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Intradermal vaccination with hollow microneedles: A comparative study of various protein antigen and adjuvant encapsulated nanoparticles

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ABSTRACT

In this study, we investigated the potential of intradermal delivery of nanoparticulate vaccines to modulate the immune response of protein antigen using hollow microneedles. Four types of nanoparticles covering a broad range of physiochemical parameters, namely poly (lactic-*co*-glycolic) (PLGA) nanoparticles, liposomes, mesoporous silica nanoparticles (MSNs) and gelatin nanoparticles (GNPs) were compared. The developed nanoparticles were loaded with a model antigen (ovalbumin (OVA)) with and without an adjuvant (poly(I:C)), followed by the characterization of size, zeta potential, morphology, and loading and release of antigen and adjuvant. An in-house developed hollow-microneedle applicator was used to inject nanoparticle suspensions precisely into murine skin at a depth of about 120 μm. OVA/poly(I:C)-loaded nanoparticles and OVA/poly(I:C) solution elicited similarly strong total IgG and IgG1 responses. However, the *co*-encapsulation of OVA and poly (I:C) in nanoparticles significantly increased the IgG2a response compared to OVA/poly(I:C) solution. PLGA nanoparticles and liposomes induced stronger IgG2a responses than MSNs and GNPs, correlating with sustained release of the antigen and adjuvant and a smaller nanoparticle size. When examining cellular responses, the highest $CD8^+$ and $CD4^+$ T cell responses were induced by OVA/poly(I:C)-loaded liposomes. In conclusion, the applicator controlled hollow microneedle delivery is an excellent method for intradermal injection of nanoparticle vaccines, allowing selection of optimal nanoparticle formulations for humoral and cellular immune responses.

1. Introduction

Skin is an attractive administration site for immunization and may act as an excellent alternative for traditional intramuscular or subcutaneous vaccination. Furthermore, intradermal vaccination may enable dose sparing, since the skin has a rich network of immune cells compared to muscle or subcutaneous tissue [1]. However, the uppermost layer of the skin, the stratum corneum, is the main barrier that prevents the transport of vaccines (> 500 Da) across the skin. Therefore, novel delivery methods need to be developed. Among various methods developed for antigen delivery *via* the skin, especially microneedle-based approaches have recently attracted increasing attention [2]. The major advantage of microneedles is their ability to pierce the skin in a minimally invasive manner and subsequently deliver their payload in the superficial skin layers potentially without pain, owing to the limited penetration depth of microneedles (typically < 500 μm) [3].

Several microneedle types have been developed for vaccine delivery, such as coated or dissolving microneedles which can release the dry antigen into the epidermis and dermis after the piercing of the skin [2]. In contrast, hollow microneedles can be used to deliver antigens or particulate formulations as solutions or suspensions into the skin. To this end, in our group a hollow microneedle device has been developed that allows precise and controlled injections into the epidermis and dermis by using etched fused-silica capillary-based microneedles [4–6]. The advantage of the hollow microneedles compared to dissolving or coated microneedles is that little time is required for modifying the dose, formulation or administration depth. This is particularly

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advantageous when studying optimization of formulations or parameters for the immunization (*e.g.* penetration depth or vaccine dose). Furthermore, if required, a higher dose can be injected into the skin compared to dissolving and coated microneedles.

Subunit antigens are based on purified antigens and are regarded safer than traditional whole bacterium- or virus-based vaccines [7]. However, these antigens have often lower immunogenicity and therefore adjuvants, such as toll-like receptor (TLR) ligands or toxoids, are needed to increase the immune response [8]. Recently, nanoparticles have gained growing attention for the delivery of subunit vaccines because of their capability of protecting antigens from degradation, forming a depot at the site of injection, and facilitating antigen uptake by dendritic cells (DCs) [9–11]. Studies have additionally shown that co-formulation of antigen and adjuvant into a nanoparticle might be crucial to improve immune responses against subunit vaccines [12–15]. However, it is not well understood how the physicochemical properties such as size, material, surface charge or release behavior of antigen/adjuvant influence the immune response. Previously, it has been proposed that positively charged nanoparticles with a size smaller than about 200 nm might be optimal for the interaction with antigen-presenting cells [9,16–18]. Moreover, sustained release of antigen and adjuvant from nanoparticles and a depot effect of nanoparticles on the cell surface could allow the co-delivery of antigen and adjuvant to antigen-presenting cells [17,19]. However, most vaccination studies have been performed by intramuscular or subcutaneous injection and no studies have directly compared different nanoparticles for intradermal vaccine delivery.

The aim of this study was to assess the potential of antigen loaded nanoparticles, with or without co-encapsulated adjuvant, to induce humoral and cellular immune responses after hollow microneedle-mediated intradermal immunization. To this end, we prepared four different nanoparticulate delivery systems with varying physicochemical properties, namely poly (lactic-*co*-glycolic) acid (PLGA) nanoparticles, liposomes, mesoporous silica nanoparticles (MSNs) and gelatin nanoparticles (GNPs). PLGA nanoparticles [10,20–24] and liposomes [12,18,22,25] have been extensively investigated as biocompatible and biodegradable nanoparticle vaccine delivery systems. MSNs gain increasing attention for vaccine delivery because of their controlled size and mesostructure, excellent *in vivo* biocompatibility and high loading capacity [26,27]. Gelatin based nanoparticles have been studied as promising vaccine carriers because of their excellent biocompatibility, stability and aptness for surface modification [28–30].

