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# Use of an enzyme immunoassay for the evaluation of entrapment efficiency and in vitro stability in intestinal fluids of liposomal bovine serum albumin

G.A. Ramaldes[a,c], J.-R. Deverre[b], J.-M. Grognet[b], F. Puisieux[a], E. Fattal[a,*]

[a] *Laboratoire de Physico-Chimie, Pharmacotechnie, Biopharmacie, URA CNRS 1218, 5 rue Jean Baptiste Clément, 92296 Châtenay-Malabry Cedex, France*

[b] *CEA Saclay, DRM, Service de Pharmacologie et d'Immunologie, F91191 Gif-Sur-Yvette, France*

[c] *Universidade Federal de Minas Gerais, Faculdade de Farmácia, Avenida Olegário Maciel 2360, 30180-112 Belo Horizonte, Brazil*

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**Abstract**

The encapsulation efficiency of bovine serum albumin (BSA) within liposomes and its stability in physiological conditions were determined by a specific enzyme immunoassay (EIA) developed for this purpose. BSA was encapsulated within liposomes composed of soyabean phosphatidylcholine (PC), cholesterol (CH), phosphatidylglycerol (PG) (molar ratio 6:3:1) or distearoylphosphatidylcholine (DSPC), CH, PG, (molar ratio 6:3:1). Vesicles were prepared according to either the thin lipid film hydration or freeze-thawing methods. EIA was directly applicable to BSA encapsulated within liposomes without the usual need for sample preparation. The high sensitivity of the method allows high dilution of samples avoiding any interference with liposome formulation as was observed with high performance liquid chromatography (HPLC) method or colorimetric assay. Using this assay it was possible to evaluate that a high entrapment efficiency of BSA was obtained when the vesicles were composed of DSPC/CH/PG and prepared by the freeze-thawing method. Free BSA was stable upon incubation at 37°C for 2 h with acidic or basic buffers and in the presence of 10 mM TC, but was degraded in the presence of a mixture of pancreatin and TC. In the presence of pancreatin alone, BSA entrapped in PC/CH/PG liposomes was less stable than the BSA entrapped in DSPC/CH/PG liposomes. When TC was added to the pancreatin, the stability of BSA (free or encapsulated in PC/CH/PG liposomes) increased, suggesting that after solubilization by TC, phospholipids rearrange forming a new structure in which BSA is protected from degradation. In conclusion, EIA might be a useful tool for the direct evaluation of the encapsulation efficiency and stability of any antigen entrapped in liposomes, without the usual need for sample preparation.

*Keywords:* Liposome; Bovine serum albumin; Enzyme immunoassay; Oral administration; Stability

* Corresponding author. Tel.: +33 146835568; fax: +33 146619334.



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## 1. Introduction

Orally administered antigens are taken up by Peyer's patches and stimulate the production of specific antibodies (secretory IgA) in mucosal sites such as gut lamina propria, respiratory tract, mammary glands, salivary glands, lachrymal glands and genitourinary tract. However, because of their peptide structure, antigens are degraded within the lumen by hydrolytic peptidases before they reach the Peyer's patches. To solve this problem, drug carriers such as liposomes were designed to protect the entrapped antigens against degradation by intestinal proteolytic enzymes (Weingarten et al., 1985; Clarke and Stokes, 1992) and to enhance uptake by the Peyer's patches, thereby improving the mucosal immune response (Fattal et al., 1995). Nevertheless, one critical point in the administration of liposomes by the oral route is their instability in the presence of intestinal bile salts (Walde et al., 1987; Ramaldes et al., 1996). Bile salts are able to disrupt and solubilize phospholipids from liposomal membrane, so that the entrapped drug is no longer protected in the gastrointestinal tract (Rowland and Wodley, 1980). The assay methods currently employed for the evaluation of the entrapment efficiency and/or the stability of proteins in liposomes are based on the use of radiolabelled (Rowland and Wodley, 1980; Gregoriadis et al., 1987, 1993; Davis and Gregoriadis, 1987; Shahum and Therien, 1988, 1989; Gregoriadis and Panagiotidi, 1989; Bejan et al., 1989; Therien and Shahum, 1989; Therien et al., 1990, 1991; Clarke and Stokes, 1992; Antimisiaris et al., 1993; Arïen et al., 1994, 1995), or fluorescently labelled proteins (Aramaki et al., 1993; Elorza et al., 1993; Gregoriadis et al., 1993; Tomizawa et al., 1993). Analysis of antigen integrity after liposome preparation or during stability studies is not possible by these methods, and alternative protein assays have to be elaborated.

