PARTICULATE CARRIERS AS IMMUNOLOGICAL ADJUVANTS

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Doctor of Philosophy

THE UNIVERSITY OF ASTON IN BIRMINGHAM

September 1993

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by

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Doctor of Philosophy

1993

SUMMARY

In recent years, much interest has focused on the significance of inducing not only systemic immunity but also good local immunity at susceptible mucosal surfaces. A new field of mucosal immunity has been established as information accumulates on gut-associated lymphoid tissue, bronchus-associated lymphoid tissue and nasal-associated lymphoid tissue (GALT, BALT and NALT, respectively) and on their role in both local and systemic immune responses. This project, following the line of investigation started by other workers, was designed to study the use of microspheres to deliver antigens by the mucosal routes (oral and nasal). Antigen-containing microspheres were prepared with PLA and PLGA, by either entrapment within the particles or adsorption onto the surface. The model protein antigens used in this work were mainly tetanus toxoid (TT), bovine serum albumin (BSA) and γ-globulins. *In vitro* investigations included the study of physicochemical properties of the particulate carriers as well as the assessment of stability of the antigen molecules throughout the formulation procedures. Good loading efficiencies were obtained with both formulation techniques, which did not affect the immunogenicity of the antigens studied. The influence of the surfactant employed on the microspheres' surface properties was demonstrated as well as its implications on the adsorption of proteins. Preparations containing protein adsorbed were shown to be slightly more hydrophobic than empty PLA microspheres, which can enhance the uptake of particles by the antigen presenting cells that prefer to associate with hydrophobic surfaces. Systemic and mucosal immune responses induced upon nasal, oral and intramuscular administration have been assessed and, when appropriate, compared with the most widely used vaccine adjuvant, aluminium hydroxide. The results indicate that association of TT with PLA microspheres through microencapsulation or adsorption procedures led to an enhancement of specific mucosal IgA and IgG and systemic IgG responses to the mucosal delivered antigens. Particularly, nasal administration of TT produced significantly higher serum levels of specific IgG in test animals, as compared to control groups, suggesting that this is a potential route for vaccination. This implies the uptake and transfer of particles through the nasal mucosa, which was further demonstrated by the presence in the blood stream of latex particles as early as 10 min after nasal administration.

Key words: Microencapsulation; Microspheres; Vaccines; Drug delivery; Tetanus toxoid.

To my Mother

In memory of my Father

ACKNOWLEDGEMENTS

I am grateful to my supervisor Dr. H. O. Alpar and my associate supervisor Professor M. R. W. Brown, for their enthusiasm and scientific guidance throughout the course of my research work.

I wish to thank Professor Doutor A. Lupi Nogueira, Professor Doutor J. Morais and Dr. M. Rodrigues Carvalho, at the Faculdade de Farmácia, Universidade de Lisboa, Portugal, for their support during my three years study in the United Kingdom.

I would like to express my appreciation to all the members of the Microbiology Research Group, particularly to Dr. Peter Lambert, and Dr. Anthony Smith for their advice and useful discussion. Special thanks to Mrs. Dorothy Townley and Mr. Roy Tilling not only for their very helpful technical assistance but also for being good friends, to Dr. Joanne Bowen for her help during my first weeks in the laboratory and to Jim Eyles for the radioactive latex particles.

I also would like to thank Dr. E. D. Williamson at the Chemical and Biological Defence Establishment, Ministry of Defence, Porton Down for her contribution to the project and useful discussion.

The Junta Nacional de Investigação Científica e Tecnológica - Programa Ciência (Portugal) and the INVOTAN (Portugal) are thanked for financing this project.

Finally, my thanks go to my family for their inestimable support, encouragement and patience during these last three years.

CONTENTS

		Page
Title		1
Thesis Summary		2
Dedication		3
Acknowledgements		4
Contents		5
List of figures		9
List of tables		15
Abbreviations		16
1.	**INTRODUCTION**	18
1.1.	Vaccine delivery systems	18
1.1.1.	Some characteristics of immunological adjuvants	19
1.1.2.	Main immunological adjuvants	20
1.1.2.1.	Mineral adjuvants	21
1.1.2.2.	Emulsions	22
1.1.2.3.	Microbial products	23
1.1.2.4.	Synthetic immunostimulants	26
1.1.2.5.	Immunostimulating complexes (ISCOMs)	27
1.1.2.6.	Liposomes	29
1.1.2.7.	Microspheres	31
1.2.	The lactide/glycolide polymers	40
1.3.	Nasal immunisation using particulate carriers	45
1.3.1.	Mucosal uptake of antigens	45
1.3.2.	The common mucosal immunity	48
1.3.3.	The nasal route of administration	51
1.3.4.	The nasal-associated lymphoid tissue (NALT)	52
1.4.	Origin and scope of the work	57
2.	**MICROENCAPSULATION OF MODEL PROTEIN ANTIGENS IN PLA/PLGA MICROSPHERES**	58
2.1.	Introduction	58
2.2.	Materials	59
2.2.1.	Polymers	59
2.2.2.	Model protein antigens	59
2.2.3.	Chemicals	59

2.3. Methods 60
2.3.1. Poly(lactide-co-glycolide) microsphere preparation 60
2.3.2. Particle size analysis 62
2.3.3. *In vitro* release studies 62
2.3.4. Determination of encapsulated protein 63
2.3.5. Studies on the degradation of PLA/PLGA microspheres 63
2.3.6. Bicinchoninic acid protein assay 64
2.3.7. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) 66
2.3.8. Western blotting (Immunoblotting) 70
2.4. Results and discussion 71
2.4.1. PLA/PLGA microsphere preparation 71
2.4.2. Protein encapsulation in PLA/PLGA microspheres 76
2.4.3. *In vitro* release studies 81
2.4.4. Protein integrity 85

3. ADSORPTION OF MODEL PROTEIN ANTIGENS ONTO PLA MICROSPHERES 88
3.1. Introduction 88
3.2. Materials 90
3.2.1. Microspheres 90
3.2.2. Model protein antigens 90
3.2.3. Chemicals 90
3.3. Methods 90
3.3.1. Adsorption of protein onto PLA microspheres 90
3.3.2. Direct determination of adsorbed protein 92
3.3.3. *In vitro* release studies 92
3.3.4. Assessment of protein integrity 92
3.4. Results and discussion 92
3.4.1. Loading onto microspheres by sorption procedures 92
3.4.2. Characterisation of the adsorption process 98
3.4.3. *In vitro* desorption studies 106
3.4.4. Stability of the antigen 109

4. SURFACE CHARACTERISATION OF PROTEIN-CONTAINING PLA MICROSPHERES 111
4.1. Introduction 111
4.2. Materials
4.2.1. PLA microspheres 113
4.2.2. Latex particles 114

4.2.3. Model protein antigens 114
4.2.4. Chemicals 114
4.3. Methods 115
4.3.1. Hydrophobic interaction chromatography (HIC) 115
4.3.2. Zeta potential determination 116
4.3.3. Statistical methods 118
4.4. Results and discussion 119
4.4.1. The HIC method - an appraisal 119
4.4.2. HIC: general results 121
4.4.3. Hydrophobicity of the protein-encapsulated formulations 123
4.4.4. Hydrophobicity of the protein-adsorbed formulations 124
4.4.5. Surface charge measurement 127

5. IMMUNISATION STUDIES WITH MICROSPHERE-ASSOCIATED TETANUS TOXOID 130
5.1. Introduction 130
5.2. Materials 131
5.2.1. Formulations 131
5.2.2. Animals 131
5.2.3. Anaesthetics 131
5.2.4. Chemicals 132
5.3. Methods 132
5.3.1. Immunisation studies using microencapsulated TT 132
5.3.1.1. Preliminary Immunisation Study 132
5.3.1.2. Study of factors influencing the immune response 133
5.3.2. Intranasal Immunisation with TT adsorbed onto microspheres 133
5.3.3. Intranasal immunisation with microencapsulated TT 135
5.3.4. Preparation of lung and gut lavages 136
5.3.5. Enzyme linked immunosorbent assay (ELISA) 137
5.3.6. Statistical methods 138
5.4. Results and discussion 139
5.4.1. Immune response to intramuscularly delivered microencapsulated TT 139
5.4.1.1. Preliminary immunisation study 139
5.4.1.2. The influence of dose, polymer composition and particle size on the immune response 140
5.4.2. Immune response to nasally delivered TT adsorbed onto microspheres 145
5.4.3. Immune response to microencapsulated TT 147
5.4.3.1. Systemic response 148

5.4.3.2. Mucosal response in the lung 150
5.4.3.3. Mucosal response in the gut 150

6. NASAL UPTAKE OF LATEX PARTICLES 155
6.1. Introduction 155
6.2. Materials 156
6.2.1. Latex particles 156
6.2.2. Anaesthetics 156
6.2.3. Animals 156
6.2.4. Chemicals 157
6.3. Methods 157
6.3.1. Particle Administration 157
6.3.2. Analysis by fluorescence microscopy 157
6.3.3. Analysis by fluorescence activated cell sorter (FACS) 158
6.3.4. Administration of radiolabelled particles 159
6.3.5. Statistical methods 159
6.4. Results and discussion 159
6.4.1. Particle counting by fluorescence microscopy 159
6.4.2. Assessment by FACS 161
6.4.3. Quantification by radioactivity 162

7. CONCLUDING REMARKS 168

8. REFERENCES 172

LIST OF FIGURES

Figure		Page
1.1.	Microsphere formation by emulsification/solvent evaporation (adapted from Watts *et al.*, 1990).	35
1.2.	Microsphere formation by the double emulsion [(w/o)/w] modification of the solvent evaporation method, as described by Ogawa *et al.* (1988) (adapted from Watts *et al.*, 1990).	36
1.3.	Degradation of PLA, PGA and copolymers (PLGA) in living systems (adapted from Literature Catalogue no BI 26, Alfa Chemicals Limited, Preston, U.K.).	41
1.4.	a) Synthesis of glycolide and DL-lactide monomers; b) Ring-opening copolymerisation of DL-lactide and glycolide monomers (adapted from Cowsar *et al.*, 1985).	42
1.5.	Half-life *in vivo* for poly(lactic-co-glycolic acid). Samples implanted in rat tissue (adapted from Miller *et al.*, 1977).	43
1.6.	Antigen processing in the gut (adapted from Bienenstock, 1988). The site of entry of bacteria, viruses and protein is also the site of absorption of particulate carriers such as liposomes and microspheres, i.e.in the M cells of the Peyer's Patches, as described by Saas *et al.* (1990).	50
1.7.	Simplified diagram of the composition of the NALT (adapted from Kuper *et al.*, 1992). Legend: B - B cell; T - T cell; M - M cell; Mϕ - macrophages; DC - dendritic cell; FDC - follicular dendritic cell; HEV - high endothelial venules.	54
1.8.	Diagram of the hypothetical mechanism of antigen processing and eliciting of the immune response after antigenic stimulation of the NALT (adapted from Kuper *et al.*, 1992). Legend: APC - antigen-presenting cell; M - M cells; PCLN - posterior cervical lymph node; SCLN - superficial cervical lymph node.	56
2.1.	Calibration curve for the estimation of proteins by the standard protocol of the bicinchoninic acid protein assay.	65
2.2.	Calibration curve for the estimation of proteins by the micro protocol of the bicinchoninic acid protein assay.	66
2.3	Calibration curve (r = 0.993) for the estimation of the molecular weight of proteins by SDS-PAGE, using the molecular weight markers described in table 2.3.	70
2.4.	Scanning electron micrograph of PLGA (90:10) microspheres. Mean particle size = 3.9 μm; σ_g = 1.7 (n = 97 particles).	73

2.5. Scanning electron micrograph of PLA (Mwt 2000 Da) microspheres prepared according to method D, using a high speed homogeniser (≥ 10000 rpm). 74
2.6. Particle size distribution of PLA (Mwt 2000 Da) microspheres prepared by method D using a high speed homogeniser (≥ 10000 rpm). Mean particle size = 0.7 μm; σ_g = 1.8 (n = 231 particles). 74
2.7. Scanning electron micrograph of PLA (Mwt 50000 Da) microspheres prepared according to method D. 75
2.8. Particle size distribution of PLA (Mwt 50000 Da) microspheres. Mean particle size = 2.1 μm; σ_g = 1.4 (n = 433 particles). 76
2.9. Scanning electron micrograph of a PLA (Mwt 2000 Da) batch of microspheres containing BSA microencapsulated. 77
2.10. Scanning electron micrograph of a PLA (Mwt 2000 Da) batch of microspheres containing TT microencapsulated. 77
2.11. Particle size distribution of PLA (Mwt 2000 Da) microspheres containing BSA microencapsulated. Mean particle size = 0.9 μm; σ_g = 1.6 (n = 176 particles). 78
2.12. Particle size distribution of PLA (Mwt 2000 Da) microspheres containing TT microencapsulated. Mean particle size = 0.9 μm; σ_g = 1.6 (n = 171 particles). 78
2.13. Variation of the amount of BSA encapsulated with the initial ratio BSA/PLA (Mwt 2000 Da): (•) - percentage of BSA encapsulated. (o) - percentage of BSA in the final composition of the formulation. 80
2.14. Release of BSA from PLA (Mwt 2000 Da) microspheres in 20 mM phosphate buffer, pH 7.5. Vertical bars indicate the standard deviation at each point (n = 4). 81
2.15. Release profile of TT from PLA (Mwt 2000 Da) microspheres in 20 mM phosphate buffer, pH 7.5 (n = 4). 82
2.16. Degradation of PLA/PLGA microspheres at different pH values: a) 0.1 N NaOH + 5% SDS, pH 11.7; b) PBS + 5% SDS, pH 7.4.; c) 0.1 N HCl + 5% SDS, pH 1.0. 84
2.17. SDS-PAGE (12% gels) of tetanus toxoid (a) and BSA (b). Lanes: 1) Tetanus Vaccine BP in Simple Solution; 2) TT before encapsulation in PLA (Mwt 2000) microspheres; 3) TT after release from microspheres; 4) BSA before encapsulation in PLA (Mwt 2000 Da) microspheres; 5) BSA after release from microspheres; 6) Molecular weight markers. 86
2.18. Western blotting of TT before and after encapsulation in PLA (Mwt 2000) microspheres. Lanes: 1) Tetanus Vaccine BP in Simple Solution; 2) TT solution before encapsulation; 3) TT after release from PLA microspheres. 86

3.1. Calibration curve for the estimation of BSA (r = 1.000) and γ-globulins (r = 0.999) by direct spectrophotometry. 91
3.2. Particle size distribution of PLA (Mwt 2000 Da) microspheres containing TT adsorbed onto the surface. Mean particle size = 0.8 μm; σ_g = 1.9 (n = 141 particles). 93
3.3. SDS-PAGE (12% gel) of TT solutions before and after adsorption on to PLA (Mwt 2000 Da) microspheres. Lanes: 1) Tetanus vaccine BP in Simple Solution; 2) and 3) 1000 μgml^{-1} before adsorption; 4) 1000 μgml^{-1} after adsorption; 5) 500 μgml^{-1} before adsorption; 6) 500 μgml^{-1} after adsorption; 7) and 8) washes. 96
3.4. SDS-PAGE (12% gel) of BSA solutions before and after adsorption on to PLA (Mwt 2000 Da) microspheres. Lanes: 1) Molecular weight markers (see table 2.3); 2 and 3) 750 μgml^{-1} before and after adsorption respectively; 4 and 5) 1000 μgml^{-1} before and after adsorption respectively; 6 and 7) 1250 μgml^{-1} before and after adsorption respectively; 8 and 9) 1500 μgml^{-1} before and after adsorption respectively. 97
3.5. SDS-PAGE (12% gel) of γ-globulins solutions before and after adsorption on to PLA microspheres. Lanes: 1 and 2) 300 μgml^{-1} before and after adsorption respectively; 3 and 4) 600 μgml^{-1} before and after adsorption respectively; 5 and 6) 900 μgml^{-1} before and after adsorption respectively; 7 and 8) 1200 μgml^{-1} before and after adsorption respectively; 9 and 10) 1500 μgml^{-1} before and after adsorption respectively; 11 and 12) 1800 μgml^{-1} before and after adsorption respectively. 97
3.6. SDS-PAGE (14% gel) of a CTB solution (500 μgml^{-1}) before and after adsorption onto PLA (Mwt 2000 Da) microspheres. Lanes: 1) Molecular weight markers (see table 2.3); 2) solution after adsorption; 3) washes; 4) solution before adsorption. 98
3.7. Langmuir isotherm of TT adsorption onto PLA (Mwt 2000 Da) microspheres (mean ± sd; n = 3). 100
3.8. Langmuir plot (r = 0.995) corresponding to the TT adsorption isotherm of fig. 3.7 (mean ± sd; n = 3). 101
3.9. Langmuir isotherm of BSA adsorption onto PLA (Mwt 2000 Da) microspheres (mean ± sd; n = 3). 101
3.10. Langmuir plot (r = 0.998) corresponding to the BSA adsorption isotherm of fig. 3.9 (mean ± sd; n = 3). 102
3.11. Langmuir isotherm of γ-globulins adsorption onto PLA (Mwt 2000 Da) microspheres (mean ± sd; n = 3). 102

3.12. Langmuir plot (r = 0.996) corresponding to the γ-globulins adsorption isotherm of fig. 3.11 (mean ± sd; n = 3). 103
3.13. Freundlich plot (r = 0.994) corresponding to the TT adsorption isotherm of fig. 3.7 (mean ± sd; n = 3). 103
3.14. a) Diagram of the ellipsoidal model of the BSA molecule calculated by Squire *et al.* (1968). b) Adsorption of BSA: I) side-on; II) end-on; III) mixed process. 104
3.15. Desorption profile of TT adsorbed onto PLA (Mwt 2000 Da) microspheres (in phosphate buffer 20 mM, pH 7.5; n = 3 ± sd). 106
3.16. Desorption profile BSA adsorbed onto PLA (Mwt 2000 Da) microspheres (in phosphate buffer 20 mM, pH 7.5; n = 3 ± sd). 107
3.17. Desorption profile of γ-globulins adsorbed onto PLA (Mwt 2000 Da) microspheres (in phosphate buffer 20 mM, pH 7.5; n = 3 ± sd). 107
3.18. SDS-PAGE (12% gel) (a) and Western blotting (b) of TT before and after adsorption onto PLA (Mwt 2000 Da) microspheres. Lanes: 1) Tetanus Vaccine BP in Simple Solution; 2 and 3) TT solution 250 μgml^{-1} before and after adsorption (supernatant); 4) Tetanus Vaccine BP in Simple Solution; 5) TT solution before adsorption; 6) TT after release from PLA microspheres. 110
4.1. Diagram of a HIC column prepared using a glass Pasteur pipette as described in the text. 115
4.2. Diagram of Malvern ZetaSizer 4 (adapted from Malvern Instruments Ltd. product information). 118
4.3. Influence of the ionic strength of the eluent on the retention of PLA (Mwt 2000 Da) microspheres (n = 3; mean ± sd). 120
4.4. Particle size dependence of the HIC method used in these studies (n = 3; mean ± sd). 121
4.5. Final retention by various stationary phases of the different protein-encapsulated formulations, compared with latex microspheres of a similar particle size (mean ± sd; n = 3). 123
4.6. Final retention by various stationary phases of the different protein-adsorbed PLA microsphere preparations, compared with latex microspheres of a similar size (mean ± sd; n = 3). 126
4.7. Comparison of the final retention values between empty PLA microspheres prepared with PVA and Tween 80, respectively (mean ± sd; n = 3). 127

4.8. Surface charge of the various formulations measured as zeta potential (mean ± sd; n = 3). Legend: A) Latex microparticles (1 μm); B) PLA microspheres prepared with Tween 80; C) PLA microspheres prepared with PVA; D) BSA-encapsulated PLA microspheres; E) BSA-adsorbed PLA microspheres; F) TT-encapsulated PLA microspheres; G) TT-adsorbed PLA microspheres; H) γ-globulins-adsorbed PLA microspheres (* n = 2). 128

5.1. Immune response to the preliminary i.m. administration of a 5 μg dose of TT (section 5.3.1.1). Animals were primed with a single dose on day 1 and similarly boosted on day 28. Results are expressed as mean ± sd; n = 4 per group; serum dilution = 1:20. 139

5.2. Scanning electron micrograph of PLA (Mwt 2000 Da) microspheres containing 8.3% (w/w) of TT (mean size = 2.4 μm, σ_g = 1.8; n = 542 particles). 141

5.3. Scanning electron micrograph of PLGA (50:50) microspheres containing 1.7% (w/w) of TT (mean size = 1.0 μm, σ_g = 1.6; n = 306 particles). 141

5.4. Scanning electron micrograph of PLGA (50:50) microspheres containing 1.8% (w/w) of TT (mean size = 2.8 μm, σ_g = 1.5; n = 347 particles). 142

5.5. Scanning electron micrograph of PLGA (50:50) microspheres containing 2.0% (w/w) of TT (mean size = 24.0 μm, σ_g = 1.7; n = 489 particles). 142

5.6. Immune response to 0.1 μg and 1.0 μg of TT microencapsulated into PLA (Mwt 2000 Da) microspheres of different particle sizes. Animals were primed (i.m.) with a single dose on day 1 and similarly boosted on day 28. Results are expressed as mean ± sd; n = 5 per group; serum dilution = 1:160 (* $p \leq 0.1$). 143

5.7. Immune response to 0.1 μg and 1.0 μg of TT microencapsulated into PLGA (50:50) microspheres of different particle sizes. Animals were primed (i.m.) with a single dose on day 1 and similarly boosted on day 28. Results are expressed as mean ± sd; n = 5 per group; serum dilution = 1:160 (* $p \leq 0.1$). 143

5.8. Serum antibody response to nasally administered TT (mean ± se; n = 3). Animals were given the vaccine by administration of soluble or PLA microsphere- adsorbed toxoid. Animals were primed with three doses (days 1, 2 and 3, respectively) and boosted with one dose on day 28. 146

5.9. Immune response of mice after immunisation by the i.m. route with 2.5 μg of microencapsulated TT (particle size = 0.8 μm ± 1.7). Gut and lung mucosal antibodies were measured in one animal at each time point (n.d. = not determined); serum antibodies were determined in pooled samples (n = 5). 149

5.10. Immune response to several doses of microencapsulated TT (particle size = 0.8 μm ± 1.7) administered by the nasal route, measured as titres of antibodies. (e. m. = empty microspheres; n. d. = not determined). Gut and lung mucosal antibodies were measured in one animal at each time point; serum antibodies were determined in pooled samples (n = 5). 151

5.11. Immune response to several doses of microencapsulated TT (particle size = 0.8 μm ± 1.7) administered by the oral route, measured as titres of antibodies. (e. m. = empty microspheres; n. d. = not determined). Gut and lung mucosal antibodies were measured in one animal at each time point; serum antibodies were determined in pooled samples (n = 5). 152

6.1. Levels of circulating fluorescent latex particles (mean size 0.51 μm ± 0.008) after nasal administration to rats (± sd; n = 4). Particle numbers were calculated using the fluorescence microscopy method, as described in the text. 160

6.2. Amount of fluorescent latex particles (mean size 0.83 μm ± 0.006) after nasal administration to rats (± sd; n = 4), as obtained by FACS analysis. 162

6.3. Micrograph of a cluster of circulating fluorescent polystyrene microspheres under UV light, obtained 60 min after administration from an animal treated using the model of Fisher *et al.* (1987). 163

6.4. Micrograph of the same preparation showed in figure 6.3, obtained under both UV and tungsten light, and where the fluorescent polystyrene microspheres (green) can be seen among the erythrocytes. 163

6.5. The levels of radioactivity in the blood after nasal administration of radiolabelled polystyrene particles (mean ± sd; n = 3). 164

6.6. Tissue distribution of radioactivity 3 hours after nasal administration of radiolabelled polystyrene particles (mean ± sd; n = 3). 165

LIST OF TABLES

Table **Page**

1.1.	Some examples of microsphere/nanosphere systems described in the literature.	32
2.1.	Modifications to the original solvent evaporation method described by Beck *et al.* (1979).	61
2.2.	Composition of running gel, stacking gel and sample buffer for SDS-PAGE.	68
2.3.	Unstained molecular weigh markers for SDS-PAGE.	69
2.4.	Mean particle size obtained with some variations of the method.	73
2.5.	Encapsulation efficiency by the solvent evaporation method.	80
3.1.	Adsorption efficiency of TT and CTB onto PLA microspheres.	94
3.2.	Adsorption efficiency of TT onto PLA (Mwt 2000 Da) microspheres (mean ± sd; n = 3).	94
3.3.	Adsorption efficiency of BSA onto PLA (Mwt 2000 Da) microspheres (mean ± sd; n = 3).	95
3.4.	Adsorption efficiency of γ-globulins onto PLA (Mwt 2000 Da) microspheres (mean ± sd; n = 3).	95
3.5.	Values obtained for TT worked out from BSA data by the Langmuir theory.	105
4.1.	Parameters used in zeta potential measurements.	117
4.2.	Elution of particles per sample and per type of agarose (% eluted ± sd; n = 3).	122
5.1.	Immunisation schedule of the study described in section 5.3.1.2.	134
5.2.	Composition of fluids for lung and gut washes.	137

ABBREVIATIONS

BALT	bronchus-associated lymphoid tissue
BCA	bicinchoninic acid
BIS	N,N' methylene bisacrylamide
BSA	bovine serum albumin
BTA	botulinum toxoid A
BTE	botulinum toxoid E
CT	cholera toxin
CTB	cholera toxin B subunit
DDA	dimethyl-dioctadecylammonium bromide (or chloride)
EDTA	ethylenediaminetetraacetate
ELISA	enzyme-linked immunoassay
FACS	Fluorescence activated cell sorter
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
GALT	gut-associated lymphoid tissue
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HSV	herpes simplex virus
i.m.	intramuscular
i.p.	intraperitoneal
ISCOMs	immunostimulant complexes
LDA	laser Doppler anemometry
Lf	Limes flocculing units
MALT	mucosal-associated lymphoid tissue
MDP	N-acetyl-muramyl-L-alanyl-D-isoglutamin
MHC	major histocompatibility complex
Mwt	molecular weight
NALT	nasal-associated lymphoid tissue
NC	nitrocellulose
OD	optical density
o/w	oil-in-water
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline/Tween 20
PGA	poly(glycolic acid)
PLA	poly(lactic acid)
PLGA	poly(lactide-co-glycolide)
PMSF	phenylmethylsulfonylfluoride
PVA	polyvinyl alcohol

RES	reticuloendothelial system
SAF	Syntex adjuvant formulation
sd	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
se	standard error of the mean
SEB	staphylococcal enterotoxin B
SEM	scanning electron microscope
SIgA	secretory IgA
SRBC	sheep red blood cells
σ_g	geometric standard deviation
TBS	tris buffered saline
Tet/Vac/Ads	Adsorbed Tetanus Vaccine (British Pharmacopoeia)
TEMED	N,N,N,'N'-Tetramethylethylene diamine
TMB	3,3',5,5'-tetramethylbenzidine
Tris	tris(hydroxymethyl)-aminomethane
TT	tetanus toxoid
(w/o)/w	(water-in-oil)-in-water
w/v	weight per volume
w/w	weight per weight
v/v	volume per volume

1. INTRODUCTION

1.1. VACCINE DELIVERY SYSTEMS

Vaccination is one of the most successful achievements of medical science. It prevents a number of infectious diseases. For example, smallpox has been eradicated and the number of cases of diphtheria, tetanus and poliomyelitis is decreasing, being currently a serious problem only in countries where vaccination is not systematically carried out (Dodet, 1987). In recent years there have been advances in molecular biology and analytical chemical techniques - mainly in the fields of biotechnology and pharmaceutical sciences - enabling the isolation, synthesis and production of new recombinant antigens from viruses, bacteria and parasites and improving existing antigens (Kersten *et al.*, 1989). Although these new antigens offer advantages in the selection of antigenic epitopes, and safety in comparison to whole-virus or whole-bacteria vaccines, they are in many cases weak immunogens (Gregoriadis, 1990; Eldridge *et al.*, 1992). Because of these considerations, potent immunoadjuvants have been sought. Edelman (1980) explains the difference between a potent and a less potent immunogen, in terms of the intrinsic adjuvanticity of the molecule itself. However, in the case of a less potent antigen, which is the case with new peptide agents, the immune response can be stimulated by the simultaneous administration of compounds that are known to enhance their immunogenicity; they are called immunoadjuvants or simply adjuvants (Kersten *et al.*, 1989).

The use of these types of compounds is limited and to obtain good results it is necessary to add them to the antigen and present the formulation to the immune system in an appropriate manner, which depends on several factors (Kersten *et al.*, 1989):

a) The antigen itself can influence the type of immune response. In the case of a humoral response against protein antigens, the molecular tertiary and quaternary conformations are usually important, whereas epitopes relevant for a cellular response are often linear. However, it is important to recognise that in some cases (e.g. viral antigens) the structure of the antigen that elicits an immunogenic stimulus in animals remains unknown and that only inferences are possible (Van Regenmortel, 1992).

b) The route of immunisation can influence the response through the production of different types of antibodies and generally a local route is used when a local response is desirable, because important infectious diseases either affect or start from the mucosal surfaces (Holmgren *et al.*, 1989).

c) The type of immune response required (humoral or cellular) depends on the type of pathology being investigated.

d) The immune status of the receiver which can be as different as it is between a neonate provided with maternal antibodies (Albrecht *et al.*, 1977) and a patient with an immune-deficiency.

Thus, the immune response may be highly influenced by vaccine formulation which can be considered as a special chapter in the area of drug development.

1.1.1. Some Characteristics of Immunological Adjuvants

An important attribute of any adjuvanted vaccine is that the benefits outweigh the risks, and according to Edelman (1980) the following list of factors should help to assure the safety of these products: The adjuvanted vaccine should maximally immunopotentiate the antigen but not the host's own tissue antigens and the vaccine should not contain antigens cross-reactive with human antigens. The adjuvant itself must not induce allergic hypersensitisation to itself or combine with naturally occurring serum antibodies to form potentially harmful immune complexes, should be chemically pure and of defined chemical composition so that its manufacture is reproducible, and should be carefully chosen to achieve the sort of immunopotentiation necessary to make the vaccine more efficacious. The adjuvant should act only to potentiate the vaccine without inducing a diverse array of other immunologic events not involved in that immunospecific response. It also must not be carcinogenic, teratogenic or abortifacient, nor should it promote tumour formation as a cocarcinogen. A biodegradable adjuvant is preferable so that, within a period of weeks or months after its adjuvant effect is exhausted, it is eliminated from the body.

1.1.2. Main Immunological Adjuvants

The new molecules isolated by biotechnology or chemically prepared are often poor immunogens because their preparation includes a high degree of purification. These processes can lead to the loss of their intrinsic adjuvanticity (Edelman, 1980) and therefore there is a need to add an adjuvant. The concept of adjuvanticity was first introduced in 1925 by Ramon who, by adding agar, starch, lecithin or even bread crumbs to the antigen, observed an increase of the antibody levels against diphtheria or tetanus. As a consequence of this, he classified as adjuvants those substances which, when used in combination with specific antigens, enhance the immunity to levels higher than those when the vaccine only is used (reviewed by Edelman, 1980 and Lindblad & Spärck, 1987). So, the use of adjuvants, by increasing the potency of antigens, leads to formulations containing smaller amounts of antigen and a decrease in the number of doses required.

According to Gregoriadis (1990), adjuvants appear to act by one of the following mechanisms. The first of these involves the formation of a depot at the site of injection, which prolongs the release and interaction of the antigen with antigen-presenting cells. After immunisation, local inflammation may occur, attracting these antigen-presenting cells. On the other hand, the adjuvant-antigen complexes may migrate to the regional lymph nodes containing T cells. The second mechanism is the activation of macrophages which release interleukin-1. The combined action of interleukin-1 and antigens on T cells induces the release of interleukin-2 and other mediators that activate effector T cells or antibody-forming B cells. The physicochemical properties of the adjuvant, e.g. both lipophilic and particulate antigens are usually better immunogens than the soluble ones, are also important and will be discussed in the following sections.

Several well known adjuvants are used in research, few are currently being used in vaccinations. In the present work, immunological adjuvants are classified either as molecules with adjuvant activity or as the so-called adjuvant formulations.

1.1.2.1. Mineral Adjuvants

Hydrated aluminium hydroxide, aluminium phosphate or calcium phosphate (British Pharmacopoeia, 1988) are currently used in medicine. Other protein precipitants that contain metal, such as cerium nitrate, zinc sulphate, colloidal iron hydroxide and calcium chloride, also increase the antigenicity of toxoids, although aluminium compounds produce the best results (Edelman, 1980). Aluminium-containing vaccines are prepared by two principal methods. The first method involves the addition of a solution of aluminium salts [e.g. $Al(SO_4)_2.12H_2O$] to the antigen, to form a precipitate of protein aluminate; the products so obtained have been termed aluminium-precipitated vaccines. In the second method, vaccines are prepared by addition of an antigen solution to aluminium compounds, most commonly $Al(OH)_3$ or $AlPO_4$, and these preparations are called aluminium-adsorbed vaccines (Nicklas, 1992). Because these salts have the ability to adsorb proteins they act mainly by a depot effect, fixing the antigen to the site of injection and delaying its release. At pH < 9 the $Al(OH)_3$ gel is positively charged and thus able to adsorb proteins, which are mostly negatively charged at physiological pH. This attraction of opposite charges is assumed to be the major cause responsible for the adsorptive capacity of the gel (Lindblad & Spärck, 1987). This results in a prolonged contact between the antigen and the immune system, enhancing the immune response. Direct and unspecific stimulation of cells involved in the immune response (Flebbe & Braley-Mullen, 1986), as well as activation of the complement system in guinea-pigs was also reported (Ramanathan *et al.*, 1979). On the other hand, there is no evidence that these compounds are themselves immunogenic or haptens (Nicklas, 1992). They attract eosinophils to the injection site and prime helper T cells for IgE production in animals (Walls, 1977). It has been also demonstrated that aluminium compounds stimulate the production of IgG_1 (Warner *et al.*, 1968). Aluminium hydroxide is reported to be more effective than aluminium phosphate because it has a higher adsorption capacity, therefore providing a better depot effect (Lindblad & Spärck, 1987).

Calcium phosphate's mode of action was reviewed by Lindblad & Spärck (1987). These authors stated that the production of IgE is not stimulated by calcium phosphate and that the adsorption of proteins is of the same magnitude as that of aluminium phosphate. Also, being a natural constituent of the organism, calcium phosphate does not seem to cause any

side effects.

However, mineral adjuvants have severe disadvantages since they are difficult to manufacture in a physicochemically reproducible way, and so variations may exist between different batches of the same vaccine (Nicklas, 1992). The structure and properties of aluminium compounds change significantly with minor alterations in production conditions and with aging, which can lead to differences in the immune response obtained (Kreuter *et al.*, 1986). Compared to water-in-oil emulsion adjuvants, the depot effect of aluminium salts is much lower, so necessitating repeated injections to obtain a more prolonged effect. In addition, some side effects were observed, e.g. formation of granuloma and nodules at the subcutaneous injection sites and, in contrast to many other adjuvants, aluminium salts are deficient in inducing cell-mediated immunity (Nicklas, 1992). Furthermore, aluminium compounds fail to increase the protection provided by some vaccines e.g. those for whooping cough and typhoid fever (Edelman, 1980).

1.1.2.2. Emulsions

Oil-based adjuvants have been used to enhance the immune response of farm animals to many attenuated vaccines (British Pharmacopoeia - Veterinary, 1985; *ibid.*, Addendum, 1992).

The studies into tuberculosis culminated in 1956 in the work of Jules Freund who developed his complete and incomplete adjuvants (FCA and FIA, respectively). The FCA is a water-in-mineral oil emulsion supplemented with killed whole mycobacteria - *Mycobacterium tuberculosis* or *Mycobacterium butyricum* (Kersten *et al.*, 1989). The mineral oil Drakeol 6VR (a mixture of hydrocarbons and aromatics) is often used as the external phase and the emulsion is stabilised with the detergent Arlacel A - a poorly defined mixture of fat and carbohydrate compounds plus mannide monoleate - (Edelman, 1980; Kersten *et al.*, 1989). The FIA consists of the same formulation except for the mycobacteria. These adjuvants are widely used in laboratory research for their extremely powerful adjuvanticity, but because of severe pain, abscess formation, fever and, possibly,

of permanent organ injury attending its use, FCA is not used in human or veterinary vaccines. The FIA is less harmful but the finding that Arlacel A is carcinogenic in mice has stopped its use in humans. Another problem with this product is the possibility of carcinogenic effects caused by the non-biodegradable mineral oil. Investigators have tried to substitute the mineral oil by vegetable oils (sesame oil, peanut oil), using Tween 80 as an emulsifier, but the immune response induced by these new formulations is reduced (Kersten *et al.*, 1989). The exact cellular and biochemical mechanisms of action of these emulsions are poorly understood, although there is a marked depot effect (Edelman, 1980).

In 1987 Byars & Allison created an emulsion-based formulation (oil-in-water or o/w) containing threonyl-muramyl dipeptide (peptide molecule with adjuvant activity), designed to be an acceptable substitute for FIA (Allison & Byars, 1992). This formulation combines the adjuvant properties of the emulsion system with the intrinsic adjuvanticity of a muramyl dipeptide derivative which has low toxicity. Apart from the peptide already referred to, it contains squalene or squalane as internal phase, 'Pluronic' L121 (polyoxyethylene-polyoxypropylene copolymer) and Tween 80 as emulsifiers, and phosphate buffered saline as external phase. Known as syntex adjuvant formulation (SAF), it increases both cellular and humoral immune responses to ovalbumin in guinea-pigs (Byars & Allison, 1987). The systemic antibody immunity was also increased after subcutaneous immunisation of guinea-pigs and mice with either hepatitis B surface antigen and hemagglutinin of influenza B virus in SAF (Byars *et al.*, 1989). When compared with aluminium-adjuvanted vaccines SAF was found to be significantly more efficacious, without side effects.

1.1.2.3. Microbial Products

A number of microbial products have been shown to possess an immunostimulating potential. Among them are whole cells from bacterial species including *Bordetella pertussis*, *Mycobacterium tuberculosis* (the mycobacterial vaccine BCG - bacille Calmette-Guérin), *Corynebacterium parvum* and *Corynebacterium rubrum*. There are also bacterial fractions, including cell wall components such as lipopolysaccharide, lipid A, wax D and muramyl dipeptide (MDP or N-acetyl-muramyl-L-alanyl-D-isoglutamine). There is also the

possibility of using DNA and RNA digests. It has been claimed that bacterial ribosomes and ribosomal RNA can be immunogenic and protect against challenge with those strains (Fontages *et al.*, 1980).

The MDP is the smallest water soluble component of the mycobacterial cell wall with adjuvant activity. It enhances humoral immune response and is relatively non toxic, although it was reported to be pyrogenic (Allison & Byars, 1992). However, the safety was further improved by the synthesis of derivatives, in which the main structure of MDP is preserved. It is a potent adjuvant when administered together with the antigen in a water-in-oil emulsion, but is not as effective when formulated in an aqueous solution because it is rapidly excreted into urine. Therefore, several hydrophobic derivatives were synthesised with improved activity (Azuma, 1992).

An approach to oral immunisation involves the use of carrier organisms as a delivery system for antigens from other human pathogens, with a view to produce protective immunity against both the carrier organism and the pathogen for which it codes (Gilligan & Li Wan Po, 1991). The ability of *Salmonella typhi* and *Salmonella typhimurium* to invade, persist and proliferate in the human or murine gut-associated lymphoid tissue (GALT) before systemic infection occurs, has been known for many years (McGhee *et al.*, 1992). Investigators are currently using attenuated or avirulent, genetically modified strains of *Salmonella* as vectors for the selective delivery to the GALT of other antigens produced by such strains as a result of the introduction of coding genes (McGhee *et al.*, 1992). Examples of such vaccines are those being studied for use against herpes simplex, hepatitis B and tetanus (Bowen *et al.*, 1990; Fairweather *et al.*, 1990; McGhee *et al.*, 1992).

Cholera toxin (CT), the exotoxin produced by *Vibrio cholerae* and responsible for causing the diarrhoea associated with cholera disease, has proved to be a potent immunological adjuvant when administered orally to animals. The response is manifested by the production of specific secretory immunoglobulin A (SIgA) and serum IgG antibodies, with extended immunological memory (Wilson *et al.*, 1989; McGhee *et al.*, 1992). It is also classified by Holmgren *et al.* (1992) as the most potent mucosal adjuvant so far identified. Because of the high affinity of CT for the monosialoganglioside GM1-expressing cells, oral immunisation

induces a strong and highly specific local (SIgA) and systemic (IgG) immune response when administered in microgram amounts (McGhee *et al.*, 1992). Although extensively studied, there are still some aspects of CT's mode of action which remain unclear. For example, Wilson *et al.* (1989) have shown differences between mouse strains in the immune response to soluble proteins administered together with CT. Another observation was that CT does not fit the classical definition of an adjuvant because it stimulates an immune response against itself, and its adjuvanticity may be related to, and depended upon, its immunogenicity (Wilson *et al.*, 1989; McGhee *et al.*, 1992). Because of its toxicity, the use of the holotoxin (the native toxin) is not feasible. Liang *et al.* (1989) obtained more than 1000-fold reduction in toxicity through the treatment of CT with glutaraldehyde, and the molecule retained the adjuvant properties.

The use of the non-toxic subunit B of the cholera toxin (CTB) has been indicated to avoid the toxicity problems associated with CT. This subunit is the part of the molecule that binds to the GM1 receptor on the brush border membrane of the intestine, so that the adenylate cyclase-activating A subunit can be introduced into the epithelial cell (Lycke & Holmgren, 1986). The CTB is also the main immunogenic component of the toxin since the neutralising antibodies are mainly directed against it, in order to prevent the binding of toxin to the mucosal receptors. Studies indicate that the administration of protein antigens covalently coupled to CTB elicits strong mucosal and systemic antibody responses to the attached antigens (McKenzie & Halsey, 1984; Czerkinsky *et al.*, 1989; Holmgren *et al.*, 1992). More recently, Dertzbaugh & Elson (1993) concluded that CTB possesses unique properties that allow it to act as more than a simple carrier protein, acting as an adjuvant. Intranasal immunisation with a protein antigen mixed or covalently coupled with CTB is an effective means of generating IgA antibody responses expressed at several mucosal sites (Wu & Russel, 1993). On the other hand, Lycke & Holmgren (1986) were unable to stimulate immunity to keyhole limpet haemocyanin (KLH) when mixed to CTB, unless they added very small amounts of holotoxin. Similar observations were made by McKenzie & Halsey (1984) and Czerkinsky *et al.* (1989) when mixtures of CTB with protein antigens (horseradish peroxidase and *Streptococcus mutans* antigen) were administered orally. So, some doubt still exists as to whether CTB is an effective adjuvant, since Tamura *et al.* (1989a, b) showed that CTB inoculated intranasally with an influenza vaccine can induce a

high level of nasal and serum antiviral antibodies, required for the protection against virus infection. Also, these protective levels of antibodies were maintained for more than 16 weeks after inoculation. Using recombinant DNA technology, it is possible to produce a modified CTB molecule provided with peptide extensions corresponding to several foreign antigen epitopes, and some vaccine candidates, prepared using this technique, are currently being tested (Sanchez *et al.*, 1990; Holmgren *et al.*, 1992).

1.1.2.4. Synthetic Immunostimulants

Some synthetic immunostimulants are being studied by the pharmaceutical industry. Two of the most successful so far investigated are levamisole (L-[2,3,5,6-tetrahydro-6-phenylimidazo(2,1-*b*)thiazole], Janssen Research Laboratories) which is currently used as an anthelminthic, and inosiplex (a complex consisting of 3 molecules of N,N-dimethylamino-2-propanol-p-acetamidobenzoate and one molecule of inosine, Newport Institute for Medical Research), used as an antiviral. Both stimulate the immune system but, although associated with human therapy, they have not been used as vaccine adjuvants (Garrett, 1984; Martindale-The Extra Pharmacopoeia, 1989). They are regarded as immunomodulators - acting by modifying the functions of the host cells involved in the defence against invaders - rather than as immunoadjuvants.

Inosiplex enhances, both *in vitro* and *in vivo*, the proliferation of B and T lymphocytes in humans and animals. Also, the antibody production is increased in man and animals, as well as the phagocytic activity of macrophages (Garrett, 1984).

The first data concerning the immunostimulant activity of levamisole were published by Renoux & Renoux (1971). Mice immunised with *Brucella abortus* vaccine were completely protected against challenge with a lethal dose of the microorganism, if they were given levamisole in the vaccine. However, when administered alone, levamisole did not confer any protection (Renoux & Renoux, 1971; Garrett, 1984). On the other hand, it can be immunosuppressive as well as immunostimulative, according to the time at which it is given in relation to the antigenic challenge (Renoux & Renoux, 1972). Levamisole influences host

defences by modulating the cell-mediated immune responses. It restores depressed T cell functions, in particular it increases activated T lymphocytes (Rosenthal, 1977) but the humoral immune response is not affected, since it does not consistently enhance the antibody titres (Renoux, 1980; Garrett, 1984).

Another molecule that was shown to be an adjuvant for both cell-mediated and humoral immune responses is the quaternary amine dimethyl-dioctadecylammonium bromide, or chloride, (DDA). The anion does not seem to interfere with the adjuvant properties of the molecule. One study indicates that DDA is effective when administered together with different antigens, e.g. whole cells, proteins, viruses, etc., conferring on laboratory animals protection against several types of infections (Hilgers & Snippe, 1992). Toxicity of DDA is not known but severe detrimental side effects were not seen (Hilgers & Snippe, 1992).

1.1.2.5. Immunostimulating Complexes (ISCOMs)

Saponins are a naturally occurring complex group of molecules with surface activity which are obtained from a number of plant families. The adjuvant properties of saponins have been studied since the 1930s and preparations have subsequently found use in veterinary vaccines, particularly those against foot-and-mouth disease (Dalsgaard *et al.*, 1977). Saponins are now included as a constituent in an increasing number of licensed vaccines for veterinary use (Lindblad & Spärck, 1987), the first commercially available being that against equine influenza (Lövgren & Morein, 1988).

