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Zein microspheres: preparation and evaluation as a carrier for vaccine delivery

BY

PEPI HURTADO-LÓPEZ

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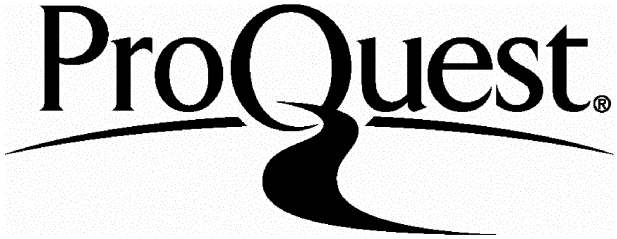

Abstract

A method for the preparation of smooth and spherical microspheres of the hydrophobic corn protein, zein, by a phase separation method, is reported in this thesis. Solubility studies on zein as well as amino acid and peptide composition analyses, together with studies on the formulation process were carried out to comprehend the mechanisms of zein microsphere formation. Microspheres were characterised in terms of guest molecule loading, size, charge, morphology, and composition. Ovalbumin, as a model protein/antigen guest, was incorporated into the zein microspheres during microsphere formation at different experimental loadings, and its *in vitro* release was examined in phosphate buffer at 37º C. Microsphere degradation studies as a function of medium pH and in the presence of enzymes were also performed *in vitro* to understand the release of the guest molecule. Zein microspheres were found to be extremely resistant to degradation in the absence of enzymes, which was reflected in negligible release of ovalbumin. Finally, zein microspheres (both blank and ovalbumin-loaded) were administered orally, intramuscularly, rectally, and vaginally to investigate whether a zein microparticulate carrier system was capable to induce an enhanced immune response to the model antigen. In these studies, adjuvanticity of zein microspheres could not be proved due to the antigenicity of the zein protein.

To my family.
Because I owe them
every single word of this thesis.

"Grown-ups are very strange",
said the little prince to himself,
continuing on his journey.

The Little Prince
Antoine de Saint-Exupéry

"Les persones grans són molt extranyes",
es va dir el petit príncep a si mateix,
durant el seu viatge.

El Petit Príncep
Antoine de Saint-Exupéry

"Guztiz arraro dira pertsona nagusiak",
zioen bere baitan printze
txikiak bere bidaian.

Printze Txikiak
Antoine de Saint-Exupéry

"Sono ben strani i grandi",
si disse il piccolo principe
durante il viaggio.

Il Piccolo Principe
Antoine de Saint-Exupéry

"Las personas mayores son muy extrañas"
se dijo el principito,
durante su viaje.

El Principito
Antoine de Saint-Exupéry

Küçük prens: " Şu büyükler çok tuhaf "
dedi kendi kendine ve yoluna devam etti.

Küçük Prens
Antoine de Saint-Exupéry

Etc.

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This thesis is dedicated to my family for their endless support. I would not like to finish this acknowledgment part without saying thank you once again.

Sometimes, gratitude cannot be expressed in a “thesis acknowledgments”, not even in a handwritten letter.

Dear Mr. Koo,

Gracias. Llegó el final. O el principio.

Pepi

Abbreviations

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)diammonium salt

APC: Antigen presenting cells

ATR: Attenuated total reflectance

BCA: Bicinchoninic acid

BSA: Bovine serum albumin

BP: British Pharmacopoeia

CE: Capillary electrophoresis

CMIS: Common mucosal immune system

CMC: Critical micellar concentration

CZE: Capillary zone electrophoresis

DMSO: Dimethyl sulphoxide

DTH: Delayed-type hypersensitivity

EM: Electron microscopy

FT-IR: Fourier transform infrared

GALT: Gut-associated lymphoid tissue

HEC: Hydroxyethylcellulose

IEF: Isoelectric focusing

IgG: Immunoglobulin G

IgA: Immunoglobulin A

IgM: Immunoglobulin M

IgE: Immunoglobulin E

i.m.: intramuscularly

i.v.: intravenous

LDV: Laser Doppler velocimetry

MW: Molecular weight

PACA: Poly(alkyl cyanoacrylate)

PBST: Phosphate buffered saline-Tween 20

PCS: Photon correlation spectroscopy

PLA: Poly(lactic acid)

PLGA: Poly(lactic glycolic acid)

PVP-40: polyvinyl pyrrolydone MW 40 000

PVP-360: polyvinyl pyrrolydone MW 360 000

RCF: Relative centrifugal force

RH: Relative humidity

s.c.: Subcutaneous

S.D.: Standard deviation

SDS: Sodium dodecyl sulphate

SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

s.e.m.: Standard error of the mean

SEM: Scanning electron microscopy

sIgA: Secretory Immunoglobulin A

TCA-BCA: Trichloroacetic acid-bicinchoninic acid

TEM: Transmission electron microscopy

USP: United States Pharmacopoeia

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Corrigendum

I wish to bring the reader' s attention to the following clarification:

Firstly, the mean of values obtained from experiments was always calculated in the work described in this thesis. There are several ways of expressing the dispersion of data, the most common ones being the standard deviation and standard error of the mean, especially in the field of particulate science. The latter statistical parameters were therefore quoted in this thesis. However, I do understand that the number of values (n=3, 5 and 10) obtained from experiments was not sufficient to determine the standard deviation. I also understand that citing the range of the data would have been a more accurate way of measuring the spread of the observations. Consequently, I would like to indicate that the use of standard deviation and standard error of the mean in this thesis was a convention; nevertheless, I wanted to convey the range.

Secondly, theoretical and experimental loadings, and loading efficiency are used in this thesis as measures of the amount of guest molecule incorporated into zein microspheres, as per convention in the field of particulate science. Thus, theoretical loading is defined as (total amount of drug / total amount of drug + polymer) * 100; experimental loading is defined as (experimental amount of drug / experimental amount of drug + polymer) * 100; and loading efficiency is defined as (experimental loading % / theoretical loading %). I understand that these definitions may be controversial to other scientists, and hence, each parameter is defined every time it has been used.

Aim and Rationale

Aim

The aim of this thesis was:

To investigate the potential of zein microspheres as vaccine delivery systems.

Therefore, the objectives were as follow:

- ✓ To characterise zein protein used in terms of solubility, amino acid composition and content, and peptide composition.
- ✓ To prepare zein particles on the basis of zein solubility-precipitability.
- ✓ To load ovalbumin as a model protein antigen into zein particles.
- ✓ To characterise zein particles, both blank and ovalbumin-loaded, in terms of size, charge, morphology, antigen loading, protein integrity, and protein composition.
- ✓ To determine *in vitro* model antigen release.
- ✓ To investigate *in vitro* zein particle erosion and degradation.
- ✓ To evaluate adjuvanticity of zein particulate antigen delivery system *in vivo*.

Rationale

For the purpose of this thesis, we uphold the hypothesis that zein meets some of the requirements to produce a vaccine delivery system, such as particulate systems.

Zein hydrophobic nature would reduce the time of circulation of particles in the bloodstream before opsonisation by macrophages and, at the same time, it would prolong the antigen release. The first assumption relates to the theory of enhanced phagocytosis of hydrophobic polymeric adjuvants by antigen presenting cells (Florence, 1997). The second assumption is supposed to increase the immune response by the depot theory, i.e. the longer interaction of the antigen with the immune system, the higher immune response (Hanes, 1995).

Zein low molecular weight would reduce the average size of its particles, making them efficient at presenting the loaded antigen at mucosal surfaces or parenterally, since it is assumed that particulate systems are opsonised by macrophages only within a certain particle size range.

Zein biodegradability, apparent inertness, and composition of only amino acids would make its degradation products safe and easily eliminated from the human body.

Zein protein nature would make it compatible with the majority of the antigenic peptides and proteins, due to their similar physicochemical properties.

Zein has been used to prepare microspheres and several zein microsphere formulations can be found in the literature; however, no trials of human or animal immunisation have been found after an extensive literature search. Natural-based polymers to produce particulate systems are supposed to be antigenic (Cleland, 1997); fact that can either be a major drawback of zein microspheres or confer additional immune adjuvant properties to the zein

microspheres. Hence, *in vivo* studies are needed in order to explore the zein particle properties as antigen delivery systems and/or vaccine adjuvants.

The use of vaccine delivery systems for mucosal immunisation is a major area in recent research in controlled release methods; due to the fact most vaccines only induce systemic immune responses to date when parenterally administered. These vaccine delivery systems, such as particulate systems, should protect labile antigens against degradation following antigen delivery. There is evidence in the literature about zein being resistant to microbiological attack and zein is used in food industry as an enteric coating. For this reason, the zein microsphere ability to pass the alimentary canal offering protection to antigens should also be explored.

Based on these justifications, a method of preparation of particulate systems using zein was proposed in this thesis, and the potential of zein particles as controlled release antigen delivery systems was then explored.

Chapter 1.

Introduction

In this thesis, the protein present in maize grain, named zein, was used to produce a vaccine delivery system. In the present chapter, cereal proteins and zein particularly are reviewed.

1.1. Brief history of cereal proteins

For thousand years, cereals have been the most important crops for human protein requirements. Even today, cereals contribute to household food sustain in many developing countries and they play a crucial role in the diet of people of many of the world's richest countries. Currently, the world annual production of maize for human consumption is 560 million metric tons approximately, half of which is produced by the United States (Shukla and Cheryan, 2001).

At the beginning of the twentieth century, Thomas B. Osborne (1908) wrote that plant proteins lie at the very foundation of plant and animal nutrition; however, scientists at that time had little knowledge of them. The only kind of plant protein known until 1805 was wheat gluten, which had been discovered by Beccari in 1746 by washing wheat flour (Osborne, 1908). In 1805, Einhof realised that similar proteins existed in other cereal grains. He also discovered that a part of the gluten was soluble in alcohol and proposed this property as a differential characteristic of all cereal proteins. It was only in 1820 that Taddei suggested a name for the alcohol-soluble part of gluten: gliadin. Later, Liebig in the 1840s and his disciple Ritthausen in the 1860s showed that four different classes of plant proteins exist, and identified and extracted some of amino acids residue from those proteins (Osborne, 1908). Despite their discovery, because neither Liebig's nor Ritthausen's work was exhaustive nor conclusive, Osborne is the scientist considered as the "father" of the chemistry of cereal proteins and his comprehensive studies on proteins of vegetable origin has provided the basis for our present knowledge of plant proteins.

The term prolamin, which will be frequently used throughout this thesis, was proposed by Osborne (1908) and later accepted by the scientific community.

Osborne's group focused all their research efforts on clarifying the uncertainties about the different proteins found in seeds. Their work was mainly undertaken during the last 20 years of the 19th century. They concluded that few proteins were constituents of the embryo, the majority being present in the endosperm of the grains as the food reserve. By the means available at that time, it was very difficult to establish the individuality of each protein, thus only the constancy of their properties and composition was established. Osborne himself believed that the method for classifying proteins according to their solubility was unsatisfactory from a chemical point of view and that identification of chemical individuals would have to await the development of newer techniques of study. However, the need for some classification was evident and the solubility classification was the only one available at that time. Hence, based on the solubility properties, he assigned the cereal proteins to commonly recognised groups previously established for proteins of animal origin, and gave modified definitions for each of these groups. Despite the lack of useful techniques to chemically characterise proteins at the time, Osborne's classification for seed proteins is still applicable today, and is used by modern scientists. Osborne's classification is as follows:

- **Globulins**: the greater part of the reserve proteins of all seeds, except those of cereals. Globulins are proteins that are soluble in solutions of neutral salts but are insoluble in water.

- **Prolamins**: a unique and differentiated group of proteins, which occur in the seeds of cereals. Prolamins are soluble in all proportions in alcohol of 70-80% and are not affected by boiling their alcoholic solutions. They are insoluble in water, but soluble in dilute solutions of acids and alkalis.

- **Glutelins**: a major component of the proteins of all cereals. Glutelins are insoluble in neutral solvents, but are dissolved by very dilute acids or alkalis.

- **Albumins**: proteins present in small amounts in nearly all seeds. Albumins are soluble in water and coagulate by heat, like the albumins of animal origin, to which they are very close in composition.

- **Proteoses**: proteins present in very small amounts in all seeds.

Other groups of animal proteins were not proved to exist in plants.

From a chemical point of view, prolamins were relatively well characterised at that time. It was known that, following hydrolysis they yielded a very small amount of arginine and histidine and no lysine or tryptophan at all. On the other hand, they were known to contain large amounts of glutamic acid. Prolamins had been found in the seeds of all cereals, but not in those of any other plant. Before Osborne's classification, they had been named simply alcohol-soluble proteins, except for the prolamin of wheat, which had the name of gliadin. The name of prolamin was related to the large proportion of proline and amide nitrogen that were yielded on hydrolysis.

Prolamins were then and still are a group of proteins characteristic of cereal seeds. Osborne said that all cereals were alike in the proportion and in the character of their proteins, except for rice. According to his work, they all contained small amounts of proteose, albumin, and globulin, and a high quantity of prolamin and glutelin. The glutelins and the prolamins of different cereals, however, were not alike. This knowledge of cereal proteins proportions is still used today.

Nowadays, cereal proteins are still fractionated on the basis of their solubility. The fractions obtained are mixtures of different proteins, each group has subgroups, and no single proteins are obtained. After this, other classifications are used, such as the ones based on biological function or based on the morphology of the cereal grains. According to the biological function, there are two groups of cereal proteins: the metabolically active or cytoplasmic proteins and the storage proteins. The former includes albumins and globulins; the latter comprises mainly prolamins and glutelins. Depending on the morphology

of the cereal grains, the proteins can be classified into endosperm proteins, proteins of the aleurone layer, and proteins of the embryo or germ (Lasztity, 1986; Shewry and Casey, 1999).

Storage proteins, thus prolamins and glutelins, are endosperm proteins. Nevertheless, amounts of storage proteins are also present in the aleurone layer and in the embryo. Albumins and globulins can be typically found in the germ and in the aleurone layer of the grain. The differences in amino acid composition of cytoplasmic and storage protein are great, which influences the nutritive value of these two groups of proteins. Since the metabolically active proteins have a higher proportion of lysine and arginine, they have a higher nutritional value than the storage proteins.

In the study described in this thesis, the prolamin present in maize grain (named zein) was used to produce a drug/vaccine delivery system. Zein is the main storage protein of corn endosperm (*Zea mays* L.). The endosperm of maize comprises about 80% of the grain and contains about 75% of the seed's protein. The endosperm also contains a high proportion of starch and a small content of lipids (Laszity, 1986). Looking into the literature, there are great differences between some authors' data about the maize protein proportions. This fact is related to the difficulties and differences in the extraction procedures used. In addition, a large variation in the protein fractions of the different plant varieties has been shown (Moueium, et al., 1996). Hence, it is not the subject of this thesis to exhaustively name the numerous proportions found in the literature, since those percentages depend on the method of extraction and fractionation of maize proteins. Instead, the general properties and composition of zein prolamin are identified.

1.2. Zein: a protein from maize

Zein is a protein, which is known for being non-toxic and for possessing GRAS status (Anonymous, 1985). Zein material has thermoplastic properties and, once purified, is non-hygroscopic. In the dry state, zein is resistant to heat (e.g. several hours at 100º C) and is stable to light. Zein is also resistant to microbiological attack. In composition and reactivity, zein protein is similar to other natural products such as casein and cellulose. United States Pharmacopoeia includes a monograph of zein (USP XXI).

Zein, the prolamin of corn, was first named by Gorham in 1821, who "discovered" it by infusing water in corn (Gorham, 1921, as cited in Shukla and Cheryan, 2001). As mentioned in section 1.1., almost all the zein is located in maize endosperm, whereas only a small amount can be found in the germ. Since its isolation in 1821, zein became of interest and, in the 1940s, it became available for commercial use as a product of the wet-milling industry (Shukla, 1992). However, its poor nutritional quality (i.e. deficiency in essential amino acids) and its insolubility in water have limited its use in food products for human consumption. Since the mid 20th century, zein has been investigated for possible uses as an industrial polymer (Leckley, 1951; Beatty and Boettner, 1984; Pelosi, 1997). In the mid 20th century, various processes coexisted to produce zein, after extensive work conducted to extract and purify zein during both the 19th and 20th centuries (Osborne, 1891; Buron and McDonough, 1936; Takahashi and Yanai, 1996). Nevertheless, the commercial production of pure zein is still expensive and research is underway to lower the cost of manufacturing and thereby increase the utilisation of zein (Shukla et al, 2000; Dickey et al., 2001; Parris and Dickey, 2001). The final product depends on the raw material (i.e. variety and part of corn), on the solvent used for the extraction of zein from the plant (generally aqueous solutions of ethanol or isopropanol), and on the method of purification. Again, it is not the intention of this thesis to evaluate the different methods of zein production, since each method produces a zein protein that

differs significantly from each another, but to review the general and universal properties of all zeins.

Until 1936, zein was considered as a homogeneous protein. Watson et al. (1936) precipitated zein solutions in 70% ethanol by adding successive amounts of water, and found that the protein could be divided into a number of fractions. The latter were characterised by their physico-chemical properties, such as sedimentation, diffusion constants and electrophoretic mobilities. Using the mobility data, the isoelectric point of zein protein was found to be between 5 and 6. The finding that zein was a complex protein composed of different fractions instead of a simple one provided new impetus for research on zein properties.

By the 1950s, several authors had demonstrated the heterogeneity of zein by gel electrophoresis (Foster et al., 1950; Mertz et al., 1958). A few years later, Turner and colleagues' work would also provide a new approach into the zein research. Turner et al. (1965) showed that zein was a heterogeneous protein, which also contained disulphide-linked aggregates. Their studies proved how important intermolecular disulphide bonds are in the structure of zein protein complex. The electrophoretic patterns of whole native zein performed by Turner showed mobile components along with a material that did not migrate. When gel electrophoresis was carried out after disulphide cleavage, the immobile origin material was no longer found. Instead, two major bands and several minor ones were found in the gels. Disulphide cleavage was performed by oxidation or by reduction. Both resulting electrophoretic patterns were identical, however the two major zein components had lower mobility than did those of the whole native zein. Zein was also reduced with mercaptoethanol and then alkylated with acrylonitrile. Turner and co-workers wanted to determine whether the change in mobility was due to disulphide cleavage or to a change in molecular charge. Alkylation had previously been demonstrated to reduce the protein without changing molecular charge. The reduced-alkylated zein also showed two major components and several minor ones, and no immobile material at the origin. In this case, the two major components had nearly the same mobility as the major components in whole

zein. The fact that the number of components and their apparent concentrations were the same for all disulphide-cleaved zeins, showed that the cleavage of disulphide bonds yielded individual polypeptide zein species. This meant that zein existed in the plant as several species which includes at least one that was a disulphide-linked aggregate of smaller components. This conclusion solved many of the contradictory observations that had been reported before the studies of Turner and colleagues.

In the 1970s, zein was again resolved into two major bands of molecular weights (MW) around 22-25 000 Da and 20-22 000 Da plus some minor components of lower MW (Misra et al., 1975; Lee et al., 1976; Paulis et al., 1975), by means of sodium dodecyl sulphate - polyacrylamide electrophoresis (SDS-PAGE). In addition, zein was fractionated into approximately 15 components by gel isoelectric focusing (IEF), which showed zein heterogeneity in terms of charge (Soave et al., 1975). It was also proved that one class of glutelins were essentially zeins, which had become insoluble in alcoholic solvents by association with other proteins through disulphide bridges (Gianazza et al., 1976). Gianazza and co-workers extracted zein from corn endosperm using alcoholic solvents and realised that alcohol extraction left some zein in the glutelin fraction. The zein in the glutelin fraction (residual zein) was able to dissolve in aqueous-alcohols following reduction of disulphide bridges with mercaptoethanol. The first zein fraction extracted was named Z_1 and was resolved by SDS-PAGE into two components of MW 23 000 and 21 000 Da, respectively. The residual zein, named Z_2, exhibited the same two components as well as two additional ones of MW 13 500 and 9 600 Da. The ratio Z_1/Z_2, as well as the extent of disulphide cross-linking, i.e. the amount of Z_2, was found to be highly variable among different maize lines and also during endosperm maturation and under different growth conditions.

Later, Gianazza et al. (1977) determined the amino acid composition of the Z_1 and Z_2 fractions, previously identified by gel electrophoresis. They affirmed that some constant variations exist between the two zein fractions. The polypeptides of MW 13 500 and 9 600 Da were found to be richer in sulphur containing amino acid residues, especially methionine, compared to the 23

and 21 000 Da ones. In addition, the two low MW polypeptides showed a decrease in hydrophobicity due to a smaller proportion in leucine, isoleucine, and phenylalanine. The 9 600 Da polypeptide was also richer in cysteine. From the amino acid composition, the authors concluded that the low MW zein polypeptides were not fragments of the higher MW chains, but had an independent origin. The 13 500 and 9 600 Da chains were found to represent 20-30% of the total zein molecules in the cell. Due to their very high content of sulphur-containing amino acids, Gianazza and co-workers speculated that these chains played a role in the formation of zein granules in the cell, by stabilising macromolecular aggregates via the formation of disulphide bridges.

In the 1980s, Landry and co-workers provided new knowledge about size and charge heterogeneity of zein. A chromatographic separation on hydroxypropylated Sephadex in 70% ethanol revealed four protein fractions and a non-protein fraction (Landry and Guyon, 1984a,b). The two major protein fractions had an apparent MW of 45 000 and 22 500 Da, respectively. After reduction with mercaptoethanol, both fractions resolved into two major components of apparent MW 22 000 and 24 000 Da and minor one of higher MW (45 000 Da and over). One of the fractions also comprised traces of a 16 000 MW component, which was only detected when gels were overloaded. Therefore, in unreduced zein, the fractions are either free or associated into oligomeric forms, predominantly into dimers. Nevertheless, these investigators concluded that oligomerisation of zein was not necessarily related to the formation of interchain disulphide bonds merely. On the other hand, the amino acid patterns of all fractions were similar to that of total zein, as well as the NH_2-termini of all of them, which were threonine and phenylalanine residues. The isoelectric points of the main fractions extracted by these researchers were found to range between 7.2 and 8.5, confirming once again the charge heterogeneity of zein subunits. Some other authors of the time gave pI values that were even more heterogeneous such as between 5 and 9 (Esen, 1987).

During the 1970s and 1980s, the studies of Paulis, Wall, Bietz, and Esen shed new light on zein polypeptides. At that time, it was known that both prolamins and glutelins were heterogeneous and that some glutelins were soluble in

alcohol upon reduction or alkylation. These researchers chromatographically fractionated the alcohol-soluble glutelins on phosphocellulose (Esen et al., 1981). The elution profile of glutelin proteins consisted of five fractions, with the first fraction being partially resolved into four portions. The first two partial portions of the first fraction contained a component having a MW of 22 000 Da, this component had the same apparent MW and relative elution volume as the 22 000 MW zein component. The second two fractions also contained two characteristic components of zein, one of 17 000 Da and another of 13 400 Da in addition to four and five larger size components, respectively, in the 20-25 000 Da range. The latter components had electrophoretic and chromatographic properties similar to those of the two major size components of zein. In these studies, the charge heterogeneity of alcohol-soluble glutelins was also proven with IEF. Later, the same authors (Esen et al., 1984) isolated and characterised the alcohol-soluble glutelin, finding that they were proteins richer in methionine, histidine, and proline and poorer in aspartic acid, leucine, and phenylalanine compared to zeins.

Esen (1982) also performed chromatography of zein on phosphocellulose. Zein was divided into 13 fractions, in the presence of a reducing agent. The first four fractions included one size component of 22 000 MW, and the smaller of the two major zein components. These fractions represented up to 45% of the total zein eluted. Fractions 5 to 9 consisted of the two major zein components (24 and 22 000 Da), while fractions 10 to 13 contained a low MW component of 15 000 Da in addition to the two major zeins. The larger component (24 000 Da) was found to be composed of two size classes, and the 22 000 Da of three size classes. The anomalous elution pattern of zein, in which a given size component was found in a number of different fractions separated in the elution order, was not fully explained by the author.

Studies by Paulis (1981) provided more insight into the disulphide structures of zein proteins and indicated that different polypeptides had different tendencies to participate in intra-molecular and inter-molecular disulphide bonds. Following SDS-PAGE analyses, the whole zein consisted mainly of 45 000 and 68 000 MW bands, with a prominent band having a MW of 24 000

Da. Some zein did not migrate in the gel, as observed by Turner and co-workers. After reduction, two subunits appeared to be of 22 000 and 24 000 Da. A small amount of 45 000 MW protein appeared in the reduced samples too, and a protein band with a MW of 14 000 approximately. The latter was associated with the alcohol-soluble glutelins and with the 13 600 Da peptide, whose amino acid composition was determined by Gianazza and was found to contain high levels of methionine. Paulis concluded that native zein occurs as single polypeptides and disulphide-linked oligomers. The proteins migrating before zein reduction would consist of monomers and oligomers of increasing MW, whereas the non-migrating ones of higher MW oligomers.

Following Turner and colleagues, Esen, Paulis, Bietz, and Wall also named zeins α and β. α-zeins being the migrating proteins before zein reduction and β-zeins the non-migrating ones. The alcohol-soluble glutelins (ASG) were referred to as glutelin-1 (Landry and Moureaux, 1971), zein-2 or Z_2 (Gianazza et al, 1976) or zein-like (Misra et al, 1975). Thus, several nomenclatures and classifications have been given to zein proteins in the course of the years leading to some confusion. In this thesis, the comprehensive nomenclature of Esen has been followed.

1.2.1. Zein classification and nomenclature

Zein polypeptides purification to homogeneity has been found to be difficult. This, along with the diversity of zein fractions extracted by different methods, has impeded a clear classification of zein proteins. Despite this, both Wilson and Esen proposed universal nomenclature for zein polypeptides. As mentioned above, this study will follow the nomenclature of Esen.

Wilson (1985), whose studies on zein using SDS-PAGE and IEF (Wilson et al., 1981; Wilson, 1984, 1985) also demonstrated the heterogeneity of zein proteins and proposed a nomenclature for the maize endosperm proteins soluble in alcoholic solutions. He defined those proteins as the two traditional zein fractions as well as the two small MW proteins that had been shown to

have different amino acid content, but which have the prolamin type amino acid composition described by himself and his co-workers. Because of disagreement regarding the exact MW of the zeins, Wilson proposed that numbers would cause confusion and he termed the four zeins as A-, B-, C- and, D-zeins. A- and B-zeins were the components found in the zein extracted without reducing agent, and C- and D-zeins were found in the zein extract produced in the presence of a reducing agent. A- and B-zeins were separated into two or more bands. The apparent MW of these proteins was determined by Wilson as follows: A-zein, 26.5 kDa; B-zein, 24 kDa; C-zein, 18 kDa; D-zein, 13.5 kDa.

Esen (1987) proposed another nomenclature for the alcohol-soluble proteins of maize. He extracted zein from the plant using alcohol-solutions and fractionated zein intro three main fractions, named α, β and γ, as shown in figure 1.2. These fractions were characterised by electrophoretic, chromatographic, and immunological procedures. α-zein was defined as the zein soluble in 50-95% propan-2-ol, which constituted 75-80% of the total zein (depending on the genotype) and was made of several 21-25 000 Da polypeptides as well as a 10 000 polypeptide (figure 1.1., lane 2). β-zein was the zein soluble in 30-85% propan-2-ol containing a reducing agent, which included two 17-18 000 Da methionine-rich polypeptides (figure 1.1., lane 3) and constituted 10-15% of the total zein. Finally, γ-zein was the zein soluble in 0-80% v/v propan-2-ol in the presence of a reducing agent, as well as in 30% propan-2-ol/30 mN sodium ethanoate, pH 6, which constituted 5-10% of the total zein and was made up of one polypeptide of 27 000 Da (figure 1.1., lane 4). γ-zein, also named proline-rich protein was not considered to be a prolamin by Wilson at that time. In addition, Esen termed one more fraction as δ-zein composed of low MW polypeptides (9-10 000 Da). However, one of these low MW peptides was included in α-zein on the basis of its solubility and immunological cross-reactivity. A few years later, Esen (1990) modified his classification and assigned the 18 kDa peptide as γ-zein$_2$, earlier denoted as β-zein, due to the immunological cross-reactivity with the 27 kDa peptide and their sequence similarity. The 27 kDa peptide was then named γ-zein$_1$. Both γ-

zeins were considered to represent the 10-15% of the total zein. γ-zein$_2$ could be a truncated version of γ-zein$_1$. γ-zein$_1$ was found to have a distinguished property: its complete resistance to *in vitro* digestion by proteolytic enzymes. The 10 kDa peptide was finally allocated to the δ-zein fraction.

Similarly, Wilson (1991) also reviewed his nomenclature some years later. He separated zein in six classes instead of four following fractionation of the proteins by reversed-phase high performance liquid chromatography (HPLC) as follow: A- and B-zeins (α-zeins), C-zeins (β-zeins), D-zeins (δ-zeins), E-zeins (γ-zeins$_1$) and F-zeins (γ-zeins$_2$). All these proteins are also named by their MW, however the numbers lead to confusion due to the different MW determined for each zein class by each author.

1.2.2. Zein structure

Concerning the primary structure of zein, a number of researchers have determined the amino acid composition and content of zein. All proportions given in the literature have similar patterns; nevertheless, zein composition varies due to batch-to-batch differences since its nature depends on the extraction method used. Briefly, zein is particularly rich in glutamic acid (21-26%), leucine (20%), proline (10%), and alanine (10%), but deficient in basic and acidic amino acid residues. It generally lacks tryptophan and lysine (Mossé, 1961; Pomes, 1971, as cited in Shukla, 2001). Gianazza et al. (1977) demonstrated that 90% of the glutamic acid and the aspartic acid were amidated in native zein.

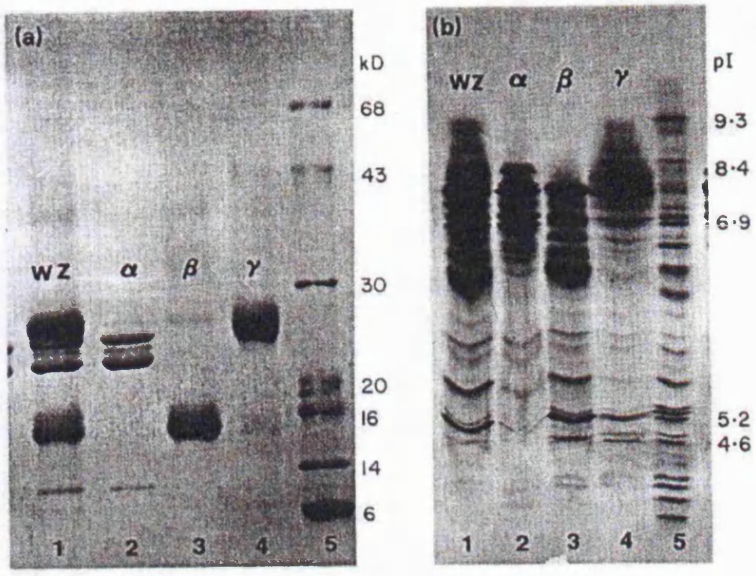


Figure 1.1. SDS-PAGE (a) and IEF (b) profiles of whole zein (lane 1) and its three fractions, α (lane 2), β (lane 3) and γ (lane 4); lane 5 being calibration standards. Each zein fraction has different polypeptide profile; however, the profile of the whole zein seems to contain all the species found in each fraction (From Esen, 1987).

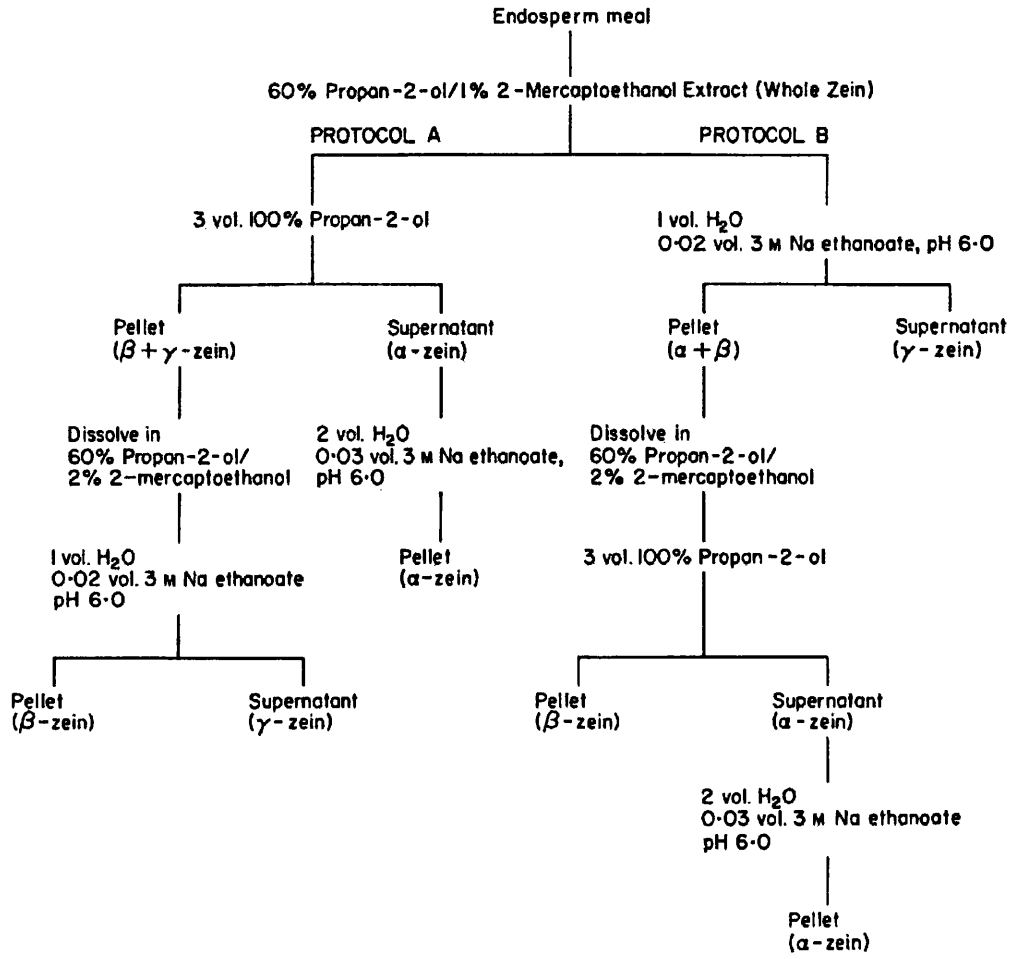


Figure 1.2. Flowsheet diagram showing the two different fractionation protocols (protocol A and B) used by Esen to separate zeins into fractions α, β and γ. Both protocols were based on differential solubility at various propan-2-ol and sodium ethanoate concentrations (From Esen, 1987).

The amino acid composition of the α, β and γ-zeins initially characterised by Esen (1987) were essentially similar, being rich in proline, glutamine and hydrophobic residues. The main differences were the high proline (25%) and histidine (8%) content of γ-zein, the high methionine (10%) and tyrosine (8%) proportion of β-zein, and the high leucine (20%) and phenylalanine (6%) content of α-zein.

The N-terminal sequence of zeins was already investigated earlier too. Threonine is the major N-terminal amino acid of major zeins. Again, N-terminal amino acid variability has been seen between corn varieties. Zein's N-terminal sequence is very hydrophobic, in fact, of the major residues identified in the first 33 positions (Bietz et al., 1979), 20 were hydrophobic: alanine, valine, leucine, isoleucine, phenylalanine, tyrosine, and methionine. The N-terminal sequence determined by Bietz and co-workers indicated significant homology among most zein subunits. Later, Esen et al. (1982) determined the N-terminal sequence of the proline-rich alcohol soluble glutelin proteins of corn, and found that these proteins have identical sequences of at least 49 amino acids. ASG protein N-terminal sequences were found extremely hydrophobic too. Because of zein's amino-terminal sequence homology, many authors have suggested that the zein polypeptides are products of homologous genes, which arose from an ancestral common gene (Marks et al., 1985).

Regarding the secondary structure of zein, the conformation studies carried out by several authors are conflicting. Recently, α-zeins have been studied by Fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR), and small-angle X-ray scattering (SAXS). These studies have shown that α-zeins have 46% of alpha helix and 22% of beta sheet (Forato et al., 2004).

The first structural model for zeins was proposed by Argos et al. (1982), using circular dichroism. These researchers showed that zein secondary structure was largely helical. They suggested a structure with nine adjacent, antiparallel helices clustered within a cylinder. In addition, it is thought that glutamine-rich

turns exist between the helices, while polar amino acids would allow intra- and intermolecular hydrogen bonds enabling zein molecules to be arranged in planes (figure 1.3.).

Matsushima et al. (1993) revised Argos' structural model by small-angle X-ray scattering. They proposed a new model: α-zeins would contain 9-10 tandem repeats units of 20 residues approximately, each tandem unit favouring the α-helix and little or no β-sheet would be present; α-zeins would take a non-globular shape and adopt an elongated molecular structure with a length of about 13 nm. In this new structural model, α-helices would also be joined by glutamine-rich turns (figure 1.4.). Matsushima and co-workers hypothesised that the elongated prism-like structure of zein may allow flexibility to the molecule to be easily packed into protein bodies in maize plant.

Tatham, Shewry and collaborators (Tatham et al., 1993) also studied zein secondary structure, and concluded that the conformation of α-zeins in solution was relatively rigid and asymmetric. This conformation is consistent with the elongated structures of zein stated by Matsushima.

All the conformations described above were determined in aqueous-alcohol solutions since these solutions are supposed to be similar to those of α-zein environment in protein bodies, in terms of low water availability and low dielectric constant.

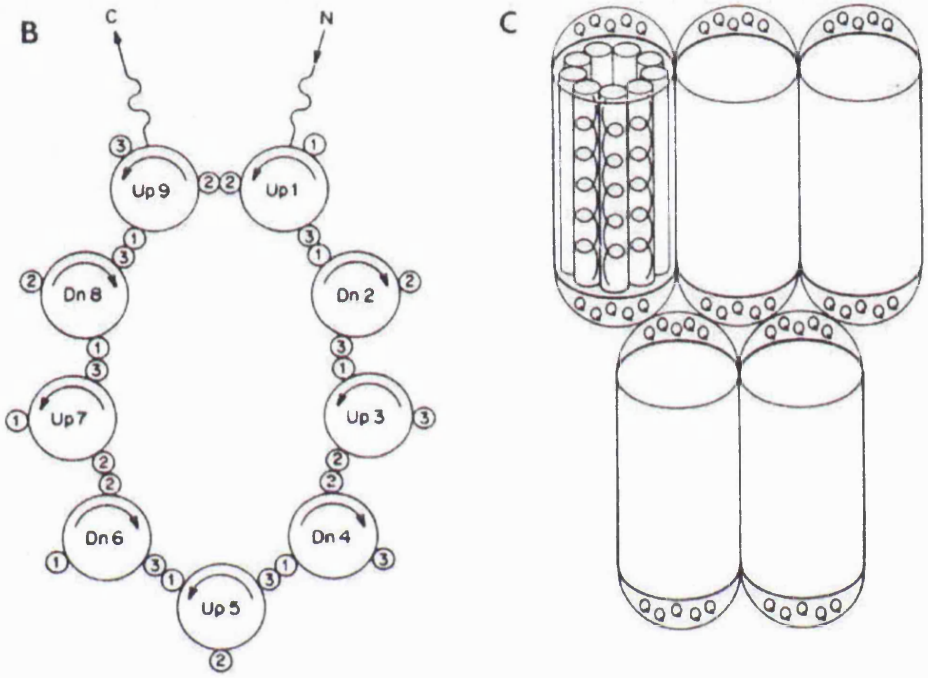


Figure 1.3. Structural model proposed by Argos and co-workers for the two major components of zein protein. (B) Organisation of nine repeated α-helices in a cross-section view of a protein molecule. (C) Model for arrangement of zein proteins within the protein body; "Q" indicates the positions of glutamine residues at the ends of the repeated peptides (From Argos et al., 1982).

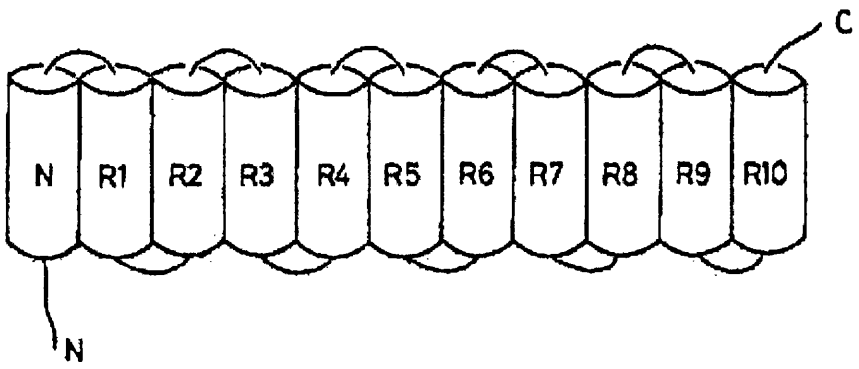


Figure 1.4. Structural model proposed by Matsushima and co-workers for the α-zeins. Each cylinder is a tandem repeat unit formed by a single α-helix, the curves joining them are glutamine-rich loops (From Matsushima et al., 1997).

Three main levels of organisation for cereal grain, including prolamins, can be mentioned: the whole grain, the endosperm, and the protein bodies. Most proteins are in protein bodies located within the starchy endosperm. The endosperm starch granules from maize have been shown to contain bound proteins, and approximately one-half of these granule associated proteins consist of zein polypeptides (Foster and Wasserman, 1998). The spatial distribution of proteins in the granules is not well known yet. However, two distributions of proteins have been found. One group of proteins are intrinsic proteins distributed within the starch granule matrix and another group is located at the surface of the granule, thus are easily accessible to proteolytic enzymes. The zeins on the surface appear in the form of aggregates and the intrinsic zein proteins as individual entities. Foster and Wasserman stated that the predominant zein species in the protein body are the α- and γ-zeins, β- and δ-zeins being present in lesser extents. This agrees with earlier observations of Esen and Wilson described earlier. In addition, the chemical cross-linking experiments of Foster and Wasserman confirmed that zein

subunits assemble into multimeric networks via disulphide-linkages and various types of non-covalent interactions.

Zein protein bodies imbedded in the starch granules are in the range of 100 to 200 μm diameter (Lopes and Larkins, 1993). The mechanisms by which zein proteins assemble into protein bodies are not clear-cut. The accumulation and the spatial distribution of zein in protein bodies must imply interactions among zein proteins. The latter have been studied by Kim et al. (2001), who showed that both γ_1-zein and γ_2-zein strongly interacted among themselves, with β-zeins and with the higher MW α-zein. Nevertheless, γ-zeins had weak interactions with the low MW α-zein and the δ-zeins. Therefore, protein bodies appear to form through an association between γ-zeins and β-zeins mainly. γ-zeins would primarily remain at the surface while the protein bodies enlarge as the α-zeins and the δ-zeins accumulate. The basis of zein protein affinities is unclear, because hydrophobic/hydrophilic interactions is expected to play a role, but this role may not be unique.

1.2.3. Zein solubility

One of the zein proteins main characteristics is their solubility, to the extent that zein has always been defined and classified on that basis, as mentioned above. Zein solvents for commercial purposes have been searched since zein started to be commercially available in the 1940s. Swallen was a pioneer in the search for solvents for zein's optimal extraction from corn (Swallen, 1941) and found ethyl and isopropyl alcohols to be the best solvents. These two solvents are still the ones that are most commonly used for zein extraction from maize. However, at the time, the heterogeneity of zein was not well known and this fact impeded the determination of universal alcohol concentrations to dissolve zein optimally. Evans and co-workers also studied zein solubility extensively (Evans and Manley, 1941, 1944; Manley and Evans, 1943; Evans et al., 1945) and took into account that the conditions under

which zein was extracted had an effect on its properties, and they searched for primary, binary, and ternary solvents for zein.

About 40 years later, once the heterogeneity of zein had been established, other studies clarified the "unusual" zein solubility, as had been named since then. Augustine and Baianu (1987) dissolved a number of commercial zeins in organic solvent systems in order to identify the best one. NMR techniques were used to monitor the mobility of the amino acid side chains in solution and SDS-PAGE was performed to identify the effects of solvents on zein subfractions. The first solvent system used was an ethanol/water mixture; the solubility of zein in this mixture was found to be highest at ethanol concentration of 70% v/v (12M), the limit of solubility being 2% w/v. Secondly, zein was dissolved in propan-2-ol/water mixtures. The optimum propan-2-ol concentration was 60% v/v (7.8M), which dissolved up to 3% w/v of zein protein. These two solvent systems were found to be the optimal ones for zein, in concordance to Swallen's studies in 1941.

It was mentioned in section 1.2. that zein extraction and production are still being researched to optimise the processes and to reduce costs. In 2001, Dickey, Parris and co-workers optimised zein extraction using ethanol/water mixtures and they rationalised the behaviour of the ethanol/water/zein solution as follows. Zein does not dissolve in either pure water or pure ethanol; however, the planar zein structure would complement the ethanol structure, which polymerises and stacks in arrays. The water molecules would then fill between the ethanol polymers around the zein solute and in the solution. A range of ethanol weight fractions can fit the planar ethanol polymer solution model determined by Matsumoto and collaborators (Matsumoto et al., 1995; as cited in Dickey et al., 2001) and these ethanol weight fractions can solubilise zein, according to them. When the water fraction exceeds the amount needed to hold the ethanol polymers together, zein precipitates. This rationale could also be applicable to other alcohol-water mixtures.

Although the main solvents for zein extraction are alcohol-water systems, zein has also been dissolved in mixtures of ketones (e.g. methyl ethyl ketone,

acetone) and water, amide solvents (e.g. acetamide), highly concentrated salts (e.g. sodium chloride, potassium bromide) solutions or glycols (e.g. ethylene glycol). In addition, the solubility of zein in water has been increased by acidic or alkaline deamidation or enzymatic hydrolysis of zein protein (Payne and Tyrpin, 1990; Mannheim and Cheryan, 1993).

1.2.4. Zein uses in pharmaceutical and other industries

Since early times zein has been cited in the literature and a great number of patents and papers have been published describing its manufacture. That may be because zein is an available industrial protein and very interesting from the commercial point of view. Zein easily forms films which are resistant to microbiological attack, flexible, and greaseproof. The use of these films for coatings and inks is one of the most promising applications of zein and, for this reason, the properties of these films have been intensively studied (Tillekeratne and Easteal, 2000; Dong et al., 2004). The pharmaceutical industry is also interested in zein since it is reported to be harmless and non-toxic for humans. Zein can act as a natural barrier material for tablet coatings and for plasters and bandages. Additionally, a process for making textile fibres from zein has been established. Shukla and Cheryan (2001), in their excellent review, summarised the most common uses of zein, as shown in table 1.1.