Grassi and Pradelles (1983), have developed an enzyme immunoassay using acetylcholinesterase (AChE) (EC 3.1.1.7) from 'Electrophorus electricus' as a tracer. AChE conjugates have been used to quantify eicosanoids (Pradelles et al., 1985), substance P (Renzi et al., 1987), thyroid releasing hormone (Grouselle et al., 1990), cyclic nucleotides (Pradelles et al., 1989), and benzyl penicilloyl groups (Wal et al., 1991) with sensitivities equal to or greater than those of conventional radioimmunoassay. We have developed an enzyme immunoassay (EIA) for the measurement of bovine serum albumin (BSA). BSA was employed as a model protein because it is highly immunogenic, and is also able to serve as a carrier enhancing the immunogenicity of many haptens. After validation, the EIA was applied to the determination of the efficiency of BSA encapsulation in fluid and solid liposomes prepared by the freeze–thawing and thin film methods. The stability of liposome-entrapped BSA exposed to conditions that simulate the gastrointestinal tract was also investigated. To our knowledge, the antigen integrity in liposomes exposed to physiological conditions has never been investigated.

In this paper, it was possible, using an EIA, to monitor the antigen integrity of BSA entrapped in liposomes in simulated intestinal fluid.

## 2. Materials and methods

### 2.1. Materials

BSA, cholesterol (CM), phosphatidylglycerol (PG), *N*-succinimidyl-*S*-acetyl-thioacetate (SATA), pancreatin, and sodium taurocholate (TC) were supplied by Sigma Chemical (St Louis, MO, USA) distearoylphosphatidylcholine (soyabean phosphatidylcholine hydrogenated, 18:0/18:0, 98.4% distearoylphosphatidylcholine) (DSPC) and phosphatidylcholine (soyabean phosphatidylcholine, 99% phosphatidyicholine) (PC), were supplied by Lipoid KG (Frigenstr 4 D-6700 Ludwigshafen). Biogel A-1.5 M was purchased from Biorad (Richmond, CA, USA). *N*-succinimidyl-4-(maleido-methyl)-cyclohexane-*l*-carboxylate (SMCC) was supplied by Calbiochem (San Diego, CA, USA). Sephadex G-25 was from Pharmacia (Uppsala, Sweden). Freund's complete adjuvant, *N,N'*dicyclohexyl carbodumide, *N*-hydroxysuccinimide, acetylthiocholine and DTNB [5,5'-dithio-

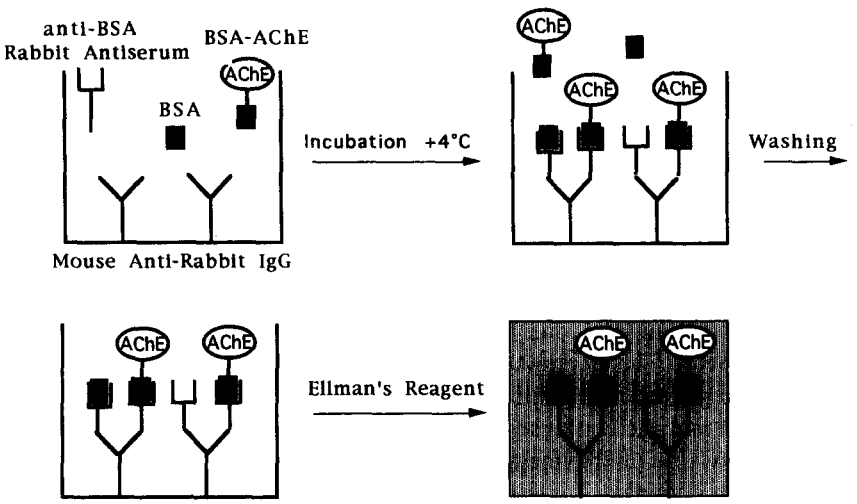


Fig. 1. Different steps of BSA enzyme immunoassay.

bis-(2-nitrobenzoic acid)] were from Sigma. Immunizations were done in Blanc du Bouscat male rabbits weighing 2.5 kg (Evic, Roquefort, France). Assays were performed on microplates (Immuno-NUNC Maxisorp) from Nunc (Denmark). Microtitration was performed using a microtiter plate washer 120 and a Multiskan MCC from Titertek Flow (Helsinki, Finland). Acetylcholinesterase (AChE) (EC 3.1.1.7) was extracted from the electric organ of the electric eel *Electrophorus electricus*, and was purified by one-step affinity chromatography as described elsewhere (Massoulié and Bon, 1976). Purified enzyme is available from SPI-BIO (Gif-sur-Yvette, France). The use of the G4 form (Massoulié and Bon, 1982) of the enzyme for the synthesis of enzymatic tracers used in enzyme immunoassays has been patented by our laboratory (Grassi and Pradelles, 1991). Enzyme activities were measured using Ellman's reagent, an AChE substrate consisting of 2.2 g of acetylthiocholine and 1 g of DTNB in 200 ml of 0.05 M phosphate buffer pH 7.4.

