

Mesoporous Silica Nanoparticles Act as a Self-Adjuvant for Ovalbumin Model Antigen in Mice

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Immunization to the model protein antigen ovalbumin (OVA) is investigated using MCM-41 mesoporous silica nanoparticles as a novel vaccine delivery vehicle and adjuvant system in mice. The effects of amino surface functionalization and adsorption time on OVA adsorption to nanoparticles are assessed. Amino-functionalized MCM-41 (AM-41) shows an effect on the amount of OVA binding, with 2.5-fold increase in binding capacity (72 mg OVA/g AM-41) compared to nonfunctionalized MCM-41 (29 mg OVA/g MCM-41). Immunization studies in mice with a 10 µg dose of OVA adsorbed to AM-41 elicits both antibody and cell-mediated immune responses following three subcutaneous injections. Immunizations at a lower 2 µg dose of OVA adsorbed to AM-41 particles results in an antibody response but not cell-mediated immunity. The level of antibody responses following immunization with nanoformulations containing either 2 µg or 10 µg of OVA are only slightly lower than that in mice which receive 50 µg OVA adjuvanted with QuilA, a crude mixture of saponins extracted from the bark of the Quillaja saponaria Molina tree. This is a significant result, since it demonstrates that AM-41 nanoparticles are self-adjuvanting and elicit immune responses at reduced antigen doses in vivo compared to a conventional delivery system. Importantly, there are no local or systemic negative effects in animals injected with AM-41. Histopathological studies of a range of tissue organs show no changes in histopathology of the animals receiving nanoparticles over a six week period. These results establish the biocompatible MCM-41 silica nanoparticles as a new method for vaccine delivery which incorporates a self-adjuvant effect.

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1. Introduction

The extensive use of inorganic mesoporous silica nanoparticles (MSN) in bionanotechnology applications is due to their capacity to generate monodispersed nanoparticles with controlled particle size and mesostructure. Silica is amenable to chemical modification with a variety of functional groups, allowing conjugation to a diverse range of biomolecules and optimization of binding efficiency and capacity. In addition, their advantageous properties of submicron size, large surface area, tuneable pore size, biocompatibility, low cytotoxicity, and relatively low cost of production together make MSN attractive vehicles for targeted delivery and release of drugs and biomolecules such as DNA, enzymes and peptides.^[1–7]

It is now well established that surface modified MSN can act as vehicles for the loading and delivery of proteins. Amino

functionalized MSN have been shown to be effective in applications such as enzyme adsorption and entrapment compared to the non-functionalized (native) counterparts.^[5,8] There are many studies on the immobilization of a variety of enzymes to different mesoporous solids.^[9] Trewyn et al.^[10] report on the applications for controlled release from MSN. Studies of the binding of model proteins such as bovine serum albumin^[11,12] and lysozyme^[11] to MCM-41 silica nanoparticles in particular showed that protein adsorption capacity is dependent on particle size, pore size distribution, and specific external surface area and functional groups.

The effectiveness of ordered mesoporous silica as a delivery system and adjuvant in mice has been reported.^[13,14] The self-adjuvant effect of carboxylated polystyrene nanobeads has been established in both mice^[15,16] and sheep.^[17,18] The studies in mice showed that cellular and humoral immunity were optimal when the model antigen OVA was covalently linked to beads of 40–50 nm in size due to preferential uptake by antigen presenting dendritic cells.^[15,16] Importantly the adjuvant effect of these nanobeads has also been demonstrated through induction of both cellular and humoral immunity in sheep which are out-bred animals.^[17] MSN are tolerated in the mammalian system at a relatively high concentration. A study in mice by Hudson et al.^[6] showed that 30 mg each of unfunctionalized MCM-41, SBA-15 and mesocellular foam were lethal when injected intraperitoneally but not when administered subcutaneously due to pharmacokinetic differences. After two or three months only trace amounts of the mesoporous silicates were detected by histological examination irrespective of the site of injection. In addition,

toxicity of MSN has been shown to differ depending on the surface adsorption of functional groups and proteins.^[19]

Studies describing the use of MCM-41 for the effective delivery of protein antigens to elicit specific adaptive immunological responses have not been reported to the best of our knowledge. The aim of the work reported here was to determine whether AM-41 nanoparticles could act as both an antigen delivery vehicle and as an adjuvant for relatively low doses (10 μ g and 2 μ g) of a model antigen. OVA was chosen as a model protein due to its well established antigenic properties and capacity to induce strong cellular immunity via T lymphocytes as well as humoral immunity via B lymphocytes. The kinetics of the adsorption of OVA onto and its subsequent desorption from AM-41 nanoparticles were characterized in vitro prior to in vivo studies in mice. The results presented demonstrate that these amino functionalized silica nanoparticles are indeed an extremely effective adjuvant capable of generating both humoral and cell-mediated immune responses, with no cytotoxic effects observed over the duration of the study.

2. Results

2.1. Characterization of MSN

Transmission electron microscopy (TEM) analysis of AM-41 shows a uniform size (90 nm) (Supporting Information Figure S1) and sphere shape with a standard deviation of 9 nm (Figure 1a) Low angle powder X-ray diffraction (XRD)