Finally, although zein has poor nutritional value, it has been discovered to have nutraceutical value, since upon hydrolysis with thermolysin, α-zeins were found to produce angiotensin-converting (ACE)-inhibitory peptides (Ariyoshi, 1993). Antioxidative activity of zein has also been proved by Wang et al. (1991).

1.2.4.1. Zein microspheres

Based on some "ideal" properties of zein, this protein has been selected in the past to prepare microspheres for drug delivery. Zein is hydrophobic and biodegradable, is metabolised in the body to peptides and/or amino acids, and can be modified proteolytically or chemically to confer desirable properties. These characteristics may or may not provide an optimal system for drug/vaccine delivery and the potential of zein protein as an optimal material to produce drug/vaccine delivery systems is still to be shown.

Nevertheless, in the 1990s, Mathiowitz and co-workers patented a method for producing protein microspheres and the preferred proteins were prolamins, such as zein. Protein microspheres were formed by phase separation in a non-solvent followed by solvent removal by extraction or evaporation (Mathiowitz et al., 1993).

At the same time, Matsuda and Suzuki groups (Matsuda et al., 1989; Suzuki et al., 1989) prepared zein microspheres for the encapsulation of antitumor agents for cancer immunotherapy. These authors used zein as a carrier matrix and conjugated both the anticancer drug and the zein protein by cross-linking with glutaraldehyde.

Advances in those original zein microspheres formulations have never been reported; however, Mathiowitz and her collaborators have patented other polymeric delivery systems where many polymers are included as examples, such as zein and other prolamins (Mathiowitz et al., 1997; Mathiowitz et al., 2004).

Recently, Demchack and Dybas (1997) and Liu et al. (2005) have used zein to encapsulate parasiticides, such as abamectins and ivermectin. These authors have prepared a novel microsphere drug delivery system by the phase separation technique. Their last results showed that zein microspheres were suitable for the sustained release of ivermectin, and that the release of the

drug in the presence of pepsin showed zero-order release. The authors concluded that ivermectin-loaded zein microspheres were appropriate for phagocytosis by macrophages due to their diameter and that they presented potential applications in tissue engineering as scaffolding material.

Uses of zein	References
Adhesives, binders	Coleman, 1944
Biodegradable plastics	Lai et al., 1997
Coating for food products	Haralampu and Sands, 1991; Wasa and Takahsahi, 1998
Delivery systems for acid sensitive drugs	Mazer et al., 1992; Ting and Hsiao, 1999
Electrophoretic coating	Korinko and Hunt, 1999
Long-acting matrix tablet formulations	Katayama and Kanke, 1992
Microspheres	Matsuda et al., 1989 ; Suzuki et al., 1989 ; Mathiowitz et al., 1993; Liu et al., 2005
Nutrient delivery systems for ruminants	Witt and Dew, 1999
Photo stabilisation of abamectins	Demachak and Dybas, 1997
Starch-based polymers	Parris et al., 1997; Wang, 1999
Surgical closure of body organs and blood vessels	Muxfeldt and Dahlke, 1981
Taste making of oral drugs	Cuca et al., 1994; Meyer and Mazer, 1997
Textile fibres	Zhang et al., 1997

Table 1.1. Commercial and research uses of zeins. (Adapted from Shukla and Cheryan, 2001).

1.3. Drug and vaccine delivery systems

Prof. S.S. Davis (Davis and Illum, 1998) highlighted the statement from a research director of a pharmaceutical company: *"our drugs deliver themselves!"*. This sort of thinking was the surprising answer of many formulation scientists to the idea of delivering drugs to the human body. However, this answer was only applicable for the "classical molecules" because compounds that were unstable, had high molecular weight, or were not well absorbed from the gastrointestinal tract, such as insulin or heparin, could only be administered by injection. New ways to deliver drugs and to control their absorption and distribution within the body were then urgently needed for these difficult molecules. Pharmaceutical formulation sciences saw a revolution when controlling the release of drugs along with selecting their delivery to their site of action was found to increase the therapeutic effectiveness of the drugs, while minimising unwanted side effects and toxicity.

Today, biotechnology is producing many natural molecules for challenging disease treatments, such as growth hormones, cytokines, etc. In addition, the emerging field of gene therapy is currently discovering hundreds of new drug targets to deliver therapeutic agents. Consequently, drug delivery still has a long research future.

The choice and development of a new delivery system will depend on the nature of the material to be delivered and on the route of administration. The research approach for drug delivery is named the "4D" approach (Davis and Illum, 1998), where the **d**isease is the prime driving force in the selection of **d**rugs, and drug **d**elivery is the scientist's tool to maximise the therapeutic efficacy (**d**estination). Today, scientists in the field of drug delivery are researching on new non-viral gene vectors for the delivery of vaccines, nanoparticles and liposomes for site specific delivery of anticancer agents and DNA, bioadhesives systems, oral delivery systems to avoid food effects, electrotransport systems for transdermal delivery, and polymer implants for

conventional drugs and polypeptides, among others. The aim of all these novel drug delivery systems is to provide one or more of the following benefits:

- ✓ Maintenance of drug concentration within an optimal therapeutic range for prolonged duration of treatment.
- ✓ Maximisation of efficacy-dose relationship.
- ✓ Reduction of adverse side effects.
- ✓ Minimisation of the needs for frequent doses.
- ✓ Enhancement of patient compliance.

Classical medicinal agents that have always been candidates for delivery are antigens. Since the conception of the idea of delivery, antigen delivery systems have been proposed as an addition to existing adjuvants for vaccination. An adjuvant would be any material that, administered with the antigen, provides a mode of presentation of the antigen that increases the humoral and/or cell-mediated immune response towards that antigen. A humoral immune response is required for the eradication of extracellular pathogens, whereas a cellular immune response is required in combating intracellular pathogens and tumour cells (Newman et al., 1998). The effectiveness of an antigen delivery system is determined by its ability to activate the immune responses. Since both definitions are conceptually similar, the terms adjuvant and delivery system have been used interchangeably in relation to vaccines. In addition, the mechanisms of action of most adjuvants are not clear-cut.

Adjuvants have been known for increasing the immune response to antigens. However, stronger adjuvants were and are still needed to reduce the number of vaccine doses required for effective immunisation. Controlled release antigen delivery systems were conceived to reduce the number of vaccine doses while protecting antigens from degradation *in vivo.*

Antigens have involved the use of peptide-based vaccines. Unfortunately, peptide antigens are poorly immunogenic and require adjuvants to increase their immunogenicity. Protein and vaccine delivery are the subject of

discussion of the present thesis. Important work on these matters has been carried out by several researchers, such as S.S. Davis, D. O'Hagan, R. Langer, and many others, who established the foundation of drug delivery. Drug and vaccine delivery and targeting issues are excellently reviewed (Panyam and Labhasetwar, 2003; Orive et al, 2004). The subject of this thesis is also the protein and vaccine delivery; however, the intention is not to review the types of delivery systems and their mechanisms of action but to provide an overview of the methods to produce the type of delivery systems used in this study, the particulate systems, and to address specific aspects of vaccine delivery.

1.3.1. Particulate systems

The idea of using biodegradable particulate systems to carry drug molecules to specific organs within the human body emerged in the 1970s. Particles for drug delivery became attractive because the methods of preparation were simple and easy to scale-up. In addition, micro- and nanoparticles were able to achieve tissue targeting of many drugs, such as cytostatics, or peptides and proteins. Since then, much research has been undertaken to understand the *in vivo* behaviour of particles as well as their physicochemical properties. Suspensions for injections consisting of a depot of microspheres containing therapeutic agents are already broadly used in clinics for sustained release (e.g. Lupron Depot® in U.S.).

Micro and nanoparticles are spherical polymeric particles with sizes in the range of micrometres and nanometres, respectively. They include micro and nanocapsules, which are reservoir-type systems where the therapeutic agent is present in a cavity surrounded by a polymeric wall; and micro or nanospheres, which are matrix-type systems where the therapeutic agent is dispersed throughout the particle (Alonso, 1996; Couvreur and Puisieux, 1993). Although the *in vitro* release behaviours of these two types are very different, the therapeutic agent is immobilised in the particle, allowing precise control over drug release profile, in both cases.

Most of the work on particles has been produced using synthetic hydrophobic biodegradable or bioerodible polymers, such as poly(ε-caprolactone), the family of poly(lactic acid) (PLA) and poly(lactic glycolic acid) (PLGA) copolymers, and poly(alkyl cyanoacrylate) polymers (PACA). Some microspheres formulations prepared with these polymers are approved for human use by both drug regulatory agencies in the U.S. and the E.U..

Several methods can be used for the preparation of particles, e.g. coacervation, emulsion polymerisation, or spray drying. Selecting the appropriate method depends on the physicochemical properties of the polymer and the drug. In turn, the procedure and the formulation conditions determine the characteristics of the delivery system. The physicochemical properties of many drugs make formulation difficult and a loss of drug stability and/or activity may occur during preparation, storage, and following *in vivo* administration. In vaccines, a loss or reduction in immunogenicity is the main concern.

Therefore, the key points for the successful development of particle formulations are polymer physico-chemical properties, method of particle production, and therapeutic agent stability, which are intrinsically related.

The majority of polymers used to prepare particles are either hydrophilic or hydrophobic macromolecules, that are obtained from natural sources or synthesised chemically. Several criteria must be taken into account when choosing the polymer: safety of the polymer, preparation conditions, drug-polymer compatibility, expected drug release behaviour, and final purpose of formulation (e.g. route of administration). A biodegradable or bioerodible polymer is preferred to avoid surgical removal of delivery vehicle after the drug has been released. Its effects in the body should be minimal. The *in vivo* degradation products of the polymer must be characterised to determine whether they are non-toxic and readily excreted. Natural polymers are attractive because they are supposed to degrade *in vivo* into innocuous amino acids or small peptides. The major drawback of natural materials is their potential antigenicity, this has not been extensively reviewed in the literature.

Thus, more studies have to be carried out to assess the safety of such carriers, if any.

The process of drug entrapment should be compatible with the handling of the drug, despite the difficulty in combining a good entrapment rate without denaturing the drug. The drug must not interact with the polymer and the latter must not alter the pharmacological properties of the drug. The release of the drug from the polymer is controlled by two major mechanisms: diffusion out of the matrix and erosion or degradation of the polymer. The rate of erosion and complete degradation are dependent on the molecular weight, composition, and chemical nature of the polymer. Water-insoluble polymers could require harsh conditions for the entrapment of the drug, but may allow a prolonged release because their degradation or erosion is slower than hydrophilic polymers, due to the complicated hydrolysis.

As mentioned above, combining high rates of loading and entrapment and little or no denaturation of therapeutic agents is complex for all the techniques currently employed to prepare particles. The drug must maintain its structure and its chemical integrity during preparation and during storage to allow delivery of active drug upon administration. In order to achieve this purpose, the formulator should consider the interactions between the drug and the polymer and also between the drug and the polymer degradation products, and finally demonstrate that the drug is not altered.

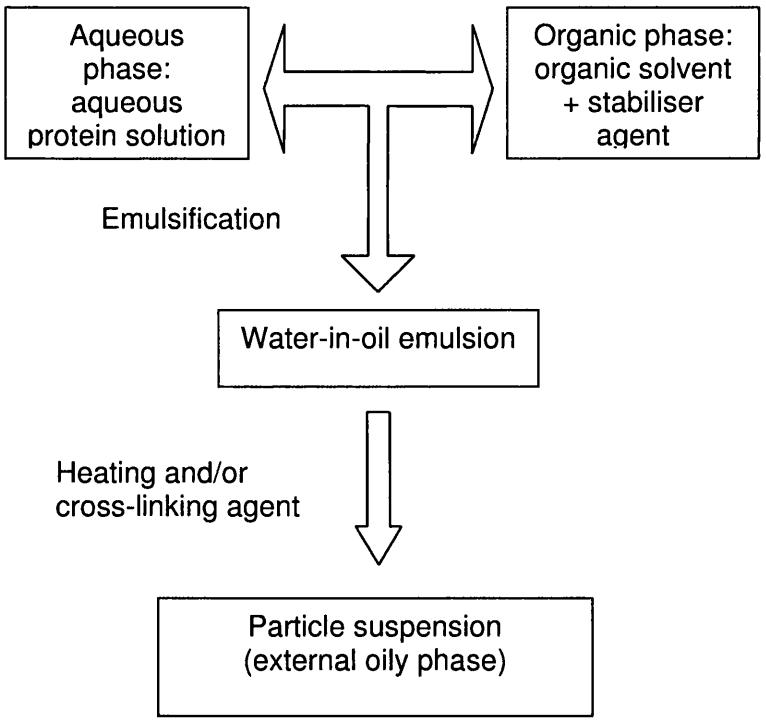
1.3.1.1. Preparation methods for particulate systems

As mentioned, most of the polymers employed to produce particulate systems have been those polymers already investigated for other biomedical applications. Some of them were proteins, e.g. albumin, polysaccharides, e.g. chitosan, or synthetic polymers, e.g. PLA or PLGA. Based on the nature of the polymers used to prepare particles, we could divide current methods of particle preparation into (i) cross-linking of macromolecules reaction during the preparation of the particles, (ii) polymerisation of monomers during the

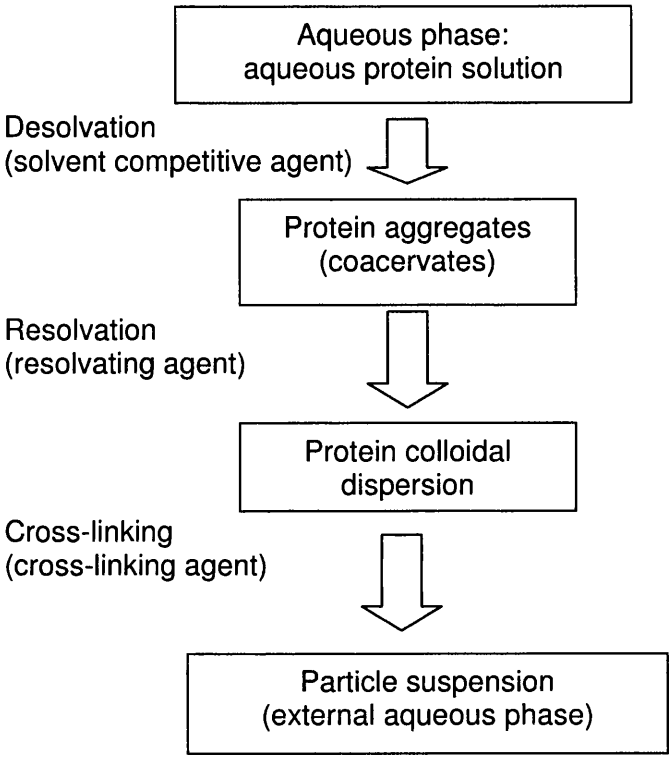
formation of the particles, and (iii) dissolution of hydrophobic polymers in organic solvents and then precipitation under controlled conditions to produce particles (Alonso, 1996).

The following examples illustrate some of the latter techniques to prepare particulate systems incorporating therapeutic agents.

i) Preparation of particles by cross-linking in a water-in-oil emulsion technique:



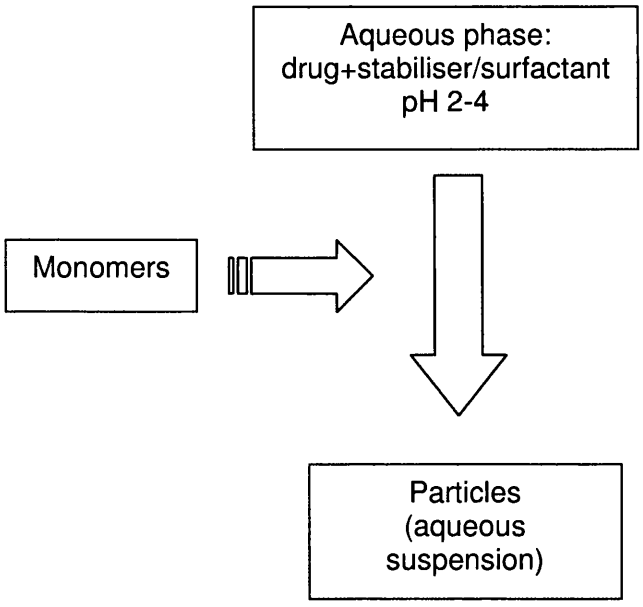
Preparation of particles by desolvation in aqueous medium technique:



Usually, the particles are prepared from amphiphilic macromolecules, polysaccharides, and proteins by these two methods. The materials (e.g. albumin or chitosan) are first induced to aggregate and are later stabilised by chemical cross-linking or heat denaturation. Cross-linking may occur in a water-in-oil emulsion or in aqueous medium. In the first case, emulsification is achieved by homogenisation or by sonication. In the latter, a phase separation process takes place as a consequence of a desolvation of the macromolecule, which changes in the pH or the presence of counterions in the aqueous medium, and thus no chemical reactions are involved. Both the emulsion droplets and the embryo particles produced by phase separation are cross-

linked by a cross-linking agent, which is most frequently glutaraldehyde (Jameela and Jayakrishnan, 1995; Ayhan et al., 2003). The water-in-oil emulsion technique is suitable for the loading of drugs and has been extensively investigated (Jeffery et al., 1993; Yamaguchi et al., 2002). However, its usefulness is limited by the use of sonication or high-speed homogenisation, as well as the use of organic solvents or oils. The phase separation technique avoids the latter, but hydrophilic drug molecules may be partitioned between the particles and the suspending aqueous medium, therefore reducing the loading efficiency. Besides, the main concern using these methods is the presence of the cross-linker, which can react with the drug or confer some degree of toxicity to the formulation.

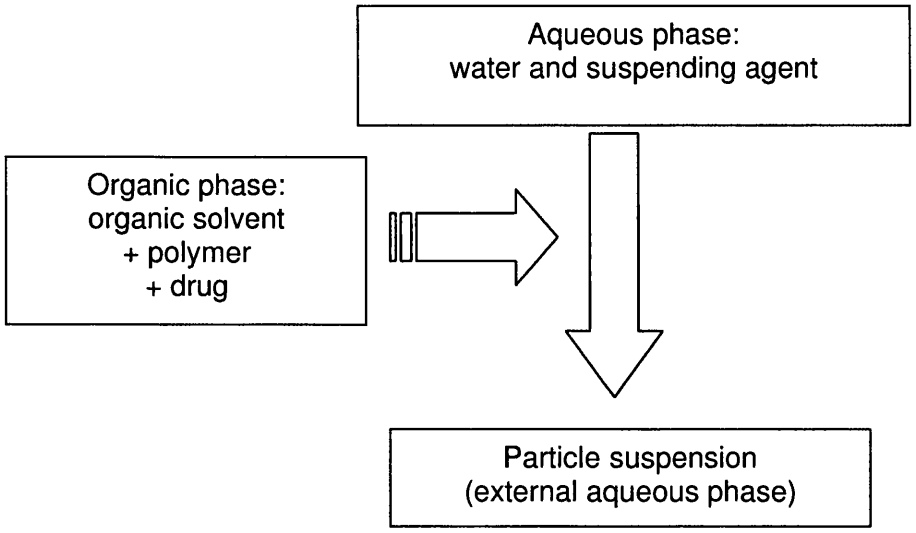
ii) Preparation of particles by the emulsion polymerisation technique:



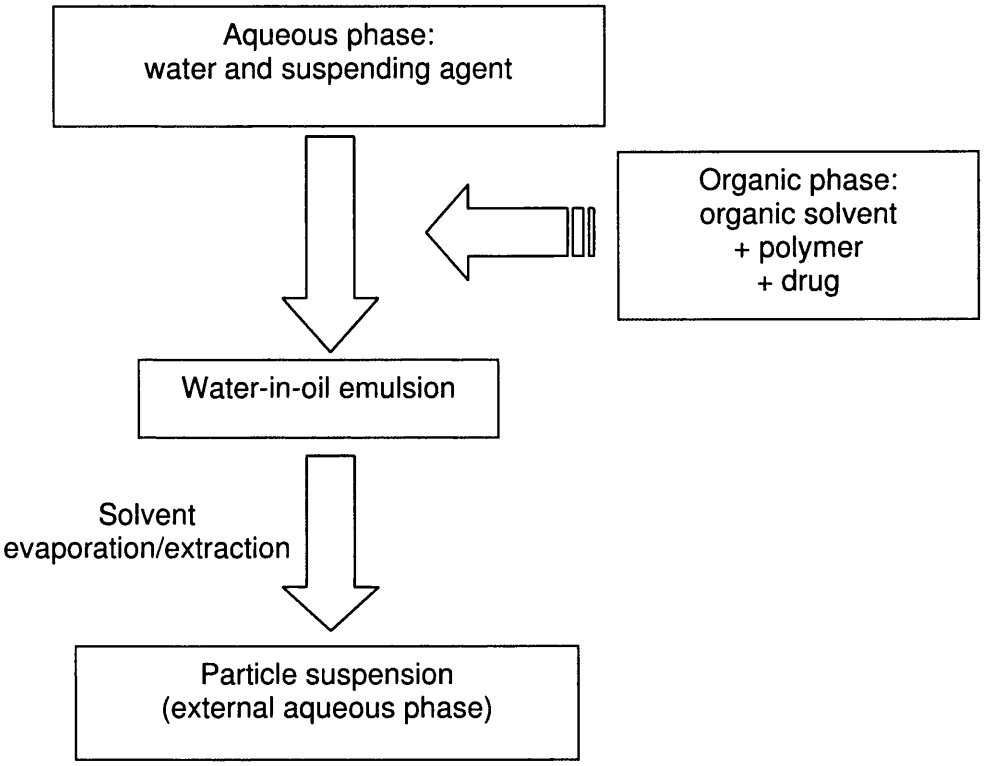
Particles are formed when water-insoluble monomers are emulsified in aqueous acidic phase plus a surfactant or other stabiliser. The polymerisation

rate will depend on the pH of the medium. The longer the alkyl chain of the monomer, the longer will be the polymerisation time. Hydrophilic and lipophilic drugs can be loaded into particles prepared by this method. When incorporating lipophilic drugs, they have to be incorporated together with a monomer solution in a polar solvent or oil plus a lipophilic surfactant. The efficiency of drug entrapment is affected by several factors, such as the drug pKa, the size and the surface charge of particles, or the drug concentration in aqueous medium. The most common polymers used with this technique are the PACA polymers (Peracchia et al., 1997; **Chauvierre et al., 2003**). Again, the main drawbacks are the use of organic solvents as well as the instability of some drugs in the acidic polymerisation medium.

iii) Preparation of particles by solvent displacement technique:



Preparation of particles by solvent evaporation-extraction technique:



The polymer precipitation term designates techniques based on the dissolution of the polymer in a solvent, followed by its dispersion in a continuous phase, in which it is insoluble. Usually, hydrophobic polymers and organic solvents are used for the inner phase. The continuous phase is an aqueous medium that contains a stabiliser. The peculiarity of these techniques is the miscibility between the organic and the aqueous phases. Several techniques are based on polymer precipitation, such as solvent extraction-evaporation or solvent displacement. The first is based on the use of solvents that have limited solubility in water and form emulsions, thus, the polymer precipitation occurs as a consequence of the solvent removal. In the second, a

solvent that is soluble in water is used and the polymer precipitation occurs because of the miscibility of the phases: the solvent diffuses instantaneously to the external aqueous phase. These techniques are useful for both hydrophilic and hydrophobic drugs, depending on the choice of the solvent. A small amount of water or oil can be incorporated into the polymer solution to improve the entrapment of very hydrophilic or very hydrophobic drugs, respectively. The solvent evaporation-extraction method has been extensively used with PLA and PLGA as reviewed in Zhou et al., 2003.

1.3.1.2. Particulate systems for vaccination

Vaccines have a critical element: memory. Commonly, immunisation takes place many years before exposure to the pathogen, so there is a necessity for a long-lived immune response. One approach to achieve this is the use of adjuvants (from the Latin word *adjuvare* = to help). Currently used adjuvants have been in use for 70 years (Hanes et al., 1995), but many of the formulations have never been accepted for routine vaccination, due to their toxicity and side effects. The need for a safe and effective adjuvant has thus become paramount. Safety of adjuvant formulations is the biggest concern; however, an ideal adjuvant preparation should also fulfil the following requirements:

- ✓ To elicit a protective immune response with weak antigens.
- ✓ To lower the dose of antigen required and to reduce the number of injections.
- ✓ To be stable without interaction with the antigen.
- ✓ To be biodegradable and non-immunogenic.

Aluminium-containing compounds were the first adjuvants approved by the Food and Drug Administration in the U.S. However, alum is not able to sufficiently enhance the immune response of poorly immunogenic antigens. Other powerful experimental adjuvants, such as MPL or quilA, are well documented in animal models, but none is approved for human use yet

(O'Hagan and Valiante, 2003). This is due, in part, to a poor understanding of their mechanisms of action.

The broad definition of adjuvants mentioned above encompasses a very wide range of materials, including a number of particulate delivery systems, such as liposomes or microspheres, whose principal mode of action is to deliver antigens to the sites responsible for the induction of immune responses. Since it is accepted in basic immunology that those antigens that do not reach the lymph nodes do not induce immune responses, these delivery systems act as adjuvants by improving antigen access to lymph nodes.

Particulate adjuvants have been shown to act (i) as a vehicle to target antigen to antigen presenting cells (APCs), which are able to take up the delivery system prior to trafficking to the local lymph nodes and (ii) as a controlled release vehicle of vaccines with the aim of reducing the number of doses for primary immunisation or of developing single dose vaccines (Gupta et al., 1998; Morein and Bengtsson, 1999; Wassef et al., 1994).

The APCs (macrophages and dendritic cells) seem to act by internalising the exogenous particulate antigen formulation, after which, the antigen is released into the cytoplasm where it is treated as an endogenous antigen. In the cytoplasm, particles can also act as intracellular reservoirs for sustained release of the therapeutic agent. This ability of intact particles (of appropriate diameter) to be uptaken directly by APCs may provide a route to evade antigen neutralisation by maternal antibodies.

The original mechanism of action attributed to adjuvants was the depot effect (Freund, 1956, as cited in Hanes, 1995), in which adjuvants increase the antigen's biological and immunological half-life at the site of injection, thus allowing longer interaction of the antigen with the immune system prior to clearance. A long time ago, particulate systems were found to provide a long-term depot, an advantage over other adjuvants, which provide a short-term depot only (Preis and Langer, 1979). From this depot, the release of the antigen may be (i) pulsatile release by selecting polymers with different rates

of hydrolysis, or (ii) continuous release over prolonged periods, which may give rise to high levels of antibodies similar to those observed after multiple injections. The promise of the depot may enable the development of single-step vaccination systems in future and this is a unique characteristic of particulate adjuvants.

Particulate adjuvants can also protect antigens form proteolytic destruction in the stomach, allowing the antigen to pass into the intestines intact for presentation to the gut-associated lymphoid system (GALT). Particles were shown to be taken up by the Peyer's patches of the gastrointestinal tract a long time ago (Eldridge et al., 1989). Nevertheless, the amount of opsonisation and thus, stimulation of antibody formation after oral administration of particulate adjuvants is still debated.

For both systemic and parenteral immunisation, the size of the particles and the degree of hydrophobicity of particle surface are important determinants of the initial uptake and cellular trafficking of loaded antigen (Morris et al., 1994).

1.4. Immunogenicity and tolerance

From fundamental immunology, we know that the ingestion of soluble protein antigens culminates in the phenomenon called oral tolerance. Oral tolerance can be defined as a T-cell mediated phenomenon that generates hyporesponsiveness to parenteral immunisation with an antigen, which was first contacted by oral route.

Single feeds of dietary antigens have been shown to inhibit antibody and cell-mediated immune responses, and cytokine production. The latter phenomenon has been well established within a week of feeding and seems to be long-lasting (Thompson and Staines, 1990; Strobel and Ferguson, 1987; Weiner et al., 1994). Strobel and Mowat (1998) highlighted, in their review about oral tolerance to dietary antigens, that there is still debate about the roles of immunological mechanisms implicated in oral tolerance. The

mechanisms seem to include clonal deletion, clonal anergy, and antigen-driven suppression. However, several other mechanisms may also be involved in this physiological phenomenon. In addition, those mechanisms may not be mutually exclusive. Which mechanism predominates in any situation may depend on the dose, the frequency of administration, and the type of antigen.

In the balance between immunity and tolerance, the dose of antigen has been shown to be an important factor, as well as the antigen formulation. Zinkernagel (2000) has proved that high intravenous (i.v.) doses resulted in immune tolerance while low dose i.v. injections or peripheral injections induced immunity. On the other hand, requirements for tolerance induction may be different for different antigens.

Oral tolerance is supposed to play a physiological role to prevent hypersensitivity to food antigens, a very important role since it explains the absence of immune responses to innocuous food derived proteins in the gut. The induction of immune tolerance could be used for the treatment of diseases. Diseases like human autoimmune disorders and food allergic conditions are the main candidates for re-induction of tolerance in the patients. Therapeutic options of immunity suppression by mucosally administered antigens are discussed in Weiner's review (1997).

Nevertheless, it has been observed that some orally administered particulates, as well as other mucosal adjuvanted antigen formulations, are able to induce immune responses without producing tolerance. Hence, secretory immune responses can be elicited at mucosal sites via oral administration of antigens if they are presented in an appropriate formulation. This fact could be due to the extended stimulation with antigen, as explained by the depot theory, or due to attraction of APCs to the site of antigen release, thus due to a more efficient antigen processing (Th. den Boer et al., 2001). Both explanations could be true for particulate system adjuvant action.

Leishman and co-workers (1998) demonstrated that tolerance to ovalbumin antigen might be induced in mice by feeding ovalbumin both after and before

immunisation; however, the scope for tolerance being greater when antigen was fed before priming. They also concluded that delayed-type hypersensitivity (DTH) responses seemed to be more easily established than humoral responses. Other investigators have also determined that cell-mediated immunity and immunoglobulin E (IgE) responses were more easily tolerated and that lower doses of antigen were required. Husby et al. (1994) demonstrated that feeding of antigen resulted in systemic T-cell tolerance, which decreased antigen-specific T-cell proliferation and produced DTH. Immunoglobulin M (IgM) and Immunoglobulin G (IgG) cells were unaffected or increased in orally fed individuals. Tolerance to IgM and IgG responses occurred at high antigen doses.

From results like the ones above, one can conclude that orally administered antigens produce tolerance. In fact, this is a generally accepted premise. Nevertheless, oral protein dietary antigens, in the absence of particulate or other type of adjuvants, have been proved to induce systemic and gut-specific antibody responses in some animals, such as birds or sheeps (Johnston et al., 1998; Klipper et al., 2001).

1.4.1. Zein immunogenicity

As mentioned in the above section, dietary protein antigens are innocuous to human and other animals, but this does not mean that the immune response to them is null. Antibodies to a variety of accepted dietary proteins are likely to be present in the serum of animals. Johnston et al. (1998) demonstrated that antibodies to zeins were present in the serum of ruminating sheep without causing abnormalities or disorders in such animals. Naim and van Oss (1992) also investigated the immunogenicity of some natural and synthetic polymers. They found that soluble zein (dissolved in 0.2% SDS) produced antibodies in rabbits within four weeks of immunisation following subcutaneous (s.c.) and intramuscular injections. The authors concluded that a protein like zein should be in a soluble state to become immunogenic.

On the other hand, it has been shown that natural-based polymers used to produce particulate systems (e.g. albumin microspheres) may cause an unwanted immune response when they are systemically administered (Cleland, 1997). Concurrently, protein-based polymers could also act as an immune adjuvant to the protein that is intended to be delivered.

1.5. Mucosal and parenteral immunisation routes

When formulating any kind of pharmaceutical formulation, the route of administration must be borne in mind from the first steps of preformulation. The most accepted route for human is the oral route; hence, oral medications are the first possibility investigated in the discovery and development of new drug entities and pharmaceutical formulations generally. Nevertheless, the classical route for immunisation is the parenteral route instead of the oral route to date. The potential for oral dosage is limited for therapeutic agents that are poorly absorbed in the gastrointestinal tract and are unstable to various enzymes, in particular to proteolytic enzymes, such as protein antigens. Developing an oral delivery system for therapeutic agents requires understanding of gastrointestinal physiology and biochemistry as well as polymer science, pharmacokinetics and pharmacodynamics.

The parenteral route is composed of primary parenteral routes (intravenous, subcutaneous, and intramuscular) and of various secondary routes such as intraabdominal, intrapleural, or intrauterine. Formulations for all these routes are relatively simple, consisting of a minimal number of excipients. In addition, if the active ingredient is unstable in solution or suspension, the product can be a dry powder. Despite the simplicity of formulating parenteral dosage forms, immunisation via the oral route presents advantages over parenteral vaccination. Oral delivery allows vaccination to patients with limited access to trained healthcare staff in developing countries, as well as improves patient compliance in rich countries. The ability of vaccines to induce protection after one oral immunisation instead of the usual two or three administrations by

injection is a priority of vaccine research, as recognised by the World Health Organisation.

The mucosal routes, such as the oral route, are known to have the potential to elicit local immune responses as a first line of defence against mucosal pathogens. The characteristic of mucosal immunity is secretory immunoglobulin A (sIgA). These immunoglobulins can prevent infection of epithelial host cells and remove antigens that cross epithelial barrier by transporting the antigens across the epithelium. It has also been shown that particles delivered via mucosal routes may be translocated to other compartments and induce a systemic response (Eyles et al., 2001). The Common Mucosal Immune System (CMIS) is long known for providing a pathway where antigens at a single mucosal surface may engender antibody production at remote lymphoid sites (McGhee et al., 1992). Even so, the majority of current vaccines induce systemic immunity by serum immunoglobulin G responses, rather than induction of sIgA at mucosal sites. Hence, oral vaccination is still under preclinical investigation.

Particulate systems, such as liposomes and microspheres, for oral and other mucosal drug/vaccine delivery, are studied due to their potential for protecting drugs and antigens in the gastrointestinal tract. The main drawback, however, is their low absorption efficiencies. Evidence in literature suggests that absorption of particulates takes place at the intestinal lymphatic tissues, particularly Peyer's patches, which are aggregates of lymphoid follicles composing the gut-associated lymphoid tissue (Hillery et al., 1994). However, despite the evidence for particle uptake in the gastrointestinal tract, controversy exists regarding the sites of particle absorption and the efficiency of particle absorption (Chen and Langer, 1998). This could be because of the different animal models used in different studies. Due to the low particle absorption efficiency observed to date, potential applications of particulates in oral delivery will be vaccines, as antigens do not require high concentrations to be effective. In addition, the inherent adjuvanticity of particulate systems explained above may compensate for the low amounts of antigens absorbed.

Several mucosal routes for the administration of therapeutic agents exist, like the buccal, nasal, ophthalmic, rectal, or vaginal. Nasal route has received much attention from the pharmaceutical industry since it provides direct access to the systemic circulation and it can be an alternative to invasive routes. In this thesis, however, the mucosal routes studied were only the oral, rectal, and vaginal routes.

Certain patient populations have difficulty with oral dosage forms and, therefore, rectal drug administration has traditionally been their alternative to oral forms. Rectal dosage forms have been used to treat local diseases and to deliver drugs systemically. However, this route has not received a great deal of attention possibly because it is not a widely accepted route by patients. An advantage of rectal delivery formulations is that the total drug load can be two-three times greater than the possible load in an oral formulation.

Vaginal dosage forms have been used clinically for many years in local therapy. The vaginal absorption capacity suggests that the vagina could provide a potential route for systemic drug delivery. Steroid hormones, which are metabolised extensively when taken orally, have been intravaginally delivered for systemic activity in clinics (e.g. vaginal rings for contraception). Vaginal route is focused on therapeutic agents that are subject to hepatic first-pass metabolism, such as proteins.

1.6. Protein antigens

One objective of this thesis was to deliver a protein as a model antigen *in vivo*. The selected protein was ovalbumin, an inexpensive, biocompatible, non-toxic, and biodegradable protein, derived from chicken eggs, that has been successfully used as an antigen in inducing antibody and cell-mediated immune responses (O'Hagan et al., 1991; O'Hagan et al., 1993; Maloy et al., 1994; Uchida et al., 1994; Puri et al., 2000). In addition, microspheres made of ovalbumin and other albumins have been produced as carrier systems for

other proteins (Ovadia et al., 1982). These microspheres are readily metabolised, especially by proteolytic enzymes.

Ovalbumin protein has been used as a model antigen due to its poor immunogenicity and the fact that it requires adjuvants to increase its immunogenicity, such as particulate systems. However, the use of the whole protein could enhance its antigenicity due to a large number of epitopes.

The main concern when loading proteins, such as ovalbumin, into microspheres as a model drug/antigen is the protein instability in the system. The term stability may have several definitions. Immunologists debate whether the conformational or physical stability of a protein antigen is essential for an immune response. The native antigenic determinants must be preserved, however.

Each protein has different thermodynamic and kinetic stability determined from its amino acid composition and sequence. Hence, each microsphere formulation loading a protein must be optimised individually. Some researchers have studied the properties of ovalbumin and other albumins loaded into particulate systems. Sah (1998) stated that ovalbumin is particularly prone to aggregate at the organic solvent/water interface used to produce PLGA microspheres using the emulsion process. However, aggregated ovalbumin was still trapped inside the microspheres.

Proteins may become inactive by chemical alteration, denaturation, and aggregation. The use of organic solvents or emulsification processes during particle preparation, and/or the freezing and drying during storage are potential causes for inactivation of proteins such as ovalbumin. In addition, during release experiments, proteins like ovalbumin may aggregate due to a difference in moisture, may unfold and adsorb onto polymer surface, or bind to erosion and degradation products. In an acidic microclimate produced by degradation products, proteins may become chemically inactivated (Schwendeman et al., 1996).

Chapter 2.

Characterisation of zein used in our studies

2.1. Introduction

The data published in the literature on the proportions of zeins in grain vary widely. The diversity of the numbers expressed by different authors may be related to the technical differences in the extraction of the various maize protein fractions. As explained before in chapter 1, the conditions under which zein is extracted will have an effect upon its properties. Since the production method of the zein protein used in our studies is propriety, it was considered important to characterise the protein in terms of primary and secondary structures firstly, and to conduct all further studies on the same batch of zein.

According to the literature, zein is a mixture of different peptides of various molecular sizes, solubilities, and charge; and whose defining characteristic is its insolubility in water, which is most probably related to its high proportion of uncharged amino acid residues. The exact and complete structure and amino acid sequence of zein has not been determined. Based on that, we determined about the zein used in our studies:

- ✓ The solubility of zein in various solvent media.
- ✓ The amino acid content and composition.
- ✓ The peptide composition.

To determine the latter, we looked back to the basic physicochemical definitions of each.

Solubility of a solute in a solvent is defined as the solute concentration in a saturated solution that is in equilibrium with the undissolved solute, at a given temperature and pressure. Pharmacopoeias and other chemical and pharmaceutical compendia use the expressions "soluble", "very slightly soluble", and "insoluble" to express solubility of a compound in a particular solvent. Nevertheless, we believed those qualitative terms were not sufficient for the purpose of our studies, and quantitative values would provide us more detailed information. Thus, a colorimetric method, Bicinchoninic acid assay,

was chosen to determine zein solubility and results were expressed as mg protein/ml solvent. Since the main objective of this work was to test zein solubility in order to understand zein precipitation during microsphere formulation and release of entrapped and/or adsorbed therapeutic agent, only solvents and solvent mixtures that had specific relation to our research were selected.

The original definition of zein by Osborne (1908) was a protein that is soluble in all proportions in 70-80% alcohol. Accordingly, in the 1940s, several authors intensively studied the solubility of zein in aqueous alcohols (Evans and Manley, 1941, 1944; Manley and Evans, 1943, Swallen, 1941). These authors established that zein is insoluble in anhydrous alcohols except methanol, and is soluble in the lower aliphatic alcohols only if a certain amount of water is present. In 1927, Dill (as cited in Evans and Manley, 1941) proposed the formation of a protein-alcohol-water complex, which is completely miscible above a certain temperature, but below which the protein has limited solubility. Even though the solubility of zein in aqueous-alcohols has been well known for decades and aqueous-alcohols are the most common solvents for zein, in this research the optimal alcohol concentration to dissolve the zein used in our studies was determined, since zein solubility depends on the method of extraction.

The primary structure of a protein is defined as **amino acid composition and content**. The first indicates which of the twenty possible amino acids are present in the protein and the latter the relative proportions of the amino acids present. Their determination is achieved by hydrolysing the amide bonds of the peptides to obtain a mixture of free amino acids, which is separated, identified, and quantified by an automatic amino acid analyser. Briefly, the instrumental consists of a negatively charged column where amino acids in an acidic solution, thus protonated, are separated by ion-exchange chromatography. Due to the opposite charge and depending on their pI, amino acids are retained in the column. Then pH is gradually increased to provoke the unprotonation of the amino acids, so they are eluted. The first amino acid being eluted will be the most acidic one, aspartic acid (Asp), and consequently

the last one will be the most basic, arginine (Arg). The quantification of each amino acid is completed by a post-column reaction with a coloured compound, the intensity of the resulting colour being proportional to the quantity of the amino acid present.

Analytical gel electrophoresis is routinely used for determining the **peptide composition** of proteins and for the estimation of the molecular masses. For this reason, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was the technique chosen to investigate the nature of zein peptides in the commercially available zein used in our experiments. The general principle and the various methods of this technique are explained in Chapter 4. For the current experiment, SDS-PAGE was carried out under denaturing and reducing conditions to ensure the complete dissociation of the protein into its polypeptides and the unfolding of the polypeptide backbones prior to the loading of protein samples on the gel. Likewise, the denatured polypeptides bind to the sodium dodecyl sulphate (SDS) and become negatively charged. The SDS-polypeptide complexes then migrate through polyacrylamide gels under an applied electric field and separate by molecular weight.

2.1.2. Bicinchoninic acid assay

Bicinchoninic acid (BCA) assay is used to measure protein concentrations. It relies on the formation of a complex, under alkaline conditions, between the peptide bonds, present in the protein sample, and Cu^{2+}, present in BCA reagents, followed by reduction of Cu^{2+} to Cu^{1+}. BCA reagents consist of a solution of copper (II) sulphate and the sodium salt of the bicinchoninic acid, which is a specific chromophore for Cu^{1+}, thus reacts with the Cu^{1+} to give a coloured complex. The reduction of copper ions is known to be produced by some amino acids present and by peptide bonds (Wiechelman et al., 1988). Therefore, the amount of reduction is proportional to the amount of protein present.

BCA method was chosen because it is applicable to a broad range of protein concentrations, is a one-step determination, and is tolerant of the presence of non-ionic detergents and simple buffer salts in the assay medium (Smith et al., 1985). However, many compounds can reduce Cu^{2+} to Cu^{1+} and consequently a few substances do interfere with the reaction. The interference by these substances may be eliminated by precipitating the protein using trichloroacetic acid and deoxycholate prior to reaction with bicinchoninic acid (TCA-BCA assay). The other option is to minimise the effect of the interfering materials by dilution (Brown et al., 1989).

In this study, some of the solvents and/or mixtures of solvents used did interfere with the BCA assay. The supplier´s instructions (Sigma, 2002) were followed to decide the maximum amount of material that could be present in the assay sample, which would not cause interference with the BCA reaction. The maximum amounts are shown in table 2.1.

The TCA-BCA assay to eliminate those incompatible substances could not be used for this experiment due to the extremely low wettability of zein after its precipitation, which impeded its redissolution. Subsequently, the effect of interfering materials was minimized by dilution.

It was difficult to prepare reference solutions of zein due to the insolubility of zein in water and in some of the other solvents being tested. For this reason, bovine serum albumin (BSA) was employed as a reference standard in all the determinations. It has been shown by Smith et al. (1985) that BSA behaves in a sufficiently nominal manner in the BCA assay so that the concentration of the unknown protein sample is not over- or underestimated.

Substances incompatible with BCA assay	**Maximum concentration of incompatible substances at which there is no interference**
Sodium acetate	200mM
Glycine	1.0M
Hydrochloric acid	100mM
Sodium hydroxide	100mM
Tween 20	5% v/v
Tween 80	5% v/v
Ethanol	10% v/v
Methanol	10% v/v
Propan-2-ol	10% v/v
DMSO	10% v/v
Sodium azide	0.2% w/v

Table 2.1. List of substances interfering with BCA assay (From Sigma, 2002).

2.2. Experimental

2.2.1. Chemicals

Zein, bicinchoninic acid test kit, sodium dodecyl sulphate, dimethyl sulphoxide (DMSO), methanol, propan-2-ol, butan-1-ol, octan-1-ol, Tween 20, Tween 80, 2-mercaptoethanol, and phosphate-buffered saline tablets were purchased from Sigma, UK. Sodium hydroxide, sodium acetate, glacial acetic acid, sodium chloride, potassium chloride, glycine, and hydrochloride acid were obtained from BDH Laboratories Supplies, UK. Coomassie Blue G-250 stain, premixed electrophoresis buffer, Laemmli sample buffer, Tris-HCl ready gels, and protein standards were obtained from Bio Rad, UK. Other chemicals were of reagent grade and were used as received. Double-distilled water was used throughout.

2.2.2. Methods

2.2.2.1. Determination of zein solubility

As mentioned in section 2.1., solubility of zein in the media used in the microsphere preparation and characterisation was determined, such as non-ionic surfactant solutions, sodium hydroxide solutions, and buffers of different pH. According to the literature, zein is totally insoluble in both water and anhydrous alcohols, but is soluble in aqueous alcohols. For that reason, zein solubility in various alcohol-water mixtures was measured to find the optimum solubilities. In addition, a few other solvents as dimethyl sulphoxide, water, and absolute alcohols were tested in order to understand zein behaviour in those media, which is related to its precipitation into zein particles (as it will be discussed in chapter 3). The different media tested are listed in table 2.2.

Absolute solvents		
	Water	
	DMSO	
	Methanol	
	Ethanol	
	Propan-2-ol	
	Butan-1-ol	
	Octan-1-ol	
Alcohol-water mixtures	Alcohol	Concentrations
	Methanol	50, 60, 70, 80 and 90% v/v
	Ethanol	50, 60, 70, 80 and 90% v/v
	Propan-2-ol	60, 70, 80 and 90% v/v
Aqueous solutions	Material	Concentrations
	Tween 20	1, 0.1 and 0.001% v/v
	Tween 80	1, 0.1 and 0.001% v/v
	Sodium hydroxide	5, 1 and 0.5% w/v
Buffers	Name	pH
	Chloride	2
	Acetate	5
	Phosphate saline	7.4
	Glycine	11.3

Table 2.2. List of solvents, mixtures of solvents, solutions and buffers tested for zein solubility.

All aqueous solutions were prepared using distilled water. The conventional buffers were prepared according to British Pharmacopoeia specifications (BP 2001). Their ionic strengths were not determined, although it is known that solubility of zein may be influenced by the nature and concentration of ions in the medium.