Saponins form micelles that can be manipulated in order to entrap antigens. They are the so-called ISCOMs (immunostimulating complexes) - first described as mixed micellar complexes between saponin and virus envelope proteins, with a defined circular structure of about 35 nm in diameter (Morein *et al.*, 1984). The general method of preparation involves the solubilisation of viral or bacterial membranes with a detergent followed by exchanging the detergent with saponin by ultracentrifugation on a saponin-containing sucrose gradient (Morein *et al.*, 1984). Later, Lövgren & Morein (1988) realised that cholesterol is essential for ISCOM formation, and indeed that ISCOMs can be prepared from this lipid in the

absence of any antigen. This is also possible with mixtures of cholesterol and phospholipids. Consequently, Bomford *et al.* (1992) classified ISCOMs as an expression of the nature of the interaction between saponins and cholesterol.

The South American tree *Quillaia saponaria* Molina produces the saponin Quil A which is a complex mixture that has been universally used as an adjuvant (Kersten *et al.*, 1989). In a recent study, it was found that apart from Quil A only the saponins from Gypsophila (e.g. *Gypsophila struthium* and *Gypsophila paniculata*) and *Saponaria officinalis* were adjuvant-active, perhaps because their structures resemble that of Quil A (Bomford *et al.*, 1992). Furthermore, a method was recently developed to solve the problem of incorporation of water soluble proteins into ISCOMs by attaching them to fatty acids (Browning *et al.*, 1992; Reid, 1992).

Several investigators have tested these compounds with a wide variety of antigens and the results appear to be very promising. The mode of action of saponins is not fully understood. Classically, saponins are recognised by their haemolytic activity, which may be related to complex formation with membrane cholesterol which is the cause of local inflammatory reactions. However, the inflammation caused by Quil A can be avoided by adsorption to cholesterol in liposomes without loss of adjuvant activity (Kersten *et al.*, 1989). This indicates that there must also be another mechanism of action other than induction of local inflammation. On the other hand, Bomford (1980) observed that the addition of liposomes containing cholesterol can abolish the adjuvant activity of Quil A, leading to the conclusion that whilst cholesterol binding is necessary for adjuvant activity, it is not sufficient (Bomford *et al.*, 1992). Several papers indicate that ISCOMs can enhance cell mediated immunity as well as humoral immunity (Kersten *et al.*, 1989; Browning *et al.*, 1992).

The obstacle to the use of ISCOMs in man is the lack of data on the safety of Quil A, since this is a mixture of many ill-defined compounds, mainly glycosides (Kersten *et al.*, 1989). Not only the haemolytic properties of saponins are involved in their toxicity. Their surface activity may lead to local necrotic reactions at the site of injection, when administered subcutaneously in too large quantities. These side effects reflect the detergent activity of

saponins (Lindblad & Spärck, 1987). Nevertheless, it should be noted that no negative side effects have been observed with the use of an ISCOM-based commercial equine influenza vaccine in horses, and that Quil A is accepted in veterinary vaccine preparations for pigs and cows at 10 to 100 mg doses, about 100 to 1000 fold higher than that used in ISCOM preparations (Claassen & Osterhaus, 1992).

1.1.2.6. Liposomes

In the past two decades the application of liposomes as a potential controlled release drug delivery system has gained wide interest. They are currently available on the market, for example, in a formulation for the delivery of amphotericin B. Liposomes are vesicles that are formed when dry phospholipids such as lecithin are confronted with excess water or aqueous salt solutions (Florence & Attwood, 1988; Buiting *et al.*, 1992). When first formed they are usually composed of several bimolecular lipid layers separated by an aqueous phase (MLV or multilamellar liposomes). Sonication of these units can give rise to unilamellar vesicles (SUV or small unilamellar liposomes). They differ with respect to their dimensions, composition (different phospholipids), charge (neutral, positive or negative), and structure (multilamellar and unilamellar). Many phospholipids, alone or in combination with other lipids (including lipid extracts from membranes), will form liposomes. Depending on their gel-liquid crystalline transition temperature (Tc - the temperature at which hydrocarbon regions change from a quasi-crystalline to a more fluid state), phospholipids determine bilayer fluidity and stability with respect to permeability to solutes *in vitro* and *in vivo* (Bangham *et al.*, 1965; Gregoriadis, 1990). Depending on the incorporation of different molecules the net charge on the liposomes can be varied. Water-soluble drugs can be encapsulated in these vesicles by dissolution in the aqueous phase while lipid-soluble drugs can be entrapped in the hydrocarbon interiors of the lipid bilayers (Shek, 1984). Liposomes were first used as a model for cell membranes, but their ability to entrap drugs, enzymes and proteins led to the formation of the concept of liposomes as drug carriers and many workers describe them as potent immunological adjuvants (Allison & Gregoriadis, 1974; Davis *et al.*, 1986). It has been established in several laboratories that liposomes can act as powerful immunological adjuvants inducing both cellular and humoral

immunity for a variety of bacterial and viral antigens relevant to human disease (reviewed by Gregoriadis, 1983, 1990, and Shek, 1984). It is generally accepted that a physical association between liposomes and antigens (as opposed to their simple mixing) is a prerequisite for adjuvanticity to occur (Gregoriadis, 1990). Such association can either be the actual encapsulation of the antigen or the simple adsorption onto their surface. Also, liposomes do not produce the side effects seen with other adjuvants. The attractiveness in the application of liposomes as carrier systems resides in the compatibility of the constituent components with the body system. They are biodegradable, nontoxic, immunologically inert, simple to prepare, and their composition can be varied to obtain more efficient antigen-liposome preparations (Buiting *et al.*, 1992). Immunity to antigens can be drastically improved, in some cases selectively, through the administration of liposomes together with other adjuvants, e.g. lipid A, MDP and DDA (Pierce *et al.*, 1984; Alving *et al.*, 1986; Kersten *et al.*, 1989). They are effective when administered by different routes such as subcutaneous, intravenous, intramuscular, intraperitoneal, intranasal and vaginal (Shek, 1984; El Guink *et al.*, 1989; Alpar *et al.*, 1992; Bowen *et al.*, 1992). It has been also reported that they increase specific SIgA immunity to antigens given orally (Pierce *et al.*, 1984; Wachsman *et al.*, 1985; Gregoriadis, 1990), as well as their uptake at the Peyer's patches (Childers *et al.*, 1990). Liposomes are able to induce cellular as well as humoral immune responses. Considering their fate *in vivo*, liposomes increase the humoral immunity through their ability to act as an antigen depot, supplying macrophages with free (released) or entrapped antigen. Their particulate form also plays an important role (see section 1.3.1.).

During the preparation of liposomes in an aqueous solution of the antigen, the latter can be encapsulated in the aqueous compartments of the vesicles, or it may be entrapped within the phospholipid bilayers themselves. In this later situation, part of the antigen molecule will probably be exposed on the outer surface of the liposomes and so recognised by lymphocytes with surface receptors for that antigen, and the immune response can be initiated. In the case of an antigen entrapped within the aqueous compartments, it will be completely protected and so recognition by antigen specific lymphocytes will be impossible (Van Rooijen & Su, 1989). In this case macrophages are required to process the liposome-entrapped antigen. The fact that macrophages are involved in the enhancement of the

immune response was shown by the lack of enhancement in animals depleted of their macrophages (Shek, 1984; Buiting *et al.*, 1992). The induction of a cell-mediated immune response appears to be related to the association of protein antigen to liposomal lipids, since hydrophobic antigens are more easily phagocytosed by macrophages and therefore their presentation to T cells is improved (Gregoriadis, 1990). The ability of liposomes to carry drugs to macrophages is demonstrated by their application in the treatment of certain intracellular infectious diseases (Croft *et al.*, 1991). Besides, the use of liposomes appears to be of great benefit in the reduction of the toxicity of some drugs e.g. amphotericin and adriamycin, and can be delivered either by the systemic or the local delivery routes (Roerdink *et al.*, 1987).

1.1.2.7. Microspheres

In the course of the last three decades microencapsulation of small particles in envelopes of polymeric, waxy, or other protective shell materials has become a well-established technology for coating and isolating substances until such time as their activity is needed (Donbrow, 1992a). Microencapsulation is a technique that involves the encapsulation of small particles of drug, or solution of drug in a polymer coat (Florence & Attwood, 1988), and any method which causes a barrier to deposit itself on the surface of a liquid droplet or a solid particle of drug, may be applied to the formation of microcapsules. Therefore, many so-called microencapsulation methods result in the formation of macroscopic 'beads' which are simply coated granules (Donbrow, 1992a). Its application to medicine and pharmacy has been extensively studied for taste masking, entrapment of irritant or unstable drugs and for sustained release of drugs.

Initially, microcapsules were produced mainly in sizes from 5 μm to as much as 2 mm, but since about 1980, a second generation of products of much smaller dimensions has been developed (Donbrow, 1992a). These include microparticles (microcapsules and microspheres) and nanoparticles (nanocapsules and nanospheres). The term microsphere can be used to describe small particles intended as carriers for drugs or other therapeutic agents. Their size can range from tens of nanometres up to one hundred microns or more.

Davis & Illum (1989) classified as nanoparticles or nanospheres the particles below 500 nm. Other scientists classify as nanoparticles the particulate carriers of a size range up to 1000 nm (Deasy, 1984; Donbrow, 1992a; Kreuter, 1992).

Table 1.1 - Some examples of microsphere/nanosphere systems described in the literature.

Material	Drug	Reference
Albumin	Mitomycin	Fujimoto *et al.* (1985)
Cellulose acetate propionate	Theophylline	Shukla & Price (1991)
Ethylcellulose	Cisplatin	Okamoto *et al.* (1986)
Ethylene-vinyl acetate copolymer	Proteins	Sefton *et al.* (1984)
Eudragit®	Ketoprofen	Goto *et al.* (1986)
Gelatin	5-fluorouracil	Oppenheim *et al.* (1984)
Polyacrylamide	L-asparaginase	Edman & Sjöholm (1982)
Polyanhydrides	Insulin	Mathiowitz *et al.* (1988)
Poly(butylcyanoacrylate)	Doxorubicin	Couvreur *et al.* (1982)
Poly(hydroxybutyric acid)	Sulphamethizole	Regina Brophy & Deasy (1986)
Poly(lactide-co-glycolide)	Progesterone	Beck *et al.* (1979)
Poly(methylmethacrylate)	Influenza virus	Kreuter & Speiser (1976)
Starch	5-fluorouracil	Lindell *et al.* (1978)

Strictly speaking, microspheres and nanospheres should be monolithic and perhaps also solid in nature. Microcapsules and nanocapsules are similar in many respects to microspheres and nanospheres respectively, but comprise small spheres that have a distinct layer enclosing a core material that could be the drug itself (Tomlinson, 1983). A rich variety of base molecules has been used to make microspheres/nanospheres which try to target specific sites in the body, releasing their payload of drug at the site of action in a

controlled manner (Oppenheim, 1988). These particulate carriers are of different physical characteristics depending on the use to which the systems are put (Tomlinson, 1983; Davis & Illum, 1989 - see table 1.1).

No description of this drug delivery system is complete without a brief introduction to the general methods of microencapsulation. The main procedures, having been extensively reviewed (Deasy, 1984; Donbrow, 1992a, b; Kreuter, 1992; Lehman, 1992; Thies, 1992) will be briefly treated in this thesis.

a) Coacervation-Phase Separation was the term used to describe the separation of macromolecular solutions into colloid-poor and colloid-rich layers (Florence & Attwood, 1988). The colloid-rich layer is present in a form of an amorphous liquid and constitutes the coacevate (Kayes, 1988). So, when this phenomenon occurs in the presence of a dispersed or dissolved drug, the latter will be encapsulated into the micelles formed. Florence & Attwood (1988) explain the process by saying that the liquid or solid to be encapsulated is dispersed in a solution of a macromolecule, such as gelatin, gum arabic, carboxymethylcellulose or poly(vinyl alcohol), in which it is immiscible. A non-solvent, miscible with the continuous phase but a poor solvent for the polymer under certain conditions, will induce the polymer to form a coacervate layer around the disperse phase (simple coacervation). Instead of stimulating coacervation by the addition of a non-solvent or salts ('salting-out'), the coacervate can be obtained by the use of another macromolecule incompatible under selected conditions with the first macromolecular species (complex coacervation). Because of its simple apparatus requirements for any operational scale, and the almost unlimited choice of polymers, coacervation-phase separation has been widely used in research. Drugs can be encapsulated as solid forms, aqueous solutions, insoluble liquids, crystal shape not being limiting (Donbrow, 1992a). The particle size of the resulting products range from large to below 40 μm for solids, and < 10 μm for liquid cores finely dispersed by mean of high-speed stirrers. Drugs may also be loaded after preparation of the carriers, by sorption procedures (Donbrow, 1992a).

b) Emulsion Polymerisation - The most important example of this method is the preparation of poly(isobutylcyanoacrylate) microspheres (Couvreur *et al.*, 1979b). A water-insoluble

monomer is polymerised as an oil-in-water emulsion. After the polymerisation the polymer will have the shape of the fine droplets of the previous emulsion. If a drug is present, polymerisation will occur around this core material. This is a method frequently used for the preparation of nanoparticles ranging from 1 to 1000 nm. The principal difficulties that are presented by this technique are the removal of contaminants such as unreacted monomer or reacting initiator at the end of the manufacturing process; its use is limited to polymers that can be formed in solution under the relatively mild conditions necessary to maintain drug stability (Watts *et al.*, 1990). Given the small size of the particles produced, the entrapment efficiency is low. So, some investigators use the adsorption of drugs onto the particles' surface to increase the load on the carriers (Harmia *et al.*, 1986; Davis & Illum, 1989; Forestier *et al.*, 1992). There is currently a formulation being assessed in clinical trials (Kattan *et al.*, 1992).

c) Spray-coating, Pan-coating and Spray-drying Methods - Generally these are called physical methods (Florence & Attwood, 1988), are among the earliest techniques used for preparing coated beads and have been improved recently (Deasy, 1984; Lehman, 1992). The technology is often used in the pharmaceutical industry because of its suitability for industrial scale production (Donbrow, 1992a). Spray-coating methods are only suitable for larger microcapsules (in the millimetre range) (Donbrow, 1992a). It was introduced several decades ago as a means of coating tablets and the method has been improved in order to produce microcapsules (Lehman, 1992). The classical pan-coating is one of the most frequently used procedures for microencapsulation (reviewed by Deasy, 1984). The spray-drying technique has been used in the preparation of microparticles (Bodmeier & Chen, 1988; Bruhn & Müller, 1991; Pavanetto *et al.*, 1992). Basically, in the process the core substance is dispersed in a solution of the coating material, which is then atomised and the solvent dried off using heated air in the spray-drier apparatus (Deasy, 1984). The solvent evaporates very quickly leaving solid microparticles. It allows the preparation of microspheres <10 μm (Pavanetto *et al.*, 1992).

d) Solidified Emulsion (Disperse Phase) - This group of techniques includes the most common method used to prepare microspheres and nanospheres such as those described in the present study. They involve the removal of volatile solvent from a multiphase emulsion,

which can occur by evaporation (solvent evaporation process) or extraction (solvent extraction process). The first of these methods was initially described by Beck *et al.* (1979) and consists of a conceptually simple procedure. First, a polymer solution-containing drug (either dissolved or in suspension) is emulsified into a second, immiscible liquid phase containing an emulsifier to form a dispersion of drug-polymer-solvent droplets. In the second step, the solvent is removed from the disperse droplets by the application of heat, vacuum, or by allowing evaporation at room temperature, to leave a suspension of drug-containing polymer microspheres that can then be separated by filtration or centrifugation, washed and dried (fig.1.1) (Watts *et al.*, 1990).

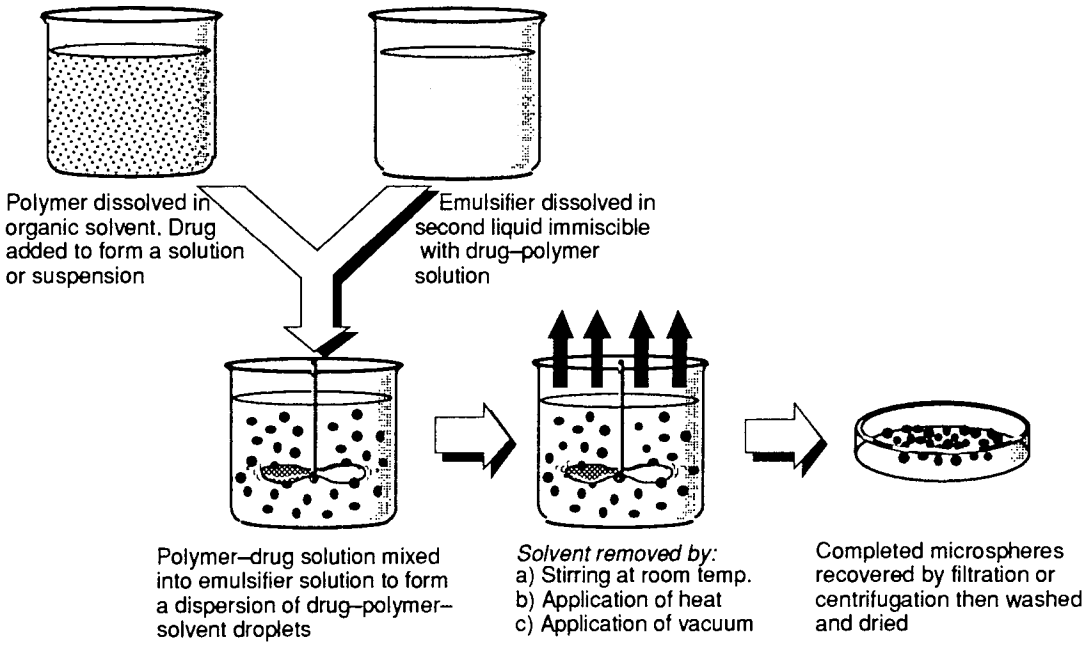

Figure 1.1 - Microsphere formation by emulsification/solvent evaporation (adapted from Watts *et al.*, 1990).

Modifications to this method have been introduced by many authors (Benita *et al.*, 1984; Cowsar *et al.*, 1985; Wichert & Rohdewald, 1990). The double emulsion technique of Ogawa *et al.* (1988) is the most interesting one because the loading of microspheres with hydrophilic drugs was improved (fig. 1.2).

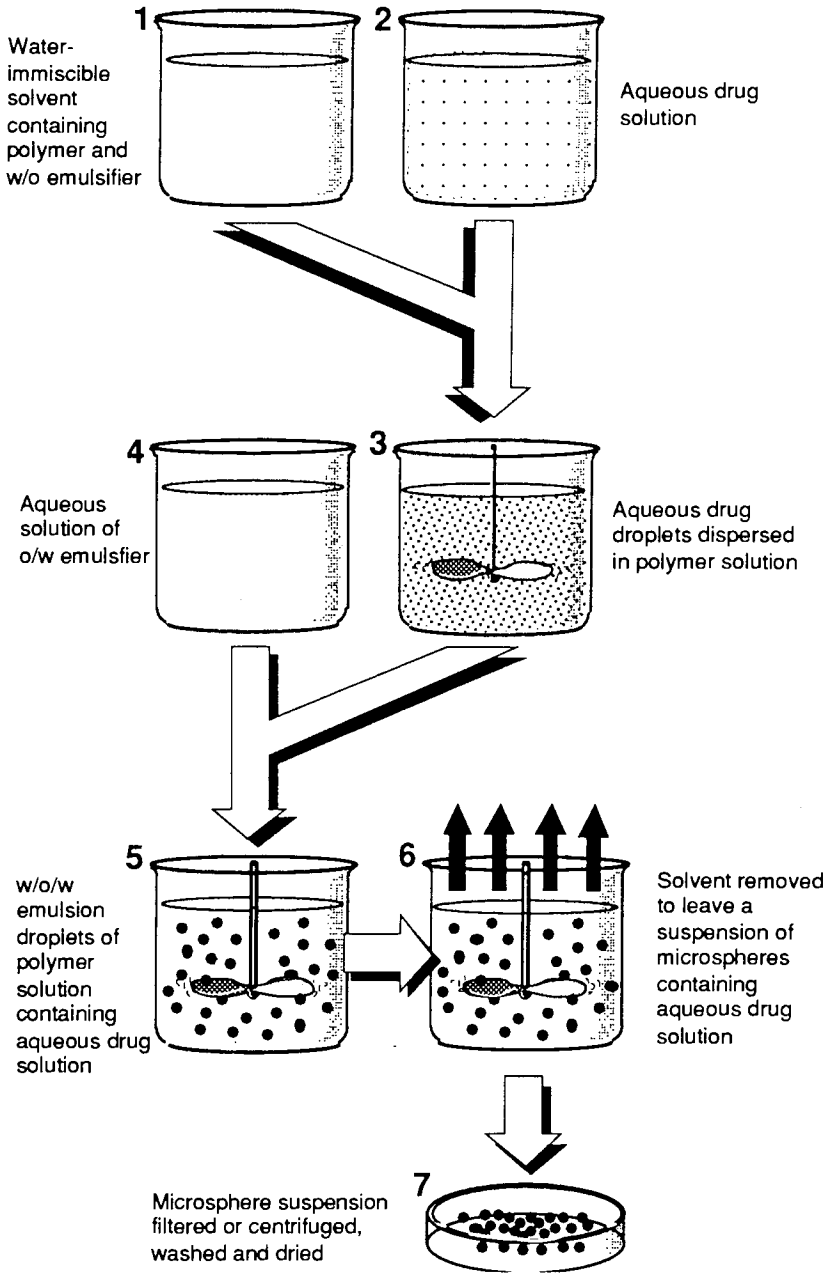

Figure 1.2 - Microsphere formation by the double emulsion [(w/o)/w] modification of the solvent evaporation method, as described by Ogawa *et al.* (1988) (adapted from Watts *et al.*, 1990).

The solvent extraction method (Cowsar *et al.*, 1985) involves the dissolution of the polymer and drug in an organic solvent, and the preparation of an emulsion as described previously. The volatile organic solvent is then evaporated until the microparticle walls become elastic. At this time the emulsion is poured into a large volume of deionised water or isopropanol to

extract quickly the remaining solvent from the microspheres (Cowsar *et al.*, 1985). These methods can be tailored to produce microspheres from less than 200 nm to several hundred microns, using a wide range of polymers and drugs with a suitable choice of solvents (Watts *et al.*, 1990).

There are several advantages resulting from the use of microspheres for the delivery of antigens. For example, given their degradation properties, the release of antigens from biodegradable microspheres can be tailored in order to achieve the best immune response (Gilligan & Li Wan Po, 1991). Two types of antigen delivery are currently under investigation within the WHO's Programme for Vaccine Development, using poly(lactide-co-glycolide) (PLGA) microspheres as vehicles and tetanus toxoid (TT) as antigen (Aguado & Lambert, 1992): continuous and pulsed antigen release, the second being closer to the conventional vaccine because one administration only can mimic the three appropriately spaced doses of TT adsorbed onto aluminium hydroxide. The experiments of Staas *et al.* (1991) with PLGA microspheres showed that copolymers of different ratios, together with an appropriate variation of particle size, can be used to deliver antigens at relatively predictable times, up to two years, resulting in a complete systemic immunisation from a single injection. A mixture of 1-10 μm and 20-50 μm PLGA microspheres stimulated both a primary and a secondary humoral responses to the microencapsulated toxoid of staphylococcal enterotoxin B (SEB), following a single injection (Eldridge *et al.*, 1991a).

Microspheres have been investigated as adjuvants with antigens adsorbed onto their surface or entrapped within the polymer matrix. The adjuvant properties of different types of microsphere preparations have been extensively studied. Birrenbach & Speiser (1976) significantly improved the antibody response in guinea-pigs against human IgG and TT by entrapping these antigens into poly(methylmethacrylate) nanoparticles. Kreuter & Speiser (1976) obtained similar results, this time using adsorption as a loading technique. These two initial reports were followed by many others on the use of polyacrylate nanoparticles as vaccine carriers, assessing several aspects of their adjuvanticity. For example, the use of nanoparticles as adjuvants for HIV-2 whole virus in mice (Stieneker *et al.*, 1991), and for ovalbumin adsorbed onto the particles' surface (O'Hagan *et al.*, 1989a) has been reported. It has been established that the particle size plays an important role on the adjuvant effect of

poly(methylmethacrylate) and polystyrene nanoparticles (Kreuter *et al.*, 1986). Working with particles of a size range of 62 to 306 nm, they found that smaller particles yielded a better immune response than larger particles, being also better adjuvants than aluminium hydroxide. When administered orally to mice, PLGA microspheres of 1-10 μm exhibited stronger adjuvant activity than those > 10 μm (Eldridge *et al.*, 1991b). The authors correlated these findings with the delivery of the 1-10 μm, but not the > 10 μm microspheres into the draining lymph nodes within macrophages. The effect of hydrophobicity was also studied by Kreuter *et al.* (1988) and it was found that the more hydrophobic particles were stronger adjuvants, probably because of a more efficient phagocytosis by antigen-presenting cells, confirming the findings obtained with bacteria by Van Oss (1978).

Another microsphere system used as a vaccine carrier was polyacrylamide which, by the intravenous and intraperitoneal routes, successfully enhanced the humoral immune response of ovalbumin and L-asparaginase, when compared to the soluble antigens (Edman & Sjöholm, 1982).

Apart from the depot effect resulting from the fixation of the antigen at the site of injection and, therefore, prolonging the contact with the immune system, microspheres when administered by the oral route will protect the antigen from proteolytic enzymes and will provide a means of targeted delivery of antigens to the mucosal associated lymphoid tissue (MALT) through their uptake by the Peyer's patches. Studies by LeFevre *et al.* (1980), Saas *et al.* (1990) and Jani *et al.* (1989, 1990, 1992a,b) have shown that latex microspheres and nanospheres were taken up from the intestinal lumen into the Peyer's patches, where M-cells pass them to the underlying lymphoid cells. Here, the antigen can trigger the mucosal and systemic immune responses. The possibility of using microspheres to stimulate the mucosal immune system has been assessed by many authors. The oral route was investigated for the delivery of polyacrylamide microspheres containing ovalbumin (O'Hagan *et al.*, 1989b) and the memory secretory IgA antibody response to the encapsulated protein was significantly raised, compared to the soluble antigen response. Following oral administration, ovalbumin associated with PLGA microspheres produced a salivary (IgA) and a serum (IgG) antibody responses approximately ten times higher than an

identical dose of soluble protein (O'Hagan *et al.*, 1992a).

Poly(lactide) (PLA) and PLGA microspheres are currently the most used particulate carrier system for vaccine delivery. A wide variety of antigens is under investigation and several routes of administration have been studied. Microencapsulated ovalbumin, the model antigen molecule studied by O'Hagan *et al.* (1991a) was shown to have an increase in serum IgG response after subcutaneous and intraperitoneal administration, even greater than that obtained with the same dose of protein in FCA. Strong antigens have also been under investigation. Diphtheria toxoid was microencapsulated into poly(DL-lactide) microspheres and implanted subdermally in mice (Singh *et al.*, 1991). The serum antibody response to this immunisation, obtained with 3 Lf of toxoid (1 Lf dose is the amount of a toxin which produces optimal flocculation with one unit of antitoxin) was found to be comparable to that obtained with three injections of toxoid (1 Lf each) with calcium phosphate as an adjuvant. As previously mentioned, increasing interest has also been focused on the microencapsulation of TT, particularly by the WHO. Recent papers have reported some formulation studies and immunisation experiments in animals (Alonso *et al.*, 1992; Esparza & Kissel, 1992; for review see Aguado & Lambert, 1992). An extensive study on the evaluation of PLGA-microencapsulated SEB toxoid has been carried out by Eldridge *et al.* (1989, 1990, 1991a,b). The first observation made was that, after oral immunisation, PLGA microspheres of 1-10 μm were taken up into the Peyer's patches. After absorption, the particles ≥ 5 μm remained in there for up to 35 days, whereas microspheres < 5 μm were transported by macrophages into the mesenteric lymph nodes and spleen. Unlike the soluble antigen, the particulate formulation induced an increase in circulating IgM and IgG specific antibodies and also enhanced the secretory anti-toxin response in saliva and gut fluid (Eldridge *et al.*, 1989). The size restriction of the absorption of microspheres at the Peyer's patches was again demonstrated by the same authors, matching the previous observations (Eldridge *et al.*, 1990). Simultaneously, they inferred that this fact would influence the type of immune response elicited by vaccine-containing microspheres administered by the oral route. So, the larger particles (> 5 μm) would remain in the site of absorption stimulating the mucosal immune response, and the smaller particles (≤ 5 μm), travelling to the lymph nodes, would be responsible for the systemic response. Although interesting, these findings are in contradiction with the values reported by Jani *et al.* (1990)

for the size of particles absorbed at the Peyer's patches (see section 1.3.1.). Further evidence of the adjuvant properties of PLGA microspheres was described by Eldridge *et al.* (1991a). After subcutaneous administration of 1-10 μm particles containing SEB toxoid, this preparation induced a serum IgG response that was approximately 500 times higher than that obtained with the soluble antigen. This effect was observed to be of a duration and magnitude similar to those induced by FCA, without any of the side effects that are intrinsic to Freund's adjuvants (Eldridge *et al.*, 1991b). After oral immunisation, and using the same type of formulation with influenza virus vaccine, Moldoveanu *et al.* (1993) could not only induce high levels of specific IgA in saliva, but also protect mice against infection. This shows that PLGA microspheres protect the antigen from degradation by the gastric acid and that the encapsulation process does not affect the immunogenicity of this antigen.

1.2. THE LACTIDE/GLYCOLIDE POLYMERS

In recent years there have been advances in the study of microspheres and nanoparticles as drug carriers. Poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and their co-polymers - poly(lactide-co-glycolide) (PLGA), are biodegradable compounds that have found particular medical application as resorbable surgical sutures and prostheses. They have also been used for surgical implants and in dental and orthopaedic applications (Tice & Cowsar, 1984). Their low toxicity allows their use as drug carriers. As described by Wade *et al.* (1977), poly(DL-lactide) caused a minimal reaction after implantation in rat muscular tissue, and no significant reaction in organ and tissue cultures were observed.

They biodegrade by undergoing random, non-enzymatic hydrolytic de-esterification to form lactic acid and glycolic acid, which are normal metabolic compounds that produce carbon dioxide and water (fig. 1.3) (Tice & Cowsar, 1984; DeLuca *et al.*, 1987).

The success of PLA as a suture and its approval by the Food and Drug Administration (FDA) for human use motivated scientists to investigate its possible application to drug delivery systems (Wise *et al.*, 1978; Rosen *et al.*, 1988), the first papers being published in the early 1970s (Yolles *et al.*, 1970). Their use as microspheres' excipients was also

described in the same decade (Beck *et al.*, 1979).

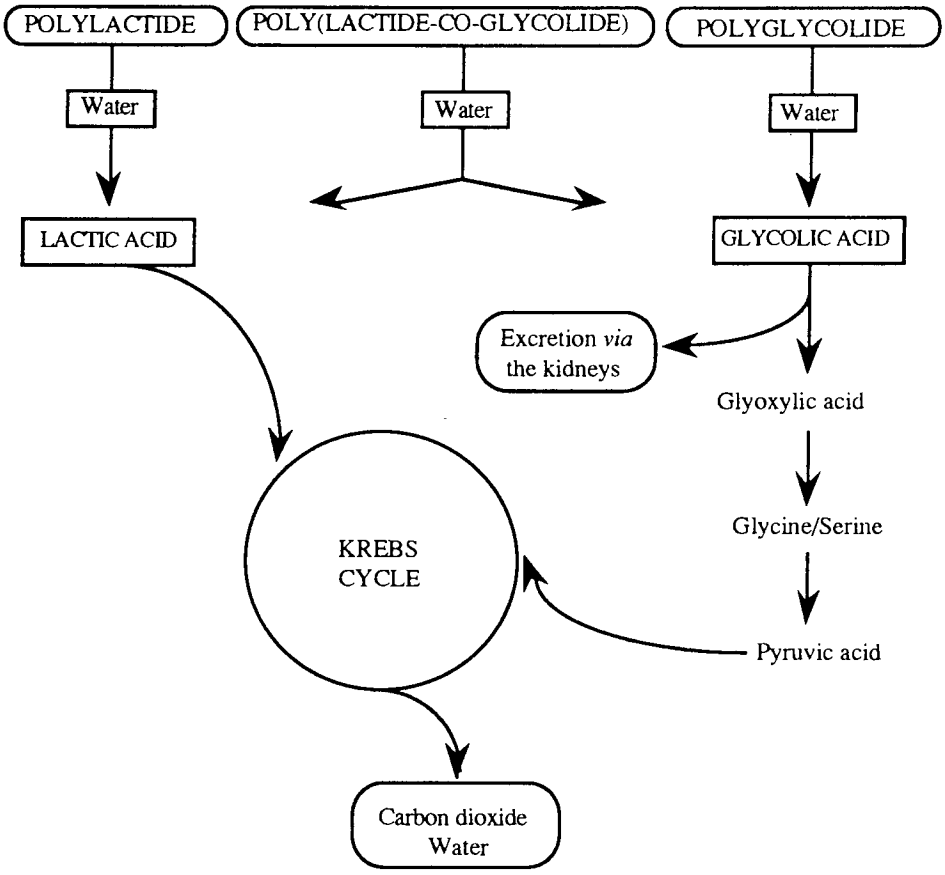

Figure 1.3 - Degradation of PLA, PGA and copolymers (PLGA) in living systems (adapted from Literature Catalogue no BI 26, Alfa Chemicals Limited, Preston, U.K.)

The preparation of these polymers is a difficult process. The production of high molecular weight polymers and copolymers of lactic acid and glycolic acid is very difficult using conventional condensation techniques due to the stepwise nature of the condensation reaction (Cowsar *et al.*, 1985). Usually the polycondensation mechanism is limited to lower molecular weights and therefore high molecular weight polymers and copolymers of these materials are prepared by ionic ring-opening polymerisations of the corresponding cyclic dimers, using stannous octoate or tetraphenyl tin as preferred catalysts (Gilding & Reed, 1979; Cowsar *et al.*, 1985; Deasy *et al.*, 1989) (see fig. 1.4). Lactide is the dimer of lactic

acid and glycolide is the dimer of glycolic acid, so the polymers synthesised from these lactones are called poly(lactide), poly(glycolide) and poly(lactide-co-glycolide).

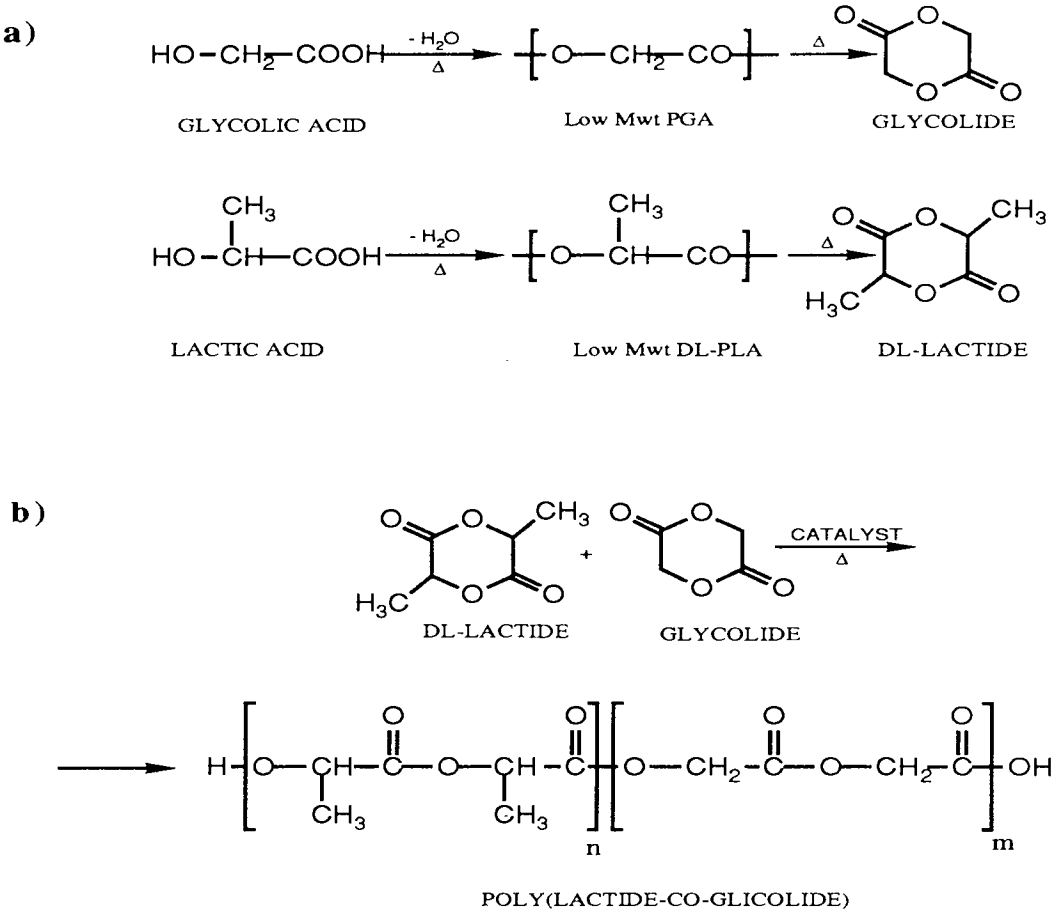

Figure 1.4 - a) Synthesis of glycolide and DL-lactide monomers; b) Ring-opening copolymerisation of DL-lactide and glycolide monomers (adapted from Cowsar *et al.*, 1985).

Because lactic acid is optically active, the homopolymer formed can be poly(D-lactide), poly(L-lactide) and the racemic poly(DL-lactide) (Rosen *et al.*, 1988). Primarily, polymers with L- and DL-lactide have been used for controlled release delivery systems (Tice & Cowsar, 1984). These polymers dissolve in common organic solvents such as chlorinated hydrocarbons, aromatic hydrocarbons and cyclic ethers, making them easily processable. Poly(glycolide) and copolymers of high glycolide content are relatively insoluble in common organic solvents which can limit their suitability for use in many manufacturing

processes (Deasy *et al.*, 1989). On the other hand, when glycolide is randomly copolymerised with DL-lactide, copolymers containing up to approximately 60% glycolide readily dissolve in common organic solvents. This is the main reason for the incorporation of glycolide in the polymers in a successful attempt to regulate the copolymer's rate of biodegradation (Tice & Cowsar, 1984). Miller *et al.* (1977) studied the difference in rate of degradation *in vivo* between PLA, PGA and several ratios of copolymers. Poly(DL-lactide) generally presents a half-life *in vivo* of about six months (fig. 1.5). The addition of up to 50% glycolide in the polymer chain will accelerate the biodegradation rate until, at its fastest, resorption takes approximately one week (Miller *et al.*, 1977).

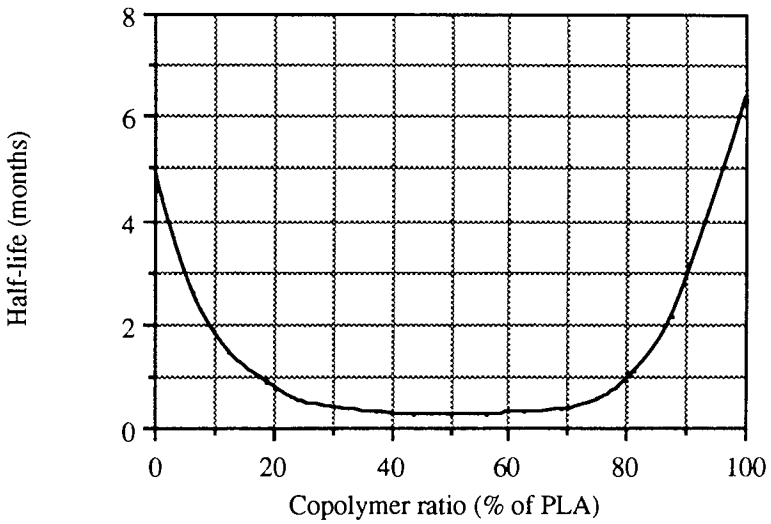

Figure 1.5 - Half-life *in vivo* for poly(lactide-co-glycolide). Samples implanted in rat tissue (adapted from Miller *et al.*, 1977).

PLGA belongs to the type III erosion polymers proposed by Heller (1984), by which high molecular weight water-insoluble macromolecules are converted to small water-soluble molecules by a hydrolytic cleavage of labile bonds in the polymer backbone. The *in vitro* degradation mechanism of PGA in the form of surgical sutures, has been also studied by Chu (1981) and hydrolysis was found to be the cause of erosion. The higher rate of degradation observed in buffered solutions, when compared to unbuffered, was described

to be due to the buffer itself, which neutralised the degradation products, shifting the reaction towards increased hydrolysis. The same type of study was carried out by Makino *et al.* (1986) using poly(L-lactide) microspheres. In strongly alkaline solutions the degradation was very fast, suggesting that the cleavage of ester bonds occurs anywhere on the polymer molecule, in this medium. These findings are in accordance with features of general acid-base catalysis, because the cleavage of ester bonds takes place by different mechanisms, depending on the pH of the medium. This is due to the reactivity of water molecules, because they are likely to penetrate into the polymer to allow OH^- and H^+ to attack the ester bonds (Makino *et al.*, 1986). The same authors state that the ionic strength of the degradation medium also plays an important role because as the concentration of salts increases, the acidic degradation products are more rapidly converted into neutral salts by combining with ions present in the medium.

Homogeneous erosion at a similar rate has been observed *in vitro* as well as *in vivo*, which implied that the degradation process is non-enzymatic (Pitt *et al.*, 1981). *In vivo*, the first stage of the degradation process involves non-enzymatic, random hydrolytic ester cleavage, and its duration is determined by the initial molecular weight of the polymer. The non-enzymatic nature of the initial chain degradation process was demonstrated by the observation of the same process in distilled water. A different opinion about the influence of the molecular weight was given by Zhu *et al.* (1991), who studied the degradation of PLA and PLGA microspheres at pH 7.4/37°C. After analysing several parameters including the amount of lactic acid and glycolic acid produced, the decrease in molecular weight of the polymers, they concluded that degradation depends mainly on the content of glycolide and not on the polymer molecular weight.

Drug permeability through this type of polyester is low and release may be measured in days, weeks or even months. The release of drug results from a combination of diffusion and polymer erosion (Watts *et al.*, 1990). Some general principles of drug release common to any microsphere system can also be observed with these polymers, e.g. the release rate will increase with a decrease in particle size as a result of the increase in surface area, and the rate of drug release also tends to increase with increased drug loading. Theoretically, this

should only apply to systems where there is some drug present in particulate form, because as drug particles dissolve, pores will be created and the higher number of dissolved particles thereby creates a more porous internal structure (Watts *et al.*, 1990). If the permeability of the drug in the polymeric excipient is extremely low, the rate of release of drug by diffusion may be too low to be effective and alternative mechanisms should be considered. Water-soluble drugs or molecules with high molecular weight, such as proteins, encapsulated in poly(lactide-co-glycolide) are two examples. For these formulations, drug release can be achieved by excipient erosion, which frees the entrapped drug, or by allowing the drug to permeate through pores or water-filled channels in the polymer before or after administration of the microspheres (Tice & Cowsar, 1984).

1.3. NASAL IMMUNISATION USING PARTICULATE CARRIERS

1.3.1. Mucosal Uptake of Antigens

It is clear that amounts of protein are absorbed at sites which have the potential to produce an immune response following oral administration. To stimulate the immune system antigens must first gain access to lymphoid tissues. As early as 1972, Walker *et al.* found that the intestinal absorption of horseradish peroxidase and bovine serum albumin was significantly decreased after oral immunisation. Presentation of antigen by the enteric route appears principally to prime the mucosal system. The nature of the orally-administered antigen may affect the secretory antibody response achieved e.g. oral immunisation with particulate antigen inducing higher salivary antibody levels than the corresponding soluble antigens (Cox & Taubman, 1982, 1984). This is perhaps due to the greater ability of particulate material to gain access to the tissue of the Peyer's patches. However, this possibility is denied by uptake studies comparing ovalbumin with bacterial cells (Wold *et al.*, 1989). Although less immunogenic, ovalbumin was more efficiently absorbed than an *Escherichia coli* strain. On the other hand, when injected directly into the Peyer's patches, the same difference in antigenicity was observed. The authors suggested either that the GALT can distinguish between food and bacterial antigens or that the particulate nature of the bacterial antigen makes it more efficiently phagocytosed by antigen presenting cells (e.g

macrophages). Soluble antigens are taken up to some extent by microfold epithelial cells (M cells) but a greater proportion will pass through the gut lamina propria into the venous system and initiate a systemic antibody response (Lim & Rowley, 1982). Large protein molecules (ferritin and adenovirus type 5 particles) were absorbed at the jejunal tissues *via* pinocytosis by enterocytes. None of them was found elsewhere in the mucosa. Only in rats deprived of dietary protein could there be observed a paracellular transport of polypeptides, perhaps because of deterioration of intercellular junctions due to protein deficiency (Worthington & Syrotuck, 1976). Absorbed protein can be detected in both intestinal lymph and systemic (peripheral) blood (Warshaw *et al.*, 1971). The fact that antigenic protein reaches the lymphatics from the intestinal lumen provides a mechanism for the direct sensitisation of the regional lymphoid tissue of the gut and results in an immunologic reaction. Concerning the respiratory tract, investigations on the absorption of soluble antigens by the respiratory epithelium overlying the BALT, showed that uptake is not restricted to BALT-associated epithelium, but it is faster at the sites where the BALT is present and only here penetration beyond the basement membrane was seen (Gregson *et al.*, 1982). In an earlier study, proteins were transported in vesicles through epithelial cells and no penetration of the intercellular tight junctions was found (Richardson *et al.*, 1976).

Antigens that are responsible for the induction of protective immunity against various pathogens may be attached to a suitable particulate carrier with adjuvant (or sustained release) properties to induce effective and long lasting local immunity by interacting with mucosal surfaces, or for inducing protective systemic immunity upon reaching immune competent organs (Gregoriadis, 1990). Evidence suggests that intragastric administration of soluble antigens associated with liposomes potentiates the humoral immune response expressed in saliva and serum (Wachsman *et al.*, 1985). This could be due to the possibility that soluble antigens, incorporated into liposomes, may stimulate the immune response in a manner similar to that of a particulate antigen. Wachsman *et al.* (1986) were also able to stimulate IgA memory, probably due to residual antigen in Peyer's patches, as well as stimulating systemic immunity. Although the interaction with bile acids in the gastrointestinal tract can be destructive (Kreuter, 1991), liposomes may be resistant and be taken up by the Peyer's patches (Childers *et al.*, 1990).