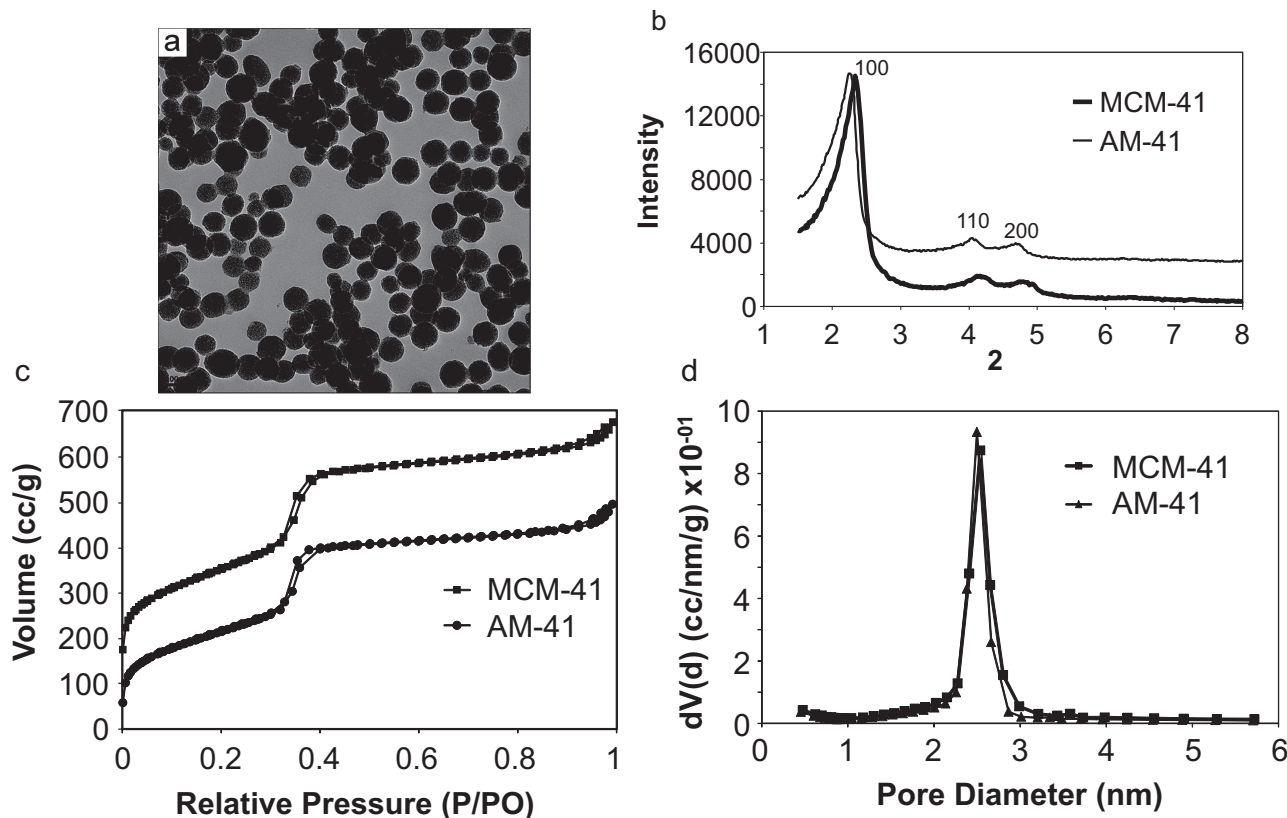


Figure 1. (a) TEM image of AM-41 nanoparticles. (b) Low-angle XRD patterns. (c) Nitrogen adsorption/desorption isotherms (Graph offset = 100 for MCM-41). (d) The BJH pore size distribution of MCM-41 and AM-41 samples.

Table 1. A comparison of the physical properties and the atomic percentages of MCM-41 and AM-41.

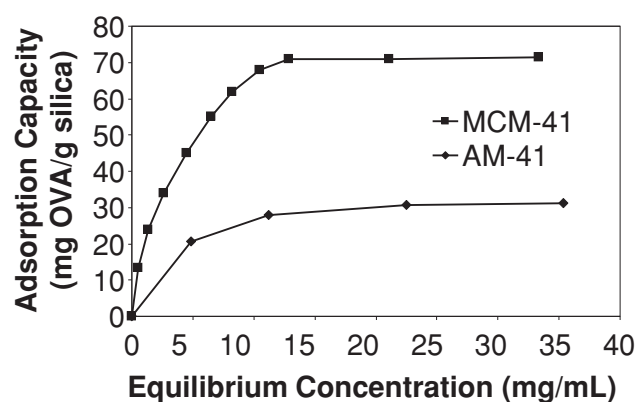
Material	MCM-41	AM-41
BET surface area [$\text{m}^2 \text{g}^{-1}$]	956.2	854.4
BJH Pore diameter [nm]	3.6	3.7
Pore volume [$\text{cm}^3 \text{g}^{-1}$]	1.01	0.79
Atomic% (determined by XPS)	Si	24.79
	O	65.58
	C	9.63
	N	0
Zeta Potential in PBS (pH 7.0) [mV]	−36.9	20.2

patterns show that both MCM-41 and AM-41 samples synthesized have significant (100), (110), and (200) diffraction peaks which are indicative of a two-dimensional hexagonal mesostructure with a space group of $p6mm$ (Figure 1b). The high peak intensity and resolution of (100), (110), and (200) can be attributed to the long-range regularity of MCM-41 and AM-41. With the addition of amino functional groups, there is no significant change in the pattern. Nitrogen adsorption isotherms of both MCM-41 and AM-41 nanoparticles are of type IV (Figure 1c) which are characteristic of mesoporous materials with 1-D cylindrical channels. The narrow pore-size distribution curves (Figure 1d) reveal that both MCM-41 and AM-41 have uniform pore size. The pore sizes, calculated from the adsorption branches of the isotherm by the Barrett-Joyner-Halenda (BJH method), pore volumes, and Barrett-Emmett-Teller (BET) surface areas are summarized in **Table 1**. It can be seen that MCM-41 has a large pore size of 3.6 nm, a large BET surface area of $956.2 \text{ m}^2/\text{g}$ and a large pore volume of $1.01 \text{ cm}^3/\text{g}$. The presence of amino groups for AM-41 sample reduces the surface area and the pore volume of the sample to $854.4 \text{ m}^2/\text{g}$ and $0.79 \text{ cm}^3/\text{g}$, respectively, but they are still very high.

Amino functionalization of the MCM-41 particles was evident due to detectable nitrogen (3.17%) in AM-41 compared to the unfunctionalized MCM-41 by X-ray photoelectron spectra (XPS) analysis (Table 1 and Supporting Information Figure S2). The presence of carbon in both samples was due to the incomplete removal of surfactant template during extraction. Zeta potential measurements for MCM-41 and AM-41 showed that there was a significant difference in the surface charges at pH 7.0. AM-41 particles have a strong positive charge (+20.2 mV), whereas MCM-41 has a negative charge (−36.9 mV). The reported isoelectric point for silica materials is $\text{pH} = 2$.^[20] After OVA adsorption, it was observed that the charges changed slightly, with measurements of −32.7 and +13.9 mV for MCM-41 and AM-41 respectively due to the effect of protein binding to the particles.