In accordance with the solubility definition mentioned in section 2.1., to calculate zein solubility, an excess of zein (1 g ± 0.05) was weighed into universal bottles (n=4), to which 20 ml of each media was added in order to produce saturated solutions. The universal bottles were placed in a water bath at 25 ºC overnight with constant shaking in order to equilibrate dissolved and undissolved protein. Due to practical reasons, all mixtures were considered to be equilibrated after 15 h of shaking. The real equilibration time was not determined due to lack of time. The next day, the undissolved zein was removed by filtration using a 0.2 µm filter paper. Solubility of zein in each media was determined by measuring zein concentration (mg/ml) in the filtrates using BCA assay.

If an interfering substance was known to be present in the filtrate, the latter was diluted with distilled water until the maximum concentration at which there is no interference was reached, as per table 2.1.

BSA standard solutions of different concentrations, all within the linear concentration range of the assay (0.2 to 1 mg/ml), were prepared starting with a 1 mg/ml stock solution of BSA in water containing 0.1% w/v of sodium azide as a preservative. The serial dilutions were prepared using the same medium as the zein test sample.

The BCA assay was performed in 96-well flat-bottomed plates. Standard curves were produced for each 96-well plate, in which zein test samples (n=6), blanks (n=10), and BSA protein standards (n=10) were always present. One part of samples (25 µl) was mixed with eight parts of BCA Working Reagent (200 µl). The 96-well plates were covered and incubated at 37 ºC for 30 min.

After incubation, the colour formation was measured by reading the absorbance at 560 nm using a multiplate spectrophotometer (Opsys MR, Dynex Technologies). Zein concentration was determined from the standard curve of net absorbance *versus* known BSA protein concentration.

Outliers results from systematic errors were rejected to achieve a standard error of the mean ≤0.1. Student t tests were conducted when two values had to be compared, in order to reveal significant differences, if any.

Due to the peculiar solubility properties of zein, the latter precipitated out when the protein sample was diluted with water for the alcohol-water mixtures and for DMSO; and these filtrates changed from solutions to fine suspensions. For this reason, the final protein concentration values obtained for these mixtures could not be used as absolute numbers, and were only treated for comparison among the same group of media. To assess the approximate amount of precipitation that occurred when diluting zein filtrates, clear solutions of known concentrations of zein (1 mg/ml) were prepared with 90% v/v methanol, 70% v/v ethanol and 70% v/v propan-2-ol. These solutions were diluted up to 5% v/v alcohols with distilled water and the resulting suspensions of zein assayed as described above. The results provided an evaluation of the underestimation of zein concentrations.

Zein concentration could also be underestimated due to protein adsorption on glass vials while equilibrating solutions. A control using a solution of zein of known concentration in a 0.1M sodium hydroxide aqueous solution was carried out. The latter solution was treated as a test sample and final determination of zein concentration was performed as detailed above.

2.2.2.2. Determination of zein amino acid composition and content

The nature and relative proportions of amino acids in the zein used in this project were determined using an automatic amino acid analyser (Biochrom 20, Amersham-Pharmacia).

A small amount of zein (unknown concentration) was dissolved in a 0.1M sodium hydroxide aqueous solution and was hydrolysed to the constituent amino acids by heating the protein solution in 6M hydrochloric acid for 48h at 110 ºC *in vacuo*. The amino acids present in the protein hydrosylate, which were positively charged, were separated by ion-exchange chromatography on a negatively charged sulphonated polystyrene column. The amino acids were identified and then quantified by a post-column reaction to a coloured compound, using ninhydrin as the colour reagent.

During the protein hydrolysis, asparagine (Asn) and glutamine (Gln) were converted to their corresponding acids, aspartic acid (Asp) and glutamic acid (Glu). Therefore, those amino acids were quantified together with their respective acids. Tryptophan, however, was degraded during hydrolysis and could not be quantified.

2.2.2.3. Determination of zein peptide composition

As zein is an oligomeric protein composed of a number of polypeptides, sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed to determine its polypeptide composition and the corresponding polypeptide molecular masses.

Zein protein samples were prepared by dissolving zein in 70% v/v aqueous ethanol at increasing concentrations (1-10 mg/ml). In addition, zein solutions

in 0.1M sodium hydroxide (2.5 and 10 mg/ml) were used in order to observe the effect of solvent, if any.

Protein samples were diluted in a Laemmli sample buffer containing 2-mercaptoethanol 5% v/v and the mixture was heated for 5 min at 95 ºC to achieve the polypeptide denaturation mentioned in section 2.1. Samples were then loaded onto a Tris-HCl gel with a linear acrylamide concentration gradient of 10-20%, containing no SDS, with an effective range of separation of 10-100 kDa (BioRad, 2001). The samples were then subjected to electrophoresis at 200 V and 100 mA in a 25mM Tris/192mM glycine/0.1% w/v SDS pH 8.3 running buffer. Gels were stained with Coomassie Blue G-250 0.1% v/v solution and destained using purified water overnight. Molecular masses of separated zein polypeptides were determined by comparing their electrophoretic mobilities to those of marker proteins of known molecular weights (from 10 to 50 kD).

2.3. Results and Discussion

2.3.1. Solubility of zein

2.3.1.1. Solubility of zein in water and in DMSO

The solubility of zein was determined in water, as solubility in water is a key functional property for proteins. Zein solubility in water was found to be almost negligible (0.620 mg/ml ± 0.002). Such a poor solubility in water is well known and has been attributed to the high concentration of uncharged amino acids residues in zein. Water is an extremely polar solvent and the old principle "like dissolves like" predicts zein insolubility in water. As the attractive forces between polar molecules are strong, affinity of water molecules for its own kind is much greater than its affinity for zein molecules. Zein is therefore excluded from water environments.

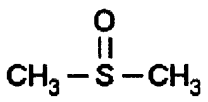
To further investigate the effect of solvent polarity on zein solubility, another polar solvent, DMSO, was tested. It is an organic liquid and is known to be a powerful solvent, with good solvating properties. DMSO molecule comprises a polarised sulphur-oxygen bond and two non-polarised methyl groups, as shown in figure 2.1.

Zein was found to be soluble in DMSO, giving a solubility of 18.688 mg/ml ± 0.080. As mentioned in the section 2.2.2.1., this value cannot be considered an absolute value, due to the effect of sample dilution on zein. Zein filtrates in DMSO were strongly yellow solutions, indicating the presence of a high concentration of zein, which is a yellow-coloured protein. When the filtrates were diluted in water, zein precipitated out of solution and a fine white suspension of zein in a water/DMSO mixture was obtained. Thus, the given result is evidently an underestimation of zein solubility in DMSO. Nevertheless, the higher solubility of zein in DMSO with regard to water is

unquestionable. Then, the cited principle "like dissolves like" becomes non-applicable to zein.

The high solubility of zein in DMSO may be due to a number of factors. Firstly, the dielectric constant of DMSO (ε=46.6) is lower than that of water (ε=80.1). This may be the reason for a lower interfacial tension between zein and DMSO (γ=0.01) compared to water (γ=1.7) (van Oss et al., 1986). As a result, the wettability (a pre-requisite for dissolution) of zein in DMSO might be greater than in water. Secondly, DMSO contains an electronegative oxygen atom that could be used for hydrogen bonding. However, DMSO is an aprotic solvent and the forces of attraction among its molecules do not arise from hydrogen bonds but from weaker van der Waals interactions, making the affinity of DMSO molecules for its own kind less strong than water-water molecules interaction. Thus, the process of zein dissolution in DMSO is a more energetically favourable than it is in water. Lastly, it can be hypothesised that the distribution of DMSO molecules in the liquid might match better with the rigid zein molecule. Consequently, the possibility of zein-DMSO interactions may be greater than zein-water interactions. Hydrophobic interactions between the non polarised methyl groups of DMSO and the nonpolar amino acid residues of zein may also be possible and may then help to the dissolution of zein in this solvent.

Figure 2.1. DMSO molecule



2.3.1.2. Solubility of zein in alcohols

The solubility profiles of zein found for methanol, ethanol and propan-2-ol/water mixtures are similar in contour, as shown in figures 2.2, 2.3., and 2.4. Solubilities of zein are low at 50 and 60% v/v alcohol concentrations; an increase in alcohol concentration results in a dramatic increase in zein solubility up to a maximum, after which further increases in alcohol concentrations result in a decrease in solubility. Anhydrous ethanol and propan-2-ol are very poor solvents for zein in contrast to anhydrous methanol, which does dissolve zein to a certain extent.

Solubilities of zein in 100% propan-2-ol, butan-1-ol and octan-1-ol were negligible. This insolubility emphasises the idea that the classical principle "like dissolves like" is not applicable to zein. These alcohols are nonpolar, their interactions with zein may then be limited to the hydrophobic groups close to the surface of zein molecules, and these weak interactions seem to be insufficient to dissolve zein.

The solubility values shown in figures 2.2. to 2.4. are underestimates due to the interference of alcohols with the BCA assay reaction. When zein filtrates were diluted with water, it was observed that precipitation of zein occurred to a certain extent. Approximate percentages of underestimation for methanol, ethanol, and propan-2-ol/water mixtures, calculated as explained in section 2.2.2.1. are listed in table 2.2.

The results obtained also agree with those of Augustine and Baianu (1987), although some differences can be found on the optimal alcohol concentrations to dissolve zein. This can be explained by the fact that the conditions under which zein is extracted from corn has an effect on its composition, in which case, different batches of zein will be composed of different polypeptides and have different solubilities. The formation of a miscible protein-alcohol-water complex proposed by Dill (1927, as cited in Evans and Manley, 1941), mentioned in section 2.1., could also be proposed for our data, even if the

effect of the temperature on the dissolution has not been studied, but the effect of the alcohol concentration. As for the latter author, in our experiments, it is observed that, at a certain alcohol-water proportion, zein is completely soluble in the mixture; as this proportion is altered, zein aggregates and falls out of solution.

The need for water in alcohols for zein to dissolve is not clear. Danzer et al. (1975) studied the relationship between the dielectric constants of alcohol-water mixtures, as well as other solvents or solutions, such as DMSO or sodium hydroxide solutions, and the α-helical content of zein dissolved in these media. No clear-cut relationship between the two could be found. The α-helical content appeared to remain constant, however some conformational changes seemed to take place when the amount of water was changed. These authors concluded that zein still shows the hydrodynamic and the conformational properties of conventional globular proteins when placed in non aqueous solvents. Later studies are in conflict with these assumptions. Tatham et al. (1993) indicated that aqueous alcohols enhance α-helical content. These authors hypothesised that alcohol-water mixtures may approximate to the environment of α-zeins in protein bodies, such as low water availability and low dielectric constant. Matsumoto et al. (1995, as cited in Dickey et al., 2001) proposed that ethanol molecules are hydrogen bonded forming polymers. These polymers can stack in planar arrays if the ethyl groups are directed to one side of the polymer, and with the ethyl group sides of adjacent polymers facing each other. Water molecules can then fill around ethanol polymer layers and between hydroxyl faces of the ethanol polymers. Dickey et al. (2001) indicated that the range of ethanol weight fractions determined to fit the planar ethanol polymer solution model of Matsumoto et al. (1995) bounds the weight fraction range of zein solubility. Then, ethanol polymer in planar arrays would be conformable to the elongated zein molecule allowing it to dissolve. When the water fraction exceeds the amount needed to fill the space between layers of ethanol polymers around the zein solute, insolubility of zein occurs. The excess of water destabilizes the hydrogen bonds, which hold the ethanol polymers together. Whether the disintegration

of ethanol polymers by water is the cause of zein insolubility is not known. Conio et al. (1970) suggested that proteins such as zein that require ethanol-water mixtures for dissolution may require stabilization of their α-helix by hydrogen bonds. Precipitation would then occur when the water-ethanol structure did not supply sufficient hydrogen bonds to stabilize the helical α-zein.

Mixed solvents are commonly used in pharmacy when drug solubility in one solvent is limited, as for zein. The addition of another component complicates any system and explanations of the solubility patterns are not easy. However, it is assumed that the solute dissolves in "pockets" of the cosolvent and that the affinity of the cosolvent for the solute is very important. Cosolvent effects for proteins may be analysed from a thermodynamic point of view, due to the cosolvent should stabilise the protein molecule and increase the free energy of the system (Florence and Attwood, 1998).

To explain our solubility results in alcohol-water mixed solvents, different factors may play a role. A crucial one might be the idea of adequate distribution of zein rigid molecule among solvent molecules, according to Dickey and co-workers (2001). In the process of dissolution, the solute molecule is relocated from an environment where it is surrounded by other solute molecules, into cavities in a liquid where solvent molecules surround it. In our case, when the alcohol-water liquid creates an adequate cavity for zein among their molecules, this will dissolve in the medium. Moreover, during the process, intermolecular attractions among both solute molecules and solvent molecules vary, being substituted to a certain extent by forces of attraction between solute and solvent molecules. The molecules of the lower aliphatic alcohols interact with their own kind of molecules and with those of water via hydrogen bonds. The forces of attraction between water and alcohol molecules are too strong to be separated by the insertion of a nonpolar solute like zein and the process is not energetically favored. It is therefore reasonable to think that the insertion of zein molecules among alcohol and water molecules is not ruled by strong forces of attraction but by having an adequate distribution for zein molecules. Thus, only a particular alcohol

concentration range will allow a water-alcohol liquid structure that favours the insertion of zein molecules. In addition, to dissolve zein molecules (i.e. create a cavity for zein molecules), water molecules should ideally organize around zein molecules in an ordered manner, resulting in a decrease of the entropy of the system. Instead, when alcohol is present, the entropy spontaneously increases, making the liquid structure more energetically favorable to insertion and thus dissolution of zein molecules.

Methanol itself is capable of dissolving zein to a certain extent. A hypothesis is the spatial distribution of the methanol molecules matching the elongated zein molecule distribution. As for DMSO, hydrophobic interactions between the non polarized methyl groups and the high number of nonpolar amino acid residues of zein may play a role stabilizing the protein in the liquid. The longer chain of butan-1-ol and octan-1-ol do not allow zein dissolution, and because their immiscibility in water they cannot be considered to dissolve zein.

In addition, both hydrophobic interactions between alkyl chains of alcohols and nonpolar zein amino acid residues (such as leucine), and hydrogen bonds between polar zein residues (such as glutamic acid) and hydroxyl groups of water and alcohols, are expected to play a role in stabilising zein molecules among alcohol and water molecules.

2.3.1.3. Influence of solvent pH on zein solubility

Zein was poorly soluble (solubility <1.5 mg/ml at all buffers) in aqueous buffers, as shown in figure 2.5. This reflects the poor solubility of the hydrophobic protein in water, as discussed earlier. Solubility was almost doubled when the solvent was highly alkaline (pH 11.3). This indicates the presence of some glutamic and aspartic acid residues in zein which can ionize in alkaline medium and increase the solubility of the protein to a certain extent. Such increase is not sufficient to consider zein soluble in the glycine buffer, the amide form of glutamic and aspartic acid residues, which do no ionize in alkaline media, may predominate, hence decreasing the number of ions able

to enhance zein solubility. On the other hand, absence of any significant increase of zein solubility in acidic media (pH 2 and 5) compared to neutral pH indicates a lack of basic amino acid residues in zein. Overall, the low solubility of zein at all pH buffers tested correlates with the fact that zein is deficient in basic and acidic amino acid residues.

The increase in zein solubility found above pH 11 agrees with the literature. Augustine and Baianu (1987) showed that carbonyl regions of zein in ^{13}C NMR spectra showed chemical shift differences for zein in pH 11.5 solutions with respect to other solvents such as aqueous ethanol or propan-2-ol. This shift was probably caused by the pH effect upon carboxyl groups, which became ionised. The carboxylate ions therefore are likely to increase zein solubility in aqueous media.

As mentioned in section 2.2.2.1., solubility is affected by the ionic concentration of buffers. In this experiment, the influence of buffer ionic strength was considered. It is expected that the consideration would have altered solubility values to a certain extent.

2.3.1.4. Solubility of zein in the presence of non-ionic surfactants

The solubility of zein in the aqueous solutions of Tween 20 and 80 was found to be very low, as shown in figure 2.6.; this can again be attributed to the hydrophobicity and poor wettability of the protein. The highest solubility was only slightly above 1 mg/ml when the polysorbates were present at 1% v/v concentration. Any zein solubility result in the polysorbates aqueous solution tested did not differ significantly.

Critical micellar concentration (CMC) of Tween 20 and 80 is 0.06 g/l and 0.014 g/l respectively, therefore between the first two concentrations used in the experiment. Results showed no significant increase in zein solubility above the

CMC, which indicates that zein was not solubilised in micelles. However, increasing surfactant concentration in the aqueous medium resulted in increased zein solubility for both polysorbates. This can be attributed, not to the solubilisation within micelles, but to a reduction in the interfacial tension between zein and water and thereby an increased wetting of zein which allows some zein dissolution.

When dry protein powder is placed in an aqueous medium, wetting of the surface is required before dissolution can occur. If the protein refolds to its tertiary configuration upon hydration, the more hydrophilic residues will be oriented outward, allowing electrostatic interactions and hydrogen bonding with water molecules. The freeze-drying process allows the conservation of the same peptide folding as that of the dissolved state. Wetting of the surface for a freeze-dried protein is then almost instantaneous. Since wetting for zein does not seem to occur, it can be safely assumed that the commercial zein used in this study had not been freeze-dried from a sole aqueous solution. In addition, only a small number of hydrophilic amino acid residues would be on the surface of zein when it assumes its tertiary configuration, due to zein's hydrophobicity. Thus, it seems that the process of wetting zein is not favored. As a result, by reducing interfacial tension, non-ionic surfactants increase zein solubility in water, but this effect is not sufficient to dissolve zein to a significant extent.

Non wetting of zein has been demonstrated by van Oss et al. (1986) who measured contact angles of zein after what he called "hydration" (3.5 h immersing in water, followed by 15 min air-drying) and on dry protein. Both contact angles barely differed, indicating that zein did not wet.

2.3.1.5. Solubility of zein in sodium hydroxide solutions

An important amount of zein dissolves in aqueous solutions of sodium hydroxide, as figure 2.7. shows. Increasing the concentration of sodium hydroxide from 0.5 % to 1 % w/v increased the solubility of zein; however, further increases in concentration up to 5 % w/v did not cause any significant change in solubility. Solubility increases as pH of medium moves away from pI of proteins. Because of the great heterogeneity of zein, its pI is established between 5 and 9 (Esen, 1987). As a result, only extremes of pH would enhance zein solubility in aqueous solutions and this is the case with sodium hydroxide aqueous solutions. As explained earlier in section 2.2.1.3., in the extremely alkaline pH of sodium hydroxide solutions, acidic amino acid residues of zein, such as glutamic acid, aspartic acid and other carbonyl groups on zein surface, such as the ones of proline residues, would be ionised to give carboxylate ions. The ionised groups increase aqueous solubility of zein via electrostatic interactions with water molecules.

It is also possible that in the highly alkaline media, zein is hydrolysed ultimately to create individual amino acids, which would be soluble in aqueous solutions. The possibility of zein hydrolysis has been investigated and is discussed in section 2.2.3.

Alcohol concentration	**% by which zein is underestimated**
Methanol 90% v/v	36.2%
Ethanol 70% v/v	21.5%
Propan-2-ol 70% v/v	27.3%

Table 2.2. Approximate percentages by which zein solubility is underestimated when measured by BCA assay. Zein underestimation due to protein adsorption on glass vials during experimental procedures was measured to be 4% approximately.

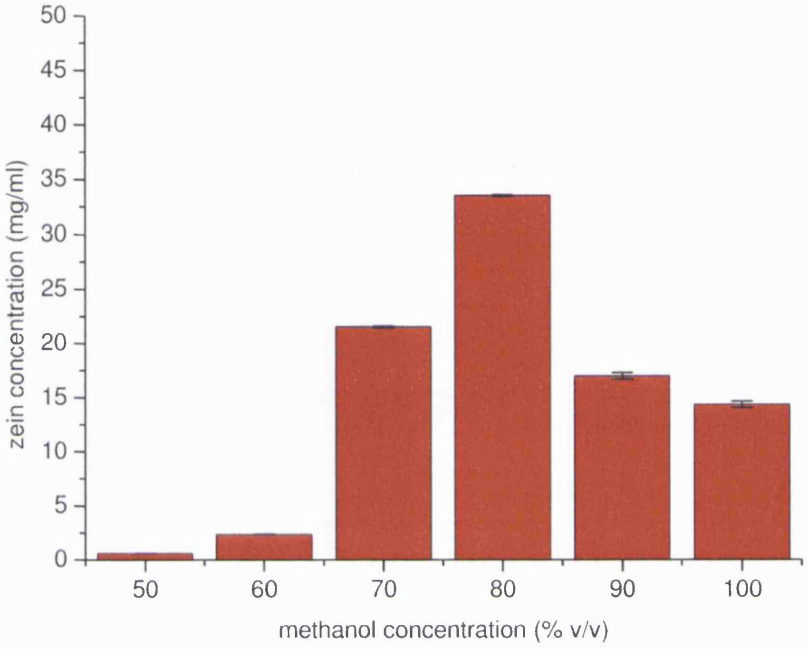


Figure 2.2. Zein solubilities (mg/ml) for 100% methanol and aqueous methanol (% v/v). The limit of solubility for each alcohol concentration was:

- 0.609 mg/ml ± 0.0053 at methanol 50% v/v.
- 2.383 mg/ml ± 0.010 at methanol 60% v/v.
- 21.543 mg/ml ± 0.063 at methanol 70% v/v.
- 33.521 mg/ml ± 0.065 at methanol 80% v/v.
- 17.013 mg/ml ± 0.289 at methanol 90% v/v.
- 14.368 mg/ml ± 0.279 at methanol 100% v/v.

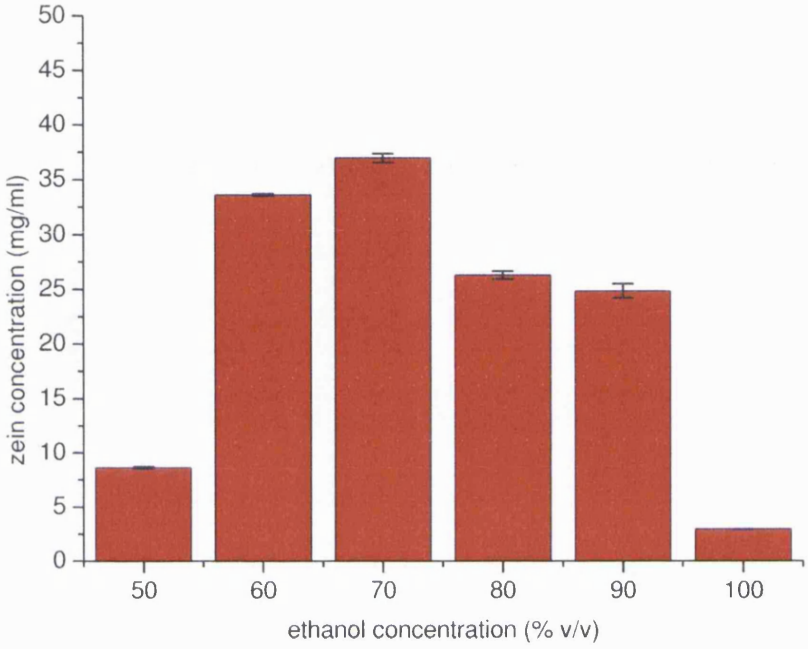


Figure 2.3. Zein solubilities (mg/ml) in 100% ethanol and aqueous ethanol (% v/v). The limit of solubility for each alcohol concentration was:

- 8.655 mg/ml ± 0.099 at ethanol 50% v/v.
- 33.618 mg/ml ± 0.097 at ethanol 60% v/v.
- 36.976 mg/ml ± 0.398 at ethanol 70% v/v.
- 26.306 mg/ml ± 0.364 at ethanol 80 % v/v.
- 24.843 mg/ml ± 0.631 at ethanol 90 % v/v.
- 2.954 mg/ml ± 0.010 at ethanol 100% v/v.

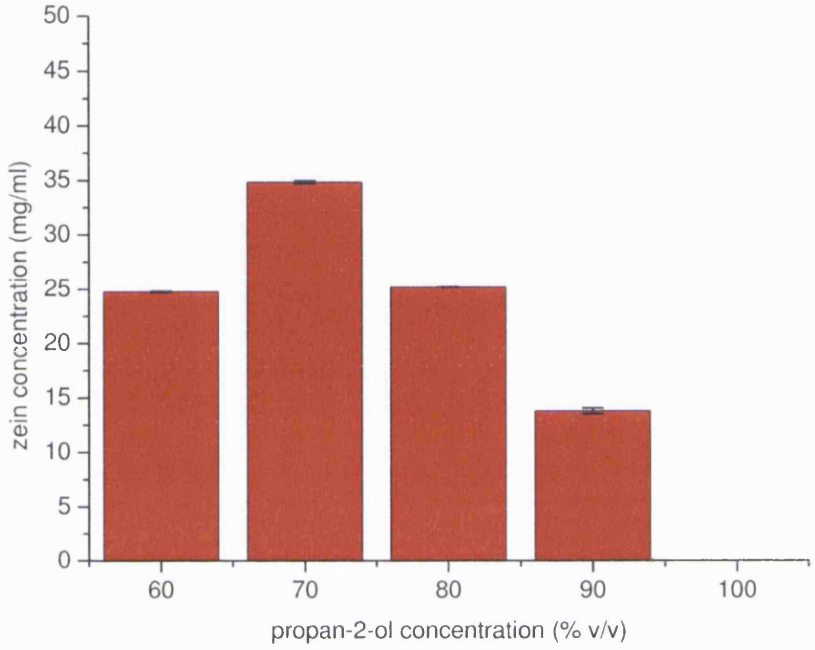


Figure 2.4. Zein solubilities (mg/ml) in 100% propan-2-ol and aqueous propan-2-ol (% v/v). The limit of solubility for each alcohol concentration was:

- 24.768 mg/ml ± 0.074 at propan-2-ol 60% v/v.
- 34.846 mg/ml ± 0.127 at propan-2-ol 70% v/v.
- 25.179 mg/ml ± 0.026 at propan-2-ol 80% v/v.
- 3.786 mg/ml ± 0.265 at propan-2-ol 90% v/v.

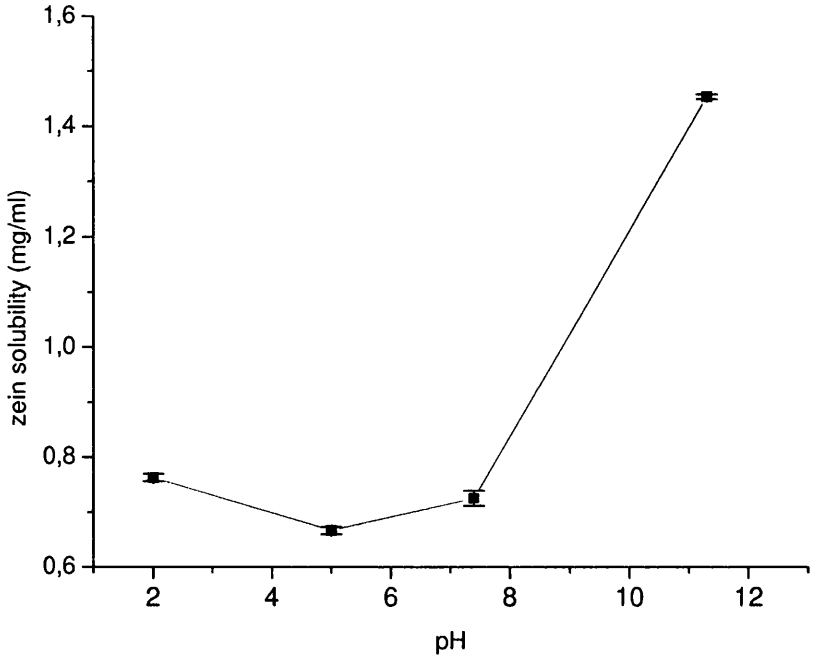


Figure 2.5. Zein solubilities (mg /ml) in aqueous buffers of different pHs. The limits of solubility were:

- 0.763 mg/ml ± 0.006 at chloride buffer pH 2.
- 0.666 mg/ml ± 0.006 at acetate buffer pH 5.
- 0.725 mg/ml ± 0.013 at phosphate saline buffer pH 7.4.
- 1.453 mg/ml ± 0.004 at glycine buffer pH 11.3.

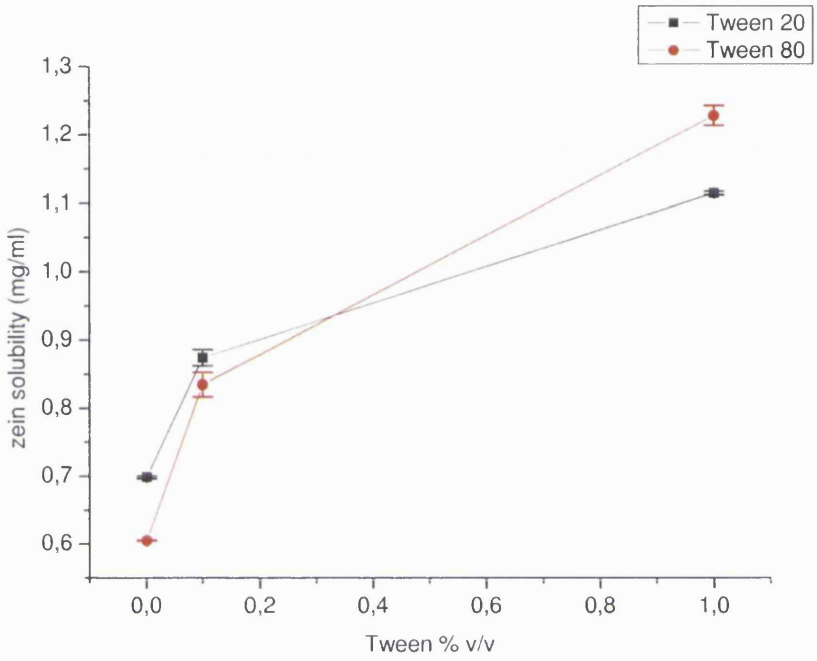


Figure 2.6. Zein solubilities (mg/ml) in Tween 20 and 80 aqueous solutions (% v/v). The limits of solubility for Tween 20 aqueous solutions were:

- 0.698 mg/ml ± 0.001 at Tween 20 10^{-3}% w/v.
- 0.874 mg/ml ± 0.005 at Tween 20 0.1% w/v.
- 1.114 mg/ml ± 0.002 at Tween 20 1% w/v.

The limits of solubility for Tween 80 aqueous solutions were:

- 0.605 mg/ml ± 0.000 at Tween 80 10^{-3}% w/v.
- 0.834 mg/ml ± 0.018 at Tween 80 0.1% w/v.
- 1.228 mg/ml ± 0.014 at Tween 80 1% w/v.

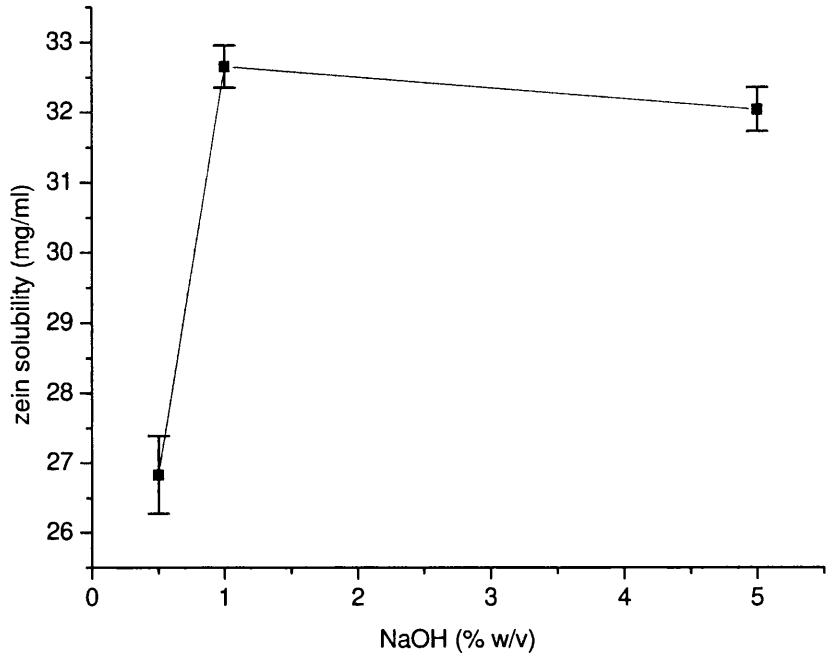


Figure 2.7. Zein solubilities (mg/ml) in aqueous sodium hydroxide solutions (% w/v). The limits of solubility were:

- 26.831 mg/ml ± 0.558 at NaOH 0.5% w/v.
- 32.657 mg/ml ± 0.301 at NaOH 1% w/v.
- 32.044 mg/ml ± 0.314 at NaOH 5% w/v.

2.3.2. Amino acid composition and content of zein

The amino acid composition and content of the commercial zein used to formulate microspheres is listed in table 2.3. Zein was found to have high proportions of neutral amino acid residues and a deficiency of basic and acidic ones. This fully agrees with findings by other authors as reviewed by Shukla (2001)

Most of the glutamic acid found in our analysis was most probably in the form of glutamine in zein initially, i.e. prior to hydrolysis. This assumption is based on a previous report that 90 % of aspartic and glutamic acids in zein are amidated (Gianazza, 1977). According to this, approximately 95.87 % of the amino acid residues in zein used in our studies would be nonpolar. However, as explained in the experimental section, both glutamic acid and glutamine residues were quantified together, due to the conversion of the amide forms into the acid forms by hydrolysis. Therefore, the exact percentage of glutamine and asparagine cannot be known with accuracy.

Other authors have shown different percentages of uncharged amino acid residues in zein (Bietz et al.,1979). This is probably due to different extraction methods used by individuals and industries, which result in different classes of zein polypeptides, which have different ratios of polar to nonpolar amino acids being extracted. For example, γ-zein is known to be cysteine-rich, β and δ-zeins have high levels of cysteine (Cys) and methionine (Met) and lower levels of glutamine (Gln) while α and β-zein lack lysine (Lys) and tryptophan (Trp) (Coleman and Larkins, 1999). From our results (low zein content in Cys and Met) and knowledge of the content of different zeins (see chapter 1), it can be said that the commercial zein used in our studies is composed of α-zein predominantly. In addition, as it is known that corn glutelins have higher Lys content than corn prolamins, the amount of glutelins contaminating zein after its extraction must be low, because of the low content of Lys found in the commercial zein.

Neutral (hydrophilic)	
Serine (Ser)	5.16%
Threonine (Thr)	2.65%
Neutral (hydrophobic)	
Leucine (Leu)	18.41%
Proline (Pro)	15.31%
Alanine (Ala)	13.26%
Phenylalanine (Phe)	5.34%
Isoleucine (Ile)	4.35%
Valine (Val)	4.06%
Tyrosine (Tyr)	3.48%
Glycine (Gly)	2.20%
Methionine (Met)	1.10%
Cystein (Cys)	0.31%
Acid	
Glutamic acid (Glu)	17.45%
Aspartic acid (Asp)	4.86%
Basic	
Histidine (His)	1.00%
Arginine (Arg)	0.70%
Lysine (Lys)	0.19%

Table 2.3. Amino acid composition and content of zein.

2.3.3. Peptide composition of zein

SDS-PAGE revealed that zein was composed of two major peptides with MW just below 25 kDa in all the samples, as shown in figure 2.8. This corresponds to the α-zein described by Esen (1987, 1990) or to the A and B zeins following the nomenclature of Wilson (1985, 1991). When zein concentration in the solutions loaded onto the gel was increased, other minor peptides, present in zein at low concentrations, were also detected (lane 3 and 5, figure 2.8.). Although zein was reduced with 2-mercaptoethanol during the procedure, a band at approximately 45 kDa was observed (lane 3 and 5, figure 2.8.), this might be related to the presence of either a dimer of one of the major peptides if reduction was not fully completed or to a minor 45 kDa peptide in zein. Bands between 15 and 10 kDa were also observed (all lanes, figure 2.8.). The peptide of MW of 10 kDa approximately probably belongs to the minor fraction δ-zein (Esen, 1990) or D zein; and the ones of 15 kDa approximately may correspond to the β-zein or to C zein. The latter zein peptides, C- or β-zein are described as peptides with a MW of 17-18 kDa. However, it has been mentioned in chapter 1 that those MW are approximate, thus the band found at 15 kDa in our sample is attributed to C- or β-zein. Zein fractions that might be present at amounts lower than 1 μg could not be detected by the staining method and therefore are not taken into account in this study.

The absence of bands between 15 and 19 kDa approximately and between 25 and 37 kDa indicates the absence of γ-zeins and of the peptides termed methionine-rich or proline-rich peptides, mentioned in chapter 1. This concords with the amino acid analysis described in the previous section 2.3.2., i.e. predominance of α-zein. Again, α-zein seems to be the main zein protein fraction present in the zein used to formulate microspheres in our experiments.

Finally, SDS-PAGE of zein dissolved in two different solvents (70% v/v aqueous ethanol solution and 0.1M sodium hydroxide aqueous solution)

revealed the same distinguishable bands of α-zeins. This indicates that these solvents, at room temperature, have no effect on the overall composition of zein. However, slight changes of concentration were observed for the low molecular weight zein peptides. The presence of α-zein peptides when zein is dissolved in sodium hydroxide seems to indicate that the hydrolysis mentioned in section 2.3.1.5. does not occur at that sodium hydroxide concentration at room temperature.

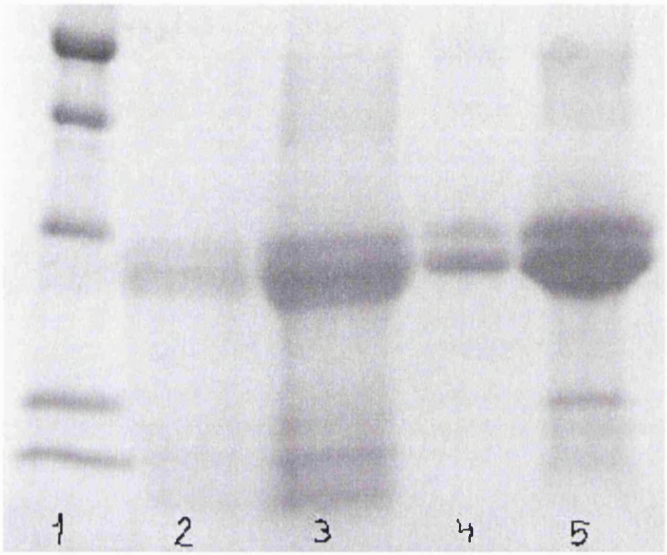


Figure 2.8. SDS-PAGE profile for zein protein. Lane 1: Molecular weight markers of 10, 15, 25, 37 and 50 kDa. Lanes 2 and 3: Zein dissolved in 0.1M sodium hydroxide (2.5 and 10 mg/ml, in that order). Lane 4 and 5: Zein dissolved in 70% v/v ethanol (2.5 and 10 mg/ml, in that order).

2.4. Conclusions

As found in the literature, the best solvents for zein are alcohol-water mixtures, principally a range of aqueous ethanol and propan-2-ol concentrations. We conclude that the exact explanation for zein solubility in these mixtures is not clear-cut. However, we speculate that the affinity of the alcohol for zein is higher than that of water (i.e. ethanol or propan-2-ol being less polar than water), and that the cosolvency favours the dissolution of zein sterically and thermodynamically.

The amino acid composition and content of the zein used throughout our studies were investigated since they determine the properties of zein, which will have an effect on drug/vaccine delivery systems prepared from zein. The amino acid analysis of the protein showed elevated content in uncharged amino acid residues, which confirms the hydrophobicity of zein found in the literature. This hydrophobicity is then consistent with zein insolubility in water found in our solubility studies. The high presence of glutamic and aspartic acid residues, which would increase the solubility in water of the protein, cannot be taken into account since the amino acid analysis did quantified together glutamine and asparagine with their respective acids. The low content in hydrophilic residues concords with zein insolubility in aqueous solutions as well, due to the low wettability of zein. In addition, the deficiency in basic amino acids confirms the insolubility in acidic buffers. On the other hand, the high content in glutamic acid, which may be initially in the form of glutamine, explains the increase in solubility when pH of the medium increases. Even though the exact ratio of hydrophilic to hydrophobic amino acids residues could not be determined (due to technical limitations of the amino acid analysis), we showed that neutral hydrophobic residues are predominant in the protein and then conclude that such amino acid composition and content play a major role in zein properties.

Zein amino acid and peptide composition lead us to conclude that the commercial zein used in this thesis is composed of α-zein predominantly, a

protein fraction that consists of two main polypeptides with MW just below 25 kDa MW. In addition, other zein protein fractions, such as of β- and δ-zeins (i.e. peptides of MW of 15 and 10 kDa) are present in minor concentrations.

Due to zein´s insolubility in aqueous media, we can predict that zein will have limited solubility in body fluids, which will be of importance when drug/vaccine delivery systems made of zein are formulated. However, from our characterisation results, we conclude that solubility of zein in water can be improved by converting glutamine and asparagine residues into glutamic and aspartic acid residues by deamidation, if necessary.

Chapter 3.

Preparation of zein microspheres

3.1. Introduction

The understanding of pharmacokinetics has historically demonstrated that drug absorption, thus its bioavailability, and not only the dose, controls the therapeutic action of the pharmaceutical agent. Over the past several years, the safety and the therapeutic efficacy of new drug products have been emphasised by regulatory activities. Efforts from pharmaceutical researchers have then moved towards the delivery of both existing and new compounds to the appropriate physiological site.

Drug delivery systems can be defined as those systems in which the drug is either associated with some other chemical or administered with the help of some device or process, in such a way that the rate of release and/or the target where it must be released can be controlled (Goldman, 1982; Robinson and Manger, 1991; as cited in Gallardo et al., 2000). Apart from the drug's bioavailability control, these systems include other advantages as follows: protection against inactivation of the drug by chemical, enzymatic, or immunological factors, less frequent dosing, increased safety because of the lower dosage and the lesser extent of drug delivery in non-target tissues, and decreased undesired effects in the gastrointestinal tract. Hence, the bioavailability of drugs can be optimised and its possible toxicity minimised.

As explained in chapter 1, since the concept of delivery of drug compounds was born, different micro- and nanoparticles have been developed to allow controlled delivery of pharmaceuticals, nutrients, and other bioactive agents. In many cases, these delivery systems are prepared using synthetic polymers. These polymers have been proven to be successful for long-term delivery applications. Biodegradable polymers are desirable because they degrade in the body to biologically inert molecules. The polymers more commonly and successfully used to prepare particulates are the polyesters that were first investigated as degradable sutures, such as poly(glycolide), poly(D,L-lactide) (PLA) and their related copolymers poly(D,L-lactide-co-glyclolide) (PLGA).

Many commercial products based on particulates are currently on the market, such as Lupron Depot® and Sandostatin LAR®. In these traditional parenteral depot formulations, biodegradable PLA and PLGA polymers have been used simply as inert carrier vehicles. Preparation of the dosage form is carried out, by various means, to incorporate the drug into the polymer matrix. Particulate preparation often involves utilisation of organic solvents and cross-linking agents. The latter agents and solvents may represent a hazard and there is a need to avoid them.

Natural biopolymers, such as zein, have also been suggested for the preparation of drug delivery systems due to their physico-chemical properties and their biodegradability. As mentioned in Chapter 1, zein has previously been used to develop microspheres (Suzuki et al., 1989; Matsuda et al., 1989; Mathiowitz et al., 1993; Demchak and Dybas, 1997; Liu et al., 2005). Among other natural proteins being investigated for their suitability to develop drug and/or nutrient delivery systems are albumins (Park et al., 1998; Rubino et al., 1993), gelatin (Levy and Andry, 1990; Oner and Goves, 1993) and caseins (Knepp et al., 1993; Santinho et al., 1999).

The aim of this chapter was to produce zein particulates as antigen delivery systems in the form of pharmaceutical suspensions. The novel formulation was intended to be as simple as the traditional PLA and/or PLGA depots mentioned above. A protein, ovalbumin, was selected as the model antigen and loaded into zein particles. The biological activity of the protein had then to be carefully protected through the whole manufacturing process. Then, emphasis was made to avoid the use of organic solvents, high temperatures, or other hazards, in order to maintain the integrity of the ovalbumin.

Therefore, the objectives of the experiments reported in this chapter were:

- ✓ To produce particulate systems made of zein protein.
- ✓ To understand the underlying mechanisms of zein particle formation.
- ✓ To load a model protein/antigen into zein particles.

- ✓ To select a general method for zein particle preparation (both blank and antigen loaded-particles).
- ✓ To test the resulting zein particles stability during manufacture and on storage.

When developing a novel formulation for pharmaceuticals, it is common that a certain amount of optimisation work is required to afford the desired product. Optimisation of a formulation or process consists of finding the best possible composition or operating conditions. Determining the latter is unfortunately an enormous task, and in this work, optimisation of zein microsphere formulation was considered as the search for a satisfactory result within a limited field. of search.

The experiments to search for the optimal result were designed in a way that they could give information about the mechanisms of microsphere formation. First, the type and the components of the zein microsphere formulation were selected in preliminary experiments. Then, the relative proportions of each component were varied to obtain the best endpoint. In addition, studies were carried out to determine the best operating conditions.

The essential characterisation parameters for pharmaceutical suspensions are particle size and size distribution (Müller et al., 2001); both were determined during the experiments for the preparation of zein particulates in the form of pharmaceutical suspensions. The mean size and width of distribution (e.g. polydispersity index) are typically determined by photon correlation spectroscopy (PCS). The measuring range of PCS is limited to approximately 3 nm-3 μm. To find which was the best endpoint, size analyses were performed for all formulations tested. On the other hand, particle charge is a stability determining parameter in aqueous suspensions. It is measured by electrophoresis and expressed as electrophoretic mobility or converted to zeta potential. Zeta potential serves as an important parameter in characterising the electrostatic interactions between dispersed particles and the properties of dispersions as affected by this electrical phenomenon. As zein particles were formulated as an aqueous suspension, analyses of zeta potential were carried

out for all formulations and used as a measure of the physical stability of the dispersed system. The discussion of these two techniques is the subject matter of chapter 4, where the characterisation of the best formulation found in this chapter is described.

