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Synthetic nanoparticle vaccines produced by layer-by-layer assembly of artificial biofilms induce potent protective T-cell and antibody responses *in vivo*

Thomas J. Powell*,[1], Naveen Palath[1], Mary E. DeRome[1], Jie Tang[1], Andrea Jacobs[1], James G. Boyd[1]

Artificial Cell Technologies, Inc., 5 Science Park, Suite 13, New Haven, CT 06511, United States

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ABSTRACT

Nanoparticle vaccines induce potent immune responses in the absence of conventional adjuvant due to the recognition by immune cells of the particle structures, which mimic natural pathogens such as viruses and bacteria. Nanoparticle vaccines were fabricated by constructing artificial biofilms using layer-by-layer (LbL) deposition of oppositely charged polypeptides and target designed peptides on $CaCO_3$ cores. LbL nanoparticles were efficiently internalized by dendritic cells *in vitro* by a mechanism that was at least partially phagocytic, and induced DC maturation without triggering secretion of inflammatory cytokines. LbL nanoparticle delivery of designed peptides to DC resulted in potent cross-presentation to CD8+ T-cells and more efficient presentation to CD4+ T-cells compared to presentation of soluble peptide. A single immunization of mice with LbL nanoparticles containing designed peptide induced vigorous T-cell responses characterized by a balanced effector (IFNγ) and Th2 (IL-4) ELISPOT profile and *in vivo* CTL activity. Mice immunized with LbL nanoparticles bearing ovalbumin-derived designed peptides were protected from challenge with *Listeria monocytogenes* ectopically expressing ovalbumin, confirming the relevance of the CTL/effector T-cell responses. LbL nanoparticles also elicited antibody responses to the target epitope but not to the matrix components of the nanoparticle, avoiding the vector or carrier affect that hampers utility of other vaccine platforms. The potency and efficacy of LbL nanoparticles administered in aqueous suspension without adjuvant or other formulation additive, and the absence of immune responses to the matrix components, suggest that this strategy may be useful in producing novel vaccines against multiple diseases.

1. Introduction

Layer-by-layer (LbL) assembly of biofilms is described most simply as sequential layering of oppositely charged polyelectrolytes on a core substrate of choice, such that the polyelectrolyes adhere to one another via electrostatic interactions resulting in the build-up of a multilayer nanofilm. The earliest demonstration of LbL technology involved assembly of colloidal particles on a solid core [1]. Beginning in the 1990s, the applications were expanded to include LbL assembly of polyelectrolyte films using organic and biopolymers, proteins, peptides, polysaccharides and DNA [2–8]. It is now recognized that LbL technology has applications in a number of industrial and medical fields (reviewed in [9,10]). Depending on characteristics of the core substrate such as size and shape, and the choice of polyelectrolytes utilized, LbL assembly of multilayer films might be used to produce cell- or virus-like particles suitable for delivery of functional biomolecules. Indeed, this approach has been used to produce artificial red blood cells [11] and controlled-release particles loaded with insulin [12,13], vitamins [13], or small antigenic peptides [14]. These examples demonstrate the potential utility of LbL nanoparticle technology as a platform for vaccine development, especially in the context of our understanding of the mechanisms of immune recognition and activation of the appropriate immune response pathways. Specifically, cells of the immune system, in particular professional antigen-presenting cells such as dendritic cells (DC), are exquisitely sensitive to recognition of micro- and nano-sized particles such as bacteria and viruses, respectively. By incorporating well-defined antigenic epitopes in micro- and nanoparticle constructs, investigators have demonstrated improved immunogenicity of both T-cell and antibody target epitopes in a number of model systems including OVA [15–17], hepatitis B antigens [18], tumor antigens [19,20], respiratory syncytial virus antigens [21], and malaria and influenza antigens [22]. The increased potency of nanoparticle vaccine constructs has been attributed to several mechanisms, including efficient phagocytosis, cross-presentation, and activa-

Abbreviations: BMDC, bone marrow dendritic cells; DP, designed peptide; LbL, layer-by-layer; OVA, ovalbumin; PGA, poly-L-glutamic acid; PGA–TR, PGA conjugated to Texas red; PLL, poly-L-lysine; PLL–FITC, PLL conjugated to fluorescein isothiocynate; TBS/T, 20 mM Tris base, pH 7.6, 140 mM NaCl, 0.1% Tween 20.

