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# Protein coated microcrystals formulated with model antigens and modified with calcium phosphate exhibit enhanced phagocytosis and immunogenicity

Sarah Jones[a,1], Catpagavalli Asokanathan[b], Dorota Kmiec[b], June Irvine[a], Roland Fleck[c], Dorothy Xing[b], Barry Moore[d,e,*], Roger Parton[a], John Coote[a]

[a] Institute of Infection, Immunity and Inflammation, College of Veterinary, Medical and Life Sciences, University of Glasgow, University Avenue, Glasgow G12 8QQ, UK
[b] Division of Bacteriology, National Institute of Biological Standards and Control (NIBSC), Blanche Lane, South Mimms, Hertfordshire EN6 3QG, UK
[c] Division of Cellular Biology and Imaging, National Institute of Biological Standards and Control (NIBSC), Blanche Lane, South Mimms, Hertfordshire EN6 3QG, UK
[d] Department of P&A Chemistry, WestChem, Thomas Graham Building, 295 Cathedral Street, Glasgow G1 1XL, UK
[e] XstalBio Ltd., CIDS, Thomson Building, University Avenue, Glasgow G12 8QQ, UK

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ABSTRACT

Protein-coated microcrystals (PCMCs) were investigated as potential vaccine formulations for a range of model antigens. Presentation of antigens as PCMCs increased the antigen-specific IgG responses for all antigens tested, compared to soluble antigens. When compared to conventional aluminium-adjuvanted formulations, PCMCs modified with calcium phosphate (CaP) showed enhanced antigen-specific IgG responses and a decreased antigen-specific IgG1:IgG2a ratio, indicating the induction of a more balanced Th1/Th2 response. The rate of antigen release from CaP PCMCs, *in vitro*, decreased strongly with increasing CaP loading but their immunogenicity *in vivo* was not significantly different, suggesting the adjuvanticity was not due to a depot effect. Notably, it was found that CaP modification enhanced the phagocytosis of fluorescent antigen-PCMC particles by J774.2 murine monocyte/macrophage cells compared to soluble antigen or soluble PCMCs. Thus, CaP PCMCs may provide an alternative to conventional aluminium-based acellular vaccines to provide a more balanced Th1/Th2 immune response.

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*Abbreviations:* ANOVA, analysis of variance; Block-B, 1% BSA in PBST; Block-G, 1% gelatine in PBST; BSA, bovine serum albumin; BSA-FITC, BSA conjugated to FITC; CaP, calcium phosphate; cRPMI, complete RPMI medium; CyaA, adenylate cyclase toxin of *Bordetella pertussis*; CyaA*, genetically-detoxified CyaA; DAPI, 4′,6-diamidino-2-phenylindole; DT, diphtheria toxoid; DTaP, diphtheria, tetanus and acellular pertussis vaccine; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; PBS-A, PBS lacking $Mg^{2+}$ and $Ca^{2+}$; PBST, PBS containing 0.05% Tween 20; PCMC, protein-coated microcrystal; PVDF, polyvinylidene fluoride; SEM, scanning electron microscopy; rt, room temperature.

* Corresponding author at: Department of P&A Chemistry, WestChem, Thomas Graham Building, 295 Cathedral Street, Glasgow G1 1XL, UK. Tel.: +44 141 330 3833.
*E-mail addresses:* sjones@biooutsource.com (S. Jones), Cathy.Asokanathan@nibsc.hpa.org.uk (C. Asokanathan), Dorota.Kmiec@nibsc.hpa.org.uk (D. Kmiec), June.Irvine@glasgow.ac.uk (J. Irvine), Roland.Fleck@nibsc.hpa.org.uk (R. Fleck), Dorothy.Xing@nibsc.hpa.org.uk (D. Xing), b.d.moore@strath.ac.uk (B. Moore), Roger.Parton@glasgow.ac.uk (R. Parton), John.Coote@glasgow.ac.uk (J. Coote).
[1] Current address: BioOutsource Ltd., 1 Technology Terrace, Todd Campus, West of Scotland Science Park, Glasgow G20 0XA, UK.

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## 1. Introduction

Conventional aluminium-containing adjuvants have been used in vaccine formulations for decades but promote poor induction of Th1 or cell-mediated immunity [1,2] and require refrigeration during transportation and storage. Approximately 50% of vaccines are discarded globally, largely due to cold chain disruption [3,4]. Therefore, a major objective of vaccine formulation *t* is to develop a safe, immunogenic composition which addresses the issues of immune bias and stability.

Protein-coated microcrystals (PCMCs) are a recent advance in vaccine formulation [5] and have the potential to by-pass the cold chain. Originally developed to stabilise enzymes for industrial applications [5–9], PCMCs are formed by rapid co-precipitation of protein(s) with an amino acid or sugar, producing particles with an inert core microcrystal coated with protein(s) [6,8,9]. Vaccine antigens, loaded onto PCMCs, exhibited much higher resistance to heat stress compared to native antigens [5,7]. These reports used PCMC formulations which were instantly soluble in aqueous buffer [5–9]. In this study, novel sustained-release PCMCs have been used which are poorly soluble due to modification of their outer surface with sparingly soluble CaP. CaP served as an adjuvant in some early acellular vaccines [10,11], and is well-tolerated in man [11–16]. CaP also enhances Th1-biased immunity although this may be antigen-dependent [11,17,18]. Here, the immunogenicity of CaP-modified PCMCs loaded with different model antigens was investigated. DT, a formaldehyde-toxoided antigen [19–21], and BSA have been used extensively as model antigens when validating new vaccine formulations [22–25].