## 2.2. Methods

### 2.2.1. BSA enzyme immunoassays (EIAs)

BSA enzyme immunoassay consisted in a competitive binding of BSA, and of BSA covalently linked to acetylcholinesterase, to a BSA antiserum bound to a mouse monoclonal anti-rabbit immunoglobulin coating microtiter plates. After separation of bound and free moieties of the tracer, acetylcholinesterase activity bound to the solid phase was measured by a colorimetric assay (Fig. 1).

*2.2.1.1. Immunization.* The immunogen BSA was emulsified in Freund's complete adjuvant and injected intra-dermally at multiple sites on the back of two adult male rabbits. Each animal was given 1.4 mg immunogen and booster injections were repeated every month. Rabbits were bled from the central ear artery 1 and 2 weeks after booster injections. Blood was centrifuged and sera were stored in 0.1% sodium azide (w/v) at 4°C.

*2.2.1.2. Synthesis of enzymatic tracer.* BSA was covalently coupled to the tetrameric form of AChE as previously described (Renzi et al., 1987; McLaughlin et al., 1987). BSA was dialysed against distilled water, and thiolated using *N*-succinimidyl-*S*-acetyl-thioacetate (SATA): 500 nm of SATA solution were added to 10 nm of BSA dissolved in 0.1 M borate buffer pH 9. After 30 min of reaction at 30°C, the thioester group was hydrolysed in the presence of 1 M hydroxylamine and the thiolated BSA (BSA-SH) was isolated from the excess thiol reagent by gel filtration on a

Sephadex G-25 column (25 × 1 cm). The thiol content of the BSA was determined using Ellman's method (Ellman et al., 1961). A maleimido group was incorporated into AChE after reaction with *N*-succinimidyl-4-(maleido-methyl)-cyclohexane-1-carboxylate (SMCC). BSA was conjugated to AChE by mixing SMCC-treated AChE (0.1 nm) with an excess of BSA-SH (2 nm). After overnight reaction at 4°C, the conjugate was purified on a Biogel A 1.5 m column (90 × 1.5 cm) from Biorad (Paris, France) as previously described (Kanaoka et al., 1981). The immunoreactivity was checked in the presence of different dilutions of BSA antiserum and the conjugate was stored at 4°C. No significant loss of enzyme activity was observed during the coupling procedure, and no significant change in the immunological binding properties of the conjugate was noted under these storage conditions over a 2 year period.

*2.2.1.3. Enzyme immunoassay.* All assays were performed in 0.1 M phosphate buffer (pH 7.4), containing 0.4 M NaCl, $10^{-3}$ M EDTA, 0.2% gelatine and 0.01% sodium azide (phosphate-gelatine buffer pH 7.4). Before use, the plates were extensively washed with 0.01 M phosphate buffer pH 7.4 containing 0.05% Tween 20 (washing buffer) using the Multiwash apparatus (300 μl/well and 10 wash cycles). The assay was performed in a total volume of 150 μl. Reagents were dispensed as followed: 50 μl of sample or standard, 50 μl of enzymatic tracer diluted 1/200 and 50 μl of BSA antiserum (diluted 1:20 000). The plates were covered with a plastic adhesive sheet and left for 18 h at room temperature. They were then washed as described above and Ellman's reagent (200 μl) was dispensed into each well using an Autodrop apparatus. During the enzymatic reaction the plates were gently agitated. When the absorbance in the 'Bo' well (bound enzyme activity in absence of competitor) reached 0.2–0.4, the absorbance at 414 nm was measured in each well using a Multiskan spectrophotometer.

Unknown concentrations were calculated from a standard curve using a four-parameter logistic transformation with the Immunofit EIA/RIA software (Beckman CA 92634-3100, USA). All measurements for standards and samples were made in duplicate. Non-specific binding (usually < 0.1% of the total enzyme activity) was determined in wells in which the antiserum was replaced by 50 μl of phosphate-gelatine buffer pH 7.4.