* Corresponding author. Tel.: +1 203 772 3430; fax: +1 203 567 8073.
E-mail address: jpowell@artificialcelltech.com (T.J. Powell).
[1] All authors are employees of Artificial Cell Technologies at 5 Science Park, Suite 13, New Haven, CT 06511, United States.

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tion of DC demonstrated by increased cytokine production and costimulatory marker expression [23–25]. In most cases, but not all, nanoparticle vaccines appear to directly activate DC and other effectors of the innate immune response, and it has been postulated that this 'adjuvant' effect of nanoparticle vaccines is key to their immune potency.

In the current study, we have adapted LbL technology to produce synthetic nanoparticles carrying designed peptide (DP) payloads. Nanoparticle delivery of ovalbumin DP induced maturation of DC without triggering secretion of inflammatory cytokines, facilitated efficient processing and presentation of the antigen payload to both CD4+ and CD8+ T-cells, and elicited potent T-cell responses including cytotoxic effector responses *in vivo*. Nanoparticle-elicited immune responses were generated in the absence of overt inflammation, injection site reactions, or other detectable adverse events, and protected mice from challenge with *Listeria monocytogenes* expressing ovalbumin as a surrogate antigen. Immunization with LbL nanoparticles also generated DP-specific antibody responses but no detectable antibody response to the matrix components of the nanoparticle vector. Since the nanoparticles are made by totally synthetic means, do not require exogenous adjuvants or innate immune stimulants, and do not trigger vector-neutralizing immune responses, this approach offers promise as a method for producing next-generation vaccines against a wide range of acute and chronic infectious diseases.

2. Materials and methods

2.1. Peptide synthesis

Standard peptide synthesis reagents were purchased from American Bioanalytical (Natick, MA), EMD Bioscience (Gibbstown, NJ), and Sigma–Aldrich. Peptides were synthesized by automated standard solid phase peptide synthesis methods on a Liberty™ microwave synthesizer (CEM) using HBTU activation, Fmoc amino acids (double coupled), and the manufacturer's standard synthesis cycles. Peptides were purified to >95% purity by reverse phase HPLC and their identities confirmed by electrospray mass spectrometry. Purified peptides were aliquoted, lyophilized, and stored at −20 °C. The designed peptide DP-2015 contains two overlapping OVA epitopes, $OVA_{257–264}$ (CD8 epitope) and $OVA_{258–276}$ (CD4 epitope), flanked by native OVA residues with a C-terminal poly-lysine tail and cysteine residue: QLE*SIINFEKL**TEWTSSNVMEER*K_9C (the CD8 epitope is underlined; the CD4 epitope is italicized). DP-2045 contains the influenza M2e epitope with a C-terminal poly-lysine tail and tyrosine residue: SLLTEVETPIRNEWGSRSNDSSDPSRK$_{20}$Y.