2.2. OVA Adsorption Isotherm on MCM-41 and AM-41

To determine the effect of amino group functionalization on the capacity of MSN to bind OVA protein, OVA adsorption isotherms of MCM-41 and AM-41 were measured

**Figure 2.** OVA adsorption isotherm to MCM-41 and AM-41.

(**Figure 2**). All isotherms show an initial rise and then reach maximum adsorption amounts (plateau) at about 12–36 mg/mL equilibrium concentration. It can be seen that the equilibrium adsorbed amount on AM-41 (72 mg OVA/g AM-41) is higher than that on MCM-41 (29 mg OVA/g MCM-41). These results indicate that an electrostatic interaction between the surface positive charge of the functional amino group of the particles and the surface negative charge of the soluble protein significantly enhances the binding of OVA to the AM-41 particles. Since amino group functionalization of MCM-41 resulted in an increase in the amount of bound OVA, all subsequent work was done using only AM-41 material.

2.3. In vitro Adsorption and Desorption Kinetics of OVA Protein

To determine whether the adsorption of OVA to AM-41 nanoparticles increased in a time dependant manner, adsorption reactions containing 20 mg AM-41 and 4 mg of OVA in 5 mL of buffer were performed. Samples of the protein and particle slurry were collected at time points between 5 min and 24 h. Total amounts of unbound OVA remaining in the supernatants for the different time points were quantified by protein assay, which shows a line of best fit ($R^2 = 0.9736$) through the data points (**Figure 3a**).

To determine whether there is desorption of OVA after binding to AM-41, desorption kinetic studies were performed at 37 °C to mimic the body temperature of mammals. OVA adsorbed nanoparticles were centrifuged and resuspended in 1 mL of pre-warmed PBS and incubated at 37 °C with agitation. Supernatants at 5, 15, 30, 60, 120, 240 min time points and overnight were run on polyacrylamide gels to visualize the amount of desorbed OVA protein. Desorption of OVA occurred rapidly during 5 to 30 min (Figure 3b), while at later time-points no OVA protein was visible indicating there was no further protein desorption. The amount of OVA protein released at the different time points was quantified by protein assay (Figure 3b insert, the corresponding polyacrylamide gel of desorbed OVA recovered at five time points). The total OVA protein released was $5.8 \mu\text{g OVA}/\text{mg AM-41}$ which is equivalent to 7.9% of the total adsorbed protein.

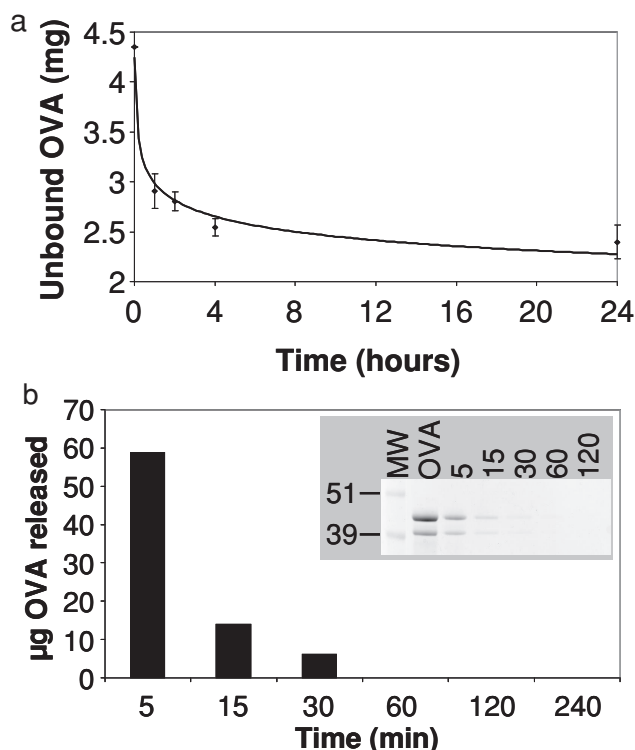


Figure 3. (a) OVA adsorption kinetics to AM-41 nanoparticles (the mean $\pm$ standard error (SE) of four separate binding experiments is shown). (b) Desorption kinetics of OVA from AM-41 nanoparticles.

2.4. Immunization of Mice with OVA bound AM-41

OVA bound AM-41 particles (2 μ g OVA/AM-41) were injected subcutaneously at the tail base of mice to investigate immune responses. The OVA+AM-41 nanoparticle doses were compared to immunization with 50 μ g OVA and 10 μ g QuilA adjuvant. The negative control group received 150 μ g AM-41 only (see the Experimental Section). A subsequent mice trial was conducted in a similar manner with 10 μ g OVA/AM-41. The total IgG anti-OVA response after three subcutaneous injections (terminal bleeds were obtained two weeks after the third injection) was determined for individual mice sera from each group by Enzyme-Linked ImmunoSorbent Assay (ELISA) (**Figure 4**). The OVA (50 μ g) plus QuilA (10 μ g) positive control group showed an excellent antibody response with an average optical density (OD) of 0.6 up to a dilution of 1:6400 (**Figure 4a**). The mice injected with 2 μ g and 10 μ g of OVA bound to AM-41 also showed detectable antibody responses up to a 1:6400 dilution (**Figure 4b,c**). Interestingly, the antibody response was not significantly higher for mice injected with the higher dose of OVA bound AM-41 (10 μ g OVA/AM-41) compared to the lower dose of 2 μ g OVA/AM-41 (**Figure 4b,c**). The antibody response of mice to OVA bound to AM-41, though not as high as OVA+QuilA, were comparable (compare titers at the higher sera dilutions in **Figure 4a–c**) as there was 5 and 25 fold less OVA antigen in the nanoformulation. Control mice receiving AM-41 nanoparticles only showed no specific antibody

