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**An investigation into the adjuvanticity and immunogenicity of zein microspheres
being researched as drug and vaccine carriers**

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Abstract

Aim: The aim was to determine whether zein microspheres can act as vaccine adjuvants, i.e., increase the immune responses to co-administered immunogens.

Methodology: Ovalbumin (model antigen)-loaded zein microspheres, blank zein microspheres and ovalbumin solution were intramuscularly administered to mice and the sera antibody levels were determined by ELISA. Another group of mice was orally dosed with blank zein microspheres, and serum and faecal antibody levels were determined.

Results: As expected, negligible antibody titres were obtained with the ovalbumin solution. Surprisingly, intramuscular administrations of blank zein microspheres elicited high levels of serum IgG which bound to the ovalbumin antigen coated on ELISA microtitre plates. This indicated that anti-zein antibodies had been elicited by blank zein microspheres and that these antibodies were cross-reacting with ovalbumin antigen coated onto ELISA plates. Such cross-reactivity inhibited the determination of the adjuvant activity of zein microspheres, if any. Additional ELISA assays, where zein was used as the coating antigen, confirmed the generation of anti-zein antibodies by blank zein microspheres, i.e. zein microspheres were immunogenic following intramuscular administration. Upon oral administration of blank zein microspheres, serum IgG levels remained low but intestinal IgA levels increased following booster doses i.e. systemic tolerance, but not mucosal tolerance, to oral zein particles was achieved.

Conclusion: Zein microspheres are immunogenic when administered intramuscularly and orally.

Keywords: zein, microspheres, adjuvanticity, immunogenicity

Introduction

In the past decades, microspheres - defined as monolithic polymeric matrix systems (Couvreur & Puisieux 1993) - have been extensively investigated as sustained/controlled release drug and vaccine carriers. Sustained release of entrapped active entity enables a reduced dosing frequency and enhanced patient compliance. The use microspheres for vaccine delivery also has the advantages of presenting the vaccine in a particulate form, which facilitates vaccine recognition and uptake by antigen presenting cells, resulting in enhanced immune responses (Storni et al 2005). Consequently, a variety of particulate vaccine delivery systems have been studied (Bramwell & Perrie 2005). Polylactide-co-glycolide (PLGA) has been the polymer of choice for the formulation of microspheres as it is already used in biomedical devices in man. However, PLGA particles do suffer from certain disadvantages, such as instability of encapsulated proteins (Putney et al 1998; Schwendeman 2002; Jiang et al 2005) and a large number of alternative polymers, synthetic and natural, have been investigated.

One such polymer is zein, a naturally occurring hydrophobic plant polymer, which is biodegradable, has GRAS (Generally Regarded as Safe) status (Anon 1985) and has been extensively used in the pharmaceutical, food, agricultural and other industries. Zein - the prolamin of maize (*Zea mays* L.), found in maize germ and endosperm – consists of a mixture of peptides, which may be divided into several classes. Each class differs in solubility, molecular weight, amino acid composition, immunological properties, etc. The classification of the different classes of zein protein has led to confusion and many nomenclatures have been proposed (Esen 1986, 1987, 1990;

Wilson 1985; Landy & Guyon 1984 a,b). In this work, the nomenclature proposed by Esen (1986, 1987, 1990) has been employed. According to this nomenclature, zein is composed of four classes of protein (α , β , δ and γ). α -zein is the most abundant, consists of two polypeptides of estimated MW of 22 and 24 kD, and its amino acid composition is similar to that of whole zein (Paulis et al 1969; Paulis & Wall 1977; Esen et al 1981). Both β -zein, a methionine-rich polymer of 17 kD, and δ -zein, a minor fraction of MW 10 kD, have similar solubility profiles to α -zein. γ -zein is composed of two components: one with MW 27 kD and the other with MW 18 kD. It is believed, however, that the 18 kD polypeptide is a truncated version of the 27 kD peptide. Microspheres of zein have been formulated as carriers of anti-cancer agents (Matsuda et al 1989; Suzuki et al 1989; Mathiowitz et al 1993), the parasiticide, ivermectin (Liu et al 2005), the pesticide, abamectin (Demchak & Dybas 1997) and essential oils (Parris et al 2005). Films composed of zein microspheres have been investigated as a biomaterial in tissue engineering and have shown good biocompatibility (Dong et al 2004). The drug release from the microspheres was reported to be low, and indicated the possibility of sustained-release of antigens entrapped within zein microspheres.