*2.2.2. Gel exclusion HPLC*

HPLC analysis was performed using a 15 × 0.78 cm QC-PAK TSK GFC 200 column supplied by TosoHaas (Stuttgart, Germany) according to the recommended operating conditions for BSA. The eluant was 0.05% sodium azide and 0.1 M $Na_2SO_4$ in 0.1 M phosphate buffer, pH 7.2. To assay BSA encapsulated in liposomes, or BSA associated with blank liposomes, samples were dispersed in phosphate buffer pH 7.2. Lipid concentration was 0.20 μm/ml.

*2.2.3. Preparation of liposomes*

Liposomes encapsulating BSA were composed of DSPC/CH/PG or PC/CH/PG (molar ratio 6:3:1), and were prepared by either the freeze–thawing or thin lipid film hydration method above the phase transition temperatures (20°C for PC/CH/PG and 60°C for DSPC/CH/PG). Liposome mean diameters were determined by quasielastic light scattering (QELS) using an N4MD nanosizer (Coultronics, France). Entrapment of BSA was estimated by EIA after dilution of liposomes in phosphate-gelatine buffer pH 7.4. Phospholipids were assayed by the method described by Bartlett (1959).

*2.2.3.1. Freeze–thawing method (FT).* Liposome-encapsulated-BSA was prepared by a modification of the method described by Kashara and Hinkle (1977). Lipids were dissolved in chloroform in a round-bottomed flask. At appropriate temperatures, the dried lipid film formed with a rotary evaporator, and containing 30–120 μm of total lipids, was dispersed in 3 ml of HEPES buffer (145 mM NaCl containing 10 mM HEPES pH 7.4). The resulting suspension was extruded by repeated filtration cycles through polycarbonate filters (pore size 0.4, 0.2, 0.1 and 0.05 μm). Small unilamellar vesicles (SUV) were then used to gen-

erate freeze–thawing vesicles in the presence of BSA. At appropriate temperatures, the suspension was mixed with an equal volume of BSA solution at different concentrations and was shaken with a vortex mixer for 5 min. Each fraction was frozen in liquid nitrogen for 5 min. The frozen mixture was thawed at room temperature for 15 min, and subsequently shaken with a vortex mixer and sonicated for 5 s. Free BSA was separated from liposome-encapsulated BSA by ultracentrifugation at $150\,000 \times g$ for 1 h at 4°C three times. After each washing step, the pellet was suspended in 3 ml of HEPES buffer.

*2.2.3.2. Thin lipid film hydration method (TLFH).* Liposomes were prepared by the conventional thin lipid film hydration method described by Bangham et al. (1965). A dried thin lipid film was dispersed in HEPES buffer pH 7.4 (for blank liposomes) or BSA-HEPES buffer pH 7.4 (for liposome-entrapped BSA), and the suspension was extruded by repeated filtration through polycarbonate filters (pore size 0.4 μm). Free BSA was separated from liposome-encapsulated BSA as described above.

*2.2.4. In vitro stability*

Our approach was to study the stability of BSA entrapped in liposomes in conditions similar to what would be expected in the gastrointestinal tract. For this study, simulated intestinal fluid (USP XXII, 1990) containing 1% w/v pancreatin, 10 mM TC as a model of bile salts, and acid and basic pHs conditions were used. For these stability studies the liposomes were prepared by the FT methods. Samples were removed every 15 min, diluted in phosphate-gelatine buffer pH 7.4 (1:105 final dilution) and analyzed by EIA.

*2.2.4.1. Stability of free BSA.* Stability of free BSA was studied at different pHs, in the presence of TC and in the presence of simulated intestinal fluid. The effect of the pH was determined by incubating 700 μl of free BSA at 37°C for 2 h in 2800 μl of HEPES buffer adjusted to pH 2, 5, 7.4, 9. The effects of bile salts were determined by incubating 700 μl of free BSA at 37°C for 2 h in 2800 μl of HEPES buffer containing 12.5 mM TC (final concentration 10 mM). The effect of the simulated intestinal fluid was determined by incubating 700 μl of free BSA at 37°C for 2 h in 2800 μl of simulated intestinal fluid containing 1.25% w/v pancreatin (final concentration 1% w/v pancreatin).