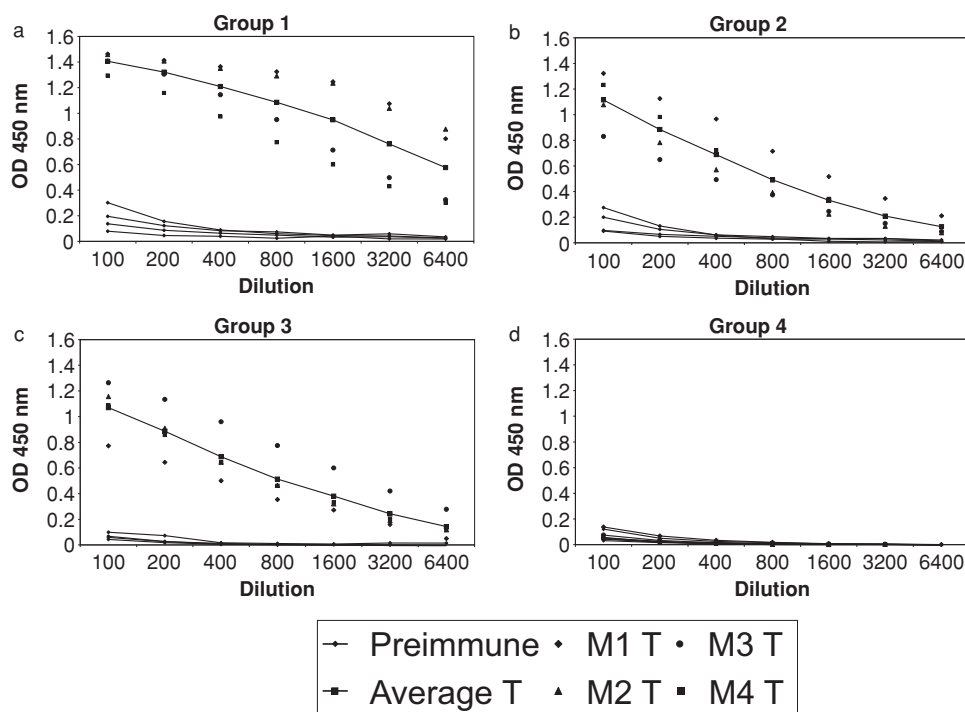


Figure 4. Immunization of mice with OVA bound AM-41 nanoparticles. Anti-OVA-specific total IgG ELISA data for the antibody response in mice ($n=4$) after 3 subcutaneous immunizations at 2 week intervals to the tail base with: (a) Group 1: 50 μ g OVA together with 10 μ g QuilA, (b) Group 2: 2 μ g OVA adsorbed to AM-41, (c) Group 3: 10 μ g OVA adsorbed to AM-41, (d) Group 4: 150 μ g AM-41 particles only. Preimmune sera were collected at the beginning of the experiment and terminal bleed sera (T) were collected two weeks following the final immunizations. Sera of individual animals (M1 to M4) were serially diluted from 1:100 to 1:6400.

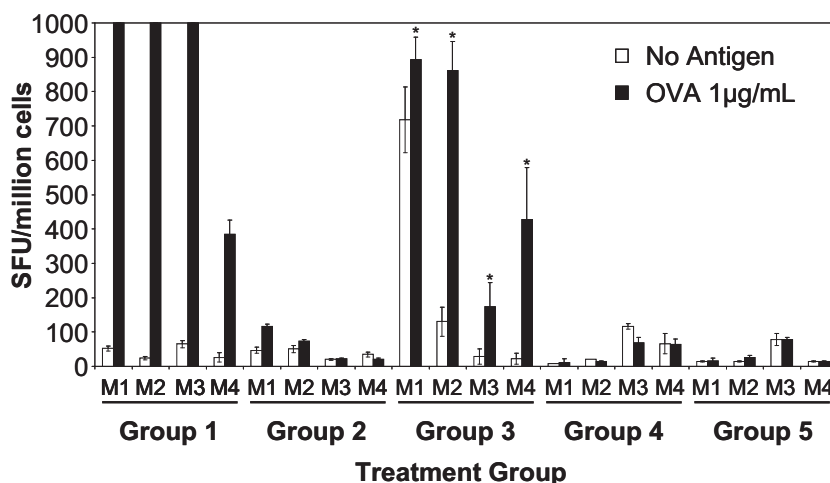


Figure 5. Antigen specific IFN- γ secretion by ELISPOT assay of murine splenocytes from OVA+QuilA and OVA+AM-41 immunized mice. Splenocytes were stimulated in vitro with the OVA CD8 peptide, SIINFEKL (1 μ g/ml, black bars) and compared to unstimulated cells (white bars). Mean SFU/million cells $\pm$ standard deviation for individual mice (M1 to M4) for each group are shown. In mice immunized with 10 μ g OVA/AM-41 (Group 3) there was a significant increase in IFN- γ response. The asterisks (*) indicate significant responses with $p < 0.05$ (unpaired t-test analysis) compared to mice immunized with the lower dose of 2 μ g OVA/AM-41 (Group 2) and the control group receiving AM-41 only (Group 4) and unimmunized mice (Group 5). The polyclonal activator, Concavalin A (ConA), was used to confirm cell viability and functionality of the assay (data not shown).

response to OVA protein (Figure 4d) and was similar to the unimmunized mice group tested (data not shown).

Splenocyte cell populations were used in an interferon- γ enzyme-linked immunosorbent spot (ELISPOT) assay to determine if there was a T-helper type 1 (Th1) cell-mediated immune response two weeks after the final immunization. All of the animals in the positive control OVA+QuilA group (Figure 5, Group 1) showed a high cell-mediated immune response to OVA epitope, SIINFEKL, as indicated by the number of cells producing interferon- γ (black bars in Figure 5 indicate the response to OVA peptide). Three of the mice in this group showed >1000 spot-forming units (SFU)/

million cells (extending beyond the axis of the graph) indicating a very strong OVA-specific response. Low cell-mediated immunity (20–116 SFU/million cells) was observed for the mice injected with 2 μ g OVA/AM-41 particles (Figure 5, Group 2). However, high levels of cell-mediated immunity to the OVA epitope were detected in animals immunized with the higher dose of 10 μ g OVA/AM-41 particles with SFU/million cells varying from 173 to 893 (Figure 5, Group 3). This is a significant finding as this group of mice received 5 fold less OVA as compared to the positive control OVA+QuilA mice (Group 1). The level of the responses were significant ($p < 0.05$) for all the mice in this group compared to the group injected with the 2 μ g of OVA bound AM-41 and the control groups injected with 150 μ g AM-41 (Figure 5, Group 4) and the unimmunized mice (Figure 5, Group 5).