We therefore investigated zein microspheres as vaccine carriers/adjuvants, using ovalbumin as a model antigen. The need for research into vaccine adjuvants remains despite much work in this field, due to the poor immunogenicity of newer and purer subunit vaccines, the actual and possible future toxicity of many adjuvants currently in research, and the fact that only a handful of adjuvants are currently used in commercial vaccines. The formulation and characterisation of zein microspheres, loaded with ovalbumin has been reported (Hurtado-López and Murdan, 2005;

Hurtado-López and Murdan *in press*). The spherical particles have a smooth and non-porous surface, a particle diameter of approximately 600nm, and released the loaded ovalbumin slowly when incubated in phosphate buffered saline.

The aim of the work reported in this paper was to determine the adjuvanticity of zein microspheres. Vaccine carriers should only increase the immune responses to the desired antigen, not to non-vaccine proteins, such as the host, food or to itself. Therefore, the generation of immune responses to blank zein microspheres, if any, following intramuscular and oral administrations were also investigated.

Materials and Methods

Animals

Female BALB/c mice, obtained from B&K Ltd, UK, were maintained on a normal mouse diet, which contains maize germ (a source of zein), and were given water *ad libitum*. All procedures had been approved by the School's Ethical Review Committee and were conducted in accordance with the Home Office standards under the Animals (Scientific Procedures) Act, 1986. The experiments were started when the mice were eight weeks old and weighed approximately 20 g.

Materials

Ovalbumin (Grade II), zein, bovine serum albumin (BSA), polyvinyl pyrrolidone MW 360 000 (PVP 360), phosphate buffered saline (PBS) tablets, Tween 20, potassium chloride, anti-mouse IgA (a-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 were used as received. ELISA 96-well microtitre plates were purchased from Dynatech, UK. Double distilled water was used throughout.

Preparation of blank and ovalbumin-loaded zein microspheres

Zein microspheres were prepared by a coacervation method based on the solubility properties of zein, as previously described (Hurtado-Lopez & Murdan, 2005). Briefly, to prepare blank (i.e. without ovalbumin) zein microspheres, zein (62.5 mg) was dispersed in 10 ml of 100% ethanol with gentle stirring. This produced a coarse dispersion. 5 ml each of Tween 20 (2.5 %v/v) and of polyvinyl pyrrolidone (PVP 40, 4 %w/v) aqueous solutions were then added to the ethanolic dispersion. Consequently, a fine aqueous-alcoholic zein suspension was formed. Ethanol was removed by rotary evaporation at 90 mBar and 40 °C for 10-15min and a fine aqueous suspension of zein microspheres was produced. The latter were stored as a suspension until needed. The average diameter of the blank microspheres was found to be 1356 ± 36.4 nm, with a polydispersity of 0.662 ± 0.218 (Hurtado-Lopez & Murdan, 2005).

Ovalbumin-loaded zein microspheres were produced as above, except for the addition of ovalbumin (50 mg) with zein (62.5mg) into 10 ml of 100% ethanol (which gave rise to a coarse dispersion upon stirring), and the addition of a small amount (0.3 ml) of 1% w/v aqueous sodium hydroxide solution. The latter was added as it was found

to prevent the aggregation of ovalbumin and zein that otherwise occurred upon the addition of the two proteins into 100% ethanol. Following the addition of Tween 20 and PVP solutions and the removal of ethanol, ovalbumin-loaded zein microspheres were produced. The average particle diameter was 607.47 ± 48.3 nm, with a polydispersity of 0.386 ± 0.166 . The experimental loading of ovalbumin in the microspheres was 23.90 %; the loaded ovalbumin is expected to be entrapped within the microsphere matrix. The unloaded ovalbumin was present either as non-uniform aggregates or dissolved in the aqueous medium. The unloaded ovalbumin was not removed from the microsphere suspension prior to administration to experimental animals; thus the latter received some free and some entrapped ovalbumin.

Intramuscular immunization

To determine the adjuvanticity of zein microspheres, mice (in groups of 5) were intramuscularly dosed with:

- i) ovalbumin-loaded zein microsphere suspension (150 μ g ovalbumin in 30 μ l suspension),
- ii) ovalbumin dissolved in saline (150 μ g ovalbumin in 30 μ l solution), or
- iii) blank zein microsphere suspension (30 μ l).