*2.2.4.2. Stability of free and encapsulated BSA in stimulated intestinal fluid and bile salts.* Free BSA (700 μl), free BSA + PC/CH/PG liposomes, BSA-PC/CH/PG liposomes, free BSA + DSPC/CH/PG liposomes, or BSA-DSPC/CH/PG liposomes were incubated at 37°C for 60 min in 2800 μl of simulated intestinal fluid (final concentration 1% w/v pancreatin), or in a mixture of TC and simulated intestinal fluid (final concentration of TC and pancreatin 10 mM and 1% w/v respectively), pH 7.5. The effect of pancreatin deactivation was also determined. Samples were incubated in 10 mM TC and every 15 min 1% w/v pancreatin was added to until a final concentration of 5%.

*2.2.5. Statistical analysis*

Analysis of variance (ANOVA) was used to establish significant differences between control (free BSA) and other experimental groups. A level of $P < 0.05$ was accepted as statistically significant.

## 3. Results

### 3.1. Validation of the EIA of BSA

The repeatability ($n = 5$), between-day reproducibility ($n = 5$), and specificity of the EIA of BSA are summarized in Table 1. EIA net the requirements in relative standard deviation of repeatability and reproducibility (less than 10%). Liposome-encapsulated BSA was quantified by diluting the liposomes by a factor of $1/10^5$ to $1/10^6$ in phosphate-gelatine buffer at pH 7.4. After dilution, all liposome suspensions were optically clear suggesting that disruption of liposomes had occurred. The results obtained after dilution or by using a membrane detergent (Triton X-100) revealed no differences between the methods, thus confirming the disruption of liposomes in the assay medium (Table 1).

Table 1
BSA enzymatic immunoassay characteristics

| | |
|---|---|
| Reference Standard | BSA |
| Minimal sample volume | 50 μl |
| Dilution buffer | Phosphate-gelatine, pH 7.4 |
| Repeatability (intra-assay variation) | CV% (50 ng/ml) = 3 ($n = 5$) |
| | CV% (100 ng/ml) = 9 ($n = 5$) |
| | CV% (200 ng/ml) = 5 ($n = 5$) |
| Reproducibility (between-day assay variation) | CV% (50 ng/ml) = 2 ($n = 5$) |
| | CV% (100 ng/ml) = 2 ($n = 5$) |
| | CV% (200 ng/ml) = 3 ($n = 5$) |
| $IC_{50}^{a}$ | 93 ng/ml (4.65 ng/well) |
| BSA concentration in liposomes diluted $x$ 100%/BSA concentration in liposomes disrupted[b] | 97 ± 9% ($n = 6$) |

[a] BSA concentration inducing 50% lowering of Bo value.
[b] Comparison between liposomes diluted with phosphate-gelatine buffer, and liposomes disrupted with Triton X-100.

Quantification of BSA by EIA gave a lower limit of quantification than HPLC (0.1 mg/ml vs. 42 ng/ml) (Table 2). Only EIA was able to detect BSA in liposomes without any sample preparation, whereas detection by HPLC required separation of BSA from the lipid components (Table 2). No interferences was generated by addition of HEPES, phosphate buffer, TC, pancreatin, Triton X-100 or blank liposomes. Calibration curves for assaying BSA in liposomes were parallel to the calibration curve of free BSA (Fig. 2). As for HPLC, EIA was able to detect degradation of BSA in different conditions (pH, temperature, bile salts, enzymes) (Table 3). However, HPLC method was not able to assay BSA when the protein was encapsulated in liposomes (Table 2). It was only possible when BSA was associated to blank liposomes (Table 2).

### 3.2. EIA evaluation of BSA entrapment in liposomes

Liposomes were prepared by both thin lipid film hydration (TLFH) and freeze–thawing (FT) methods. TLFH yielded liposomes of diameter 500 ± 140 nm, and FT liposomes of diameter 120 ± 60 nm independently of the composition and concentration of BSA. In both cases, the diameter was not influenced by lipid composition or the presence of BSA (data not shown). Fig. 3 shows the effect on BSA encapsulation of initial BSA concentration, liposome preparation

Table 2
Comparison between EIA and HPLC in the assay of BSA under different conditions

| | EIA | HPLC |
|---|---|---|
| BSA standard curve | Yes | Yes |
| BSA-liposomes[a] | Yes | No |
| BSA + blank liposomes[b] | Yes | Yes |
| Non-specific interference[c] | No | No |
| Limit of quantification | 42 ng/ml (2.1 ng/well) | 0.1 mg/ml |
| Accuracy (1 mg/ml) | E% = 111 ($n = 5$) | E% = 105 ($n = 5$) |

Yes, the signal correspond to the theoretical amount; No, no signal is detected for BSA.
[a] BSA into liposomes (BSA-PC/CH/PG or BSA-DSPC/CH/PG-molar ratio 6:3:1).
[b] Blank liposomes (PC/CH/PG or DSPC/CH/PG-molar ratio 6:3:1).
[c] Blank liposomes (PC/CH/PG or DSPC/CH/PG-molar ratio 6:3:1), HEPES buffer, phosphate buffer, taurocholic acid, pancreatin, and Triton X-100 (< 0.1% w/v).