PAGES 47 & 48

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called follicle-associated epithelium (FAE) (Bienenstock, 1988). This epithelium selectively absorbs soluble antigens and particulate matter by endocytosis (at the M cells), with subsequent transport into the follicle of apparently undigested material. Above the basement membrane within the epithelium are intraepithelial B and T lymphocytes. Also within this compartment are non-adrenergic, non-cholinergic nerves. The mast cells found below the basement membrane and sometimes in the epithelium are of a specific phenotype known as the mucosal mast cell which differs from its connective tissue counterpart. The complex interactions between these components together regulate homeostasis in a mucosa and provide efficient protection (Bienenstock, 1988) (fig. 1.6).

The principal mechanism of protection at the mucosal sites relies on the action of secretory IgA (SIgA), that is produced *in situ*. The SIgA molecules exert an important function on mucosal immunity. They resist proteolysis and mediate antibody-dependent T cell-mediated cytotoxicity; inhibit microbial adherence, colonisation and penetration, as well as food antigen uptake; neutralise biologically active antigens, such as toxins; activate complement (reviewed by Holmgren *et al.*, 1992; McGhee *et al.*, 1992). Figure 1.6 shows the basic mechanism of entry of an antigen into the body through the gut, and the subsequent SIgA production. The SIgA is synthesised as a dimer by B lymphocytes, that enter the Peyer's patches through the endothelial venules. This synthesis occurs in the lamina propria and dimeric SIgA is transported *via* its specific ligand, called the secretory component, across the epithelium to the surface where it is incorporated into the mucous blanket. Excess secreted IgA in the tissue fluid finds its way to the lymphatics and thence to the circulation. The secretory component is a peptide expressed on the basolateral and apical epithelial cell membranes. Its interaction with SIgA results in endocytosis and transport across the epithelial cells until release at the luminal surface. Here, the secretory component protects SIgA from proteolysis (Bienenstock, 1988; Holmgren *et al.*, 1992).

The mucosal immune response was first considered as a local phenomenon occurring only at the site of contact with the antigen, with no effect at the other mucosal surfaces. However, this is a more generalised process from which derives the name 'common mucosal immune system' (Mestecky & McGhee, 1987). Antigens enter the MALT at the lymphoid aggregates, being phagocytosed or pinocytosed by the M cells, and are

transported to the lymphoid tissue which contains T and B lymphocytes as well as antigen presenting cells (macrophages, dendritic cells and B cells). After antigenic stimulation, B and T cells migrate to the regional lymph nodes and then, after further differentiation, to the circulation, through the thoracic duct. They will eventually home on to the various mucosal surfaces and exocrine glands, e.g. BALT, mammary glands, salivary glands, lacrimal glands and urogenital mucosa, where they start the production of the specific SIgA (for review see McGhee *et al.*, 1992). Macrophages can also migrate to the regional lymph nodes contributing to the enhancement of the immune response (Corry *et al.*, 1984).

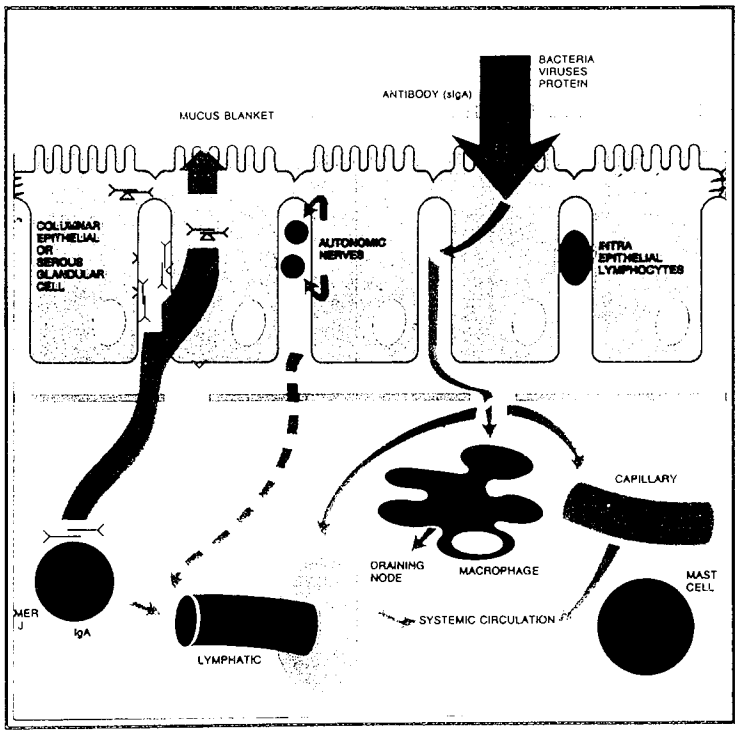


Figure 1.6 - Antigen processing in the gut (adapted from Bienenstock, 1988). The site of entry of bacteria, viruses and proteins is also the site of absorption of particulate carriers of antigens such as liposomes and microspheres, i.e. the M cells of the Peyer's patches, as described by Saas *et al.* (1990).

The common mucosal immune system includes, apart from the SIgA production, a cell-mediated immune response which, like the humoral mucosal immunity, seems to function,

at least partially, independently of its systemic counterpart. Although not completely understood, it is reported to include MHC-restricted cellular toxicity, natural killer cell activity and antibody-dependent cell-mediated cytotoxicity. This occurs particularly in the intestinal tract, acting against invasive pathogens (Holmgren *et al.*, 1992).

1.3.3. The Nasal Route of Administration

The use of the nasal route was, until recently, limited to the administration of locally active drugs, e.g. vasoconstrictors and antihistamines. However, some workers have tried to use the nasal mucosa as a route for systemic delivery and, in some cases, the results appear to be as good as those obtained with the parenteral route. For example, the bioavailability of dobutamine was found to be theoretically equivalent to the intravenous infusion route (Su *et al.*, 1987). Similar results were reported by Illum *et al.* (1988), who studied the nasal absorption of gentamicin. Nitroglycerin is another example of a drug considered for nasal delivery (Hill *et al.*, 1981). The nasal administration of drugs exploits the higher permeability of the nasal mucosa when compared to other mucosal surfaces (Watanabe *et al.*, 1980; Illum *et al.*, 1988). It was classified by Cremaschi *et al.* (1991) as a leaky epithelium that allows restricted passive permeation of high molecular weight molecules through intercellular pathways. Among the advantages offered by this route are the valuable mucosal surface of approximately 150 cm^2, the accessibility and easy administration that increases patient compliance. The mucosa is highly vascularised and the venous flow that drains it escapes the portal system, allowing the blood to reach various tissues and organs before the liver, thus preventing a first-pass metabolism. There is also typically a rapid onset of pharmacological action after nasal administration. Finally, it can be an ideal route for the administration of products that undergo degradation in the gastrointestinal tract or gut wall metabolism (for review see Bond, 1987; Duchêne & Ponchet, 1993).

Much of the research in this field has been focussed on the delivery of peptides of both low and high molecular weight that usually show poor oral absorption. Some of them were investigated nearly 30 years ago, e.g. oxytocin was nasally delivered to human volunteers

by Talledo *et al.* (1964). More recently, studies were reported on the nasal absorption of insulin (Rydén & Edman, 1992), interferon (Greenberg *et al.*, 1978) and encephalin analogues (Su *et al.*, 1985). However, due to the natural mechanisms of defence and maintenance of the nasal mucosa, several problems arise when attempts are made to deliver proteins. The mucociliary clearance and the presence of enzymes, particularly proteases, although being important non-immunological ways to prevent microorganisms from colonising the mucosa, can become a problem difficult to solve. Nevertheless, it should be noted that the level of proteolytic enzymes in the nasal mucus is lower than in the gastrointestinal tract (Zhou & Li Wan Po, 1991). Because such mechanisms represent a barrier against the penetration of microorganisms and toxic molecules (even drugs or excipients), they must be preserved.

To improve the uptake of peptides at the nasal mucosa, several absorption enhancers have been investigated, acting through different mechanisms such as avoiding or delaying mucus clearance through the increase of viscosity of the formulations. This approach produces a more sustained effect of the drug, but the onset of activity is delayed and no enhancement was achieved in the total bioavailability (Harris *et al.*, 1989). Mucoadhesive powders (hydroxypropyl cellulose, carbopol®) were also successfully used to enhance the absorption of insulin, the decrease in plasma titers of glucose being superior to that obtained with the intravenous control (Junginger, 1990). Bioadhesive microspheres (serum albumin, starch or diethylaminoethyl-dextran) were described as nasal dosage forms, acting on the basis of the gelling properties of the microsphere system (Illum *et al.*, 1987b). Some bile acids (e.g. sodium glycocholate, sodium dehydrocholate) increase the uptake of drugs and were found to cause no membrane damage and no effect on ciliary movement (Gizurarson *et al.*, 1990). Dimethyl-β-cyclodextrin (Schipper, *et al.*, 1990) and phosphatidylcholine (Gizurarson *et al.*, 1990) were also studied as absorption enhancers.

1.3.4. The Nasal-Associated Lymphoid Tissue (NALT)

The discovery of a nasal-associated lymphoid tissue appears to be an obvious consequence

of the research efforts described in sections 1.3.1 to 1.3.3, since the nasal mucosa is the first site of contact with inhaled antigens. However, the study of the mucosal immunity of the upper respiratory tract was neglected for a long time, perhaps due to the absence of a suitable animal model (Kuper *et al.*, 1992). The emphasis was on the lower respiratory airways i.e. the BALT, so extensively investigated (Tenner-Rácz *et al.*, 1979; Bienenstock, 1984; Van der Brugge-Gamelkoorn *et al.*, 1986). Evidence that the nasal administration of antigens can be a useful route of immunisation has been provided since the 1920's, when Peters & Allison (1929) investigated the possibility of inducing immunity to scarlet fever by repeated applications of erythrogenic toxin to the nasal mucosa. From the group of 62 humans involved in the study, 46 showed immunity to some extent. An attempt to immunise male volunteers against an inactivated strain of rhinovirus resulted in protection of the individuals given vaccine nasally, whereas the frequency with which illness developed in the volunteers administered intramuscular vaccine did not differ from that of the seronegative controls (Perkins *et al.*, 1969). These had developed only serum antibodies, while the volunteers who were immunised nasally developed both serum and secretory antibodies. Vaccination was investigated by administering TT together with colloidal silicon dioxide, as a gelling agent, and high concentrations of SDS, as a penetration enhancer; the results were comparable with parenteral immunisation (reviewed by Junginger, 1990, and Duchêne & Ponchet, 1992). Studies like these and some descriptions of lymphoid aggregates in the rat nasal mucosa led to the works of Spit *et al.* (1989), who characterised the lymphoid accumulations that exist at the left and right side of the nasal entrance of the pharyngeal duct. The scanning electron microscopic analysis of the lymphoid tissue showed the presence of both B and T lymphocytes that were closely associated with the epithelium. The presence of M cells was also observed and due to the similarities in structure and function to both the Peyer's patches and the lymphoid aggregates of the BALT, Spit *et al.* (1989) introduced the concept of NALT (fig. 1.7).

In humans, tissues equivalent to the NALT are known as Waldeyer's ring and consist of the adenoid or nasopharyngeal tonsil, the bilateral pharyngeal lymphoid bands, the bilateral tubal and palatine tonsils and the the bilateral lingual tonsils (Kuper *et al.*, 1992). Similar tissues have been found in several other animal species, including monkeys, horses, sheep and cattle (Chen *et al.*, 1991; Kuper *et al.*, 1992). The absorption of 1-5 μm diameter

particles by the tonsils in calves (Payne *et al.*, 1960), together with the penetration of mucosal surfaces by pathogenic bacteria, including experimental infections (Moxon *et al.*, 1974; Salit *et al.*, 1984), indicated the existence of a particle uptake pathway at the nasal mucosa. Active transport of polypeptides (≈4000 Da) in an area of the rabbit nasal mucosa with structures "resembling Peyer's patches" was observed by Cremaschi *et al.* (1991), leading to their hypothesis relating active transport of proteins to antigen sampling. The NALT exists in mice and hamsters (Kuper *et al.*, 1992). However, much of the function of the NALT and its place in the general mucosal immunity remains obscure (Spit *et al.*, 1989).

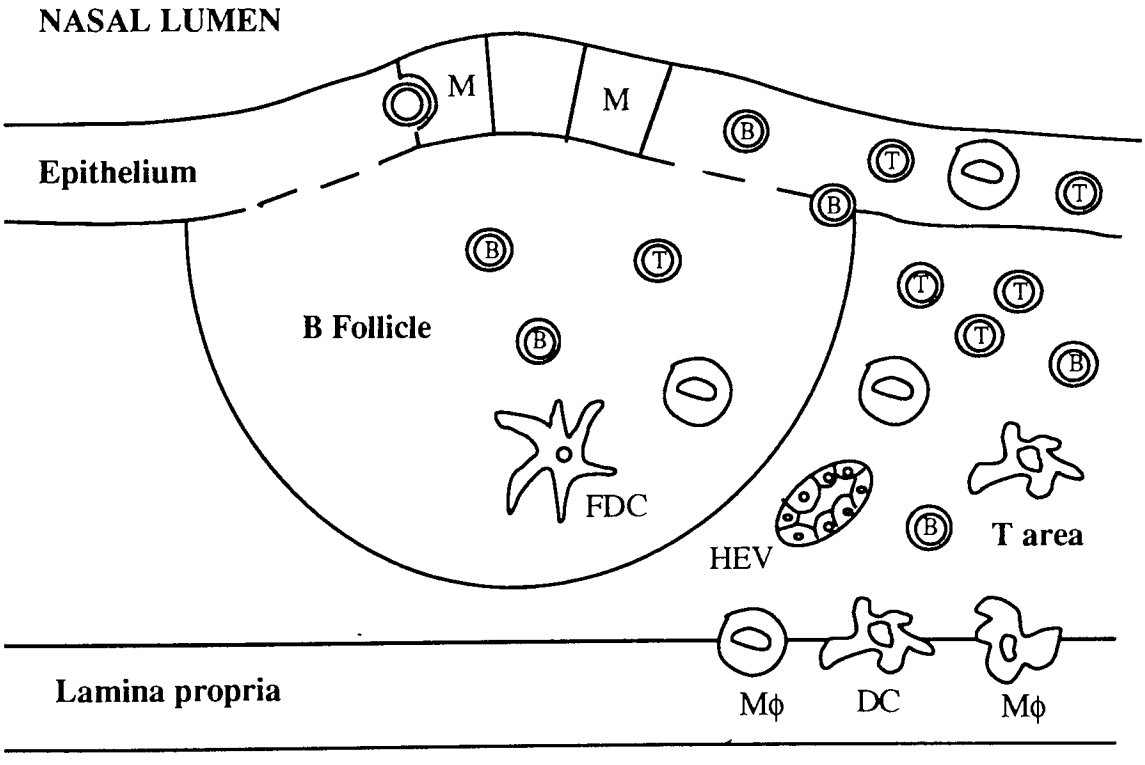


Figure 1.7 - Simplified diagram of the composition of the NALT (adapted from Kuper *et al.*, 1992). Legend: B - B cell; T - T cell; M - M cell; Mϕ - macrophages; DC - dendritic cell; FDC - follicular dendritic cells; HEV - high endothelial venules.

Briefly, nasal inoculation results in uptake of particulate antigens mainly by the M cells of the lymphoid tissue, while soluble antigens are mainly absorbed at the nasal epithelium (fig.

1.8). Antigens of the first type will be processed at the NALT and preferentially drained to the posterior cervical lymph nodes (PCLN). After uptake at the nasal epithelium, soluble antigens contact antigen presenting cells that will carry them to the superficial cervical lymph nodes (SCLN), which in turn drain to the PCLN. At the SCLN soluble antigens may induce a systemic immune response or a status of specific tolerance. On the other hand, as PCLN are involved in the enhancement of a secretory immune response these antigens can also induce this type of immunity. The final result of a NALT stimulation will depend on the balance between the activation in the posterior or superficial cervical lymph nodes (Kuper *et al.*, 1992). Therefore, given the sites of preferential uptake, the nature of the antigen plays an important role in the ultimate response.

Attempts to immunise animal models using the nasal delivery route have been made recently, some of them successful. The research efforts have been centred around infections that affect the respiratory tract. An influenza vaccine composed of liposome-entrapped glycoproteins from the envelope of influenza virus was administered nasally, inducing a strong systemic immunity and a local (nasal wash) SIgA response that protected against virus challenge (El Guink *et al.*, 1989). The nasal administration of CTB as a vaccine adjuvant can produce persistent local and systemic antibody responses to influenza vaccine (Tamura *et al.*, 1989b). Using the same adjuvant, Wu & Russel (1993) successfully enhanced specific antibody titres in serum, saliva, and in the tracheal, gut and vaginal washes of mice, against a streptococcal antigen. The nasal dosing with TT-containing liposomes produced serum IgG titres similar to those obtained with a $\times 10$ lower dose given by intramuscular route (Alpar *et al.*, 1992). In the same study, liposomes were absorbed and found in liver, spleen and lungs until 48 hours after administration.

The difference in function between the NALT and the other MALT structures, as well as the hypothetical mechanisms of antigen processing and elicitation of the immune response were reviewed by Kuper *et al.* (1992).

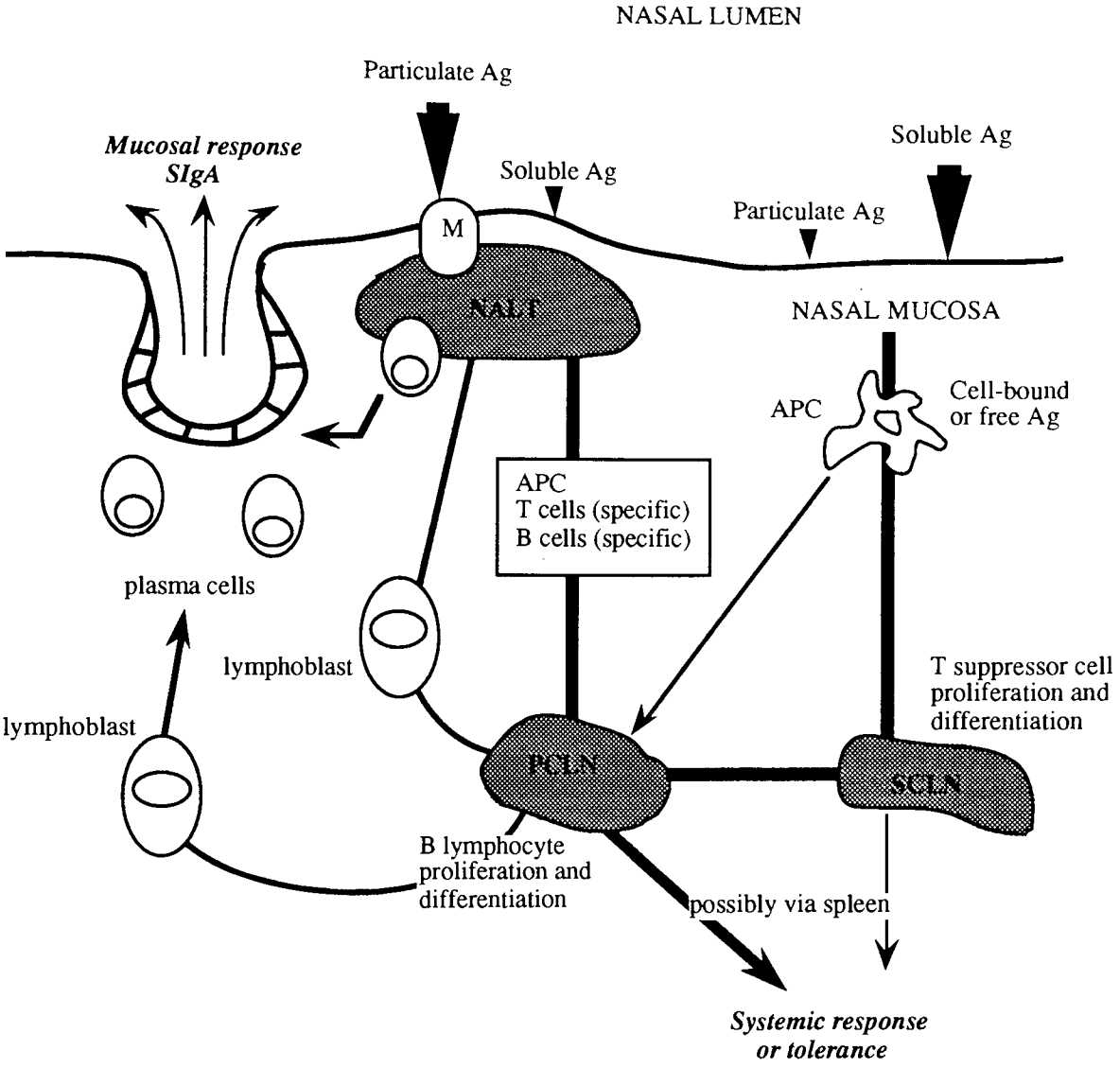


Figure 1.8 - Diagram of the hypothetical mechanism of antigen processing and eliciting of the immune response after antigenic stimulation of the NALT (adapted from Kuper *et al.*, 1992). Legend: APC - antigen-presenting cell; M - M cells; PCLN - posterior cervical lymph node; SCLN - superficial cervical lymph node.

The role of the NALT in eliciting systemic and local immunity to soluble and particulate antigens was studied by Hameleers *et al.* (1991). The results obtained with the soluble antigen confirmed the mechanism proposed by Kuper *et al.* (1992) by which the immune response is mainly started at the PCLNs. However, no immune response was induced by the sheep red blood cells (SRBC), studied as a model particulate antigen. The authors suggested that the lack of response was due to: a) SRBC are non-replicating antigens and therefore unable to colonise the mucosa in order to produce a strong immune response,

attributing to the NALT the ability to distinguish between replicating and non-replicating antigens; b) the very efficient mucociliary clearance of particles. Since SRBC are particles of about 7 μm in diameter, they are too large to be taken up by M cells, as shown by Jani *et al.* (1989, 1990, 1992a, b) in a similar MALT component. Even the publications that describe the uptake of larger particles (Eldridge *et al.*, 1990), report a cut-off point of 5 μm for the stimulation of a systemic immune response. Therefore, this may be the main reason for the poor immunogenicity of SRBC, not as suggested by the authors.

1.4. ORIGIN AND SCOPE OF THE WORK

Drug delivery systems are designed to enhance the efficiency of therapeutic agents which may result in enhanced bioavailability, enhanced therapeutic index, reduced side-effects and improved acceptance. Conventional vaccine delivery presents problems which are related both to patient compliance and to the adjuvants employed. For example, aluminium adjuvants are widely used and are difficult to manufacture in a physicochemically reproducible way, and so variations may exist between different batches of the same vaccine. Furthermore, as previously stated, aluminium compounds do not increase the protection provided by some vaccines. On the other hand, because mucosally-delivered vaccines are in general easier to produce and to administer to the population than parenteral vaccines - they do not require to be produced under sterile conditions or to be administered by medically trained personal using sterile medical materials.

This project, following the line of investigation started by other workers (e.g. Alpar *et al.*,1989, 1992; Bowen, 1990; Bowen *et al.*, 1990, 1992), was designed to study the use of particulate carriers to deliver antigens by the oral and nasal routes. It was also designed to assess the possibility of targeting antigens to lymphoid tissue either enterically, through Peyer's patches, or through the respiratory tract, particularly the nasal mucosa. As its name suggests, particulate carrier technology involves the encapsulation of small particles of drugs, or solutions of drugs, in a polymer coat. Investigations included production and optimisation of physicochemical properties of colloidal delivery systems as well as systemic and mucosal immune responses induced upon oral and nasal administration.

2. MICROENCAPSULATION OF MODEL PROTEIN ANTIGENS IN PLA/PLGA MICROSPHERES

2.1. INTRODUCTION

Protein delivery has become an important area of research as a result of the therapeutic potential of an increasing number of new molecules of biotechnological and chemical origin. Microspheres have been studied, not only as a means of protecting the peptides from degradation and prolonging their short half-lives *in vivo*, but also because they are an excellent slow release adjuvant for the delivery of protein antigens (Eldridge *et al.*, 1990). Several methods have been used to microencapsulate proteins in various polymer coatings. When vaccine formulation is concerned, the PLA/PLGA polymers are the most commonly used for the advantages already described in section 1.2. The good results obtained with the solvent evaporation method of microencapsulation (reviewed by Watts *et al.*, 1990) led to a generalised use of this procedure for the entrapment of proteins. Although the original technique of Beck *et al.* (1979) is designed for the encapsulation of lipophilic drugs, it has been used for protein entrapment (Kwong *et al.*, 1986; Eldridge *et al.*,1990; Hora *et al.*, 1990; Moldoveanu *et al.*, 1993). However, the improvement of the encapsulation efficiency of hydrophilic molecules by the double-emulsion technique of Ogawa *et al.* (1988), opened up new perspectives for a better formulation of therapeutic proteins and antigens (see sections 1.1.2.7 and 1.2). Currently, much of the work carried out on antigen microencapsulation is based on this method (O'Hagan *et al.*, 1991b, 1993; Singh *et al.*, 1991; Alonso *et al.*, 1992; Edelman *et al.*, 1993).

The aim of this study was to prepare of PLA or PLGA microspheres of a particle size appropriate to mucosal immunisation according to the findings of Jani *et al.* (1989, 1990) and Eldridge *et al.* (1990) and, simultaneously, to optimise the encapsulation efficiency of protein antigens by the single-emulsion solvent evaporation method, while preserving their molecular integrity and antigenicity throughout the procedure.

2.2. MATERIALS

2.2.1. Polymers

The polymers used in these experiments were poly(L-lactide) (PLA), molecular weights (Mwt) 2000 Da, 50000 Da and 300000 Da and poly(lactide-co-glycolide) (PLGA) of different compositions (90:10; 70:30) and were purchased from Polysciences, Inc., Northampton, U.K.. These polymers were also a gift from the Chemical and Biological Defence Establishment, Porton Down, Salisbury, U.K. and the Centre for Applied Microbiology and Research (PHLS), Porton Down, Salisbury, U.K. PLGA (50:50), also used, was a gift from ICI Pharmaceuticals plc., U.K.

2.2.2. Model Protein Antigens

Bovine serum albumin (BSA) - fraction V (Mwt 60000 Da), myoglobin - type III (Mwt 17000 Da) and cholera toxin B subunit (CTB) (Mwt 11000 Da), from Sigma Chemical Co., Poole, U.K. Tetanus toxoid (TT) (Mwt ≈150000 Da) obtained by dialysis and freeze-drying from Tetanus Vaccine BP in Simple Solution (The Wellcome Foundation Ltd., Beckenham, U.K). Botulinum toxoid A (BTA) and botulinum toxoid E (BTE) (both Mwt ≈150000 Da) were a gift from the Centre for Applied Microbiology and Research (PHLS), Porton Down, Salisbury, U.K.

2.2.3. Chemicals

All chemicals and reagents not specified in the text were supplied by BDH Chemicals Ltd. (Poole, U.K.), Sigma Chemical Company (Poole, U.K.) and Fisons (Loughborough, Leics.U.K.) and were of Analar grade or equivalent.

2.3. METHODS

2.3.1. Poly(lactide-co-glycolide) Microsphere Preparation

The PLA and PLGA type polymers are predominantly used in the preparation of microspheres by the solvent evaporation method described by Beck *et al.* (1979) (see fig. 1.1). The polymer and drug are dissolved together in an organic solvent (e.g. dichloromethane) and this solution is poured into a vigorously stirred aqueous phase containing an oil-in-water emulsifier to help the short-lived stabilisation of the dispersed polymer droplets (e.g. polyvinyl alcohol). An oil-in-water emulsion is formed. Evaporation of the organic solvent from the droplets results in the hardening of polymer microspheres.

This is a method designed for the microencapsulation of lipophilic drugs, which dissolve in the organic phase at the same time as the polymer. In the present study, the protein molecules used are hydrophilic and do not dissolve in the organic solvents as required for this technique. Therefore the proteins are suspended in the organic phase with the help of a sonication bath and vigorous mixing, or, by previous grinding of the protein powder in a mortar followed by sonication of the suspension.

Modifications of this method (Benita *et al.*, 1984; Cowsar *et al.*, 1985; Ogawa *et al.*, 1988, and Wichert & Rohdewald, 1990) were used to develop a suitable technique which was adopted for ensuing preparations (table 2.1, D).

Drug (30 to 150 mg) was dispersed in a solution of PLA or PLGA (500 mg) in 10 ml of dichloromethane. The aqueous phase consists of 150 ml of a solution of 1.5% polyvinyl alcohol (PVA, Mwt 10000, 88% hydrolysed; Aldrich Chemical Company, Inc., Dorset, U.K.) in double-distilled water. Both phases were previously cooled in an ice bath for 1 hour. The oil-in-water emulsion was prepared by adding the organic phase through a Pasteur pipette to the aqueous solution 1.5% PVA with stirring (≥10000 rpm, Silverson Mixer model STD1). After stirring for 5 min, the mixture was magnetically stirred at room temperature overnight, until the organic solvent had evaporated. The particles were recovered by centrifugation at 10000 rpm for 30 min (JA-14 rotor, Beckman J2-21

Centrifuge; Beckman Instruments Ltd., Bucks., U.K.) washed three times with double-distilled water, and freeze dried (Edwards Modylo freeze drier; Edwards High Vacuum Ltd., Sussex, U.K.).

Table 2.1 - Modifications to the original solvent evaporation method described by Beck *et al.* (1979).

Method	Aqueous Phase (ml)	Organic Phase (ml)	PLA/PLGA (g)	Emulsifier (PVA %)	Temperature (°C)	Stirring Rate (rpm)	Time (hours)
Benita *et al.* (1984)	250	CH_2Cl_2 (20)	0.927	1.5	22	450	7-17
Cowsar *et al.* (1985)	475	$CHCl_3$/Acetone (29:16)	7.5	5.0	1	800	24
Wichert & Rohdewald (1990)	40	CH_2Cl_2 (1)	0.12	0.5	room temp.	≈10000	1
A	150	CH_2Cl_2 (10)	0.5	1.5	room temp.	500	17
B	150	CH_2Cl_2 (10)	0.5	1.5	room temp.	2000	17
C	150	CH_2Cl_2 (10)	0.5	1.5	4	2000	1
D	150	CH_2Cl_2 (10)	0.5	1.5	room temp.	≥10000	17

Various molecular weights of PLA and PLGA composed of different ratios of lactide and glycolide were used for the preparation of microspheres. Both unloaded and protein-loaded microspheres were prepared by the solvent evaporation method in an attempt to obtain very small particles. The size should be preferably under 10 μm in diameter to facilitate particulate uptake by the gut as described by Eldridge *et al.* (1990). In this study, particles smaller than 1 μm were used in an attempt to improve uptake. Microspheres were loaded with the model antigen proteins described in section 2.2.2.

2.3.2. Particle Size Analysis

Particle size was determined by scanning electron microscopy (SEM; Cambridge Instruments Stereoscan 90). Microsphere samples were photographed and slides were made so that particles ($n \geq 97$) could be measured on a screen. For SEM, particles were fixed to a SEM stub and a fine gold film was electrolytically deposited (sputtered) onto the sample in a (Emscope SC 500) sputter coater. The particle size was statistically analysed in order to assess the size distribution and to determine the geometric standard deviation (σ_g).

2.3.3. *In Vitro* Release Studies

Microspheres (20 mg) were placed in a conical flask containing release medium (20 ml of either 20 mM phosphate buffer, pH 7.5 or 0.1 M HCl), and shaken ($\approx$120 cycles min^{-1}) in a water-bath at 37°C. Aliquots (1 ml) were collected at different intervals and centrifuged in Eppendorf tubes at 13000 rpm for 20 min (Eppendorf 5412 bench centrifuge; Baird and Tatlock, Leics., U.K.). The supernatants were analysed by bicinchoninic acid protein assay (section 2.3.6) and SDS-PAGE (section 2.3.7). After each sample collection an equivalent volume of fresh buffer was added to maintain the volume at 20 ml.

2.3.4. Determination of Encapsulated Protein

The amount of protein encapsulated per unit weight of microspheres was determined by digestion of the microspheres at room temperature in a solution of 0.1 M NaOH containing 5% sodium dodecyl sulphate (SDS), as previously described by Hora *et al.* (1990). Sodium hydroxide hydrolyses the polymer allowing the release of entrapped protein; SDS increases its solubility.

Microspheres (20 mg) and digestion medium (20 ml) were magnetically stirred in a conical flask, until the solution was completely clear (about 5 hours for PLA, Mwt 2000 Da). The resultant solution was neutralised to pH 7 with hydrochloric acid and the bicinchoninic acid protein assay (section 2.3.6) was used to quantify the protein loading.

2.3.5. Studies on the Degradation of PLA/PLGA Microspheres

The influence of pH and lactide/glycolide ratio on the hydrolysis of PLA/PLGA was studied by comparing the rate of degradation of microspheres of PLA (Mwt 2000 Da), PLGA (70:30) and PLGA (50:50) in different media. Based on the digestion medium described by Hora *et al.* (1990) (section 2.3.4), the following three degradation media were used: 0.1 M NaOH containing 5% SDS, pH 12.0; 0,1 M HCl containing 5% SDS, pH 1.0; isotonic phosphate-buffered saline (PBS - 0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4) containing 5% SDS, pH 7.4. Suspensions of microspheres in the degradation media (20 mg in 20 ml) were prepared in conical flasks and sonicated for 30 seconds, before being shaken in a water-bath ($\approx$120 cycles min^{-1}) at room temperature ($\approx$20°C). The turbidity of suspensions (OD_{600}) was measured at 1 hour intervals using a spectrophotometer (LKB Ultrospect 4050) and results were expressed as percentage decrease in OD_{600}.

2.3.6. Bicinchoninic Acid Protein Assay

The basis of this method is in attempts made to improve the sensitivity and reproducibility of the method of Lowry *et al.*, (1951). It is similar to that of Lowry's in that a protein-copper complex is formed. Proteins react with copper (II) to produce copper (I). The 4,4'-dicarboxy-2,2'-biquinolone (bicinchoninic acid, or BCA), the key component of this protein assay (Smith *et al.*, 1985), forms alkali metal salts which are soluble in water. The purple reaction product is also water-soluble enabling the spectrophotometric measurement of an aqueous protein solution. Unlike the Lowry method very few interferences were observed and it is more sensitive than the Lowry assay (10 $\mu g ml^{-1}$; 0.1 μg of protein in sample). It can be performed in a microtitre plate (flat bottom microtitre trays from L.I.P. - Equipment and Services, Ltd., West Yorkshire, U.K.) effectively reducing the sample volume to 10 μl.

a) <u>The Standard Assay Protocol</u>

The assay procedure involves mixing on a microtitre plate, 1 volume (10 μl) of sample or standard (20 to 2000 $\mu g\ ml^{-1}$) with 20 volumes (200 μl) of freshly prepared protein reagent (see composition below). Colour development proceeded at 60°C for 1 hour and plates were allowed to cool at room temperature before the absorbance was measured at 550 nm (Anthos Reader 2001; Anthos Labtec Instruments, Austria). A standard curve was constructed (see examples in fig. 2.1). For the small volume of samples and standards used, a minimum of four samples of each solution were prepared for all solutions analysed, the final value represents the average.

The BCA protein reagent is prepared by mixing 50 volumes of reagent A with 1 volume of reagent B. This solution should be freshly prepared. Reagent A consists of an aqueous solution of 1% bicinchoninic acid (disodium salt), 2% $Na_2CO_3.H_2O$, 0.16% disodium tartarate, 0.4% NaOH, and 0.95% $NaHCO_3$. Where necessary, appropriate addition of NaOH (50%) or solid $NaHCO_3$ was made to reagent A to adjust the pH to 11.25. Reagent B consists of 4% $CuSO_4.5H_2O$ in double-distilled water.

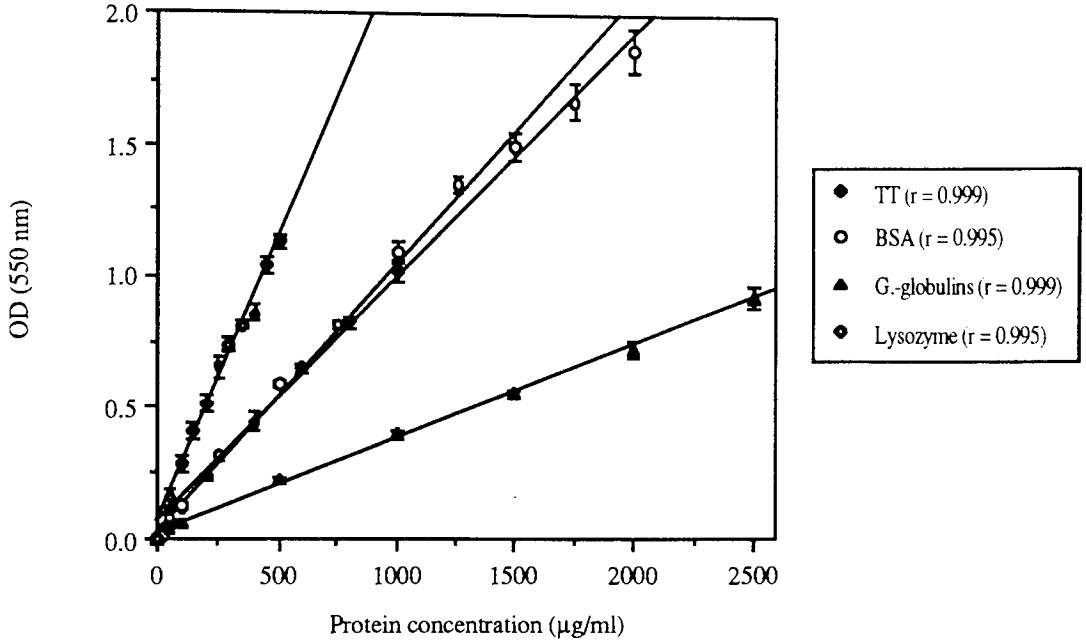


Figure 2.1 - Calibration curve for the estimation of proteins by the standard protocol of the bicinchoninic acid protein assay.

b) The Micro Assay Protocol

Extremely dilute protein solutions (0.5-10 μgml^{-1}) can be efficiently assayed by a protocol which employs a more concentrated reagent formulation. Micro-reagent A (MA) consists of an aqueous solution of 8% $Na_2CO_3.H_2O$, 1.6% NaOH, 1.6% disodium tartarate and sufficient $NaHCO_3$ to adjust the pH to 11.25. Micro-reagent B (MB) consists of 4% of bicinchoninic acid (disodium salt) in double-distilled water. Micro-reagent C (MC) consists of 4 volumes of 4% aqueous $CuSO_4.5H_2O$ plus 100 volumes of MB. Micro-working reagent (M-WR) consists of 1 volume of MC plus 1 volume of MA. The reagents MA and MB are stable indefinitely at room temperature, but MC and M-WR should be freshly prepared.

To carry out the assay, samples and standards (100 µl, 1 to 20 μgml^{-1}) were mixed in a microtitre plate with an equal volume of M-WR and incubated at 60°C for 60 min. Plates were allowed to cool at room temperature before the measurement of absorbance at 550 nm

(Anthos Reader 2001; Anthos Labtec Instruments, Austria) and a standard curve was constructed (see examples in fig. 2.2). For the small volume of samples and standards used, a minimum of eight samples of each solution assayed were prepared for all solutions analysed, the final value represents the average.

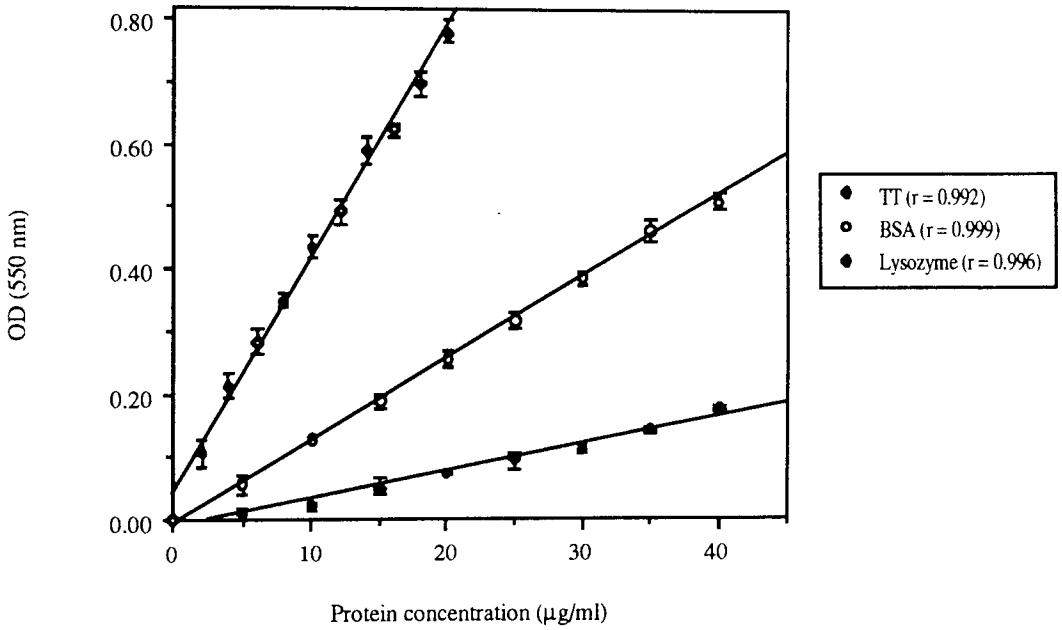


Figure 2.2 - Calibration curve for the estimation of proteins by the micro protocol of the bicinchoninic acid protein assay.

2.3.7. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

a) Separation Procedure

Electrophoretic methods of separation are based on the movement of charged particles in an external electric field. The rate of movement of the particles in a standard electric field will depend on their charge and mass and the electrophoresis medium. These properties can be controlled to achieve different types of separation. Proteins in their native state behave in general as charged particles. If the pH is above their isoelectric point they will have a net negative charge and move towards the anode. If it is below their isoelectric point, they will

have a net positive charge and move towards the cathode. At the isoelectric point they have zero net charge and do not move in an electric field. This is the basis of the various routine electrophoretic techniques which involve the separation of molecules by charge only.

As proteins possess ionisable groups they can exist as electrically charged species in solution (cations or anions). Differences in charge to mass ratios resulting from differences in molecular weight cause a differential migration of the ions in solution when subjected to an electric field. Cations move to the cathode and anions to the anode at rates depending on the impelling force of the electric field on the charged ion and the frictional and electrostatic retarding effects between the sample and the surrounding medium. The sample must be dissolved or suspended in buffer for electrophoresis to take place and any supporting medium must also be saturated with buffer to conduct the current and to maintain a constant state of ionisation since any changes in pH would alter the charges on the molecules being separated.

Polyacrylamide gel electrophoresis (Laemmli, 1970) is a particularly powerful technique for the determination of molecular weights of proteins. The macromolecules have first to be treated so as to produce uniform charge/mass ratios for the subsequent separation in gels to be achieved solely on molecular size. This is accomplished by treating the proteins with SDS and 2-mercaptoethanol which breaks disulphide bonds, assists solubilisation, and attaches an ionic group at regular intervals along the polypeptide chain. Each constituent (i.e. sample mixture and reference standards) must be run under the same conditions. Calibration curves for SDS-PAGE electrophoresis show a linear relationship between the migration distance and $\log_{10}$ of the molecular weight of the protein.

Separation of proteins was carried out by gel electrophoresis using the Mini-Protean system (Bio-Rad Laboratories Ltd., Herts., U.K.) according to the methods described by Lugtenberg *et al.* (1975), as modified by Anwar *et al.* (1983). The running and stacking gels were prepared as described in table 2.2 and polymerisation was initiated by the addition of N,N,N,'N'-tetramethylethylene diamine (TEMED). The running gel was poured between the glass plates separated by 1.5 mm plastic spacers and allowed to set for 10 min. A spray of electrode buffer on top of the gel produced anaerobic conditions and so ensured

complete polymerisation. This buffer solution was removed and the stacking gel was cast by the same method. A teflon comb was inserted between the plates to create wells for sample application. Samples were denatured by maintaining a temperature of 100°C, for 10 min in the presence of an equal volume of a denaturing mix (sample buffer, see table 2.2) before loading onto the gel. The electrode buffer contained 0.025 M Tris, 0.19 M glycine and 0.1% SDS. A constant voltage of 200 V (power pack Bio-Rad model 500/200; Bio-Rad Laboratories Ltd., Herts., U.K.) was applied across the gel and electrophoresis continued until the tracking dye had migrated to within 0.5 cm of the bottom of the gel.

Table 2.2 - Composition of running gel, stacking gel and sample buffer for SDS-PAGE.

Constituent	Running Gel 12%	Running Gel 14%	Stacking Gel	Sample Buffer
Stock 1[1]	6 ml	7 ml	—	—
Stock 2[2]	—	—	2 ml	—
1.5M Tris[3]	7.5 ml	7.5 ml	—	—
0.5M Tris[3]	—	—	3 ml	5 ml
10% (w/v) SDS	0.6 ml	0.6 ml	0.12 ml	10 ml
Distilled Water	9.5 ml	8.5 ml	6.4 ml	10 ml
10% (w/v) AMPS[4]	80 µl	80 µl	40 µl	—
TEMED[5]	56 µl	56 µl	32 µl	—
Glycerol	—	—	—	5 ml
Mercaptoethanol	—	—	—	0.5 ml
5% (w/v) Bromophenol blue	—	—	—	0.4 ml

1) 44% (w/v) acrylamide and 0.8% (w/v) N,N'-methylene-bis-acrylamide (BIS)
2) 30% (w/v) acrylamide and 0.8% (w/v) BIS
3) Tris (hydroxymethyl) aminoethane
4) Ammonium persulphate (freshly prepared)
5) N,N,N,'N'-Tetramethylethylene diamine

b) Visualisation of Protein

Gels were stained for protein using 0.1% Coomassie brilliant blue R-250 in 50% methanol/10% acetic acid for 60 min. Gels were subsequently destained in 10% methanol /20% acetic acid and photographed using diffuse transmitted light and finally dried onto filter paper using a gel dryer (Bio-Rad model 224; Bio-Rad Laboratories Ltd., Herts., U.K.). In some cases, before drying the gels, proteins were quantitatively scanned using a laser densitometer (LKB 2202 Ultroscan), against a standard preparation of the same protein.

c) Molecular Weight Determination

Denatured polypeptides bind SDS in a constant ratio and have essentially identical charge densities and therefore migrate in polyacrylamide gels according to their size. The molecular weights of the proteins separated by SDS-PAGE can be predicted by comparison of their Rf values with the Rf values of standard molecular weight marker proteins (Dalton Mark VII-L, from Sigma Chemical Company, Poole, U.K.; see table 2.3).