The microsphere suspensions were vortexed immediately before injection and a single injection in the quadriceps was used to administer the dose. Booster intramuscular administrations were given twelve weeks after the primary immunisation, in an identical way. To determine immune responses, the animals were bled from the tail vein, 7 weeks after the first dose and again, 1, 4 and 7 weeks after the booster dose. The blood samples were allowed to clot overnight in a refrigerator, after which, they

were centrifuged at 21 000 rpm for 10 min in a table-top centrifuge. Sera were collected and stored at -70°C until assayed.

Oral immunisation

To determine whether oral administration of zein microspheres gives rise to immune responses against zein, mice were orally dosed with 100 μl of blank zein microsphere suspension, containing 625 μg of zein protein, on three consecutive days following overnight fasting. Identical booster doses were administered on days 29, 30 and 31 of the study. The animals were bled 4 weeks after priming and again, 4 weeks after boosting, and serum samples were obtained as described above. At the same time intervals, fresh faecal samples were collected from individual mice placed in metabolic cages. The faeces were added to PBS pH 7.4, the mixtures were homogenized, centrifuged at 21 000 rpm for 15 min in a table-top centrifuge, and the supernatants were collected and stored at -70°C until assayed. A control group of non-immunised animals was used to obtain control faecal and blood samples.

Determination of antibody levels by ELISA

Serum and faecal samples were analysed for anti-ovalbumin and anti-zein IgG and IgA antibodies respectively by a standardised ELISA method. Microtitre ELISA plates were coated with 100 μl per well of antigen solution. The latter was either ovalbumin 1 %w/v in PBS pH 7.4 or zein 1% w/v in 60% v/v ethanol. Zein, a water-insoluble protein, was dissolved in 60% ethanol as the adsorption of zein to polystyrene microtitre plates when dissolved in aqueous alcohols has been demonstrated, as has the subsequent quantitative measurement of the protein following a regular ELISA assay (Conroy & Esen 1984; Chirido et al 1995).

Following overnight incubation at 4 °C, the plates were washed three times using phosphate buffered saline-Tween 20 (0.05% Tween 20), then once again with double distilled water. The plates were then blocked at 37 °C for 1 h, with 100 µl per well of aqueous blocking solution: PVP 360 1% w/v (when ovalbumin was the antigen) or bovine serum albumin 1 % w/v (when zein was the antigen). Plates were washed as described. Serum or faecal samples (100 µl /well, diluted 16x) were placed in the top row of ELISA plates, serial double dilutions were conducted in the other rows and the plates were incubated for 1 h at 37 °C. Following washing, 100 µl per well of anti-mouse IgA or IgG peroxidase conjugate (diluted 1:1 000 in PBS pH 7.4) was added to the ELISA plates, which were incubated at 37 °C for 1 h. Plates were washed, freshly prepared ABTS/hydrogen peroxide solution was added (50 µl per well) and the plates were incubated at 37 °C for 30 min. The reaction was stopped by the addition of 1% w/v sodium dodecyl sulphate aqueous solution (50 µl/well) and the plates were read at 405 nm in an ELISA reader (Opsys MR, Dynex Technologies). To compare the levels of antibodies generated by the different formulations, the optical density readings obtained for the serum that was diluted 16x were used.

Statistical analyses

One-Way ANOVA, followed by post-hoc Tukey HSD tests were used to compare the anti-ovalbumin IgG titres generated by intramuscularly administered ovalbumin solution, and ovalbumin-loaded and blank zein microspheres. Kruskal-Wallis, followed by Nemenyi tests were conducted to compare the primary and secondary anti-zein IgG titres generated by intramuscularly administered ovalbumin-loaded and

blank zein microspheres. Mann Whitney U tests were conducted to compare the primary and secondary immune responses to orally administered blank zein microspheres. Results were considered statistically significant when $P < 0.05$.

Results and Discussion

The zein microspheres (blank and ovalbumin-loaded) were prepared by a simple coacervation method. Spherical zein particles with a smooth surface were produced, as observed by scanning electron microscopy (Figure 1). The average diameter of the blank microspheres was found to be 1356 ± 36.4 nm, with a polydispersity of 0.662 ± 0.218 , while that of ovalbumin-loaded particles was 607.47 ± 48.3 nm, with a polydispersity of 0.386 ± 0.166 . The experimental loading of ovalbumin in the microspheres was 23.90 % (Hurtado-Lopez & Murdan, 2005).