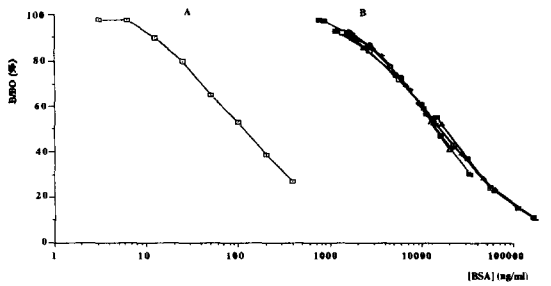

Fig. 2. Standard curve of free BSA (A) and dilution curve of BSA encapsulated in liposomes samples (B).

method, and lipid composition. Increasing BSA concentration resulted in an overall increase in the amount of protein entrapped (Fig. 3). For large amounts of BSA (10 and 20 mg/ml), no differences were observed for PC/CH/PG and DSPC/CH/PG liposomes prepared by TLFH (Fig. 3). Only DSPC-containing liposomes efficiently encapsulated BSA when prepared by FT (Fig. 3).

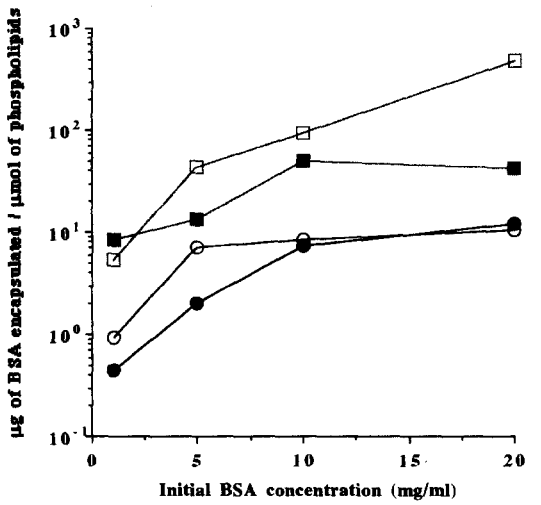


Fig. 3. Efficiency of BSA entrapment in liposomes prepared by the thin lipid film hydration method, PC/CH/PG (closed circles), DSPC/CH/PG (open circles) or the freeze–thawing method PC/CH/P (closed squares), DSPC/CH/PG (open squares).

### *3.3. EIA evaluation of the in vitro stability of free and liposomal BSA*

An important pre-requisite for the oral administration of vaccines is the maintenance of antigen integrity in the presence of intestinal fluids for long enough to ensure efficient interaction with antigen-presenting cells. BSA stability was estimated by EIA. Prior to the study of in vitro stability of liposomal BSA, free BSA stability was examined. Table 3 shows the effects of pH and taurocholic acid on BSA stability. At pH 2.0, 5.0, 7.4 and 9.0, or in the presence of 10 mM TC, no degradation of BSA at 37°C was observed after 2 h of incubation. At pH 2.0, 10% of the BSA was degraded under the same incubation conditions. This data were obtained by EIA assay and confirmed by HPLC.

Table 3
Effect of pH, taurocholic acid, and simulated intestinal fluid/TC on BSA stability (initial BSA concentration = 1 mg/ml)

| Incubation medium | % BSA degraded EIA | % BSA degraded HPLC |
|---|---|---|
| 2 h/37°C/pH 2 | 7 ± 3 | Zero |
| 2 h/37°C/pH 5 | Zero | Zero |
| 2 h/37°C/pH 7.4 | Zero | Zero |
| 2 h/37°C/pH 9 | Zero | Zero |
| 2 h/37°C/10 mM TC | Zero | Zero |
| 3 min/70°C/pH 13 | 93 ± 7 | 66 ± 1 |
| 2 h/37°C/SIA-TC | 45 ± 12 | 33 ± 5 |

Enzyme immunoassay (EIA) and HPLC results are expressed as the percentage ± S.D. of BSA degraded in relation to the initial BSA concentration ($n = 4$).