To determine whether there were deleterious side effects associated with injection of AM-41 particles, tissue from the injection sites and organs were harvested from two mice for each group at the time of necropsy. Tissues including heart, lung, kidney, liver, thymus, spleen, and lymph nodes (local and peripheral) were examined. Hematoxylin and eosin staining of fixed tissues showed that there were no discernible morphological differences in the tissue at the site of injection in mice receiving the nanoformulations compared to the unimmunized controls (Figure 6a,b). Similarly, no morphological changes could be detected between all organs derived from animals in the unimmunized control group compared to the treatment groups receiving subcutaneous injections of nanoformulations. Representative stained sections of liver, kidney and lymph nodes are shown in Figure 6c–h.

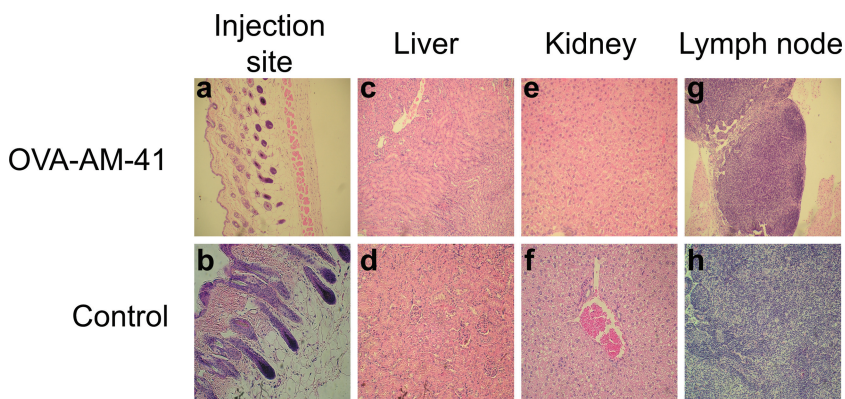


Figure 6. Histopathological studies of tissue organs from a mouse injected with OVA+AM-41 (top panel) and a non-immunized control animal (bottom panel). Formalin fixed organs were harvested from two mice for each treatment group and embedded in paraffin. Sections were stained with hematoxylin and eosin stain. Representative images of the injection sites (a) in transverse section, (b) in longitudinal section, liver (c,d), kidney (e,f) and lymph nodes (g,h) are shown.

3. Discussion

The need of eliciting both humoral and cell-mediated immunity has acted as a limiting factor in developing subunit vaccines comprised of proteins and peptides. This stems from the fact that they can be degraded by proteases, may have limited bioavailability and present relatively low immunogenicity. These issues can be addressed by use of a vaccine delivery system and/or inclusion of adjuvants. The most widely used saponin based adjuvants are QuilA and its derivative QS-21 which can induce both the Th1 immune response as well as the production of cytotoxic T-lymphocytes making them ideal for use in subunit vaccines.^[21] QuilA remains

the most widely used adjuvant in research for the development of novel vaccines. We also used QuilA in our system as the positive control to elicit strong antibody and cell-mediated immune responses to determine the self-adjuvanting capacity of AM-41 nanoparticles loaded with OVA. However, QuilA has been reported to have serious drawbacks such as high toxicity, undesirable hemolytic effect and instability in aqueous phase which limits its use as an adjuvant in clinical vaccines by preventing registration.^[21] Aluminum salts and derivatives, often called alum, have been widely used as adjuvants in vaccines for the last 80 years and till recently was the only adjuvant approved for human use in the USA.^[22] However, alum salts have been reported to have poor induction of cell-mediated immunity.^[23–25] Alum has also been reported to have side effects such as formation of granulomas when administered subcutaneously or intra-dermally rather than intramuscularly.^[26] Alum is now being replaced or used in conjunction with other adjuvants to improve vaccine efficacy.^[27]

Nanoparticle based delivery systems for subunit vaccines can increase the presentation of the proteins to antigen presenting cells. In addition, nanoparticles can also act as self-adjuvants to assist in generation of enhanced immune response. The use of a nanoparticle based delivery system and strategies to increase immunogenicity for synthetic peptides as vaccines has recently been reviewed by Salvador et al.^[27] reporting the induction of both humoral and cellular immune responses using polymeric nanoparticles and nanobeads for peptide based vaccine delivery. Silica nanoparticles are biologically compatible and therefore an ideal candidate for a new generation of adjuvants. The role of the adjuvant is to enhance the immune response by increasing the production of cytokines and chemokines by antigen presenting cells. This activation of cell signals in turn recruits more antigen presenting cells to the area of injection, thereby increasing the subsequent immune response. In the present study utilizing the well-characterized OVA protein as a model antigen we investigated the use of AM-41 mesoporous silica nanoparticles as both a method of delivery and providing an adjuvant effect in vivo. Previous reports on the use of mesoporous silica nanoparticles have concentrated on their effectiveness as vehicles for drug delivery due to their large surface area and targeting of cell types through the incorporation of cell surface receptors.^[28,29]

Mesoporous silica particles MCM-41 and AM-41 have been successfully synthesized and our characterization data is consistent with published results.^[30–32] The effect of the functionalization on the MCM-41 shows no physical differences as demonstrated by the low angle powder XRD (Figure 2), although nitrogen adsorption data showed a slight decrease in the surface area and pore volume of AM-41 (Table 1). The removal of surfactant was incomplete as trace amounts of carbon were still present in the sample which has not been fully removed from the micropores by a solvent washing method (Table 1). This observation is consistent with published data that solvent extraction of the surfactant is not 100% effective.^[33] However, with a high level of the surfactant removed and no traces of bromine present in the sample, these materials were not toxic in vitro

whereas unfunctionalized MCM-41 nanoparticles were found to be toxic in in vitro cytotoxicity assays by trypan blue dye staining (data not shown).