Table 2.3 - Unstained molecular weight markers for SDS-PAGE.

Protein	Molecular Weight (Da)
Bovine albumin	66000
Egg albumin	45000
Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase subunit	36000
Bovine erythrocyte carbonic anhydrase	29000
Bovine pancreas trypsinogen PMSF treated	24000
Soybean trypsin inhibitor	20100
Bovine milk α-lactoglobulin	14200

The Rf value (relative mobility) was calculated by dividing the distance migrated from the origin of the separating gel to the centre of the protein band by the migration distance of the bromophenol blue tracking dye from the top of the separating gel. A calibration curve was then constructed by plotting the Rf values against the $\log_{10}$ of the known molecular weights (fig. 2.3). The molecular weights corresponding to the Rf values for the unknown proteins were read from the graph.

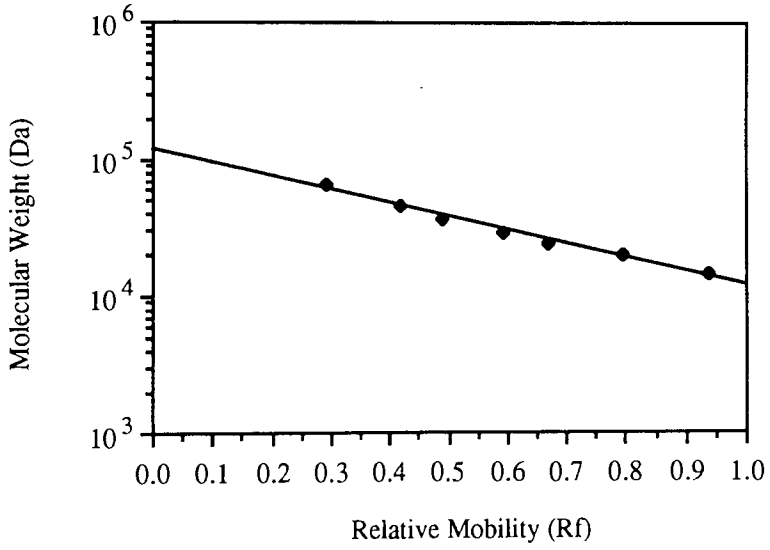


Figure 2.3 - Calibration curve (r = 0.993) for the estimation of the molecular weight of proteins by SDS-PAGE, using the molecular weight markers described in table 2.3.

2.3.8. Western Blotting (Immunoblotting)

The transfer of TT, separated by SDS-PAGE, to a nitrocellulose (NC) membrane for immunoreaction was performed according to the method of Towbin *et al.* (1979), as modified by Anwar *et al.* (1984). Following electrophoresis the gel and NC (Trans Blot Membrane, pore size 0.45 μm, Bio-Rad Laboratories Ltd., Herts., U.K.), were sandwiched between chromatography paper (Whatman Ltd., Kent, U.K.), and Scotchbrite pads (Bio-Rad Laboratories Ltd., Herts., U.K.), in a Trans Blot cassette. Electroblotting was performed in a Trans Blot cell at 100 V for 1h filled with transfer buffer (192 mM

glycine, 25 mM Tris, 20% methanol, pH 8.3), at 4°C. Efficiency of protein transfer is known to be a function of molecular weight (Burnette, 1981) and these conditions were necessary to ensure complete transfer of high molecular weight proteins. The transfer of TT was confirmed by staining the gel after blotting with coomassie blue.

After transfer the NC paper was soaked in Tris buffered saline [TBS - 10 mM Tris HCl, 0.9% (w/v) NaCl, pH7.4] containing 0.3% (v/v) Tween-20 (TBS-Tween), for 1 hour, to saturate non-specific binding sites on the NC (Batteiger *et al.*, 1982). The NC was incubated for 4h at 37°C in anti-tetanus immunoglobulin (Humotet®, The Wellcome Foundation Ltd., Beckenham, U.K.) purified from the sera of healthy human donors known to have high levels of tetanus antitoxin following active immunisation with tetanus vaccine (used as a standard antitoxin), diluted 1 in 2000 in TBS-Tween. Following this the immunoblot was washed in TBS and incubated for a further 2h at 37°C with protein A horseradish peroxidase (HRP) conjugate (0.25 $\mu g ml^{-1}$; Miles Scientific, Rehovot, Israel) diluted 1 in 1000 in TBS-Tween.

After further washing in TBS, the HRP conjugates were visualised using a solution containing 25 $\mu g ml^{-1}$, 4-chloro-1-naphthol and 0.01% (v/v) hydrogen peroxide in TBS. On reaching the desired intensity the reaction was terminated by placing the immunoblot in double-distilled water.

2.4. RESULTS AND DISCUSSION

2.4.1. PLA/PLGA Microsphere Preparation

For effective antigen carriers designed for mucosal delivery, particle size must be optimally controlled. As Eldridge *et al.* (1990) described, particle size should be smaller than 10 μm, and preferably ≤ 5 μm, in order to be absorbed in the Peyer's patches. These results contradict those obtained by Jani *et al.* (1990) who studied the absorption of particles in the size range 50 nm to 3 μm, and concluded that absorption improves with a decrease in

particle size. The 3 μm particles showed a very slow absorption and were not detected in the majority of the organs analysed. However, Eldridge *et al.* (1990) stated that particles < 5 μm in diameter were transported through the efferent lymphatics within the macrophages, while the majority of those > 5 μm remained fixed in the Peyer's patches. So, as opinions differ, the aim of this study was to prepare small microspheres, preferably under 1 μm, to make sure that they would be absorbed.

PLA of several molecular weights and different ratios of PLA/PGA were studied in order to reduce particle size. For the same reason, a few modifications of the original method (Beck *et al.*, 1979) were tried. Table 2.4 shows some of the mean particle sizes, measured by SEM, obtained with modifications of some aspects of the solvent evaporation technique. One of these consisted of the decrease in temperature of both the oil and the aqueous phases by keeping them in an ice-bath for at least 1 hour, so that the resulting increase in viscosity could act as an additional factor for stabilisation of the final emulsion. The low temperature also slows the evaporation of the organic solvent which contributes to the formation of regular shaped spherical microspheres with a smoother surface. Another modification consisted of increasing the speed of mixing by using an homogeniser (Silverson Mixer model STD1) at its maximum speed (≥ 10000 rpm), which divides the internal phase into smaller droplets so originating smaller particles. As described by Benita *et al.* (1984) and Babay *et al.* (1988) the particle size tends to decrease with increasing mixing speed, accompanied by a narrowing of the particle size distribution.

The first modification resulted in microspheres that presented a very smooth surface although the particle size was still too large for the purposes of the present work (fig. 2.4). The final method of preparation (D) produces spherical particles with an apparently smooth surface. In addition, the results obtained under the same experimental conditions are reproducible (n ≥ 3). When observed under SEM, these batches show small spherical particles with a slightly skewed size distribution (figs. 2.5 and 2.6).

Table 2.4 - Mean particle size obtained with some variations of the method.

Polymer	Preparation Method	Mean Size (μm) (geometric)
PLGA (90:10)	Benita *et al.* (1984)	≈100*
PLGA (90:10)	Cowsar *et al.* (1985)	8.5 ±1.4
PLGA (90:10)	C	3.9 ± 1.7
PLGA (70:30)	Cowsar *et al.* (1985)	9.8 ± 1.7
PLA (Mwt 2000)	D	0.7 ± 1.8
PLA (Mwt 50000)	D	2.1 ± 1.4
PLA (Mwt 300000)	D	≈20*

* - Particles viewed under light microscopy and values estimated using the graticule described in the British Standard 3406, Part IV (1961).

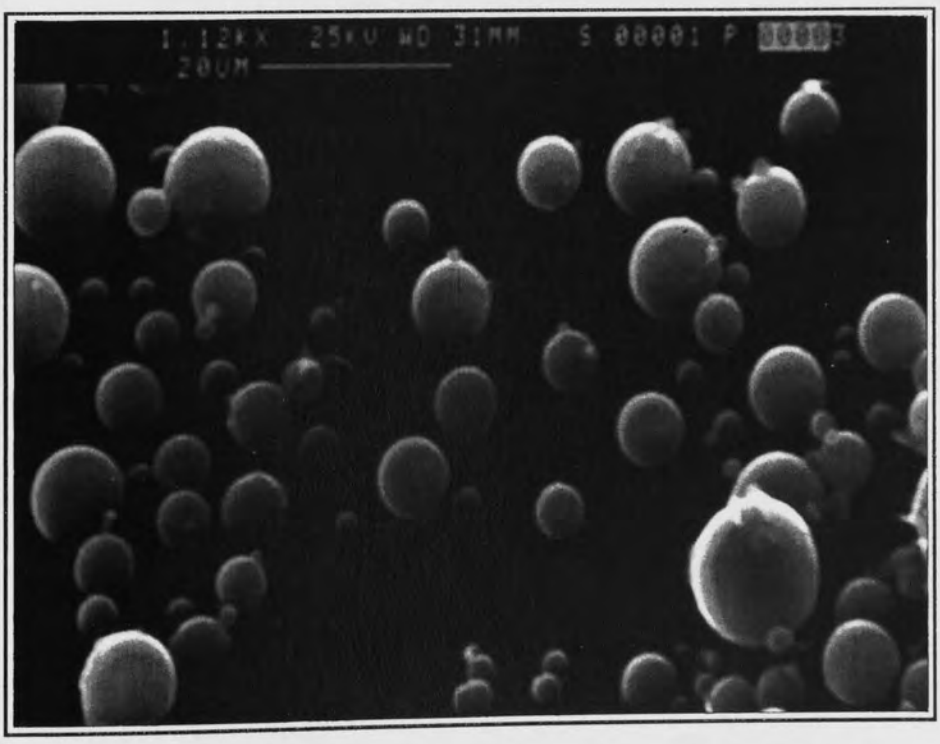


Figure 2.4 - Scanning electron micrograph of PLGA (90:10) microspheres. Mean particle size = 3.9 μm; σ_g = 1.7 (n = 97 particles).

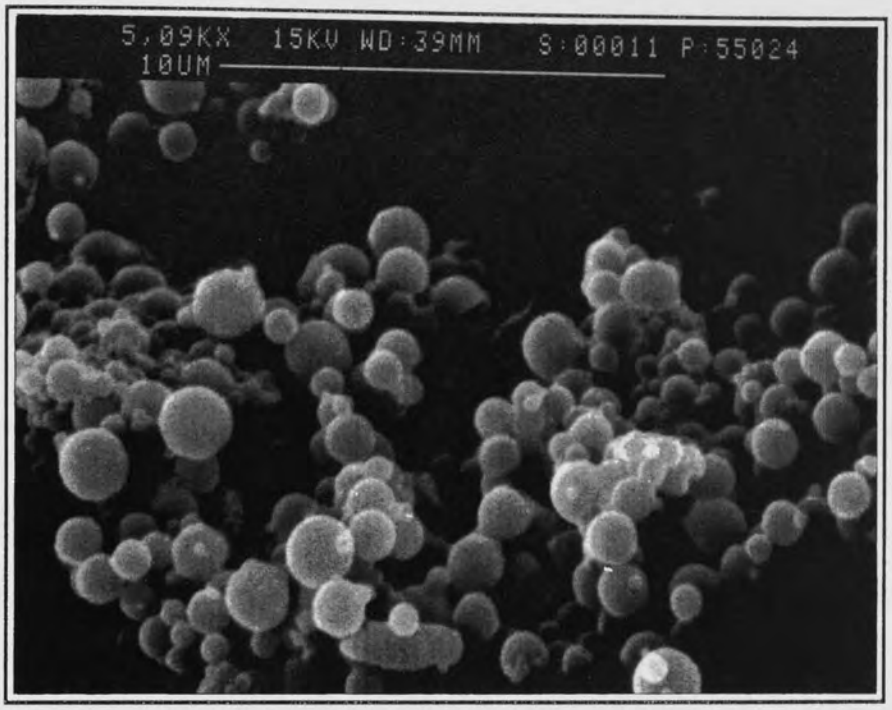


Figure 2.5 - Scanning electron micrograph of PLA (Mwt 2000 Da) microspheres prepared according to method D, using a high speed homogeniser (≥ 10000 rpm).

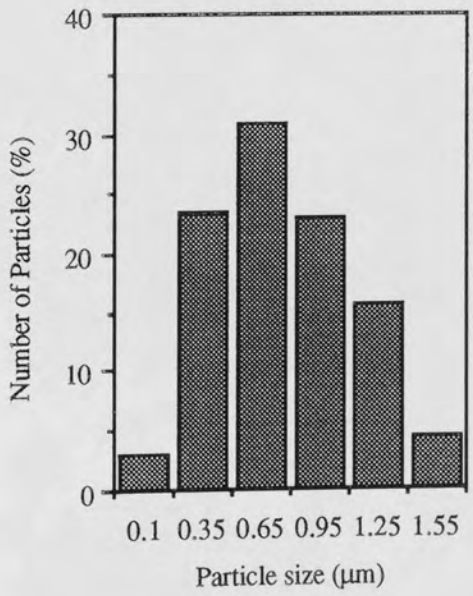


Figure 2.6 - Particle size distribution of PLA (Mwt 2000 Da) microspheres prepared by method D using a high speed homogeniser (≥ 10000 rpm). Mean particle size = 0.7 μm; σ_g = 1.8 (n = 231 particles).

With respect to the different molecular weights of polymers used, the results reached are similar to those of Wichert & Rohdewald (1990), i.e. an increase in PLA or PLGA molecular weight leads to an increase in particle size of microspheres. Using the same method of preparation the mean particle size for PLA (Mwt 2000 Da) varies between 0.7 and 0.9 μm, whereas PLA (Mwt 50000 Da) microspheres show a mean size of 2.1 μm and PLA (Mwt 300000 Da) presents a value of approximately 20 μm. However, particles maintain the spherical shape, the smooth surface and present a similar particle size distribution (figs. 2.7 and 2.8). The solution of PLA (Mwt 2000 Da) in dichloromethane has a lower viscosity than those of PLA (Mwt 50000 Da) and PLA (Mwt 300000 Da). The increase in viscosity of the internal phase, provoked by higher molecular weight polymers, is particularly large with PLA (Mwt 300000 Da). This may result in a larger droplet size during homogenisation and in a larger particle diameter. A similar effect is observed when the polymer concentration in the disperse phase is increased (Watts *et al.*, 1990). This effect on viscosity is boosted by the decrease of temperature, prior to the addition of both phases. Consequently, the division of the internal phase in fine droplets is more difficult when the viscosity is higher and the droplets formed will be bigger with a 50000 Da than with a 2000 Da polymer.

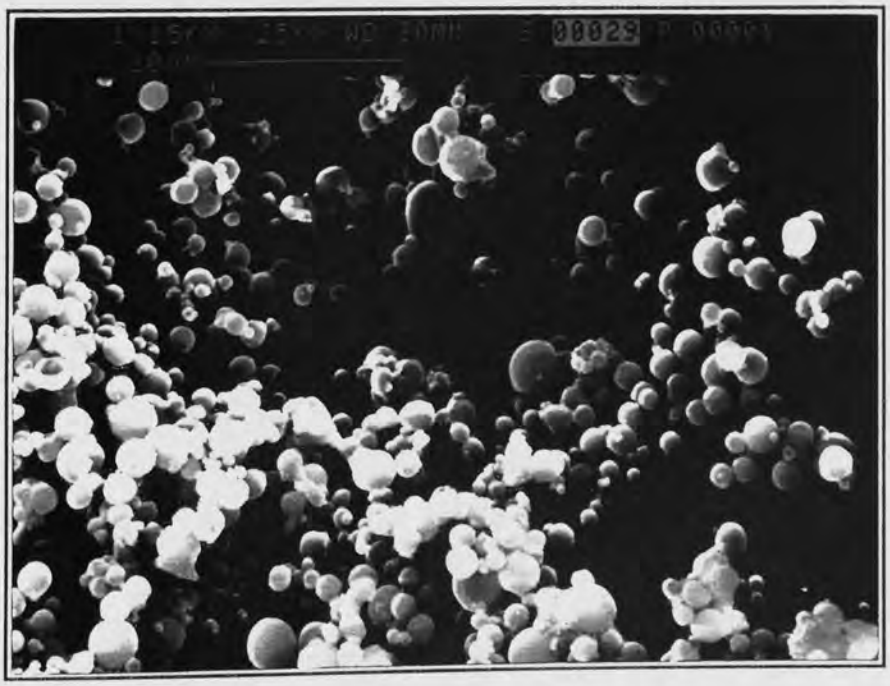


Figure 2.7 - Scanning electron micrograph of PLA (Mwt 50000 Da) microspheres prepared according to method D.

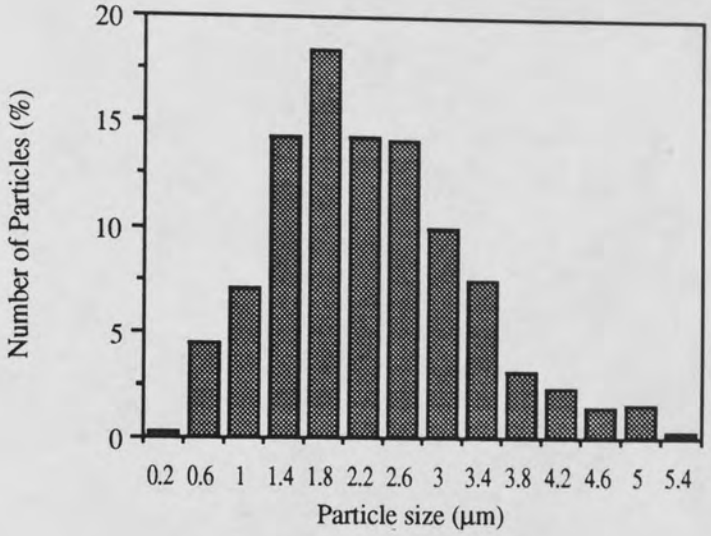


Figure 2.8 - Particle size distribution of PLA (Mwt 50000 Da) microspheres. Mean particle size = 2.1 μm; σ_g = 1.4 (n = 433 particles).

2.4.2. Protein Encapsulation in PLA/PLGA Microspheres

Six different proteins were encapsulated using this procedure: BSA, TT, CTB, myoglobin, BTA and BTE. The resulting preparations, when observed under SEM, show very small and spherical particles, although in some cases the surface is not smooth perhaps because of protein crystals deposited at the microspheres surface (figs. 2.9 and 2.10). Nevertheless, particles maintain the same pattern of distribution (figs. 2.11 and 2.12).

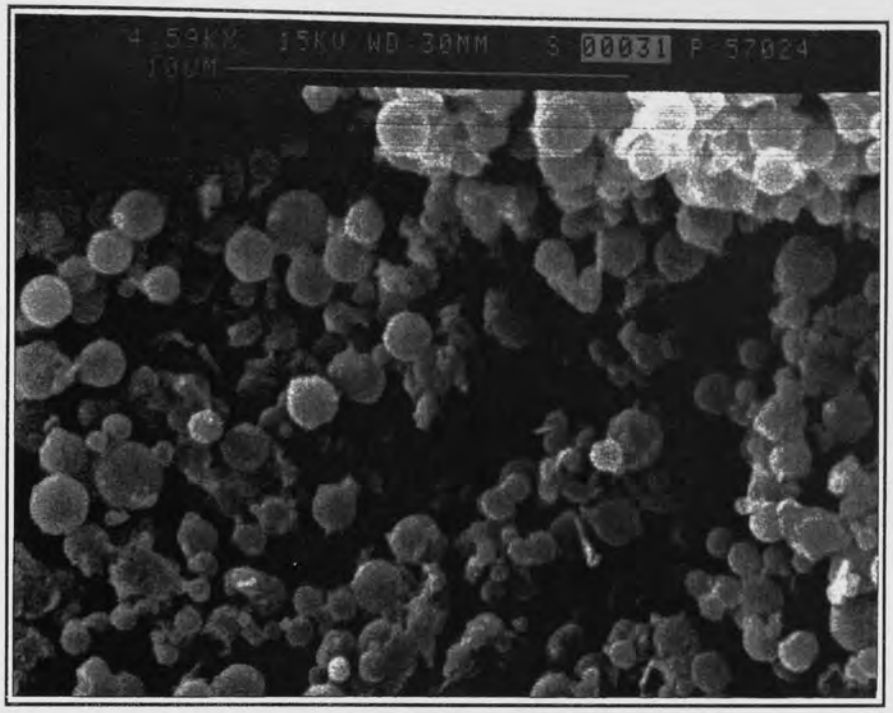


Figure 2.9 - Scanning electron micrograph of a PLA (Mwt 2000 Da) batch of microspheres containing BSA microencapsulated.

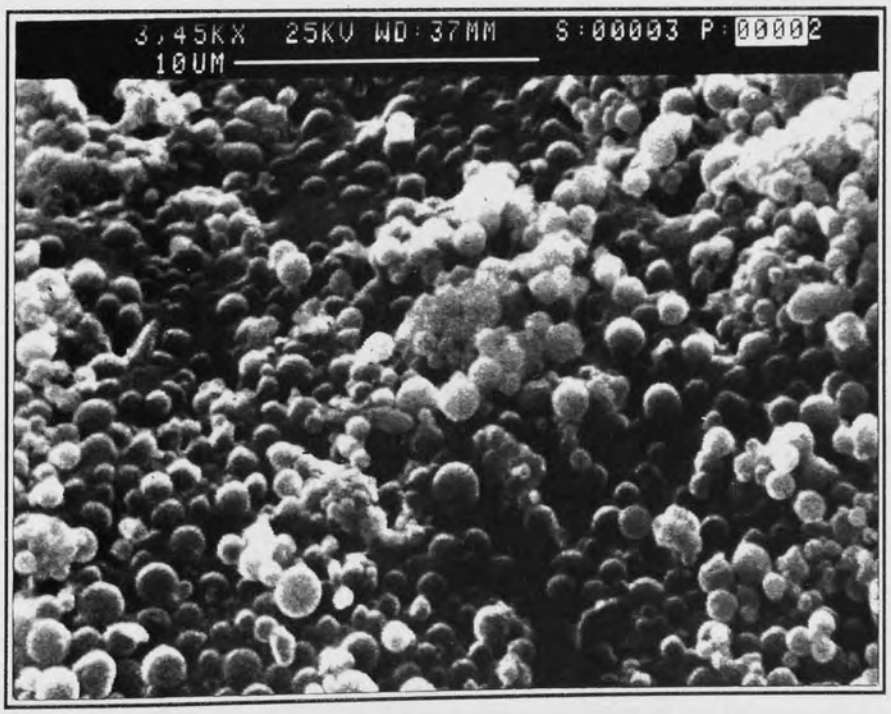


Figure 2.10 - Scanning electron micrograph of a PLA (Mwt 2000 Da) batch of microspheres containing TT microencapsulated.

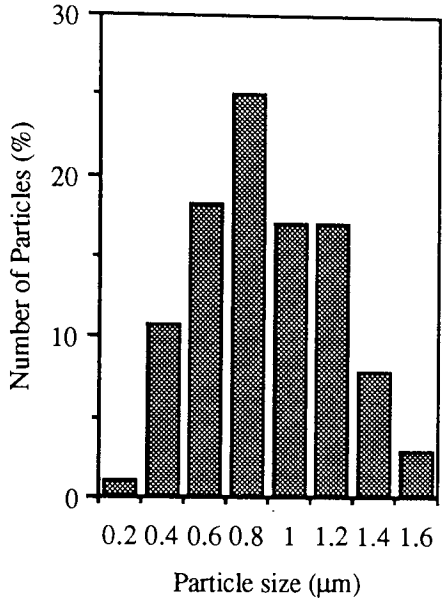


Figure 2.11 - Particle size distribution of PLA (Mwt 2000 Da) microspheres containing BSA microencapsulated. Mean size = 0.9 μm; σ_g = 1.6 (n = 176 particles).

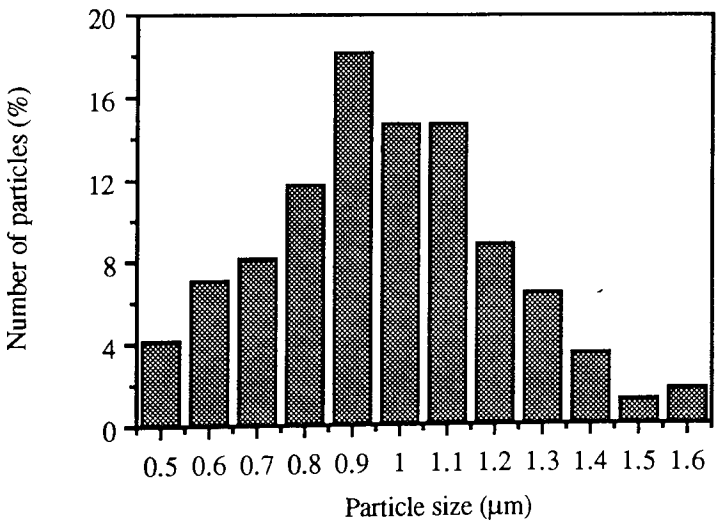


Figure 2.12 - Particle size distribution of PLA (Mwt 2000 Da) microspheres containing TT microencapsulated. Mean size = 0.9 μm; σ_g = 1.6 (n = 171 particles).

To determine the efficiency of encapsulation [measured in % (w/w)], supernatants of the final particles' suspension were assayed by the BCA protein assay (section 2.3.6) and microspheres were digested using the procedure described in section 2.3.4, followed by the same protein assay. The amounts found either from the assay of supernatants or after extracting from particles were in agreement and the loading efficiency values are described in table 2.5. The encapsulation efficiency is high even though the method used for the preparation is more appropriate to lipophilic molecules and the aim of this work is to microencapsulate water-soluble molecules (proteins). Drugs with high water solubility rapidly partition from the more hydrophobic polymer-solution phase into the aqueous phase (Watts *et al.*, 1990). For example, small water-soluble molecules such as theophylline, caffeine and salicylic acid could not be entrapped within the microspheres because of complete partitioning into the continuous phase (Bodmeier & McGinity, 1987a). Besides, the preparation procedure includes the washing of microspheres with distilled water to remove the surfactant (PVA) employed to stabilise the initial o/w emulsion. During these steps protein can also be washed from the surface. Most of the molecules entrapped near the particles' surface may be removed; perhaps this is an explanation for the smooth surface observed in some batches (fig. 2.9).

The values for the encapsulation efficiency of water-insoluble drugs described in the literature are about 25% (w/w) in the final formulation (Watts *et al.*, 1990). An encapsulation of 15% (w/w) for BSA is relatively high since O'Hagan *et al.* (1991b) claim to have obtained excellent results in immunisations with preparations containing only 1% (w/w) of ovalbumin, which is a weak antigen.

The encapsulation efficiency depends upon the initial amount of drug added to the polymer. Therefore, the values presented in table 2.5 are dependent on the initial amount of protein loaded i.e. the ratio protein/polymer. Figure 2.13 shows the variation of the microspheres payload with the ratio BSA/PLA. There is an increase in the percentage of protein encapsulated when the initial amount added to the preparation is increased from 6% to 25% related to the weight of PLA. However, above 25% the amount entrapped by the polymer remains approximately constant, but an increase of the final preparation content in protein can still be observed.

Table 2.5 - Encapsulation efficiency by the solvent evaporation method.

Polymer	Protein	Amount Encapsulated [%(w/w)] related to the initial amount of protein	Amount Encapsulated [%(w/w)] in final spheres composition
PLA (Mwt 2000)	Tetanus Toxoid	65.2 to 90.6	1.3 to 11.7
PLA (Mwt 2000)	Cholera Toxin B Sub.	37.6	0.15
PLA (Mwt 2000)	Botulinum Toxoid A	>80	>1
PLA (Mwt 2000)	Botulinum Toxoid E	40.4	0.23
PLA (Mwt 2000)	Myoglobin	65.8	14.0
PLA (Mwt 2000)	BSA	15.3 to 73.7	0.9 to 15.0

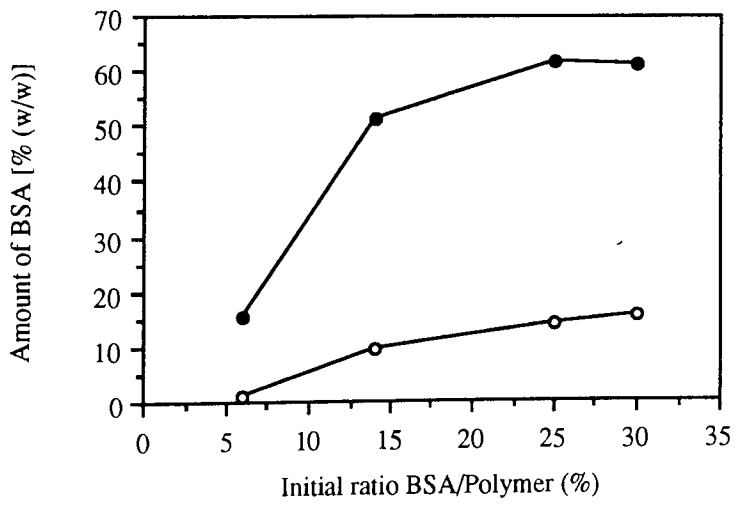


Figure 2.13 - Variation of the amount of BSA encapsulated with the initial ratio BSA/PLA (Mwt 2000 Da): (•) - percentage of BSA encapsulated. (o) - percentage of BSA in the final composition of the formulation.

2.4.3. *In Vitro* Release Studies

Results from the release tests of the formulations are in general agreement with the findings of Cohen *et al.* (1991) who used a similar receiving medium (20 mM phosphate buffer, pH 7.5). An initial fast release of large amounts of the protein (58% within 3 hours for BSA, and 59% within 5 hours for TT) is then followed by a steady stage of release during several days (figs. 2.14 and 2.15). For TT the release was complete after 3 days regardless of the total erosion of the polymer. Microspheres could still be observed after the release of 100% of the drug. Cohen *et al.* (1991) described a burst effect of approximately 80% for BSA and the release was also completed before total degradation of the microspheres. PLGA films containing 5 to 30% (w/w) of BSA release 90% of their content into PBS within 24 hours (Shah *et al.*, 1992). A reduction in drug loading is known to decrease the burst effect. The initial burst of the drug, as observed with BSA and TT, has been attributed to the cleavage of the very low molecular weight polymer resulting from the bulk hydrolytic degradation of the ester bonds, but it may also be due to incompletely incorporated drug at the polymer surface, which is rapidly released (Shah *et al.*, 1992). In addition, the presence of plasma proteins (e.g. albumin and γ-globulins) in the aqueous medium accelerates the cleavage of ester bonds in poly(L-lactide) molecules as measured by the increase of the amount of lactic acid produced (Makino *et al.*, 1987a).

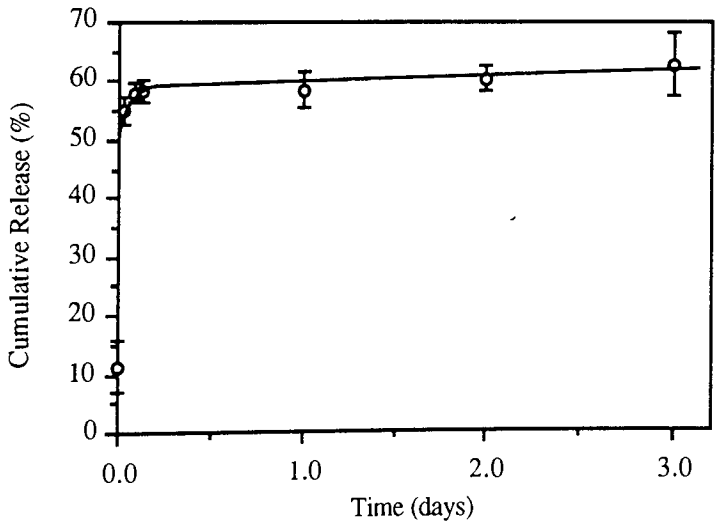


Figure 2.14 - Release of BSA from PLA (Mwt 2000 Da) microspheres in 20 mM phosphate buffer, pH 7.5. Vertical bars indicate the standard deviation at each point (n = 4).

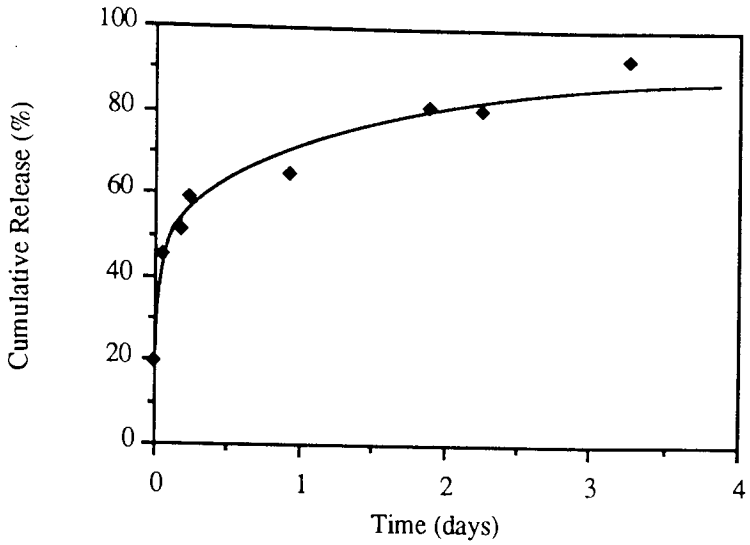


Figure 2.15 - Release profile of TT from PLA (Mwt 2000 Da) microspheres in 20 mM phosphate buffer, pH 7.5 (n = 4).

Release characteristics were more consistent with the expected release pattern when the experiments were carried out using phosphate buffer than when using a lower pH medium. In 0.1 M HCl drug release was reduced (< 30% in three days) which can be due to the lower solubility of BSA and TT in acidic solutions. Under such conditions these proteins tend to precipitate and so give atypical release profiles. Coincidentally, Boquet *et al.* (1984) reported that low pH solutions (< 4) induce a hydrophobic domain in the native tetanus toxin and the molecule tends to precipitate. Wichert & Rohdewald (1990) found a pH dependent drug release for PLA (Mwt 2000 Da) microspheres, in contrast to higher Mwt PLA. The release was slower in acidic media when compared to neutral or alkaline solutions. Also, according to the reports of by Chu (1981) and Makino *et al.* (1986), PLA/PGA are degraded by hydrolysis giving lactic acid and glycolic acid respectively, and these reactions occur better in a non-acid medium that neutralises the two acids formed. So, in alkaline conditions the hydrolysis will be faster. The same conclusions can be inferred from the incorporation of basic drugs (e.g. amines) into PLGA microspheres which cause a very fast release due to drug-accelerated hydrolysis of the polymer (reviewed by Watts *et al.*, 1990). As mentioned in section 1.2 these observations agree with the fact that the cleavage of ester bonds takes place by different mechanisms, depending on the pH of the medium. Also, the penetration of water molecules into the polymer, resulting in the attack of

the ester bonds by OH^- and H^+ (Makino *et al.*, 1986), is facilitated by the large size of these protein molecules. The molecules that are released from the polymer matrix will produce large pores that allow the penetration of increasing numbers of water molecules. On the other hand, the pore formation will increase the surface area exposed to the water and, consequently, a more rapid degradation of the polymer matrix will occur.

These factors concerning the influence of pH in the degradation of PLA/PGA polymers were taken into consideration in the method described by Hora *et al.* (1990) in which a strongly alkaline solution is used to digest the microspheres (see section 2.3.5). In alkaline conditions the degradation of both PLA and PLGA microparticles is faster than in neutral or acid media (fig. 2.16-a) and the lactide homopolymer shows a slower degradation than PLGA (70:30) and PLGA (50:50). In the conditions described by Hora *et al.* (1990) (0.1 M NaOH + 5% SDS, pH 11.7), the PLGA microspheres were rapidly degraded. Within 3 hours PLGA (50:50) microspheres were reduced to 0.8% and PLGA (70:30) to 26.5%, while PLA (Mwt 2000 Da) microspheres still presented 44.3% in suspension. The total degradation of microspheres was achieved within approximately 9 hours for both PLGA (70:30) (0.9% remaining) and PLA (Mwt 2000 Da) (0.6% remaining). This similar time of degradation is due to the differences in molecular weight, because the homopolymer used was a smaller molecule. Thus, under these conditions the rate of degradation of the three polymers can be ordered as PLGA (50:50) > PLGA (70:30) > PLA (Mwt 2000 Da), confirming the data previously published (reviewed by Rosen *et al.*, 1988). When the degradation was carried out in neutral (PBS + 5% SDS, pH 7.4) or acidic (0.1 N HCl + 5% SDS, pH 1.0) solutions no differences were observed between the polymers and the microspheres were shown to be quite stable (fig 2.16-b and c). These results correlate with those published by Makino *et al.* (1986).

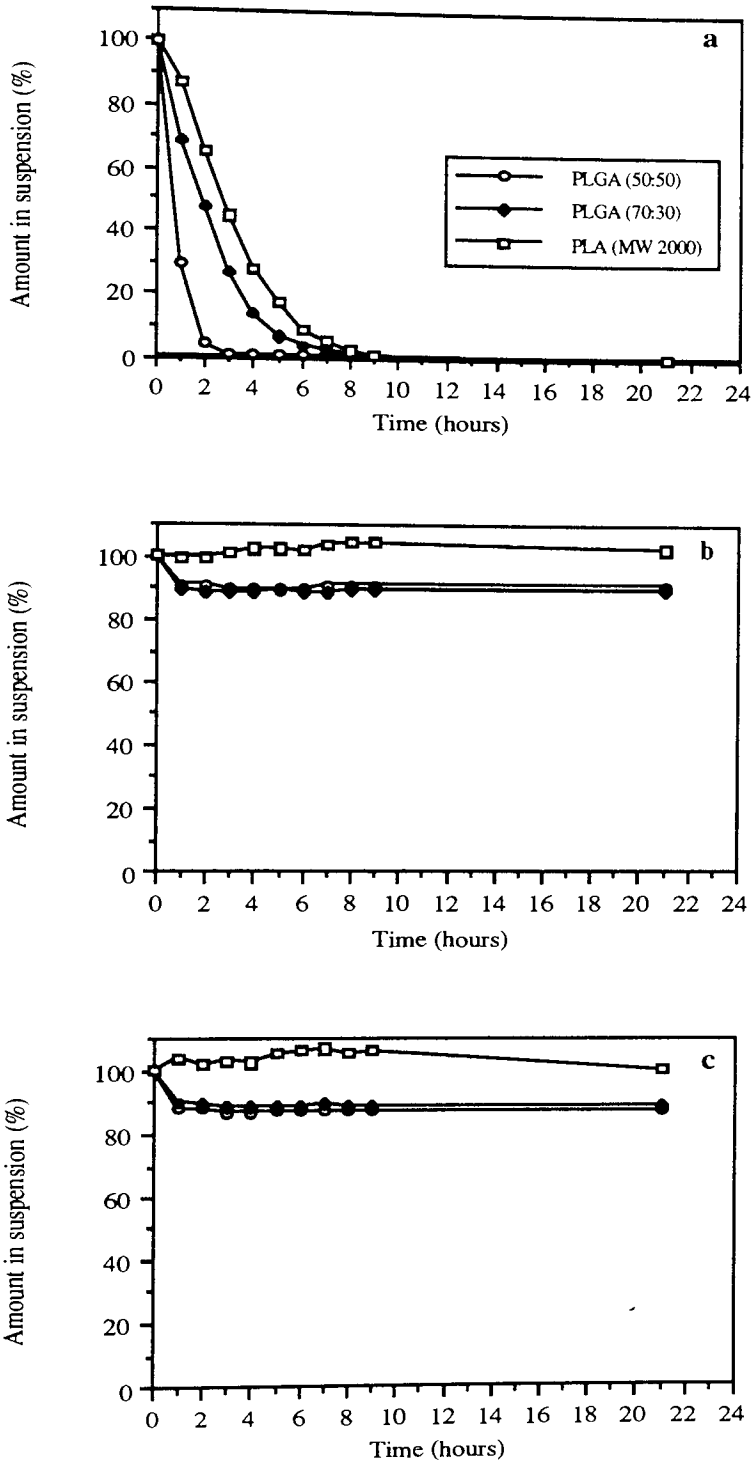


Figure 2.16 - Degradation of PLA/PLGA microspheres at different pH values: a) 0.1 M NaOH + 5% SDS, pH 11.7; b) PBS + 5% SDS, pH 7.4; c) 0.1 M HCl + 5% SDS, pH 1.0.

2.4.4. Protein Integrity

The dispersion of protein molecules in an organic solvent such as dichloromethane and the vigorous mechanical agitation during microparticle preparation, may cause damage to their structure or self-aggregation with consequent losses in physiological activity (Cohen *et al.*, 1991; Hora *et al.*, 1990; Jeffery *et al.*, 1993). For example, albumin can be denatured at the oil/water interface during the preparation of microspheres and the dichloromethane molecules may brake hydrophobic bonds which maintain the albumin tertiary structure (Verrecchia *et al.*, 1992). To assess whether such alterations occur during the microencapsulation method, the integrity of structures of TT and BSA were analysed by SDS-PAGE (section 2.3.7) before and after the encapsulation. In addition, the antigenic properties of the TT molecule after encapsulation and release were assessed *in vitro* by immunoblotting (section 2.3.8).

The SDS-PAGE analysis does not present any evidence of degradation during the preparation procedure as demonstrated by the fact that the characteristic bands of the proteins do not suffer any modification in the migration pattern and there were no additional bands to indicate the presence of aggregates or fragments (figs 2.17-a and b). Furthermore, this rules out the existence of chemical interactions, leading to a modification of the molecular weight of any band, between the polymer and BSA or TT, thus showing that these molecules are only retained physically by the PLA matrix. Therefore, the residence of these model proteins within the hydrophobic microspheres does not affect their stability.

The immunoblotting of TT shows that the molecule can react with the specific antibodies after being encapsulated and released from the microspheres, thus keeping its antigenicity. These properties are not significantly affected and the regions of the TT molecule that are responsible for its antigenicity (i.e. the epitopes) were not destroyed. The differences observed in the intensity of the bands before the encapsulation and after the release are due to the low concentration of TT in the release medium. To achieve the same intensity in SDS-PAGE bands, the samples would have to be concentrated by freeze-drying, which could cause some further denaturation. So, the alternative was to analyse the maximum possible volume of sample, which led to the diffusion of the released samples in some of the gels

and immunoblotting (fig. 2.18), and can be seen by the widening of the respective band.

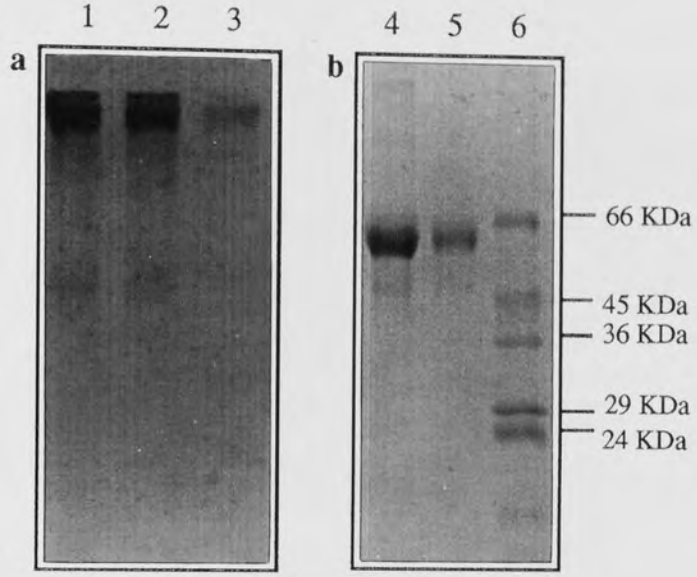


Figure 2.17 - SDS-PAGE (12% gels) of tetanus toxoid (a) and BSA (b). Lanes: 1) Tetanus Vaccine BP in Simple Solution; 2) TT before encapsulation in PLA (Mwt 2000 Da) microspheres; 3) TT after release from microspheres; 4) BSA before encapsulation in PLA (Mwt 2000) microspheres; 5) BSA after release from microspheres; 6) Molecular weight markers.

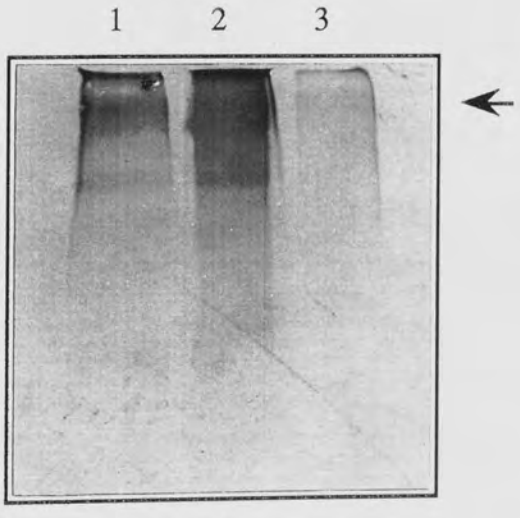


Figure 2.18 - Western blotting of TT before and after encapsulation in PLA (Mwt 2000) microspheres. Lanes: 1) Tetanus Vaccine BP in Simple Solution; 2) TT solution before encapsulation; 3) TT after release from PLA microspheres.

As the gels and the immunoblotting do not present any evidence of degradation occurring during the formulation, the stability of proteins appears to be maintained and antigenic activity was unaffected by the entrapment procedure and residence within the hydrophobic polymer. These results are in accordance to the work of Cohen *et al.* (1991), Hora *et al.* (1990) and Jeffery *et al.* (1993), who used similar *in vitro* approaches to assess the stability of proteins throughout the solvent evaporation microencapsulation procedure.