Immune responses to intramuscularly administered formulations

To determine whether zein microspheres enhance the immunogenicity of ovalbumin, and thereby act as an adjuvant, the serum anti-ovalbumin IgG levels generated following administration of ‘free’ and ‘microsphere-loaded’ ovalbumin were measured. Blank zein microspheres were used as controls. The primary and secondary (at weeks 1, 4 and 7 after the booster dose) IgG titres of the 3 preparations are shown in Table 1. To determine statistical differences, if any, between the titres of the 3 preparations, One-Way ANOVA was conducted for each time-point. From the table, it can be seen that:

i) as expected, administration of free, non-encapsulated ovalbumin gave rise to very low levels of primary and secondary antibodies, ovalbumin being poorly

immunogenic in the free form. Booster doses of the free antigen significantly increased the levels of anti-ovalbumin IgG; these levels however decreased with time. At all time points, the antibody titres generated by the solution were significantly lower than those of the microsphere formulations.

ii) administration of both blank and ovalbumin-loaded zein microspheres elicited a higher IgG response, especially following the booster administrations. Serum IgG levels remained high for several weeks following the booster administration. Interestingly, there was an IgG response to blank zein microspheres. The primary IgG levels were higher ($P < 0.05$) than those elicited by the ovalbumin-loaded zein microspheres, while the secondary IgG levels (at all time points) were not significantly different to those elicited by ovalbumin-loaded zein microspheres ($P > 0.05$).

The high antibody responses generated by blank zein microspheres indicate that anti-zein antibodies had been generated and that these antibodies were binding (non-specifically) to ovalbumin antigen coated onto the ELISA microtitre plate wells. The non-specific binding of anti-zein antibodies to ovalbumin antigen coated on ELISA microtitre plate walls meant that any possible increase in anti-ovalbumin IgG titres, due to microencapsulation of the antigen into zein particles could not be detected. Thus, from this study, the adjuvant activity of zein particles, if any, cannot be ascertained.

Generation of antibodies by blank zein microspheres was surprising, given that the experimental mice were fed a standard mouse diet which includes zein. It is well

known that feeding of dietary proteins predominantly induces tolerance i.e. feeding does not result in immune responses to the proteins; in addition when the same protein is subsequently administered via an immunogenic route, such as the intramuscular one, injurious local or systemic immune responses are not elicited (Mowat & Weiner, 1999). To investigate into the immune responses generated to zein particles further, additional ELISA assays, where the microtitre plate wells were coated with zein antigen, were conducted to measure the primary and secondary anti-zein IgG levels elicited by blank and ovalbumin-loaded zein microspheres. The serum IgG levels are shown in Table 2. To determine significant differences, if any, between the primary and the secondary titres of the two formulations, Kruskal-Wallis, followed by Nemenyi's tests were conducted. It was found that the anti-zein IgG titres generated by blank and by ovalbumin-loaded zein microspheres were not significantly different from each other, for both primary and secondary response ($P > 0.05$). In addition, for both formulations, the anti-zein IgG titres increased significantly following the booster dose ($P < 0.05$).

The results confirm that feeding of dietary zein did not induce tolerance to zein particles when the latter were administered intra-muscularly. This could be due to the fact that zein was administered in the particulate form, the latter is known to be more immunogenic than the soluble state. The generation of antibodies by zein particles show that zein, when administered intramuscularly in a particulate form, is immunogenic and raises concerns over the use of these particles as drug and vaccine delivery systems. Drug carriers should be non-immunogenic to avoid concerns about their biocompatibility (Hillery, 2001). For the same reason, vaccine delivery systems

(some of which, such as microspheres, also act as adjuvants) should also be non-immunogenic.

Immune responses to orally administered zein microspheres

Oral tolerance (partial or complete immunological non-responsiveness to orally administered antigen) to zein particles would allow their use as oral drug/vaccine delivery vehicles. In order to determine the possibility of oral tolerance to zein microspheres, a group of 5 mice was orally dosed with blank zein microspheres and the immune responses (serum IgG and intestinal mucosal IgA to zein antigen) were monitored. Significant differences between the primary and secondary responses, if any, were determined using Mann Whitney U tests.