OVA protein adsorbed to AM-41 nanoparticles at a capacity of 72 μg OVA/mg AM-41 (Figure 2) which was 2.5 fold greater than the unfunctionalized MCM-41. This high binding capacity is due to strong electrostatic interactions between the negative carboxylic groups present on the protein and the positive amino groups on the particles. The change in the zeta potential of AM-41 following OVA adsorption (Table 1) confirmed this interaction. In vitro desorption studies of OVA at 37 °C showed that once bound the protein does not easily dissociate from the particles. There was only minimal desorption of OVA (accounting for 7.9% of the total bound protein) which occurred rapidly within 30 min (Figure 3b). The minimal in vitro desorption of OVA from AM-41 confirms its usefulness as both an adjuvant and a delivery vehicle with bound protein remaining stable and biologically functional.

To demonstrate that AM-41 nanoparticles can deliver OVA in vivo and elicit an immune response we injected mice subcutaneously, three times at fortnightly intervals with two doses of OVA+AM-41 (2 μg OVA or 10 μg OVA). 50 μg of OVA plus QuilA adjuvant was chosen in our study as the positive control to ensure that the mice had a good immune response to OVA to allow for development of OVA-specific ELISA and ELISPOT assays. Establishment of these assays in our laboratory was essential to determine the immune response of mice immunized with the nanoformulation. Li et al.^[34] also used 50 μg of OVA with the adjuvants Alum and Freund's complete adjuvant.

The injections of OVA+AM-41 into mice at the tail base site did not result in localized skin redness or swelling therefore AM-41 nanoparticles are well tolerated in mice. An immune response which was readily measurable with an OVA-specific ELISA demonstrated development of specific antibodies following two injections (data not shown) with a higher response in the terminal bleeds obtained two weeks after the third injection (Figure 4). The effectiveness of AM-41 as a self-adjuvant was demonstrated by the ELISA assay results which showed good OVA-specific antibody responses for both groups injected with 2 μg and 10 μg OVA bound to AM-41 particles without any adjuvant. Although the overall antibody responses to OVA nanoformulations were not as strong as that observed for the OVA+QuilA positive control group, there was 5 and 25 fold less OVA antigen respectively in the nanoformulations. This clearly demonstrates the nanoformulations were effective in generating immune responses to OVA in the absence of a conventional adjuvant.

The process of eliciting an immune response to a vaccine antigen depends on efficient uptake of the antigen by dendritic cells at the peripheral site of administration and subsequent presentation of the antigen in the major lymphoid organs. The nanoparticles used in this study were 90 nm in diameter and the development of readily detectable antibody responses to OVA+AM-41 demonstrates this sized particle is suitable for in vivo immune studies. This fits with published studies of polystyrene nanobeads conjugated to OVA

in which mice were injected with a range of different sized beads (from 20 nm to 2 μm), with the optimal size identified within the viral size range of 30 to 80 nm.^[15] The adjuvant effect of this size of nanoparticles is thought to be due to the capacity of the dendritic cells to develop a size specific response in mice to injected particles.^[15] A similar finding has been published in sheep in which polystyrene beads of 50 nm were most effective at inducing both cellular and humoral immunity when administered through intradermal and subcutaneous routes.^[17] The type of immunity seen in response to a vaccine antigen is dependent on the antigen dose, route of administration, the adjuvant and the type of antigen itself. In our investigation mice injected with 10 μg of OVA bound to AM-41 showed high SFU indicating presence of cell-mediated immunity. This is an important finding since it demonstrates that the AM-41 is an efficient self-adjuvant that can provide immunological memory which is essential for the recognition and elimination of invading pathogens. The fact that cell-mediated responses were observed when using 10 μg OVA and not with 2 μg OVA indicates that there is a minimal threshold amount of the antigen and AM-41 adjuvant required to elicit both cellular and humoral adaptive immunity.

A study of SBA-15 (a silica cylinder mesostructure of 2 μm in length) as an adjuvant and delivery vehicle for bovine serum albumin (BSA) showed both humoral and cell-mediated immunity in mice.^[14] In this study the BSA adsorbed/encapsulated in SBA-15 particles was administered via intramuscular and oral routes in high and low antibody responder mice lines, with an increase in the immunogenicity in low responder mice. The anti-BSA responsiveness was found to be higher in mice injected via the intramuscular route presumably due to greater degradation of the adsorbed/encapsulated antigen via the highly acidic gut route.

Silica nanoparticles have excellent potential to overcome antigen degradation by enhancing the co-transport and co-delivery of antigen via encapsulation from the peripheral injection site via the lymphatic system to the main lymphoid organs. However like all potential adjuvants the in vivo safety of silica nanoparticles is a primary consideration as it has previously been demonstrated that the liver and kidney have the highest uptake of intravenously injected MSN (dosage of 16 mg kg⁻¹).^[35] A recent study on the in vivo biodistribution of fluorescein isothiocyanate labelled MSN and PEGylated MSN after tail-vein injection in mice showed that the particles were mainly distributed in the liver and spleen with no abnormality in gross morphology of the tissues.^[36] Importantly in our study no morphological changes were observed in the major organs from mice injected subcutaneously with AM-41 nanoparticles (Figure 6) at the relatively low doses tested providing evidence that silica nanoparticles can be safely used as an effective vaccine delivery platform.

4. Conclusion

We have shown that OVA+AM-41 nanovaccine given via the subcutaneous route elicited both antibody and cell-mediated immune responses thus demonstrating the self-adjuvanting

potential of these particles for vaccine delivery applications both for human and animal health. The capacity to stimulate both arms of the mammalian immune system is a considerable advantage over conventional vaccine/adjuvant systems which often prime only one arm of the immune system.