3. ADSORPTION OF MODEL PROTEIN ANTIGENS ONTO PLA MICROSPHERES

3.1. INTRODUCTION

The use of adsorption onto a preformed system as a loading technique is an approach extensively used for small particulate carriers, i.e. within the submicron range (Harmia *et al.*, 1986; Davis & Illum, 1989). With encapsulation the smaller the particle, the more difficult it becomes to achieve a high drug loading efficiency and a good sustained release effect. To circumvent this problem, the drug can be adsorbed onto the particle surface rather than encapsulated within (Watts *et al.*, 1990). Usually, nanoparticles prepared with acrylate polymers by the emulsion polymerisation method (section 1.1.2.7) are loaded by this technique (Couvreur *et al.*, 1979a; Harmia *et al.*, 1986; Brasseur *et al.*, 1991; Forestier *et al.*, 1992). Recently, PLA and PLGA microspheres have also been studied as carriers for adsorbed therapeutic peptides, e.g. calcitonin (DeLuca *et al.*, 1992).

Due to their amphipathic nature, proteins are known to adsorb onto glass or polymer surfaces in a wide range of biological and non biological processes (Soderquist & Walton, 1980). This physicochemical behaviour, leading to adsorption, can negatively affect the stability of the medicines of which they are the active ingredients, particularly at low protein concentrations (Burke *et al.*, 1992). The classical example is insulin administered by intravenous infusion, which can be 78% adsorbed by the glass bottles and the polyvinylchloride infusion containers and intravenous tubing (Petty & Cunningham, 1974). On the other hand, scientists have used this tendency to adsorption as a means to load particulate carriers with proteins. As reported in section 1.1.2.7 many papers describe the adsorption of antigens to poly(methylmethacrylate) microspheres and subsequent *in vivo* studies demonstrate their efficacy as vaccine delivery systems (Birrenbach & Speiser, 1976; Kreuter & Speiser, 1976; O'Hagan *et al.*, 1989a; Stienecker *et al.*, 1991).

Structural changes and loss of activity of protein molecules during adsorption and desorption from polymer surfaces have been described (Soderquist & Walton, 1980; Norde, 1984; Arai & Norde, 1990). The loss of activity of immobilised enzymes as a function of

time is often mentioned as an example of the change in the structure of adsorbed proteins (Soderquist & Walton, 1980). The three-dimensional structure presented by a protein molecule is the result of various interactions, e.g., hydrophobic interaction, hydrogen bonding, electrostatic interactions, S-S bonds, inside the molecule as well as between the protein and the environment (Arai & Norde, 1990). Therefore, the adsorption behaviour of proteins is related to its structure stability. Adsorption from aqueous solutions usually involves dehydration of hydrophobic areas of the adsorbate at the solid-liquid interface, which can modify the interfacial properties and the structure of the peptides. This is the reason why proteins in aqueous solutions can adsorb onto hydrophobic surfaces even when these present the same electrostatic charge (Norde & Favier, 1992). With globular proteins that have a strong internal coherence ('hard' proteins, e.g. lysozyme), the rearrangements do not contribute significantly to the adsorption process, whereas globular proteins with a much lower internal stability ('soft' proteins, e.g. BSA) undergo structural rearrangements upon adsorption, which, in turn, contribute to the adsorption affinity (Norde & Anusiem, 1992). For example, on hydrophilic surfaces the 'hard' proteins only adsorb to surfaces that present the opposite charge. Nevertheless, structural rearrangements enable the 'soft' protein molecules to adsorb even onto electrostatically repelling surfaces. However, when proteins are displaced from the surface, the original native structure may not be fully regained and the characteristics of the desorbed molecule may be different to those of the native protein (Norde & Favier, 1992).

In the present work the use of adsorption was designed to avoid the possible degradation of the protein molecules caused by the contact with the organic phase (dichloromethane) during the solvent evaporation technique of microencapsulation (Verrecchia *et al.*, 1992). However, TT and BSA keep their integrity throughout the process (chapter 2). Nevertheless, this approach was also proposed as a way of increasing the loading of the antigen-containing microspheres. The characterisation of the antigen-adsorbed preparations, as well as the physicochemical study of the adsorption process itself, were undertaken. To assess whether the formulation by adsorption affected the molecular stability of TT, *in vitro* studies of protein integrity were also carried out.

3.2. MATERIALS

3.2.1. Microspheres

Empty PLA (Mwt 2000 Da) and empty PLA (Mwt 50000 Da) microspheres, were prepared using the polymer described in section 2.2.1 The method for the preparation of empty PLA and PLGA microspheres is described in chapter 2, section 2.3.1 The particle size analysis was carried out as in section 2.3.2.

3.2.2. Model Protein Antigens

The proteins used in the adsorption experiments were BSA, TT and CTB, already described in section 2.2.2, and γ-globulins (bovine, Cohn, fractions II, III) purchased from Sigma Chemical Company, Poole, U.K.

3.2.3. Chemicals

All chemicals and reagents not specified in the text were supplied by BDH Chemicals Ltd. (Poole, U.K.), Sigma Chemical Company (Poole, U.K.) and Fisons (Loughborough, Leics.U.K.) and were of Analar grade or equivalent.

3.3. METHODS

3.3.1. Adsorption of Protein onto PLA Microspheres

Empty microspheres (50 mg) were incubated (shaking water bath, ≈100 cycles min^{-1}) for 24 hours with a protein solution in saline (5 ml, 25 to 3000 μg ml^{-1}, depending on the protein used - see tables 3.2 to 3.4). The temperature of the shaking water bath during the

incubation also depended on the protein: 20°C for TT and CTB; 37°C for BSA and γ-globulins. After incubation, the microspheres were centrifuged at 15000 rpm for 60 min (Ti-70 rotor, Beckman L8-60M Ultracentrifuge; Beckman Instruments, Bucks., U.K.) and the pellet was washed twice in saline solution (5 ml). A protein solution in saline, without microspheres, was used as a control.

To determine indirectly the amount of protein adsorbed, the initial supernatant and washes were analysed by the BCA protein assay (section 2.3.6). The measurement of the amounts of BSA and γ-globulins adsorbed was also carried out by direct UV spectrophotometry at 280 nm, using a Philips UV/Vis Spectrometer PU 8730. Standard solutions of the proteins in normal saline were prepared (50 μgml^{-1} to 2500 μgml^{-1}). Examples of the standard curves obtained are shown in figure 3.1.

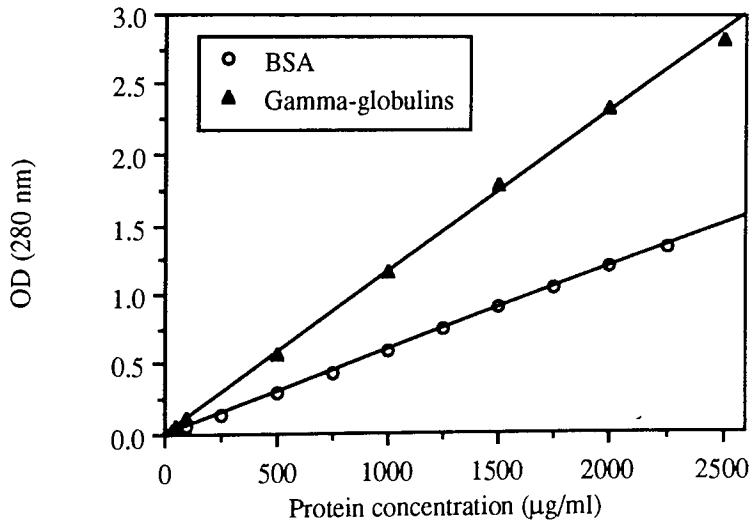


Figure 3.1 - Calibration curve for the estimation of BSA (r = 1.000) and γ-globulins (r = 0.999) by direct spectrophotometry.

The adsorption procedure was also monitored by SDS-PAGE (section 2.3.7) of starting solution, supernatant and washes. Pellets were dried in a desiccator under vacuum at room temperature. The type of adsorption that occurred was determined mathematically by using the models of Langmuir (1916) and Freundlich (1926) (reviewed by Carstensen, 1980 and Florence & Atwood, 1988).

3.3.2. Direct Determination of Adsorbed Protein

The amount of protein adsorbed per unit weight of microspheres was determined directly by the digestion method of Hora *et al.* (1990), described in section 2.3.4.

3.3.3. *In vitro* Release Studies

Desorption of protein from microspheres was studied *in vitro* using the method described in section 2.3.3.

3.3.4. Assessment of Protein Integrity

The assessment of protein integrity was investigated using SDS-PAGE (section 2.3.7). The immunogenic properties of TT after adsorption and release were analysed *in vitro* by immunoblotting (section 2.3.8).

3.4. RESULTS AND DISCUSSION

3.4.1. Loading onto Microspheres by Sorption Procedures

As an alternative to the direct incorporation method, adsorption appears to be the ideal technique for the loading of proteins onto microspheres. Proteins were adsorbed onto empty

PLA microspheres as previously described (section 3.3.1). The size distribution of the particles is not modified after adsorption, as shown in figure 3.2 (compare with figure 2.6). Results from protein adsorption onto PLA microspheres show amounts adsorbed were up to 97.3% of the initial amount. For the proteins studied the method allows a maximum loading efficiency of 13.0 ± 0.5 % for BSA, 10.2 ± 0.7 % for γ-globulins and 1.3% for TT. The adsorption studies carried out with CTB were not so detailed and the preliminary value found is shown in table 3.1.

As adsorption from solution depends on the solute concentration, the final composition of the formulation will depend on the concentration of protein added to the microspheres (tables 3.1 to 3.4). Plasma proteins seem to have a higher affinity for the PLA surface than TT or CTB.

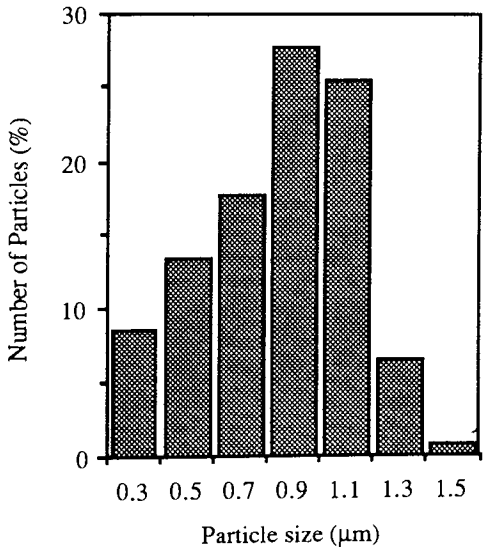


Figure 3.2 - Particle size distribution of PLA (Mwt 2000 Da) microspheres containing TT adsorbed onto the surface. Mean particle size = 0.8 μm; σ_g = 1.9 (n = 141 particles).

Table 3.1 - Adsorption efficiency of TT and CTB onto PLA microspheres.

Polymer	Protein	Amount Adsorbed [%(w/w)]	
		related to the initial amount of protein	in final spheres composition
PLA (Mwt 50000)	TT	45 to 75	2 to 6 (n = 2)
PLA (Mwt 2000)	CTB	41.6 ± 19.4	0.10 ± 0.05 (n = 3)

Table 3.2 - Adsorption efficiency of TT onto PLA (Mwt 2000 Da) microspheres (mean ± sd; n = 3).

Protein ($\mu g ml^{-1}$)	Microspheres (mg)	Amount Adsorbed [%(w/w)]	
		related to the initial amount of protein	in final spheres composition
50	50.4 ± 0.2	50.8 ± 25.0	0.6 ± 0.1
100	50.3 ± 0.3	50.1 ± 6.7	0.8 ± 0.1
150	50.0 ± 0.2	46.0 ± 3.6	1.1 ± 0.1
200	50.2 ± 0.2	42.6 ± 5.3	1.2 ± 0.3
250*	50.4	41.0	1.3

* n = 2

Table 3.3 - Adsorption efficiency of BSA onto PLA (Mwt 2000 Da) microspheres (mean ± sd; n = 3).

Protein (μgml^{-1})	Microspheres (mg)	Amount Adsorbed [%(w/w)]	
		related to the initial amount of protein	in final spheres composition
500*	50.2	95.6	4.3
750*	50.0	97.3	6.6
1000	50.2 ± 0.4	93.9 ± 0.4	8.4 ± 0.2
1250	50.2 ± 0.4	92.3 ± 0.3	10.6 ± 0.0
1500	50.2 ± 0.6	81.5 ± 1.3	11.3 ± 0.2
1750	49.8 ± 0.2	73.7 ± 2.9	11.7 ± 0.8
2000	49.8 ± 0.1	64.1 ± 2.7	11.6 ± 0.9
2250	49.9 ± 0.1	63.5 ± 1.6	12.6 ± 0.5
2500	50.2 ± 0.1	56.3 ± 0.7	12.1 ± 0.5
2750	49.9 ± 0.6	51.8 ± 1.1	12.9 ± 1.1
3000	50.2 ± 0.2	49.1 ± 0.2	13.0 ± 0.5

* n = 2

Table 3.4 - Adsorption efficiency of γ-globulins onto PLA (Mwt 2000 Da) microspheres (mean ± sd; n = 3).

Protein (μgml^{-1})	Microspheres (mg)	Amount Adsorbed [%(w/w)]	
		related to the initial amount of protein	in final spheres composition
500	49.8 ± 0.4	91.5 ± 2.3	4.3 ± 0.0
750	50.1 ± 0.3	92.1 ± 0.8	6.8 ± 0.0
1000	50.0 ± 0.5	85.4 ± 2.0	8.7 ± 0.4
1250	50.4 ± 0.2	71.6 ± 6.8	8.9 ± 0.1
1500	50.2 ± 0.4	69.4 ± 5.9	10.2 ± 0.7
1750	50.4 ± 0.2	54.7 ± 1.8	9.4 ± 0.6

When a high loading is required, both the direct use of the solvent evaporation procedure and the adsorption technique involve large initial amounts of drug. The amounts are approximately identical. For example, in the case of TT, to achieve a loading of about 1% (w/w) in 50 mg of microspheres the adsorption procedure requires an initial amount of 0.75 mg, whereas 0.85 mg will be needed to obtain the same loading with the same amount of microspheres by the solvent evaporation method. Nevertheless, the contact between the protein and the organic solvents is avoided, which may represent an advantage.

Adsorption of proteins was visually confirmed by SDS-PAGE. In figures 3.3 to 3.6 the difference in concentration between the solutions of protein, before and after adsorption, shows, by the clear loss of protein after incubation with the microspheres, that adsorption occurred.

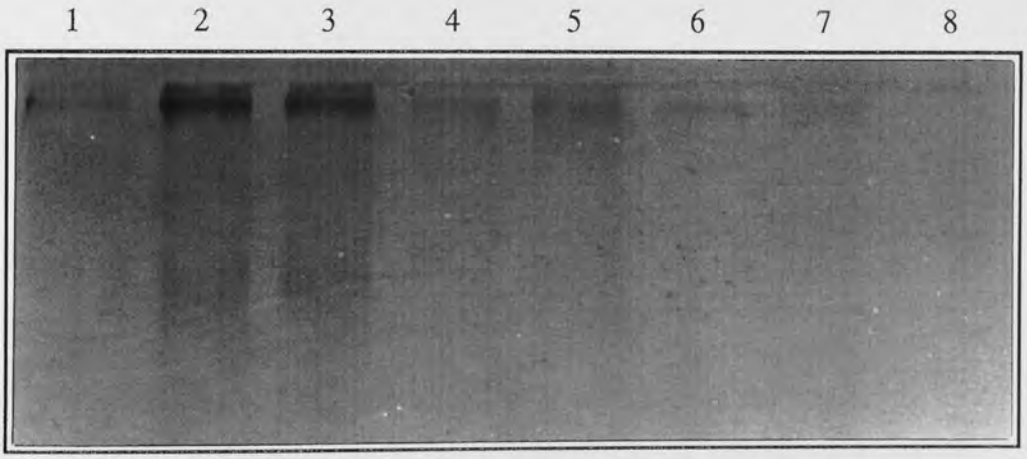


Figure 3.3 - SDS-PAGE (12% gel) of TT solutions before and after adsorption onto PLA (Mwt 2000 Da) microspheres. Lanes: 1) Tetanus Vaccine BP in Simple Solution; 2) and 3) 1000 $\mu g ml^{-1}$ before adsorption; 4) 1000 $\mu g ml^{-1}$ after adsorption; 5) 500 $\mu g ml^{-1}$ before adsorption; 6) 500 $\mu g ml^{-1}$ after adsorption; 7) and 8) washes.

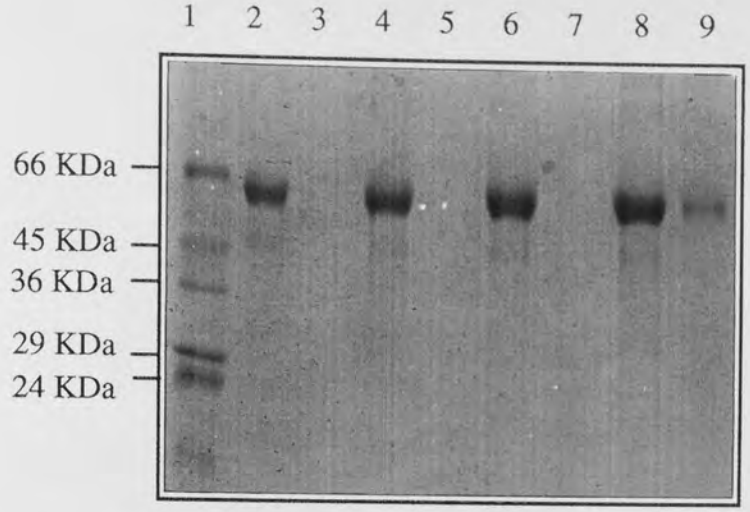


Figure 3.4 - SDS-PAGE (12% gel) of BSA solutions before and after adsorption onto PLA (Mwt 2000 Da) microspheres. Lanes: 1) Molecular weight markers (see table 2.3); 2 and 3) 750 μgml^{-1} before and after adsorption respectively; 4 and 5) 1000 μgml^{-1} before and after adsorption respectively; 6 and 7) 1250 μgml^{-1} before and after adsorption respectively; 8 and 9) 1500 μgml^{-1} before and after adsorption respectively.

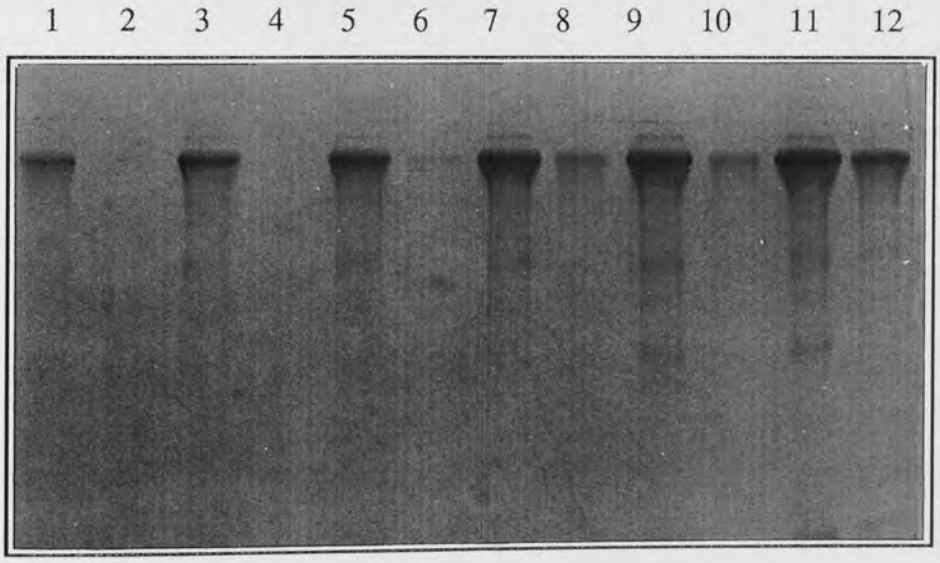


Figure 3.5 - SDS-PAGE (12% gel) of γ-globulins solutions before and after adsorption onto PLA microspheres. Lanes: 1 and 2) 300 μgml^{-1} before and after adsorption respectively; 3 and 4) 600 μgml^{-1} before and after adsorption respectively; 5 and 6) 900 μgml^{-1} before and after adsorption respectively; 7 and 8) 1200 μgml^{-1} before and after adsorption respectively; 9 and 10) 1500 μgml^{-1} before and after adsorption respectively; 11 and 12) 1800 μgml^{-1} before and after adsorption respectively.

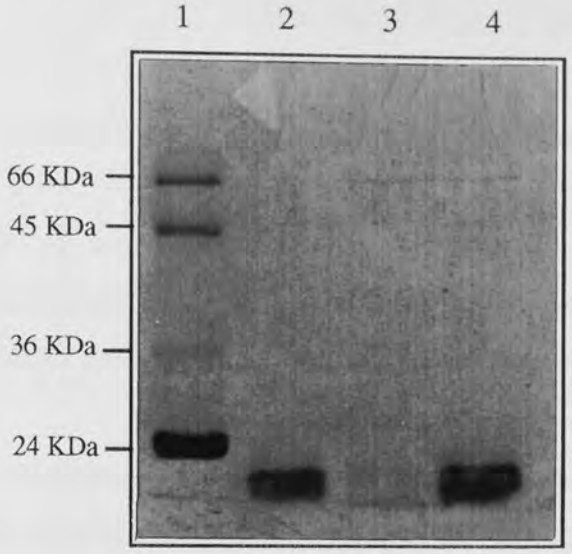


Figure 3.6 - SDS-PAGE (14% gel) of a CTB solution (500 $\mu g ml^{-1}$) before and after adsorption onto PLA (Mwt 2000 Da) microspheres. Lanes: 1) Molecular weight markers (see table 2.3); 2) solution after adsorption; 3) washes; 4) solution before adsorption.

3.4.2. Characterisation of the Adsorption Process

The methods of Langmuir (1916) and Freundlich (1926) (reviewed by Florence & Attwood, 1988), were used to characterise the adsorption of the model protein antigens onto the microspheres' surface.

Briefly, the equation of Langmuir was originally established for the adsorption of gases onto solid surfaces and is the most frequently used method for the interpretation of adsorption from solutions. The theory supporting this equation assumes that the contact between the solution and the solid surface will result in a final dynamic equilibrium in which the number of molecules that are adsorbed equals the number of those that abandon the solid, and that molecules or atoms are adsorbed on specific points of attachment on the solid surface (Hem *et al.*, 1986). It is also assumed that the adsorbed layer is unimolecular (monolayer). When it is applied to the adsorption from solutions the Langmuir equation can be written as follows:

$$x/m = abc/(1 + bc)$$

Where c is the equilibrium concentration of the solute; x is the amount of solute adsorbed per weight m of adsorbent; b is a constant related to the enthalpy of adsorption, also called adsorption coefficient; a measures the specific adsorptivity of the solute for that particular solid surface and represents the amount of adsorbate that constitutes a complete monolayer (Florence & Attwood, 1988).

Examples of the isotherms originated by this equation are shown in figures 3.9 and 3.11. By rearranging the equation and plotting c against $c/(x/m)$ a straight line is obtained:

$$c/(x/m) = 1/ab + c/a$$

The values a and b can be determined from the slope ($1/a$), and from the intercept on the ordinate ($1/ab$), respectively. Usually the deviations from this theory are attributed to the formation of multilayers and are treated by the equation of Freundlich which does not predict a limiting value for adsorption (Fell, 1988):

$$x/m = kc^n$$

In this equation, x is the amount of solute adsorbed per mass m of adsorbent; c is the concentration at equilibrium; k is a constant for the system whose index n is usually <1, so that the amount adsorbed increases less rapidly than the concentration.

The Freundlich isotherms can be handled more conveniently when written in the logarithmic form, which yields straight lines:

$$\log(x/m) = \log k + n \log c$$

The constant $\log k$ is the intercept on the ordinate and n is the slope of the straight line.

For the proteins studied the adsorption process appears to follow a Langmuirian type of equilibrium with specific adsorptivity values of 14.0 μgmg^{-1} for TT, 128.6 μgmg^{-1} for BSA and 99.4 μgmg^{-1} for γ-globulins (figs. 3.7 to 3.12). In the case of the plasma proteins the isotherms reach very clear plateaux of equilibrium (figs 3.9 and 3.11). Also, the Langmuir plots show good regression coefficients (0.998 for BSA and 0.996 for γ-globulins), while the adsorption behaviour of TT is not so well-defined, even with a regression coefficient of 0.995 (figs. 3.7 and 3.8). It happens that the isotherm of TT seems not to reach a state of equilibrium over the concentration range studied. However, lack of reliable data at concentrations of TT above 250 μgml^{-1} due to the tendency of soluble TT to aggregate, prevented extending the isotherm any further. Makino *et al.* (1987a) and Kondo (1992) reported similar types of isotherms as those obtained for BSA and γ-globulins but data on adsorption of TT could not be found in the literature. So, the confirmation of the data obtained with TT will be the subject of the next few pages.

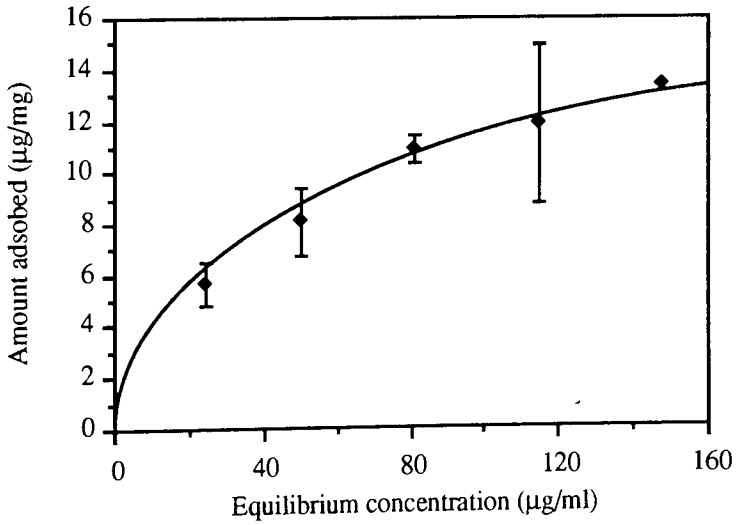


Figure 3.7 - Langmuir isotherm of TT adsorption onto PLA (Mwt 2000 Da) microspheres (mean ± sd; n = 3).

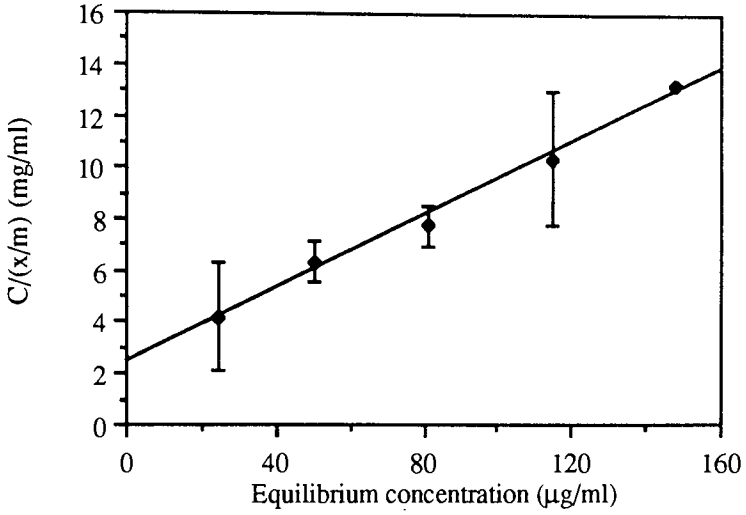


Figure 3.8 - Langmuir plot (r = 0.995) corresponding to the TT adsorption isotherm of fig. 3.7 (mean ± sd; n = 3).

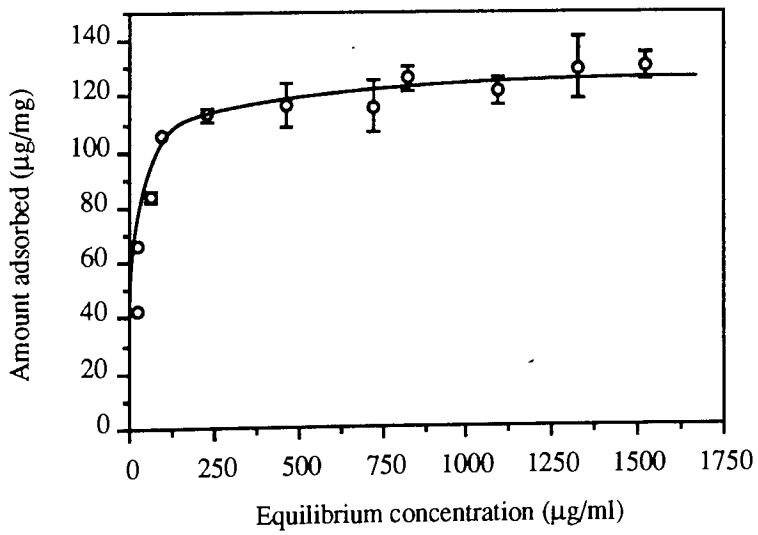


Figure 3.9 - Langmuir isotherm of BSA adsorption onto PLA (Mwt 2000 Da) microspheres (mean ± sd; n = 3).

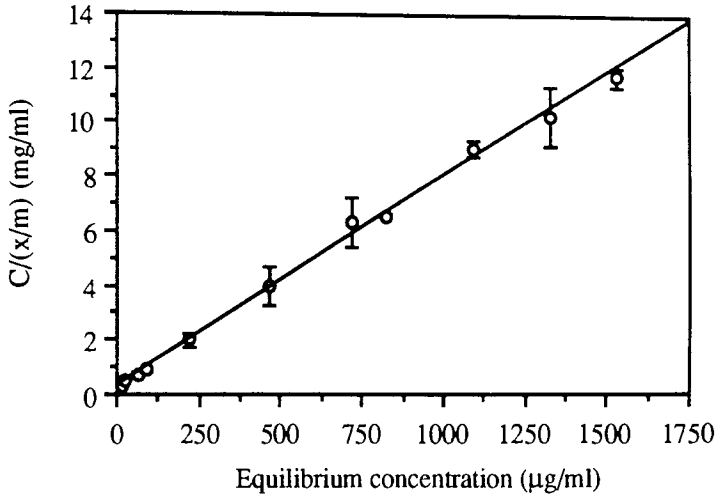


Figure 3.10 - Langmuir plot (r = 0.998) corresponding to the BSA adsorption isotherm of fig. 3.9 (mean ± sd; n = 3).

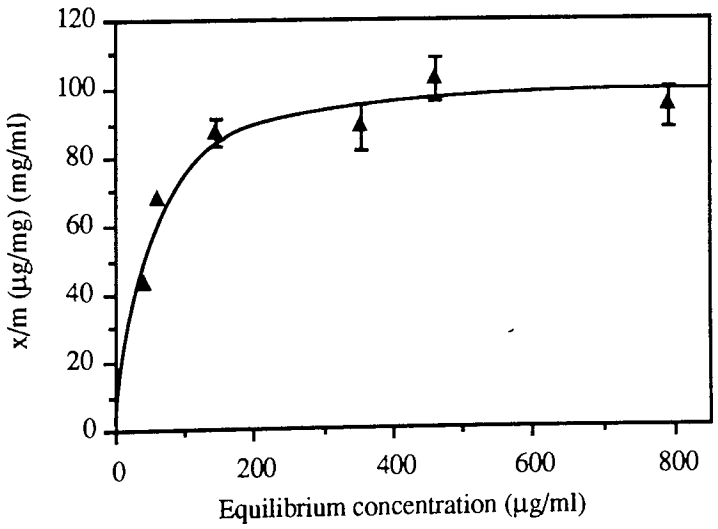


Figure 3.11 - Langmuir isotherm of γ-globulins adsorption onto PLA (Mwt 2000 Da) microspheres (mean ± sd; n = 3).

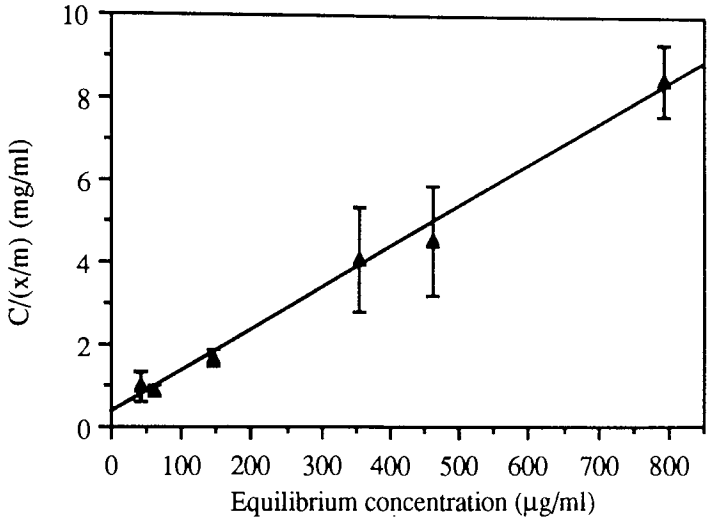


Figure 3.12 - Langmuir plot (r = 0.996) corresponding to the γ-globulins adsorption isotherm of fig. 3.11 (mean ± sd; n = 3).

The TT data were also treated by the equation of Freundlich and the agreement with this interpretation is as good as with the Langmuir theory (fig. 3.13). Thus, the type of adsorption obtained with TT under the experimental conditions used does not allow any immediate conclusions concerning the mathematical model that it follows.

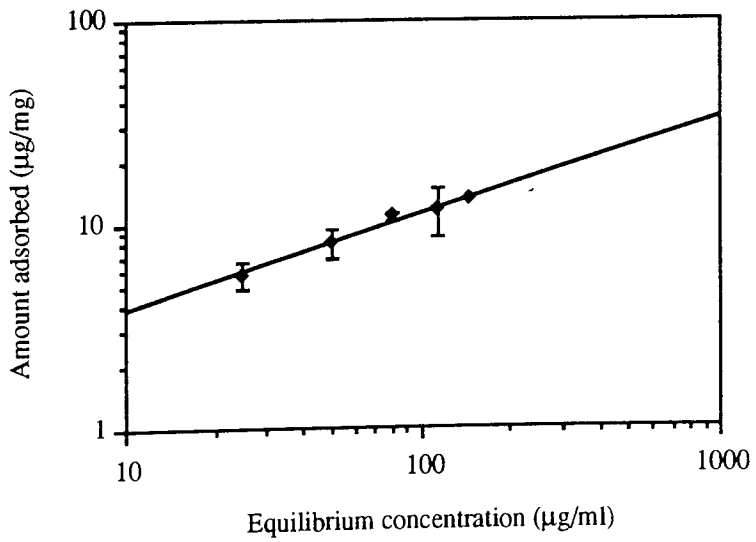


Figure 3.13 - Freundlich plot (r = 0.994) corresponding to the TT adsorption isotherm of fig. 3.7 (mean ± sd; n = 3).

Given the uncertainty concerning the model of adsorption followed by TT, the data were analysed by the Langmuir theory starting by the determination of the surface area of PLA microspheres using the values obtained with the adsorption of BSA. However, the value for projected surface area of the BSA molecule, which is the basis of the calculations, is not a fully accepted parameter because the molecular configuration, or three-dimensional structure, of albumin is not completely solved, being still the object of discussion. In this thesis, the model followed was that calculated by Squire *et al.* (1968) for BSA, used by Soderquist & Walton (1980) and confirmed by Carter *et al.* (1989) for human serum albumin (fig. 3.14a). Three ideal cases were taken into consideration for the adsorption of BSA, i.e. all molecules adsorbed side-on, all molecules adsorbed end-on and a mixed process in which the number of molecules adsorbed side-on is equal to the number of molecules adsorbed end-on (fig. 3.14b).

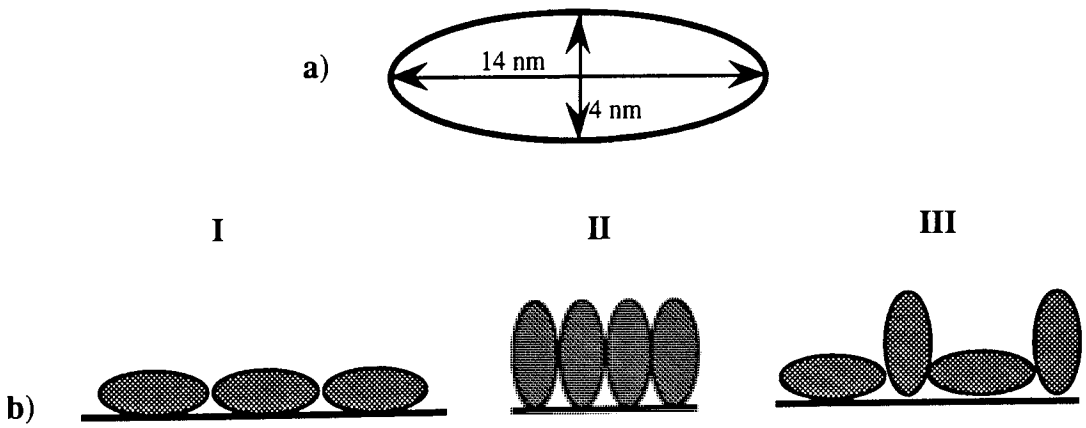


Figure 3.14 - a) Diagram of the ellipsoidal model of the BSA molecule with the approximate dimensions calculated by Squire *et al.* (1968). b) Adsorption of BSA: I) side-on; II) end-on; III) mixed process.

As the particle size distribution of the microspheres prepared by the method discussed in chapter 2 is reproducible, the projected surface area of one molecule of TT can be calculated from the surface area of the particles. Then the ratio between this value and the projected surface area of the BSA molecule was calculated for each case (see table 3.5) and compared to the ratio of specific adsorptivity values expressed in $molg^{-1}$ (BSA/TT = 20.9). The factor

of approximately 21 that relates the specific adsorptivity values was also found in the ratio of molecular projected surface areas (TT/BSA = 20.8).

Table 3.5 - Values obtained for TT worked out from BSA data by the Langmuir theory.

Orientation of BSA molecule	BSA projected surface area (nm^2)	PLA microspheres surface area (m^2g^{-1})	TT projected surface area (nm^2)	Ratio of molecule surface areas (TT/BSA)
side-on	44.0	51.5	91.6	20.8
end-on	12.6	14.7	26.2	20.8
mixed-process	—	33.1	58.8	—

However, these results are arguable. As Singh (1992) stated, there are a number of reasons why the apparently straightforward approach of Langmuir does not provide a totally reliable method for the determination of the surface area. For example, if the plateau of the isotherm is not well defined (e.g.TT in this study), the equation of Langmuir is unlikely to yield the true value of the specific adsorptivity of the solute. In addition, if the surface is heterogeneous, and interacts specifically with the solute molecules, the surface coverage will not be uniform. As it will be discussed in chapter 4, the surface of microspheres may be partially covered with PVA molecules remaining from the preparation by the solvent evaporation method. Therefore, their surface is likely to be heterogeneous. Furthermore, the orientation of the adsorbate molecules may vary and for large molecules the interactions adsorbent-adsorbate can influence the structure of adsorbed layer, which may extend beyond the monolayer or involve the adsorption of micelles (Singh, 1992). Finally, the same author mentions the incorporation of solvent in the monolayer, which may result in an appreciable variation of the effective molecular area.

3.4.3. *In Vitro* Desorption Studies

The release of adsorbed protein from microspheres (desorption) was also studied. As the molecules of polymers are attached to the adsorbent at several contact points, desorption would imply the simultaneous detachment of all these bonds, which is statistically improbable (Norde, 1984). Therefore, the desorption of proteins from the PLA microspheres should be a slow process. Also, due to molecular rearrangements during adsorption, their desorbability towards dilution is likely to be poor. However, the formulations exhibited fast release profiles. Approximately 30% of TT are released in the first 30 min, then reaching a stage of slower release (40% within 48 hours; fig. 3.15). Desorption is similarly fast for BSA (54% within 2 hours; fig. 3.16), while γ-globulins were completely desorbed within 3 hours (fig. 3.17).

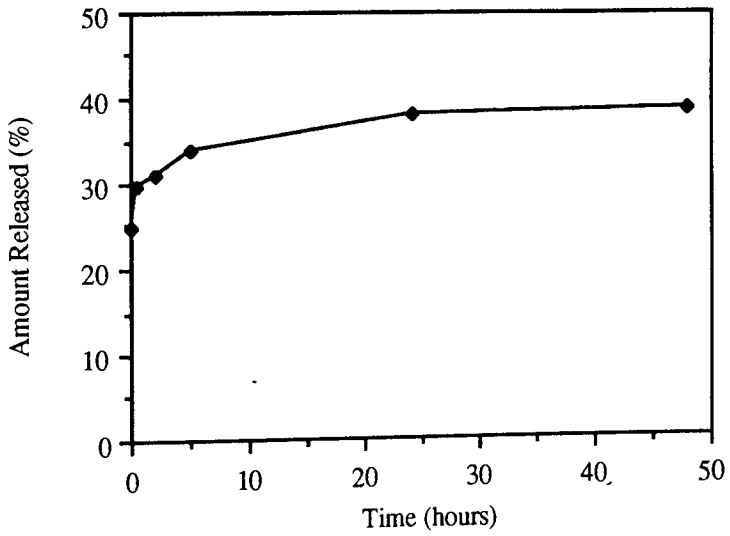


Figure 3.15 - Desorption profile of TT adsorbed onto PLA (Mwt 2000 Da) microspheres (in phosphate buffer 20 mM, pH 7.5; n = 3 ± sd).

The differences observed in the desorption rate for these proteins can be explained by their bonding and packing characteristics at the interface between solution and PLA microspheres

and their behaviour when in contact with an aqueous medium of different ionic moiety, strength and pH. Properties such as molecular configuration, chain length, presence and concentration of hydrophilic/hydrophobic 'anchor' groups in the polymeric protein and their orientation at the surfaces will affect their inherent affinity for the adsorption site and, therefore, the extent of their adsorption and desorption at interfaces.

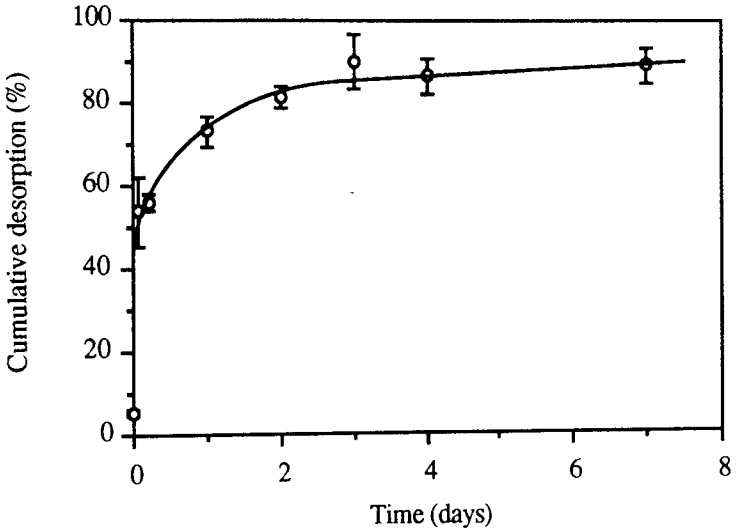


Figure 3.16 - Desorption profile BSA adsorbed onto PLA (Mwt 2000 Da) microspheres (in phosphate buffer 20 mM, pH 7.5; n = 3 ± sd).

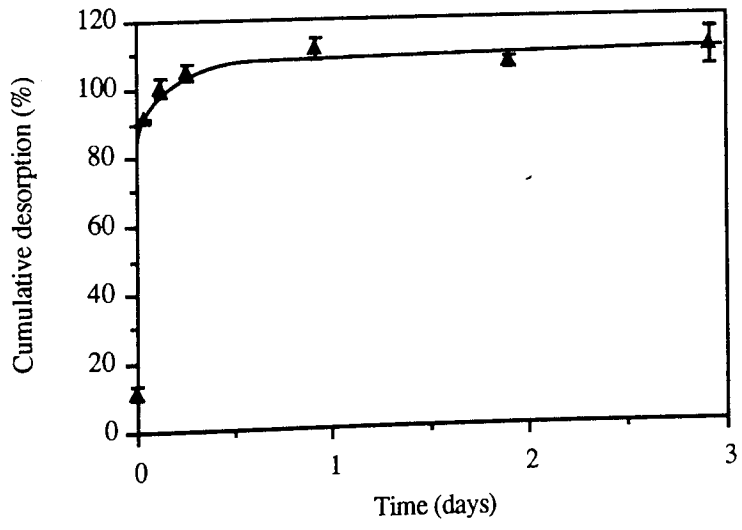


Figure 3.17 - Desorption profile of γ-globulins adsorbed onto PLA (Mwt 2000 Da) microspheres (in phosphate buffer 20 mM, pH 7.5; n = 3 ± sd).

Although the adsorption at the interfaces is generally considered as being through weak interaction forces (van der Waals) between the molecules of adsorbate and adsorbent and completely reversible, in the case of polymers (e.g. proteins) the presence of strong enough hydrophobic interactions and hydrogen bonds would result in the some irreversible adsorption taking place. Slow and incomplete desorption observed in the case of TT could be an example for such interactions. As the proteins were adsorbed in a physiological saline solution (0.9% NaCl, pH 5.6) and desorbed in 20 mM phosphate buffer, pH 7.4, the simple change of ionic strength and pH could have promoted such a relatively rapid desorption for BSA and γ-globulins. On the other hand, it should be mentioned that, in addition to the general effect of the ionic strength of the medium, the adsorption/desorption equilibrium may be influenced by the type of small ions that are present in the solution. For example, phosphate ion ($H_2PO_4^-$) can prevent protein adsorption of proteins onto glass and some plastic surfaces (Anik & Hwang, 1983). Phosphate ion also interferes with the adsorption of antigens onto $Al(OH_3)$ (Lindblad & Spärck, 1987).