3.1.1. Pharmaceutical suspensions

Zein microspheres were formulated in an aqueous suspension form, a type of disperse system. Of the pharmaceutical dosage forms, liquid disperse systems are one of the most complexes. Their challenges are related to their non-equilibrium state, as they are continuously seeking to reach thermodynamic equilibrium. A suspension is a class of dispersed system in which the internal or suspended phase is dispersed uniformly by mechanical agitation throughout the external phase, that is, the suspending medium. The suspended phase, composed of solid particles, must be maintained dispersed with the aid of a single or a combination of suspending agents. The suspended particles exhibit a minimum degree of solubility in the external phase. Usually, the size of dispersed particles for pharmaceutical suspensions ranges in the colloidal domain (0.1-1 μm), then the principles of the colloid and surface science have to be used to deal with their properties. The spontaneous tendency of these dispersed systems is the aggregation of single particles to minimise the total energy, unless an energetic barrier hinders it. The Gibbs free energy change, ΔG, associated with the formation of a dispersed phase is positive, and for this reason, a colloidal dispersion is thermodynamically unstable.

In addition to the solvent and the insoluble solute, the following additives are included in pharmaceutical suspensions: wetting agents, compounds that control stability and sedimentation, additives used to regulate the flow behaviour, pH regulators, other additives to ensure taste, colour and fragrance, etc.

The first requirement in the production of a suspension is the wetting of the solid particles by the liquid vehicle. The difference between the surface tension of a solid-air and a solid-liquid interface is the driving force determining the ease of immersion of solid particles in a liquid. The drug particle will be wetted by the liquid if the solid-air interface is spontaneously replaced by a solid-liquid one when the drug is immersed in the vehicle. This concept is related to the thermodynamic quantity known as W^s, work of spreading. A positive value of W^s means that the immersion of the solid particle is spontaneous; otherwise, work must be done on the system to wet the solid properly. Once the particles are wetted, they can either show a tendency to form aggregates or to remain as individual entities in the liquid vehicle. In order to ensure suspension stability, particles should remain separated. The irreversible aggregation of suspended particles, called caking, must be avoided. One of the most widely used procedures to prevent it is the controlled flocculation of suspended particles. Flocculation produces a network of loosely aggregated particles held together by the van der Waals forces or interparticle bridges. The achievement of such stability requires the addition of components such as surfactants. Surfactants are additives used to improve the wettability of hydrophobic powders in aqueous suspensions. The surfactant molecules adsorbed at the solid-liquid interface modify the surface tension of the surface. The reason of this is that interfacial interactions play a role in controlling the tendency of particles in suspension to remain stable or to aggregate or coagulate.

In addition to interfacial interactions, electrostatic repulsion does also contribute to the interaction energy between dispersed particles. A solid immersed in a polar liquid acquires a net surface charge. The overall neutrality of the dispersion requires the surface charge being compensated for ions of opposite charge in the solution close to the particle. A diffuse layer of counterions is thus formed around the particle, giving rise to a distribution of charge and potential known as electric double layer. The zeta potential, ζ, is the potential at the electrokinetic or shear plane that ideally separates the mobile and the immobile parts of the diffuse ionic layer. Thus, another possible way of achieving the flocculation behaviour is by using inorganic salts

in solution. By changing the salt concentration, the ζ potential will be modified as well. As a consequence, the interaction between the particles can change from a stable suspension to a coagulating one and viceversa.

Another way of controlling stability of suspensions is the addition of polymers. They can interact with the dispersed particles, giving rise to polymer-particle complexes. Polymers can stabilise the suspension by hindering particle-particle contact, or controlling the formation of aggregates (bridging or depletion flocculation). This strategy can create an energy barrier strong enough to make a pharmaceutical suspension stable. In addition, additives to increase viscosity of the dispersion medium reduce the frequency of suspension particle collisions, while hindering their sedimentation and thereby additives can increase the stability of disperse systems. Of course, the viscosity cannot be increased until the flow of the system is too low. This latter aspect is related to the rheological behaviour of the system that must be controlled for practical shear stress.

Ideally, a pharmaceutical suspension should be stable in a wide pH range, but the chemical stability of the active compound requires the pH of the suspension to be maintained within specific values. pH regulators may be added to pharmaceutical suspensions, if needed. The remaining components of the suspension must produce their effects at that pH range.

No additives for a pharmaceutical suspension must have therapeutic activity by themselves or be toxic or irritant. It is determinant to bear in mind that pharmaceutical suspension final destiny is to be administered to human beings. In pharmaceutical sciences, suspensions can also be used as delivery systems.

3.2. Experimental

3.2.1. Chemicals

Dimethyl sulphoxide (DMSO), zein, ovalbumin (Grade II), Tween 20, Tween 80, polyvinyl pyrrolydone MW 40 000 (PVP 40), sodium chloride, potassium chloride, and Bicinchoninic acid test kit were purchased from Sigma, UK. Other chemicals were of reagent grade and were used as received. Double-distilled water was used throughout.

3.2.2. Methods

3.2.2.1. Preparation of blank zein particles

In these studies, zein particles were prepared by precipitation of zein in an insoluble media. By understanding zein solubility behaviour, the precipitation was controlled to certain extent. Before the general method for their preparation was found, different preliminary approaches were completed to prepare blank microspheres (i.e. composed of zein protein only). The main intention of all preliminary experiments was to prepare a controlled flocculated suspension of zein, which had been precipitated into particles. Conventional ways of formulating pharmaceutical suspensions as delivery systems (see chapter 1) were followed for this purpose. Zein was first dissolved or dispersed in a volatile liquid. Aqueous solutions of stabilisers (e.g. surfactants, polymers, or electrolytes) were then added to the resulting solution or dispersion. Moreover, the zein particles ultimately formed upon solvent removal, if needed. Particle formation was confirmed under microscopic examination (light and electronic microscopy) after harvesting particles by centrifugation of the liquid in a table-top centrifuge. The whole process was primarily considered to avoid hazards such as organic solvents, high shear forces, high temperatures, or extreme pH. Ethanol due to its low toxicity in humans, and

dimethyl sulphoxide, due to its ability to dissolve zein (see chapter 2), were tried as the volatile liquids.

When DMSO was used, a solution of zein was obtained. Water was then used as a non-solvent for zein. In some experiments, aqueous solutions of surfactant Tween 20 at several concentrations were added (instead of only water) in order to observe the effect, if any. The aqueous phase, with or without surfactant, was slowly added until turbidity was observed, which was assessed only visually. In all cases, macroscopic phase separation was evaded. Slow stirring was maintained during the whole process. Since DMSO boiling point is higher than that of water, solvent evaporation was not feasible. Centrifugation (13 000 rpm, 5 min) of the liquid system was carried out and a pellet was collected, which was observed under light and scanning electron microscopy (SEM).

100% ethanol cannot be used as a solvent for zein and a coarse dispersion of zein was obtained when zein was added to this alcohol, upons low stirring. The addition of Tween 20 in an aqueous solution was found to convert the coarse dispersion into a fine disperse system. A range of alcohol-aqueous solution proportions was tried as well as Tween 20 aqueous solutions. When the ethanol by rotatory evaporation was removed, a finer suspension could be observed. As above, a pellet was harvested by centrifugation, and light and scanning electronic microphotographs were undertaken. When adding aqueous solutions to the ethanol, the contraction of final volume, owing to contraction of ethanol in water, was taken into account and the volume was consequently adjusted.

Unfortunately, suspensions and dispersions are known to have a major problem of coalescence and sedimentation, and so did happen with the suspension of particles found using the latter ethanol-surfactant aqueous solution mixture. In order to meet these challenges, different strategies were taken: (i) adding a mixture of polysorbates, (ii) adding electrolytes, and (iii) adding a hydrophilic polymer plus a polysorbate.

Two polyoxyethylene sorbitan fatty acid esters, Tween 80 and Tween 20 were the mixture of polysorbates selected. Tween 20 aqueous solution was used at the concentration that had been proved to be optimal to help to the formation of zein particles, so it was used. Tween 80 concentration range used was from 0.1 to 2% v/v. Equal volumes of polysorbate aqueous solutions were added to the ethanolic dispersion of zein, ethanol was subsequently removed, and a pellet was collected by centrifugation as above.

Both potassium chloride and sodium chloride were tried as electrolytes. Electrolyte aqueous solutions at different concentrations were added to the zein protein dispersed in ethanol. In this case, no surfactant or other stabiliser was added. Following ethanol removal and liquid centrifugation, as above, a very small pellet was collected.

For the combination of a polysorbate and a hydrophilic polymer, Tween 20 (whose efficiency in helping zein particles formation had been proved) and polyvinyl pyrrolydone (PVP 40) were chosen. Various proportions of Tween 20 and PVP 40 were tried. Again, ethanol was removed by rotatory evaporation and the liquid was centrifuged in a table-top centrifuge.

Each collected pellet was examined under SEM to prove the absence or the presence of microspheres. The suspensions of zein microparticles were kept at room temperature for 20 days and their sedimentation was followed with time and assessed visually. After 20 days, suspensions were centrifuged again as above and the collected pellet was examined under SEM to see the changes in particle morphology.

For all formulation trials, the best proportions of additives were searched for and the optimal formulation was established as that one leading to the formation of spherical, smooth particles, with a diameter in the range of microns. Controls using no additives were also carried out.

3.2.2.2. Preparation of protein-loaded zein particles

Ovalbumin was the protein selected as a model guest molecule. Ovalbumin is soluble in water; then aqueous solutions of ovalbumin or ovalbumin in powder form, were incorporated to the ethanol dispersion of zein. However, due to the insolubility of both zein and ovalbumin in absolute ethanol, strong macroscopic aggregation rapidly occurred and no disperse system was formed in any case. Increasing or decreasing the amount of stabilisers (i.e. PVP 40 and Tween 20) did not avoid aggregations. Neither did the addition of ovalbumin to the aqueous solutions of the stabilisers. After a number of approaches had been tried, sodium hydroxide was found to increase the dispersibility of both macromolecules in the non-solvent ethanol. Therefore, ovalbumin and zein were added to 100% ethanol, a coarse dispersion was again formed upons low stirring, and a small amount of sodium hydroxide 1% w/v aqueous solutions were added, followed by equal volumes of the two stabiliser solutions (i.e. PVP 40 and Tween 20). The proportions of ethanol-water and the concentration of the stabilisers which provided an optimal result (i.e. microsphere formation) were used for the preparation of blank zein microspheres. The amount of sodium hydroxide was decreased (from 1 ml to 0.3 ml) until optimal microspheres were formed. When the ethanol was removed by rotary evaporation, a fine aqueous suspension of zein-ovalbumin microspheres was obtained. As above, a table-top centrifuge was used to collect a pellet and this was examined under SEM to observe under which conditions zein-ovalbumin microspheres were formed. The amount of ovalbumin added to the system was increased. This excess of ovalbumin was primarily added in order to maximise entrapment, if possible. Entrapment of ovalbumin in zein microspheres is subject of matter in chapter 4.

A control of the procedure was also carried out adding ovalbumin protein without zein to the ethanol-aqueous solutions and subsequently evaporating the ethanol.

3.2.2.3. Studies on zein microsphere formulation variables

To formulate a disperse system as a pharmaceutical dosage form, thus a stable disperse system (e.g. a flocculated suspension), the formulator can rely on the mechanical energy applied and on the inherent energy of the system. The mechanical energy applied will be the focus of the next chapter's section. The issue of inherent energy is related to the components of the formulation and the present study was carried out to determine the conditions under which zein microspheres are best formed and to understand the role of suspension components on particle formation.

Screening methods are used to identify important and critical effects in the manufacturing process. Since the one-factor-at-a-time method, in which one factor is varied and then another, cannot be used when there are strong interactions between factors; a factorial design was developed to identify important and critical effects in the manufacturing process. It was believed that spherical, uniform zein particle formation was due to a number of variables and to the interactions among them. Factor influence studies are closely related to optimisation. However, as mentioned in this chapter's introduction and due to the viability, optimisation of the formulation was merely considered as the search for a satisfactory result.

The preliminary experiments explained above to prepare both blank and loaded zein microspheres led to the identification of the main components of the formulation of zein microsphere suspension. They were Tween 20, PVP 40, 100% ethanol and sodium hydroxide in the case of ovalbumin-loaded zein microspheres, along with the suspended proteins. Then, these components were identified as the significant variables that can have an effect on microsphere formation and final characteristics. Those effects must be determined empirically and the results will help to understand the role of each component in the formulation.

The independent variables (quantitative factors) selected were (i) zein and ovalbumin mass (g), (ii) amount of sodium hydroxide added (expressed as sodium hydroxide concentration in solution), and (iii) proportion of Tween 20 and PVP 40 additives (expressed as concentration ratio). Other variables that could affect the microsphere final characteristics, such as stirring speed or alcohol evaporation conditions, were kept constant for the duration of the study. They were considered as other type of energy and will be the subject of discusison for the next chapter's section. The dependent variables (results) determined were (i) particle morphology, (ii) particle size and size polydispersity, (iii) zeta potential of the suspension as a relative measurement of particle charge, and (iv) proportion of protein precipitated (zein and ovalbumin) as a virtual measurement of the yield.

Then, the resulting factorial design was a 3^3, where three factors were considered and each of which took three different levels. The number (N) of the distinct combinations among all levels of all factors (runs or trials) was 27. Each combination was then a different formulation of zein microspheres. For each formulation, results were determined and different effects on results were searched. The effect of a factor is defined as the change in response caused by varying the levels of the factor. Those changes that were considered consistently different between levels of a factor were assigned as main effects. Those changes between factors and not levels were identified as interactions effects. In statistics, interaction is defined as a lack of additivity of factor effects. Lastly, those treatments that had no effect on particle characteristics were considered null cases. Table 3.1. shows the 3^3 model matrix for the calculation of effects.

Results were determined as follows. The proportion of protein precipitated (zein and ovalbumin) and zeta potential were analysed on day 1; while particle morphology, particle size, and size polydispersity were measured on days 1, 2, and 7. The techniques for the examination of particle morphology under scanning electronic microscopy, and measurement of particle size, size distribution, and zeta potential using Malvern Zetasizer instrumental will be fully described in next chapter. Briefly, to examine the shapes and surfaces of

particles under scanning electron microscopy, zein particles were collected from a suspension by centrifugation (13 000 rpm, 5 min), a sample of the pellet was dried, sputter-coated with a thin layer of gold and observed microscopically. The particle size and size distribution were determined using photon correlation spectroscopy technique. Then, an aliquot of zein microsphere suspension was suspended in purified water prior to analysis. The mean particle diameter was expressed in nm. As a relative measure of the particle surface charge, the zeta potential was determined using laser Doppler velocimetry. Phosphate buffered saline pH 7.4 diluted in water (0.1mM phosphate buffer solution) was used as the dispersing medium for the measurements.

To determine the proportion of protein precipitated, thus the yield, the particle suspension was centrifuged at 6 000 rpm for 20 min to separate soluble protein, ensuring that the temperature never reached a maximum of 25º C, considered to be room temperature. The supernatant, containing soluble protein, was removed and replaced with fresh water. Centrifugation was repeated as before, and the supernatant was removed once more. The microsphere pellet was then dissolved in 80% v/v propan-2-ol aqueous solution. The concentration of protein in the supernatants and in the dissolved pellet solutions was measured by BCA assay (see chapter 2 for BCA assay). The protein precipitated was expressed as a percentage of total protein used (i.e. (protein concentration in pellet / protein concentration in pellet + protein concentration in supernatants) x 100). This analysis was conducted to determine the total mass of zein and ovalbumin that precipitated when ethanol was removed from the aqueous medium. From the results, we cannot distinguish whether ovalbumin and zein co-precipitated into the same particles or into different particles. Further studies were carried out to clarify this aspect and are described in chapter 4.

3.2.2.4. Studies on zein microsphere formulation procedure

3.2.2.4.1. Standardisation of formuiation procedure

Since the experiments described in the last section focussed on the components of the formulation and how they could affect microsphere formation; the present experiments rely on the mechanical energy applied to the disperse system, and how this energy could affect the uniformity of zein particles. After establishing a formulation procedure to form zein microspheres loading ovalbumin as guest protein, there was a need to produce particles of better quality in terms of size distribution. Analysis of particle diameter always showed a broad range of sphere diameter. Therefore, in order to improve the quality of the particles, if possible, efforts were made to standardise the procedure of particle preparation. Firstly, since ethanol removal was thought to be the crucial moment of particle formation, the conditions of its evaporation were normalised. Secondly, it was suggested that homogenisation and/or sonication of the zein-ovalbumin disperse system before and after ethanol evaporation could reduce size and/or size polydispersity. Previously, sonication had been avoided to reduce the chances of loosing ovalbumin biological activity, a major concern when producing protein-loaded delivery systems.

As mentioned before, a rotavapor instrument (Büchi Rotavapor R-200) was used to evaporate ethanol from the aqueous-ethanolic suspensions of zein and ovalbumin. To homogenise the parameters under which ethanol was removed, the water temperature was kept constant at 40 ºC and the vacuum pressure was controlled as follows, using Büchi Vacuum Controller V-800. For a starting vacuum pressure of 175 mBa, the pressure was carefully reduced until ethanol started its slow evaporation; the pressure was then kept constant for the time necessary to reduce to 50% of the initial volume (volume contraction when mixing ethanol and water had previously been taken into

account). Batches of microspheres prepared in this way were analysed in terms of average size and size polydispersity and were compared with those produced with initial longer times and higher vacuum pressures on ethanol evaporation.

In order to preserve ovalbumin biological activity, sonication had been avoided initially, and only stirring had been used to maintain the dispersion of zein and ovalbumin in the liquid mixture. For the same reason, stirring had been kept to a minimum speed and for the minimum possible time. Stirring was not manually but magnetically, and the size of the stirrers along with the vials used were chosen to reduce the extent of turbulence in the liquid. Still, the possible effect of stirring and sonication on average particle size and size distribution was studied.

Batches of ovalbumin-loaded zein particles were prepared (controlling the evaporation parameters as detailed above) and were subjected to one of the following conditions:

- Stirring of protein suspension for 1h at the minimum speed before ethanol evaporation.
- Bath-sonication of protein suspension for 5min before ethanol evaporation.
- Bath-sonication of microsphere suspension for 5min after ethanol evaporation.

Size distribution of particles in the final microsphere suspensions of all batches was determined. The average particle size and the size polydispersity of particles present in the protein suspension before ethanol evaporation without sonication and after 5min of sonication were also analysed.

3.2.2.4.2. Scaling-up of formulation

The standardised procedure described so far was used to produce bigger batches of ovalbumin-loaded zein microsphere suspensions in terms of volume. 40 ml microsphere suspensions were prepared using the same proportions of ingredients than for previous batches. Particle size analyses were carried out as before.

3.2.2.4.3. Storage of zein microsphere suspension

Since zein microsphere average size and polydispersity were found to increase with time at room temperature batches zein microsphere suspensions were stored at –70 ºC within experiments. Then, the influence of freeze-thaw cycles on average size and morphology of zein particles was studied. Batches of zein microspheres were subjected to three freeze-thaw cycles, average size and size polydispersity of particles analysed after each cycle and morphology of microspheres observed under scanning electron microscopy.

In addition, other batches of zein suspensions were centrifuged and the obtained pellets (i.e. the zein microspheres) were oven-dried for 24 h. The dried pellet was gently pulverised in a mortar and the resulting powder examined under SEM. In this case, the centrifugation was carried out in a table-top centrifuge for blank zein microsphere suspension, following sucrose gradient centrifugation technique for ovalbumin-zein microsphere suspension. Sucrose gradient centrifugation, a technique that allow the separation of free and precipitated protein by density, will be fully detailed in next the chapter.

3.2.2.5. Statistical analyses

In all cases, experiments were replicated and analyses were carried out in triplicate. Standard deviation (S.D.) was given as a measure of error for all analysis. Student t tests were conducted when two values had to be compared, in order to reveal significant differences, if any.

Variable level in coded form

Formulation No.	X_1	X_2	X_3
1	-1	-1	-1
2	-1	-1	0
3	-1	-1	1
4	-1	0	-1
5	-1	0	0
6	-1	0	1
7	-1	1	-1
8	-1	1	0
9	-1	1	1
10	0	-1	-1
11	0	-1	0
12	0	-1	1
13	0	0	-1
14	0	0	0
15	0	0	1
16	0	1	-1
17	0	1	0
18	0	1	1
19	1	-1	-1
20	1	-1	0
21	1	-1	1
22	1	0	-1
23	1	0	0
24	1	0	1
25	1	1	-1
26	1	1	0
27	1	1	1

Coded values	Actual values		
	X_1	X_2	X_3
-1	0.25/0.05	10/1	2
0	0.125/0.05	5/2	1
1	0.0625/0.05	2.5/4	0.5

X_1 = Ratio of zein/ovalbumin (g/g)

X_2 = Ratio of Tween 20/PVP 40 (% v/v/% w/v)

X_3 = Concentration of sodium hydroxide (% w/v)

Table 3.1. 3^3 factorial design to explore the effects of formulation factors on the characteristics of the resulting zein particles.

3.3. Results and discussion

3.3.1. Preparation of zein microspheres

The fundamental requirement of protein and/or antigen delivery technologies is to maintain the integrity of the protein or antigen during the delivery system manufacture; hence, the maintenance of their biological or immunological activity. Thus, this chapter's concern and primary objective was to avoid undesirable stress, such as high shear force to form particles, exposure to polymerisation reactions, high temperature, or extreme pH, while producing zein microspheres. A simple, rapid, and secure method to produce zein microspheres as vaccine delivery systems was searched.

Zein microspheres have been prepared in the past by phase separation (Mathiowitz et al., 1993) and cross-linked in solution using glutaraldehyde (Matsuda et al., 1989; Suzuki et al., 1989). Further investigations on zein microsphere characteristics were not reported. More recently, zein microspheres have been formulated, in order to encapsulate parasiticides, by a phase separation process and by a solvent depletion procedure in a non-aqueous zein emulsion (Demchak and Dybas, 1997; Liu et al., 2004). Zein microspheres formulated by cross-linking showed aggregated particles with extremely low release. Instead, phase separation method results were more promising. Due to the poor results by cross-linking, this method was discarded since the beginning of these studies. Instead, the idea of forming a stable disperse system, which comprises a phase rich in zein microspheres, was preferred.

The solvent evaporation method to develop micro- or nanoparticulates has been commonly used (Pavanetto et al., 1992; Jeffery et al., 1993; McGinity and O'Donnell, 1997; Yamaguchi et al., 2002). Among other solvent evaporation techniques, such as spray-drying, solidification of droplet emulsions is one of the most widespread processes to harden polymer particles. Nevertheless, there are problems associated with the use of these

methods to produce systems for protein delivery like the protein instability during the preparation of protein-loaded microspheres. Frequently, an aqueous protein solution is dispersed in an organic polymer solution by using a homogeniser or a sonicator to create a water-in-oil (w/o) emulsion. Consequently, the exposure of the protein to the organic solvent or to the aqueous-organic interface, and/or the stress of sonication or homogenisation may produce adverse effects on the stability of the proteins. Thus, the solvent evaporation technique was used in this study, but avoiding as much as possible the organic solvents and sonication used in other encapsulation techniques. To achieve this, solvent evaporation technique was combined with a phase separation or coacervation process, to produce zein microspheres.

Firstly, a simple phase separation method was tried, where zein was dissolved in DMSO to which an aqueous solution was added afterwards. DMSO totally dissolves zein, but zein is insoluble in an aqueous medium (see chapter 2 for zein solubility results). Both DMSO and water are miscible. When using DMSO, a solution of zein was obtained. Therefore, no surfactants were needed to wet zein solid; however, the surfactant Tween 20 was added to the aqueous phase in some samples to observe its effects, if any. Following the gradual introduction of the aqueous solution, it is believed that macromolecular nuclei of high concentrations of zein protein were formed. This gradual addition of the aqueous medium ultimately led to the formation of a macroscopic phase separation or precipitation. Therefore, when turbidity was visually observed, the addition of the aqueous solution, with or without Tween 20, was stopped. It was observed that in the absence of Tween 20 (which acts as a stabiliser) and stirring, the precipitation tended to occur more rapidly. Some spheres could be observed under scanning electronic microscopy (SEM) for the samples having 0.25 g of zein dissolved in 5 ml of DMSO, to which an aqueous solution of 5% v/v Tween 20 was added (figure 3.1), being the latter the optimal formulation to produce zein microspheres (i.e. formulation that produced smooth, spherical particles, having a diameter in the range of microns). It is hypothesised that the zein nuclei may form the spheres observed under SEM, but that indefinite addition of aqueous phase, with or

without surfactant, would bring about the precipitation of the protein because of the decrease of zein solubility in the DMSO-water mixtures.

The procedure above is the basis for the precipitation technique used to form polymer particles: dissolving the drug in a solvent and adding this solvent to a non-solvent (Arshady, 1990; Alonso, 1996; Haas et al., 1996). According to the latter authors, the challenge of this technique is that the growth of the drug crystals has to be limited by surfactant addition; therefore, Tween 20 was included in the formulation even though the power of the solvent DMSO was sufficient to wet zein protein completely. The requisites of the precipitation technique are drug solubility in at least one solvent (e.g. zein in DMSO) and solvent miscibility with a non-solvent (e.g. DMSO and water). The precipitation technique has also been called coacervation or phase separation. There are small differences between them, but all these techniques are based on polymer precipitation. The term coacervation is meant to describe macromolecular aggregation (or phase separation) processes brought about by partial desolvation of fully solvated macromolecules (Arshady, 1990). The polymer-rich phase is called the coacervate phase or simply coacervate and the polymer-poor phase is called the coacervation medium. The macromolecular aggregates, or coacervates, tend to gradually approach each other to form a macroscopic coacervate phase. For the three-component mixture zein-DMSO-water used in our studies, the phase separation process in zein solution takes place because the DMSO-water interactions become more favourable and reduce zein solvation. Then, interactions between zein molecules increase, and therefore, the formation of coacervates does.

Although, the coacervation technique to produce zein microspheres was found to be simple and rapid, the SEM microphotograph only did show a few microspheres (figure 3.1.). Other approaches were therefore investigated.

The next approach used a volatile liquid that produced a disperse system instead of a solution, such as 100% ethanol. The selection of 100% ethanol to prepare zein microspheres were made based on our previous knowledge of zein solubility (see chapter 2 for solubility studies). Since it was known that

zein phase separates at certain water-ethanol proportions, zein was first dispersed in 100% ethanol and water was added to the dispersion to form a suspension, avoiding zein dissolution. However, in order to produce a disperse system as stable as possible, not only water and 100% ethanol were used, but some additives where included in the aqueous phase. Tween 20 was again used as a wetting and stabiliser agent. Tween 20 was found to be capable of converting zein coarse dispersion in ethanol into a fine suspension in water-ethanol mixtures by decreasing interfacial tension. The gradual removal of the ethanol by rotatory evaporation from the aqueous-alcoholic suspension of zein was obviously accompanied by a corresponding decrease in the volume (up to a 50% of the total V), and thus by an apparent increase in the viscosity of the resulting suspension, which was assessed visually. Following ethanol evaporation, SEM photographs showed the presence of spherical, non-porous uniform microparticles. Before ethanol evaporation, those particulates were not found under SEM examination. It was then found that the process of zein precipitation into microspheres primarily occurred while ethanol rotatory evaporation. The optimal formulation (i.e. formation of uniform microspheres) was found using 0.125 g of zein, 10 ml of 100% ethanol and 10 ml of 5% v/v Tween 20 aqueous solution. Light and scanning electronic microphotographs revealed the presence of spherical particles only in the final suspension (figure 3.2. and 3.3.). Thus, this formulation procedure was selected for further studies.

In this preparation of zein microspheres, zein is initially dispersed in 100% ethanol, a medium in which zein is insoluble, and a very coarse dispersion is formed as the mixture is gently stirred. With the addition of the stabiliser aqueous solution, some of the zein dissolves and/or wets. Before alcohol removal by rotatory evaporation, ethanol-aqueous proportion is of 50%, and Tween 20 is present in the medium. Although the exact solubility of zein in such media is unknown, we accept as true that some zein is dissolved and most of the zein remains undissolved, thus suspended. This certainty comes from our solubility studies (see chapter 2 for solubility discussion) were it was proved that surfactants were able to wet zein to certain extent and to increase slightly zein solubility in water; and that solubility of zein at 50% ethanol

aqueous solution was 0.8 mg/ml after equilibrating the media for 24 h. The undissolved zein is finely suspended and stabilised by the surfactant molecules in the medium and by the constant gentle stirring. When ethanol is removed by rotatory evaporation, zein slowly precipitates out in the form of microspheres. The present hypothesis is that undissolved zein before ethanol evaporation may be then used as beds or nuclei to allow more and more zein to precipitate on top. If all zein is dissolved previously (as when using DMSO), less zein may be able to precipitate in the form of spheres. The non-static conditions and the presence of stabilisers prevent particle aggregation to a certain extent, a phenomenon that is thermodynamically favourable, and a suspension of zein precipitated into microspheres can then be produced.

The rationale behind the precipitation of zein in water-ethanol mixtures must be remembered at this point. On their own, ethanol molecules form 'polymers' via intermolecular H bonds while water molecules form three-dimensional clusters. When mixed together at certain concentrations, they form a structure in which zein's elongated α-helical molecule can fit and zein dissolves (Dickey et al., 2001; Matsushima et al., 1997). When the water fraction of the water-ethanol mixture increases, the number of hydrogen bonds between water and ethanol molecules increases. This interferes with the optimal solvent structure needed to dissolve zein. As a result, zein molecules cannot fit in the water-ethanol network and zein precipitates out. Subsequently, as zein solubility decreases as ethanol evaporates, zein precipitates in the form of spheres.

Precipitation of zein into microspheres can also be attributed to the self-agglomeration of zein, as other authors stated (Korus et al., 2003; Kim et al, 2004). These authors hypothesise that zein molecules aggregate when producing films by cross-linking. They established two stages for agglomeration: at the first stage, aggregation takes place from individual molecules, and at a second stage, zein aggregates on smaller particles. Therefore, the concentration of zein in solution would play a central role in the first stage and size, instead, will become more important during the second stage.

Why do zein molecules self-agglomerate or precipitate out creating a protein matrix of spherical form? Kim and co-workers (Kim et al., 2004) attributed the aggregation of zein molecules when producing films to electrostatic interactions and they do not believe zein films are produced by molecular collision. It must be borne in mind that zein proteins are stored in maize protein bodies along with glutelins. Both zeins and glutelins create protein matrices by some kind of interactions that have spherical form (Kim et al., 2002; Forster and Wasserman, 1998). Hence, forming spheres may be the natural way of zein protein aggregations due to electrostatic interactions. The same electrostatic interactions among zein molecules may occur when zein precipitates out to produce microspheres in our method of production.

The zein microsphere suspensions stored at room temperature were found to sediment within 24 h. The sediments were loosely packed and easily resuspended by hand shaking, giving an idea of the flocculated state of the suspension. However, since disperse systems are thermodynamically unstable systems, additives were changed in order to produce a suspension of zein microspheres as stable as possible for long periods.

The additives tried were (i) a mixture of polysorbates, (ii) electrolytes, and (iii) a hydrophilic polymer plus a polysorbate.

Tween 80 and Tween 20 (which had been proved an optimal additive for producing zein microspheres) were the selected mixture of polysorbates. Following the previous formulation procedure, a pellet was collected and its microscopic examination revealed similar spherical particles in number and diameter than when using Tween 20 only. The capability of both surfactants to solubilise zein found in the solubility studies (see chapter 2) had no significant difference. It was not expected that their wetting effect on zein was additive, therefore the addition of Tween 80 would not improve the formulation of zein microspheres.

No pellet (i.e. no zein particles) was formed when electrolytes were used to create a stable suspension, neither potassium chloride nor sodium chloride, at any of the concentrations tried. The solubilisation zein by electrolytes was not of great magnitude, preliminary experiments were carried out in our laboratory (data not shown) and resulted in a very low solubility of zein protein in potassium chloride and sodium chloride aqueous solutions at concentrations 1, 5 and 10% w/v. Therefore, it is hypothesised that the effect of charge on zein protein creates electrostatic repulsion among molecules and they do not self-agglomerate to produce spheres

Instead, when a hydrophilic polymer such as PVP 40 was used together with the surfactant Tween 20, a pellet was collected and its microscopic examination showed microspheres. The suspensions of microspheres were found to take more time to sediment when stored at room temperature. PVP-40 is commonly used to prepare particulate systems. It is known that particle coalescence and/or coagulation can be overcome by stabilisers. The stabiliser may provide a thin protective layer around the particles and hence reduce the extent of their collision and coalescence (Arshady, 1990). That could be the effect of PVP 40, which may act sterically stabilising the particles, thus preventing as much as possible adjacent suspended particles from coming close enough to join.. Light and SEM microphotographs showed spherical, smooth surface microparticles, when adding two equal volumes (5 ml) of 5% v/v Tween 20 and 2% w/v PVP 40 aqueous suspension to the ethanolic dispersion of zein (0.125 g zein in 10 ml 100% ethanol). The latter additives were found to be the optimal ones to produce zein microspheres and selected for further experiments (figure 3.4.).

Zein microsphere suspensions using Tween 20 and PVP 40 as stabilisers sedimented rapidly and caused a clear supernatant and voluminous sediment, which was easily resuspended by hand-shaking at all times, therefore zein microsphere suspensions were flocculated. After storing suspensions for 20 days at room temperature, SEM photographs showed well-formed, spheres, which showed no porosity (figure 3.5.). However, size of these particles was clearly of greater magnitude, showing that some Ostwald ripening-like

phenomena had occurred. Ostwald ripening can be defined as the growth of large particles at the expense of small ones. It is a term generally used for the growth of drug crystals. Owing to the difference in dissolution rates of different size of particles and to the self-agglomeration of zein molecules and particles, it is believed that such phenomenon occurs in zein microsphere suspensions. It is known that shaking disintegrates aggregates into smaller particles, but only loosely bound aggregates, thus those aggregates found in flocculated suspensions. Repeated shaking would help in the formation of better-organised and more-densely packed aggregates (Kim et al., 2004).

In order to summarise, the optimal formulation to obtain smooth, spherical microparticles composed of zein protein was found to be the following: 1.25 g of zein were dispersed in 10 ml of 100% v/v ethanol, and then 5 ml of each stabilizer aqueous solutions (5% v/v Tween 20 and 2% PVP 40) were added, upon gently stirring. Then, ethanol was removed by rotatory evaporation and a fine zein suspension was obtained and centrifuged using a table-top centrifuge (13 000 rpm, 5 min) to harvest the zein microspheres formed.

When adding stabilisers to a pharmaceutical suspension, the concentrations of these are usually advised to be low, Nash (1988) advises a wetting agent concentration of no more than 0.1-0.2% of the final concentration and Billany (2001) suggests a concentration of wetting agents up to 0.1% approximately. Nevertheless, the final choice will depend on the properties of the material used and the class of suspension desired. Commonly, very low concentrations of wetting agents result in incomplete wetting, and great concentrations solubilise very fine particles, leading then to changes in particle size. For our formulation, low proportions of surfactants were not capable to sufficiently wet zein protein and high concentrations of additives had then to be used due to the extreme insolubility of zein protein in aqueous media. In other experiments (see next section 3.3.3.), the changes in particle characteristics by changing the additives concentration were explored. The ultimate effect of the high concentration of a polymer such as PVP 40 *in vivo* was considered in later experiments.

On the other hand, it was believed that a zein-ethanol-water mixture could produce zein particles by zein precipitation following ethanol removal without using any surfactant or additive. Controls without additives were undertaken, but a film was found at the bottom of the vial used, which was extremely difficult to resuspend. Whether the film was composed of zein microspheres was not investigated, since a suspension of zein microspheres was preferred to evaluate the characteristics of zein microspheres *in vitro* and *in vivo.*

3.3.2. Preparation of protein-loaded zein microspheres

Smooth, spherical, non-porous microparticles, apparently smaller in size than the blank microspheres produced before, were observed when ovalbumin was added to the ethanolic dispersion of zein protein (figure 3.6.). These zein-ovalbumin microspheres were prepared in the same way and using the same additives as blank zein particles, except for the addition of a small amount of sodium hydroxide in the ovalbumin-zein-ethanol mixture. It is known that albumins bind some cations (Purohit et al., 2003; Oshima and Nagasawa, 1973), such as sodium or calcium cations, and the solubility of zein in alkaline solutions has already been noted in chapter 2. It was then hypothesised that sodium hydroxide would act as a dispersing agent by creating some repulsion between the proteins (i.e. ovalbumin binding to sodium cation), thus preventing the aggregation of ovalbumin and zein that would otherwise occur upon the addition of the two proteins into 100% ethanol due to the insolubility of both proteins in such media. Certainly, it was found that a small amount of sodium hydroxide aqueous solution (0.3 ml of 1% w/v solution to 1.25 g of zein and 0.05 g of ovalbumin) increased the dispersibility of both macromolecules in the non-solvent ethanol. Hence, in the presence of sodium hydroxide, ovalbumin and zein remained dispersed in the liquid medium until ethanol removal; at this stage zein precipitated into microspheres and ovalbumin (which is water-soluble) may have become entrapped and/or adsorbed onto the zein microspheres.

Controls of the formulation procedure were performed using ovalbumin by itself. Ovalbumin microspheres were not found using this formulation procedure, but ovalbumin was found to aggregate into coarse, non-uniform assemblies.

The smaller size and the narrower size distribution of ovalbumin-loaded microspheres compared to blank zein microspheres could be due to the adsorption of ovalbumin onto newly formed particles. This adsorption leads to a reduction of the interfacial tension and to the stabilisation of the surface, thus, the existence of small particles. This effect of ovalbumin (i.e. reduced particle size when ovalbumin is loaded into particles) has been previously reported (Krishnan, 2004).

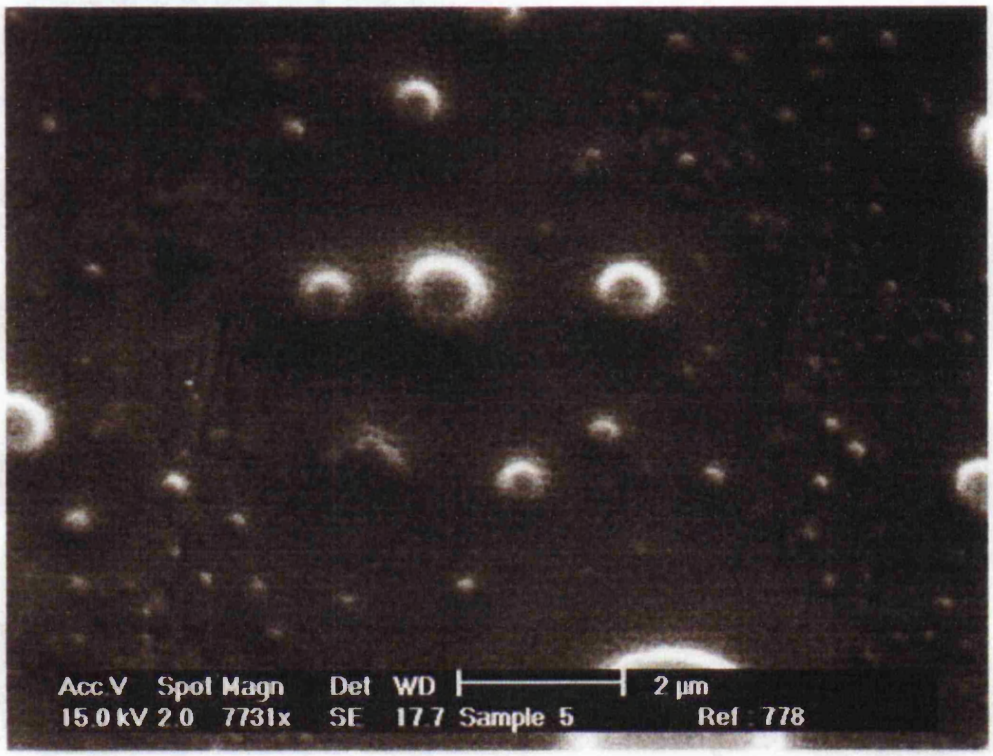


Figure 3.1. Zein particles formed by coacervation using DMSO as a solvent. 0.25 g of zein dissolved in 5 ml of DMSO, coacervates formed after the addition of 5% v/v Tween 20 aqueous solution, which acts as a non-solvent.

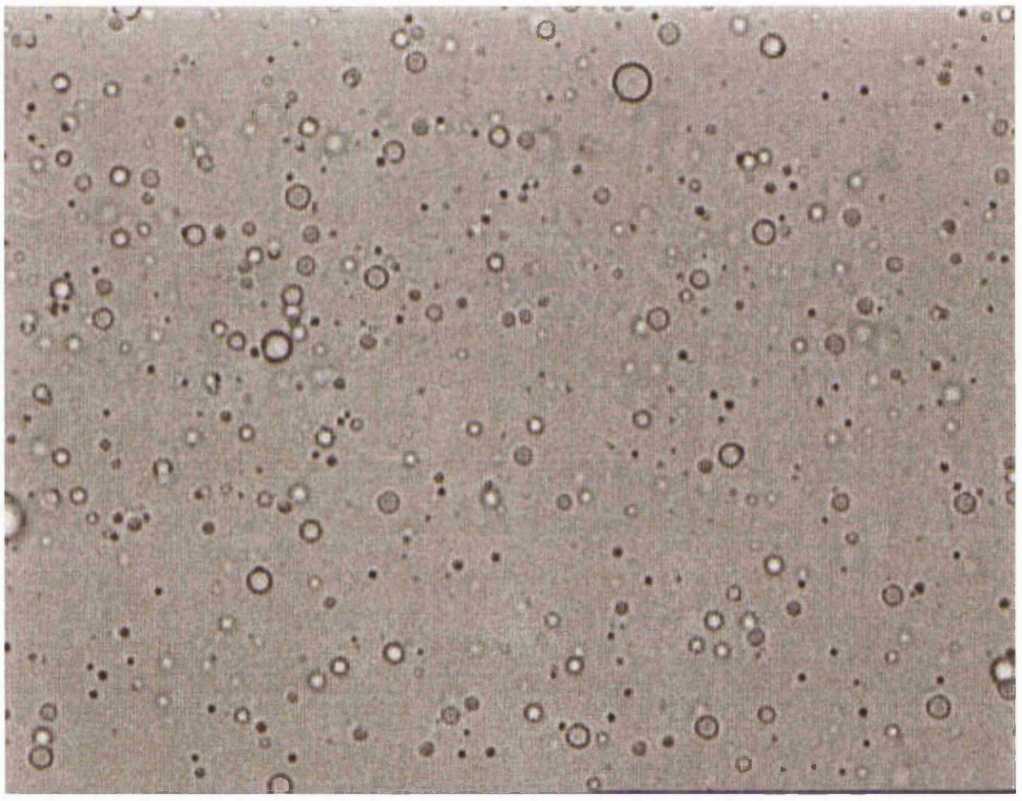


Figure 3.2. Light microscopy* photograph of zein particles formed by dispersing zein in 100% ethanol, adding a Tween 20 aqueous solution, and subsequently removing the ethanol to precipitate zein in the form of microspheres.

*Nikon Microphot FXA (Objective: x40)

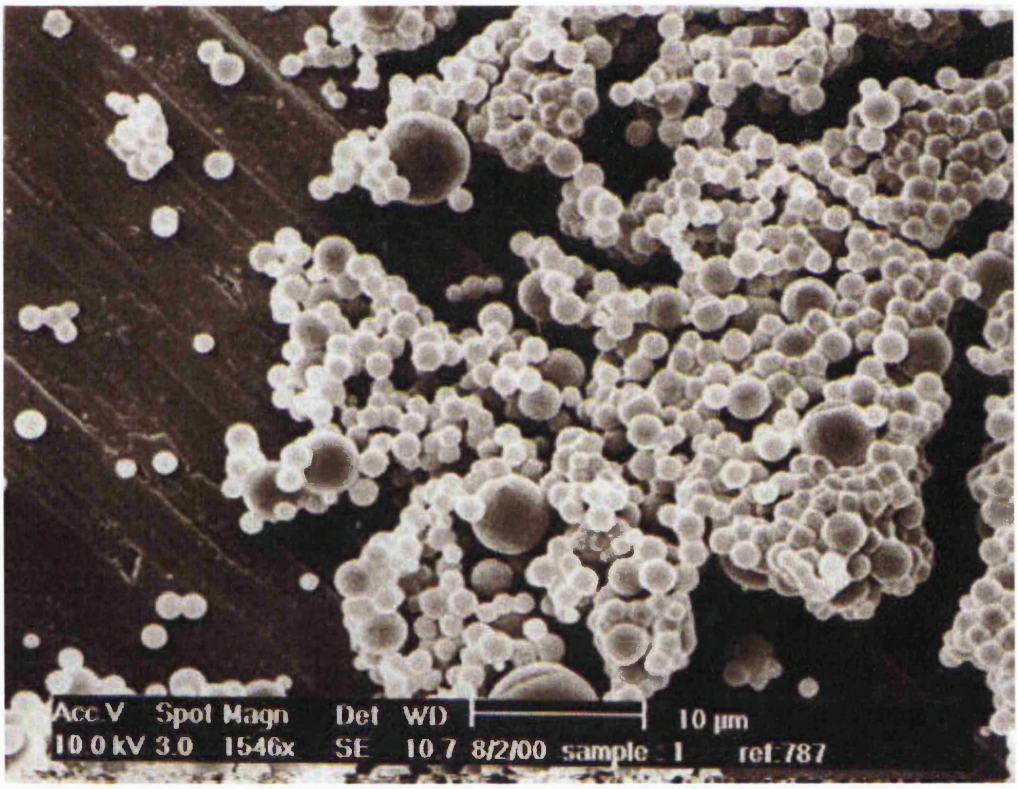


Figure 3.3. Scanning electron microphotograph of zein particles formed by dispersing zein in 100% ethanol, adding a Tween 20 aqueous solution, and subsequently removing the ethanol to precipitate zein in the form of microspheres.