5. Experimental Section

Materials: Octadecyltrimethylammonium bromide (C₁₈TAB, 99%), tetraethylorthosilicate (TEOS, purity >98%), 3-Aminopropyltriethoxysilane (APTES, 99%) and OVA Grade III, were all obtained from Sigma-Aldrich (St. Louis, USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and an antibiotic-antimycotic (containing penicillin G sodium, streptomycin sulphate, Fungizone) were obtained from Life Technologies (Carlsbad, USA). PBS (NaCl (137 mM), KCl (2.7 mM), Phosphate buffer (10 mM), pH 7.2) was obtained from Amresco (Solon, USA). An ELISPOT^{PLUS} kit for mouse Interferon- γ detection by splenocytes was obtained from MabTech (Sweden).

Mesoporous Silicate Syntheses: MCM-41 was synthesized according to previously published protocols.^[37] Briefly, in a typical synthesis, 0.2 g C₁₈TAB was mixed with water (96 mL) and 0.7 mL sodium hydroxide (2 M). This mixture was heated to 80 °C and stirred in a water bath. Once at 80 °C, the solution was left for 20 min to ensure a uniform temperature, then 6 mmol TEOS was added. The solution was allowed to stir vigorously for 2 h. The surfactant was removed by collecting the white precipitates by centrifugation and washing twice in ethanol (100 mL) and hydrochloric acid (32%, 2 mL) at 60 °C for 24 h. The washed silica was then dried at 50 °C.

Amino Functionalization of MCM-41: Functionalized MCM-41 was synthesized by a co-condensation method. C₁₆TAB (0.2 g) was mixed with water (96 mL) and NaOH (2 M, 0.7 mL) and heated to 80 °C and allowed to stir for an additional 30 min. TEOS (5.45 mmol) and APTES (0.55 mmol) was mixed together and then added to the heated solution. Subsequent collection and washing steps were identical to those for the MCM-41 synthesis described above.

Nanoparticle Suspensions: Suspensions of the silica particles in PBS were created to ensure maximum availability of the external surface area. Typically, powdered silica particles (100 mg) and PBS (10 mL) were added into a glass vial and ultrasonicated (1 min, room temperature) using a probe (Hielscher UP100H, Teltow, Germany) set at 80% amplitude.

Characterization of MSN: Small angle XRD was carried out on a Rigaku Miniflex diffractometer (Tokyo, Japan) using CoK α radiation ($\lambda = 1.792 \text{ \AA}$) operated at 30 kV with a variable slit width. The scanning rate was set at 1 °min⁻¹ over a 2 θ range of 1.5–8°. Nitrogen adsorption was performed on a Quadrasorb SI (Quantachrome, Boynton Beach, USA) at the liquid nitrogen temperature (77 K). Surface area was calculated by the Brunauer–Emmet–Teller (BET) method using multiple points over the linear part of the plot (P/P₀ range 0.05–0.25). Pore diameter distribution was determined by the Barrett–Joyner–Halenda (BJH) method and pore volume was estimated from the amount of nitrogen adsorbed at a relative pressure of 0.99. TEM images of AM-41 MSN were collected using a JEOL JEM-1010 (Tokyo, Japan) with an acceleration voltage of 100 kV. Amino functionalization was characterized using XPS using AlK α radiation to determine the elemental composition

of the materials. Zeta potential of the nanoparticles was measured on a Nanosizer Nano ZS analyzer (Malvern Instruments, Worcester-shire, UK).

OVA Adsorption Isotherm: To determine the OVA adsorption isotherm on MCM-41 and AM-41, 5 mL of OVA (0.4 mg mL⁻¹) in PBS was mixed with the particles (0.2–20 mg mL⁻¹) for 4 h. The amount of OVA protein remaining in the supernatants was quantified using a micro bicinchoninic acid (BCA) protein assay (Sigma Aldrich) following the manufacturer's instructions.

OVA Adsorption Kinetics to Amino-Functionalized MSN: Adsorption kinetic experiments used AM-41 particles (20 mg) with an OVA solution (0.8 mg mL⁻¹, in a final volume of 5 mL) in PBS. This particle-protein slurry was placed in a shaker at 25 °C. At each time point of 5, 15, 30, 60, 120, 180, 240 min and 24 h, a 200 µL sample of the particle-protein slurry was removed and centrifuged (16.2g, 1 min). The amount of OVA protein remaining in the supernatants following adsorption was quantified by protein assay (Biorad DC Kit) following the manufacturer's instructions.

In vitro Desorption of OVA Protein: To determine the kinetics of OVA protein release from MSN in vitro desorption studies were carried out. OVA loaded nanoparticles were suspended in of PBS solution (1 mL, pH 7.2) and incubated with shaking (37 °C). At the 5 min time point, the sample was centrifuged and the supernatant collected. Particles were then resuspended in PBS (1 mL) and incubated with shaking (37 °C) until the subsequent time point. The procedure was repeated at the following time points 15, 30, 60, 120, 240 min and overnight. Quantification of OVA protein released into the supernatant at each time point was performed using the Biorad DC Kit protein assay and visualized with the electrophoresis of the supernatants on SDS-PAGE gels.

Polyacrylamide Gel Electrophoresis (PAGE): MSN samples were prepared by taking the particle slurry (5–20 µL) and centrifuging (16g, 2 min). Supernatant samples (5 µL) were combined with sample reducing buffer (Life Technologies) and heated (70 °C, 10 min) before electrophoresis on 10% Bis-Tris gels (Life Technologies). Nanoparticle samples were resuspended in SDS Reducing Buffer (Tris-HCl (62.5 mM, pH 6.8), DTT (117 mM), Glycerol (10%), SDS (2%), Bromophenol blue (0.02%, 20 µL) and incubated (85 °C, 2 min) then subject to electrophoresis on 10% Tris-Glycine gels (Life Technologies). Electrophoresis of Life Technologies XCell SureLock Mini-Cell PAGE gels was according to the manufacturer's instructions. The gels were visualized by staining in methanol (50%), acetic acid (10%), Coomassie Blue R250 (0.25%) for 30 min, followed by destaining in methanol (30%), acetic acid (10%) for three 30 min washes.