## 2. Materials and methods

### 2.1. Source of antigens

The DT preparation was the 2nd international standard for use in flocculation tests (02/176, NIBSC, UK). CyaA* was purified and characterised as described previously [26–28]. BSA was from Sigma and BSA-FITC was from Life Technologies, UK.

### 2.2. PCMC preparation

All reagents were of the highest grade available and were used at rt. The aqueous solution was prepared in endotoxin-free, sterile water (Sigma) and contained 30 mg/ml L-glutamine as the core component of the PCMCs, trehalose and the test antigens, sufficient to give final loadings of 10% and 0.2–0.4%, respectively, in the PCMC preparation. To precipitate PCMCs, 3 ml of the aqueous solution was added drop-wise to 60 ml of rapidly stirred isopropanol and stirring continued for 1 min at 1500 rpm. For CaP-modified PCMCs, the required concentration of $NaH_2PO_4$ was included in the aqueous solution and $CaCl_2$ was included in the isopropanol at a 2-fold molar excess compared to $NaH_2PO_4$. PCMCs were collected by vacuum filtration onto PVDF hydrophilic 0.45 μm filters (Millipore, UK) and dried overnight for storage as a dry powder.

### 2.3. Quantification of antigen loading by ELISA

PCMCs were dissolved at 10 mg/ml in sodium citrate buffer [50 mM sodium citrate, 20 mM Tris, 1 mM EDTA, pH6.8]. The PCMC solution was diluted 1:3 v/v in carbonate coating buffer [15 mM $Na_2CO_3$, 30 mM $NaHCO_3$, pH9.5] and serially diluted in a flat-bottom 96-well ELISA plate (MAXISorp, Nunc, UK). Plates were incubated overnight at 4 °C prior to washing 3 times in PBST. Non-specific binding was blocked by addition of 100 μl/well of block-B and incubation for 1 h at 37 °C. For BSA-containing PCMCs, block-G was used in place of block-B. After further washing, samples were incubated (2 h, 37 °C) with 50 μl/well of the appropriate primary antibody [anti-DT (NIBSC, 1/1000), anti-CyaA* (in-house, 1/500)] or anti-BSA (Sigma, 1/1000)] diluted in the appropriate blocking buffer. After washing, 50 μl/well of peroxidase-conjugated secondary antibody (Sigma) diluted 1/1000 in the appropriate blocking buffer was added and plates incubated for 1.5 h at 37 °C. Plates were washed again and protein binding was visualised using 50 μl/well of O-phenylene-diamine. After incubation for 10–15 min at rt, colour development was stopped with 3 M HCl and absorbance at 492 nm was measured. Protein loading onto PCMCs was quantified by comparison to a stock antigen standard curve.

### 2.4. Determination of PCMC morphology

For SEM, dry PCMCs were gold-plated prior to visualisation with a JEOL6400 electron microscope operating at 6 kV.

### 2.5. Characterisation of antigen release in vitro

PCMCs were suspended at 10 mg/ml in 1.5 ml of either 0.1 mM sodium citrate (pH 6.0) or PBS and incubated at rt or 37 °C with gentle agitation. At intervals, the PCMC suspension was centrifuged for 1 min at 2400 × *g* and 1 ml of supernate removed to determine protein release. More buffer was then added to the pelleted PCMCs to readjust the volume to 1.5 ml and the incubation continued. Supernates were stored at −20 °C prior to quantification of protein release by ELISA as described above.

### 2.6. Adsorption of antigens to $Al(OH)_3$

Soluble antigens were dissolved in sterile PBS containing 10% $Al(OH)_3$ (A8222, Sigma), mixed thoroughly and incubated overnight at 4 °C. Adsorbed antigens were then used for immunisation.

### 2.7. Immunisation of mice

Groups of 8 inbred, female 6–8 week old NIH mice (Harlan, UK) were injected subcutaneously at days 0 and 28 with 0.5 ml volumes of the desired formulation or PBS as a control. Immediately prior to immunisation, the required doses of PCMCs were suspended in sterile PBS. Mice were sampled for sera at 28 d and 42 d post-immunisation, as described previously [28]. All animal experiments were performed under UK Home Office License and in accordance with EU Directive 2010/63/EU.

### 2.8. Determination of antigen-specific serum IgG titres

Antigen-specific IgG, IgG1 and IgG2a titres were determined using ELISA as described previously [26] with the use of block-G when determining anti-BSA responses. Geometric mean titres were calculated by comparison to reference sera.

### 2.9. Culture of J774.2 cells

Murine monocyte/macrophage J774.2 cells were maintained in 75 $cm^2$ tissue-culture flasks (Corning, UK) (37 °C, 5% $CO_2$) in complete RPMI [cRPMI; RPMI 1640 medium (Life Technologies, UK), 10% foetal calf serum (Sigma), 10 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies, UK)].

### 2.10. Uptake of PCMCs by J774.2 cells

#### 2.10.1. Flow cytometry

Each well of a 24-well tissue-culture plate (Corning, UK) was supplemented with $10^6$ J774.2 cells and incubated (2 h, 37 °C, 5%

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