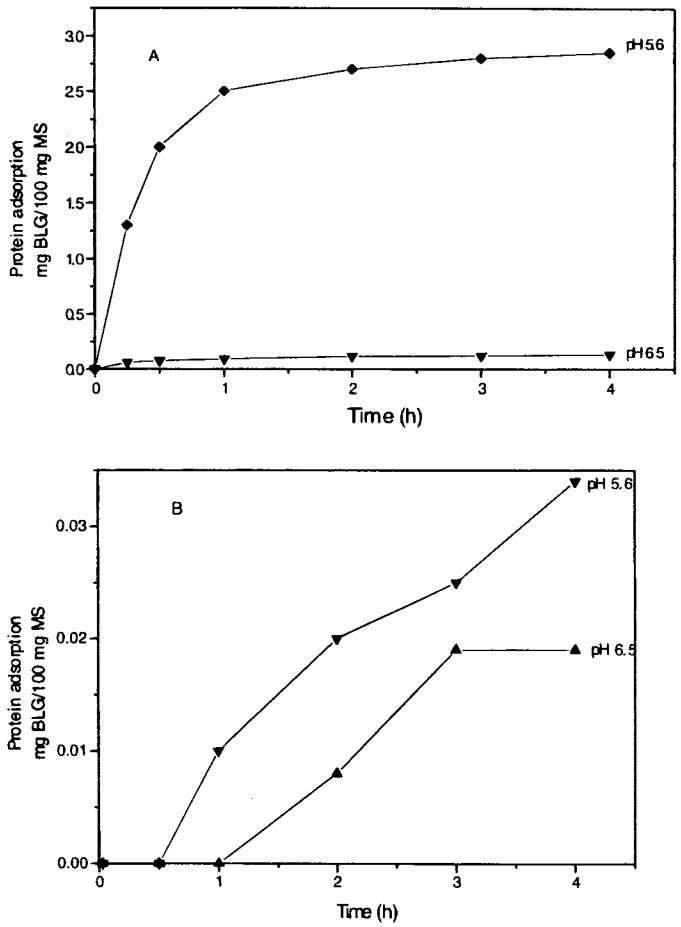

Fig. 4. (A): Adsorption of BLG onto PLGA microspheres (MS) prepared without Tween 20 at pH 6.5 (closed down triangles) and pH 5.6 (closed diamonds). (B): Adsorption of BLG onto Tween 20 PLGA microspheres at pH 6.5 (closed up triangles) and pH 5.6 (closed down triangles). Before one hour, at pH 5.6 and two hours at pH 6.5, BLG was not detectable.

Table 3. Effect of Tween 20 on Zeta Potential of BLG Microspheres (n = 3)

BLG mg/ml of inner aqueous phase	Protein:Tween 20 (molar ratio)	Zeta potential (mV)
50	1:0	− 8.9
50	1:0.01	−10.1
50	1:1	−15.5
50	1:4	−14.5
50	1:8	−22.3
0[a]	0:0	−24.8
0[a]	[b]0:8	−25.6

[a] Unloaded microspheres.
[b] 26 mg/ml of Tween 20.

CONCLUSIONS

BLG microspheres made of biodegradable PLGA were prepared with an adequate quantity of a polysorbate-based nonionic surfactant, Tween 20. This surfactant was able to displace protein from microsphere surface and to increase the encapsulation efficiency. Reduction of burst effect could improve the control of drug release constituting a strategy for the delivery of proteins from PLGA microspheres.

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