A model antigen, ovalbumin (OVA), with and without a TLR3 agonist, poly(I:C), was encapsulated into the nanoparticles. First, the physicochemical properties and the *in vitro* release of antigen and adjuvant of the different nanoparticulate formulations were characterized. Next, mice were immunized with the formulations by using a hollow microneedle device followed by the analysis of humoral and cellular immune responses. The results reveal that the immune response depends on encapsulation of antigen/adjuvant and the characteristics of nanoparticles. Furthermore, we demonstrate that the hollow microneedles together with the applicator are excellent tools for intradermal vaccination and screening of nanoparticulate formulations.

2. Materials and methods

2.1. Materials

PLGA (acid terminated, lactide glycolide 50:50, Mw 24–38 kDa), gelatin from porcine skin (bloom 300), OVA for *in vitro* studies (albumin from chicken egg white, lyophilized), bovine serum albumin (BSA) ≥ 96%, gluteraldehyde, glycine, cholamine chloride hydrochloride (2-aminoethyl)-trimethylammoniumchloride hydrochloride, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC), cholesterol (≥ 99%) and hydrofluoric acid ≥ 48% were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Polyvinyl alcohol (PVA) 4–88 (31 kDa) and ethylenediaminetetraacetic acid (EDTA) were purchased from Fluka (Steinheim, Germany). 1-step™ ultra 3,3′,5,5′-tetramethylbenzidine (TMB) was obtained from Thermo-Fisher Scientific (Waltham, MA). Endotoxin-free OVA, polyinosinic-polycytidylic acid (poly(I:C)) (low molecular weight) and its rhodamine-labeled version were purchased from Invivogen (Toulouse, France). Egg phosphatidylcholine (EggPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-[phosphor-L-serine](sodium salt) (DOPS), 1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were ordered from Avanti Polar Lipids (Alabaster, AL). HRP-conjugated goat anti-mouse total IgG, IgG1 and IgG2a were purchased from Southern Biotech (Birmingham, AL). Fluorescently labeled antibodies specific for CD4, CD8 and CD45.1 were ordered from eBioscience (San Diego, The Netherlands). Sulfuric acid (95–98%) was obtained from JT Baker (Deventer, The Netherlands). Ethyl acetate and silicone oil (AK350) were ordered from Boom Chemicals (Meppel, The Netherlands). Dimethylsulfoxide (DMSO) was ordered from Biosolve BV (Valkenswaard, The Netherlands). Sodium dodecyl sulfate (SDS) was purchased from Merck Millipore (Hohenbrunn, Germany). Vivaspin 2 centrifugal concentrators (PES membrane, MWCO 1000 kDa) were obtained from Sartorius Stedim (Nieuwegein, The Netherlands). Sterile phosphate buffered saline (PBS, 163.9 mM Na^+, 140.3 mM Cl^-, 8.7 mM HPO_4^{2-}, 1.8 mM H_2PO^{4-}, pH 7.4) was obtained from Braun (Oss, The Netherlands). Cell culture medium was prepared by mixing Roswell Park Memorial Institute medium (RPMI) with 10% Fetal bovine serum (FBS), 1% L-glutamine and 1% Penicillin-streptamycin. 1 mM phosphate buffer (PB, 0.77 mM Na_2HPO_4, 0.23 mM NaH_2PO_4, pH 7.4), 10 mM PB (7.7 mM Na_2HPO_4, 2.3 mM NaH_2PO_4, pH 7.4), 5 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, pH 7.4) buffer, lysis buffer (150 mM ammonium chloride, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2), and FACS buffer (2% FBS in PBS, pH 7.4) were prepared in the lab. All the other chemicals used are of analytical grade and Milli-Q water (18 MΩ/cm, Millipore Co.) was used for the preparation of all solutions.

2.2. Preparation of nanoparticles

2.2.1. Preparation of PLGA nanoparticles

OVA loaded PLGA nanoparticles (PLGA-OVA) were prepared by double emulsion with solvent evaporation method as previously reported with modifications [31]. Briefly, 75 μl OVA (20 mg/ml) in PBS was dispersed in 1 ml ethyl acetate containing 25 mg/ml PLGA by using a Branson sonifier 250 (Danbury, CT) for 15 s with a power of 20 W. The obtained water-in-oil emulsion was emulsified with 2 ml aqueous solution containing 2% (w/v) PVA with the sonifier (15 s, 20 W). The water-in-oil-in-water double emulsion was added dropwisely into 25 ml 0.3% (w/v) PVA (40 °C) under stirring. The ethyl acetate was evaporated by a rotary evaporator (Buchi rotavapor R210, Flawil, Switzerland) for 3 h (150 mbar, 40 °C). The nanoparticles were collected by centrifugation (Avanti™ J-20XP centrifuge, Beckman Coulter, Brea, CA) at 35000 *g* for 10 min. Finally, they were washed twice with 1 mM PB to remove the excess OVA and PVA and dried in an ice condenser (Alpha 1–2, Osterode, Germany) in freeze vacuum (− 49 °C, 90 mbar) overnight for further use and storage.

To prepare OVA and poly(I:C) co-encapsulated PLGA nanoparticles (PLGA-OVA-PIC), 18.75 μl OVA (40 mg/ml) and 75 μl poly(I:C) (46.7 mg/ml, including 0.03% fluorescently labeled equivalent) were emulsified with 1 ml PLGA (25 mg/ml) in ethyl acetate to obtain the water-in-oil emulsion. The rest of the procedure was identical to that of PLGA-OVA.

2.2.2. Preparation of liposomes

Liposomes were prepared by a film hydration method [32]. A thin lipid film of EggPC: DOPE: DOTAP in a molar ratio of 9:1:2.5 was