The stability of liposomal BSA from PC/CH/PG and DSPC/CH/PG vesicles prepared by FT was examined at 37°C in the presence of 1% w/v pancreatin alone or mixed with TC. In the absence of TC, BSA encapsulated in liposomes was more stable in solid DSPC-containing liposomes, as compared with fluid PC-containing liposomes (Table 4). Stability was also studied in the presence of a mixture of TC and pancreatin. In the presence of TC mixed with 1% (Table 4), the BSA degradation rate was slower with PC/CH/PG liposomes than with DSPC/CH/PG liposomes. As shown in Table 4, the degradation of BSA was significantly higher for the free protein than for BSA associated with, or entrapped in, PC/CH/PG liposomes. However, BSA degradation essentially occurs during the first 15 min of incubation, and

Table 4
BSA degradation at 37°C in the presence of (a) simulated intestinal fluid (1% w/v pancreatin) and 10 mM taurocholic acid or (b) simulated intestinal fluid (1% w/v pancreatin)

| | Initial BSA (mg/ml) | % BSA degraded | | | | Significance vs. free BSA (1.5 mg/ml) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 15 min | 30 min | 45 min | 60 min | 15 min | 30 min | 45 min | 60 min |
| Free BSA | 1.5[a] | 40 ± 9 | 42 ± 13 | 45 ± 14 | 43 ± 12 | — | — | — | — |
| | 20[a] | 42 ± 14 | 56 ± 3 | 61 ± 3 | 67 ± 1 | | | | |
| Free BSA + DSPC/CH/PG | 1.5[a] | 41 ± 3 | 44 ± 3 | 54 ± 8 | 55 ± 6 | NS | NS | NS | NS |
| | 20[a] | 42 ± 8 | 45 ± 3 | 60 ± 7 | ND | | | | |
| Free BSA + PC/CH/PG | 1.5[a] | 17 ± 8 | 25 ± 5 | 20 ± 12 | 35 ± 7 | $P<0.001$ | $P<0.01$ | $P<0.01$ | NS |
| | 20[a] | 12 ± 7 | 18 ± 10 | 20 ± 7 | 32 ± 13 | | | | |
| BSA-DSPC/CH/PG | 1.5[a] | 31 ± 3 | 37 + 5 | 38 + 9 | 45 ± 8 | NS | NS | NS | NS |
| | 1.5[b] | 0 | 9 | ND | ND | | | | |
| BSA-PC/CH/PG | 1.5[a] | 27 ± 5 | 28 ± 3 | 36 ± 8 | $P<0.001$ | $P<0.01$ | $P<0.05$ | NS | |
| | 1.5[b] | 32 ± 10 | 37 ± 17 | ND | ND | | | | |

NS, not significant.
ND, not determined.
Enzyme immunoassay (EIA) results are expressed as the percentage of BSA degraded ± SD in relation to the initial BSA concentration. BSA-liposomes and blank liposomes (molar ratio 6:3:1) were prepared by freeze–thawing as described in Section 2. Initial lipid concentration was 20 μm/ml ($n = 4$).

there was little further degradation up to 60 min (Table 4). Thus, this apparent stability of BSA would be dependent on the concentration of pancreatin employed. However, the results of Table 5 show that in more drastic conditions, the BSA degradation rate was also slower for BSA associated with, or entrapped in, PC/CH/PG liposomes. No differences were found between free BSA and BSA associated with, or entrapped in, DSPC/CH/PG liposomes (Table 4). The percentage of BSA degraded was also independent of the initial BSA concentration (1 or 20 mg of BSA/ml) (Table 4).

Table 5
BSA degradation in the presence of 5% w/v pancreatin and 10 mM taurocholic acid

| | % BSA degraded | | |
|---|---|---|---|
| | 15 min | 30 min | 45 min |
| Free BSA | 100 | 100 | 100 |
| DSPC/CH/PG + Free BSA | 88 ± 2 | 100 | 100 |
| PC/CH/PG + Free BSA | 73 ± 1 | 74 ± 4 | 85 ± 4 |
| DSPC/CH/PG-BSA | 100 | 100 | 100 |
| PC/CH/PG-BSA | 87 ± 4 | 91 ± 3 | 95 |

Incubation at 37°C in the presence of 5% w/v pancreatin and 10 mM taurocholic acid. Initial lipid concentration was 20 μm/ml. Initial BSA concentration was 1.5 mg/ml. Enzyme immunoassay (EIA) results are expressed as the percentage of BSA degraded ± S.D. in relation to the initial BSA concentration ($n = 4$).