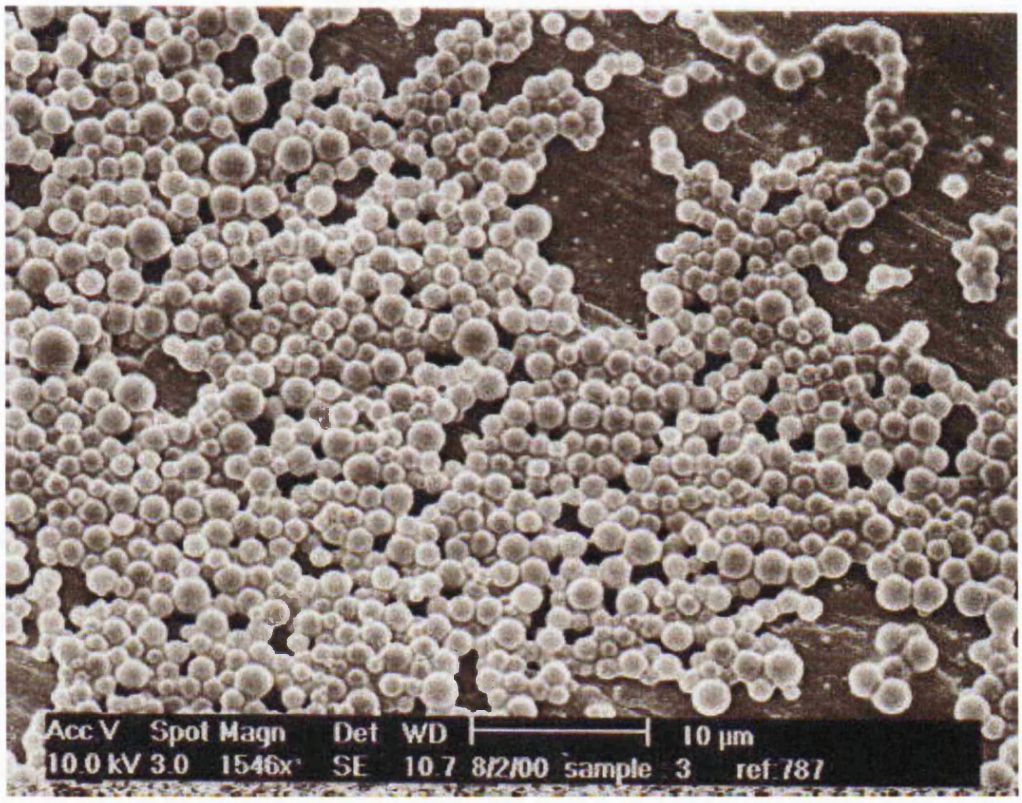


Figure 3.4. Scanning electron microphotograph of zein particles formed by dispersing zein in 100% ethanol, adding Tween 20 and PVP-40 aqueous solutions, and subsequently removing the ethanol to precipitate zein in the form of microspheres.

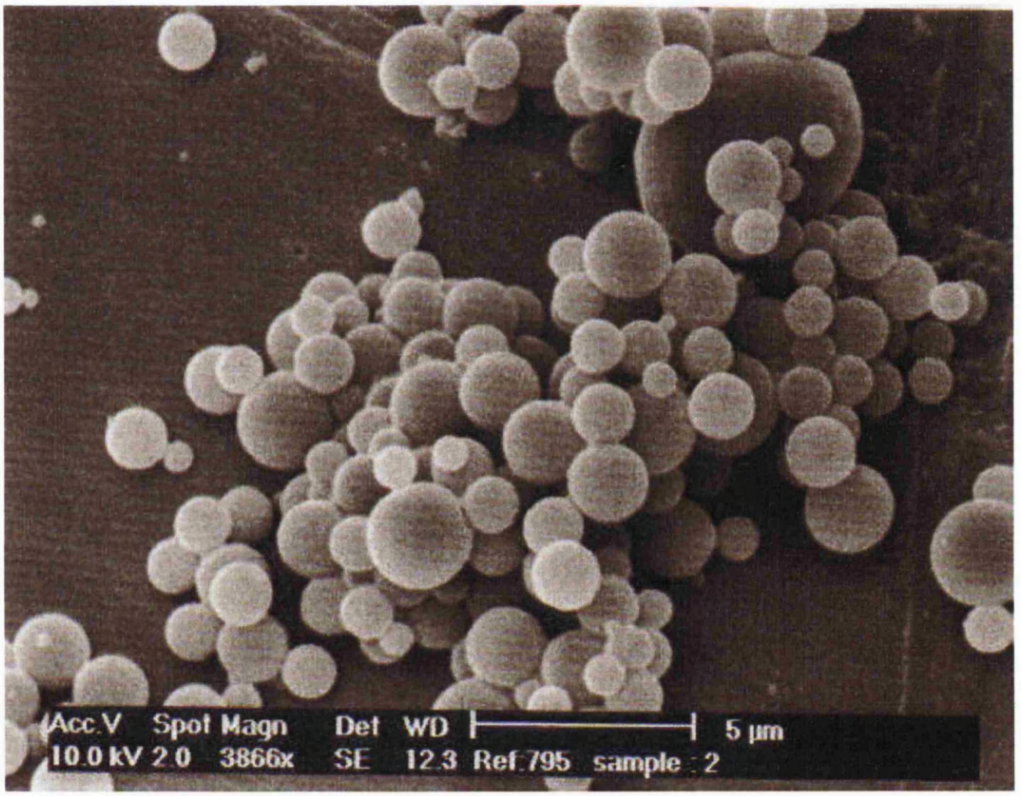


Figure 3.5. Zein microspheres showed in figure 3.4. following storage at room temperature for 20 days.

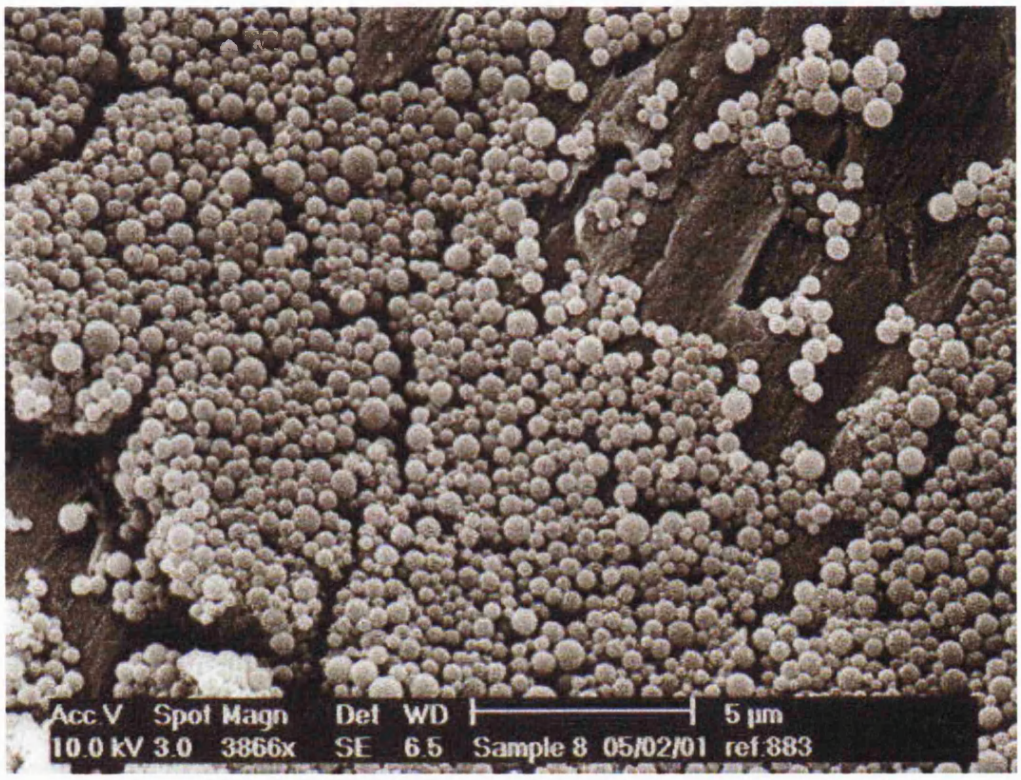


Figure 3.6. Zein-ovalbumin particles formed by dispersing zein and ovalbumin in 100% ethanol, adding 0.3 ml of 1% w/v sodium hydroxide aqueous solution, 5 ml of 5% v/v Tween 20 aqueous solution and 2% w/v PVP-40 aqueous solution, and subsequently removing the ethanol to precipitate zein in the form of microspheres.

3.3.3. Studies on zein microsphere formulation variables

3.3.3.1. Factorial design studies

Factorial designs are widely used to study the joint effect of several factors on a response. The difference between a factorial design and a one-factor-at-a-time method is that variables can be changed concurrently in the former. If the variables are found to act additively, the factorial design will provide an answer. On the other hand, when the variables do not act additively, the factorial design can detect the interactions. The purpose of using a factorial design was to achieve two of this study's objectives: to understand the underlying mechanisms of zein microsphere formation and to select a general method for zein-ovalbumin microsphere formulation.

The results found after performing factorial design studies were as follow and the corresponding values are shown in table 3.2.

- Size changes and changes in protein precipitated were the main effects found.

- Increasing sodium hydroxide concentration in the medium resulted in a decrease in particle size and in the mass of protein precipitated (figures 3.7. and 3.8.). Both effects may be due to an increase in zein solubility in aqueous media when the alkali content is increased. The less zein dissolved, the bigger the particle size is due to the self-agglomeration of undissolved zein molecules, which act as beds for dissolved zein precipitating on top when ethanol is removed. The more zein dissolved, the smaller the particle size is up to a certain extent, at which almost all zein is dissolved. At that point, no sufficient particles are able to form.

- Increasing the mass of zein and hence increasing zein/ovalbumin ratio resulted in an increase in microsphere size (figure 3.9.), but had no effect on the mass of protein precipitated. The latter was the same percentage at every level. Because more zein (rather than ovalbumin) is expected to precipitate when the mass of zein is increased, the lack of change in mass of protein precipitated may mean that less ovalbumin had precipitated. It is hypothesised that reduced ovalbumin precipitated might be due to increased zein-zein interactions compared to zein-ovalbumin interactions. However, at this point we must take into account the limitations of the BCA assay to quantify both proteins co-precipitating. An increase in particle size could be due to increased of self-agglomeration of undissolved zein molecules; and it could be due to a smaller amount of ovalbumin adsorbed on microsphere surface which would lead to an increase in interfacial tension and, therefore, to an increased particle diameter.

- Increasing Tween 20/PVP 40 ratio resulted in an increase in microsphere size, however, that size increase is not significant as it was the effect of sodium hydroxide (figure 3.10.). The effect of PVP 40 on particle size is related to particle stabilisation rather than particle division. At low PVP 40 concentration, the particles are very poorly stabilised and they tend to come together at a great rate. At higher concentration of stabiliser, the particles are better stabilised and their tendency to join is lowered. Therefore, increasing PVP 40 concentrations has only a limited effect in reducing particle size. On the other hand, Tween 20 acts as a stabiliser by decreasing interfacial tension and allowing some dissolution of the zein protein in the aqueous-ethanolic medium. It has been seen in the solubility studies (see chapter 2) that Tween 20 does not significantly improve zein solubility, and then does not significantly change particle size, in contrast to the effect of sodium hydroxide concentration on particle size. The ability of the surfactant to wet zein in the liquid is sufficient to allow the maintenance of zein dispersed in the medium only, which is

the requirement for the formation of zein microspheres in subsequent steps.

- In all the formulations examined, a smaller particle size was followed by a smaller size polydispersity; however, the size distribution seemed to be fairly broad for all the formulations. SEM showed that all particles were spherical.

- The zeta potential of all formulations was between -20 and 0, a range that is thought to be adequate for flocculated suspensions. For a physically stable suspension solely stabilised by electrostatic repulsion, a minimum of ± 30 mV zeta potential is required. In the case of a combined electrostatic and steric stabilisation, a range of ± 20 mV is sufficient (Müller, 1996; as cited in Müller et al., 2001). Other authors suggest that stability is achieved when the zeta potential is between ± 30 mV and ± 60 mV, and good to excellent physical stability is achieved when the zeta potential is between ± 60 mV and ± 100 mV. (Nash, 1988). There was no obvious relationship between particle zeta potential and the experimental variables.

- An interactive effect was established, i.e. microsphere formation cannot be explained without mentioning all the factors simultaneously. The proportion of sodium hydroxide to zein/ovalbumin ratio and the concentration of PVP 40 used were found to be very important. Microspheres were not formed in the presence of high concentrations of sodium hydroxide due to insufficient protein precipitation, unless the amount of zein and surfactant were high as well. On the other hand, microspheres were not formed at low sodium hydroxide concentrations because of macroscopic aggregation of zein and ovalbumin, unless the amount of zein was also low and the concentration of PVP 40 had been increased.

- Upon standing microsphere suspensions at room temperature for a week, size and size polydispersity were found to increase for all the formulations, up to the maximum limit of the instrumental used. Therefore, Ostwald ripenning-like phenomena seemed to occur for all samples due to particle decrease in solubility. It is believed that the bigger the particles, the less soluble they are in media (Augustine and Baianu, 1987). The microsphere suspension also sedimented upon standing at room temperature for a week, but it could easily be redispersed with gentle agitation, indicating the suspension was flocculated.

- No null cases were identified.

Variable level in coded form

Formulation No.	X_1	X_2	X_3	Size (nm)	Size polydispersity	Protein precipitated (%)
1	-1	-1	-1	559.1 ± 17.9	0.415 ± 0.509	56
2	-1	-1	0	1450.1 ± 209.3	1.0	63
3	-1	-1	1	*	*	*
4	-1	0	-1	658.4 ± 7.0	0.949 ± 0.08	62
5	-1	0	0	1575.2 ± 314.8	0.743 ± 0.44	81
6	-1	0	1	*	*	*
7	-1	1	-1	210.2 ± 3.0	0.097 ± 0.06	48
8	-1	1	0	991.8 ± 162.5	1.0	70
9	-1	1	1	1162.3 ± 101.1	1.0	85
10	0	-1	-1	501.8 ± 12.7	0.187 ± 0.08	51
11	0	-1	0	744.9 ± 11.2	0.796 ± 0.35	79
12	0	-1	1	*	*	*
13	0	0	-1	**	**	**
14	0	0	0	813.3 ± 16.2	0.696 ± 0.33	79
15	0	0	1	1246.2 ± 179.2	0.735 ± 0.45	85
16	0	1	-1	**	**	**
17	0	1	0	554.8 ± 8	1.0	79
18	0	1	1	684.3 ± 42.0	0.806 ± 0.33	86

19	1	-1	-1	**	**	**
20	1	-1	0	761.4 ± 98.9	0.962 ±0.6	73
21	1	-1	1	999.6 ± 138.1	0.896 ± 0.181	45
22	1	0	-1	**	**	**
23	1	0	0	388.8 ± 6.2	0.149 ± 0.140	79
24	1	0	1	832.5 ± 38.8	0.594 ± 0.36	86
25	1	1	-1	**	**	**
26	1	1	0	497.1 ± 1.5	0.223 ± 0.234	82
27	1	1	1	707.8 ± 10.2	0.780 ± 0.382	53

Coded values	Actual values		
	X_1	X_2	X_3
-1	0.25/0.05	10/1	2
0	0.125/0.05	5/2	1
1	0.0625/0.05	2.5/4	0.5

X_1 = Ratio of zein/ovalbumin (g/g)

X_2 = Ratio of Tween 20/PVP 40 (% v/v/% w/v)

X_3 = Concentration of sodium hydroxide (% w/v)

Table 3.2. 3^3 factorial design to study the effects of formulation factors on the characteristics of the resulting microspheres.

*Macroscopic aggregation of proteins observed.

**Absence of zein precipitation into microspheres.

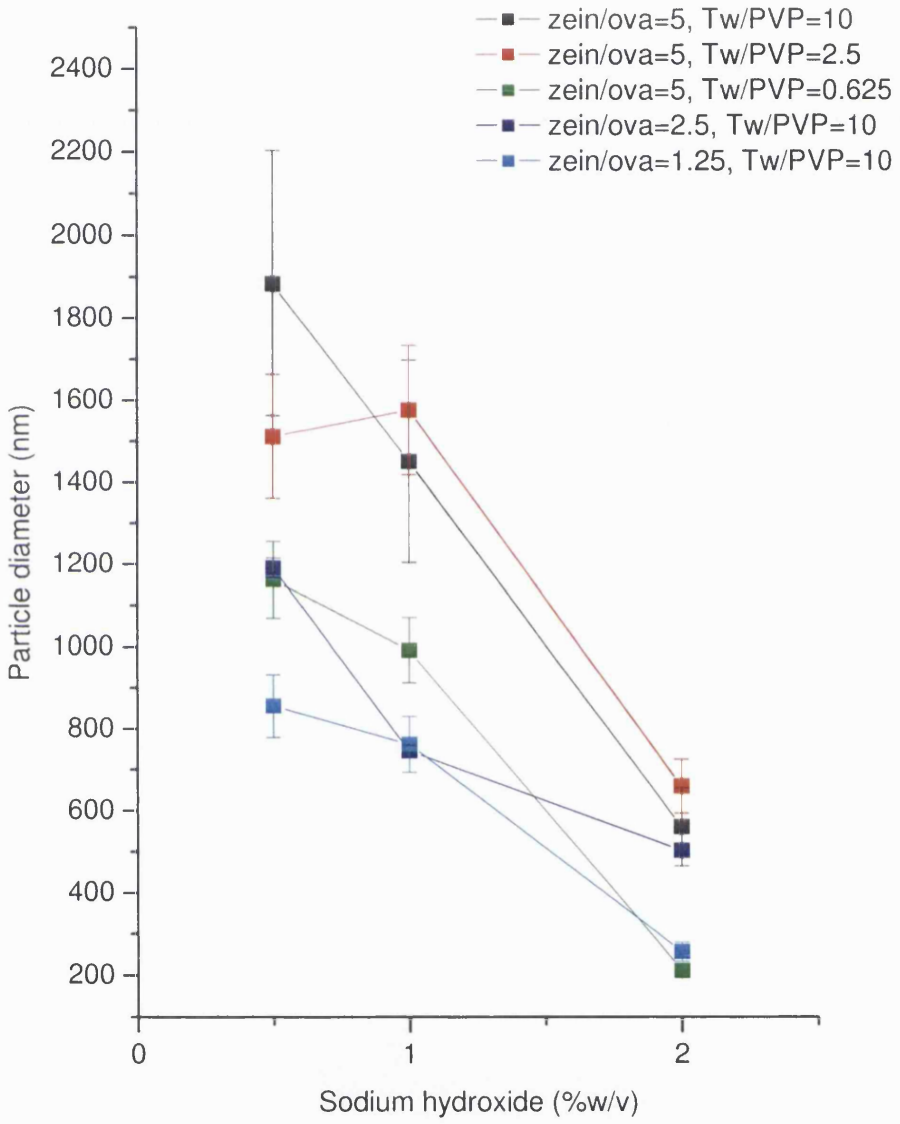


Figure 3.7. Effect of sodium hydroxide concentration on microsphere size.

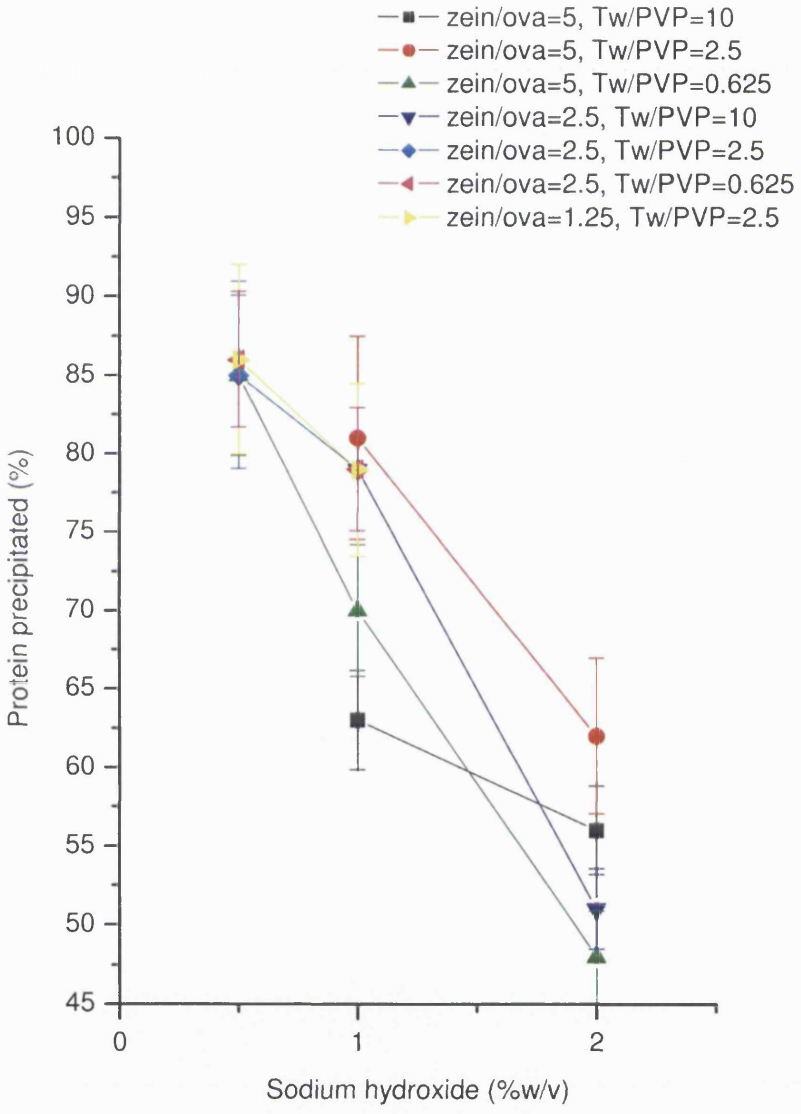


Figure 3.8. Effect of sodium hydroxide concentration on microsphere protein precipitated.

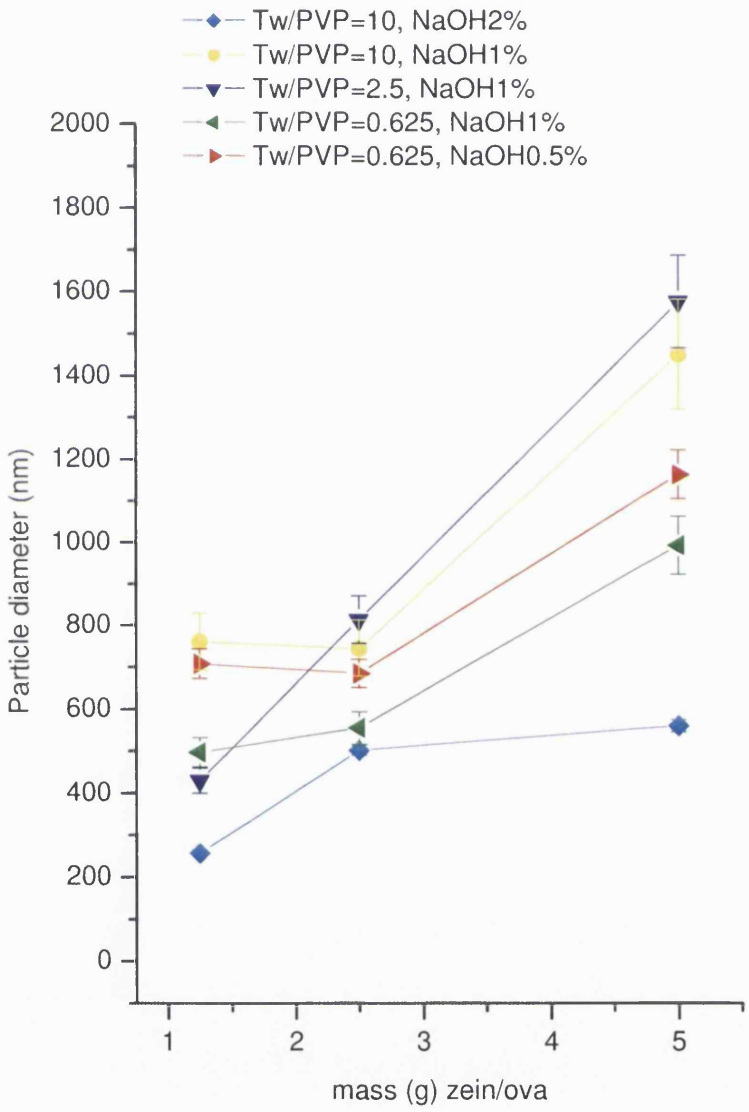


Figure 3.9. Effect of zein/OVA mass ratio on microsphere size.

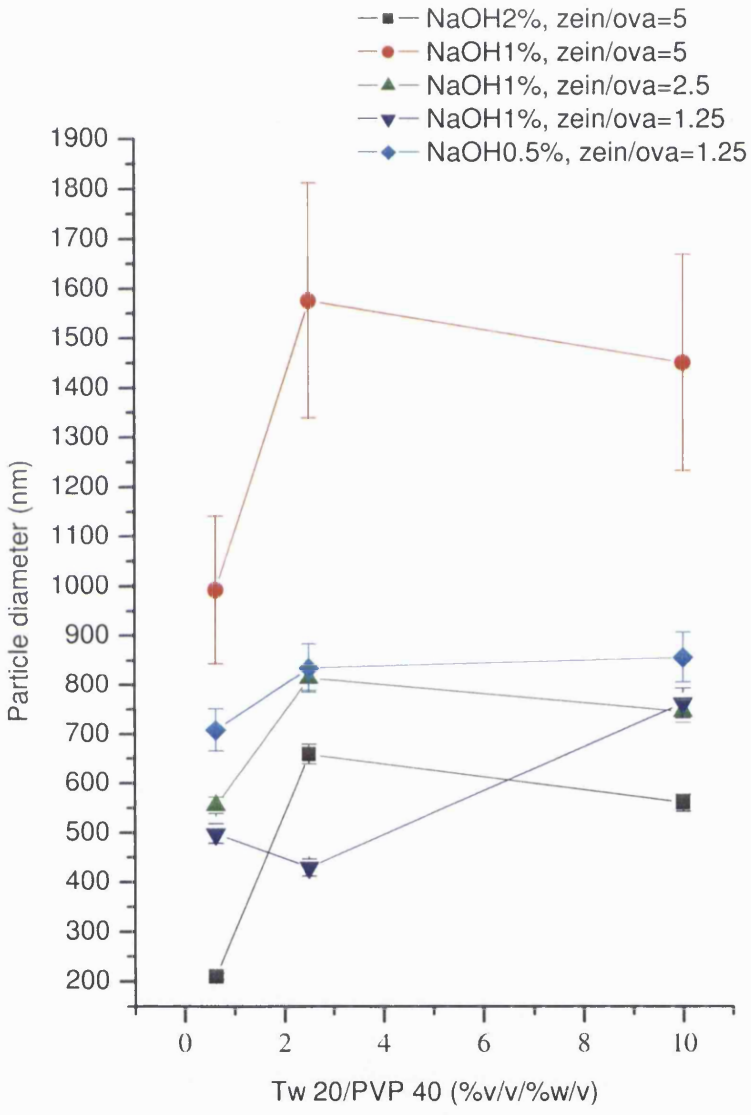


Figure 3.10. Effect of Tween 20/PVP 40 concentration ratio on microsphere size.

According to the results following factorial design studies, the concentration of additives were changed, thus the general method selected to produce zein microspheres, both blank and ovalbumin-loaded. In order to summarise, the optimal formulation to obtain smooth, spherical zein microparticles incorporating ovalbumin as guest molecule was found to be the following: 0.0625 g of zein and 0.05 g of ovalbumin were dispersed in 10 ml of 100% ethanol, and then 0.3 ml of a 1% w/v sodium hydroxide aqueous solution plus 5 ml of each stabiliser aqueous solutions (2.5% v/v Tween 20 and 4% PVP 40) were added, upon gently stirring. Then, ethanol was removed by rotatory evaporation and a fine zein suspension was obtained and centrifuged using a table-top centrifuge (13 000 rpm, 5 min) to harvest the zein microspheres formed. The zein-ovalbumin microspheres obtained are shown in figure 3.11.

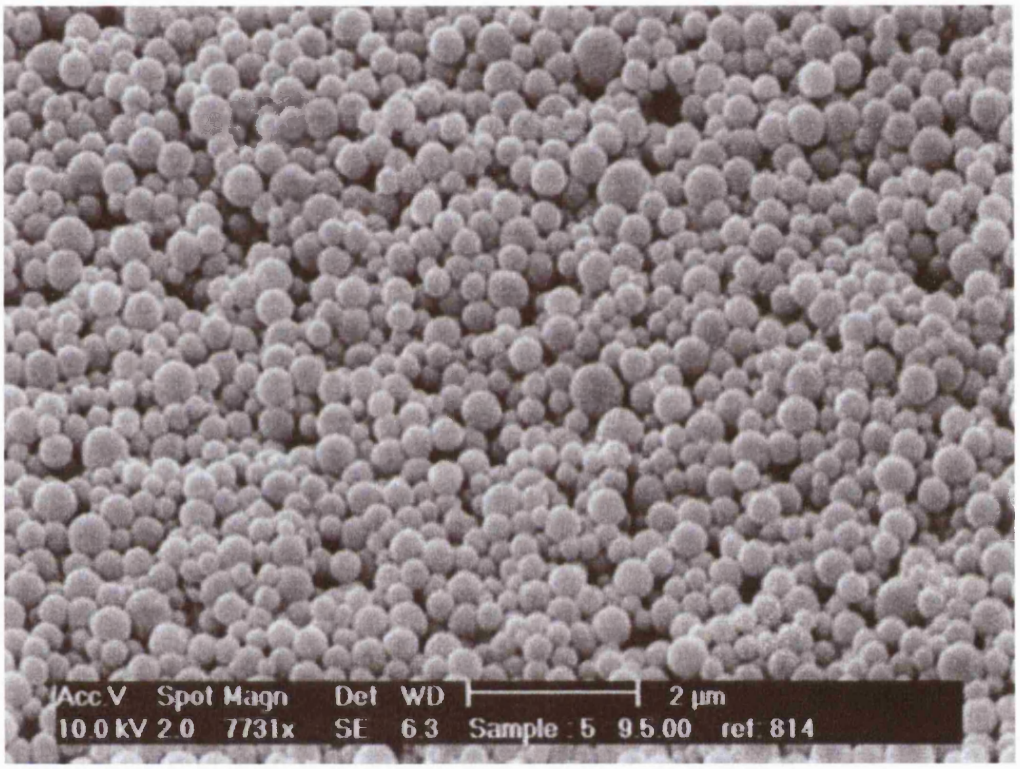


Figure 3.11. Zein-ovalbumin particles formed by dispersing 0.0625 g of zein and 0.05 g of ovalbumin in 100% ethanol, adding 0.3 ml of 1% w/v sodium hydroxide aqueous solution, 5 ml of 2.5% v/v Tween 20 aqueous solution and 5 ml of 4% w/v PVP-40 aqueous solution, and subsequently removing the ethanol to precipitate zein in the form of microspheres.

3.3.4. Studies on zein microsphere formuiation

3.3.4.1. Standardisation of formulation procedure

There is evidence for an empirical relationship between average particle size and manufacturing parameters (Arshady, 1990). Therefore, ethanol evaporation conditions and stirring *versus* sonication before and after ethanol removal were studied in an attempt to improve particle size distribution, which was found to be broad in previous experiments. The purpose of these experiments was to standardise the manufacturing procedure, thus to select a general procedure to produce zein microspheres once again.

On the other hand, it is widely stated that the size of final particles depend pretty much on stirring for those particulates prepared by solidification of droplet emulsions (Tice and Gilley, 1985). There is a question mark, however, on whether stirring has a significant effect on zein microsphere size as well.

Ethanol was removed until the initial volume was reduced by half. The initial vacuum pressure when evaporating ethanol was decreased to 90 mBa, which was the pressure at which liquid evaporation could be observed. Since vacuum pressure to evaporate water at 40 ºC is 72 mBa, at 90 mBa only ethanol is supposed to be removed. By keeping this pressure value constant, the total volume was reduced to 50% in 15 min.

In preceding experiments, ethanol evaporation pressure and time were not controlled. Water temperature was then kept at 25 ºC (considered room temperature). Then, vacuum pressures were higher than 90 mBa and evaporation times resulted to be fairly long. The microspheres produced by controlling ethanol evaporation parameters had an average diameter of 918.3 ± 37.08 nm and a size polydispersity of 0.421 ± 0.19. These results represent an increase in particle size compared to the preceding ones. It is hypothesised that this increase may be due to the reduced speed used in rotating the

suspension and to the reduced time used in evaporating the ethanol, in consequence, due to the reduced time and rotation while microspheres are being formed.

Before solvent evaporation, some particles could be observed under light microscopy, probably due to undissolved protein, as previously explained. The average size of these particles was 158.9 ± 2.8 nm and the size polydispersity was 0.194 ± 0.07. When the protein suspension was bath-sonicated for 5min before ethanol removal, the particle average size was 172.2 ± 4.3 nm and the size polydispersity was 0.188 ± 0.13. The difference on particle size with or without sonication was then not significant at this stage. This showed that sonication before ethanol evaporation, thus before microsphere formation, do not change the size of undissolved zein, which acts as nuclei for the precipitation of zein in the form of spheres when ethanol is being removed.

When the protein suspension was left stirring for 1 h before alcohol evaporation, average particle size and size distribution were not significantly different from the previous ones (mean size: 936,6 ± 8.02 nm, size polydispersity: 0.566 ± 0.03).

The size of stirrers and vials to stir the proteins magnetically in the liquid mixture were carefully selected, because it was believed that reducing the turbulence in the liquid would increase the uniformity of the mixing force throughout the suspension mixture. This was supposed to enhance the stability of the suspension and perhaps the particle uniformity. However, since the crucial particle formation was found to occur upon ethanol removal, the final size distribution of the particles was not greatly influenced by the stirring before ethanol evaporation. Similarly, sonication (before and after ethanol evaporation) did not have an effect on the average size of the resulting zein microspheres.

Nevertheless, when examining size distribution, a broad distribution. A broadly distributed population of particles could also be measured when protein

suspension was bath-sonicated for 5 min before ethanol evaporation, and again no significant changes were seen in average diameter and polydispersity. Sonication or stirring for 1 h before alcohol removal did not reduce protein nuclei and, consequently, did not have an effect on final particle average size. However, the resulting broad distribution of sphere size revealed a different effect on final particle size. Since solutions need to equilibrate, more zein possibly becomes soluble upon stirring or sonication and less zein nuclei are suspended before ethanol evaporation within certain time or with strong agitation (i.e. sonication). When removing ethanol, some zein would precipitate on top of undissolved zein nuclei and some would not, creating then different sphere sizes.

Instead, when aqueous microsphere suspension (i.e. after ethanol evaporation) was bath-sonicated for 5min, average microsphere size was found to be smaller than without sonicating (average particle size: 511.6 ± 30.59 nm, size polydispersity: 0.525 ± 0.41) and distribution seemed to be narrower. However, size polydispersity was still a high value due to a small population of particles under 100 μm of diameter that could be observed in one out of two batches of zein microspheres. This population was only observed occasionally when microsphere suspensions were not bath-sonicated.

Since the only noticeable difference when sonicating microsphere suspensions was a reduction in size, this procedure was not selected to form protein-loaded zein microspheres, due to the possibility of guest protein denaturation. It is hypothesised that the population of particles having a diameter under 100 μm is related to ovalbumin denaturation while sonication, an issue that will be discussed in chapter 4. On the other hand, particles of approximately 1 μm, as the ones produced by this method, have an adequate size to be administered by several routes, which will be the subject of further discussion in chapters 4 and 5.

As mentioned above, Kim et al. (2004) stated that aggregation of zein molecules in solution was not affected by stirring when zein was cross-linked to form films. Similarly, stirring does not seem to play a crucial role in the formation of zein microspheres by the technique used in this study.

Zein microspheres were then produced by slight stirring while suspending proteins in the selected media and, after that, by evaporating the alcohol present at 90 mBa and 40 ºC for 15min. The moment of ethanol rotatory evaporation is the crucial moment of zein microsphere formation, thus temperature, pressure, time, and rotation are crucial variables affecting final zein microsphere characteristics, such as particle size and size distribution.

3.3.4.2. Scaling-up of formulation

When batches of ovalbumin-loaded zein microsphere suspensions were of 40 ml, thus containing 0.25 g of zein and 0.2 g of ovalbumin, size was found to increase and size polydispersity was the same range as for 10 ml microsphere suspension batches. Examination of size distribution showed one population of particles principally. Average microsphere size was 1585.7 ± 72.03 nm, size polydispersity being 0.603 ± 0.44. This result was expected due to the increase in protein mass. A similar result had been found when zein mass was increased during factorial design studies.

3.3.4.3. Storage of zein microsphere suspension

After three freeze-thaw cycles at –70 ºC, the morphology of the particles was found to be spherical, smooth and non-porous as it was before storing the suspensions (figure 3.12.). Average microsphere size did not significantly change. However, size polydispersity did increase slightly, even though in a non-significant manner, and again a small population of particles under or about 100 nm was observed in the size distribution. The appearance of this

population again may suggest other possible rationalization apart from the formation of zein particles, which will be further discussed in chapter 4. Briefly, it is hypothesised that denaturation of ovalbumin during freeze-thaw cycles is related. However, it is also believed that increase in size polydispersity during freeze-thaw cycles is due to Ostwald ripening-like phenomena too, which was found to occur in microsphere suspensions before.

Harvested pellet had been proved to contain the microspheres. Then, after separating this pellet by centrifugation, microspheres were oven-dried and pulverised in a mortar. SEM microphotographs still showed smooth, non-porous spherical particles, in the same range of sizes as previously (figure 3.13.). The present experiment was carried out after centrifugation of blank zein microsphere suspensions in a table-top centrifuge and after sucrose gradient centrifugation of ovalbumin-zein microsphere suspensions. Sucrose gradient centrifugation allows the separation of unloaded ovalbumin from ovalbumin-loaded zein microspheres. Since oven drying wanted to be a mean of final storage for zein microspheres, this way of centrifugation was undertaken at this point. Nevertheless, this method of separation will be thoroughly discussed in chapter 4.

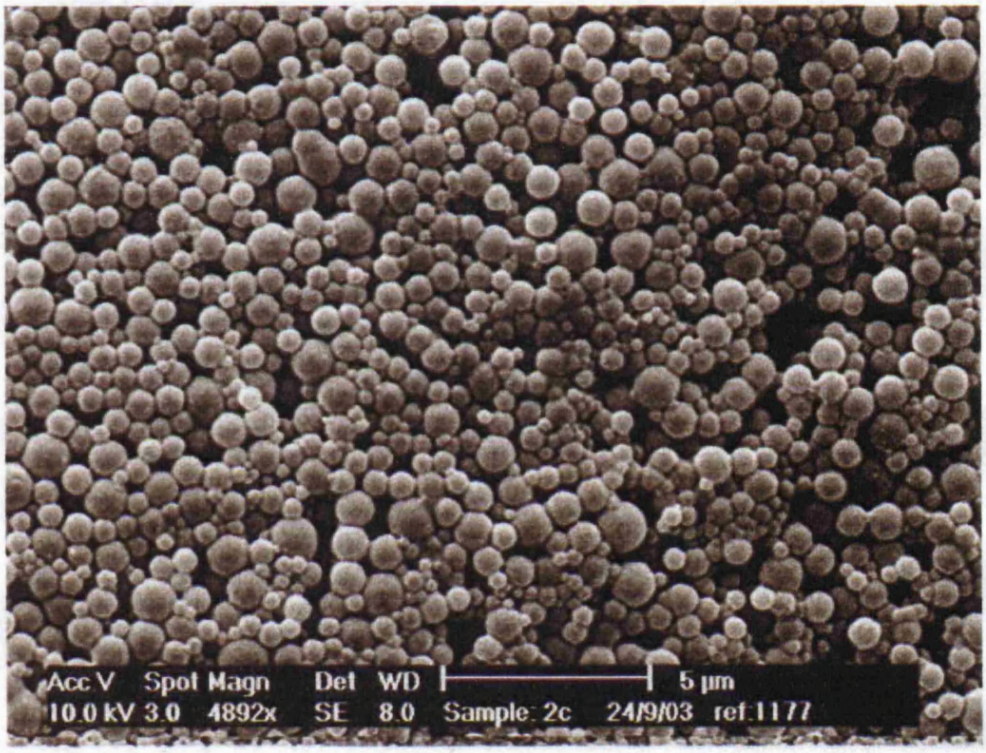


Figure 3.12. Zein microspheres following one freeze-thaw cycle.

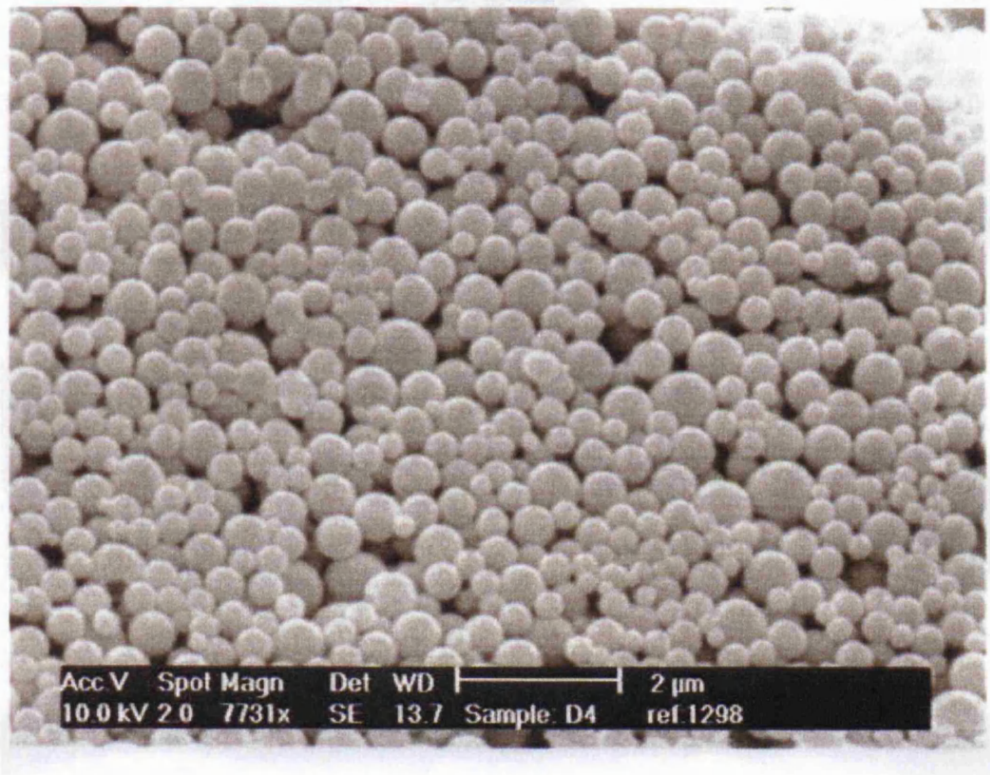


Figure 3.13. Zein microspheres harvested by centrifugation using a table-top centrifuge, oven dried overnight, and pulverised in a mortar.

3.4. Conclusions

In this study, microspheres as vaccine delivery systems were prepared using zein as the polymeric material. Following results reported in chapter 2, zein was allowed to precipitate in a controlled manner in an ethanol-water mixture, to which additives were added to produce a flocculated suspension containing zein precipitated into microspheres.

For a given protein or polymer, a particular liquid may be a solvent, a precipitant or neither, depending on the difference between the respective solubility parameters. For zein, neither ethanol nor water are solvents. Both precipitate zein on their own. On the contrary, their mixture can act as a solvent for zein. Avoiding complete zein dissolution and controlling its subsequent precipitation when changing ethanol-water mixture proportions, raises in zein precipitation, thus in the formation of zein microspheres.

We hypothesised that zein precipitates in the form of microspheres because zein molecules self-agglomerate producing matrices of spherical form in the endosperm of corn, thus spherical particles is a natural way of aggregation for zein.

To the ethanol-water mixture, we added different concentrations and types of stabilisers in order to maintain zein microspheres in a suspension as stable as possible. We conclude that these additives are necessary, even though precipitation of zein into microspheres most probably would occur without the additions of surfactants or polymers. The obtained zein microsphere suspensions were found to be flocculated and microsphere did not morphologically change over long periods. However, due to the inherent instability of disperse systems, growth in microsphere size and size distribution (i.e. Ostwald ripening-like phenomena) occurred even in the presence of stabilisers.

When investigating the mechanisms of zein microsphere formation, we found that the moment of zein precipitation into microspheres was during ethanol removal. The latter was achieved by rotatory evaporation. Standardisation of the evaporation conditions (as well as other parameters of manufacturing, such as stirring) was then attempted. However, a broad microsphere size distribution was also found after standardisation of the microsphere preparation procedure. We conclude that a more accurate control of ethanol evaporation time, pressure, and temperature as well as environmental conditions (such as laboratory temperature) and timing could reduce size polydispersity only to a certain extent. Whether this broad size distribution is not convenient for the *in vivo* activity of antigen delivery systems needs to be researched.

In order to understand the role of each additive added and how they affect the final microsphere characteristics, factorial design studies were carried out. The latter studies and the standardisation of the microsphere preparation procedure, led to the selection of a general method of zein microsphere production, for both blank and ovalbumin-loaded zein microspheres.

Freezing the microsphere suspension did not affect the microsphere morphology according to SEM photographs, nevertheless freezing did not avoid Ostwald-ripening phenomena. Hence, we conclude that harvesting zein microspheres by suspension centrifugation and oven drying them overnight could be a mean of microsphere storage. We conclude that once the potential of zein microspheres as vaccine delivery systems are explored, other ways of final storage can be investigated, such as freeze-drying.

Finally, we conclude that the studies reported in this chapter can provide an understanding of the formulation process and the effects of the experimental conditions on microsphere formation and properties. Such an understanding is an invaluable tool that can be used by formulation scientists to produce zein microspheres suitable for different purposes.

Since the method of preparation of zein microsphere suspensions was proved to be simple and rapid, zein microspheres can be further investigated as drug and/or vaccine delivery systems.

Chapter 4.

In vitro characterisation of zein microspheres

4.1. Introduction

The selected formulation of zein microspheres reported in chapter 3, both blank and ovalbumin-loaded, was further characterised. This characterisation is reported in the present chapter.

Even though high temperatures and/or sonication, among other hazards, were avoided when preparing zein microspheres; whether ovalbumin integrity was maintained during microsphere manufacture was not known. Since ovalbumin was used as a model protein antigen, we believed that structural integrity was of importance, but antigenicity and/or bioactivity of ovalbumin had to be tested later. However, some studies on protein integrity were carried out at this stage.

The amount of guest molecule, ovalbumin, incorporated to the formulation was elevated in order to maximise the entrapment. Investigations on protein microsphere composition and loading efficiencies were then undertaken. We hypothesise that microsphere matrix is composed of zein and ovalbumin; however, whether ovalbumin is entrapped within the matrix, adsorbed, and/or covalently bound to the surface is not known. It is hypothesised that ovalbumin became entrapped within zein microsphere matrix while zein precipitated into microspheres. That is why, the term loading is used instead entrapment or adsorption, and why ovalbumin-loaded zein microspheres are called zein-ovalbumin in this study too.

Microsphere matrix was supposed to be composed of zein and ovalbumin, thus ovalbumin (i.e. model protein antigen) had to be released from microsphere matrix. The release of drugs entrapped within microspheres depends on a number of factors including size and density of the particle, loading and distribution of the drug within the particle, interactions between the drug and the matrix, type of matrix, release environment, for example the presence of enzymes, etc. The most common mechanisms of drug release from polymeric micro or nanoparticles are (i) liberation due to polymer erosion

and/or degradation, (ii) drug-diffusion through matrix pores and (iii) release from polymer surface (Courvreur and Puisieux, 1993). Release *in vitro* experiments are reported in this chapter as well.