For TT, the interaction between microspheres surface and protein may be more of a hydrophobic type, being affected less by the ionic changes. The adsorption phenomenon may be driven by non-polar or polar forces, e.g. hydrogen bonds also reported by Makino *et al.* (1987b). This type of strong interaction may have arisen from the orientation of protein molecules at the particle surface, leading to a situation where the polar moiety of the molecule established an interaction with the polar sites at the polymeric surface, and some of the hydrophobic sites folding outwards, while some interacting with similar sites on the available surface.

The relatively quick total desorption of BSA and γ-globulins from the microspheres confirm the Langmuirian type of adsorption for these two proteins as previously reported by Makino *et al.* (1987a) and Kondo (1992). These reports are contradicted by Verrecchia *et al.* (1992) who could not obtain a complete desorption of human serum albumin from PLA microspheres in several media (e.g. phosphate buffer and foetal calf serum) , attributing this fact to the modification of the first albumin layer that remains irreversibly adsorbed, thus classifying that system as presenting a non-Langmuirian type of physicochemical behaviour.

Although protein adsorption has been proposed as a dynamic process in which partial exchange of protein molecules between the adsorbed and dissolved states takes place (Norde & Anusiem, 1992), these authors obtained results with the adsorption of BSA onto hydrophilic surfaces (i.e. silica and haematite), similar to those described by Verrecchia *et al.* (1992). The present study with BSA and γ-globulins suggests exactly the opposite, i.e. the association of these proteins with PLA microspheres is completely reversible and follows the model of Langmuir. However, such a clear and complete desorption profile does not occur with TT, leading to doubts about whether the Langmuirian equilibrium does occur, or whether it behaves as described by Verrechia *et al.* (1992). Nevertheless, whatever the physicochemical process, it must be stated that the incomplete desorption of TT does not mean that the protein is being degraded, as will be discussed in section 3.4.3.

3.4.4. Stability of the Antigen

As reported in section 3.1, adsorption of proteins at solid-liquid interfaces may affect their secondary and tertiary structures and, consequently, some physicochemical properties. There is consequently concern about the possibility of irreversibly damaging the antigen during the formulation procedures.

The stability of the TT was investigated by SDS-PAGE before adsorption onto and after release from microspheres. No changes in the pattern of migration of the antigen could be detected. Also, no additional bands revealing fragmentation or aggregation were seen, which indicates that the structure of the molecule was maintained.(fig. 3.18a). Therefore, within the scope of this analytical method, the adsorption procedure appears not to affect the whole structure of TT. However, such observation does not necessarily indicate that the TT molecule retains its immunogenicity intact. For this reason Western blotting analysis of TT before adsorption and after release was carried out. The antigenic activity was also unaffected by the adsorption procedure as suggested by the results shown in figure 3.18b. The binding of the hyperimmune anti-tetanus serum to the desorbed toxoid demonstrates that the antigen can still be recognised by specific antibodies, which suggests that, at least, some epitopes remain intact. This immunoblot shows a lower colour intensity of the

desorbed sample (lane 6), when compared to the original TT solution (lane 5) which, as in figure 2.18 (section 2.4.4) and for the same reasons, is due to the low concentration of TT in the release medium.

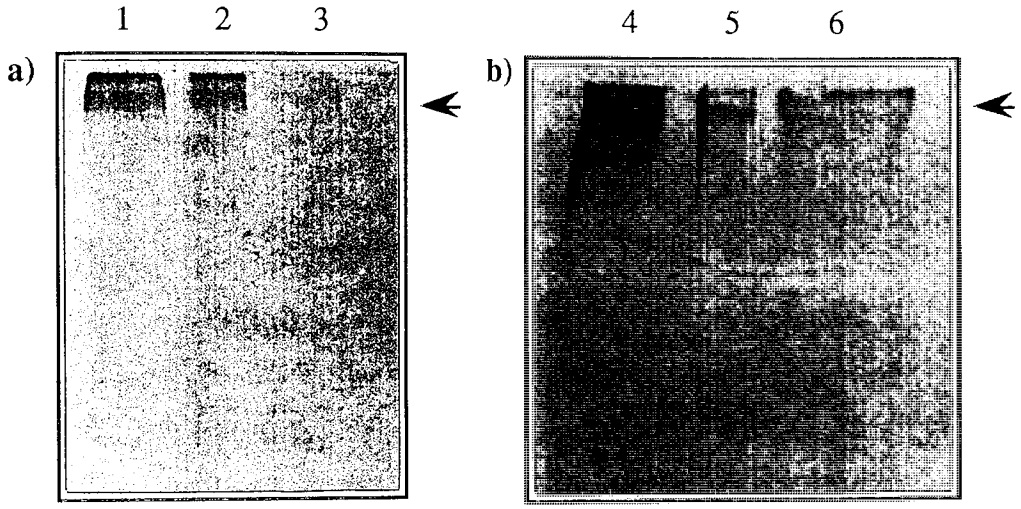


Figure 3.18 - SDS-PAGE (12% gel) (a) and Western blotting (b) of TT before and after adsorption onto PLA (Mwt 2000 Da) microspheres. Lanes: 1) Tetanus Vaccine BP in Simple Solution; 2 and 3) TT solution 250 $\mu g ml^{-1}$ before and after adsorption (supernatant); 4) Tetanus Vaccine BP in Simple Solution; 5) TT solution before adsorption; 6) TT after release from PLA microspheres.

4. SURFACE CHARACTERISATION OF PROTEIN-CONTAINING PLA MICROSPHERES

4.1. INTRODUCTION

After intravenous injection, the fate of colloidal particles in the body is determined mainly by their physicochemical characteristics, i.e. particle size and shape, particle charge and surface hydrophobicity (Müller *et al.*, 1986). They tend to be rapidly cleared from the blood stream and accumulate at tissue sites, such as liver and spleen, after being phagocytosed by cells of the reticuloendothelial system (RES) also called mononuclear phagocytic system (MPS) (reviewed by Bradfield, 1984 and Juliano, 1988). Therefore, particles can be targeted to the RES, mainly the Kupffer cells of the liver, macrophages of the spleen and bone marrow, but if the target is another organ it will be necessary to prevent the clearance. Müller (1991) defined the general requirements, which must all be fulfilled, to avoid RES recognition: *a)* uncharged particles; *b)* hydrophilic surface; *c)* little interaction with serum components, i.e. low zeta potential in serum; *d)* absence of complement activating groups; and *e)* appropriate conformation of the coating polymers on the surface. There are other requirements which are still unknown and for a successful outcome each parameter must be appropriately designed.

Avoidance of the RES has been studied by blocking the system with a prior injection of microspheres (Illum *et al.*, 1986a) and changing the particles' physicochemical properties, particularly their hydrophobicity. Van Oss (1978) reported that particles that are more hydrophobic than the phagocytes readily become phagocytosed, whereas particles which are more hydrophilic resist phagocytosis. However, the latter can also be taken up after opsonisation with plasma proteins. This phenomenon results from the adsorption of proteins (e.g. immunoglobulins, complement components, albumin, clotting factors, etc.) onto particles, which not only render them more hydrophobic but can also interact with receptors on the macrophages' surface, thus promoting phagocytosis (Juliano, 1988). The more hydrophilic the particles are, the better they resist opsonisation (Van Oss, 1978).

The influence of surface charge on the blood clearance of particulate matter has also been

studied. However, it is pertinent to emphasise that the relation between phagocytosis and zeta potential seems to be far from simple and its measurement does not result in a direct correlation between the two phenomena. It has been acknowledged for over twenty years that non-charged colloids are more likely to escape recognition by the RES (Wilkins & Myers, 1966). Due to opsonisation, particles with different surface properties present, after incubation with serum, a similar surface charge (≈ -11 mV to -18 mV) (Müller *et al.*, 1986). Therefore, since the surface charge is similar, the subsequent interaction between particles and phagocytic cells will be of hydrophobic nature and will depend upon the differences in hydrophobicity (Müller *et al.*, 1986). In any case, zeta potential is important because phagocytosis can be altered by charge effects. In fact, the composition and thickness of the coating layer of opsonins will depend not only on hydrophobicity of particles but also on the surface charge (Müller *et al.*, 1986).

Coating of particulate carriers with materials that produce a hydrophilic surface would reduce the uptake by the RES, extending their circulation times (Illum *et al.*, 1986b). Several authors have described the use of non-ionic surfactants, such as the block copolymers of the poloxamer series and, indeed, a substantial reduction in liver uptake and delayed clearance of microspheres from the blood was observed (Illum *et al.*, 1987a; Müller *et al.*, 1992; Tröster *et al.*, 1992). Adsorption of serum proteins onto the particulate carriers has also been used to avoid the clearance from the blood stream, as their endogenous nature would help to mask the foreign particles. PLGA and PLA microspheres (Tabata & Ikada, 1989; Bazile *et al.*, 1992), liposomes (Torchilin *et al.*, 1980) and poly(methylmethacrylate) nanospheres (Borchard & Kreuter, 1993) have been treated by this process with some success. The latter study involved the coating of the nanospheres with serum albumin, serum, or inactivated serum, and demonstrated that albumin coating leads to no significant change in the body distribution of particles. However, incubation with either native or inactivated whole serum significantly reduced the RES clearance. A slight improvement in the biodistribution of albumin coated PLA nanospheres has been reported (Bazile *et al.*,1992) although it was not efficient enough to allow the use of such preparations in the targeting of non-RES organs. Both papers claim that a possible change of albumin structure during the adsorption procedure could be responsible for the recognition of the coated particles by the RES (Bazile *et al.*, 1992; Borchard & Kreuter, 1993).

Apparently, the uptake of microspheres at mucosal sites and the subsequent phagocytosis by macrophages are also influenced by carrier hydrophobicity (Alpar, unpublished results). Probably because of a more efficient interaction with these antigen-presenting cells, more hydrophobic particles are often stronger adjuvants (Kreuter *et al.*, 1988), which is in accordance with the observations of Van Oss (1978).

The association of antigens with particulate carriers is usually achieved by entrapment within the microspheres. However, adsorption may be used as an alternative loading technique to avoid the harsher procedure of antigen-encapsulation, important when dealing with labile proteins (see chapters 2 and 3), and since the 1970's there is experimental evidence (Kreuter & Speiser, 1976) for the efficacy of antigen-adsorbed microspheres. The coating of microspheres with proteins will alter their surface characteristics. On the other hand, during the microencapsulation procedure, protein molecules can be deposited at the surface, so influencing the physicochemical properties of the carriers. Therefore, when compared to empty PLA microspheres, the formulations herein studied can present a distinct pattern of uptake, depending on the surface properties that result from encapsulation or adsorption. In addition, in the sequence of studies described in chapter 3, it was expected to obtain more information about the type of interactions that are responsible for the adsorption of the model protein antigens. Hence, the changes in surface characteristics of the microspheres after encapsulation or adsorption of model protein antigens were monitored. The characterisation of these formulations included the assessment of hydrophobicity by a modification of the hydrophobic interaction chromatography (HIC) method and zeta potential analysis.

4.2. MATERIALS

4.2.1. PLA Microspheres

The empty and protein-containing PLA (Mwt 2000 Da) microspheres used in these experiments were prepared by the solvent evaporation method described in section 2.3.1. Empty PLA (Mwt 2000 Da) microspheres prepared by the same method but using

polysorbate 80 (Tween 80) as an emulsifier instead of PVA were also used. PLA (Mwt 2000 Da) microspheres prepared using PVA as an emulsifier and loaded by adsorption using the method described in section 3.3.1, were also analysed.

4.2.2. Latex Particles

Latex particles (polystyrene, monodisperse, 1 μm ± 0.009 sd and 0.51 μm ± 0.008 sd; manufacturer's information) were used as a known hydrophobic control and purchased from Polysciences, Inc., Northampton, U.K. Prior to use, particles were washed several times and then dialysed with double-distilled water to remove surfactants or preservatives present in the suspension.

4.2.3. Model Protein Antigens

The proteins used in the preparation of the formulations herein studied were TT, BSA and γ-globulins described in sections 2.2.2 and 3.2.2.

4.2.4. Chemicals

Agarose, propyl-agarose, pentyl-agarose, hexyl-agarose and octyl-agarose used as stationary phases in HIC were purchased from Sigma Chemical Company (Poole, U.K). All the other chemicals and reagents not specified in the text were supplied by BDH Chemicals Ltd. (Poole, U.K), Sigma Chemical Company (Poole, U.K) and Fisons (Loughborough, Leics. U.K.) and were of Analar grade or equivalent.

4.3. METHODS

4.3.1 Hydrophobic Interaction Chromatography (HIC)

The procedure of Smyth *et al.* (1978), and Mozes & Rouxhet (1987), has been used to measure the degree of hydrophobicity of bacteria by comparing adsorption to hydrophobic groups in an uncharged bed of sepharose. The main work on HIC of microparticles was carried out by Müller (1991) who used several agarose derivatives to distinguish between batches of very similar hydrophobicity.

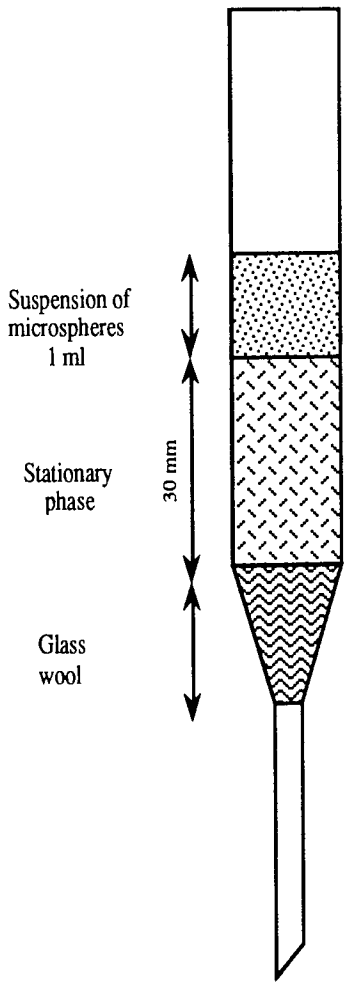


Figure 4.1 - Diagram of a HIC column prepared using a glass Pasteur pipette as described in the text.

The chosen method used in this study followed the procedures described by Smyth *et al.* (1978), and Mozes & Rouxhet (1987), combined with the approach of Müller (1991) and Carstensen *et al.* (1991).

For the chromatography columns, glass Pasteur-pipettes, plugged with glass wool, were used (fig. 4.1). The agarose, propyl-agarose, pentyl-agarose, hexyl-agarose and octyl-agarose (Sigma Chemical Company, Poole, U.K), were washed several times in double-distilled water to remove any traces of preservative and then resuspended in 0.6 M NaCl, pH 7.4 (adjusted with 0.01 M NaOH) to produce a thick slurry of approximately 70% sepharose. The agarose and derivatives were degassed under vacuum for 15 min, and then layered on top of the glass wool sinter in the Pasteur pipette to a height of 30 mm (approximately 1 ml of packed gel volume) and the column was washed with 10 ml of 0.6 M NaCl, pH 7.4 to keep the bed hydrated and to expose the hydrophobic surface.

Three identical columns were run per each type of agarose used. Suspensions of microspheres were adjusted to an OD_{600} of 0.5 (Philips UV/Vis spectrometer PU 8730) and 1 ml volumes were loaded onto columns, followed by 2 x 1 ml of 0.6 M NaCl, pH 7,4, and 2 x 1 ml of solution 0.1% Triton X-100 (Sigma Chemical Company, Poole, U.K) in double-distilled water. The OD_{600} of the eluates (five fractions of 1 ml each) were compared to the OD_{600} of 1 ml of the original suspensions. The results were expressed as percentage decrease in OD_{600}, and graphs of cumulative elution and final retention values were plotted. The hydrophobicity of the formulations determined by this method was calculated as a percentage of microspheres eluted from the column and the total percentage retained onto the packing. The percentage of particles eluted by the 0.6 M NaCl solution and that washed off with aqueous 0.1% Triton X-100 were also calculated.

4.3.2. Zeta Potential Determination

The charge carried by the microspheres can be determined by measuring the particle mobility in an electric field, then using the equations of Smoluchowski, Henry or Debye-Hückel (Müller *et al.*, 1986) to calculate the zeta potential (ζ). The electrophoretic mobility (μ_E) is defined as the particle's velocity under unit electric field and usually expressed in relation to the particle velocity (v) and the the applied field strength (E), as in the equation:

$$\mu_E = \frac{v}{E}$$

where v is measured in ms^{-1}, and E in Vm^{-1}, so that μ_E has the dimensions $m^2s^{-1}V^{-1}$ (Kayes, 1988). The equation used for converting the electrophorectic mobility into zeta potential depends upon the values of the Debye-Hückel parameter (k), which depends on electrolyte concentration, and of the particle radius (a). At large values of ka the Smoluchowski equation can be applied. In the present work, given the actual conditions of measurement (aqueous medium, moderate electrolyte concentration and an average particle radius of approximately 1 μm), this was the only equation used:

$$\zeta = \mu_E \frac{4\pi\eta}{\varepsilon}$$

where η is the viscosity of the medium (in poises) and ε is the respective dielectric constant. The value of ζ is measured in mV. It follows from this equation that the electrophoretic mobility of a non-conducting particle for which k*a* is large at all points on the surface, should be independent of its size and shape provided the zeta potential is constant (Shaw, 1980).

The microsphere preparations were dispersed by a 2 min sonication in a solution of 1 mM KCl, pH 5.0, and analysed by laser Doppler anemometry (LDA), using a zetameter (Malvern ZetaSizer 4, Malvern Instruments, Ltd., Malvern, U.K.) provided with a digital correlator (Series 7032 Multi-8, Malvern Instruments, Ltd.). Determinations were undertaken using the following parameters (table 4.1):

Table 4.1 - Parameters used in zeta potential measurements.

Parameter	Value
Cell type	AZ104
Cell voltage	136.0 ± 0.9 V
Current	0.5 mA
Conductivity	0.15 ± 0.01 mS
Temperature	25.5 ± 0.1°C
Dielectric Constant	79.0
f(ka)	1.50 (Smoluchowski)

According to McFadyen (1986) and Müller (1991), a LDA set up (fig. 4.2) consists of two coherent laser beams derived from the output of a low power laser that intersect within the

sample cell, forming a beam crossover with a pattern of interference fringes. Particles moving across the fringes in response to the applied electric field scatter light with an intensity which fluctuates at a frequency related to their velocity. The frequency of the scattered laser light differs from the frequency of the initial laser beam. This shift is caused by the Doppler effect and is a function of the particle velocity. The signal from individual photons of scattered light are detected by a photomultiplier and analysed by a digital correlator to give a frequency spectrum from which the particle mobility and the zeta potential are calculated.

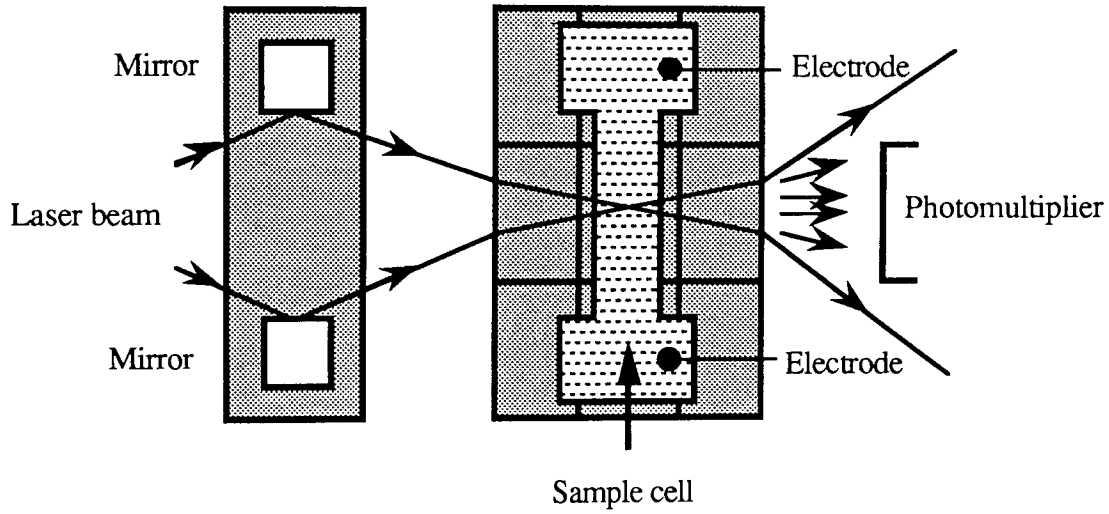


Figure 4.2 - Diagram of Malvern ZetaSizer 4 (adapted from Malvern Instruments Ltd. product information).

4.3.3. Statistical Methods

Comparison between the amounts of particles retained in, or eluted from, the HIC columns were analysed for significance using Student's unpaired t-test. Differences with $p < 0.05$ were considered significant.

4.4. RESULTS AND DISCUSSION

4.4.1. The HIC Method: an Appraisal

The origins and the final version of the HIC method used in these studies have already been described in section 4.3.1. This is a modification of a procedure first designed for the measurement of the hydrophobicity of bacteria (Smyth *et al.*, 1978) and the conditions used are different from those of the method employed to assess the hydrophobicity of microspheres (Müller, 1991). Therefore, it is not possible to make any direct correlations between the results obtained with this technique and those of the other two used as references. All the HIC results presented in this thesis are valid as comparative values between samples analysed under the same conditions.

Three aspects had to be investigated before the final adoption of the technique. The first was related to the the ionic strength that would allow the detection of differences in hydrophobicity between the various preparations. A characteristic feature of all hydrophobic interactions is that they diminish upon decreasing the ionic strength of the medium (Hjertén, 1973). Therefore, addition of salts can promote or reduce the hydrophobic interactions between matrix and solute. Anions such as Cl^- and PO_4^{3-} increase hydrophobic interactions ('salting-out' effect), whereas the addition of cations such Ca^{2+} and Ba^{2+} disrupts the structure of water ('chaotropic effect') and leads to a decrease in interactions (Müller, 1991). Figure 4.3 shows the variation in total retention of a standard preparation of PLA microspheres with the ionic strength of the eluent, in both the least hydrophobic (agarose) and the most hydrophobic (octyl-agarose) stationary phases. An optimal concentration of 0.5 M NaCl, giving the larger difference in retention ($p \leq 0.0005$) for PLA (Mwt 2000 Da) microspheres was found. After further adjustments, the working eluent used in all the subsequent determinations was a solution of 0.6 M NaCl. Elution can be accomplished by adding a component (e.g. non-ionic surfactants such as Triton X-100) that binds to the stationary phase and displaces the particles bound to the matrix. They also interact with the particles' surface making them less hydrophobic, which facilitates displacement (Hjertén, 1973).

The second aspect to take into consideration was the influence of particle size in the retention of microspheres in the columns. They should be retained only by hydrophobic binding with the stationary phase and not by a mere filtration mechanism. The dependence on particle size was studied with polystyrene particles of 0.5 μm and 1 μm in diameter (fig. 4.4).

There is no difference in retention between the two particle sizes when the columns were packed with pentyl-agarose, hexyl-agarose and octyl-agarose. The retention is though significantly different in columns of agarose ($p < 0.005$) and propyl-agarose ($p \leq 0.005$). However, the pattern of retention is similar for both particle sizes, i.e. the retention reaches approximately 100% when the hydrophobicity of the stationary phase increases from propyl-agarose to pentyl-agarose, the difference in retention being highly significant for both particle sizes ($p \leq 0.0005$). On the other hand, since the several agarose derivatives present a particle size (60 to 140 μm, dried bead diameter; manufacturer's information), which is big enough to allow the polystyrene particles to permeate through the column packings, the difference in retention observed with agarose and propyl-agarose may be due to actual physicochemical differences between the two commercial batches of polystyrene particles.

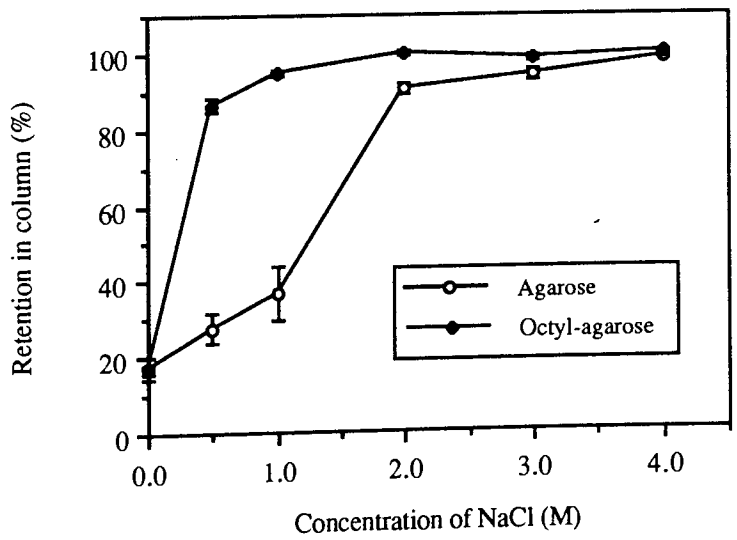


Figure 4.3 - Influence of the ionic strength of the eluent on the retention of PLA (Mwt 2000 Da) microspheres (n = 3; mean ± sd).

The third aspect was the number of particles (i.e. the size of the sample) which must be appropriate for the dimensions of each column used. Each should be loaded with such an amount of microspheres so that both the saturation of the stationary phases' binding sites by a too large sample and the total and irreversible retention of a too small sample could be avoided. It was found that 1 ml of a suspension presenting an OD_{600} value of 0.5 was the appropriate size.

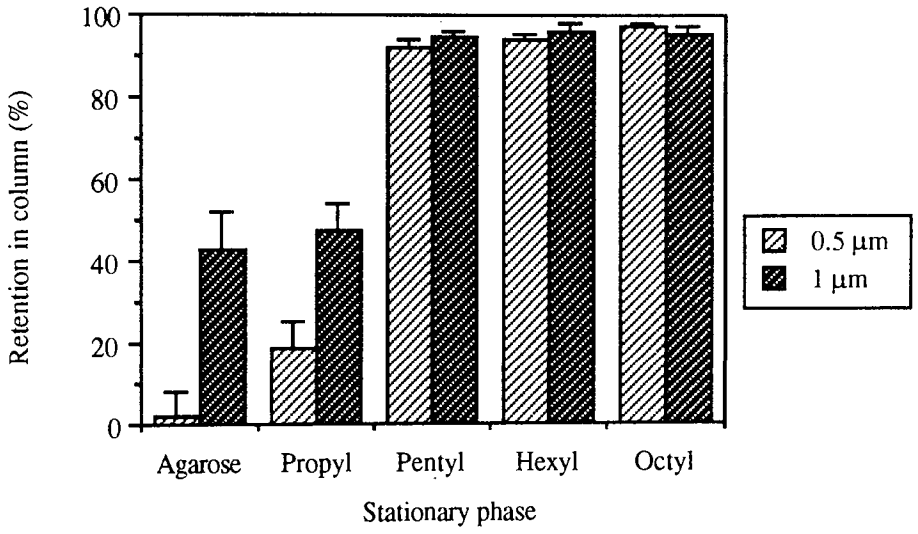


Figure 4.4 - Particle size dependence of the HIC method used in these studies as measured using two different sizes of polystyrene particles (n = 3; mean ± sd).

4.4.2. HIC: General Results

Table 4.2 shows the percentage of microspheres eluted per type of column and the amount washed off with the surfactant. The main feature observed is that, even after washing, the columns may retain a significant amount of microspheres, suggesting the occurrence of irreversible binding to the stationary phases, which is more frequent in the more hydrophobic packings. This means that some binding sites will remain permanently

occupied by irreversibly bound particles, which could affect further results if the column was to be reused. For this reason each column was used only once.

Table 4.2 - Elution of particles per sample and per type of agarose (% eluted ± sd; n = 3).

Formulation		Stationary Phase				
		Agarose	Propyl-	Pentyl-	Hexyl-	Octyl-
TT-enc.	Elution	50.6±3.0	50.2±0.9	33.6±2.3	32.4±2.5	27.0±1.0
	Wash	7.0±1.9	5.9±0.7	4.7±1.1	4.0±0.4	5.1±0.6
BSA-enc.	Elution	63.3±2.9	67.2±4.2	66.4±2.7	58.8±2.2	11.1±1.7
	Wash	8.1±2.8	6.5±0.3	8.6±0.8	11.3±2.1	5.1±1.7
TT-ads.	Elution	57.0±2.9	54.3±4.3	52.5±3.9	50.0±6.2	30.4±2.6
	Wash	2.9±0.4	3.4±0.2	3.6±0.5	5.4±2.7	3.2±0.4
BSA-ads.	Elution	37.3±4.5	44.8±4.2	29.3±4.1	32.9±3.9	7.6±1.1
	Wash	3.0±0.4	5.0±1.3	2.2±0.8	1.7±0.4	2.0±0.4
γ-G.-ads.	Elution	42.5±2.3	32.7±3.2	28.2±6.3	33.5±5.6	25.1±1.4
	Wash	4.9±0.3	11.9±3.7	7.4±2.3	8.0±2.9	3.3±0.6
PLA	Elution	34.6±2.9	36.1±4.4	44.6±1.5	42.2±6.3	28.2±3.8
(PVA)	Wash	13.1±5.2	11.3±1.1	9.8±2.7	12.7±0.5	9.9±2.9
PLA	Elution	47.2±8.4	39.3±7.3	37.7±1.6	36.8±0.9	5.8±1.2
(Tween 80)	Wash	39.4±10.2	48.5±9.7	42.2±1.5	41.9±1.7	36.6±3.2
Latex	Elution	0.0±0.0	0.0±0.0	1.9±1.1	1.7±1.3	3.2±1.8
(1 μm)	Wash	57.0±9.1	52.5±6.4	3.2±0.7	2.6±1.9	1.7±1.0
Latex	Elution	1.3±0.7	1.3±1.5	0.4±0.6	0.4±0.3	0.6±0.3
(0.5 μm)	Wash	96.5±5.8	80.2±6.4	7.7±1.9	5.7±1.4	2.4±0.4

With the exception of latex particles, which were sometimes completely retained, all the PLA batches were partially retained in the columns. Both the 0.6 M NaCl used as eluent and the aqueous Triton X-100, did not remove all the particles. Nevertheless, the comparison with latex particles shows that empty PLA microspheres and protein-associated formulations are less hydrophobic. A detailed discussion of these results is given in the following sections 4.4.3 and 4.4.4.

4.4.3. Hydrophobicity of the Protein-Encapsulated Formulations

In section 4.1 it was mentioned that the microencapsulation technique is likely to produce preparations that present some protein molecules deposited at the surface, so influencing the surface characteristics of the carriers, which in turn will differ from those of the empty PLA microspheres. To guarantee that such differences could be detected, the preparations used in these experiments were some of those with higher encapsulation values [10.7% (w/w) of BSA and 3.0% (w/w) of TT].

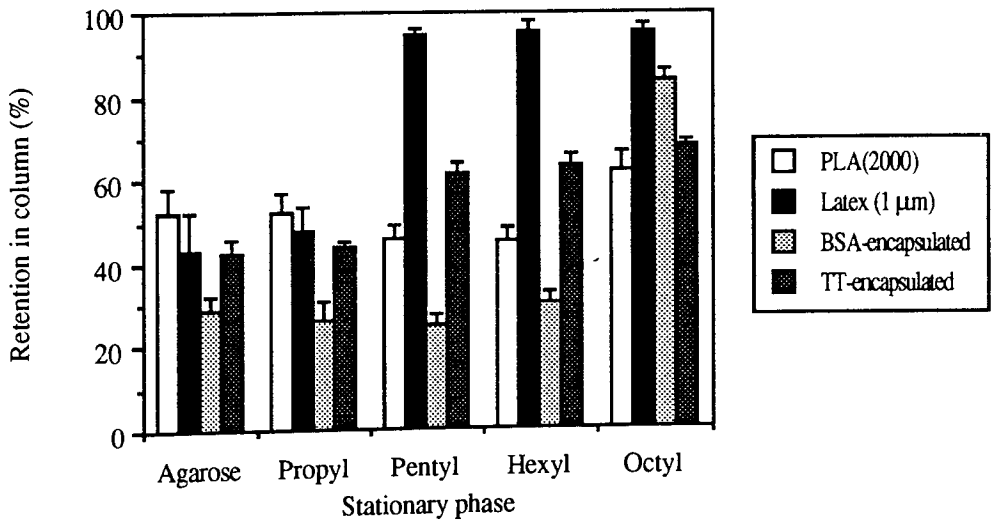


Figure 4.5 - Final retention by various stationary phases of the different protein-encapsulated formulations, compared with latex microspheres of a similar particle size (mean ± sd; n = 3).

Except in octyl-agarose, BSA-encapsulated microspheres are retained less than empty PLA particles (fig. 4.5). Considering only the most hydrophobic packings, the difference between these two preparations is significant ($p \leq 0.0005$ for pentyl-agarose, $p \leq 0.025$ for hexyl-agarose and $p \leq 0.01$ for octyl-agarose). The fact that the BSA-encapsulated preparation is more retained by the octyl-agarose than the control (i.e. empty PLA microspheres) is surprising since it was less retained by propyl-agarose, pentyl-agarose and hexyl-agarose, so changing its pattern of behaviour.

On the other hand, the results from retention of the TT-encapsulated preparation seem to be more consistent throughout the series of stationary phases. It shows always a pattern of retention more similar to that of the empty PLA microspheres and it is gradually more retained when the hydrophobicity of the packings is increased. This means that TT encapsulation results in preparations slightly more hydrophobic but the overall situation is that the hydrophobicity was more affected by BSA than by TT. This can be due to the protein loading of the preparation since the BSA formulation contains a higher amount of protein than the TT-containing microspheres. Therefore, it is likely to affect more the hydrophobicity of the particles. In addition, the efficiency of the washing step after preparation of the microspheres and the amounts of both protein and PVA remaining at the surface are also important. However, all the encapsulated formulations are far less hydrophobic than latex particles

4.4.4. Hydrophobicity of the Protein-Adsorbed Formulations

To study the changes in surface properties, it is important that the proteins form a coverage as complete as possible on the surface of the particles, which is normally only obtained in the plateau of the adsorption isotherms (Illum *et al.*, 1987a). The preparations used fulfilled this condition and contained 11.5% (w/w) of BSA, approximately 1.5% (w/w) of TT and approximately 10.3% (w/w) of γ-globulins. The HIC characterisation of these preparations suggests that the hydrophobicity of a PLA microsphere surface is only slightly modified after adsorption with either BSA or γ-globulins, but unchanged with TT (fig. 4.6). The results obtained with the two more hydrophilic columns (agarose and propyl-agarose) do

not allow any type of distinction between the several protein-adsorbed preparations and the controls. Therefore, only the retention in the more hydrophobic stationary phases (pentyl-agarose, hexyl-agarose and octyl-agarose) shows some difference between the several batches.

Adsorption of BSA renders the particle surface slightly more hydrophobic than empty PLA microspheres ($p \leq 0.005$ for pentyl-agarose, $p \leq 0.01$ for hexyl-agarose and $p \leq 0.005$ for octyl-agarose). The coating with γ-globulins produced an even lower increase in hydrophobicity, whereas TT does not affect significantly the retention of the microspheres throughout the series of stationary phases.

These results may appear surprising since coating hydrophobic microspheres with hydrophilic proteins results in an increase of the surface hydrophobicity. There are two possible explanations for such physicochemical behaviour. The first is the solvent-evaporation technique for the preparation of PLA or PLGA microspheres which includes the emulsification of the organic phase that contains the polymer. PLA (Mwt 2000 Da) is a hydrophobic molecule that, during the process, will orientate itself so that its more hydrophilic moiety will be exposed to the aqueous external phase. In the case of poly(lactide) the carboxyl groups of the molecule would be exposed, which may explain the involvement of dipolar interactions (e.g. hydrogen bonds) between the proteins and the particles' surface. The possibility of involvement of hydrogen bonds was also reported by Makino *et al.* (1987b). During adsorption some polar groups of the protein would then stay in contact with the solid surface and some of the hydrophobic regions of the molecule would be exposed to the liquid, so contributing to an increase of hydrophobicity.

The second explanation is also related to the same process of emulsification and to the use of a non-ionic emulsifier (PVA, Mwt 10000 Da) to stabilise the emulsion. This large molecule accumulates at the oil/water interface, its hydrophobic segments have affinity for the organic solvent and its polar groups for the aqueous phase. During the evaporation process it will remain at the interface in close contact with the hydrophobic PLA. Further washing steps are usually unable to remove completely the surfactant. Nevertheless, this is not a classical example of adsorption because the entanglement of some molecules of the

emulsifier in the polymer cannot be withdrawn (Bazile *et al.*, 1992). The surface of the resultant microspheres will be at least partially covered with PVA molecules which are likely to have their hydrophilic groups exposed. This causes a reduction in surface hydrophobicity and affects the process of adsorption of proteins, that may bind to the particles through polar interactions, so exposing some hydrophobic regions.

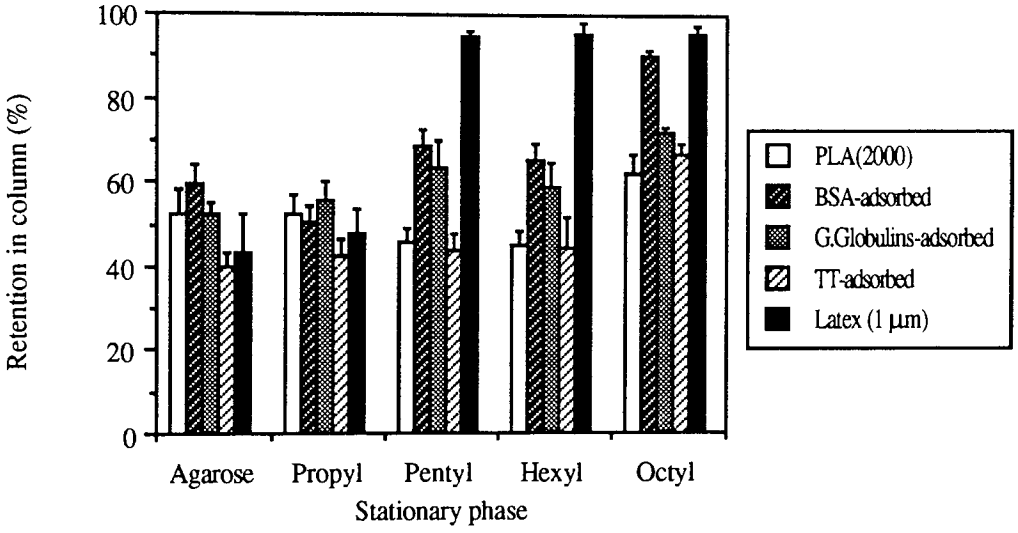


Figure 4.6 - Final retention by various stationary phases of the different protein-adsorbed PLA microsphere preparations, compared with latex microspheres of a similar size (mean ± sd; n = 3).

To investigate the role of the surfactant during the preparation and its influence on the surface characteristics, PLA microspheres were prepared by the same method but using, instead of PVA, a chemically distinct non-ionic surfactant. Differences in hydrophobicity were observed when the emulsifier employed during the fabrication was changed from PVA to Tween 80 (fig 4.7). The latter was chosen because it has been used in the preparation of microspheres (Bodmeier & McGinity, 1987b), and is chemically different from PVA. The microspheres prepared with Tween 80 are in general more hydrophilic than those prepared with PVA. The two preparations present the same hydrophobicity towards octyl-agarose, but the PVA-stabilised preparation is significantly more retained in propyl-agarose ($p \leq 0.0005$), pentyl-agarose ($p \leq 0.0005$) and hexyl-agarose ($p \leq 0.005$). The influence of the

surfactant employed on the microspheres' surface properties is thus demonstrated, and its implications on the adsorption of proteins are an obvious consequence.

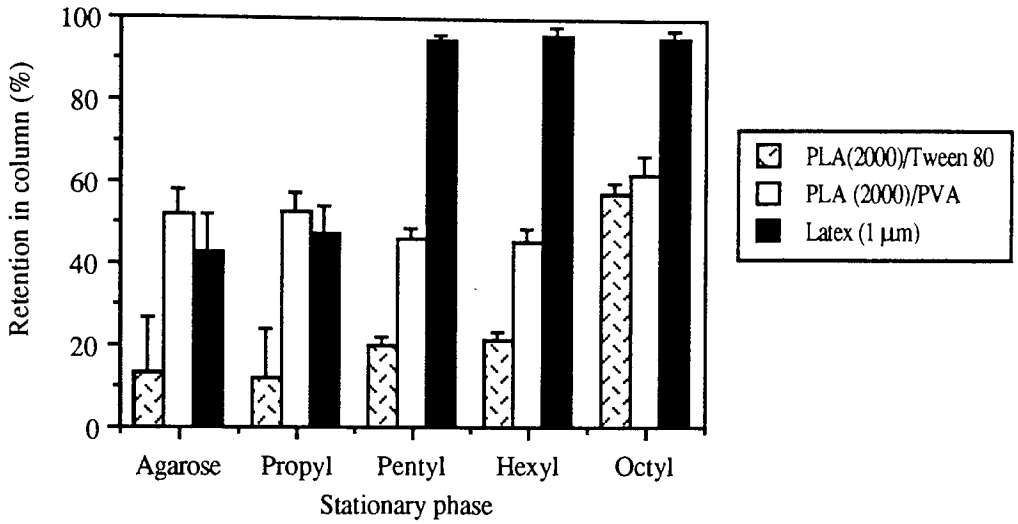


Figure 4.7 - Comparison of the final retention values between empty PLA microspheres prepared with PVA and Tween 80, respectively (mean ± sd; n = 3).

This study has shown that polar interactions are probably involved in this process, probably hydrogen bonds. However, the results did not contribute to characterise the model of adsorption followed by TT which, after adsorption, shows a hydrophobicity similar to that of empty PLA microspheres and, therefore, remains unclear.

4.4.5. Surface Charge Measurement

The zeta potential of a colloidal particle is the measure of its effective electrostatic charge (Martin *et al.*, 1983). The analysis of the surface charge and the assessment of the changes caused by the encapsulation or the adsorption procedures were carried out by the measurement of zeta potential (section 4.3.2). Given the experimental conditions, only general trends regarding the surface charge can be assessed. For example, the fast release of

protein reported for both the encapsulation and adsorption preparations can influence the final results, as well as the conditions under which the redispersion of the microspheres is carried out. To obtain a fine dispersion it may involve sometimes a light sonication of the suspension that can contribute for the desorption of the protein. Again, the influence of the surfactant used to prepare the microspheres cannot be excluded, as demonstrated by the difference of zeta potential between the PVA-stabilised and the Tween 80-stabilised preparations (fig 4.8).

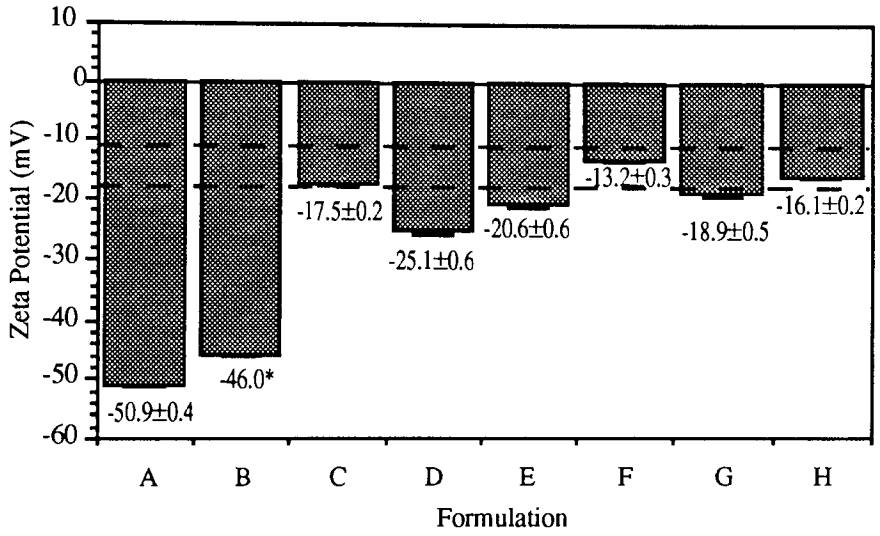


Figure 4.8 - Surface charge of the various formulations measured as zeta potential (mean ± sd; n = 3). Legend: A) Latex microparticles (1 μm); B) PLA microspheres prepared with Tween 80; C) PLA microspheres prepared with PVA; D) BSA-encapsulated PLA microspheres; E) BSA-adsorbed PLA microspheres; F) TT-encapsulated PLA microspheres; G) TT-adsorbed PLA microspheres; H) γ-globulins-adsorbed PLA microspheres. (* n = 2).

All the PLA microspheres analysed were less negatively charged than the latex controls and the variation observed in PLA particles after both of the formulation procedures is small (fig. 4.8). The batches obtained by microencapsulation do not show any consistent trend. BSA renders the microspheres more negative but TT does exactly the opposite. Possibly responsible for this variation are not only the chemical differences between BSA and TT but also differences in the total amount of protein encapsulated and its distribution at the

surface, the amount of emulsifier remaining deposited onto the surface and the experimental conditions above described.

A general increase of the net negative charge of the microspheres after adsorption was found, which is in accordance with the conventional model for the potential distribution around a charged particle (Shaw, 1980). It assumes that charges are located at the particular surface, and predicts that, with the exception of some cases of adsorption of surface active or polyvalent counter-ions, the zeta potential and the net charge of the particle are always of the same sign. The adsorption of surface active co-ions can create a situation in which the magnitude of zeta potential is increased (Shaw, 1980). Under the experimental conditions, BSA (isoelectric point = 4.7) carries a net negative charge and therefore increase the negative charge of the particles. A similar type of behaviour was shown by TT, but conclusions cannot be drawn because its isoelectric point is unknown. On the other hand, this is confirmed by γ-globulins [average isoelectric point ≈ 6.4 (Elgersma *et al.*, 1992) to 6.8 (Makino *et al.*, 1987b)] that also bear some positive charge under the conditions of assay and renders the particles slightly less negative. Probably in the case of the microspheres prepared using Tween 80, the surfactant is attached to the surface mainly by the alkyl chains leaving the negative part of the dipole (the ethylene dioxide chain) directed into the solution.