The primary and secondary serum IgG titres were low and similar to those in naïve mice. The serum IgG titres can therefore be assigned to the presence of zein in the mouse feed, rather than to the zein microspheres. Anti-zein IgG titres did not increase following the booster dose ($P > 0.05$) i.e. orally administered zein particles did not induce a systemic immune response and instead, systemic tolerance to oral zein particles was induced.

In contrast, the intestinal mucosal IgA response did not show the induction of tolerance. The primary IgA levels were low and similar to the levels in naïve mice. However, following the booster dose, IgA levels increased significantly ($P < 0.05$; titre doubled from 0.32 ± 0.001 to 0.62 ± 0.05). The generation of IgA antibodies to zein particles is expected to limit the application of the latter as oral drug/vaccine carriers, as the secretory IgA molecules will bind to zein particles, and thereby

prevent the latter's adherence to the mucosal surface and subsequent uptake by Peyer's Patches.

Our results - the absence of a strong serum IgG response concomitantly with the presence of a significant IgA response - reflect reports by other researchers (Challacombe & Tomasi 1980; McGhee et al 1999) of systemic unresponsiveness, concomitant with mucosal responsiveness to oral antigens. IgA responses seem to be more resistant to the induction of oral tolerance (McGhee et al 1999).

Conclusions

The aim of the study reported in this paper was to investigate the potential of zein microspheres as vaccine delivery systems. Ovalbumin was used as a model antigen. The adjuvanticity of zein microspheres could not be ascertained in this study. However, their immunogenicity was demonstrated. Following the intramuscular administration of blank and ovalbumin-loaded zein microspheres, a significant IgG response to zein was elicited. Following oral administration of blank zein microspheres, a significant anti-zein IgG response was not observed, but the intestinal anti-zein IgA titres was found to double, i.e. oral administration induced systemic, but not mucosal, tolerance to zein particles. Thus, this study, which shows the immunogenicity of zein particles, raises questions about their potential application as drug and vaccine delivery vehicles.

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Figure legends

Figure 1: Scanning electron micrograph of ovalbumin-loaded zein microspheres.

Table 1: The primary and secondary anti-ovalbumin IgG titres generated following the intramuscular administration of ovalbumin solution, ovalbumin-loaded zein microspheres and blank zein microspheres in mice. Mean \pm S.D are shown, n=5.

Formulation	Anti-ovalbumin IgG titre			
	Primary response	Secondary response (week(s) after booster dose)		
		1 week	4 weeks	7 weeks
Ovalbumin solution	0.11 \pm 0.01	0.89 \pm 0.09	0.38 \pm 0.04	0.34 \pm 0.13
Ovalbumin-loaded zein microspheres	0.15 \pm 0.03	1.74 \pm 0.32	1.36 \pm 0.37	1.36 \pm 0.25
Blank zein microspheres	0.21 \pm 0.03	1.59 \pm 0.21	1.64 \pm 0.34	1.11 \pm 0.09

Table 2: The primary and secondary anti-zein IgG titres generated following the intramuscular administration of blank and of ovalbumin-loaded zein microspheres in mice. Mean \pm S.D are shown, n=5.

Formulation	Anti-zein antibody titre	
	Primary response	Secondary response
Ovalbumin-loaded zein microspheres	0.25 \pm 0.03	0.91 \pm 0.08
Blank zein microspheres	0.24 \pm 0.03	0.82 \pm 0.13