We hypothesised that one of the advantages of using zein was its biodegradability *in vivo*. Degradation of zein would in turn result in release of ovalbumin. It is then important to understand the drug release profile and associated mechanism from zein microsphere delivery systems before one can attempt to manipulate them. Given that diffusion of proteins and peptides, such as ovalbumin, through hydrophobic materials like zein is supposed to be negligible (Bezemer et al., 2000) and the fact that zein microspheres prepared in our laboratory showed no visible surface porosity, we hypothesised that ovalbumin release might be mainly governed by microsphere surface erosion or matrix degradation. In an attempt to establish the relationship, if any, between microsphere degradation and drug release, we investigated the degradation and erosion of zein microspheres, in the presence and absence of enzymes.

To date, controlled release systems of zein are limited. Some examples include tablets produced by direct compression of spray-dried drug-zein particles or by direct compression of a physical zein-drug mixture (Katayama and Kanke, 1992), zein microspheres produced by cross-linking zein with glutaraldehyde (Matsuda et al., 1989) and zein microspheres produced by phase separation (Mathiowitz et al., 1993). The tablets did not disintegrate in an acid milieu and showed zero-order drug release following a brief burst effect, which was large when a physical drug-zein mixture was directly compressed (Katayama and Kanke, 1992). The presence of pepsin in the medium facilitated drug release. From this, the authors concluded that release of drug from directly compressed tablets depended on zein solubility and on the nature of the molecular interactions between zein and the drug. Drug release from the two zein microspheres formulations (Matsuda et al., 1989; Mathiowitz et al., 1993) was extremely low; however drug release from glutaraldehyde cross-linked microspheres was increased in the presence of the enzyme actinase E.

For the studies reported in this chapter, a number of techniques were used. The principles of them are briefly explained in the following sections.

4.1.1. Polyacrylamide gel electrophoresis

When centrifuging the suspensions obtained following ethanol removal, we harvested a pellet, which was found to be composed of microspheres under microscopic examination. When blank zein microspheres were produced there was no doubt about the protein composition of microspheres, however, whether ovalbumin was present in those microspheres or not (i.e. present in the pellet or in the supernatant following centrifugation) was not known. Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to investigate the protein composition of microspheres when ovalbumin was incorporated as a guest molecule. On the other hand, SDS-PAGE would give us information about ovalbumin integrity.

The general principle of electrophoresis is the following: under the influence of an electrical field, charged particles dissolved or dispersed in an electrolyte solution migrate in the direction of the electrode bearing the opposite polarity. Due to different physico-chemical properties, different macromolecules of a mixture (e.g. zein and ovalbumin in microsphere suspensions) will migrate at different velocities and will be then separated into different fractions.

Polyacrylamide gel electrophoresis, a technique which stationary phase is a gel made of a mixture of acrylamides, is appropriate to identify the heterogeneity of proteins in pharmaceutical preparations and to estimate protein molecular masses. When using sodium dodecyl sulphate (SDS), the technique is named denaturing polyacrylamide gel electrophoresis. SDS is an anionic detergent that is able to dissociate proteins before they are loaded in the gel to carry the analysis. Denaturing gel electrophoresis can be carried out under reducing conditions (i.e. reducing disulphide bonds) or non-reducing conditions. Proteins are ultimately detected in the gels using staining solutions.

4.1.2. Fourier transform infrared spectroscopy

Structural changes in secondary structure of both zein and ovalbumin during microsphere manufacture were investigated using Fourier transform infrared spectroscopy (FT-IR). However, we believed that this technique would not give us information about changes in ovalbumin immunogenicity (i.e. changes in ovalbumin epitopes) and the latter was tested *in vivo* later.

Infrared spectroscopy measures the vibrational energy changes of molecules. The vibrations oscillate with frequencies that comprise the infrared spectral region. It is a technique widely recognised for providing information regarding protein secondary structure (Cooper and Knutson, 1995).

4.1.3. Capillary electrophoresis

Heterogeneity of zein protein has been well stated in chapter 1. To investigate the degradation of zein microspheres, related to the *in vitro* release of ovalbumin, it was decided to investigate the degradation of zein polypeptides. As zein is not a unique protein, zein polypeptides were first separated.

A variety of separation techniques has been used by researchers to separate and identify proteins and other analytes from mixtures. Capillary electrophoresis (CE) is a relatively new method that provides an efficient separation of peptides and proteins. It is an electrophoresis technique, which principle has been explained above, performed in a capillary tube. CE was selected for our studies, since several types of electrophoresis has been successfully used to identify zein polypeptides in the past (see chapter 1).

A typical capillary electrophoresis system consists of a fused-silica capillary that connects two buffer reservoirs, a power supply, and a detector. Molecules are separated as an electrical force drives them at different rates through the capillary filled with electrolyte solutions. The electrolyte solutions, buffers, and

additives will be chosen depending on the nature of the molecules to be separated. Depending on the types of capillary and electrolytes used, there are several methods of capillary electrophoresis. For our experiments, Capillary zone electrophoresis (CZE) was selected due to it is the simplest form of CE. Recent methods for the separation of peptides using capillary electrophoresis are reviewed by Kašička (2001).

4.1.4. Particle size and zeta potential analyses

Particle size and zeta potential parameters are used here to investigate the characteristics of the zein microsphere suspensions, as it is believed that these characteristics lead to suspensions instability or stability.

There are several ways of determining particle size. A method commonly used is Photon correlation spectroscopy (PCS). This method determines particle size in terms of particle diameter; and size distribution using a parameter named polydispersity index. PCS is a laser light scattering technique. Briefly, the apparatus consists of a laser, a temperature controlled sample cell and a photomultiplier for the detection of the light scattered at a certain angle (90º for our measurements). The signal is transferred to a correlator for calculation of the correlation function. Size is obtained from the correlation function by using algorithms in the PCS software (Malvern website).

In pharmaceutical suspensions, electrical charges are developed at the interface between the dispersed phase and the aqueous medium. These electrical charges play an important role in the physical stability of the disperse systems. If the dispersed systems are intended to use as delivery systems (like zein microsphere suspensions), the therapeutic activity will be affected by these surface charges. Zeta potential serves as an important parameter in characterising the electrostatic interactions between particles in dispersed systems and the properties of the dispersion. As mentioned in chapter 3, we used zeta potential results as a measurement of the suspension flocculation. Zeta potential was measured using laser Doppler velocimetry (LVD)

technique. LVD measures the velocity of particles moving through a fluid in an electrophoresis experiment. Small particles are illuminated by a laser beam and the light scattered to various angles is compared to light in a reference beam to determine the Doppler shift of the scattered light. For the majority of samples, the mobility of particles is directly related to the zeta potential of the particles (Malvern website).

4.1.5. Electron microscopy

Electron microscopy is used in a variety of ways in biology and material sciences. Commonly, it is used to measure particle size too. Since the size distribution of zein microsphere seemed to be fairly broad, we relied on PCS measurements for particle size and used electron microscopy to examine zein microsphere morphology. In addition, erosion of zein microsphere surface was also followed under scanning electron microscope examination.

The two basic electron microscopes are the transmission electron microscope (TEM) and scanning electron microscope (SEM). Proper sample preparation is essential for optimal image quality when using both microscopes. The main difference between the two instruments is the process of image formation. Electron microscopes function exactly as their optical counterparts except that they use a focused beam of electrons instead of light to image the specimen and gain information as to its structure and composition (Flegler et al., 1993).

4.1.6. Densitometry

Densitometry may be defined as measuring the optical density of a substance by shining light on it and measuring its transmission. After visualising the separated components within an electrophoretic gel using stains, bands can be measured to determine the amount of the relative purity of separated molecules (Scion website). Uncalibrated densitometry is rarely adequate.

Calibration in units of concentration, using external standards, provides the most accurate concentration measurements. As external standards, a set of protein standards on a gel can be used.

In our studies, we used calibrated densitometry to quantify the amount of ovalbumin released *in vitro* following incubation of ovalbumin-loaded zein microspheres and to study ovalbumin loading efficiencies. We understand that densitometry is a semi-quantitative method only, however, approximate values were given, which were assumed to be true due to technical limitations.

4.1.7. Density centrifugation

The density gradient centrifugation may be defined as a method of separating macromolecules by their (i) differential rate of sedimentation in a centrifugal gradient, or (ii) by their differential buoyancy in a density gradient. The density gradient centrifugation differs from the classical centrifugation (differential centrifugation, where a suspension is separated in two fractions: pellet and supernatant) because it allows separation of many or all components in a mixture and allows measurements to be made. However, both kinds of centrifugation are based on Stokes' law.

Density gradients are very useful in separating a large array of biological material, such as proteins. The most common way of performing density gradient centrifugation is named rate zonal centrifugation. It is carried out creating a density gradient. If the sample has a density that is greater than all the layers in the solution, it will be poured on top of the density gradient and spun. Over a specific length of time, the sample will separate and stratify according to weight and density. Over a prolonged period of centrifugation, the sample will ultimately settle to the bottom. However, when withdrawn at the appropriate time, the stratification can be achieved. Separation is based on mass (i.e. larger particles will sediment faster).

Sucrose density gradient centrifugation was used in this study to separate ovalbumin loaded from ovalbumin unloaded, since it has been used to separate fractions containing ovalbumin in the past (Suzuki et al., 1997), and sucrose additive is supposed to have little effect on macromolecules.

4.2. Experimental

4.2.1. Chemicals

Zein, ovalbumin (Grade II), sodium dodecyl sulphate (SDS), polyvinyl pyrrolydone MW 40 000 (PVP-40), sucrose, phosphate buffered saline tablets, Tween 20, pepsin A, pancreatin, monobasic potassium phosphate, sodium acetate, and 2-mercaptoethanol were purchased from Sigma, UK. Sodium hydroxide, sodium chloride, and potassium chloride were obtained from BDH Laboratory Supplies, UK. Coomassie Blue G-250 stain, premixed electrophoresis buffer, Laemmli sample buffer, Tris-HCl ready gels, and protein standards were obtained from Bio Rad, UK. Urea, aspartic acid, and hydroxyethylcellulose were purchased from Fluka Biochemica, Switzerland. All chemicals were of reagent grade and were used as received. Double-distilled water was used throughout.

4.2.2. Methods

4.2.2.1. Particle size analysis

The particle size and size distribution of blank and ovalbumin-loaded microspheres was determined using Photon Correlation Spectroscopy (Malvern Zetasizer). Prior to size analysis, an aliquot of zein microsphere suspension was suspended in double-distilled water. The mean particle diameter was expressed in nm. Results were the average of three different samples, each of which was measured three times. Standard deviation (S.D.) was given as a measure of error for all analyses

4.2.2.2. Zeta potential determination

As a relative measure of the surface charge of the microspheres, the zeta potential was determined using Laser Doppler Velocimetry (Malvern Zetasizer). 0.1 mM phosphate buffered saline pH 7.4 was used as the dispersing medium for the measurements. Results were the average of three different samples, each of which was measured three times. Standard deviation was given as a measure of error for all analyses.

4.2.2.3. Particle morphology examination

The shapes and surfaces of both blank and ovalbumin-loaded zein microspheres were examined under scanning electron microscopy (Philips XL-20). Zein microspheres were collected from the microsphere suspension by centrifugation (13 000 rpm, 5 min), a sample of the pellet was dried, sputter-coated with a thin layer of gold and observed microscopically.

4.2.2.4. Determination of ovalbumin and zein integrity

It is known that the integrity of proteins loaded into microspheres may be adversely affected during the preparation, due to harsh conditions such as organic solvents, high temperature, etc. To determine the effects of the microsphere preparation on the integrity of zein and ovalbumin proteins, gel electrophoresis and infrared studies were conducted.

SDS-PAGE analysis was undertaken to determine the changes, if any, on the structures of ovalbumin and of zein. SDS-PAGE was carried out under denaturing and reducing conditions to ensure the complete dissociation of the zein and ovalbumin into their polypeptides. Protein samples were diluted in a Laemmli sample buffer containing 2-mercaptoethanol 5% v/v and the mixture was heated for 5 min at 95 ºC to achieve the polypeptide denaturation

mentioned. Samples were then loaded onto a Tris-HCl gel with a linear acrylamide concentration gradient of a 4-15%, containing no sodium dodecyl sulphate (SDS), with an effective range of separation of 10-100 kDa (BioRad, 2001). The samples were then subjected to electrophoresis at 200 V and 100 mA in a 25mM Tris/192mM glycine/0.1% w/v SDS pH 8.3 running buffer. Gels were stained with Coomassie Blue G-250 0.1% v/v solution and destained using purified water overnight. Molecular masses of separated zein polypeptides were determined by comparing their electrophoretic mobilities with those of marker proteins of known molecular weights.

Protein samples were supernatant and solutions of pellet harvested following sucrose gradient centrifugation of zein-ovalbumin microsphere suspensions. Pellets were dissolved in 1% w/v aqueous SDS solutions. The samples were loaded onto the gels together with protein markers and untreated ovalbumin dissolved in 1% w/v aqueous SDS solution. Sucrose gradient centrifugation procedure is fully explained in section 4.2.2.5.

To detect changes in zein and ovalbumin secondary structures, FT-IR spectra, of blank and zein-ovalbumin microspheres, were obtained. IR spectra of zein microspheres were obtained using a FT-IR spectrometer equipped with a single bounce horizontal ATR accessory (Avatar 360 FT-IR), using a Ge crystal. Each spectrum was corrected from the background and the wavenumber range was scanned from 4000 to 500 cm^{-1}. Microspheres harvested following sucrose gradient centrifugation of microsphere suspensions (both blank and ovalbumin-loaded), and ovalbumin subjected to microsphere formation conditions (e.g. suspension in aqueous-alcohol mixture containing Tween 20 and PVP 40, followed by ethanol removal at 40º C and 90 mBar), were pressed onto the Ge crystal ATR unit and spectra were collected.

Supplier's zein IR spectrum was compared to blank zein microsphere spectra obtained in our studies.

4.2.2.5. Determination of ovalbumin loading

To determine whether ovalbumin was co-precipitated with zein and to measure ovalbumin loading in the microspheres, sucrose gradient centrifugation was carried out as follows (Antimisiaris, 1993): 3 ml of each aqueous sucrose solutions of decreasing concentrations (40-10% w/v) were carefully layered in centrifuge tubes, followed by the addition of a last layer of zein-ovalbumin microsphere suspension. The tubes were centrifuged for 1h at 14 000 rpm (18 407 Relative Centrifugal Force (RCF)) at 22 ºC. After centrifugation, a pellet and a fine suspension were obtained, at the bottom and at the very top of the centrifuge tube, respectively. The fine suspension and the pellet were examined under transmission and scanning electron microscopy (Philips CM 120 Biotwin) respectively to determine which one consisted of microspheres. To investigate which proteins were present in the two fractions, the pellet was dissolved in 1% w/v aqueous SDS solutions. Then, the pellet and the fine suspension were loaded onto a Tris-HCl gel of 4-15% gradient and analysed by SDS-PAGE, as detailed in section 4.2.2.4. The latter revealed the protein composition of the microspheres and protein integrity too.

The ovalbumin present in the microspheres was quantified via densitometry (Scion Image for Windows), after Coomassie Blue staining, which was carried out as explained in section 4.2.2.4. Ovalbumin solutions in 1% w/v aqueous SDS solutions of known concentrations were used as standards. Each gel contained a full set of ovalbumin standards. Results of ovalbumin content in microspheres determination are expressed as experimental loading, i.e. (experimental mass of ovalbumin / total mass of ovalbumin + zein) x 100.

In initial experiments, ovalbumin had been added in large amounts for the preparation of ovalbumin-loaded zein microspheres, to maximise loading. Theoretical loadings, i.e. (total mass of ovalbumin / total mass of ovalbumin + zein) x 100) was of 44%. However, it is known that solute load in

microspheres is influenced by the amount of solute added as well as other factors. To investigate the relationship between theoretical and experimental loading in zein microspheres, microsphere formulations with 22 and 11% theoretical ovalbumin loadings were prepared and the experimental loading was determined as explained previously.

The microsphere suspensions were replicated five times and each measurement was made per triplicate. Results were expressed as average and standard deviation was given as a measure of error for all analyses.

4.2.2.6. In vitro release studies

Zein-ovalbumin microsphere suspensions were placed in PBS pH 7.4 (1:1 ratio, 50% v/v) containing 0.1% w/v sodium azide aqueous solution to avoid microbial contamination. Then, suspensions were incubated in a shaking water bath at 37ºC for seven days. At time intervals, aliquots of 1 ml were withdrawn, the volume replaced with fresh buffer, and the aliquots were centrifuged (13 000 rpm, 5 min) in a table-top centrifuge. The pellets obtained were examined under SEM, and ovalbumin content of the supernatant was analysed by SDS-PAGE via densitometry scans (Scion Image for Windows), as explained in 4.2.2.5. For ovalbumin quantification, a complete set of protein standards (solutions of ovalbumin in SDS 1% w/v aqueous solution) were prepared and loaded onto each gel. Electrophoretic analysis was carried out as detailed in 4.2.2.4., using 4-15 % linear gradient precast gels. Release was expressed as cumulative release *versus* incubation time. Experiments were replicated and each measurement was made per triplicate. Results were expressed as average and standard deviation was given as a measure of error for all analyses.

4.2.2.7. Studies on erosion and degradation of zein microspheres

Blank zein microsphere suspensions were incubated in a 1:1 (50% v/v) ratio in chloride buffer pH 2.0 or acetate buffer pH 5.0 or phosphate buffered saline pH 7.4 at 37 ºC, for seven days, with occasional shaking to avoid sedimentation. The buffers were prepared according to BP 2001 specifications. Litmus paper was used to follow changes in pH of the incubation media, if any, during the incubation time. At time intervals, aliquots of the incubation media were withdrawn and replaced with fresh medium. The aliquots were used to investigate changes in particle size (by PCS) and microsphere surface properties (by SEM) as described in section 4.2.2.1. and 4.2.2.3. In addition, the liquid medium (supernatant obtained after centrifuging the aliquot at 13 000 rpm for 5 min) was analysed for degradation products of zein by Capillary Zone Electrophoresis (Bio Focus 3000) using a 100 cm long 50 µm ID uncoated capillary and 40mM aspartic acid (Asp), supplemented with 0.5% w/v hydroxyethylcellulose (HEC) and with 6M urea (Asp/HEC/urea, pH≤3) as the running buffer. Samples were injected onto a silica capillary (thermostated at 30 ºC) by pressure at 20 psi x s, runs were performed at 20 kV and detection was at 214 nm. For reliable peak assigment, marker proteins (chimiotrypsinogen MW 25 kDa and ovalbumin MW 45 kDa) were also injected.

An uncoated silica capillary was used for the study to minimise protein adsorption on the capillary wall, which could occur with the use of an acidic buffer like Asp. HEC was added to the Asp buffer as it acts as a dynamic coating to reduce protein adsorption on the capillary walls (Righetti et al., 1999). A high concentration of urea was also used to dissolve the protein (Righetti et al., 1998). Because zein has a low number of acidic residues, it had a low mobility in the acidic environment of the running buffer (Shukla and Cheryan, 2001), and the elution times were fairly long - around 30 min.

CZE was chosen for the degradation studies, due to its minimum requirements in terms of sample volume (nL), protein/peptide quantity (submicrograms) and for its high resolution, which makes it a reliable method for the separation of a group of peptides with small MW distribution.

The electropherograms were compared with that of fresh zein microspheres (obtained after centrifugation of zein microsphere suspensions at 13 000 rpm, 5 min), which were dissolved in 0.1M sodium hydroxide. Collection of zein microspheres following centrifugation resulted in poor wettability of these spheres and the latter were placed in 0.1M sodium hydroxide aqueous solutio overnight to enable dissolution. 0.1M sodium hydroxide (a different solvent to that used as the incubation media) could be used as, at this concentration, sodium hydroxide does not alter the integrity of the two major zein polypeptides (see chapter 2, section 2.2.3.).

Blank zein microsphere suspensions were incubated in simulated gastric and intestinal fluids (prepared following USP 24 specifications) in a 1:1 (50% v/v) ratio at 37 ºC for seven days. Aliquots of the media were removed, replaced with fresh fluids, and centrifuged as above. Microsphere pellets and supernatants were examined using SEM and CZE respectively, as described above.

In addition, SDS-PAGE analysis of the supernatants, freshly prepared solutions of zein in 0.1M sodium hydroxide aqueous solutions, and protein markers was undertaken. The sample solutions were prepared by incubating one part of sample with two parts of sample buffer containing 10% w/v sodium dodecyl sulphate plus 5% v/v β-mercaptoethanol. Then, they were loaded onto a Tris-HCl precast gel with an acrylamide linear concentration gradient of 10-20%. Tris-glycine containing 0.1% w/v SDS was used as the running buffer and the gels were stained with Coomassie Blue G-250 and destained overnight using purified water. More detailed SDS-PAGE procedure is explained in 4.2.2.4.

4.3. Results and Discussion

4.3.1. Particle size

Particle size and size polydispersity of blank microspheres were higher than those loaded with ovalbumin were. This was confirmed by PCS, the average diameter of blank and zein-ovalbumin microspheres being 1356 nm ± 36.4, polydispersity 0.662 ± 0.218, compared to 607.47 nm ± 48.3, polydispersity 0.386 ± 0.166, respectively. Although particles are statistically different, qualitatively they are not very different. The reduced size and narrower size distribution of ovalbumin-loaded particles could be due to the adsorption of ovalbumin onto newly formed particles which leads to a reduction in the surface tension of particles, and thereby stabilisation of the surface and hence, the existence of small particles. This effect of ovalbumin i.e. reduced particle size when ovalbumin is loaded into particles has been previously reported (Krishnan et al., 2004).

The optimal zein microsphere formulation could be used as a vaccine carrier as it presents an adequate diameter for phagocytosis by macrophages (Jenkins et al., 1994).

The sub-micron size of nanoparticles offers advantages over microparticles. Nanoparticles have in general higher intracellular uptake compared to microparticles (Desai et al., 1996, 1997). The particle absorption pathway as well as absorption efficiency in the gastrointestinal tract has been shown to be affected by the size of the particles administered. It is suggested that submicron colloidal particles can be absorbed and transported via the intracellular pathway through the enterocytes, while larger particles (several microns) are absorbed exclusively by the Peyer's patch M cells (Chen and Langer, 1998). Eldridge et al. (1990) demonstrated that the particles greater of 10 microns were not taken up. They also proved that the nature and surface

characteristics of the particles affect particle uptake as well. Hydrophobic particles were absorbed more readily than more hydrophilic ones.

4.3.2. Zeta potential

The zeta potential was -7.85 mV ± 1.8 and -6.41 ± 1.2 for blank and zein-ovalbumin microspheres, respectively. Thus, values did not differ markedly from each other. It is believed that molecules of ovalbumin present on the surface of the microspheres only cause slight or no changes in the surface charge, as both proteins are negatively charged at the pH of the microsphere suspension. It has been already mentioned in chapter 3, that this value of zeta potential helps to the stability of a disperse system in terms of flocculation (see section 3.3.3.1.). However, zeta potential values are relatively low and this may be a reason for the high aggregation tendency of the zein microspheres.

The effect of surface charge on phagocytosis is not clear-cut; while it has been reported that macrophage phagocytosis is enhanced when the absolute value of zeta potential increases for both negatively and positively charged surfaces, it has also been reported that positive charges aid phagocytic uptake (Tabata and Ikada, 1988; Florence, 1997).

4.3.3. Particle morphology

Scanning electron microscopy on blank and ovalbumin-loaded zein microspheres revealed that microspheres were spherical in shape, but polydisperse in size and had non-porous, smooth surfaces (figure 4.1. and 4.2.). Size and size polydispersity of blank microspheres were higher than those loaded with ovalbumin, as was seen following particle size analyses by PCS.

4.3.4. Zein and ovalbumin integrity

During microsphere formation, ovalbumin and zein were exposed to ethanol, sodium hydroxide and to mechanical agitation, i.e. potentially harsh conditions, which could theoretically lead to irreversible denaturation of these proteins.

We found no such denaturation however for ovalbumin protein; SDS-PAGE analysis of untreated ovalbumin (figure 4.3.) and of microsphere formulations (figure 4.4.) showed identical bands, related to the monomeric and dimeric species of ovalbumin. In addition, FT-IR spectra of ovalbumin subjected to microsphere formation conditions showed no differences in peak location for amide I and II bands with respect to native ovalbumin spectra known from the literature (figure 4.5.). Therefore, the microsphere formation process did not extensively affect the structural integrity of ovalbumin.

SDS-PAGE and FT-IR also showed that zein structure was not greatly affected by the microsphere preparation. Two bands related to the 22 and 24 kDa peptides of α-zein were observed upon SDS-PAGE (figure 4.4.). These bands were the same as those observed for untreated zein (see chapter 2, figure 2.8.). FT-IR spectra of zein formulated into microspheres showed no remarkable changes for the two major peaks in amide I and II regions (figure 4.6.). Some minor changes were however observed. The zein IR spectra obtained from suppliers (figure 4.7.) showed a peak at 1730 cm^{-1} approximately next to amide I band. This peak disappeared and a broad band appeared between 3300 and 3400 cm^{-1}. The latter is probably due to OH groups from residual ethanol. The reason for the disappearance of the peak at 1730 cm^{-1} is unclear.

The FT-IR spectra of zein-ovalbumin microspheres were almost identical to that of blank microspheres (figure 4.6.). This shows that loading ovalbumin into zein microspheres had no effects on zein structure.

4.3.5. Ovalbumin loading in zein microspheres

To determine the content of ovalbumin in zein microspheres, the latter were subjected to sucrose gradient centrifugation. A pellet and a fine suspension (supernatant) were obtained at the bottom and at the top of the centrifuge tube, respectively. An examination of the supernatant under TEM showed aggregates ranging in size from 50 to 100 nm (figure 4.8.) while the pellet consisted of spherical particles of uniform size (figure 4.9.).

Electrophoresis showed the absence of zein and the sole presence of ovalbumin in the supernatant aggregates, in the form of ovalbumin monomers and dimers, while the pellet consisted of both ovalbumin and zein proteins (figure 4.4.). Aggregation of unloaded ovalbumin into the structures observed in TEM is probably due to an increase in ovalbumin mobility when ethanol was evaporated off (i.e. when the water content of the suspending medium was increased) and the inherent tendency of ovalbumin to aggregate (Sah, 1999). In chapter 3, it was reported that two particle size populations were often observed when carrying out PCS analyses. One of the particle size populations had a diameter less than 100 nm. It is believed that these ovalbumin aggregates could be included in this population along with zein precipitated into very small microspheres.

Analysis of the pellet for ovalbumin content showed an experimental ovalbumin loading of 23.90%. The theoretical loading [(total amount of drug / total amount of drug + polymer) * 100] was 44%, thus a loading efficiency of 53.90% was achieved. The loading efficiency was calculated as (experimental loading % / theoretical loading %). Such a relatively low loading was thought to be primarily due to the formation of ovalbumin aggregates, which could

reduce the amount of ovalbumin available for loading into zein microspheres. To determine the relationship between theoretical loading and the loading efficiency, the ratio of ovalbumin to zein was decreased to theoretical loadings of 22 and 11% and the loading efficiencies were found to increase (table 4.1.). It seems that increasing the amount of ovalbumin above a certain point results in a smaller percentage of ovalbumin that is loaded into zein microspheres.

4.3.6. *In vitro* release of ovalbumin from microspheres

SEM showed an almost identical morphology for ovalbumin loaded-zein microspheres after seven days of incubation in PBS (figure 4.10.). Zein microspheres seemed to degrade slowly in pH 7.4. Therefore, released ovalbumin did not seem to increase after a week of incubation either.

Release of ovalbumin from zein microspheres seems to follow a zero order kinetics (figure 4.11.). However, the amount of ovalbumin determined in the supernatant respect to the total amount of ovalbumin present was found to be of no consequence. Although, at 120 h a little increase of ovalbumin release can be observed in figure 4.11., this did not represent an important increase; due to it represented only a 20% of the total ovalbumin amount. This percentage is even lower than the percentage of unloaded ovalbumin, which had not been removed prior incubation. According to this result, no significant amount of ovalbumin seemed to be released from zein microspheres. This agrees with other low release rates described in literature previously from zein formulations.

Considering that release is mainly governed by formation of pores, matrix degradation and solubility of the polymer forming the particles (see section 4.1.), the irrelevant release can be explained by the no remarkable erosion in terms of pore formation on zein microsphere surface, by the incomplete

degradation of zein microspheres, and by the insolubility of zein in aqueous media.

The latter release results are not clear-cut. The aggregation of ovalbumin shown above following sucrose gradient centrifugation studies means some kind of protein denaturation. Thus, the ovalbumin used (grade II) may suffer a kind of degradation *in vitro* and cannot be assayed properly. Then, it was decided to assay release *in vivo* as a result of antibody response to ovalbumin antigen.

4.3.7. Erosion and degradation of zein microspheres

4.3.7.1. Changes in incubation medium and in microsphere morphology upon incubation of zein microspheres in different media

To study their degradation and erosion, blank zein microspheres had to be suspended in a suitable medium. PBS pH 7.4 was not an appropriate suspending fluid, when blank zein microsphere suspensions were placed in PBS pH 7.4, phase separation occurred, and a microsphere-rich film was formed. In contrast, microspheres could be suspended in chloride and acetate buffers. Incubation of zein microspheres for seven days did not cause any changes in the pH or turbidity of the three buffers. The absence of any pH change may be due to zein's composition of mainly neutral amino acids and non-acidic degradation products. Gastric and intestinal simulated fluids were found to be suitable suspending media for zein microspheres. However, upon incubation of microspheres, a noteworthy decrease in turbidity of the suspending media was observed. If decrease of turbidity was a result of particle erosion and/or degradation, it can be said that zein microsphere

erosion and/or degradation did not take place in the simple buffers, but did occur in simulated gastric and intestinal fluids containing proteolytic enzymes.

Upon incubation in chloride and acetate buffers, particle size and size polydispersity of microspheres increased within 24 h of incubation. Size polydispersity increased up to 1.0, the upper limit for quality size measurement by the equipment used. The increase in microsphere size is thought to be due to Ostwald ripening-like phenomena of suspended microspheres. Examination of particles under SEM confirmed the increase in size, nevertheless zein particles remained spherical in shape and no visible surface porosity and/or roughness was observed, even after a week of incubation (figure 4.12.). Lysis time (defined as the time of disappearance of all microspheres from the medium) could not be determined for the simple buffers, because microspheres were still present after one week of incubation.

In contrast, the presence of enzymes in the incubation media had a very different effect on the microspheres. When microspheres were incubated in intestinal simulated fluids, most of the microspheres disappeared after approximately 5 h of incubation, to be replaced by elongated tubules (figure 4.13.). This change in shape might be due to a change of zein protein. After two days of incubation both the spherical particles and the elongated tubules had disappeared as zein was degraded by pancreatin. The presence of pepsin had a different effect on microspheres. The latter disappeared completely following incubation in simulated gastric fluid within 1 h; however, following a week of incubation, a small pellet could still be harvested by centrifugation. This pellet was not composed of microspheres or other shapes of particles, but consisted of a non-uniform agglomeration of protein (figure 4.14.).

4.3.7.2. Changes in zein protein structure upon incubation of zein microspheres in different media

CZE and SDS-PAGE analyses of zein protein and of freshly prepared, non-incubated zein microspheres were first conducted to obtain the control results.

SDS-PAGE analysis of zein protein (dissolved in 0.1M sodium hydroxide) showed two major bands just below 25 kDa, which correspond to α-zeins (figure not shown). When sample concentration was high, minor bands were also detected, one at approximately 45 kD and two at around 14 and 10 kD. The band at 45 kD may be either a dimer of one and/or both α-zein peptides or a minor peptide component of zein. The low MW bands would correspond to the other classes of zein, such as β- and δ-zein (see chapter 2, section 2.3.3.).

CZE electropherograms of freshly prepared, non-incubated zein microspheres showed two main peaks, the first one being partially resolved (figure 4.14,f). CZE separates peptides based on their differences in mass/charge ratio. It is known that zein is highly heterogeneous in terms of size and charge, and isoelectric focusing has revealed that zein contains four or five times as many components as the ones resolved by SDS-PAGE (Esen et al., 1981). The unresolved peak obtained on our electropherogram might then occur due to the mass/charge ratio heterogeneity of the protein. The first peak is thought to correspond to the isoforms of the α_1- and α_2-zein peptides (approximately 22 and 24 kD, respectively), which migrate at similar rates and the second peak probably corresponds to the 45 kD peptide. The smaller third peak might be a tetramer; it is known that zein occurs as oligomers in the native protein form (Landry and Guyon, 1984).

The baseline was found to be irregular for all samples, as a result of the urea-based buffer, which it is known to increase background absorbances (Bean et al, 2000), and the different pHs of the incubation media.

CZE analysis of zein microspheres that had been incubated at pH 2 showed that the shape of the first peak (corresponding to α-zeins) stayed the same, but the height gradually decreased with time (figure 4.15,a). This may mean that α-zein was being hydrolysed in the acidic medium by the effect of the hydrochloric acid present in the chloride buffer. By the end of the first week of incubation, the α-peak had almost completely disappeared (figure 4.15,b). In contrast, the disulphide structure peak remained relatively unchanged. This shows that the dimer was resistant to acidic breakdown; this concurs with the knowledge that disulfide structures are strongly resistant to degradation (Fahmy et al., 1991).

When microspheres were incubated in buffers at pH 5 (figures 4.15,c and 4.15,d) and at pH 7.4 (figures 4.15,e), the α-zein peak did not disappear but it lost heterogeneity, suggesting that some, but not all, isoforms of α-zeins were degraded. Concurrently, the second peaks, thought to represent dimers of α-zeins, increased from the first hour of incubation until the end of the study, as it is shown in table 4.2.

Following incubation of zein microspheres in gastric fluids, SDS-PAGE analysis revealed only one band at 45 kD approximately (figure 4.16.). This shows that pepsin digested α-zein, but not the dimer. This may be attributed to formation of disulphide bonding resulting in the folding of the zein molecule in such a way that became less susceptible to the attack of to digestive enzymes. CZE electropherogram revealed a major peak and a minor one, both of which remained until the end of the study (figure 4.17.). The major peak can be assigned to the dimer and we think that the second peak is a tetramer, which could not be detected by SDS-PAGE analysis, probably due to its very low concentration in the medium.

The effect of pancreatin on zein was very different to that of pepsin described above. Electrophoretic gel (figure 4.16.) showed three bands between 26 and 30 kD (not α-zein whose band occurs just below 25 kD), and one band above 50 kD (not an α-zein dimer whose MW is around 45 kD). The absence of

bands for α-zein and of its dimer is a result of digestion of these two peptides by pancreatin. The appearance of new bands is probably a result of polymerisation of low MW (present in small quantities in zein) zein peptides to these new species. The triplet of bands shown in figure 4.16. has been reported previously, but only when zein was extracted together with methionine-rich zeins (zeins with MW between 14 and 16 kD) from some maize inbreds (Esen, 1986). CZE analysis showed two peaks (figure 4.18.); the major one eluted last, thus it probably corresponds to the disulphide linkage, structures that seem to have longer elution times than monomers. The first, smaller peak did not have the same shape as the α-zeins peaks seen when zein microspheres had been incubated in the other media.

Zein microspheres were being investigated as vaccine carriers. Hence, the discussion of the latter results was made in order to predict the fate of microspheres when administered *in vivo* and to understand the mechanisms of release of loaded antigens, as follows.

When blank zein microspheres were incubated in simple buffers without enzymes, an increase in particle size, presumably due to Ostwald ripening-like phenomena, seems to be the only major change. The particle shape and surface properties, and the suspension pH and turbidity remained the same. Ovalbumin-loaded zein microspheres were incubated in simple buffers for a week as well (data not shown) and the results were identical in terms of particle shape. SDS-PAGE analysis showed bands corresponding to α-zein and to the dimer (figure not shown). This reflected the two peaks obtained on the CZE electropherograms, corresponding to α-zein and to its disulphide structures, the same peaks observed for freshly prepared zein microspheres. With time, there was a reduction in the height or heterogeneity of the α-zein peak as the α-zein was slowly reduced. In contrast, the disulphide structure peak was not reduced (the disulfide linkage being resistant to degradation), but increased in height. This implies that during incubation of the microspheres, disulphide cross-linking among sulphur-containing amino acids in α-zein, such as Cys and Met, occurs which result in increased dimer (thus a

disulphide aggregate of one or both of the α-zeins) peak. The polymerisation of α- β- and γ- zeins, via the formation of disulphide bridges, has been reported previously, where the effect was assigned to increased temperature and stirring during cooking (Duodu et al., 2002, 2003). Polymerisation, via non-disulphide crosslinks, such as coupling of Tyr residues, has also been suggested.

Disulphide formation during microsphere incubation seems to inhibit degradation of zein microspheres and the latter were still present in the incubation media after one week of incubation. The absence of an obvious microsphere degradation, of surface erosion and the aqueous insolubility of zein explain the negligible release of ovalbumin from zein particles found in previous experiments, given that release of a loaded solute is mainly due to the formation of pores, matrix degradation, the solubility of the polymer which makes up the particles and the nature of the interactions between polymer and drug.

In contrast to the relatively mild effect of incubation in simple buffers, incubation in the presence of the enzymes, pepsin and pancreatin, had a marked effect on zein microsphere suspension. There was a marked reduction in suspension turbidity, which shows some zein particle dissolution. Zein degradation into amino acids did not, however, lead to changes in suspension pH due to the small number of acidic and basic amino acid residues in zein.

When particles were incubated in simulated gastric fluids, pepsin digested zein, as a result of which all zein particles disappeared within 1h of incubation. SDS-PAGE analysis showed only one band corresponding to zein disulphide structure, while CZE electropherogram showed two peaks. The absence of an α-zein band in SDS-PAGE analysis showed that pepsin digested α-zein, but not the disulphide structure. This implies that the first peak of the CZE electropherogram corresponds to the dimer while the second peak may correspond to a tetramer. The latter was not picked up by SDS-PAGE,

probably because of its low concentration. An agglomeration of the zein tetramers and dimers is thought to make up the small pellet, which could be harvested by centrifugation of the suspension after one week of incubation.

Pancreatin had a very different effect on zein microspheres. SDS-PAGE analysis showed the absence of α-zeins and of α-zein dimers, but the presence of three proteins of MW between 26 and 30 kD, whose MW were above those of α-zein monomers, and another protein, whose MW was 50 kD approximately. Absence of α-zein is linked to the disappearance of microspheres, while the appearance of the other proteins is thought to result in the emergence of elongated tubules in the incubation media. It could be that some low MW zein peptides, present in minor quantities in zein, were polymerised in the incubation media producing the triplet (i.e. the three bands present between 26-30 kD) and the high MW structure (above 50 kD).

The degradation of zein particles by the enzymes pepsin and pancreatin suggests that when antigen-loaded zein particles are administered in vivo, release of the active agent via particle degradation is likely in the gastro-intestinal tract.

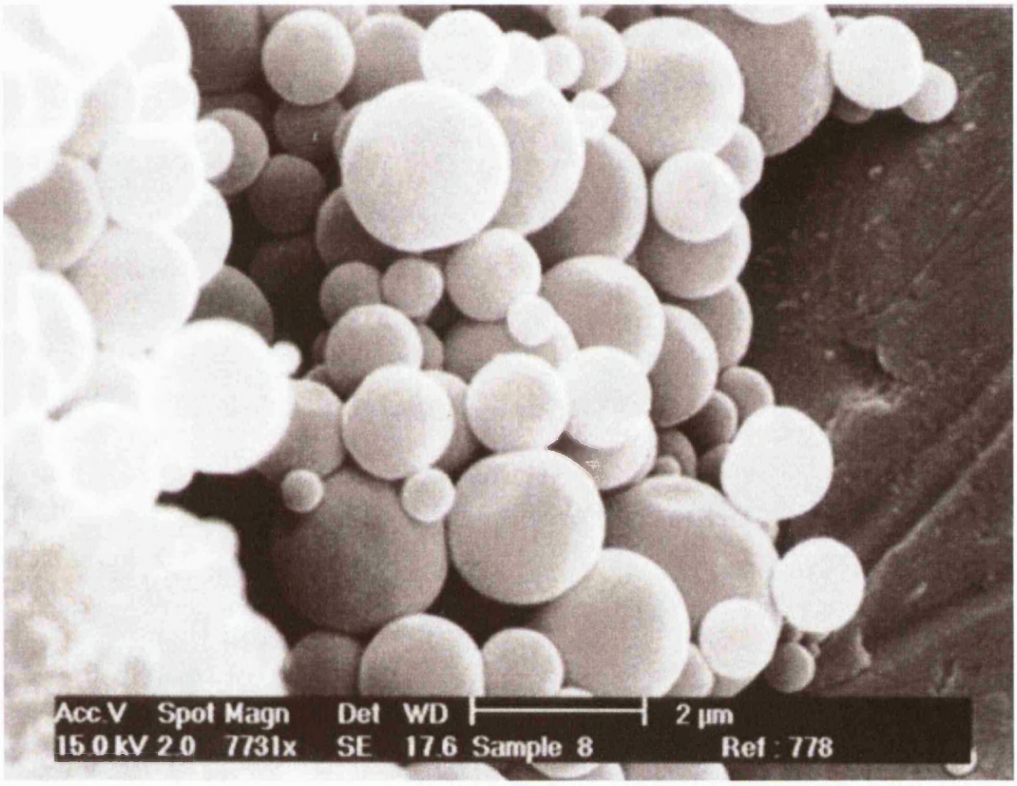


Figure 4.1. . SEM photograph of blank zein microspheres.

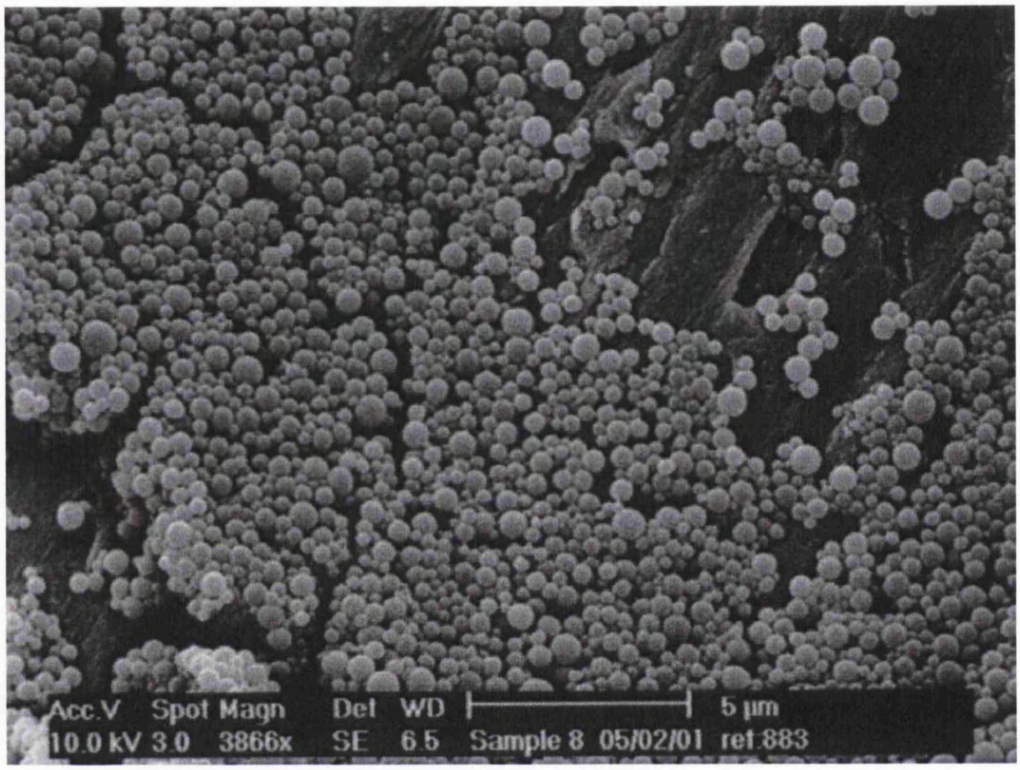


Figure 4.2. . SEM photograph of ovalbumin-loaded zein microspheres.

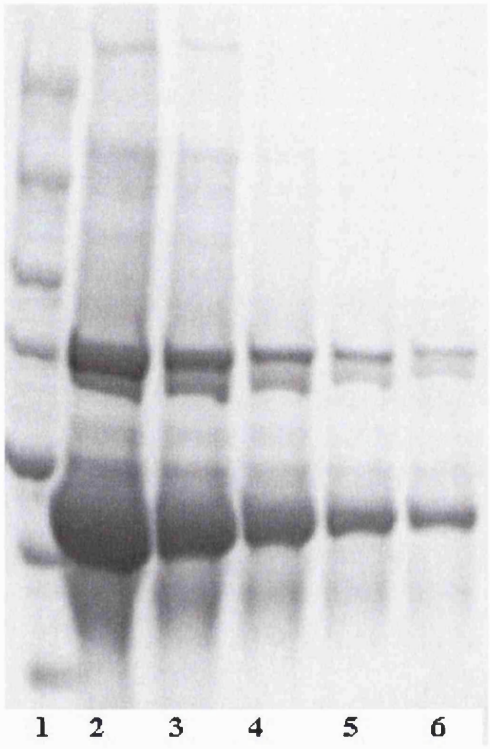


Figure 4.3. SDS-PAGE profile of untreated ovalbumin. Lane 1: Molecular weight markers of 25, 37, 50, 75, 100, 150, and 250 kD. Lane 2 to 5: Decreasing concentrations of untreated ovalbumin protein.

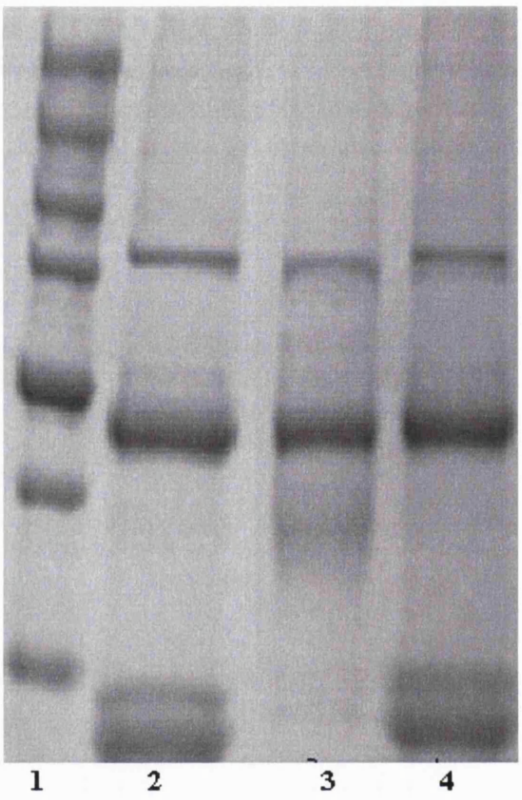


Figure 4.4. SDS-PAGE profile for ovalbumin-loaded microspheres. Lane 1: Molecular weight markers of 25, 37, 50, 75, 100, 150, and 250 kD. Lane 2: ovalbumin-loaded zein microsphere suspension before sucrose gradient centrifugation (presence of zein bands and ovalbumin monomers and dimers). Lane 3: Supernatant obtained after sucrose gradient centrifugation (no zein bands sole presence of ovalbumin monomers and dimers). Lane 4: Pellet obtained after sucrose gradient centrifugation and dissolved in SDS 1%, (zein and ovalbumin bands).