2.2. Nanoparticle fabrication

$CaCO_3$ nanoparticles (NPCC-111) were obtained from NanoMaterials Technology (Singapore). Polypeptides poly-L-lysine 15 kDa (PLL, catalog # P6516), FITC labeled poly-L-lysine 15–30 kDa (PLL–FITC, catalog # P3543), poly-L-glutamic acid 14.5 kDa (PGA, catalog #P4636), and 1 M HEPES buffer solution (catalog #H-3662) were obtained from Sigma–Aldrich (USA). Oppositely charged polypeptides were allowed to self-assemble into a multilayer film on $CaCO_3$ nanoparticle cores in successive adsorption steps essentially as previously reported [9,11,26,27], and as depicted schematically in Fig. 1. Briefly, PLL, PGA and DP (where indicated) were dissolved to 1 mg/ml in 10 mM HEPES, pH 7.4, and filtered through a 0.22 μm filter. $CaCO_3$ nanoparticle cores were washed three times with endotoxin-free water and centrifugation at 16,000 × *g* for 1 min in a microcentrifuge. Nanoparticle cores were resuspended to 6% (w/v) in 1 mg/ml PGA as the first layer. At neutral pH, PGA exhibits a net negative charge while the $CaCO_3$ particles are net positive, thus enabling electrostatic interaction and successful deposition of the first layer. The mixture was incubated for 10 min at room temperature, then washed twice with 10 mM HEPES buffer and centrifugation at 48,700 × *g* for 1 min (TL-100 Ultracentrifuge, Beckman). For second layer deposition, the nanoparticles were resuspended to 6% (w/v) in 1 mg/ml PLL (positive charge) and processed as for the first layer. Each subsequent layer was deposited by the same method, using PGA and PLL in alternating layers; where indicated, DP (positive charge) was used in place of PLL. Following the final layer deposition, the mature nanoparticles were washed twice with 10 mM HEPES, pH 7.4, and aliquots were spun down, aspirated, and stored as dry pellets at 4 °C until use. Several designs were produced using essentially the same procedure and altering the sequence and location of the designed peptide; all nanoparticles contain a total of eight layers in the polypeptide biofilm. Nanoparticle designs are summarized in Table 1.

The concentration of polypeptide or DP on the nanoparticles was determined by amino acid analysis (W.M. Keck Foundation Biotechnology Resource Laboratory, New Haven, CT). Nanoparticle size was estimated by dynamic light scattering (Malvern Nano S-90) of 6% (w/v) suspensions diluted 1:100 or 1:1000 in HEPES, pH 7.4, and sonicated for 20 min in an ultrasonic water bath (Branson 1510, USA) immediately prior to measurement. Morphology was examined by scanning electron microscopy (FEI Model XL-30) of gold-coated nanoparticles. SEM images were acquired at magnifications indicated in the figures. Levels of endotoxin in the nanoparticles were determined by Limulus Amebocyte Lysate assay, an endpoint chromogenic assay (#50-647U, Lonza, Walkersville, MD). The prepared nanoparticles were stored as a damp pellet at 4 °C until ready for use.

2.3. Cells

The B3Z T-cell hybridoma specific for H-2K^b:$OVA_{257–264}$ (SIINFEKL) was obtained from Dr. N. Shastri (University of California, Berkeley). The MF2.2D9 T-cell hybridoma specific for I-A^b:$OVA_{258–276}$ (IINFEKLTEWTSSNVMEER) and the DC2.4 dendritic cell line (H-2^b) were obtained from Dr. K. Rock (University of Massachusetts, Amherst). The JAWSII dendritic cell line (H-2^b) was obtained from ATCC (CRL-11904). All cell lines were cultured according to the supplier's specifications. Bone marrow-derived dendritic cells (BMDCs) were prepared by harvesting bone marrow cells from the tibia of C57BL/6J mice and culturing in RPMI supplemented with 10% FBS, 20 ng/ml rGM-CSF and 7.5 ng/ml rIL-4. Medium was refreshed on day 3 and every other day thereafter. BMDC were harvested on day 8 and used for *in vitro* assays.

2.4. Mice

Female mice, 6–8 weeks of age, were obtained from Jackson Laboratories. C57BL/6J (TLR4+), C57BL/10J (TLR4+), and C57BL10/ScNJ (TLR4-deficient), all of the H-2K^b haplotype, were used for OVA studies while BALB/c mice were used for the M2e experiments. Mice were housed in standard microisolator cages and given food and water *ad libitum*. All animal studies were approved by the Northeast Life Sciences (New Haven, CT) Institutional Animal Care and Use Committee.

2.5. Phagocytosis assays

JAWSII cells (5×10^4/well) were incubated with ACT-1008 (PGA–TR) for 30 min or overnight at 37 °C, then washed to remove free nanoparticles. Trypan blue (250 μg/ml) was added to the wells to quench extracellular fluorescence [28], and the samples were examined on a Zeiss Axiovert 40 CFL (Carl Zeiss, Thornwood, NY)