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Freeze-drying of ovalbumin loaded mesoporous silica nanoparticle vaccine formulation increases antigen stability under ambient conditions

Karishma T. Mody[a,1], Donna Mahony[a,1], Antonino S. Cavallaro[a], Frances Stahr[b], Shi Zhang Qiao[b,2], Timothy J. Mahony[a], Neena Mitter[a,*]

[a] Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Queensland, Australia
[b] Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, Queensland, Australia

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ABSTRACT

Amino functionalised mesoporous silica nanoparticles (AM-41) have been identified as a promising vaccine delivery material. The capacity of AM-41 to stabilise vaccine components at ambient temperature (23–27 °C) was determined by adsorbing the model antigen ovalbumin (OVA) to AM-41 particles (OVA-41). The OVA-41 was successfully freeze-dried using the excipients 5% trehalose and 1% PEG8000. The immunological activity of OVA and the nanoparticle structure were maintained following two months storage at ambient temperature. The results of immunisation studies in mice with reconstituted OVA-41 demonstrated the induction of humoral and cell-meditated immune responses. The capacity of AM-41 particles to facilitate ambient storage of vaccine components without the loss of immunological potency will underpin the further development of this promising vaccine delivery platform.

1. Introduction

The role of vaccines is to elicit protective immunity against infectious agents. While improvements have been made with the development of protein and peptide based subunit vaccine formulations, many challenges still remain. Key challenges include the complexities associated with physical and chemical stability of the vaccine antigen components (de la Fuente et al., 2008; Kammer et al., 2007; Koping-Hoggard et al., 2005; Lee, 2002; Mody et al., 2012). The freeze-drying process and the novel delivery systems can address the issues associated with conventional and subunit vaccines.

Mesoporous silica nanoparticles (MSNs) have emerged as excellent carriers for delivery of biologically active molecules such as drugs, genes, therapeutic proteins and enzymes. The ease of MSNs synthesis and its characteristics such as porous structure, large surface area, flexible surface chemistry and *in vivo* biocompatibility contribute to the utility of this carrier system (Carvalho et al., 2010; Giri et al., 2005; Trewyn et al., 2008; Wendorf et al., 2006). The efficiency of ordered silica nanoparticles (SBA-15) and MSNs as adjuvants and protein carriers have already been explored in mice immunisation studies (Carvalho et al., 2010; Guo et al., 2012; Mahony et al., 2013; Mercuri et al., 2006). Silica nanoparticles have emerged as a new approach to address the limitations of current technologies such as high toxicity, severe side effects, and inflammation at the site of injection associated with conventional adjuvants and it also aims to improve the overall safety profile of the vaccine formulations (Mody et al., 2013).

The current investigation focused on the development of a freeze-dried MSN based vaccine delivery system. The freeze-drying process can preserve unstable molecules such as proteins and nanoparticles (Abdelwahed et al., 2006; Hsu et al., 1995). During the freeze-drying process water is removed from the frozen sample by sublimation and desorption under specific vacuum pressure and temperature conditions. The freeze-drying process can damage biological molecules leading to loss of activity; therefore excipients such as sugars, surfactants, amino acids and polymers are added to the formulation to protect and enhance the overall

* Corresponding author at: Queensland Alliance for Agriculture and Food Innovation, Ritchie Building 64C, Research Road, The University of Queensland, St Lucia, Queensland 4072, Australia. Tel.: +61 7 3346 6513; fax: +61 7 3346 6501.
E-mail addresses: k.mody@uq.edu.au (K.T. Mody), d.mahony1@uq.edu.au (D. Mahony), a.cavallaro@uq.edu.au (A.S. Cavallaro), s4075611@student.uq.edu.au (F. Stahr), s.qiao@adelaide.edu.au (S.Z. Qiao), t.mahony@uq.edu.au (T.J. Mahony), n.mitter@uq.edu.au (N. Mitter).
[1] Contributed equally.
[2] School of Chemical Engineering, The University of Adelaide, SA 5005, Australia.

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stability of proteins (Baheti et al., 2010; Mody et al., 2012). The excipients used to protect from the freezing stress are known as cryoprotectants, whereas the ones protecting from drying stress are known as lyoprotectants (Wei, 2000). The physico-chemical property of freeze-dried human serum albumin nanoparticles was retained by addition of 3% trehalose (Anhorn et al., 2008). It has been established that physical characteristics of freeze-dried polymeric nanoparticles such as poly(lactide-co-glycolide), poly(gamma-glutamic acid) and poly(D,L-lactic acid) have been preserved by the addition of excipients (Layre et al., 2006; Morris et al., 2011; Quintanar-Guerrero et al., 1998). However, since the discovery of Mobile Crystalline Materials No. 41 (MCM-41) type MSNs in 1992 by Mobil scientists (Kresge et al., 1992), no information has been reported on the freeze-drying of protein loaded MCM-41 type MSNs or the capacity of these formulations to elicit immunogenicity following reconstitution.

In this paper we report the successful freeze-drying of amino functionalised MCM-41 nanoparticles (AM-41) following the loading of the model antigen Ovalbumin (OVA). The OVA loaded AM-41 nanoparticles are termed as OVA-41. The OVA antigen was chosen for this study, as OVA degradation was observed when stored at ambient temperature for periods as short as 16 h. We investigated the impact of the freeze-drying process and the effect of excipients trehalose and PEG8000 on the stability of OVA. The freeze-dried samples were stored at ambient temperature for two months, following which the immunogenicity of the formulation was tested in mice. We have demonstrated the capacity of the freeze-dried OVA-41 nanoformulation to induce humoral and cell-mediated immune responses.