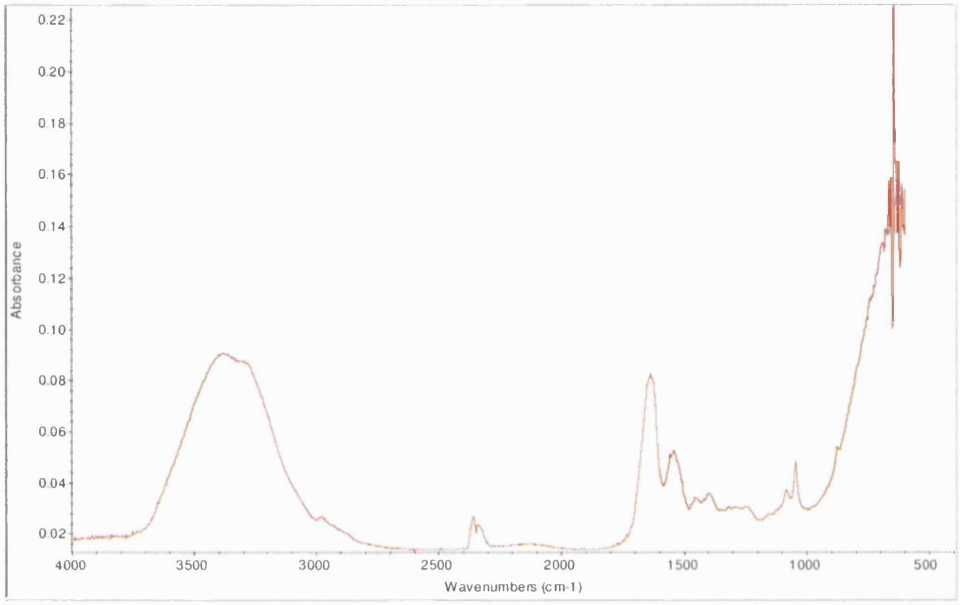


Figure 4.5. FT-IR spectrum of ovalbumin alone subjected to microsphere formation conditions.

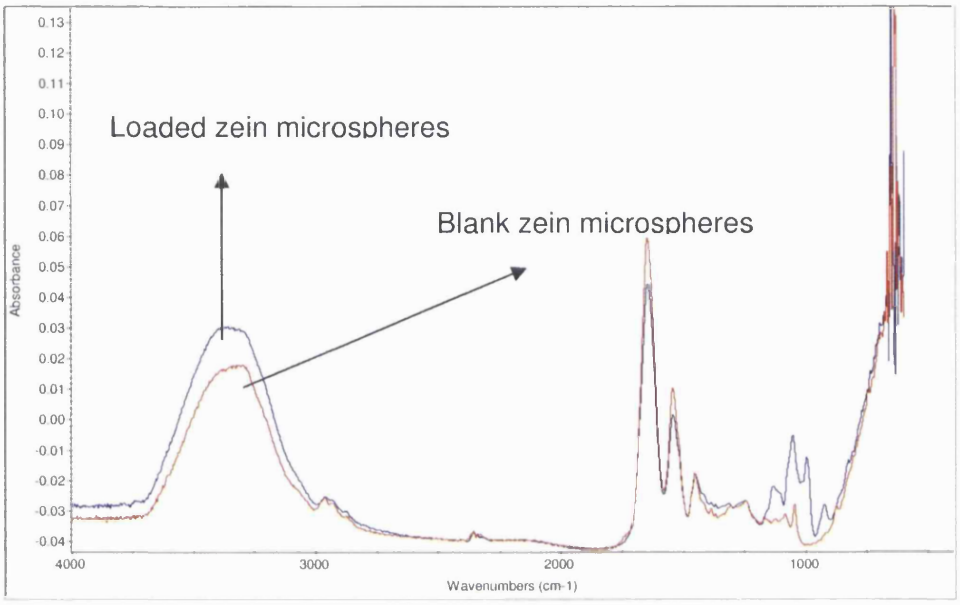


Figure 4.6. FT-IR spectra of blank and ovalbumin-loaded zein microspheres.

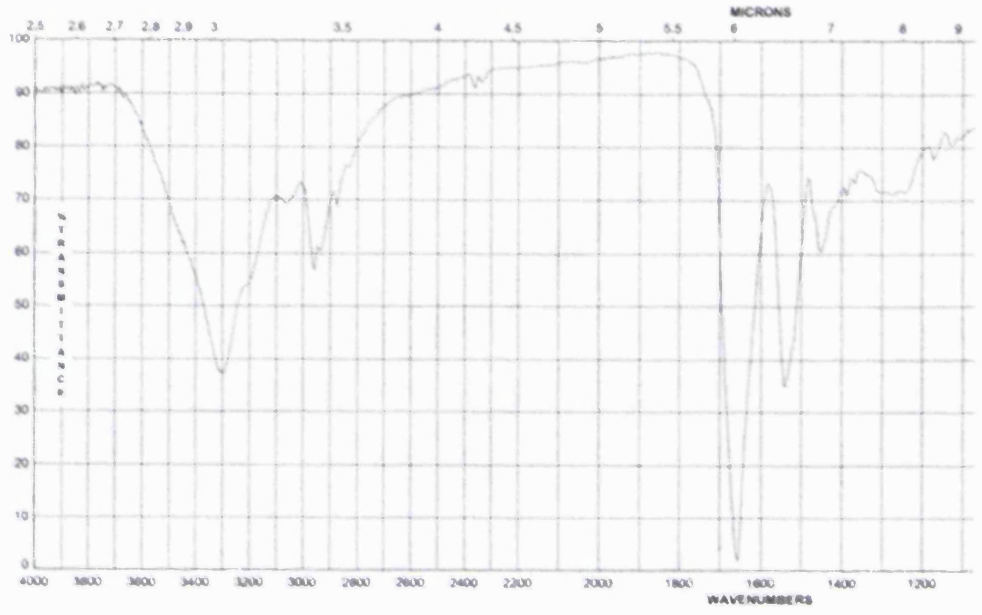


Figure 4.7. IR spectrum of zein purchased from Sigma (From Sigma webpage).

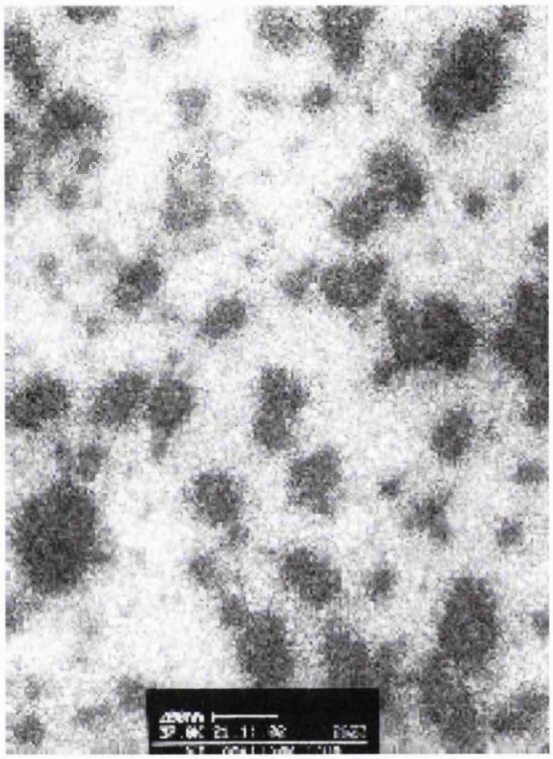


Figure 4.8. TEM photograph of ovalbumin aggregates present in supernatant obtained after sucrose gradient centrifugation.

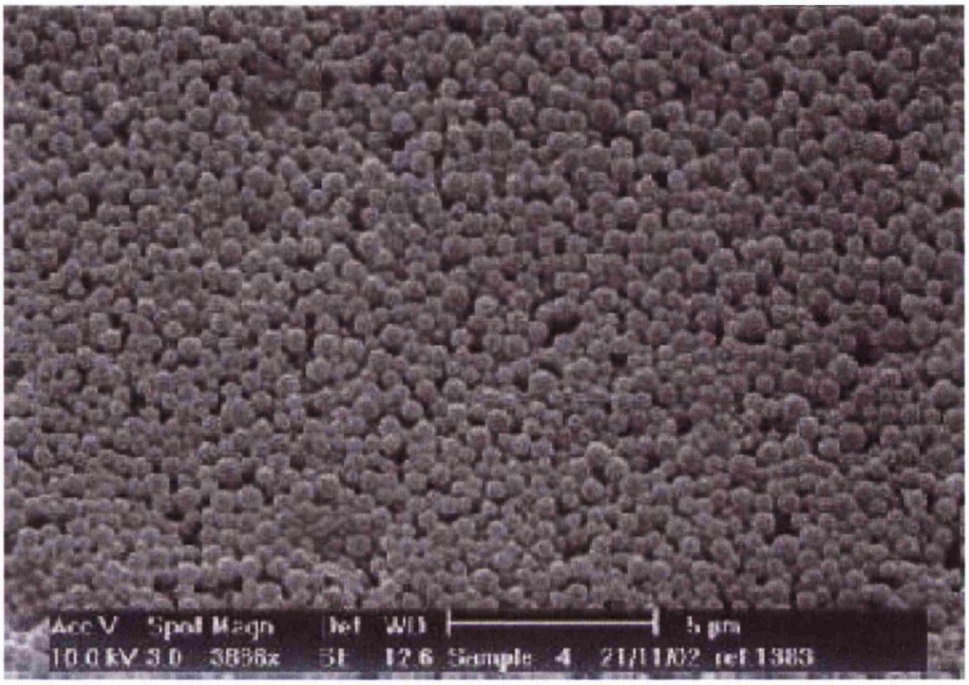


Figure 4.9. SEM photograph of microspheres present in pellet obtained after sucrose gradient centrifugation.

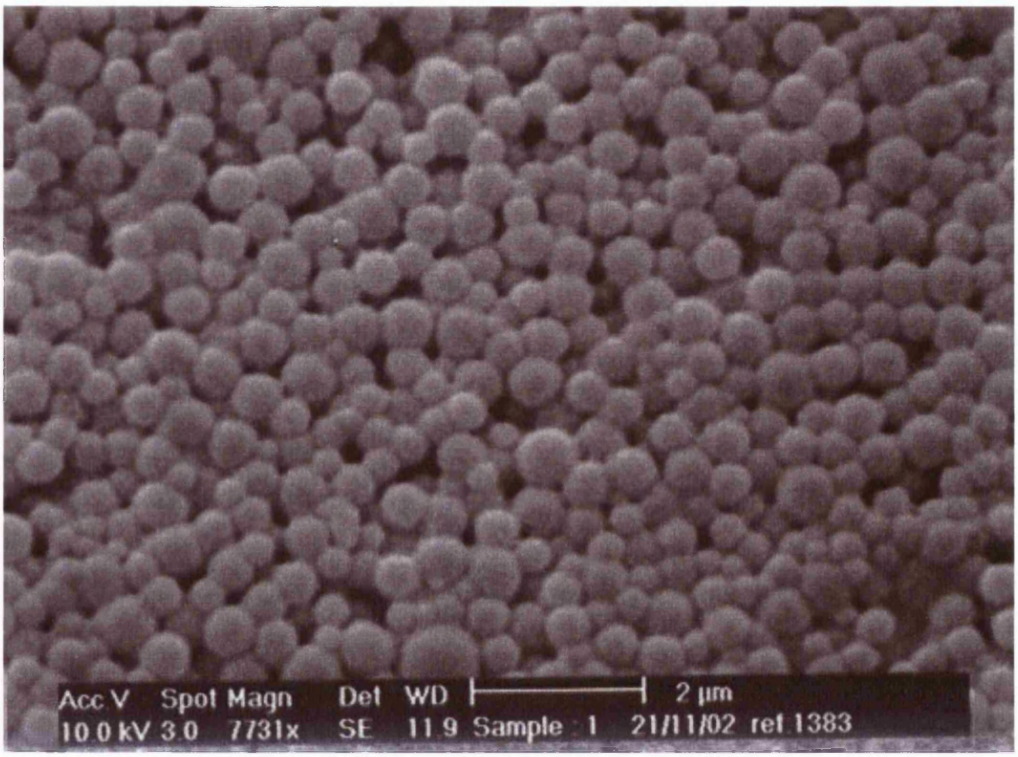


Figure 4.10. SEM photograph of ovalbumin-zein microspheres following seven days of incubation in PBS pH 7.4.

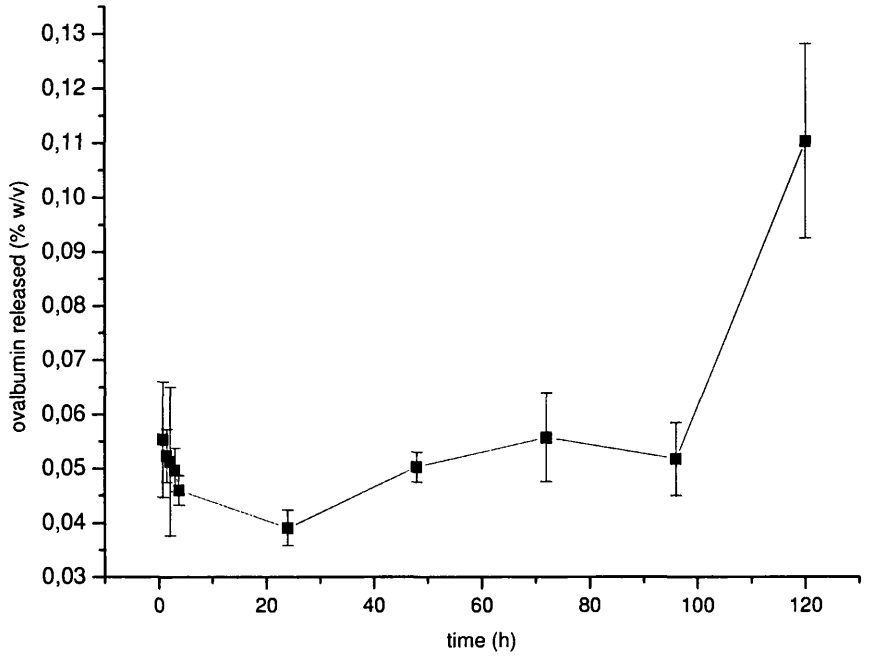


Figure 4.11. Release profile of ovalbumin from ovalbumin-loaded zein microspheres following incubation in PBS pH 7.4.

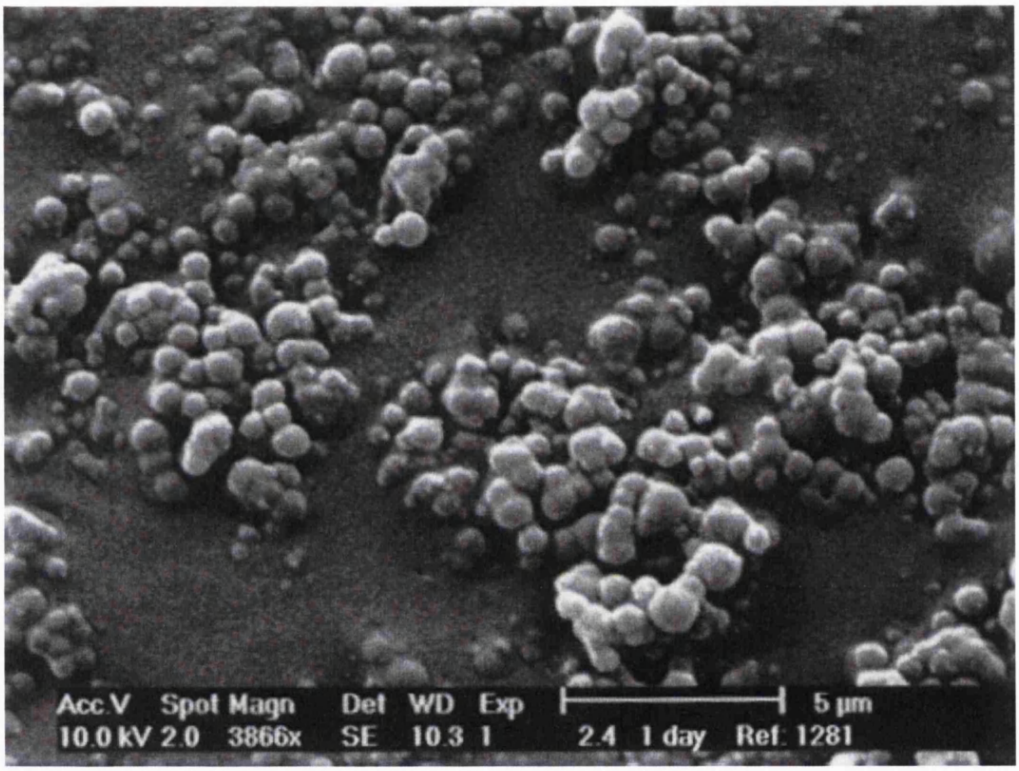


Figure 4.12. SEM picture of blank zein microspheres after incubation in acetate buffer for one day.

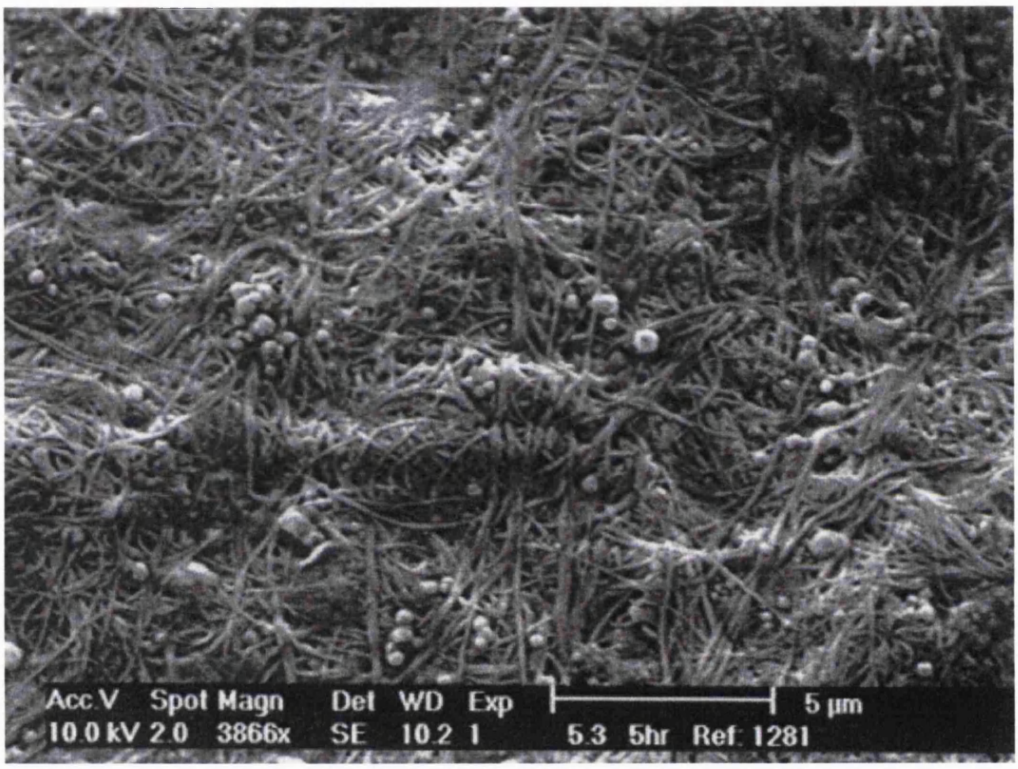


Figure 4.13. SEM picture of blank zein microspheres after incubation in intestinal simulated fluids for 5 h.

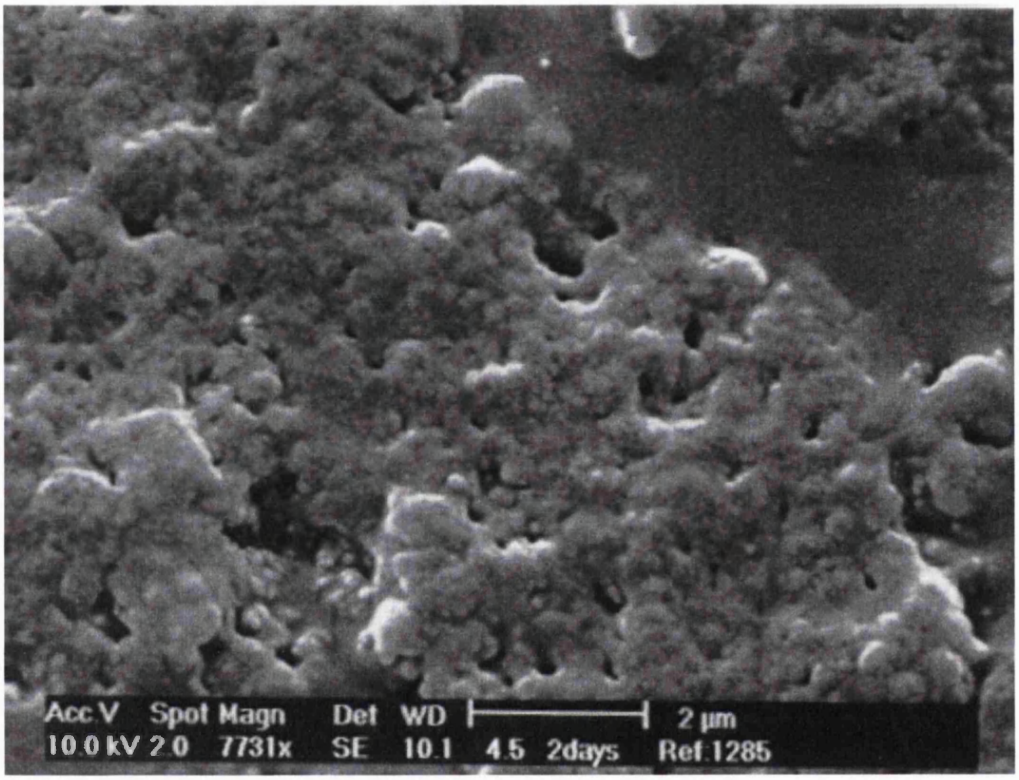


Figure 4.14. SEM picture of blank zein microspheres after incubation in gastrointestinal simulated fluids for two days.

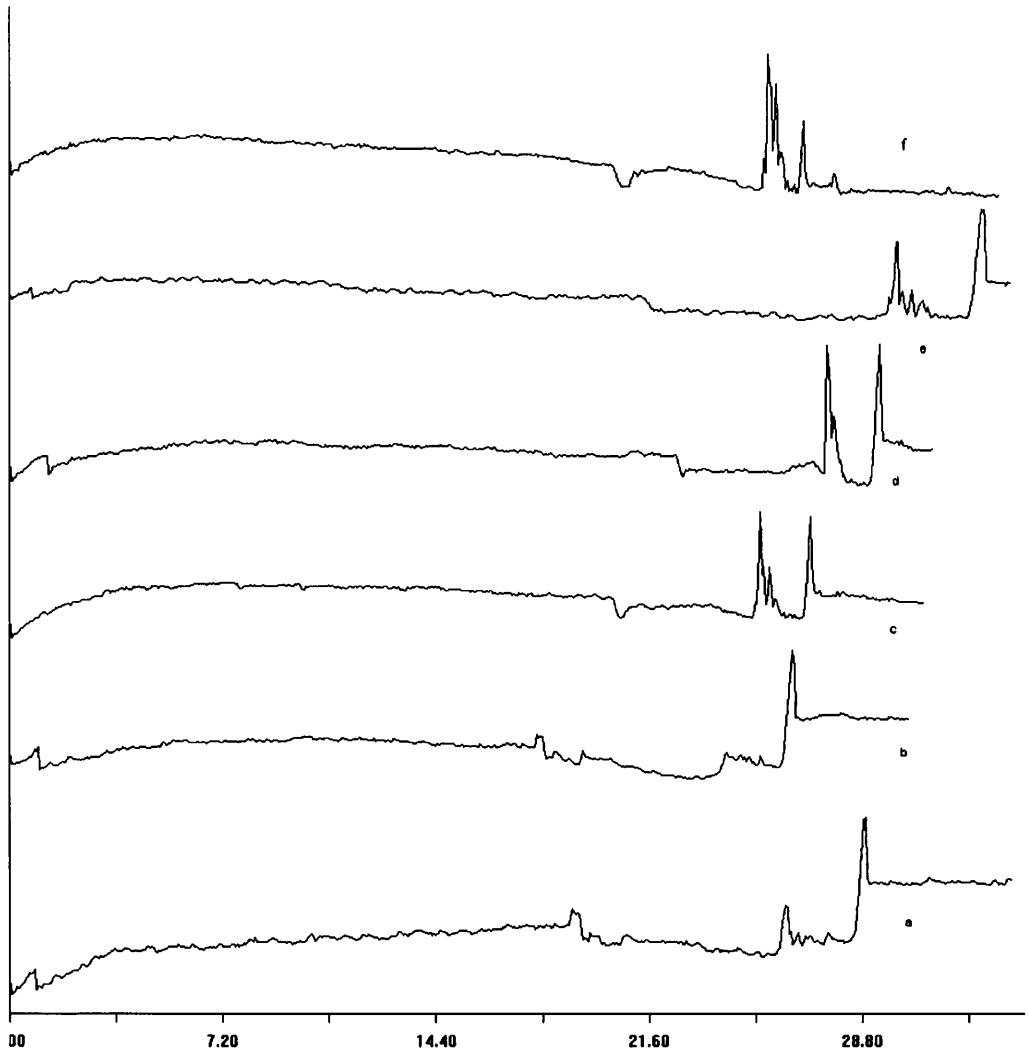


Figure 4.15. (a) Electropherogram of blank zein microspheres after incubation in chloride buffer for 5 h. (b) Electropherogram of blank zein microspheres after incubation in chloride buffer for 6 days. (c) Electropherogram of blank zein microspheres after incubation in acetate buffer for 5 h (d) Electropherogram of blank zein microspheres after incubation in acetate buffer for 6 days. (e) Electropherogram of blank zein microspheres after incubation in phosphate saline buffer for 6 days. (f) Electropherogram of non-incubated blank zein microspheres (sample solvent: 0.1M sodium hydroxide).

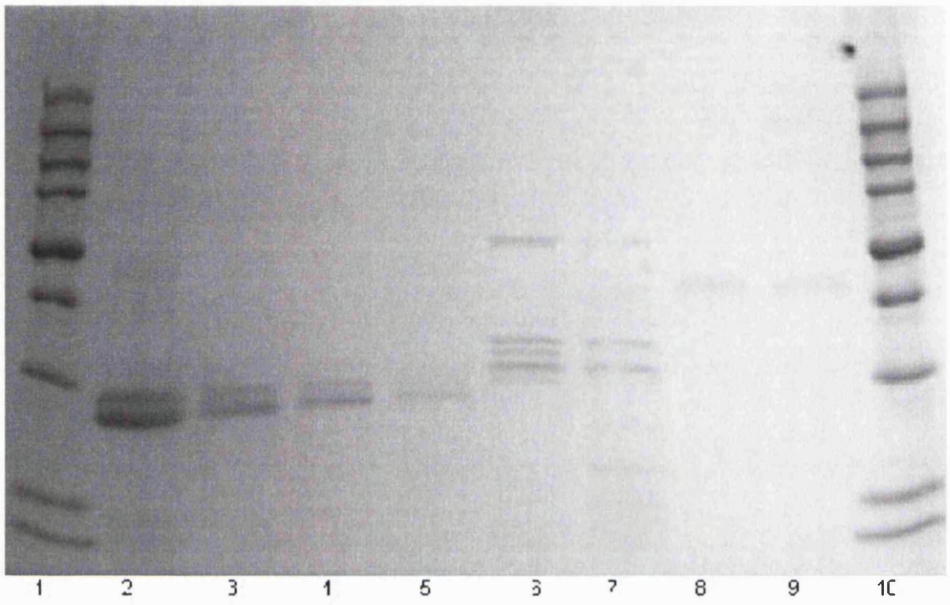


Figure 4.16. SDS-PAGE showing zein microspheres degradation. Lane 1 and 10: Protein standards with MW of 250, 150, 100, 75, 50, 37, 25, 15, 10 kD. Lane 2 to 5: Zein protein at different concentrations (sample solvent: 0.1M sodium hydroxide). Lane 6: Blank zein microspheres after incubation in intestinal simulated fluids for 3 h. Lane 7: Blank zein microspheres after incubation in intestinal simulated fluids for 1 day. Lane 8: Blank zein microspheres after incubation in gastrointestinal simulated fluids for 3 h. Lane 9: Blank zein microspheres after incubation in gastrointestinal simulated fluids for 1 day.

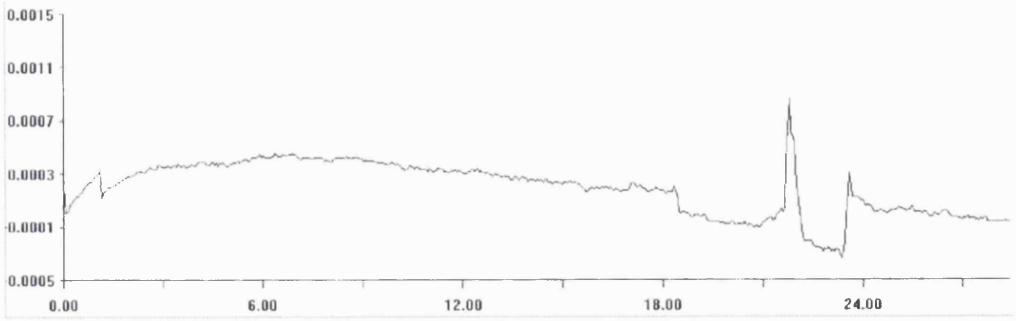


Figure 4.17. Electropherogram of blank zein microspheres after incubation in gastrointestinal simulated fluids for two days.

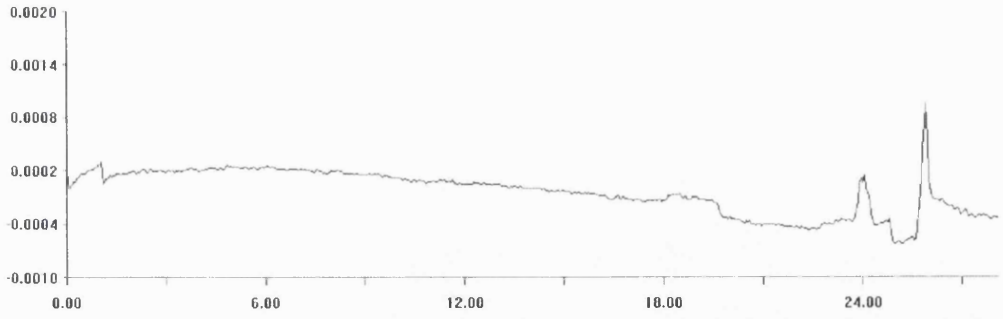


Figure 4.18. Electropherogram of blank zein microspheres after incubation in intestinal simulated fluids after 5 h.

theoretical loading (% w/w)	**experimental loading (% w/w)**	**loading efficiency (% w/w)**
44	23.90	53.90
22	19.05	85.73
11	8.25	74.25

Table 4.1. Relationship between theoretical, experimental loadings, and loading efficiencies of ovalbumin-loaded zein microspheres.

BUFFER	**Incubation period**	**Peak**	**Elution time (min)**	**AR/MT***
Acetate pH 5	5h	Disulphide structure	22.56	9 950
	6 days	"	24.50	20 627
Phosphate pH 7.4	5h	Disulphide structure	32.89	21 317
	6 days	"	30.47	26 027

Table 4.2. Integrated areas for disulphide peaks of zein microsphere electropherograms after their incubation in acetate and phosphate buffers. It can be observed that area under the curve increased for these peaks, which can be due to more zein is in the form of dimers.
*Area/Migration time

4.4. Conclusions

From the characterisation of zein microspheres, we believe there are three key factors: the particle diameter, the release of ovalbumin from zein microsphere matrix, and the erosion and degradation of zein microspheres.

Surface hydrophobicity as well as particle size are considered as important parameters affecting the *in vivo* organ distribution after parenteral administration of particulate drug delivery systems. As mentioned in the discussion, we believed that our zein microspheres are of adequate size to be uptaken by APCs *in vivo.* Since zein material is hydrophobic and we have reported in this chapter that zein microsphere matrix is composed of zein protein, hydrophobicity of zein particles is ensured. Thus, hydrophobicity of particles will also help to their phagocytosis *in vivo.*

We conclude that negligible ovalbumin release in PBS, which was found to be of no consequence, is due to the mechanism of degradation and erosion of zein microspheres. From the degradation and release studies, we can conclude that zein microspheres are not degraded in the absence of enzymes, but are degraded by gastro-intestinal enzymes via cleavage of peptidic bonds. However, the more resistant disulphide linkages are not completely degraded either by pancreatin or by pepsin. In addition to degradation of α-zeins during incubation of zein microsphere, polymerisation of certain zein peptides into larger structures also occurs, which makes total degradation of the microspheres more difficult. From these studies, we expect vaccine-loaded zein microspheres to release their drug load following digestion by gastro-intestinal enzymes following oral administration.

The absence of significant release of ovalbumin from zein microspheres may or may not be a drawback. Release also depends on the nature of the interaction between polymer and drug. In this case, we hypothesise that non-covalent interactions are established between zein and ovalbumin, and then release may only vary as a function of the distribution of the drug throughout

particle matrix. Placing then ovalbumin on the surface of zein microspheres might improve the release rate.

On the other hand, we believe that in order to explore the potential of zein microspheres as vaccine delivery systems, further characterisation *in vivo* is needed, since the present results *in vitro* are not conclusive. Whether the ovalbumin epitopes integrity is kept during zein microsphere manufacturing, or whether the lysis time of microspheres in the gastrointestinal tract is sufficient to protect ovalbumin protein degradation must be explored in further *in vivo* experiments.

Chapter 5.

In vivo evaluation of zein microspheres

5.1. Introduction

The generation of antibodies is essential for a vaccine; in contrast, antibodies to a protein that is not meant to be a vaccine may result in unwanted autoimmune responses and other adverse side effects. If a protein is administered to humans, there is a possibility that this protein will raise an antibody response. A microparticle formulation containing a protein may enhance this response due to its adjuvanticity action, as previously described (O'Hagan, 1991).

The aim of this study was:

- ✓ To evaluate the adjuvanticity of zein microspheres.
- ✓ To evaluate the immunogenicity of zein microspheres.

Adjuvanticity of zein microspheres administered intramuscularly and via mucosal routes (oral, rectal, and vaginal) was investigated.

The principal route of administration for medicinal products is the oral route. Particulate systems are required to be resistant in the gastrointestinal tract and to protect the drugs from degradation. However, *in vivo* conditions were expected to induce enzymatic degradation of zein since zein microspheres were found to degrade faster in the presence of pepsin and pancreatin (see chapter 4). On the other hand, the classical route to induce immune tolerance is also the oral route.

Apart from oral administration, other mucosal routes seem also promising and the action of proteolytic enzymes is not central. Rectal immunisation shows potential due to the abundance of lymphoid tissue present in the rectum and colon (Hillery, 1998). Instead, the vagina appears not to have much epithelial lymphoid tissue aggregates and little antibody response to intravaginal delivery of drugs has previously been reported from some authors (O'Hagan, 1993). However, the genital tract is a component of the common mucosal

immune system (CMIS) and, according to other authors, there must be a potential to induce immune responses by the local application of immunogens (Wu et al., 2000).

Immunogenicity of zein microspheres was investigated following parenteral and mucosal routes too. Induction of oral tolerance and the responses to native zein was also followed with time.

5.1.1. Enzyme-linked immunosorbent assay (ELISA)

Enzyme immunoassays combine the specificity of antibodies with the sensitivity of simple spectrophotometric enzyme assays. In order to detect antibodies against zein and ovalbumin, enzyme-linked immunosorbent assay was selected for our study due to it is suitable to be operated in small laboratories and its lack of the hazards present in other immunoassays, such as radio immunoassays (RIA). Nonetheless, the reading of results for ELISAs is as objective, specific, and sensitive as for RIA or other immunoassays.

The performance of ELISA depends on the following major principles:

- ✓ Most antigens and/or antibodies, (i.e. proteins in our studies), bind spontaneously to solid phases, such as the wells of polystyrene microtitre plates. Once they are bound, they become resistant to vigorous washing in detergent buffer.
- ✓ Antigens and antibodies can form immune complexes to which enzyme conjugates of antibodies and/or antigens are able to bind leaving the enzyme component available for substrate interaction.
- ✓ Enzymes can be coupled to antibodies and/or antigens under conditions that retain their biological properties (i.e. antigenicity).
- ✓ Addition of substrates result in a progressive substrate solution colour change, which can be determined by either visual comparison with standards or by optical density measurements.

Based on these antibody or antigen solid phase coating principles, various alternative assay techniques can be used for the detection of antibodies, such as indirect antibody ELISA system, which is the one used in our studies. The latter is very popular for the screening of serum or other body fluid samples for the presence of specific antibodies. Antigens are used to coat the ELISA plate wells followed by the application of samples. By using an antiglobulin-enzyme conjugate, the specific antibody bound can be revealed. The antiglobulin may be of broad specificity to detect all antibody classes or it may be class- or subclass-specific. In addition, universal anti-species immunoglobulin-enzyme conjugates can also be utilised. The principle of indirect antibody ELISA assay is illustrated in figure 5.1.

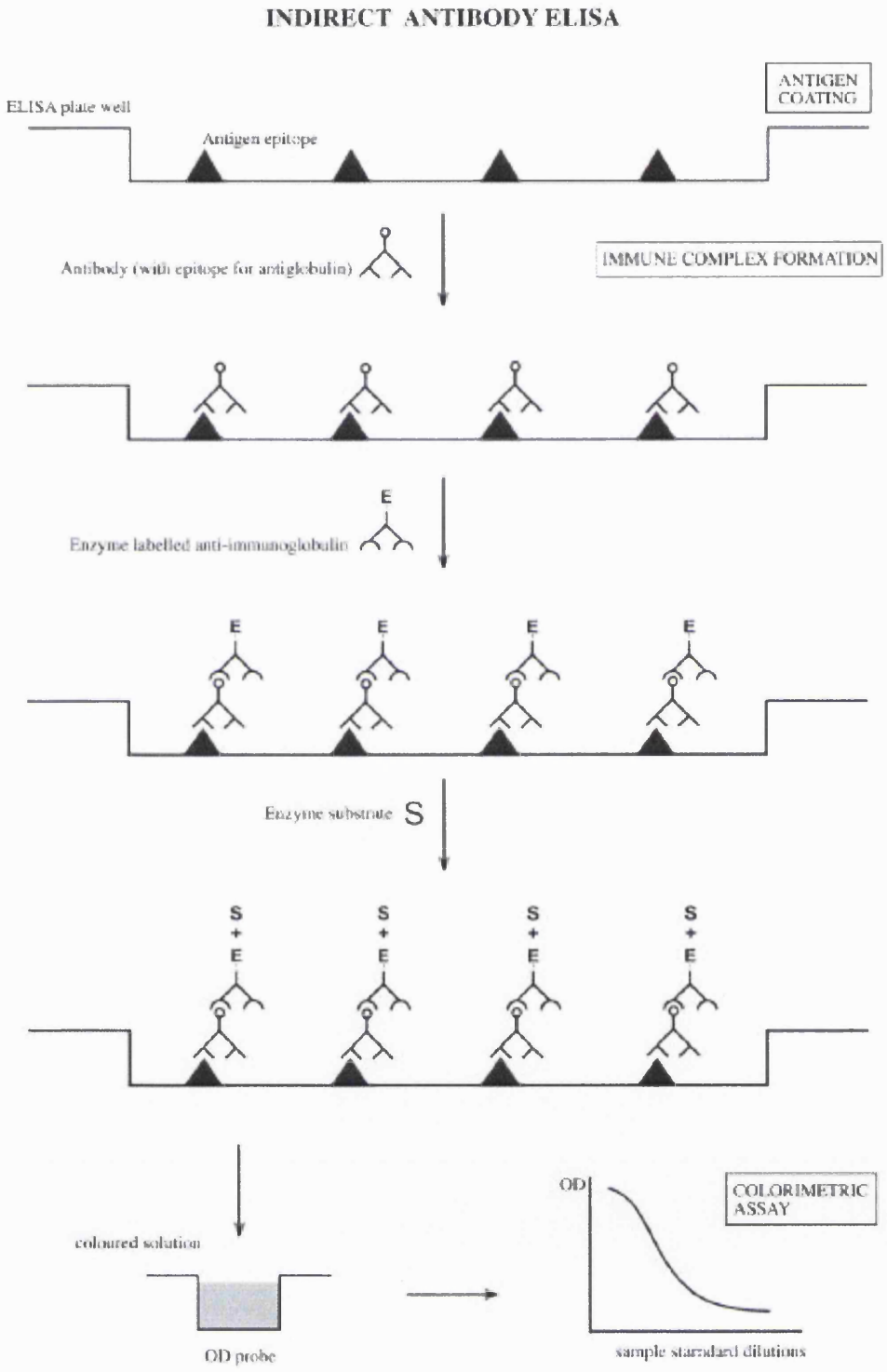


Figure 5.1. Principles of the indirect method of enzyme immunoassay. Antigens are coated onto ELISA plate wells, and then one or more layers of

immune complex are formed on the solid phase. The reaction between fixed enzymes and substrates leads to coloured products, colour formation is then measured and it is proportional to the amount of antibodies in the serum or other body fluid samples.

5.2. Experimental

5.2.1. Materials

5.2.1.1. Animals

Female BALB/c mice were obtained from B&K Ltd, UK and were acclimatised for seven days before the beginning of the study. All animals procedures were conducted in accordance with the Home Office standards under the Animals (Scientific Procedures) Act, 1986. The animals were caged in a room with standardised environmental conditions (20 ± 2 ºC, 35-45% Relative Humidity (RH)) and a constant day/night cycle. Animals were maintained on a normal mouse diet, which contains maize germ, and given water *ad libitum* throughout the studies. The first administration of formulations was conducted when mice were eight weeks old and weighed approximately 20 g.

5.2.1.2. Chemicals

Ovalbumin (albumin, chicken egg, Grade II), zein, bovine serum albumin (BSA), polyvinyl pyrrolydone MW 360 000 (PVP 360), phosphate buffered saline (PBS) tablets, Tween 20, potassium chloride, anti-mouse IgA (α-chain specific) peroxidase conjugate, anti-mouse IgG (whole molecule) peroxidase conjugate, hydrogen peroxide (30% v/v aqueous solution), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) tablets, heparin and sodium dodecyl sulphate (SDS) were obtained from Sigma, UK. Di-sodium hydrogen orthophosphate, citric acid, potassium hydrogen orthophosphate and sodium chloride were purchased from BDH Laboratory Supplies, UK. Other chemicals were of reagent grade and all of them were used as received. Diphtheria toxin was kindly donated by Serum Laboratories of India and dissolved in PBS pH 7.4 solution at a concentration of 14.5

mg/ml. Double distilled water was used throughout. ELISA 96-well microtitre plates were purchased from Dynatech, UK.

5.2.2. Methods

5.2.2.1. Intramuscular immunisation

All the immunisation studies were performed under a project license. Animals were cared for and treated according to an approved animal use protocol (Wolfensohn and Lloyd, 1998). Mice were randomly assigned to immunisation groups. Each animal group was composed of five mice and they were kept in one cage.

Mice were dosed intramuscularly (i.m.) with ovalbumin-loaded microsphere suspensions, as possible positive treatment, along with blank zein microsphere suspensions and ovalbumin dissolved in saline solution (sodium chloride 0.9% w/v), as possible negative controls. The microsphere suspensions were vortexed before injection. The dose of ovalbumin was kept constant at 150 μg, contained in 30 μl of ovalbumin-loaded zein microsphere suspension. Any unloaded ovalbumin had not been removed prior to particle suspension. The formulations and doses administered to each group is summarised in table 5.1.

All groups of mice were each immunised i.m. in the quadriceps with a single dose. Booster i.m. immunisations were administered twelve weeks after the primary immunisation, in an identical way.

For each group of mice, sampling was made at seven weeks after priming and one, four and seven weeks following boosting.

Blood was collected from the tail vein of mice. Tail veins were dilated with heat to aid visualisation and superficially cut using a scalpel. 100 μl of blood was

collected from each mouse into heparinised capillary tubes (using a 0.1% w/v aqueous heparin solution). Then, blood samples were refrigerated and allowed to clot overnight and sera were collected following centrifugation at 21 000 rpm for 10 min in a table-top centrifuge. Sera were stored at –70 ºC until assayed.

5.2.2.2. Oral immunisation and tolerance studies

To determine whether oral administration of zein microspheres gives rise to immune responses against zein, each individual animal of a group of five mice was orally administered (using a feeding needle) 100 µl of blank zein microsphere suspension, containing approximately 625 µg of zein protein. Mice were dosed on three consecutive days following overnight fasting of animals. Three identical booster doses were administered on days 29, 30 and 31 of the study (Fattal et al., 2002).

It has been shown that feeding protein antigens, such as corn proteins, raises an immune response in some animal species (Johnston et al., 1996; Klipper et al., 2001). To investigate whether serum IgG antibodies to zein proteins or other maize proteins could be detected in mice at normal concentrations of dietary proteins; animals were bred and raised on a normal mice diet, where contains maize germ as one of its main ingredients. Maize germ is composed of approximately 5% zeins and 23% glutelins (Lasztity, 1986). Since there is a certain primary structure homology among glutelins and zeins, one might expect antigenic cross-reactions between both types of proteins.

Serum samples were obtained and harvested as described above four weeks after priming and four weeks after boosting from animals immunised. Additional serum samples from non-immunised animals, raised on a diet containing maize germ, were taken when mice were four, eight and ten weeks old.

At the same time intervals, faecal samples were harvested for both groups of mice. Individual animals were placed in metabolic cages and their faecal material was collected after spontaneous defecation. Faeces were placed in PBS pH 7.4 solutions at a concentration of 10 µg/ml and homogenised. Then they were centrifuged at 21 000 rpm for 15 min in a table-top centrifuge. Supernatants were collected and stored at –70 ºC until assayed.