## 4. Discussion

The use of liposomes for the oral administration of vaccines is highly dependent on their ability to ensure antigen stability in the presence of intestinal fluids. We looked at the stability of a model protein (BSA) encapsulated in liposomes composed of PC/CH/PG or DSPC/CH/PG. An original method allowing specific measurement of the antigen integrity of BSA was developed. Polyclonal antibodies were used in order to ensure 'a mapping' of BSA epitopes. BSA enzymatic immunoassay was shown to be a highly reliable method which is reproducible, highly specific, and has a very low limit of quantification. The high sensitivity of the method allows high dilution of samples avoiding any interference with liposome formulation as it was observed with HPLC method or colorimetric assay. It was therefore possible to assay BSA

directly in liposomes, avoiding radiolabelling procedures. As BSA was easily detectable in the medium used for stability studies, it was also possible to assay BSA in its native form, without resorting to the use of gel electrophoresis.

EIA was first applied to the study of BSA entrapment in liposomes. The efficiency of BSA encapsulation in liposomes was highly dependent on lipid composition (PC or DSPC liposomes) and on the preparation method employed. DSPC/CH/PG liposomes entrapped BSA more efficiently than PC/CH/PG liposomes particularly when vesicles were prepared by the FT method. These values are consistent with those reported by Gregoriadis et al. (1987) for the entrapment of tetanus toxoid into DSPC/CH liposomes prepared by dehydration–rehydration (DRV), as compared with PC liposomes. This great difference is probably due to the interactions of BSA with saturated phospholipids. It might be that antigens of high molecular weight, in addition to being passively entrapped, also interact in some way with DSPC, perhaps hydrophobically (Gregoriadis et al., 1987). The preparation obtained by FT, for DSPC vesicles, again proved to have a higher encapsulation capacity than the TLFH preparation. It is noteworthy that during freeze–thawing of SUV, the bilayer structure collapses and reforms during the thawing step. This results in increasing the trapped volume and trapping efficiencies, as described by Mayer et al. (1985).

EIA was also utilized to determine the stability of BSA. In the presence of pancreatin alone, degradation was greater in BSA-PC/CH/PG liposomes than in BSA-DSPC/CH/PG liposomes. This result is in agreement with the findings of Rowland and Wodley (1980), and Op Den Kamp et al. (1974), who showed that phospholipases present in pancreatin caused greater lysis of liposomes of unsaturated phospholipids than of liposomes of saturated phospholipids with a high transition temperature. Therefore, BSA was more easily released from fluid liposomes and subsequently degraded by proteases contained in pancreatin.

Degradation of liposomal BSA can be related to an increase in membrane permeability due mainly to the presence of bile salts. As reported by various authors (Richards and Gardner, 1978; Rowland and Wodley, 1980; Chiang and Weiner, 1987; Clarke and Stokes, 1992), a rapid release of drugs encapsulated in liposomes occurs in the presence of bile salts. The stability of liposomal BSA was very similar in its free and encapsulated forms in both fluid and solid liposomes, suggesting that rapid disruption of the liposomes occurs in the presence of TC. However, compared with free BSA, the stability was improved in the case of liposome-associated BSA, whether free or encapsulated in PC/CH/PG liposomes. These results suggests that there is a relationship between the solubilization of the vesicles and the molecular reorganization of the BSA into structures that would be less accessible to proteases. Thus, it is possible that in spite of large-scale disruption of liposomes in bile, some sort of complex between BSA-phosphatidylcholine-taurocholic acid could explain the protector effect observed in vitro. This type of preservation observed in presence of taurocholic acid is not unique: similar conclusions were also noted in vitro by Arïen et al. (1995), and in vivo by Fukunaga et al. (1991). In the case of DSPC/CH/PG liposomes, their highly rigid structure is poorly permeable to bile salts (Ramaldes et al., 1996), but it is likely that TC induces the formation of pores in membranes (Schubert et al., 1986) enhancing the access of proteases to BSA that has leaked out through these pores. At a similar condition, Chiang and Weiner (1987) observed that liposomes of DSPC/CH (molar ratio 2:1, 12 $\mu$m) in the presence of TC mixed with phospholipases released 81% of carboxyfluorescein after 5 min of incubation.

## 5. Conclusions

BSA enzyme immunoassay was validated and was shown to be a useful tool for characterizing drug carriers such as liposomes. The proposed method permits the direct observation of antigen integrity in liposomes. BSA was efficiently encapsulated in liposomes, but was poorly stable in the presence of intestinal artificial fluids. In vivo stability studies are required to confirm these results.

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