One last comment about the surface charge is that most of the values of the model antigen formulations are within the range described by Müller *et al.* (1986) for particles after incubation in serum, i.e. ≈ -11 mV to -18 mV (dashed lines in fig. 4.8) and, except for latex particles and for the Tween 80-stabilised PLA microspheres, the rest of them are very close. In this situation particles are easily phagocytosed by macrophages, which could be a good indication for their uptake at mucosal sites by tissues belonging to the MALT and the subsequent translocation to the lymphatics or blood stream by the macrophages (see section 1.3.1).

5. IMMUNISATION STUDIES WITH MICROSPHERE-ASSOCIATED TETANUS TOXOID

5.1. INTRODUCTION

The exploitation of new generation vaccines and the delivery of peptides and proteins in general has been impeded by a lack of appropriate delivery systems. Most protein molecules show poor transport characteristics across the epithelial barrier and are most commonly administered parenterally, but an alternative route would increase patient compliance. Due to its easier accessibility, the nasal cavity is a potential alternative to the parenteral route for peptide drugs. On the other hand, although nasal and pulmonary absorption routes are potentially important in the administration of immunogenic substances (O'Hagan & Illum, 1990) few reports have indicated their full potential. For some infections, the mucosal route is the most appropriate way of immunisation (Bienenstock, 1988). The antigens may be administered locally to the immunoactive tissues, such as BALT and NALT, at certain areas of the respiratory tract for the induction of an immune response. Nasal administration of antigens could produce effective vaccines both for protection of the upper respiratory tract and for induction of systemic immunity. Antigens that are responsible for the induction of protective immunity against various pathogens may be attached to a suitable carrier with adjuvant (or sustained release) properties, to induce effective and long lasting local immunity by interacting with the MALT, or for inducing protective systemic immunity upon reaching immunocompetent organs, e.g. spleen (Gregoriadis, 1990). Microspheres prepared with PLA and PLGA have been widely used to deliver antigens (reviewed in sections 1.1.2.7 and 1.3.1). The paucity of information in the area of nasally administered carrier-associated antigens, led to the investigation of the advantages of using this route.

Experiments were designed to study the nasal route for delivering antigens associated with particulate carriers and the immune responses subsequently induced. In this study, microspheres containing TT encapsulated or adsorbed were used. Mice and guinea-pigs were immunised by the nasal route with TT, which was employed as a model. For comparison, mice were also treated by the oral and intramuscular routes. Part of the *in vivo* work was carried out at the laboratories of the Ministry of Defence, Porton Down, U.K.

5.2. MATERIALS

5.2.1. Formulations

The PLA (Mwt 2000 Da) microspheres containing TT, were formulated either by microencapsulation (section 2.3.1) or by adsorption (section 3.3.1). Empty and TT-containing PLA (Mwt 2000 Da) and PLGA (50:50) microspheres of different particle sizes were prepared by the solvent evaporation method (section 2.3.1). All the preparations were analysed by the methods described in chapters 2 and 3.

A commercially available vaccine consisting of TT adsorbed onto aluminium salts (Tet/Vac/Ads), was used as a positive control in the studies involving the parenteral route, and was purchased either from The Wellcome Foundation Ltd., Beckenham, U.K. (Wellcome Adsorbed Tetanus Vaccine BP®), or from the Institut Mérieux, Lyon, France (Mérieux Tetavax®).

The free (also called soluble) TT used in these experiments was obtained by dialysis and freeze-drying from Tetanus Vaccine BP in Simple Solution (The Wellcome Foundation Ltd.), as described in section 2.2.2.

5.2.2. Animals

Female Dunkin-Hartley guinea pigs (≈300 g), male and female Balb/C mice (≈25 g) were used. During the experiments all animals were allowed food and water *ad libitum*.

5.2.3. Anaesthetics

In the *in vivo* experiments carried out at Aston University, halothane (Fluothane®, ICI Pharmaceuticals, Macclesfield, U.K.) was used as an inhalation anaesthetic for intranasal

dosing of guinea-pigs, and withdrawal of blood, by cardiac puncture, from guinea-pigs and mice.

5.2.4. Chemicals

All chemicals and reagents not specified in the text were supplied by BDH Chemicals Ltd. (Poole, U.K.), Sigma Chemical Company (Poole, U.K.) and Fisons (Loughborough, Leics. U.K.) and were of Analar grade or equivalent.

5.3. METHODS

5.3.1. Intramuscular Immunisation Studies with Microencapsulated TT

5.3.1.1. Preliminary Immunisation Study

Four groups of Balb/C mice (n = 4 per group) were treated with TT *via* the i.m. route. The four different preparations administered included: a standard preparation (see section 2.3.1.) of PLA microspheres containing TT encapsulated [10% (w/w)] suspended in normal saline; aluminium-adsorbed TT (Mérieux Tetavax®); empty PLA microspheres; and normal saline. A single vaccine dose of 5 μg of TT, dispersed or diluted with normal saline to a volume of 0.1 ml, was administered on days 1 (priming) and 28 (booster). The vaccine preparation was carried out just before injection. Blood samples were collected by cardiac puncture before dosing and on weeks 4, 6, 7 and 9 into the experiment. The blood was allowed to clot, centrifuged at 1000 ×*g* for 10 min at room temperature and the serum collected and stored at -20°C until analysis by the ELISA method described in section 5.3.5. The results were expressed as OD values at 450 nm for a serum dilution of 1:20.

5.3.1.2. Study of Factors Influencing the Immune Response

The influence of dose, polymer composition, and particle size on the immune response to intramuscularly-administered microencapsulated TT was assessed through the following *in vivo* study. Microspheres of three different sizes were prepared by the solvent evaporation method (section 2.3.1) during which the rotation speed of the mixer was adjusted to obtain mean particle sizes of approximately 0.8 μm (≥ 10000 rpm; Silverson Mixer model STD 1), 2 μm (2000 rpm; Silverson Mixer model STD 1) and 20 μm (800 rpm; Heidolph Mixer type RZR 50).

Eighty Balb/C mice (n = 5 per group) were injected i.m. with 0.1 ml of various amounts of TT microencapsulated in PLA (Mwt 2000 Da), or PLGA (50:50) microspheres of different sizes, or adsorbed onto aluminium (Tet/Vac/Ads; Wellcome Adsorbed Tetanus Vaccine BP®), dispersed or diluted with PBS just before use. These included groups of control animals treated with empty PLA (Mwt 2000 Da) microspheres, empty PLGA (50:50) microspheres or PBS (see table 5.1). Primary immunisation was carried out with a single injection on day 1 and animals were boosted with the same dose of antigen on day 28. Blood samples were collected at intervals (days 0, 11, 39, and 84) and treated for ELISA analysis as described above (section 5.3.1.1). The results were expressed as OD values at 450 nm for a serum dilution of 1:160.

5.3.2. Intranasal Immunisation with TT Adsorbed onto Microspheres

Three groups of guinea pigs (n = 3) were used in these experiments. A single dose of 60 μg of TT was used, group 1 being treated with microspheres containing TT adsorbed onto the surface. This preparation contained 1% (w/w) loading in the final composition of the microspheres and a mean particle size of 0.8 μm ± 1.8 (σ_g). Group 2 was treated with free TT dissolved in PBS and group 3 (control) was given only buffer solution. Vaccine was obtained by suspending the microsphere preparation in a calculated volume of vehicle in order to obtain the required concentration of TT. This preparation was carried out just before

administration. Simultaneously, a solution of free TT in the same vehicle and of the same concentration was prepared. Animals were given the vaccine by introducing 100 µl of the appropriate preparation into each nostril by means of an automatic pipette (Gilson pipetman P-200; Anachem Ltd., Luton, U.K.).

Table 5.1 - Immunisation schedule of the study described in section 5.3.1.2.

Group	Formulation	Amount Encap. [%(w/w)]	Mean Particle Size ($\mu m \pm \sigma_g$)	Dose (µg of TT)
1	Microspheres PLA/TT	3.4	0.8 ± 1.4	1.0
2	"	"	"	0.1
3	"	8.3	2.4 ± 1.8	1.0
4	"	"	"	0.1
5	"	4.7	18.5 ± 1.5	1.0
6	"	"	"	0.1
7	Microspheres PLGA/TT	1.7	1.0 ± 1.6	1.0
8	"	"	"	0.1
9	"	1.8	2.8 ± 1.5	1.0
10	"	"	"	0.1
11	"	2.0	24.0 ± 1.7	1.0
12	"	"	"	0.1
13	Tet/Vac/Ads	—	—	1.0
14	"	—	—	0.1
15	Empty PLA microspheres	—	0.8 ± 1.7	—
16	Empty PLGA microspheres	—	0.8 ± 1.6	—

Priming consisted of three doses on days 1, 2, and 3, respectively, and the booster consisted of one dose one month after the third dose of priming (week 5). Serum samples were obtained by cardiac puncture before immunisation and at weeks 2, 4, 7 and 15. The

blood was allowed to clot, centrifuged at 1000 $\times g$ for 10 min at room temperature and the serum collected and stored at -20°C until analysis by ELISA.

5.3.3. Intranasal Immunisation with Microencapsulated TT

This *in vivo* study was carried out by Dr. E. D. Williamson, at the Chemical and Biological Defence Establishment, Porton Down, Salisbury, UK., using formulations prepared by microencapsulation (section 2.3.1) at Aston University. The PLA microspheres used were of a mean size 0.8 μm ± 1.7 (σ_g) and contained 4.4% (w/w) of TT encapsulated.

Three delivery routes were compared: nasal, oral and i.m.. Adult female Balb/C mice, were used at 6-8 weeks of age. Oral dosing was effected through an especially-adapted blunt-ended gavage needle in a volume of 100 μl of 1.5% $NaHCO_3$. Intranasal dosing was performed in a total volume of 5 μl of 1.5% $NaHCO_3$ under light sedation [a mixture of medetomidine hydrochloride (Dormitor®; SmithKline Beecham Animal Health, Surrey, U.K.) and ketamin hydrochloride (Ketalar®; Parke Davis Research Laboratories, Hampshire, U.K.)], administered i.m.. Animals were revived by administration of the antidote atipamzole (Antisedan®; SmithKline Beecham Animal Health, Surrey, U.K.) subcutaneously.

Animals were assigned to either an oral, intranasal or i.m. schedule in which they were primed on days 1, 2 and 3 and boosted on day 28. For the oral and intranasal routes, a number of different dose levels of TT encapsulated in microspheres was administered. A group of 5 animals was assigned to each dose level. Doses of TT of 5, 15, 25 and 40 μg were administered intranasally. Oral dosing was carried out with doses of 10, 25, 50 and 75 μg. A single dose level of 2.5 μg TT was administered i.m. (day 1). Control groups received empty PLA by only one of the three routes.

Blood samples were collected from lightly sedated animals by superficial venepuncture of the tail vein on days 1, 25, 35, 43, and 53 of the immunisation schedule and pooled by

group for analysis. Larger samples were collected from individual animals by cardiac puncture under terminal anaesthesia.

Secretions from the gut and lung were collected by washing with a lavage solution, as described below (section 5.3.4), on days 1, 25, 35, and 43 into the experiment. At each of these time-points, one animal per group was sacrificed.

5.3.4. Preparation of Lung and Gut Lavages

To measure the mucosal immune response (both SIgA and IgG) in the gut and the respiratory tract, gut and lung secretions were collected from the sacrificed animals, by washing with a lavage solution described in table 5.2.

The animals were sacrificed, the trachea and lungs were exposed by removing the thymus. Using a butterfly needle (Venisystems Butterfly-19), 5 ml of lavage fluid were gently injected into the trachea to inflate the lungs. The liquid was extracted immediately afterwards. If a larger volume is required an injection of 2 ml may be used for further recovery. After centrifuging the samples at 20000 $\times g$ for 30 min, the supernatants were collected and stored at -70°C. Lung washes were concentrated by freeze-drying. Samples were reconstituted with 100 µl of PBS immediately prior to analysis by ELISA (section 5.3.5).

Similarly, the gastrointestinal tract was exposed and approximately 2 inches of small intestine incorporating the Peyer's patches were isolated and transferred to a Petri dish. The gut was sectioned longitudinally and 4 ml of lavage fluid added. The exposed gut was scraped to disperse the mucus. The liquid was placed in a container and stored in an ice-bath. After sonication for 30 seconds, to release the proteins from bound mucin, the liquid was then treated as described for the lung lavages

Table 5.2 - Composition of fluids for lung and gut washes.

Composition*	Lung	Gut
PMSF[1]	1 mM	1 mM
Sodium chloride	0.9 % (w/v)	___
Tween 20	0.5% (v/v)	___
Sodium Azide	0.1% (w/v)	___
Iodoacetic acid	___	1 mM
Trypsin inhibitor soybean type 1-5[2]	___	0.1% (v/v)
EDTA (disodium salt)	___	10 mM

1) Phenylmethylsulfonylfluoride; previously dissolved in 400 µl ethanol.
2) Added in last, just before use.
* The constituents were dissolved in distilled water.

5.3.5. Enzyme Linked Immunosorbent Assay (ELISA)

The principle of this test (Avrameas & Guilbert, 1971) is that the antigen or antibody, immobilised on a solid phase, is used to separate the complementary antibody or antigen from a specimen. The presence and quantity of the complex, bound to an enzyme, is determined by a colour change in an appropriate substrate and read on a spectrophotometer. The protocol can be slightly modified depending on the particular antigen or antibody. This method was used to detect antibodies and quantify the immune response in samples collected during *in vivo* experiments with various vaccine formulations.

Antibody responses to the tetanus toxoid in immunised animals were monitored by the microplate ELISA described by Davis & Gregoriadis (1989). Tetanus toxoid (5 $\mu g ml^{-1}$) in 0.05 M sodium carbonate-bicarbonate buffer (pH 9.6) was added to the microelisa plates (Immulon 2 flat-bottom plates, purchased from Dynatech, Sussex, U.K.) and incubated overnight at 4°C. The wells were washed three times with PBS containing 0.05% Tween 20

(PBST), pH 7.4. Dilutions of serum in PBST containing 0.1% BSA were incubated in plates for 2 hours at room temperature. The total amount of bound IgG was estimated by the addition of a specific anti-IgG-horseradish peroxidase conjugate.

The anti-tetanus immunoglobulin (Humotet®, The Wellcome Foundation Ltd.; see section 2.3.8) was used as a standard, serially diluted from 1:100, in doubling dilutions, on each plate. Protein A peroxidase (0.5 $\mu g ml^{-1}$) was used to estimate the total amount of IgG bound to the tetanus toxoid. After incubation for 1 hour at room temperature the enzyme substrate 3,3',5,5'-tetramethylbenzidine (TMB, Sigma) was added (0.15 mM TMB in 0.1 M sodium acetate/citrate pH 6, containing 0.003% (v/v) hydrogen peroxide) and the reaction was allowed to proceed for 3 min. The reaction was stopped by the addition of 2 M sulphuric acid and the absorbance at 450 nm measured (Anthos Reader 2001, Anthos Labtec Instruments, Austria). The endpoint of each titration was defined as the dilution at which OD_{450} was 0.2.

In the studies carried out at the Chemical and Biological Defence Establishment, Porton Down, UK. (see section 5.3.3), the biological samples collected were analysed using a version of the same ELISA method, described by Farzad *et al.* (1986).

5.3.6. Statistical Methods

Comparison between the immune responses of the different groups of animals were analysed for significance using Student's unpaired t-test. Differences with $p < 0.05$ were considered significant.

5.4. RESULTS AND DISCUSSION

5.4.1. Immune Response to Intramuscularly Delivered Microencapsulated TT

5.4.1.1. Preliminary Immunisation Study

Microspheres apparently possess adjuvant properties when introduced by the subcutaneous and i.m. routes (Eldridge *et al.*, 1990, 1991; O'Hagan *et al.*, 1989a, b). On the basis of the preliminary immunisation studies (section 5.3.1.1), after administration by the i.m. route the microencapsulated TT induced a higher immune response to the antigen, when compared to the aluminium-adsorbed vaccine (fig. 5.1).

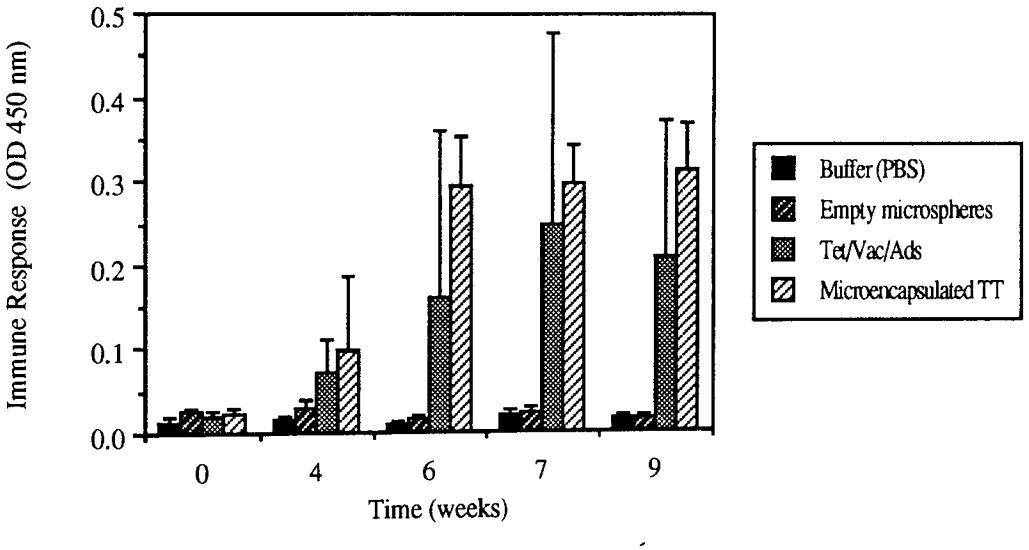


Figure 5.1 - Immune response to the preliminary i.m. administration of a 5 μg dose of TT (section 5.3.1.1). Animals were primed with a single dose on day 1 and similarly boosted on day 28. Results are expressed as mean ± sd; n = 4 per group; serum dilution = 1:20.

Although the increase in specific serum IgG titre is higher in the group treated with the PLA-microencapsulated TT than in the animals dosed with the Tet/Vac/Ads, the difference is not statistically significant ($p > 0.05$). Nevertheless, a humoral response as high as that

obtained with the commercially available vaccine suggests that the formulation with PLA microspheres has an adjuvant capacity at least equivalent. Furthermore, the group dosed with the aluminium-adsorbed vaccine shows a decrease in the antibody titre 7 weeks after the priming, whereas the group treated with the encapsulated TT presents an apparently sustained immune response. In addition, the administration of empty PLA microspheres did not increase the IgG titre, showing that the immune response results only from the association of TT with the carriers which do not present immunogenic activity. Therefore, these preliminary studies agree with the results described by Eldridge *et al.* (1990, 1991a) and O'Hagan *et al.* (1991b), who have also demonstrated the adjuvant properties of PLGA microspheres.

5.4.1.2 The Influence of Dose, Polymer Composition and Particle Size on the Immune Response

The study was carried out using two doses of TT (0.1 and 1.0 μg) and polymers of two different compositions [PLA (Mwt 2000 Da) and PLGA (50:50)] formulated in microspheres of three mean particle sizes (section 5.1). The microscopic appearance of the particles obtained with PLA did not differ from those of the preparations shown in chapter 2 (figs. 2.10 and 5.2) and PLGA (50:50) produced the microspheres presented in figures 5.3 to 5.5.

No increase in immune response to the toxoid was detected in any group between priming (day 1) and booster in day 28 (figs. 5.6 and 5.7). On day 39 all groups of animals treated with 1.0 μg of TT showed an increase in specific circulating IgG, irrespective of the formulation used. This indicates that the priming, although not increasing the immune response, could stimulate a serum IgG memory. These results are in general agreement with the previous findings of O'Hagan *et al.* (1991a), who reported that PLGA-microencapsulated ovalbumin elicited low primary antibody titres followed by a potent secondary immune response. Hence, they proposed that microencapsulated antigen injected alone may not function as an effective primary vaccine, but may serve well as a vaccine for booster injections.

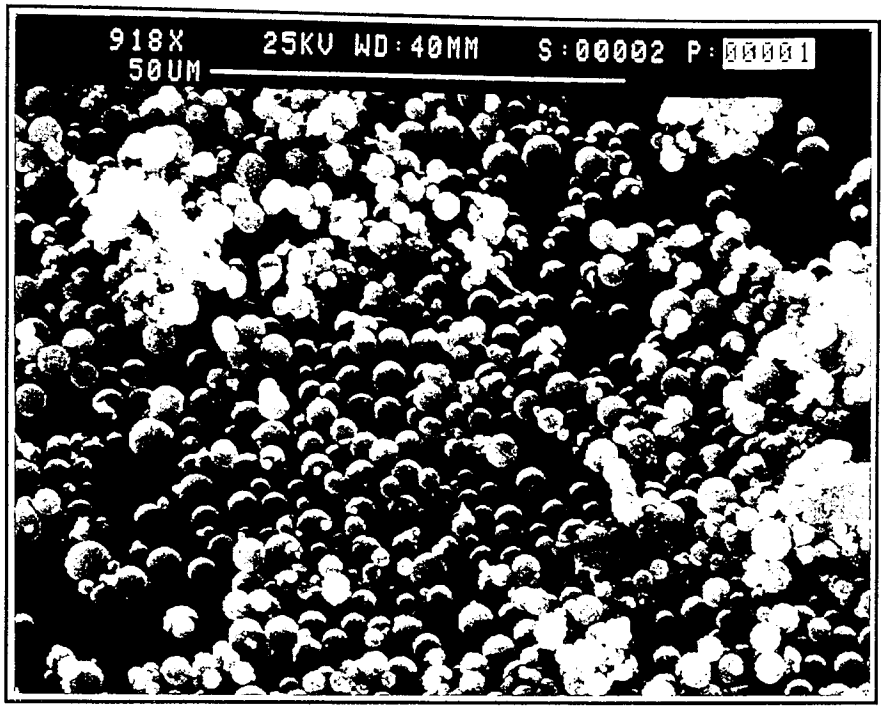


Figure 5.2 - Scanning electron micrograph of PLA (Mwt 2000 Da) microspheres containing 8.3% (w/w) of TT (mean size = 2.4 μm, σ_g = 1.8; n = 542 particles).

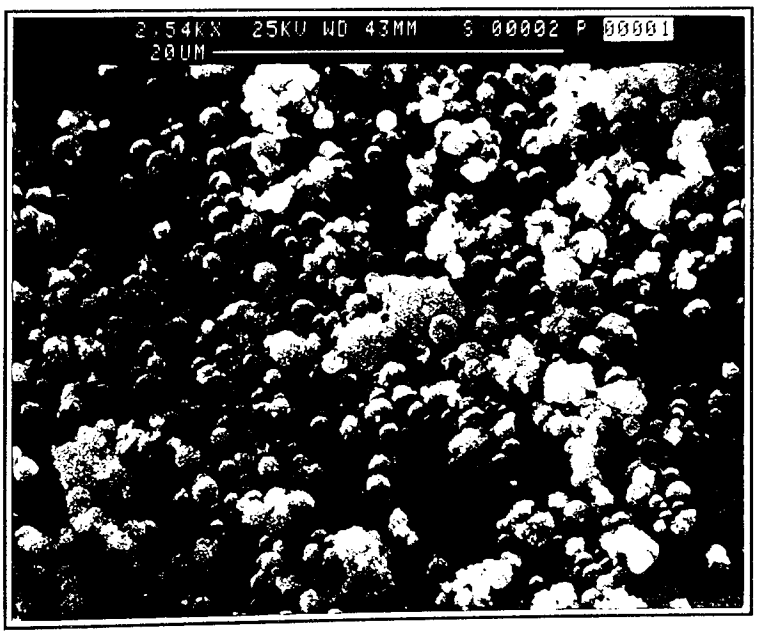


Figure 5.3 - Scanning electron micrograph of PLGA (50:50) microspheres containing 1.7% (w/w) of TT (mean size = 1.0 μm, σ_g = 1.6; n = 306 particles).

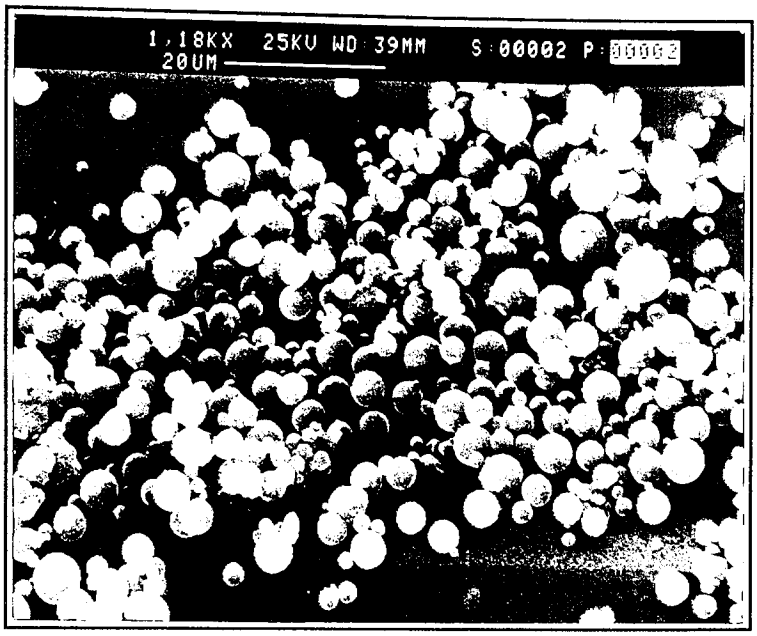


Figure 5.4 - Scanning electron micrograph of PLGA (50:50) microspheres containing 1.8% (w/w) of TT (mean size = 2.8 μm, σ_g = 1.5; n = 347 particles).

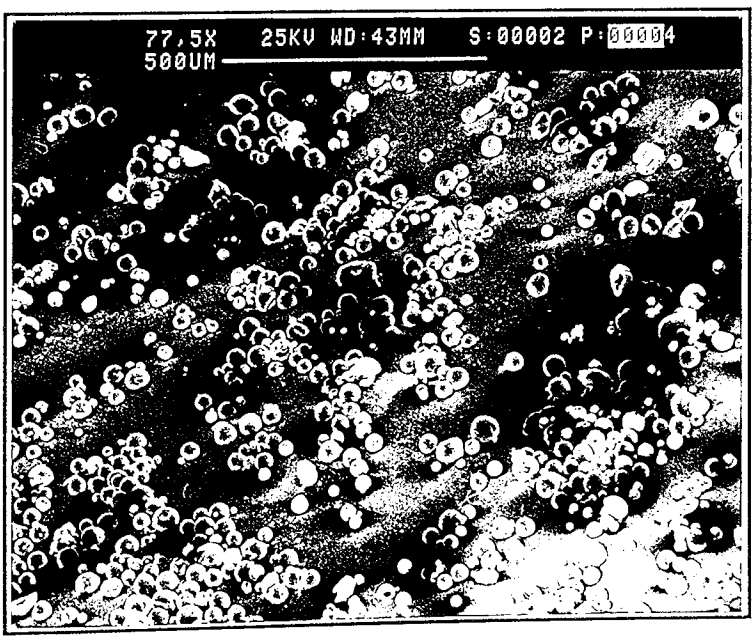


Figure 5.5 - Scanning electron micrograph of PLGA (50:50) microspheres containing 2.0% (w/w) of TT (mean size = 24.0 μm, σ_g = 1.7; n = 489 particles).

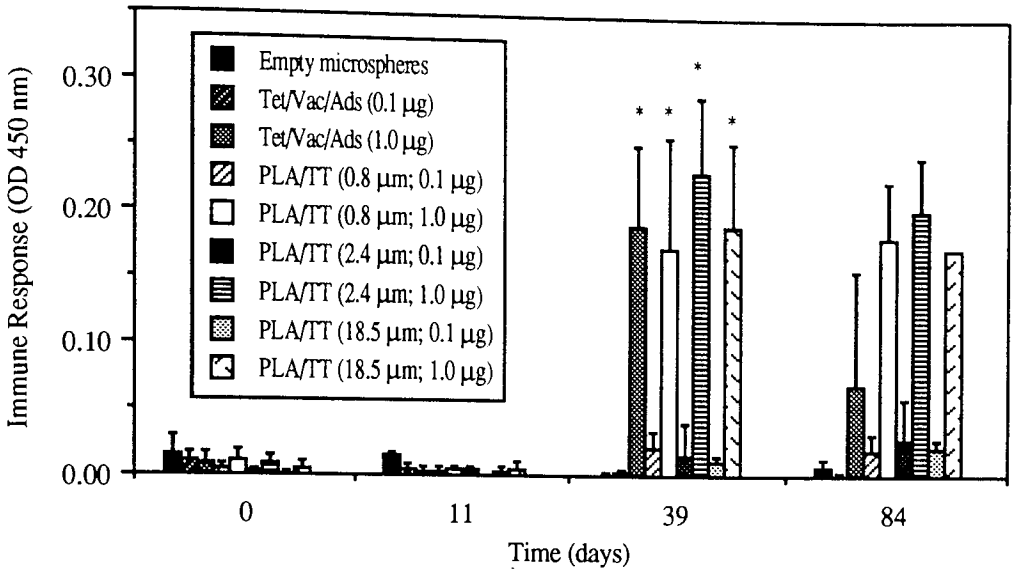


Figure 5.6 - Immune response to 0.1 µg and 1.0 µg of TT microencapsulated into PLA (Mwt 2000 Da) microspheres of different particle sizes. Animals were primed (i.m.) with a single dose on day 1 and similarly boosted on day 28. Results are expressed as mean ± sd; n = 5 per group; serum dilution = 1:160. (* $p \leq 0.1$).

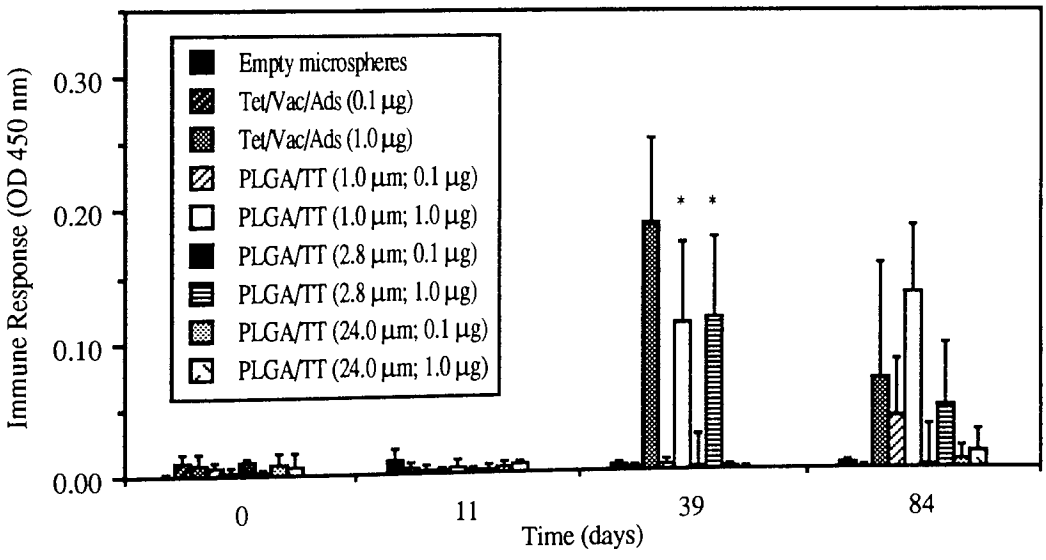


Figure 5.7 - Immune response to i.m. 0.1 µg and 1.0 µg of TT microencapsulated into PLGA (50:50) microspheres of different particle sizes. Animals were primed (i.m.) with a single dose on day 1 and similarly boosted on day 28. Results are expressed as mean ± sd; n = 5 per group; serum dilution = 1:160. (* $p \leq 0.1$).

Also Esparza & Kissel (1992) observed weak antibody titres after the primary immunisation of mice with microencapsulated TT, whereas the booster elicited a strong secondary response.

The immune response to the 0.1 μg dose was low or non-existent. Until day 39, the immune response obtained with most of the formulations was not significantly different ($p \leq 0.1$, at the greatest) from the preimmune serum. An increase in the serum specific IgG titre was observed on day 84 (12 weeks) for some of the microsphere formulations, when compared with the previous titres ($p \leq 0.005$ for both PLA 2.4 μm and PLA 18.5 μm). Although these values are statistically significant, the magnitude of the antibody titres achieved is nevertheless low.

The dose of 1.0 μg of TT increased the immune response to values of OD_{450} similar to those obtained with a 5 μg dose in the preliminary immunisation experiment (section 5.4.1.1). Given that the serum dilutions used in the ELISA analysis of the blood samples were 1:160 for 1.0 μg dose and 1:20 for the preliminary 5 μg dose, one can say that the antigen dose of 1.0 μg enhanced a higher antibody production, compared to that obtained with a five times higher dose in the preliminary study. This probably suggests the existence of an optimal ratio polymer/TT at which the adjuvant effect of the microspheres is maximum. A similar characteristic is presented by other adjuvant formulations, such as aluminium hydroxide (Lindblad & Spärck, 1987) and particularly liposomes, which have a better adjuvant effect when the phospholipid/toxoid mass ratios are high (Davis & Gregoriadis, 1987).

All the batches prepared using PLA and injected at the dose of 1.0 μg showed an increase of immune response which was maintained until 12 weeks after the priming (fig. 5.6). The same dose given as Tet/Vac/Ads initially increased the antibody titres (day 39) to levels similar to those of the particulate preparation. However, at day 84 the amount of circulating antibodies had decreased to a value of approximately 36%, which suggests a better long-term adjuvant effect of the PLA preparations. This observation confirms the early decrease during the preliminary studies, described above (section 5.4.1.1).

The PLGA microsphere vaccines did not, at any time, elicit humoral immune responses as high as those of the PLA formulations (fig. 5.7). Moreover, no immune response was detected in the group treated with 1.0 μg of TT microencapsulated in 24.0 μm PLGA particles which, even after 84 days, showed an antibody titre similar to some of the preimmune values. The 1.0 μm/1.0 μg preparation was the only PLGA preparation that presented a sustained increase in specific serum IgG. Besides, the response to Tet/Vac/Ads (1.0 μg of TT) after 39 days was higher than that elicited by the PLGA particulate carriers. The fact that PLGA (50:50) suffers a rapid degradation *in vivo* when compared to PLA (see section 1.2, fig. 1.5) may be responsible for the difference in response observed after 84 days.

Finally, an overall appraisal of this study suggests that a 0.1 μg dose of TT, either adsorbed onto aluminium or microencapsulated, is too low to induce the production of specific IgG and only the 1.0 μg dose elicited a measurable increase of antibodies. The PLA preparations are better immunoadjuvants than the PLGA-based formulations. Although the primary immune response could not be detected, the booster with the same dose elicited higher IgG titres. On the other hand it seems that under the experimental conditions used in the study, the particle size does not influence significantly ($p > 0.05$) the immune response to the vaccines that gave the best results, i.e. the PLA-microencapsulated TT.

5.4.2. Immune Response to Nasally Delivered TT Adsorbed onto Microspheres

Animals were immunised with TT in solution or adsorbed onto PLA microspheres, and circulating anti-tetanus IgG was assayed by the ELISA method described above (section 5.3.5), in blood samples collected at weeks 2, 4, 7 and 15. The increase in IgG titre is almost immediate in the group treated with the adsorbed TT (time zero = 140; week 2 = 1550; week 4 = 2760), reaching 36000 two weeks after the booster (week 7), whereas the soluble antigen produced an immune response similar to that found in non-treated animals (control, given PBS only). On the other hand, at week 15 (10 weeks after the booster) the titre produced by the microsphere preparation had decreased to 1200, which still is 8.5

times higher than the prebleed value of 140 (fig.5.8).

The results indicate that association of TT with PLA microspheres through the adsorption procedure has increased systemic IgG responses to nasally delivered antigen over free antigen delivered in solution. It is now accepted that the form of an antigen determines the immune response. Thus, it is important to evaluate suitable adjuvant/antigen combinations which are administrated mucosally but still increase the serum antibody (and/or mucosal antibody) levels according to the type of infection or type of protection required. Increased saliva and serum antibody levels have been observed following oral administration of various soluble antigens delivered as colloidal systems when compared to soluble antigen delivery (Wachsman *et al.*, 1985; O'Hagan *et al.*, 1989a, b; Eldridge *et al.*, 1990). There have been few previous studies using the nasal route for the administration of particulate vaccines (El Guink *et al.*, 1989; Hameleers *et al.*, 1991). In the present study we have shown that the nasal administration of PLA-associated TT gave an antibody response higher than that from the administration of the same quantity of soluble antigen.

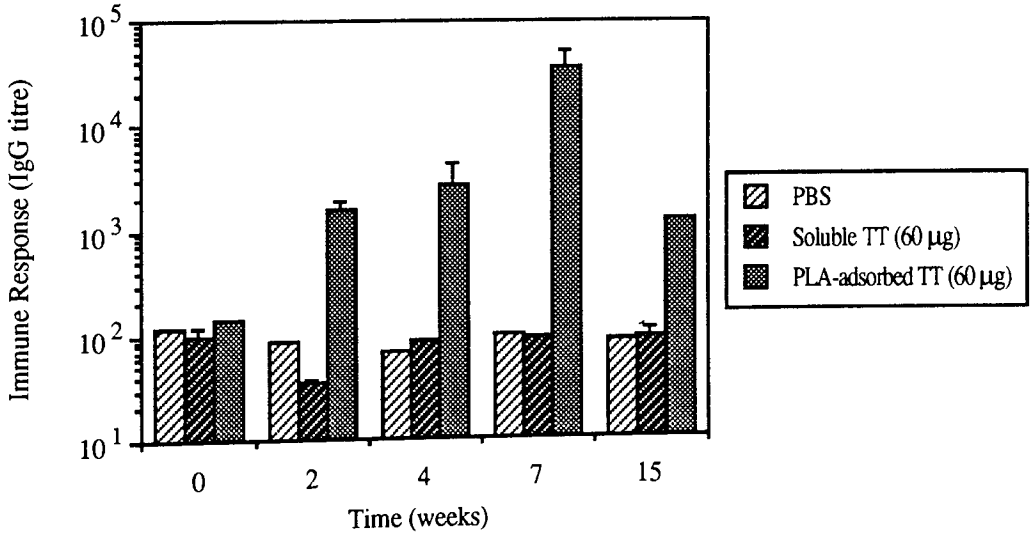


Figure 5.8 - Serum antibody response to nasally administered TT (mean ± se; n = 3). Animals were given the vaccine by administration of soluble or PLA microsphere- adsorbed toxoid. Animals were primed with three doses (days 1, 2 and 3, respectively) and boosted with one dose on day 28.

Maximal IgG titres occurred in serum 40 days after the priming. The primary response lasted about 20 days, but even after 30 days the titres did not reach the preimmune levels, i.e. it was not a short lived response, considered to be the main disadvantage of mucosal immunisation (Challacombe *et al.*, 1992). The secondary response was rapid (occurring only 10 days after a single delivery) suggesting that the mode of immunisation stimulates serum IgG memory. The observed serum IgG antibody production stimulated by particulate TT may indicate an interaction of this antigen with non-NALT lymphatic tissues (e.g. spleen) after being drained from the nasal cavity *via* the lymphatics. This would imply that uptake and transfer of particles through the nasal mucosa has occurred: indeed, latex particles have been taken up following nasal administration, as shown in chapter 6. Also, the particulate nature of antigens may give rise to enhanced immune responses after interaction with immunocompetent cells. The data agree with the findings of Perkins *et al.* (1969) who stimulated both secretory IgA and serum antibody production by the intranasal administration of killed rhinovirus.

The induction of the immune response also remains obscure although in the gut, antigens, pathogens and particles are taken up by M-cells in the GALT (Wolf & Bye, 1984; McCluggage *et al.*, 1986). In the respiratory area where the more complex mechanisms are involved, it has been shown that lymphoepithelial cells of BALT are also involved in antigen sampling and uptake (Gregson *et al.*, 1982; Van der Brugge-Gamelkoorn *et al.*, 1985). Many similarities between the lymphoid follicles of BALT and GALT suggest that similar fundamental mechanisms may be involved in the uptake process. Furthermore, a mechanism of antigen processing and eliciting of the immune response after antigenic stimulation of the NALT has been recently proposed by Kuper *et al.* (1992).

5.4.3. Immune Response to Microencapsulated TT

The present section describes and discusses some results of an *in vivo* study carried out in collaboration with an independent research group mentioned above (section 5.3.3.), using microencapsulated TT. Such study was designed to confirm some of the results discussed in the previous section (5.4.2), particularly the increase in circulating anti-tetanus antibodies

after immunisation by the nasal route. However, several parameters were different from those used before, i.e. the animal species (mice instead of guinea-pigs) and the antigen doses. In addition, two other administration routes were studied (oral and i.m.) and the ELISA method used (Farzad *et al.*, 1986) was slightly different from that described in this thesis (Davis & Gregoriadis, 1989; section 5.5). It should be remembered that the mucosal immune response in the gut and lungs was measured from one animal at each time point, which only allows the establishment of some trends. All these factors taken together make comparisons difficult. Nevertheless, some interesting indications may be inferred from these immunisations.

To assess the dose dependency of the humoral immune response, several amounts of antigen were administered by the oral and intranasal routes. For comparison purposes a low dose of TT (2.5 μg) was given intramuscularly, which is the conventional route of immunisation against tetanus. Figures 5.9 to 5.11 show the most relevant results obtained by the three routes.

5.4.3.1. Systemic Response

The systemic IgA response was maximum at day 43 for all administration routes, but the intranasal immunisation was more effective than the oral route. Nasal doses of 25 and 40 μg gave antibody titres higher than 75 μg delivered orally, which did not elicit any significant immune response when compared with the nasally-administered empty microspheres (figs. 5.10 and 5.11). The peak of circulating specific IgG was achieved at day 53 and again the nasal route elicited better antibody titres than oral administration. The latter failed to stimulate the production of serum IgG throughout the study and the IgA levels attained were inconsistent and low (fig. 5.11). Although the type of formulation used is different, these maximum levels of serum IgG at day and 53 after nasal administration correlate with that obtained well with guinea-pigs at week 7 (section 5.4.2).

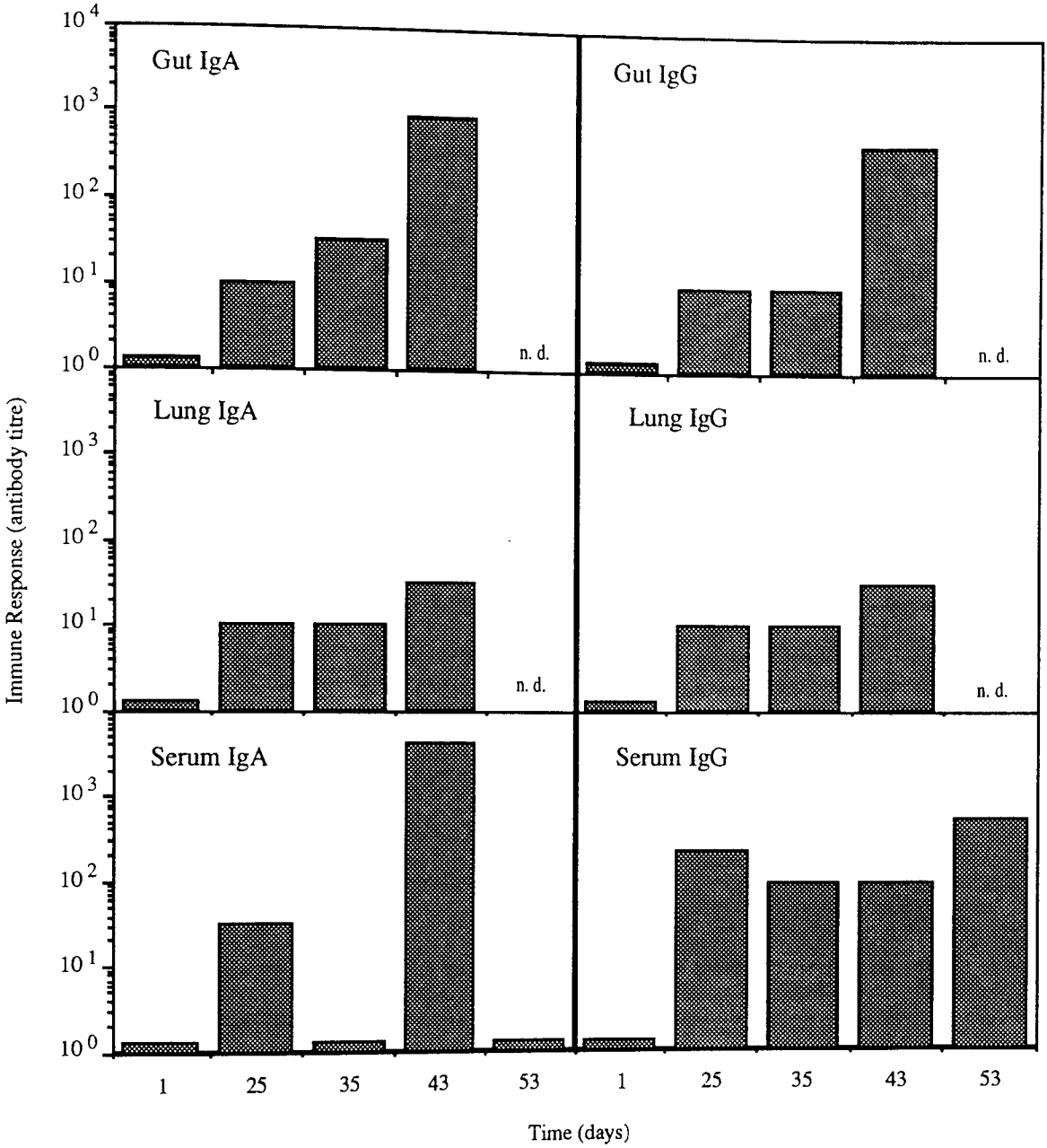


Figure 5.9. - Immune response of mice after immunisation by the i.m. route with 2.5 μg of microencapsulated TT (particle size = 0.8 μm ± 1.7). Gut and lung mucosal antibodies were measured in one animal at each time point (n.d. = not determined); serum antibodies were determined in pooled samples (n = 5).