Animals: C57BL/6J mice were purchased from and housed in the Biological Resource Facility, The University of Queensland, Brisbane, Australia under specific pathogen-free conditions. Eight week old female mice were housed in HEPA-filtered cages with 4 animals per cage in an environmentally controlled area with a cycle of 12 h of light and 12 h of darkness. Food and water were given ad libitum. All procedures were approved by The University of Queensland Ethics Committee. Animals were closely monitored throughout the study. All the animals remained in good health for the duration of the study with no visible deleterious health effects.

Immunization of Mice: Pre-immunization blood samples were collected by retro-orbital bleeds using heparin coated hematocrit tubes (Hirschmann Laborgeräte, Heilbronn, Germany). Pre-immunization blood samples collected prior to the first immunization

Table 2. Immunization groups in mice study. All doses were administered at the tail base.

Treatment Group	Group Description	Injected dose [100 µL]
1	OVA Control	50 µg OVA + 10 µg QuilA
2	OVA+AM-41	2 µg OVA/62 µg AM-41
3	OVA+AM-41	10 µg OVA/150 µg AM-41
4	AM-41 only	150 µg AM-41
5	Unimmunized Control	N/A

were referred to as the preimmune (PI) samples. **Table 2** shows the different treatment groups in the study. OVA protein adsorption to AM-41 particles was performed within 48 h of animal immunization. Adsorption reactions were prepared aseptically as described above, the injection doses administered were OVA (2 µg) and OVA (10 µg) in AM-41, (Table 2). QuilA (2 mg mL⁻¹, Superfos Biosector, Vedback, Denmark)^[38] was resuspended in sterile injectable water (Pfizer, Brooklyn, USA). Doses prepared in saline (0.9%, 100 µL, Pfizer) were administered by subcutaneous injection at the tail base using a sterile 27 gauge needle (Terumo, Tokyo, Japan). Three injections were administered at 2 week intervals and mice were euthanized 14 days after the final immunization.

Detection of OVA-Specific Antibody Responses: ELISA for the detection of OVA-specific antibodies were performed by coating microtitre plates (96 well, Nunc, Maxisorb, Roskilde, Denmark) with OVA antigen solution (2 ng µL⁻¹, 50 µL) in PBS overnight at 4 °C. The coating solution was removed and the plates were washed once with PBS-T (PBS (1x), Tween-20 (0.1%), Sigma-Aldrich) and blocked with Bovine Serum Albumin (BSA, 5%, Sigma-Aldrich) and skim milk (5%, Fonterra, Auckland, New Zealand) in PBS (200 µL) for 1 h with gentle shaking at RT. Plates were washed three times with PBS-T.

Mouse sera samples were diluted from 1:100 to 1:6400 in PBS (50 µL) and each dilution was added to the wells of the blocked plates followed by incubation for 2 h at 25 °C. To detect mouse antibodies Horse Radish Peroxidase (HRP) conjugated polyclonal sheep anti-mouse IgG antibodies (Chemicon Australia, Melbourne, VIC, Australia) diluted in PBS to 1:1000 were added to each well and incubated for 1 h at 25 °C with gentle shaking. Plates were washed three times in PBS-T. TMB substrate (100 µL, Sigma-Aldrich) was added to each well and incubated for 15 min at room temperature; HCl (1N, 100 µL) was added to wells to stop the chromogenic reaction. The plates were read at 450nm on a Labsystems Multiskan RC plate scanner.

Isolation of Murine Splenocytes and IFN-γ ELISPOT Assays: Spleens were aseptically removed following euthanasia and placed into ice cold DMEM media (5 mL) supplemented with fetal bovine serum (FBS, 10%), Hepes (20 mM, pH 7.3), sodium pyruvate (1 mM), Glutamax (1 mM), penicillin G (100 units mL⁻¹), streptomycin (100 µg mL⁻¹), Fungizone (0.25 µg mL⁻¹). Spleens were gently disrupted and passed through a nylon mesh (100 µm, Becton Dickinson, Franklin Lakes, NJ) using a syringe plunger. Cells were washed with DMEM (5 mL) and centrifuged (800g, 5 min, 4 °C) and then resuspended in lysis buffer (NH₄Cl (0.15 mM), KHCO₃ (10 mM), Na₂-EDTA (0.1 mM), 1 mL) for 5 min at room temperature. Repeat wash steps twice with DMEM (9 mL and 5 mL) each time. Cell pellets were resuspended in DMEM (2 mL) and cell numbers

determined by staining with trypan blue (0.2%). Cells from each mouse spleen were seeded at $1.0\text{--}1.5 \times 10^5$ cells/well in triplicate into Polyvinylidene fluoride (PVDF) ELISPOT plates precoated with monoclonal interferon- γ (IFN- γ) (Mabtech) capture antibody. Cells were incubated in complete DMEM medium at 37 °C and 5% CO₂ for 40 h in the presence or absence of synthetic OVA peptide ($1 \mu\text{g mL}^{-1}$, SIINFEKL, Auspep, Parkville, VIC, Australia) or the polyclonal activator ConA ($1 \mu\text{g mL}^{-1}$, Sigma Aldrich) as a positive control. IFN- γ ELISPOT assays were performed according to the manufacturer's specifications. The ELISPOT plates were read on an ELISPOT reader (Autoimmun Diagnostika, Strassburg, Germany).

Histological Examination: Tissues recovered at the necropsy including heart, lung, liver, spleen, kidney, lymph nodes (local and peripheral) and the injection site area (including skin and underlying tissue) were immediately fixed in neutral buffered formalin (10%), (Sigma-Aldrich). Subsequent paraffin embedding, sectioning and staining with hematoxylin and eosin for histological examination was performed using standard techniques by Cerbrus Sciences (Adelaide, SA, Australia).

Statistical Analysis: The mean $\pm$ SE for adsorption studies was calculated using Microsoft Excel. Statistical analysis was performed on the SFU/million cells for individual animals by ELISPOT using an unpaired two-tailed Student's *t*-test (Microsoft Excel).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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