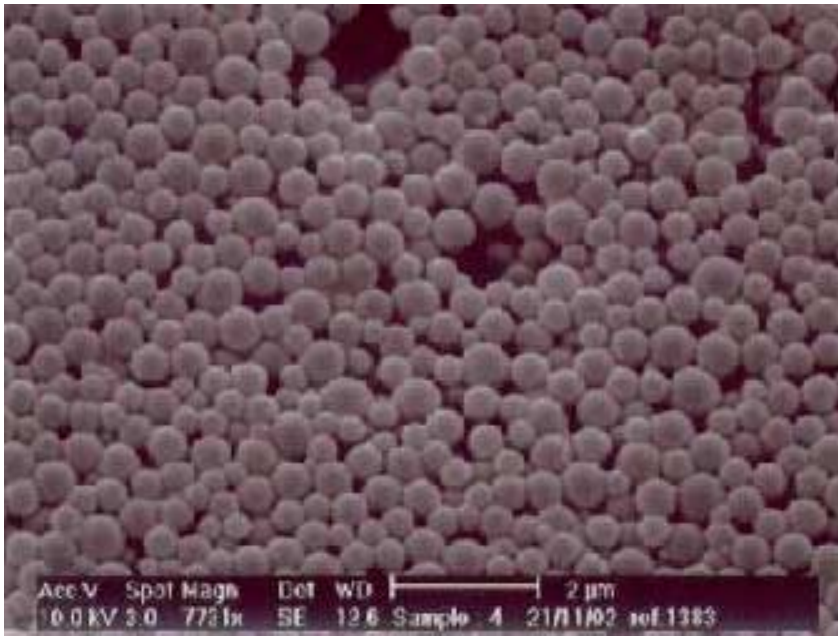


Figure 1

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Figures 1-3 have been changed to 2 tables (1 for anti-ovalbumin IgG, the other for anti-zein IgG). Figures 4 and 5 have been deleted and data is presented in the text.

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The Journal of Pharmacy and Pharmacology

STATISTICAL EDITOR'S REPORT

In this current paper statistical methods have been defined, although some of these methods are not appropriate to the experimental design. These details are described below:

General Comments Concerning Your Experimental Design

(i) In the section entitled Statistical Analysis you have described the use of a series of two sample tests for the quantification of the outcomes of this study. Unfortunately as some of the figures/tables contain more than two treatments, this approach is incorrect as it will indirectly increase the likelihood of producing a type 1 error, i.e. rejecting the null hypothesis whenever it is indeed correct. Therefore, to negate this problem, a multiple hypothesis test should be used. If the sample size is greater than 5, the data are normally distributed and the variances of the different treatments are equal then the parametric Analysis of Variance (ANOVA) should be performed. If these assumptions cannot be

fulfilled then a non-parametric multiple comparisons test, e.g. the Kruskal-Wallis test, should be employed.

Suggested examples of these instances are provided below.

(ii) In Figure 1 the effect of the various formulations on the titre should be analysed using a Kruskal-Wallis test. Individual differences between the formulations may be then evaluated using a suitable post hoc test, e.g. Nemenyi's test or Dunn's test. As stated above it is incorrect it is incorrect to perform a series of two sample tests.

(iii) In figure 2 the effect of the various formulations and time on the titre should be analysed using Friedman's test. Individual differences between the formulations and time may be then evaluated using a suitable post hoc test, e.g. Nemenyi's test or Dunn's test. As stated above it is incorrect it is incorrect to perform a series of two sample tests.

Alternatively, the effect of formulation on titre at each time point may be statistically analysed using the Kruskal-Wallis test. Individual differences between the formulations may then be determined using Dunn's test.

This suggestion was taken up. The effect of the different formulations on titre at each time point was analysed. One-way ANOVA, followed by post hoc Tukey tests was used (instead of Kruskal-Wallis and Dunn's test) as the number of replicates was 5, the data were normally distributed and the variances of the different treatments were equal.

(iv) The effect of formulation and immunisation number should be analysed using a Kruskal-Wallis test. Individual differences between the formulations may be then evaluated using a suitable post hoc test, e.g. Nemenyi's test or Dunn's test. As stated above it is incorrect it is incorrect to perform a series of two sample tests.

Kruskal-Wallis test, followed by Nemenyi's have been conducted to analyse differences between the primary and secondary anti-zein titres of blank and ovalbumin-loaded zein microspheres.

(v) The data presented in Figures 4 and 5 may be statistically analysed using the Mann Whitney U test.

This is now done.

If you require further assistance may I recommend that you consult the following text:

Pharmaceutical Statistics by Jones, DS, The Pharmaceutical Press (2002).

Alternatively, as part of this service, you may contact the JPP office (jpp@qub.ac.uk) with any questions.

Reviewer #1: In present, aluminium adjuvant is used as most vaccine carriers. It is necessary that find a new and effective adjuvant in place of traditional aluminium adjuvant. The paper must be corrected as follows before it is accepted.

(1) Authors should add data on the immunity effect compared aluminium adjuvant with zein microspheres in the paper.