5.2.2.3. Rectal and vaginal immunisations

Three groups of mice were immunised rectally or vaginally using the same doses and formulations as used for i.m. immunisation (see table 5.1.). Since mucosal immunisations are supposed to raise smaller immune responses than parenteral immunisations, frequency of dosing was increased for rectal and vaginal administration. Mice were dosed on days 1, 5, 30, and 35.

Due to the low weight of the animal model used for our studies (approximately 20 g), the total volume of samples administered (30 µl), even if small, was introduced carefully in 10 by 10 µl using a 10 µl pipette, to allow its absorption.

Blood, faecal, and vaginal wash samples were collected to assay for antibodies. For all groups of mice, faecal and vaginal wash samples were taken one week after the last immunisation, while blood was taken two weeks after the last boosting. Serum and faecal samples were obtained as described above.

Vaginal washes were obtained by introducing 50 µl sterile PBS pH 7.4 solution into the vagina with a graduated pipette and flushing the solution in and out of the vagina four times. The obtained fluid was centrifuged at 21 000 rpm for 10 min in a table-top centrifuge and supernatants were collected and stored at –70 ºC until assay.

5.2.2.4. Determination of anti-ovalbumin and anti-zein antibody levels

Serum, vaginal wash and faecal samples from individual mouse were analysed for specific anti-ovalbumin and/or anti-zein IgG and IgA antibodies by a standardised ELISA method, as follows:

- Solutions of antigens (ovalbumin and zein) were prepared in coating buffers (PBS pH 7.4 solution and 60% v/v ethanol, respectively) at 10 µg/ml (1% w/v) and 100 µg/ml (10% w/v).
- Solutions of PVP 360 and BSA (1 and 4% w/v) were also prepared in PBS pH 7.4 solution.
- Microtitre ELISA plates were coated at 4 ºC overnight with:
    - 100 µl per well of ovalbumin 1% w/v solution in PBS pH 7.4 solution, or
    - 100 µl per well of ovalbumin 10% w/v solution in PBS pH 7.4 solution, or
    - 100 µl per well of zein 1% w/v solution in 60% v/v ethanol, or
    - 100 µl per well of zein 10% w/v solution in 60% v/v ethanol.
- Plates were washed-out three times in phosphate buffered saline-Tween (PBST), then once in double distilled water (see formula in Appendix).
- Plates were blocked with 100 µl per well of PVP 360 1% w/v or BSA (1 and 4% w/v) aqueous solution for 1 h at 37 ºC.
- Plates were washed-out three times in phosphate buffered saline-Tween (PBST), then once in double distilled water.
- Serum, vaginal wash and/or faecal samples (100 µl) were added on coated and blocked ELISA plates and incubated for 1 h at 37 ºC. The initial dilution of samples was 1:16 and subsequent 1:2 serial dilutions of that were made.
- Plates were washed-out three times in phosphate buffered saline-Tween (PBST), then once in double distilled water.
- 100 µl of anti-mouse IgA or IgG peroxidase conjugate diluted 1:1 000 in PBS pH 7.4 were added to the wells and incubated at 37 ºC for 1 h.

- Plates were washed-out three times in phosphate buffered saline-Tween (PBST), then once in double distilled water.
- Peroxidase substrate (ABTS) was dissolved to a concentration of 60 mg/100 ml in citrate buffer (see formula in Appendix). Then, 2 µl of hydrogen peroxide 30% v/v solution was added to each 15 ml of ABTS buffer solution immediately before use and mixed.
- 50 µl of ABTS/hydrogen peroxide solution were added to ELISA plate wells and incubated at 37 ºC for 30 min.
- The reaction was stopped by the addition of 50 µl of SDS 1% w/v aqueous solution.
- Plates were read at 405 nm in an ELISA reader (Opsys MR, Dynex Technologies).

Positive readings were considered those absorbances above the mean ± 2 S.D. of the negative controls. Negative controls were wells coated with body fluids samples of the same animal but before antigen administration. The antibody titre was expressed as the reciprocal of the serum, vaginal wash or faecal samples dilution that gave a positive absorbance.

Zein, as a highly hydrophobic protein, was not totally amenable to standard ELISA assays because of its insolubility in aqueous buffers. For our experiments, however, indirect ELISA assay was not changed when zein was used as a coating antigen instead of ovalbumin, a water-soluble protein. It has been previously demonstrated the adsorption of zein to polystyrene microtitre plates in conventional prolamin solvents such as aqueous alcohols, and the subsequent quantitative measurement of the protein following a regular ELISA assay (Conroy and Esen, 1984; Chirdo et al., 1995).

Antiserum and/or other body fluid samples adsorbed on solid phases should show a minimum of cross-reactivity with other antigens concurrently present. Zein is not a single protein, but a mixture of proteins containing multiple antigens with different abilities of inducing immune responses. It has been shown immunochemical cross-reactivity among different classes of zein and

between zein and other prolamins (Conroy and Esen, 1984; Esen, 1987; Dierks-Ventling and Cozens, 1982). Thus, non-specificity of anti-zein antibodies or cross-reactivity between zein and ovalbumin protein was plausible. Subsequently, microtitre ELISA plate wells were coated with diphtheria toxin in PBS pH 7.4 solution (1% w/v), a specific antigen to which the anti-zein antibody may not cross-react, designed for quality control. In addition, routine control assays were performed, such as (i) plate wells without antigen overnight coating, (ii) plate wells without blocking, (iii) plate wells without the addition of serum, vaginal wash, and faecal samples and (iv) plate wells without the addition of anti-mouse antibody-enzyme conjugate.

5.2.2.4.1. Statistical analyses

The results are expressed as mean ± S.D. for each group of mice. Student's t test was used to compare the means of each study group and to assess statistical significance. Results were considered statistically significant if $p < 0.05$.

Group	Formulation	Microspheres administered	Ovalbumin administered
1	Ovalbumin-loaded zein microspheres	30 µl suspension	150 µg
2	Blank zein microspheres	30 µl suspension	none
3	Free ovalbumin	none	150 µg

Table 5.1. Dose of ovalbumin, amount of microsphere suspension and type of formulation, which was administered intramuscularly, rectally and vaginally to three groups of mice.

5.3 Results and discussion

5.3.1. Immune responses to intramuscular administration of zein microspheres

5.3.1.1. Systemic antibody response

These studies were primarily set out to investigate the specific serum antibody responses generated after the administration of antigen-loaded zein microspheres. The chosen antigen was ovalbumin, known for being a poor immunogen when administered without the presence of adjuvants (Haas et al., 1996). Then, ovalbumin-loaded zein microspheres were administered and the anti-ovalbumin IgG antibody responses compared with those responses generated after administration of blank zein microspheres and of ovalbumin solution (see table 5.1.). However, mice immunised with both blank and loaded zein microspheres were found to develop significant IgG responses after boosting.

When ELISA plates were coated with ovalbumin, primary antibody responses to soluble ovalbumin, was not detectable. In contrast, positive readings were obtained after priming with both ovalbumin-loaded and with blank zein microspheres (figure 5.2). Even though absorbance readings for all three formulations and for negative controls were not significantly different, unexpectedly, the highest absorbance was obtained for mice immunised with blank zein microspheres.

In contrast, secondary immunisation did induce a significant response only a week after boosting, as shown in figure 5.3. Serum antibodies were obtained after immunisation with both blank and with ovalbumin-loaded zein microspheres even at week seven after boosting and their respective responses were not significantly different from one another. Secondary IgG

response to ovalbumin solution in saline, however, significantly decreased at week four following boosting.

The high antibody responses generated by blank zein microspheres indicated that anti-zein antibodies had been generated following administration and that these antibodies were binding to ovalbumin antigen coated onto the ELISA microtitre plate wells. This could be due to non-specific binding of anti-zein IgG antibodies and/or similarities between ovalbumin and zein antigens, which give rise to cross-reacting anti-ovalbumin and anti-zein antibodies. To investigate into the latter further, additional ELISA assays where the microtitre plate wells were coated with zein antigen were conducted. The primary anti-zein immune responses were found to be insignificant, as seen before for ovalbumin coating. Nevertheless, after secondary immunisation, immune responses were high, with the highest anti-zein IgG antibody response was obtained with blank zein microspheres. Even if ovalbumin-loaded zein microspheres also elicited an antibody response, the one generated by blank zein microspheres was significantly greater than the response generated by ovalbumin-loaded zein microspheres (figure 5.4.). This is a discrepancy with the results obtained when ovalbumin was adsorbed on plate wells, where responses from both microsphere formulations were not significantly different. Therefore, it can be assumed that anti-ovalbumin antibodies generated by ovalbumin-loaded zein microspheres do not cross-react with zein antigen adsorbed onto ELISA microtitre plate wells, in contrast to anti-zein antibodies, which do bind to ovalbumin antigen to a great extent. This assumption emphasises the hypothesis of the non-specificity and cross-reactivity properties of anti-zein IgG antibodies. Then, due to some structural homology between ovalbumin and zein proteins, anti-zein IgG recognised cross-reacting antigenic determinants on ovalbumin.

Cross-reactivity of zein with other antigens has been previously reported by various authors. Dierks-Ventling and Cozens (1982) tested different prolamins for immunochemical reactivity and found anti-zein IgG reacting with hordeins and gliadins but did not find the reverse action. These investigators speculated that antigenic recognition by anti-zein antibodies was possibly based on

structural similarity. Conroy and Esen (1984) assayed cross reactivity of three homogeneous polypeptides and their results showed extensive immunological cross-reactions among them. The more structural similarities the more cross-reactivity, subsequently zein ELISA tests were used by Conroy and Esen in defining homology groups for zein proteins. Later on, Esen (1987) studied immunological relationships among zein fractions. His results suggested that one or more epitopes were shared by all α-zeins. However, α-zein showed slight reactivity towards β- and γ-zein.

The non-specificity of zein antibodies could be the cause of the noise detected when ELISA plates were assayed and read. Those readings that were higher than readings for negative controls were considered high background readings (noise). In our studies, absorbance readings after ELISA assays had very high backgrounds, even if empty microtitre ELISA plate wells gave minimal values (<0.10). For this reason, it was difficult to find the linear part of the absorbance dilution curve for microsphere formulation samples. Instead, when using the same initial serum dilution and conjugate working dilution for ovalbumin solution samples we could easily worked on that linear part. To find a compromise, high positive readings-to-noise background ratios (>6:1) were required for assays of both microsphere formulations (highest OD_{405nm} was 2.0-1.5 after 30 min of colour development). The cause of high backgrounds is frequently the size of immune complexes (Catty and Raykundalia, 1988). Blockers, such as BSA or PVP 360, may reduce the backgrounds. For this reason, BSA was used when plate wells were coated with zein antigen and the PVP 360 was used when ovalbumin antigen was adsorbed on wells. Backgrounds resulted satisfactory for samples from mice administered with ovalbumin solution when wells were ovalbumin-coated, however results were not acceptable for samples collected after administering both microsphere formulations, neither when zein nor ovalbumin were used for antigen coating. Another cause of high backgrounds is the absence of an optimal antigen coating level. To solve this, two different antigen concentrations (1 and 10% w/v) were used to coat the ELISA plate wells. Both concentrations led to high backgrounds when microspheres formulation samples were tested and

adequate backgrounds for ovalbumin solution samples were again found for both of them.

The performance of ELISA assay was then not very acceptable for serum samples obtained after administering mice with both loaded and blank zein microsphere formulations. Therefore, routine control assays results were analysed. Control rows were first read by eye and then by the ELISA plate reader:

- Control wells without antigen coating did not show a contrast to the antigen-coated wells. Instead, an IgG antibody response was found. This shows that the conjugate may have non-specific binding properties. Conjugate could also be too concentrated, however, previous assays performed in our laboratories (data not shown) showed this conjugate working dilution had optimal binding properties for ovalbumin antigen.
- When microtitre plates were coated with antigen and were not blocked, background readings were higher than with when wells were blocked. This may indicate that the cause of high backgrounds could be related to the size of enzyme-protein polymers and quenching with a polymer or a weak protein reduces the problem.
- When plate control wells were antigen-coated and blocked, but serum samples were omitted, absorbance readings were of the same gradation or weaker than those of negative serum samples. The fact that negative serum samples give higher readings than those given when no sera was added could be a sign of the presence of anti-zein antibodies in mice before administration of any formulation. The anti-zein antibodies would react with ovalbumin antigen. In addition, antigens may have IgG to which the conjugate cross-reacts.
- When the antibody-enzyme conjugate was not added, readings were minimal, as expected.

Since zein antigen was shown to be an antigen of poor quality for ELISA assays, when assayed together with ovalbumin antigen; a structurally different and specific antigen (diphtheria toxin) was used to coat ELISA microtitre plate

wells and serum samples obtained after blank zein microspheres immunisation were tested. Theoretically, no anti-diphtheria toxin IgG antibodies should be present in the sera assayed. However, cross-reactions were observed and high backgrounds readings were given. Even though positive sera readings were significantly higher that those of negative controls, the noise was sufficiently high to impede establishment of antibody titres. Once more, the non-specific anti-zein antibodies bound to a different antigen adsorbed on plate wells.

5.3.1.2. Antigenicity of zein microspheres

After our results, an antibody response, which lasted long time, was raised after the second administration of both microsphere formulations. Although proteins from natural sources, such as zein, should not be immunogenic, their systemic administration may cause an unwanted immune response. This immune response, however, did not seem to cause related diseases. Animals were watched and records were kept on parameters such as body weight and food and water intake, parameters that did not change thorough the studies. In addition, alterations in the normal behaviour of the animals, distress, or pain were not observed. Prolamins are known to be plant food allergens and can produce some kind of food intolerance. However, allergic or toxic prolamines have not been reported in maize (Denery-Papini et al., 1999; Mills et al., 2003).

Immunogenicity of zein has been formerly reported (Naim and van Oss, 1992). These authors' findings proved that highly hydrophobic and highly hydrophilic polymers were not immunogenic and that solubility in water favoured immunogenicity. Zein, which was previously dissolved with 0.2% w/v SDS, easily produced anti-zein antibodies within four weeks of immunization. They concluded that a hydrophobic protein could be readily rendered immunogenic once it is dissolved. Upon dissolution, the epitopes situated on the dissolved antigen molecules become separated from each other and are readily

available for immune recognition. However, our studies resulted in particulate zein activating an immune response without the need to be in soluble state.

The generation of anti-zein antibodies was surprising and unexpected. The antibody response was raised only after the secondary administration; this is probably due to the maturation of the immune response (change in antibody expression from IgM to a predominant IgG isotype) and the presence of memory lymphocytes generally occurs after at least two doses of antigen administration (Lofthouse, 2002). However, as mice feed includes zein, it was anticipated that mice might have developed tolerance to zein. In this case, zein microspheres would have been non-immunogenic, and therefore could have been used as vaccine adjuvants. The zein ingested by mice is in the form of native protein and, during microsphere formation, the secondary structure of zein changes to a certain extent (see chapter 4). These changes may expose new zein epitopes on the surface of the protein, which were previously buried in the native folded protein. Then, the second administration of zein in the form of microspheres may lead to the anti-zein antibody production when zein microspheres are administered parenterally. This may not occur when zein microspheres are administered orally, as it is generally assumed that tolerance occur via the gastrointestinal tract.

Other authors reported that proteinoid microspheres induced significant serum IgG titers within two weeks when microspheres were composed of poorly defined amino acid mixtures. Instead, when microspheres were made from derivatised amino acids little serum IgG was detected until at least eight weeks following dosing (Haas et al., 1996). Selecting and purifying zein polypeptide mixture used to produce microspheres could reduce the generation of systemic immune responses and zein microsphere immunogenicity could be decreased.

Johnston et al. (1996) demonstrated that antibodies to a variety of commonly accepted dietary proteins are present in the serum of ruminating sheep. In their experiments, lambs were either fed with corn proteins at the recommended amounts or inoculated subcutaneously with saline extracts of

each protein. While the serum IgG concentrations to zein in fed lambs remained the same over the duration of the experiment, the IgG anti-zein antibodies in the subcutaneously injected lambs increased eight times after 4-6 weeks. Zein protein ingested and injected s.c. were both in the form of native protein, this suggests that the change in the secondary structure of zein during microsphere formation in our experiments may or may not have had an impact on the immunogenicity of our zein microspheres. The route of administration may play a more important role.

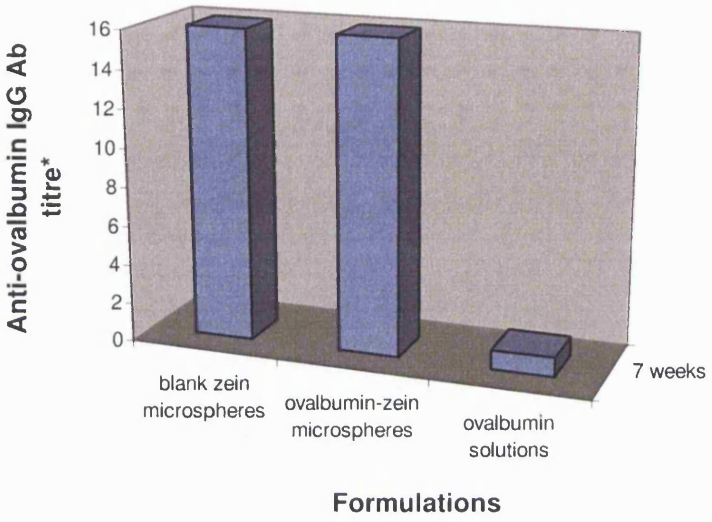


Figure 5.2. Anti-ovalbumin IgG antibody response after the primary immunisation of ovalbumin-loaded zein microspheres, blank zein microspheres and ovalbumin in saline solution (see table 5.1. for treatment groups).

* Ab titre (average of five mice) is the reciprocal of the sera dilution that gave a positive reading (absorbance of negative control ± 2 S.D.).

Mean ovalbumin-loaded zein microspheres formulation: 0.1468 ± 0.025.

Mean blank zein microspheres formulation: 0.213 ± 0.025.

Mean ovalbumin solution formulation: 0.110 ± 0.006.

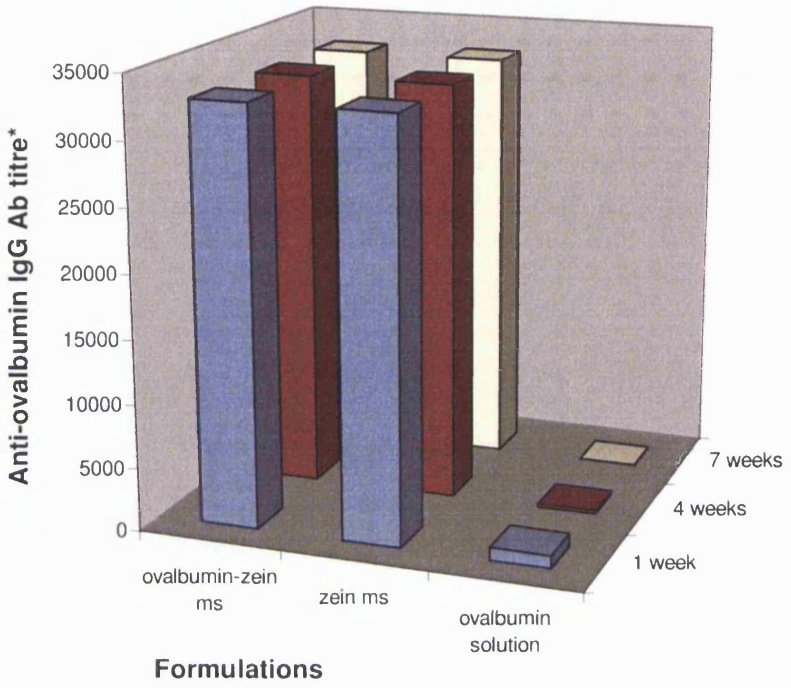


Figure 5.3. Anti-ovalbumin IgG antibody response in mice after the secondary immunisation of ovalbumin-loaded zein microspheres, blank zein microspheres and ovalbumin in saline solution (see table 5.1. for treatment groups).

*Ab titre (average of five mice) is the reciprocal of the sera dilution that gave a positive reading (absorbance of negative control ± 2 S.D.).

Mean ovalbumin-zein microspheres formulation, one week after boosting: 0.355 ± 0.128.

Mean ovalbumin-zein microspheres formulation, four week after boosting: 0.460 ± 0.119.

Mean ovalbumin-zein microspheres formulation, seven week after boosting: 0.343 ± 0.072.

Mean zein microspheres formulation, one week after boosting: 0.491 ± 0.141.

Mean zein microspheres formulation, four week after boosting: 0.637 ± 0.148.

Mean zein microspheres formulation, seven week after boosting: 0.396 ± 0.159.

Mean ovalbumin solution formulation, one week after boosting: 0.253 ± 0.035.

Mean ovalbumin solution formulation, four week after boosting: 0.281 ± 0.029.

Mean ovalbumin solution formulation, seven week after boosting: 0.235 ± 0.042.

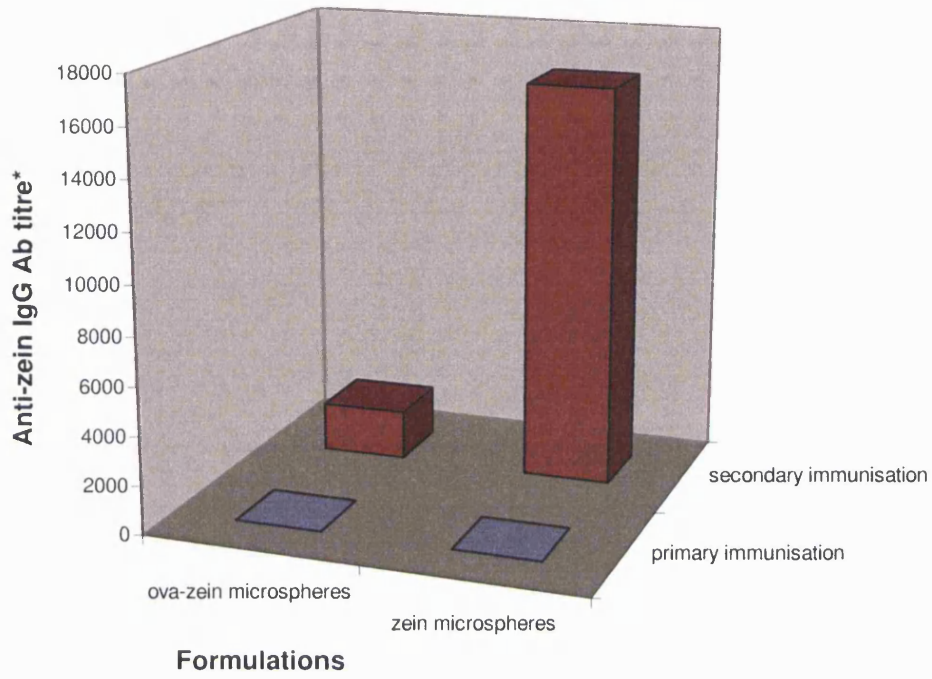


Figure 5.4. Anti-zein IgG antibody response in mice seven weeks after primary and secondary immunisation of ovalbumin-loaded and blank zein microspheres (see table 5.1. for treatment groups).

*Ab titre (average of five mice) is the reciprocal of the sera dilution that gave a positive reading (absorbance of negative control ± 2 S.D.).

Mean ovalbumin-zein microspheres formulation, after priming: 0.248 ± 0.028.

Mean ovalbumin-zein microspheres formulation, seven weeks after boosting: 0.688 ± 0.078.

Mean zein microspheres formulation, after priming: 0.235 ± 0.025.

Mean zein microspheres formulation, seven week after boosting: 0.749 ± 0.024.

5.3.2. Immune responses to oral administration of zein microspheres

5.3.2.1. Systemic antibody response

To determine whether orally administered blank zein microspheres induce an immune response, a group of mice were orally dosed with blank zein microspheres and the primary and secondary responses were monitored.

Anti-zein IgG antibody responses elicited after oral administration of blank zein microspheres are shown in figure 5.5. An immune response was observed, which, in contrast to the i.m. administration, did not significantly increase after secondary immunisation. The antibody response after boosting was of the same gradation as that observed after primary parenteral administration. The unwanted immune response after boosting observed following i.m. immunisation was then not detected. Hence, whichever changes are produced in zein protein during microsphere formation do not play an important role in generating an antibody response after oral administration, in contrast with i.m. administration. This fact may be due to the phenomenon of oral tolerance and/or to the enzymatic processing of zein via proteolytic enzymes (as it has been observed *in vitro*, see chapter 4) present in the gastrointestinal tract, which removes the protein antigenicity.

Since oral feeding of protein antigens has been demonstrated to elicit an antibody response in other animal species, anti-zein IgG antibodies were assayed on serum samples of mice fed at recommended concentrations of zein as a dietary protein. In effect, colour formation (absorbance reading >0.2) could be observed for all samples tested. To follow if the latter antibody response increased with time, samples from mice aged four, eight, and ten weeks old were assayed. No significant increases could be detected at any time. A certain level of anti-zein IgG antibodies seems to be circulating in blood since mice are at puberty. Therefore, serum samples from non-

immunised mice (negative controls) contain antibodies against zein, which show non-specific binding and which may be responsible of the high backgrounds previously observed in ELISA assays.

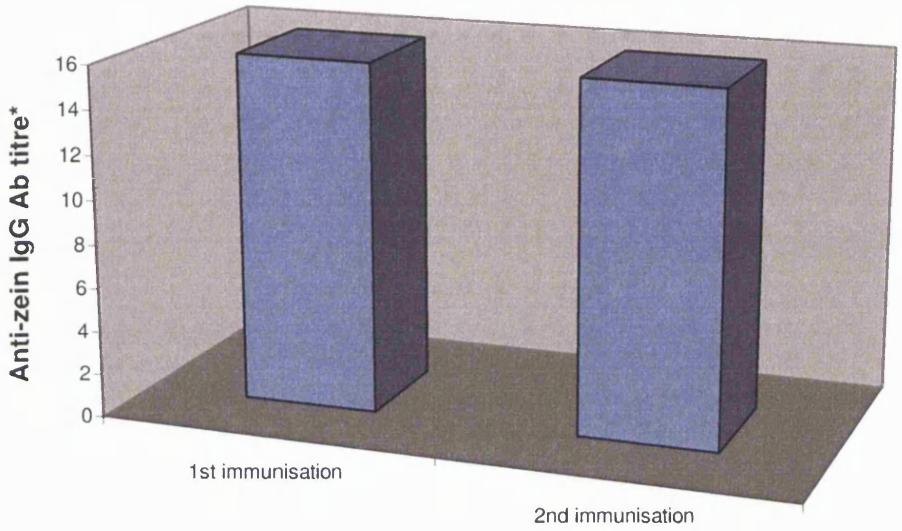


Figure 5.5. Anti-zein IgG antibody response in mice after orally immunisation of blank zein microspheres.

*Ab titre (average of five mice) is the reciprocal of the sera dilution that gave a positive reading (absorbance of negative control ± 2 S.D.).

Mean priming: 0.462 ± 0.048.

Mean boosting: 0.520 ± 0.068.

5.3.2.2. Mucosal antibody response

The corresponding ELISA routine assay controls were performed when assaying anti-zein IgA isotypes as well. For all controls, results were equivalent to those obtained when testing IgG antibodies. Backgrounds, however, were smaller than those obtained when assaying anti-zein IgG, thus non-specific binding properties of anti-zein IgA antibodies may be of less importance.

As for IgG antibodies, after oral administration of blank zein microspheres, IgA antibodies against zein present in faecal material were assayed for non-immunised mice at four, eight and ten weeks of age and for mice immunised with blank zein microspheres.

Anti-zein IgA in non-immunised mice, fed with zein at regular protein amounts, could be observed from the time when animals were four weeks old, the same as it occurred for IgG antibodies. However, noise in ELISA assays was found to be smaller, it can be reaffirm then the reduction in non specific binding with respect to anti-zein IgG antibodies.

Samples from mice orally immunised with blank zein microspheres oral resulted in significant IgA antibodies against zein after priming and particularly after boosting a month later, as shown in figure 5.6. IgA antibody responses are characteristic of mucosal immunity, although IgG responses also develop after mucosal immunisation. In our findings, the immune response was both of mucosal and systemic type, but the IgG response did not increase following boosting as the IgA antibody response did. This may be due to the different mechanisms of generating both immunoglobulins.

Absorption of particulates in the intestine following oral administration is possible to occur via several mechanisms (Chen and Langer, 1998). The absorption pathways are affected by the size of the particles administered. The size of blank zein particles (about 1 μm) suggests that the majority of

them must be absorbed exclusively by the intestinal lymphatic tissues (i.e. Peyer's patch M cells), as well as the hydrophobic nature of zein. The amount of particles taken up after oral administration is controversial (Florence, 1997); however, this may be explained by the different animal models used in the different studies. Current knowledge from mice may or may not be extendable to human beings. We hypothesise that the ability of zein microspheres to elicit a mucosal immune response may be due to the particle uptake by intestinal lymphoid tissue, together with the existence of mucosal antibodies due to zein included in mice diet. The systemic response might be due to small particles leaving Peyer's patches after particle uptake and transported to elicit a systemic anti-zein antibody response. However, because the systemic response generated is small and it is not significantly enhanced after boosting, as it occurs for mucosal response, it is possible that anti-zein IgG detected is that which is elicited by zein native protein of mice diet only.

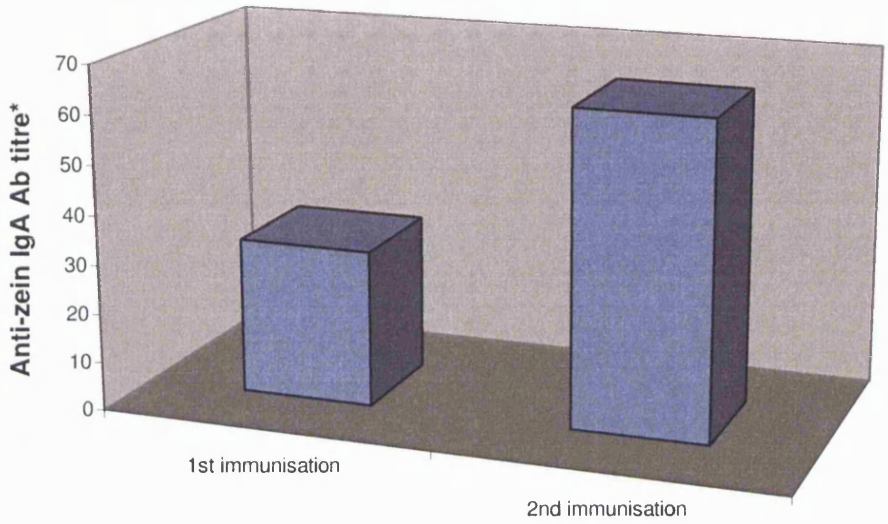


Figure 5.6. Anti-zein IgA antibody response in mice after orally immunisation of blank zein microspheres.

*Ab titre (average of five mice) is the reciprocal of the sera dilution that gave a positive reading (absorbance of negative control ± 2 S.D.).

Mean priming: 0.183 ± 0.025.

Mean boosting: 0.163 ± 0.022.

5.3.2.3. Oral tolerance

Oral administration is the classical route to induce immune tolerance. Nonetheless, the generally accepted premise that proteins orally administered induce oral tolerance is in contradiction with studies in different animal species, which showed that dietary proteins induced antibody responses (Klipper et al., 2001; Johnston et al., 1996). On the other hand, the phenomenon of oral tolerance is usually initiated by the ingestion of soluble protein antigens, rather than orally administered particulate antigens, such as microspheres, which elicit immune responses as they are taken up by the M cells of the small intestine. Our results confirm with this theories.

Our studies were conducted to determine whether orally administered zein protein, either as a native molecule (present in maize germ, which is part of mice diet) and/or in a particulate manner (our zein microsphere formulation), depresses systemic and/or enteral antibody response to zein microspheres and induces oral tolerance.

Zein as a native protein seems to be non-tolerogenic because mice fed with zein as a native protein as part of their in mice diet, raised an extensively immune response after secondary parenteral immunisation. Unfortunately, in the case of zein and other prolamins, it exists an additional dilemma to investigate into tolerance. As these kinds of cereal proteins are not a single protein but a mixture of proteins, multiple antigens exist that may hamper the identification of single tolerogenic molecules. The fact that secondary structure of zein protein changes when zein is formulated into microspheres may also impede the generation of tolerance by the native zein ingested by mice daily.

Oral tolerance, however, seems to occur after mucosal administration since secondary responses to oral immunisation were found to be much more depressed than those to i.m. immunisation. However, it has been previously reported that particulate antigens, such as zein in our case, often induce active immunity instead of tolerance, caused by their uptake by M cells,

leading to more efficient antigen processing (Strobel and Mowat, 1998). Then, the IgG unresponsiveness after secondary oral immunisation may be due to the phenomena of oral tolerance, but also to the degradation of zein protein at the gastrointestinal tract.

If certain extent of tolerance occurs, with all probability this is not generated by the native protein included in the mice diet (due to the change in secondary structure, which may expose different epitopes) but by the primary administration of zein in the form of microspheres. A selective nature of tolerance must be affected by the primary route of the antigen entry.

5.3.3. Immune responses to rectal and vaginal administration of zein microspheres

As certain extent of tolerance affected by the route of entry was observed after mucosal immunisation, it was decided to immunise model animals via a mucosal route where particles can be uptaken, but lacks proteolytic enzymes to fast degrade zein and ovalbumin proteins as it occurs orally. Different immunisation strategies have been previously reported to induce local induction of IgA or IgG antibodies after vaginal immunisation, but dissemination to the systemic compartment was limited, probably because the genital tract lacks organised mucosal inductive sites as the gastrointestinal tract (Haneberg et al., 1994; Wu et al., 2000). However, other studies have shown that both rectal and vaginal routes elicit systemic and mucosal responses, along with cell-mediated immune responses (Mitchell and Galun, 2003; Hamajima et al., 2002). Rectal and vaginal immunisation using ovalbumin as antigen and other microspheres formulations than zein as delivery systems, had previously given positive results in our laboratories (data not shown). Thus, those routes seemed encouraging to elicit antibody responses when administering ovalbumin-loaded zein microspheres. The rectum and colon contains abundance of lymphoid tissue, which should be able to uptake zein particles and elicit an IgA antibody response, as it was

shown to occur after oral administration of blank zein microspheres. The female genital tract is a component of the common mucosal immune system (CMIS), also ease of access to the vagina makes the local immunisation simple. Unfortunately, immune responsiveness of the female tract varies during the estrous cycle (Russell and Mestecky, 2002).

Blank and loaded zein microspheres and ovalbumin in saline solution were administered as for i.m. immunisation, and ELISA assays were performed to search for anti-ovalbumin IgG and IgA in serum, faecal and vaginal wash samples.

From our results after i.m. immunisation IgA and IgG antibodies could be both anti-zein and anti-ovalbumin, however, the priming did not produce significant levels of IgA in faecal material and/or vaginal secretions or IgG in sera for all formulations, with respect to negative controls. Unfortunately, after boosting, levels of IgA and IgG antibodies were still not significant.

The insignificant IgG response in sera agrees with our findings after oral immunisation with blank zein microspheres, where systemic response against zein was neither of importance, most probably due to some tolerance created by the route of first entry. On the other hand, the immune IgA unresponsiveness after immunisation by these routes may be due to deficiency in particle uptake. Both reasons may cause the non-existence of anti-zein antibodies present in samples and cross-reacting with ovalbumin antigen adsorbed onto ELISA microtitre plate wells, which occurs after i.m. immunisation.

The aqueous environment of both tracts may not allow sufficient release of ovalbumin from zein microspheres for the generation of immune responses. Being ovalbumin a poor immunogen, the exposure of low levels ovalbumin antigen to antigen presenting cells after rectal and vaginal immunisation may not suffice for the generation of significant both mucosal and systemic immune responses.

5.4. Conclusions

Parenteral administration of blank and ovalbumin-loaded zein microspheres showed that antibodies against zein and against ovalbumin were generated. Anti-zein IgG antibodies had non-specific binding properties and they cross-reacted with ovalbumin antigen, probably as a result of the sharing of epitopes, due to structural similarities. The raise of anti-zein IgG antibodies occurred after secondary administration and hampered the quantification of anti-ovalbumin IgG antibodies, which were possibly also raised after boosting. The presence of antibodies against zein converts zein microspheres a deficient delivery system for parenteral immunisation.

The question if the phenomena of oral tolerance could overcome the antigenicity of zein was addressed. Native zein protein was included in normal mice diet and the protein was shown to be non-tolerogenic since an extensive immune response was induced after intramuscular boosting. That was supposed to occur owing to two reasons: i) the changes in the secondary structure when zein was formulated into microspheres, and ii) the fact that native zein is a non-purified mixture of proteins with multiple antigens.

Oral administration of blank zein microspheres did not induce significant increase of IgG antibodies after boosting. However, we hypothesise that this sort of tolerance was affected by the primary route of entry along with the degradation of zein by gastrointestinal proteolytic enzymes, removing protein immunogenicity. However, local mucosal immune response after oral boosting did occur and some extent of anti-zein IgG antibodies were found in non-immunised mice since they were at puberty, due to the ingestion of native zein protein. Once again, immune responses against zein were generated after oral administration (even it was much smaller than after i.m. immunisation), which would cross-reacted with other antigens and hamper its identification. This fact means that zein microspheres would not be an ideal delivery system for oral immunisation either.

After rectal and vaginal immunisation, neither anti-zein nor anti-ovalbumin antibodies were generated. The reason for the absence of anti-ovalbumin antibodies could be due to negligible ovalbumin release from zein microspheres, in an aqueous media such the one in rectal and vaginal tissue. It could be assumed that antibodies against zein were not present, otherwise they would have cross-reacted with ovalbumin antigen coating the ELISA microtitre plate wells. Absence of anti-zein antibodies could be due to a minor zein microspheres uptake in those tissues.

We conclude that zein microsphere formulation is not an acceptable delivery system due to its inherent antigenicity. To overcome this, a zein protein can be studied to identify a single component, which down regulates the immune responses elicited by the entire zein polypeptide mixture, as it has been investigated for gliadins (Senger et al., 2003). The one or more purified zein polypeptides could be formulated into microspheres to be used as vaccine delivery systems. Then, the properties of zein microspheres as vaccine delivery system could improve.

The response to native zein as a dietary protein in human should be elucidated. However, even if oral zein antigens induce immune responses, it is obvious that additional mechanisms must account for the absence of related allergies or toxicities, since the latter diseases for maize prolamins have not been detected in contrast to other cereal prolamins.

Before evaluating zein microspheres *in vivo*, the proportion of PVP 40 present in the zein microspheres formulation (see chapter 3) was expected to affect the immunogenicity of the delivery system. However, the effect of the hydrophilic polymer PVP 40 has not been evaluated and it is concluded that zein microspheres are a deficient delivery system due to its intrinsic antigenicity.

Chapter 6.

Conclusion

6. Conclusion

The aim of the current thesis was to prepare microspheres made of zein and to explore the capacity of such zein microspheres to act as controlled antigen delivery systems *in vitro* and *in vivo*. For this purpose, we first investigated the ability of zein protein to act as a microsphere matrix

Zein properties were first examined. Since zein peptides vary from batch to batch depending on plant extraction, zein composition was determined to know the nature of zeins used. The same batch of zein was then used in all the experiments. The solubility properties of zein in relation to its precipitability were considered very important since the formulation of microspheres was likely to be based on zein solubility. Thus, zein solubility was determined in the solvents that were used in microsphere preparation method.

From the solubility studies on zein, insolubility of zein in water was found to be extremely high and this finding agrees with the data found in literature. Amino acid composition of zein revealed a high number of uncharged residues, which would confer hydrophobicity to zein protein. The latter also corresponds to previous literature.

Once zein properties were known, a method was searched to produce zein particulate systems. The main objective of this method was to avoid hazards and to be simple and rapid, due to the practical production limitations in the laboratory. A technique based on zein precipitation due to zein's insolubility in aqueous media was developed, and zein microsphere aqueous suspensions were prepared. Studies were carried out to understand the underlying mechanisms of microsphere formation and/or the effects of experimental conditions (e.g. the role of suspension additives) on microsphere formation and properties. The understanding of the formulation process is a tool that could be used by formulation scientists to produce zein microspheres suitable for different purposes. However, high variability was found in the size distribution of zein microspheres. This fact may be due to the mechanism of

microsphere formation and we conclude that optimisation studies should be performed in an environment able to control room temperature (which would affect zein solubility), and experimental conditions of solvent removal (the moment at which zein microspheres are supposed to be formed) to a maximum extent. By controlling solvent removal and determining the maximum residual ethanol permitted in each case, we hypothesise that variability of zein microsphere properties could be reduced and so an optimal formulation could be found.

However, a rapid and simple method to produce zein microspheres was found and we conclude that zein microspheres can be further investigated as drug and/or vaccine delivery systems.

The characteristics of the final formulation of zein microspheres were determined. It is generally accepted that the size of microspheres affects the potentiation of an immune response, with the greatest immune response occurring with microspheres <10 microns in diameter. From the size of our zein microspheres, we conclude that they would undergo phagocytosis and transportation of lymph nodes so they could possess an adjuvant activity.

Technical limitations were found in experiments on loading of ovalbumin and *in vitro* release, since interactions between both proteins did occur probably due to similarities between them. The techniques at disposal could only be semi-quantitative and they were used to give an approximation of both characteristics. Since the purpose of the present study was to explore the potential of zein microspheres as antigen delivery systems, that approximation was assumed to be true. We conclude that further experiments will have to be carried out with an optimised formulation when other techniques are available. Using a different model protein as antigen will help to achieve an accurate result.

Protein integrity was also determined for both ovalbumin and zein. Zein seemed to have changes in secondary structure; however, this would not

represent a setback since activity of zein did not have to be kept for adjuvancy. On the other hand, it is not a clear-cut whether these changes could boost the antigenicity of zein microspheres that was found later when they were administered parenterally. Ovalbumin integrity seemed to be preserved, and its biological activity was probably retained. However, the activity of ovalbumin as an immunogen is its capacity of producing an immune response. We could not demonstrate ovalbumin epitopes were untouched by the techniques used (i.e. SDS-PAGE and FTIR), but this fact was considered unimportant since its immunogenicity was going to be determined *in vivo* while demonstrating adjuvanticity of the whole delivery system.

Peptides and proteins are rapidly cleared from the body. Since zein is commercially used for enteric coating in food industry, it was hypothesised that zein microspheres would offer the advantage of resisting the action of proteolytic enzymes and thus protecting ovalbumin from degradation and clearance in a physiological environment. Zein microspheres were found to be extremely resistant to the pH action and to storage. However, proteolytic enzymes did cause enzymatic degradation on zein microspheres and we conclude that they would not allow sufficient protection of ovalbumin in the gastrointestinal tract. On the other hand, the extreme resistance of zein microspheres in the absence of proteolytic enzymes impeded ovalbumin release *in vitro*. This fact would not be a drawback and we hypothesised that it could be used for prolonged release of guest molecules when using other drugs for different therapeutic conditions. In this laboratory, we have incorporated small molecules, such as aspirin, into zein microspheres. Further experiments on small molecules *in vitro* release may be performed to explore potential of zein particulate systems as sustained release delivery systems.

The effectiveness of an antigen delivery system is determined in part by its ability to activate the humoral and/or cell-mediated immune responses. In this thesis, the humoral immune response to the weak antigen ovalbumin was determined when ovalbumin was administered into zein microspheres, which would act as adjuvants. When the latter was attempted to be measured in mice, the major drawback of zein microspheres as controlled antigen delivery

system was found. The drawback was the antigenicity of zein microspheres. Zein immunogenicity was found to be especially high when microspheres were administered parenterally. In addition, such antigenic properties interacted with the technique employed and the immune response to ovalbumin could not be proved. The latter response was not further investigated since the result was found already valid, i.e. the main hypothesis to be investigated was the adjuvanticity of the system, and such an antigenic system had to be rejected as an adjuvant. The possibility of tolerance to zein by mucosal routes was then explored; nevertheless, we conclude that mice immune system does respond to zein administered either by mucosal and parenteral routes or simply by gavage. It is known that extracting zein from plant with ethanol yields a mixture of zein and maize lipid. We hypothesise that purifying zein could reduce zein antigenicity.

We finally conclude that to produce the present zein particulate system, environmental conditions have to be controlled in order to optimise the formulation process and to reduce the amount of additives, if possible. Other drugs, such as small molecules may be incorporated and zein microspheres ability to act as a sustained delivery system investigated. A modified zein protein in terms of antigenicity should be produced to prepare antigen delivery systems, probably by purifying. Once a modified zein is used, its antigenicity as a single protein and precipitated into microspheres should be explored. If zein antigenicity is reduced, a stronger and purer immunogen could then be incorporated in the system. The current method of production was proved to be rapid and simple and probably do not alter the integrity of the epitopes, since it avoids hazardous organic solvents, high temperatures, and high shear.

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Appendix

Preparation of simulated fluids and buffers:

Acetate Buffer pH 5.0

To dissolve 13.6 g of sodium acetate and 6 ml of glacial acetic acid in sufficient water to produce 1 l.

Chloride buffer pH 2.0, 0.1M

Dissolve 6.57 g of potassium chloride in water, add 119.0 ml of 0.1m hydrochloric acid and dilute to 1 l with water.

Phosphate buffer pH 7.4

Add 250 ml of 0.2M potassium dihydrogen orthophosphate to 393.4 ml of 0.1M sodium hydroxide.

Or

Dissolve a ready phosphate buffered saline tablet in 200 ml of water.

Glycine buffer pH 11.3

Mix a solution containing 0.75% w/v of glycine and 0.58% w/v of sodium chloride with an equal volume of 0.1M sodium hydroxide and adjust the pH if necessary.

Simulated gastric fluid

Dissolve 2 g of sodium chloride and 3.2 g of purified pepsin in 7 ml of hydrochloric acid and sufficient water to make 1 l.

Simulated intestinal fluid

Dissolve 6.8 g of monobasic potassium phosphate in 250 ml of water, mix, and add 77 ml of 0.2N sodium hydroxide and 500 ml of water. Add 10 g of purified pancreatin, mix, and adjust the resulting solution with either 0.2N sodium hydroxide or 0.2N hydrochloric acid to a pH of 6.8 ± 0.1. Dilute with water to 1 l.

Phosphate buffered saline-Tween 20 (0.05%)

Dissolve and mix 40 g of sodium chloride, 1 g of potassium chloride, 7.21 g of disodium hydrogen orthophosphate, and 1 g of potassium hydrogen orthophosphate in water. Add 2.5 ml of Tween 20 and sufficient water to make 5 l.

Citrate buffer

Dissolve 4.6 g of citric acid and 9.78 g of disodium hydrogen orthophosphate in sufficient water to make 500 ml.