2. Experimental details

2.1. Materials

OVA Grade III, D-(+)-trehalose dihydrate, PEG8000, Bovine Serum Albumin (BSA) and PBS-T [PBS (1×), Tween-20 (0.1%)] were all obtained from Sigma–Aldrich (St. Louis, USA). PBS (137 mM NaCl 2.7 mM KCl 10 mM Phosphate buffer pH 7.2) was obtained from Amresco (Solon, USA). Dulbecco's Modified Eagle Medium (DMEM), antibiotic/antimycotic (containing penicillin G sodium, streptomycin sulphate, Fungizone) and Foetal Bovine Serum (FBS) were obtained from Life Technologies (Carlsbad, USA). ELISPOT^{PLUS} kit for the detection of the mouse Interferon (IFN)-γ by splenocytes was obtained from MabTech (Sweden).

2.2. Preparation of mesoporous silicate nanoparticles

The nanoparticles used in this investigation were unfunctionalised and amino functionalised MCM-41. The nanoparticles were prepared by co-condensation method as described previously (Mahony et al., 2013). To ensure maximum availability of the external surface area of the nanoparticles, the nanoparticle suspension was prepared in PBS. Nanoparticles (100 mg) and PBS (10 ml) were combined and ultrasonicated in a glass vial for 1 min at room temperature (RT) using probe (Hielscher UP100H, Teltow, Germany) set at 80% amplitude.

2.3. Trypan blue staining for in vitro cytotoxicity assay

Madin–Darby bovine kidney (MDBK) cells (ATCC) were seeded at 80–90% confluency onto glass coverslips in a 24 well plate and allowed to adhere overnight at 37 °C, 5% CO_2. To investigate the effect of nanoparticle concentration on the cells a dilution range (0.5 mg/ml, 0.1 mg/ml and 0.01 mg/ml) of MCM-41 and AM-41 particles in Earle's Minimum Essential Media (containing 5% FBS) were prepared and gently added drop wise to the adherent cells. The cells and nanoparticles were incubated at 37 °C, 5% CO_2 for 20 h. Media was carefully removed and the wells were gently washed three times with PBS to remove the nanoparticles. To determine the cell viability 0.2% trypan blue stain (Life Technologies) was added for 2 min. Trypan blue stain was carefully removed and the wells were washed once with PBS. Cells were fixed in 4% paraformaldehyde (PFA) pH 7.4 for 15 min, and then washed three times with PBS. Coverslips were mounted with 5 μl of MOWIOL (Sigma). Cell viability was determined by imaging on a Zeiss HAL100 microscope under bright field.

2.4. OVA loaded AM-41 nanoparticles

Loading reaction consisted of 20 mg of AM-41 particles and OVA at 0.8 mg/ml in a 5 ml final volume of PBS at pH 7.0 as previously described (Mahony et al., 2013). This particle–protein slurry was placed in a shaker at 25 °C for 240 min. A 200 μl sample of the particle-protein slurry was removed and centrifuged 16.2 × *g* for 5 min. The amount of unbound OVA protein remaining in the supernatant was assessed by Biorad DC kit protein assay and visualised on SDS-PAGE gels.

2.5. Freeze-drying process

Following OVA loading, the sample was centrifuged at 16.2 × *g* for 1 min and the supernatant was removed. The protein–nanoparticle (OVA-41) (4 mg OVA: 20 mg AM-41) pellet was freeze-dried with different combinations of excipients: (1) OVA-41 alone, (2) OVA-41 + 20% trehalose, (3) OVA-41 + 1% PEG8000, (4) OVA-41 + 5% trehalose + 1% PEG8000. The final formulation was obtained by mixing the excipients to the nanoparticle–protein pellet at fixed concentration to make up the final volume to 1 ml. Samples were frozen in liquid nitrogen, then placed in the Christ freeze-dryer (Model LPC-32, Martin Christ, Osterode AM Harz, Germany) at 24 °C, 0.1 mbar for 17 h. Freeze-dried samples were stored in a vacuum desiccator at ambient temperature (23–27 °C).

2.6. Desorption studies on the freeze-dried samples

Following 3 days of storage at ambient temperature, the freeze-dried samples were resuspended in 200 μl of PBS and centrifuged for 5 min at 16.2 × *g* to remove the supernatant. Vaccinations were prepared by resuspending the freeze-dried samples in 500 μl of saline. Hence, the nanoparticle pellet obtained was resuspended in 500 μl of pre-warmed PBS and incubated at 37 °C for 120 min on a shaker at 200 rpm. The samples were centrifuged as above and the supernatant was assessed for desorbed protein by electrophoresis on SDS-PAGE gels.

2.7. Polyacryalamide gel electrophoresis (PAGE)

To prepare the particulate and supernatant samples, particle slurry (20 μl) was taken and centrifuged at 16.2 × *g* for 5 min. The supernatant and nanoparticle samples were resuspended in SRB (SDS Reducing Buffer consisting of 62.5 mM Tris–HCl (pH 6.8), 117 mM DTT, 10% Glycerol, 2% SDS, 0.02% Bromophenol blue), incubated at 85 °C for 2 min then subjected to electrophoresis on 10% Tris–Glycine gels (Invitrogen). The gels were visualised by staining in 50% methanol, 10% acetic acid, 0.25% Coomassie Blue R250 for 30 min, followed by destaining in 30% methanol, 10% acetic acid with three 30 min washes.