It is true that aluminium is used at present. However, it is known that alum has limitations, such as, failure to enhance the immune response in certain vaccines e.g. influenza and alum is thought not to be the best adjuvant for small-size peptide vaccines (Lindblad, in *The Theory and Practical Application of Adjuvants*, ed; D E S Stewart-Tull, Wiley, Chichester, 1995, pp21-35). Thus, alum is not a 'gold standard' that all new adjuvants must compare with. The reviewer is right in pointing out that a traditional adjuvant could have provided comparisons; this would somewhat enable the comparisons of the myriad of adjuvants currently in research. Some researchers in the field use Freund's Adjuvant as a positive control. There is no consensus on a 'gold standard' and we did not conduct experiments using aluminium, therefore, cannot compare the immunity of zein microspheres with that of alum.

(2) Authors should add laser particle distribution figure and scanning electron microscopy (SEM) of zein microspheres in order that readers can understand the content of the paper.

OK, SEM is now provided.

Laser particle distribution is not added as an average particle size is given: 3 different microsphere samples were sized; each sample was sized 3 times.

A new (short) paragraph (page 11, para 2) has been added in the results and discussion section to help readers understand the content of the paper.

(3) Authors should add error trendline on fig. 1, 2, 3 and 4.

The figures have been changed to tables. Standard deviations are shown in all cases.

Reviewer #2: Referee Report
Comments for authors

Contribution to the field
Moderate - will make a limited contribution to the field

Soundness of methodology
More information required regarding statistical analysis
[This is now provided](#)

More information required regarding regulatory approval for experimental work
[This is now given in the Materials section.](#)

Soundness of interpretation
Issues associated with interpretation

Has been addressed

Quality of written manuscript
Figures and Tables require revision
These have been revised.

Please include a detailed written commentary below:

This is a preliminary work on the immunogenicity of zein microspheres, but should be publish (after massive review), since describe the vainness of zein based system for drug/vaccine delivery. The observations of course undermine the value of the authors two publications as well, which are "in press".

The following points should be addressed:

1 Define zein!

Zein is a prolamin of maize. This was included in the original manuscript. Some more information regarding zein's nature has now been included in the revised manuscript, but it is quite difficult to *define* zein.

2 Abstract: Results - rewrite and explain!

Has been rewritten and explained.

3 P7: purpose of NaOH?

Sodium hydroxide was added as it was found to prevent the aggregation of ovalbumin and zein that otherwise occurred upon the addition of the two proteins into 100% ethanol. In the presence of sodium hydroxide, ovalbumin and zein remained dispersed in the liquid medium until the removal of ethanol, at which point zein precipitated into microspheres. The purpose of NaOH has now been added in the text.

4 Justification of the doses (very big)

Ovalbumin is often used as a model antigen due to its availability and its low cost, certainly compared to antigens such as hepatitis B surface antigen. The large dose of ovalbumin is fairly common as it is poorly immunogenic, again compared to antigens such as hepatitis surface antigen and influenza subunits.

5 P 10: paragraph 2; P 11: paragraph 3, 4, 5: explain!

Page 10, para 2 – because mice feed includes zein, the experimental animals had a low and constant levels of anti-zein antibody in the sera. The inherently present anti-zein antibodies led to background readings that were higher than empty cells and therefore each animal's pre-immunisation serum was assayed every time. I agree that the paragraph is not very clear. I've gone back to the raw data and decided to use the

absorbance readings for the revised version (instead of the serum dilution that gives a reading below an arbitrary value).

Page 11, para 3-5

Now on page 12 , sections are re-written.

6 P 12; last paragraph: similar levels of "anti-ovalbumin"!
The sections have been re-written, after revised calculations

7 P 13: first paragraph; p 14 second paragraph: explain!

P13, first para – revised calculations and interpretations have led to a revised paragraph – see page 12, para 3.

P 14, para 2:

I have re-written to explain: In the revised manuscript, see page 12, last para, 13, para 1-2, page 14, para 2 and page 15 para 1-2.

8 Error bars!
OK, Standard deviations are now shown.

9 What are the numbers in the figs (especially Figs 4 and 5)
Numbers are now shown in tables and in the text

10 There are unnecessary repetitions from the authors' "in press" papers
OK, sorry. Repetitions have been removed.

11 Meaningless speculations (P 12-15)
The speculations have been removed.

12 P 17: paragraph 2: what type of immune response?
The type of immune response has been clarified.

Importantly the manuscript should be massively cut back and published as a "Short communication".

Manuscript has been massively cut back. I have not changed the format to that of a 'Short communication' as it is perhaps too long for a short communication and this has not been suggested by the Editor.