At day 53 the titres of both circulating IgA and IgG elicited after intranasal immunisation show a dose-response effect, which is higher in the case of IgG. However, the i.m. route is the most effective one in increasing systemic immunity (fig. 5.9).The IgA titres reached their maximum at day 43 and the systemic IgG show a sustained increase throughout the

experiment. An i.m. dose of 2.5 μg of TT could produce antibody titres similar to those of a sixteen times higher dose given nasally (40 μg) and its efficacy is even superior when compared to an oral dose thirty times higher (75 μg). Therefore, the depot effect that results from an i.m. injection of the particulate vaccine appears to exert a stronger action on the systemic immunity than the mucosal delivered microspheres.

5.4.3.2. Mucosal Response in the Lung

The IgA response in the lung was remarkably low for all routes, when compared with the values found in serum and gut. Some of the dose levels show a minimum response, or absence of it. Intranasal doses of 15 and 25 μg and oral doses of 25 and 50 μg did not produce an immune response higher than that induced by the control (empty microspheres). The IgG levels found in the lung washes were slightly higher than the IgA ones, particularly for 40 μg intranasal.

Not surprisingly, the lung IgA levels resultingt from the i.m. immunisation, were not higher than those obtained with nasally administered empty microspheres (fig. 5.9). On the other hand, it was more efficient in increasing lung IgG titres, e.g. greater than those obtained with 25 μg delivered nasally. It is well established that the effect of this parenteral route is mainly systemic and, therefore, the antibodies present in the washes may result from migration to the lung from the blood stream, rather than local production. Besides, the values found in the lungs may be affected by a low efficiency of recovery of antibodies in the lung washes. When a more drastic treatment was applied (gut washes), the IgA titres found were higher for all delivery routes.

5.4.3.3. Mucosal Response in the Gut

As mentioned in the preceding section, the response measured at this mucosal site was better than that of the lung. The nasal route of immunisation elicited in the gut, at all dose levels, antibody responses at least as high as those resulting from the oral administration.

However, the general assessment shows a low response when compared to the values obtained with the control (empty microspheres).

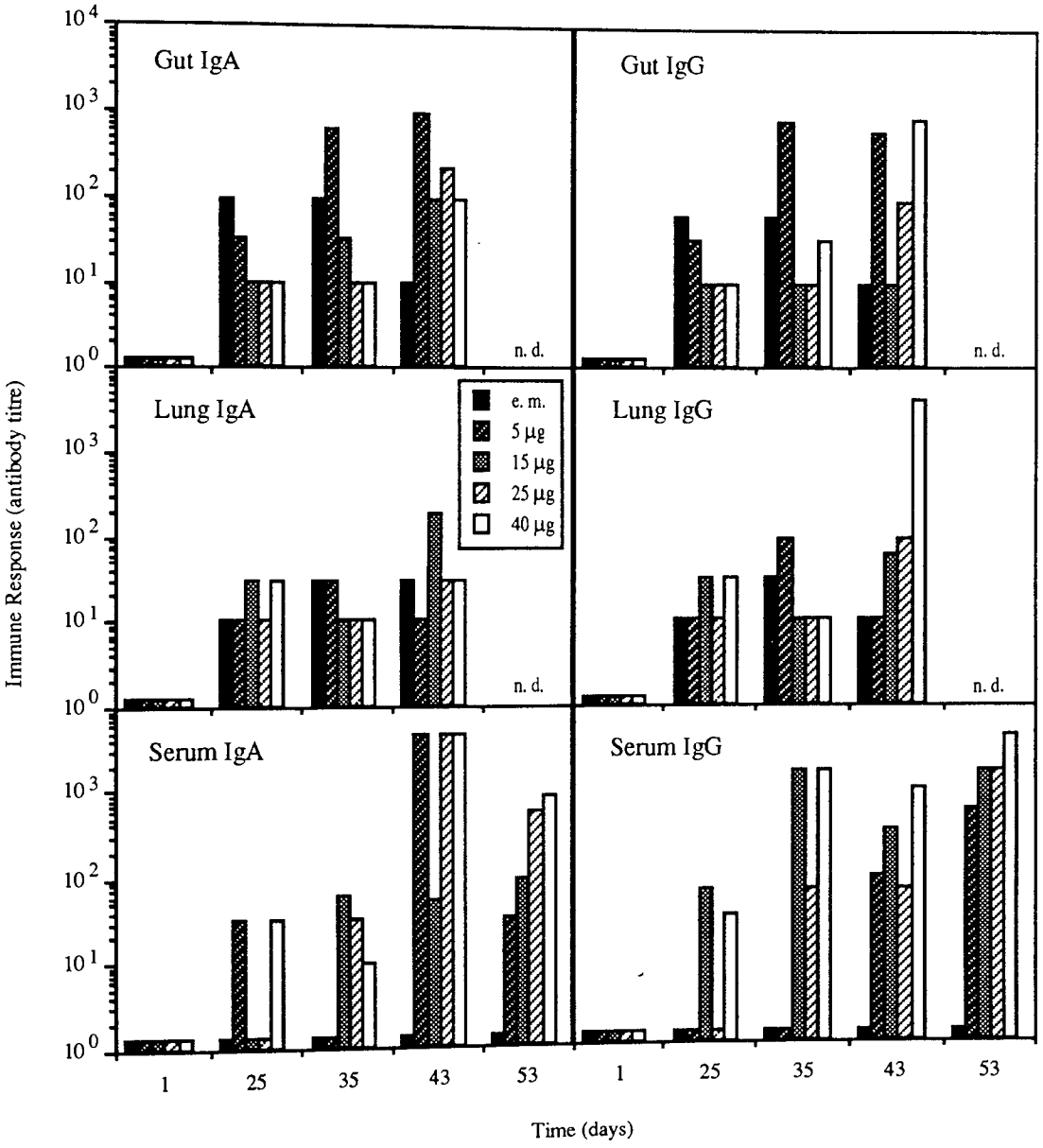


Figure 5.10 - Immune response to several doses of microencapsulated TT (particle size = 0.8 µm ± 1.7) administered by the nasal route, measured as titres of antibodies. (e. m. = empty microspheres; n. d. = not determined). Gut and lung mucosal antibodies were measured in one animal at each time point; serum antibodies were determined in pooled samples (n = 5).

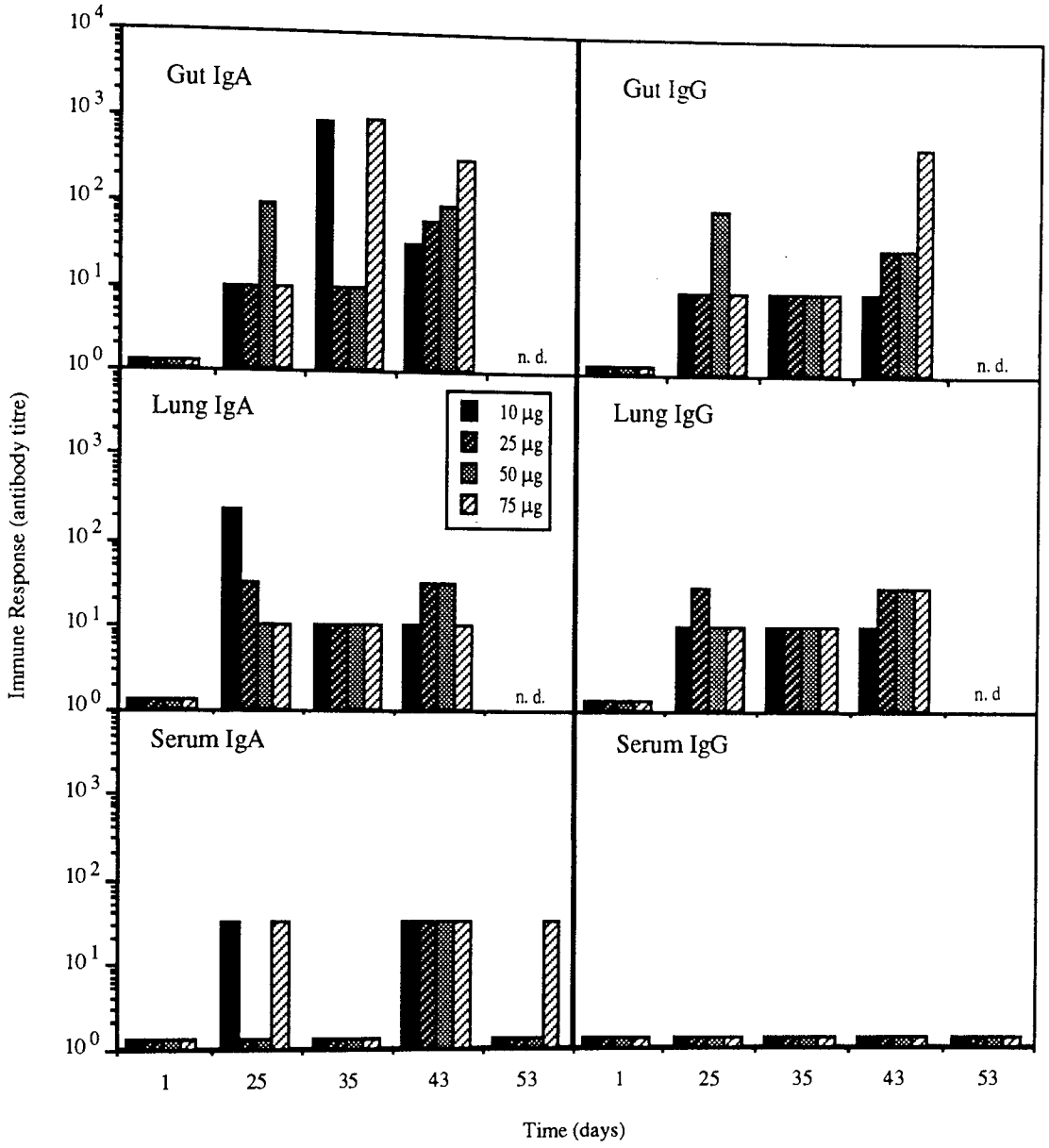


Figure 5.11 - Immune response to several doses of microencapsulated TT (particle size = 0.8 µm ± 1.7) administered by the oral route, measured as titres of antibodies. (e. m. = empty microspheres; n. d. = not determined). Gut and lung mucosal antibodies were measured in one animal at each time point; serum antibodies were determined in pooled samples (n = 5).

This was the only compartment where oral immunisation resulted an increase of the antibody levels. In the gut at day 43 (maximum) the response to oral administration follows a dose-response pattern for both IgA and IgG (fig 5.11), as previously seen in the lungs after nasal delivery (fig 5.10).

The IgA and IgG levels in the intestine were also increased by the injection of 2.5 μg of microencapsulated TT, which could induce at day 43 responses similar to those obtained with 40 μg intranasal and 75 μg oral. This induction of mucosal immunity through a parenteral route of vaccination is not confirmed by the lower titres induced in the lungs. It was already discussed in section 5.4.3.2 that this may be due to migration of antibodies to the gut instead of production of mucosal antibodies.

The comparison between oral and nasal delivery is clearly favourable to the latter. Apart from the systemic immunity, where the nasal route gave better results, the intranasal administration of microencapsulated TT not only increased the mucosal antibody levels in the respiratory tract but also was more effective in the gut (fig 5.10), whereas the orally delivered antigen only acted efficiently in the gastrointestinal tract (fig.5.11). Even at this mucosal site the levels elicited by 75 μg of oral TT were similar to those produced by 40 μg given nasally. Similarly, the intranasal application of a protein antigen together with CTB (Wu & Russel, 1993) resulted in an increase of specific antibodies in several sites, such as serum, saliva, and tracheal, gut and vaginal washes, which was at least as good as that obtained using the oral route.

One of the main features of this study is the low response to the orally delivered vaccine. Although some publications have reported good results obtained after oral immunisation with particulate carriers (Challacombe *et al.*, 1992; Moldoveanu *et al.*, 1993), these studies suggest that the nasal route can be even better. In several immunisation studies against viral and bacterial infections, carried out with monkeys, Staas *et al.* (1993) found that animals treated by a respiratory mucosal delivery route (intratracheal) were better protected from challenge than those treated by the oral or parenteral routes.

Being obtained in association with the CBDE laboratories, and taking into consideration the difference of experimental conditions used, the results confirmed some preliminary observations made at Aston University following the oral immunisation of mice, which revealed a weak systemic immune response to the microencapsulated antigen (results not shown). Furthermore, the effectiveness of the nasal immunisation in two different species (guinea-pigs and mice) with two distinct particulate formulations (adsorbed and

microencapsulated) of the same antigen implies that this is not an isolated phenomenon and it may be a means of generating antibody responses in serum and at several mucosal sites where protective immunity may be beneficial.

6. NASAL UPTAKE OF LATEX PARTICLES

6.1. INTRODUCTION

The use of a mucosal route for immunisation and of particulate carriers for mucosal delivery of antigens has been extensively reviewed in sections 1.3 and 5.1. Briefly, the uptake of particles by some MALT components, such as the Peyer's patches, was demonstrated (Jani *et al.*, 1989) and several mechanisms of absorption have been reported (reviewed by Kreuter, 1991). Many similarities between the lymphoid follicles of BALT and GALT suggest that similar fundamental mechanisms may be involved in the uptake process. Particles can penetrate the gut mucosal barrier by way of the M cells. These cells were more recently found in the nasal mucosa of rats (Spit *et al.*, 1989), which led to the characterisation of the NALT. Penetration of the nasal mucosa by pathogenic bacteria, including experimental infections in rats (Moxon *et al.*, 1974; Salit *et al.*, 1984), suggests the existence of a particle uptake pathway at the nasal mucosa. Although Kuper *et al.* (1992) proposed a mechanism for the sampling of particulate antigens at the nasal mucosa (fig.1.8), Hameleers *et al.* (1991) had already failed in their attempt to demonstrate that fact, perhaps by using an antigen of a too large particle size (see section 1.3.4).

In the sequence of the immunisation experiments described in chapter 5, a study on the uptake and translocation of solid particles through the nasal epithelium was designed to assess whether the results obtained were due to absorption of free or microsphere-associated antigen at the nasal mucosa. Uptake of microspheres by a MALT component in the nasal cavity, like Peyer's patches, would allow the observation of these particles in the blood stream. This would contribute to confirm the previously cited suggestions (Moxon *et al.*, 1974; Salit *et al.*, 1984; Spit *et al.*, 1989 and Kuper *et al.*, 1992). Also, the experiments described in this chapter constitute attempts to quantify the uptake of particles in the nasal mucosa. The difficulty in quantifying the *in vivo* uptake of colloidal particles is related to the techniques used in the determination. Usually the amount absorbed is very small and, therefore, sensitive methods are required for an accurate measurement.

6.2. MATERIALS

6.2.1. Latex Particles

Fluorescent latex particles (fluoresbrite carboxylated microspheres, 0.87 μm ± 0.085 sd, 0.83 μm ± 0.006 sd and 0.51 μm ± 0.008 sd in diameter; manufacturer's information) were obtained from Polysciences, Inc., Northampton, U.K., and used as received or after appropriate dilution with normal saline. Latex particles (polystyrene non-carboxylated microspheres, 0.825 μm in diameter) were purchased from Sigma Chemical Company, Poole, U.K., and radiolabelled with ^{125}I at the Drug Development Research Group, Pharmaceutical Sciences Institute, Aston University.

6.2.2. Anaesthetics

For some procedures with rats and guinea-pigs, e.g. nasal administration of microspheres and blood withdrawal by cardiac puncture, halothane was used as an inhalational anaesthetic (Fluothane®; ICI Pharmaceuticals, Macclesfield, U.K.). For surgical purposes, animals were anaesthetised with 0.3 $mlkg^{-1}$ (rats) or 1 $mlkg^{-1}$ (guinea pigs) of Hypnorm® (0.315 $mgml^{-1}$ phenyl citrate, 10 $mgml^{-1}$ fluanisone; Janssen Pharmaceutical Ltd.) by the i.m. route (Green, 1982). Simultaneously, an intraperitoneal (i.p.) injection of 2.5 $mgkg^{-1}$ (rats and guinea-pigs) of diazepam (Diazepam Injection B.P.; Phoenix Pharmaceuticals, Ltd., Gloucester, U.K.) was administered as a muscular relaxant.

6.2.3. Animals

For the determination of nasal uptake of microspheres, male Wistar rats (≈400 and ≈500 g) and female Dunkin-Hartley guinea pigs (≈500 g) were used. All animals were freely allowed food and water during the experiments.

6.2.4. Chemicals

All chemicals and reagents not specified in the text were supplied by BDH Chemicals Ltd. (Poole, U.K.), Sigma Chemical Company (Poole, U.K.) and Fisons (Loughborough, Leics.U.K.) and were of Analar grade or equivalent.

6.3. METHODS

6.3.1. Particle Administration

The nasal administration of polystyrene particles to rats and guinea-pigs, was carried out by means of an automatic pipette (Gilson pipetman P-200; Anachem Ltd., Luton, U.K.). The volume of particle suspension administered was 100 µl (50 µl per nostril). Blood samples for particle counts were taken into heparinised tubes immediately before dosing and at intervals after administration, from the tail vein (rats) and by cardiac puncture (guinea-pigs). Three *in vivo* experiments using different quantification methods were performed and are described in sections 6.3.2 to 6.3.4.

6.3.2. Analysis by Fluorescence Microscopy

Male Wistar rats (≈400 g; n = 4) were anaesthetised with halothane and dosed intranasally, without any further treatment, with 1.38×10^{10} fluorescent latex particles (mean size 0.51 µm ± 0.008) suspended in saline. Blood samples were obtained at 10, 30, 60, 120 min, 5 and 24 hours after dosing. Particle counts were made using a microscope illuminated by ultraviolet and tungsten light (Zeiss Standard Microscope provided with an IV, FI Epi-Fluorescence Condenser, Germany). The blood samples were diluted with an appropriate volume of normal saline and both latex particles and erythrocytes were counted in a Neubauer chamber (haemocytometer). In each blood sample, cells and particles were counted in 16 squares of 0.04 mm^2 (total area = 0.64 mm^2). The mean values for particles

counted per 100 erythrocytes in blood samples were calculated and expressed as percentage of total administered particles. An example of the calculations is as follows: the average number of erythrocytes in rats is 7.0×10^9 cell ml^{-1} (Green, 1982). Since the blood volume of a rat is 5 ml per 100 g of body weight (Green, 1982), for a dose of 1.38×10^{10} particles, a value of 1 particle per 1250 erythrocytes would represent approximately 0.96% of the administered particles in circulation.

6.3.3. Analysis by Fluorescence Activated Cell Sorter (FACS)

A group of three male Wistar rats (407 to 410 g) were dosed intranasally with 7.95×10^9 fluorescent latex particles (mean size 0.83 µm ± 0.006) suspended in saline (100 µl). They were anaesthetised with Hypnorm® as described in section 6.2.2, and prepared for intranasal dosing using the experimental model of Fisher *et al.* (1987). The animals were placed on their backs, submitted to tracheotomy (a cannulae was inserted into the trachea to maintain respiration) and the oesophagus sealed with a suture. They were kept under these conditions until the end of the experiment, i.e. 2 hours after dosing. Blood samples were collected into heparinised tubes at 10, 30, 60, and 120 min. Micrographs of the samples were obtained using a fluorescence microscope described in section 6.3.2.

The blood samples were analysed for microsphere content by FACS at the Scientific Services, Medical School, University of Birmingham. Prior to analysis the blood samples were treated using a previously described method (Ebel, 1990). An appropriate volume of blood (minimum 100 µl) was dissolved by adding a solution of 1% Triton X-100 and 1% of KOH (5 ml) and heating to 60°C for 48 hours. Dissolved samples were centrifuged at 25000 $\times g$ for 4 hours to pellet the latex particles. The supernatant (4ml) was decanted and the pellet was resuspended in additional Triton X-100 and KOH solution (4 ml). Centrifugation and decantation were repeated, the pellets were finally suspended in 1 ml of normal saline (this volume depends on the concentration of particles) and analysed by FACS. A blood sample from a non-treated animal was used for background correction. Results were expressed as a percentage of dose administered.

6.3.4. Administration of Radiolabelled Particles

Female Dunkin-Hartley guinea pigs (≈500 g; n = 3 per group) were anaesthetised by i.m. injection of Hypnorm® (see section 6.2.2). The animals were tracheotomised and the oesophagus sealed (model of Fisher *et al.*, 1987). A volume of 50 µl of a suspension of ^{125}I-polystyrene microspheres (mean size 0.825 µm) in saline was introduced into each nostril (total dose 22.6 KBq ≡ 1.41×10^{10} particles). They were kept under these conditions until 3 hours after dosing, when they were sacrificed. A control group (n = 3) was similarly treated but with omission of the surgery. Blood samples (0.5 ml) were collected from each animal, into heparinised tubes, at 10, 60, 120 and 180 min after dosing. All the animals were sacrificed after 3 hours and liver, spleen, lungs and small intestine were collected, weighed and immediately analysed, together with the blood samples, for radioactivity content, in a gamma counter (LKB CompuGamma, model 1282). Results were expressed in percentage of dose administered.

6.3.5. Statistical Methods

The results obtained with control groups and tracheotomised animals were analysed for significance using Student's unpaired t-test. Differences with $p < 0.05$ were considered significant.

6.4. RESULTS AND DISCUSSION

6.4.1. Particle Counting by Fluorescence Microscopy

The investigation of the particle uptake was first carried out by fluorescence microscopy and the total numbers of polystyrene microspheres counted in the haemocytometer were related to the total counts of erythrocytes (see section 6.3.2). Four rats were individually dosed with fluorescent polystyrene particles (0.51 µm ± 0.008), without any previous

surgical treatment. Ten minutes after dosing, the number of particles in tail vein blood samples was found to be 0.96% ± 0.42 (sd) of the number administered; at 30 min 0.53% ± 0.25; at 60 min 0.39% ± 0.10; at 2 hours 0.20% ± 0.14; at 5 hours 0.29% ± 0.22, and at 24 hours < 0.14% (fig. 6.1). This experiment shows a fast transfer of 0.51 μm particles to the circulation but, as it happens to the uptake at the Peyer's patches, a cut-off point may exist for larger particles. However, since the animals were dosed without any previous treatment, the absorption of particles at other mucosal sites, such as the lungs and the gastrointestinal tract after leakage from the nasal cavity cannot be excluded after nasal administration. In fact, preparations of the small intestine collected and analysed immediately after the sacrifice revealed the presence of some fluorescent particles.

Direct counting of particles *via* microscopy is one of the most appropriate methods of measuring the absorption of particulates but, because the particles usually are not uniformly distributed, counting can be difficult. In addition to the low amounts of particles found in blood after a single dose, it is necessary to dilute the samples in order to count the erythrocytes, which makes the detection of particles even more difficult.

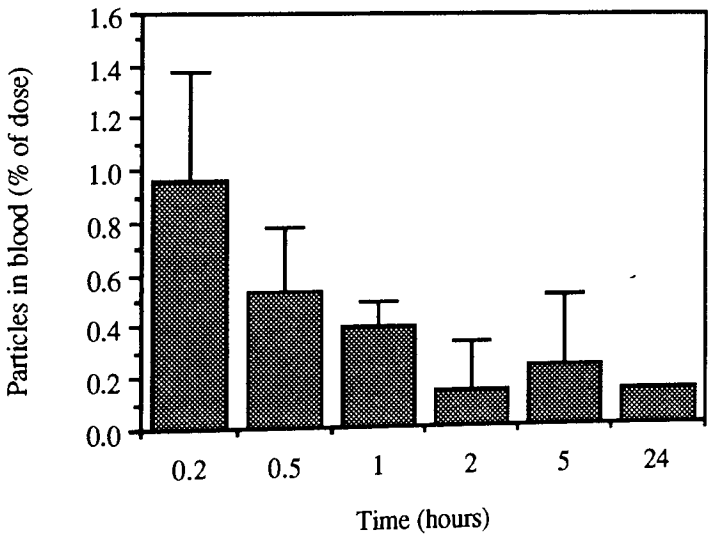


Figure 6.1 - Levels of circulating fluorescent latex particles (mean size 0.51 μm ± 0.008) after nasal administration to rats (± sd; n = 4). Particle numbers were calculated using the fluorescence microscopy method, as described in the text.

To check that nasal absorption of particles occurred in species other than the rat, the experiment was repeated by Alpar (published in Almeida *et al.*, 1993) with rabbits (n = 2) which received particles with a diameter of 0.83 μm, suspended in saline (100 μl per nostril). At 10 min the blood samples contained 1.9% of particles; at 60 min 3.4%, and at 2 hours 1.7% of administered particles. These results show that the nasal uptake of particulate systems after nasal administration is not a feature exclusive to the rats.

6.4.2. Assessment by FACS

To make sure that the absorption is exclusively from the nasal cavity, the experimental model of Fisher *et al.* (1987) was used. The method described by Ebel (1990) was used to treat the blood samples obtained in the experiment described in section 6.3.3, and the analysis of the blood samples by FACS resulted in the data shown in figure 6.2. The levels of circulating particles now obtained are lower than those presented in figure 6.1. This can be due to the experimental model used, which avoids the leakage of particles from the nasal cavity to the lungs and intestine. Microscopic observation of lung and small intestine samples immediately after sacrifice revealed the presence of rare latex particles. On the other hand, the particles used (0.83 μm) were larger than those used in the previous study, which can influence their uptake at a MALT component (Jani *et al.*, 1990). This particle size was chosen for being similar to that of the PLA microspheres employed in the intranasal immunisations with TT.

FACS analysis, like microscopy, gives equivocable results because of the treatment samples have to be submitted to before analysis. Ebel (1990) claimed that the treatment of biological samples with an aqueous solution of 1% Triton X-100 and 1% of KOH at 60°C/72 hours results in efficient dissolution of the tissues and preserves the integrity of the latex particles, 94% of which could be recovered after treatment. Such extreme conditions of pH and temperature during a period of three days seem to be quite destructive, not only for tissues but also for the fluoresceine and polystyrene. Even after applying the treatment for a shorter period of time (60°C/48 hours) than that recommended, the samples were very deteriorated and the calculated average recovery of particles was approximately 1%. So, the

quantification presented below (fig. 6.2) can be subjected to different interpretations, and the extent of uptake remains uncertain. Nevertheless, the fact that particles are indeed taken up at the nasal mucosa is demonstrated by the micrographs of the same blood samples obtained under a fluorescence microscope (figs. 6.3 and 6.4) in which fluorescent particles can be seen among the red blood cells.

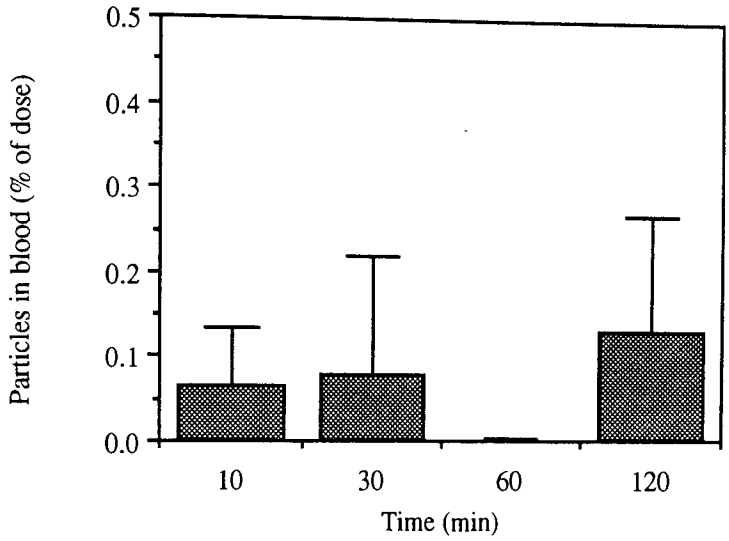
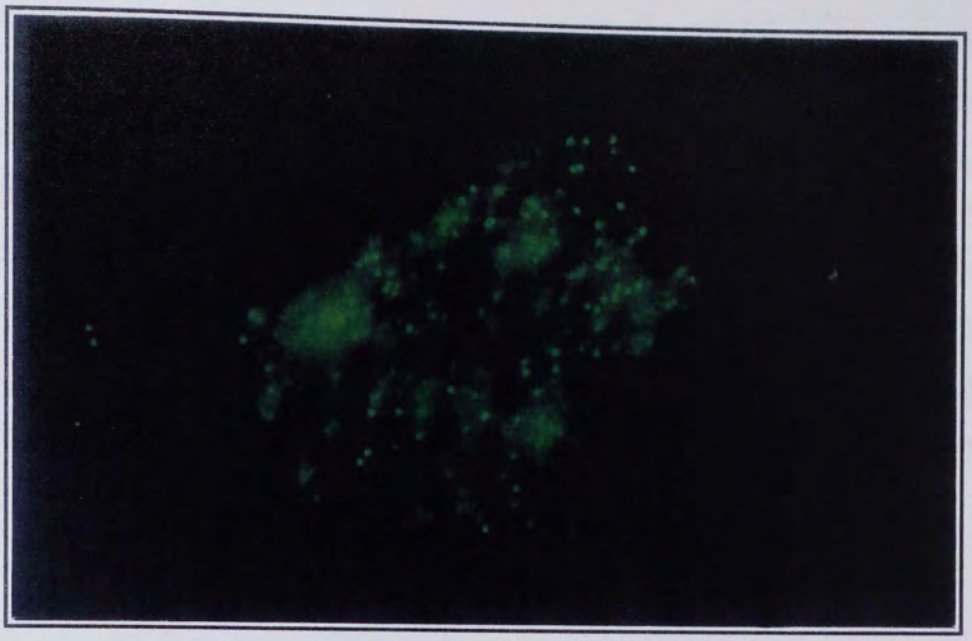


Figure 6.2 - Amount of fluorescent latex particles (mean size 0.83 μm ± 0.006) after nasal administration to rats (± sd; n = 4), as obtained by FACS analysis.

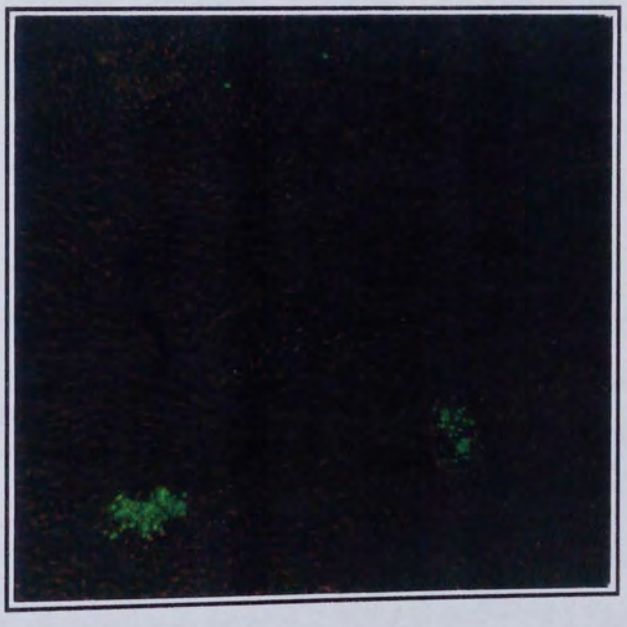
6.4.3. Quantification by Radioactivity

Guinea-pigs were used in these studies. The quantification of nasal uptake of microspheres was also attempted by the administration of ^{125}I-polystyrene microspheres prepared by the chloramine-T method (Hunter & Greenwood, 1962). After preparation, free ^{125}I in samples of labelled microspheres was removed by dialysis until the supernatants of particulate suspensions had reached a constant low value of radioactivity.



20 μm

Figure 6.3 - Micrograph of a cluster of circulating fluorescent polystyrene microspheres under UV light, obtained 60 min after administration from an animal treated using the model of Fisher *et al.* (1987).



50 μm

Figure 6.4 - Micrograph of the same preparation showed in figure 6.3, obtained under both UV and tungsten light, and where the fluorescent polystyrene microspheres (green) can be seen among the erythrocytes.

The circulating levels of particles obtained with the animals treated by the model of Fisher *et al.*, (1987) did not differ significantly ($p > 0.05$ for all samples, except for 180 min which presented $p \leq 0.05$) from those detected with the control animals (non surgically-treated). The first presented radioactivity levels of 1.55% ± 0.48 after 10 min; 2.25% ± 0.79 after 60 min; 2.16% ± 0.67 after 120 min, and 2.36% ± 0.48 after 180 min. The values detected in the control group were 1.58% ± 0.68 at 10 min; 1.80% ± 0.89 at 60 min; 1.53% ± 0.64 at 120 min, and 1.31% ± 0.62 at 180 min (fig. 6.5). Similarly, the differences between the amounts found in the organs studied were again non significant ($p > 0.1$ for all tissues; see fig. 6.6).

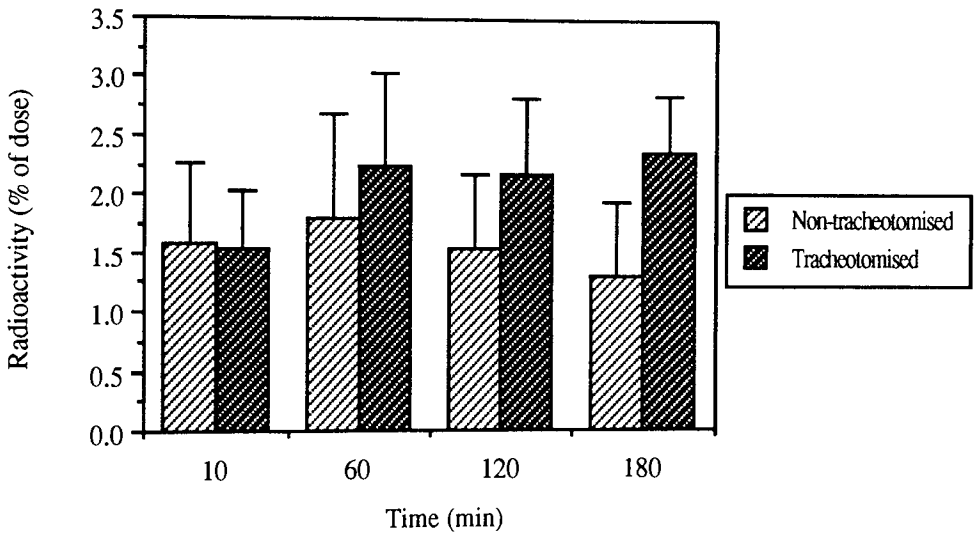


Figure 6.5 - The levels of radioactivity in the blood after nasal administration of radiolabelled polystyrene particles (mean ± sd; n = 3).

The low concentrations of particles in the liver 3 hours after dosing may be explained by the mechanism suggested by Kuper *et al.* (1992) for particulates, by which particles would reach the blood stream *via* the lymphatics and the spleen (see fig. 1.8). This implies that the particules will reach these tissues before attaining the liver. Retention in spleen and lymphatics can be the cause of the low amounts in the liver. Furthermore, the venous blood

draining the nasal mucosa can directly enter the systemic circulation and reach other organs and tissues before passing through the liver, which should potentially decrease the retention by this organ (Wüthrich & Buri, 1989). The percentage of radioactivity found in the small intestine is surprisingly high (approximately 0.74% in both groups) when compared to the amount retained in liver (approximately 0.35%) and spleen (0.02% for both groups). After injection in the rat's tail vein show a large number of particles was detected in the gut wall (H. O. Alpar, unpublished results), suggesting a certain degree of accumulation of particles in that compartment.

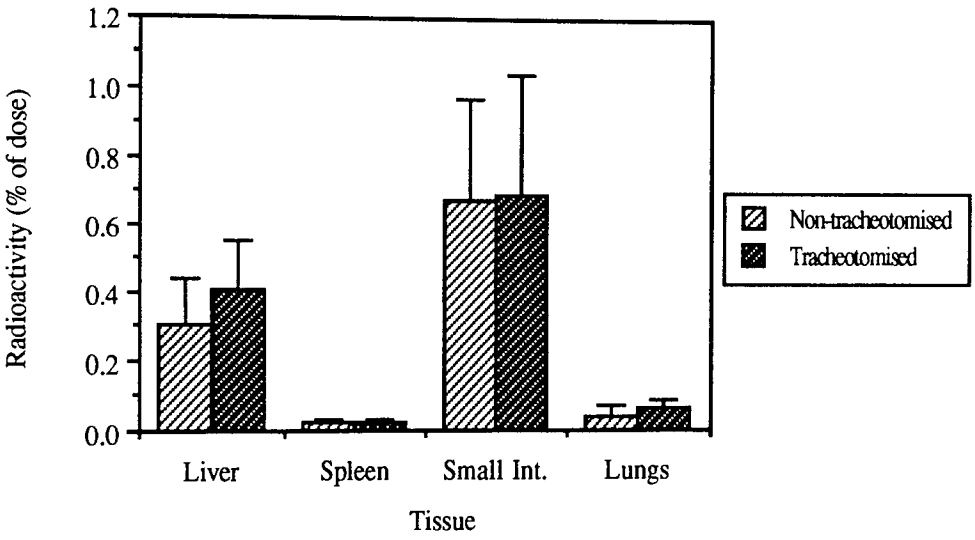


Figure 6.6 - Tissue distribution of radioactivity 3 hours after nasal administration of radiolabelled polystyrene particles (mean ± sd; n = 3).

A major observation results from the data described in figure 6.5, i.e. using the Fisher's experimental model did not produce any significant alterations in the amount of particles absorbed or in the levels detected in tissues. A decrease in uptake would be the expected consequence of both tracheotomy and sealing of the oesophagus. This can be attributed to the volume of 50 µl per nostril (total 100 µl) administered which may be small enough to remain in the nasal cavity without leaking to the lungs or the gastrointestinal tract, so that no differences would be observed by the use of the model. This is supported by the similar

percentages of radioactivity found in lungs and small intestine of both tracheotomised and non-tracheotomised animals. On the other hand, the limited stability of the radioactive preparation may have also contributed to the final results of this study. Given the difficulty involved in the preparation of the experimental model, the investigation was carried out for several days, the surgically-treated animals being studied 8 days after the controls. During this time the radioisotope, could have been partially released. In fact, by the end of the investigation, the supernatant of the particulate suspension presented approximately 8% of free ^{125}I. Therefore, the values obtained with the model animals may have been increased by the uptake of free iodine. Jani *et al.* (1990), detected high levels of radioactivity in the urine of animals treated orally with ^{125}I-labelled microspheres. Although the high levels of radioactivity in the urine may result from the presence of undialysed free iodine, the authors expressed doubt on the stability of the labelled latex.

Several methods to assess the mucosal uptake of particulate matter have been reported (LeFevre *et al.*, 1978; Pappo & Ermak, 1989; Ebel, 1990; Eldridge *et al.*, 1990; Jani *et al.*, 1990) and in the present study three of them were assessed, i.e. microscopic counting, FACS analysis and the use of radiolabelled particles. These three techniques present the above mentioned problems that can affect their reliability, making the quantification of the uptake an extremely difficult process. For this reason, this thesis cannot clearly conclude quantitatively about the extent of nasal absorption of particles, although the uptake as a phenomenon is demonstrated by the presence of fluorescent beads in the blood stream (figs. 6.3 and 6.4). Perhaps an appropriate alternative method would be gel permeation chromatography, which could have been useful in this determination.

These results show that particles (mean sizes of 0.51, 0.825 and 0.83 μm), when administered nasally can penetrate into the blood circulation. The mode of entry of nasally administered particles into the circulation is unknown and few investigators have published on putative mechanisms (Kuper *et al.*, 1992). The BALT from a number of animal species and the ovine pharyngeal tonsil (tissue equivalent to NALT) have been demonstrated experimentally to be capable of taking up and transporting particulate and soluble antigens (Gregson *et al.*, 1982; Chen *et al.*, 1989). However, Chen *et al.* (1991) reported that

attempts to demonstrate the ability of NALT to deal with inhaled antigens, such as microorganisms and allergens under natural conditions have been made, but the results are generally disappointing. The similarities between the follicle-associated epithelium in the ovine pharyngeal tonsil and other MALT components implies that they have a similar role in mucosal immunity. This is supported by the observation that MALT in the ovine pharyngeal tonsil was able to take up intranasally administered colloid carbon (Chen *et al.*, 1989).

Irrespective of the mode of entry, these findings concerning the uptake of particulate carriers by the nasal route suggest that the nasal mucosa is a potential administration route for the delivery of particulate vaccines.

7. CONCLUDING REMARKS

The control of infectious diseases by vaccination is a preferred alternative to chemotherapy, for medical, economic and logistic reasons. While the vaccines in current use have had a substantial beneficial impact, it is necessary to improve some vaccines and to develop new ones. Despite the efforts of WHO, UNICEF and others over the years, more than a million infants still die each year of diseases preventable by existing vaccines. Conventional vaccine delivery presents problems which are related both to patient compliance and to the adjuvants employed. A new generation of vaccine antigens are being identified and produced in the form of subunits and synthetic peptides, which although offering the advantage of safety, are in many cases weakly immunogenic. Therefore, there is an urgent need for pharmaceutically acceptable delivery systems and adjuvants for these antigens. Publications have emphasised the adjuvant properties of PLA and PLGA microspheres when administered by parenteral and non-parenteral routes and a great effort has been put into the development of these antigen carriers. The advantages of such a system are its safety, biocompatibility and the possibility of non-parenteral administration. This project was designed to study the use of microspheres to deliver antigens by the mucosal routes (oral and nasal).

The production of PLA and PLGA microspheres of a particle size convenient for mucosal delivery ($\approx$1 μm) was carried out by the o/w single emulsion version of the solvent evaporation method, which is a simple technique that results in spherical and well formed microspheres. Once the other parameters, such as the volume ratio of the liquid phases, and the concentration of polymer were chosen, the particle size could be controlled mainly by the speed of rotation of the mixer employed.

The microencapsulation method produced formulations containing protein with good encapsulation efficiencies, even though the method had been developed for the entrapment of hydrophobic drugs. Moreover, the need for a high encapsulation efficiency of antigens is arguable. From a purely scientific point of view it is known that a few micrograms of some antigens can make a protective dose, particularly when administered together with a potent adjuvant. On the other hand, given the cost of some protein antigens the economic factor

must also be taken into consideration. Therefore, the waste of antigen created by a low encapsulation efficiency must be avoided. Nevertheless, the 15% (w/w) obtained for the encapsulation of BSA is relatively high since other authors (O'Hagan *et al.*, 1991b) found good immune responses in immunisations with preparations containing only 1% (w/w) of weak antigens. The need for a good encapsulation efficiency of water-soluble molecules led to the development of more suitable preparation methods. The main example is the (w/o)/w double emulsion solvent evaporation method (Ogawa *et al.*, 1988) that has been successfully used. However, the solvent evaporation process has the general disadvantage of a difficult scaling-up, that makes it unlikely to be employed as an industrial method. Currently, some research groups (Pavanetto *et al.*, 1992) are trying to solve the problem by using a spray-drying technique that appears to generate microspheres with characteristics suitable for vaccine production.

Antigen-containing microspheres were also prepared by adsorption onto the particles' surface. The results obtained show that this may be a useful alternative means of loading preformed microspheres, simultaneously avoiding the contact with the organic solvent. Also, it may be applied to previously loaded microspheres as a way of increasing the payload, particularly in the case of nanoparticles that present a low encapsulation capacity. The adsorption process will depend on the system microspheres/protein/solvent as well as the temperature and time of incubation. This study has shown that the adsorption of some proteins (e.g. BSA and γ-globulins) at the surface of PLA microspheres follow the classical Langmuirian model of equilibria and that polar interactions are probably involved in this process. The adsorption of TT onto PLA (Mwt 2000 Da) microspheres did not fit the same mathematical model as well, which is also suggested by the different desorption profile shown by this protein.

The contact between the model antigens and the organic dichloromethane appears not to damage the protein molecules or, in the case of TT, it does not act on the immunogenicity. *In vitro* studies showed that BSA and TT maintain their integrity and the immunisations revealed that the production of anti-tetanus antibodies was not affected. As the stability of protein molecules is likely to be affected by the contact with organic solvents, the effect of this exposure is probably both antigen and solvent dependent, and a range of combinations

may need to be assessed for their suitability for each individual antigen. Therefore, depending on the antigen to be microencapsulated, exposure to organic solvents is not necessarily a detrimental step during vaccine production. Correspondingly, the adsorption procedure, although described as affecting some properties of the peptidic molecules did not affect the whole structure of TT. We have also found by immunoblotting and *in vivo* immunisation that the antigenicity of this protein was not destroyed

The surface characterisation of the particulate preparations obtained by adsorption showed that the adsorption process slightly alters the surface hydrophobicity of particles, rendering them more hydrophobic, the magnitude of effect depending on the protein employed. The influence of the surfactant employed on the microspheres' surface properties and its implications on the adsorption of proteins was also shown. Hence, in designing antigen delivery systems these properties could be exploited in obtaining formulations with an initial rapid release of the peptides and also in enhancing the uptake of particles by antigen-presenting cells (e.g. macrophages) which prefer to associate with hydrophobic surfaces.

Systemic and mucosal immune responses induced upon nasal, oral and intramuscular administration have been assessed and, when appropriate, compared with the most widely used vaccine adjuvant i.e. aluminium hydroxide. The results indicate that association of TT with PLA microspheres through stable microencapsulation or adsorption procedures led to an enhancement of specific mucosal and systemic antibody responses (IgG and IgA) to the delivered antigens. Particularly, nasal administration of two distinct formulations containing TT produced significantly higher serum levels of specific IgG in two species of test animals, as compared to control groups, suggesting that this is a potential route for vaccination.

In contrast, there was an apparent failure of the oral route in inducing both high mucosal and systemic antibody responses which is inconsistent with some publications describing high humoral immune responses obtained after oral immunisation with particulate carriers. However, in similar formulations, different drugs can present totally different patterns of pharmacological behaviour *in vivo*. So, the same can occur with different antigens and yet their mechanisms of action are not fully understood. This can explain the difference between

the results obtained by different investigators with various antigens, using distinct formulations.

This thesis cannot clearly conclude quantitatively about the extent of nasal absorption of solid particles, although the uptake as a phenomenon, was demonstrated by the presence of fluorescent beads in the blood stream, which confirms that the immune response to the nasally delivered particulate antigen can be due to the uptake of microspheres by the NALT. The observations made in two different species of animals (rats and guinea-pigs) imply that this is not an isolated phenomenon and involves the uptake and transfer of particles through the nasal mucosa. These findings suggest that the nasal mucosa is a potential administration route for the delivery of particulate vaccines.

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