Published in final edited form as:

Biomaterials. 2016 November ; 106: 228–239. doi:10.1016/j.biomaterials.2016.08.028.

The next-generation nicotine vaccine: a novel and potent hybrid nanoparticle based nicotine vaccine

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Abstract

Owing to the urgent need for more effective treatment against nicotine addiction, a hybrid nanoparticle-based nicotine vaccine (NanoNiccine) was developed in this study. NanoNiccine was composed of a poly(lactide-co-glycolide) acid (PLGA) core, keyhole limpet hemocyanin (KLH) as an adjuvant protein enclosed within the PLGA core, a lipid layer, and nicotine haptens conjugated to the outer surface of the lipid layer. In contrast to the traditional nicotine vaccine, NanoNiccine is not a nicotine-protein conjugate vaccine. Instead, the nicotine hapten and protein are separately located in the nanostructure to minimize antibody production towards KLH. The cellular uptake study demonstrated that NanoNiccine was ideal for internalization and processing by dendritic cells (DCs). Mice immunized with NanoNiccine produced much lower IgG level against KLH as compared to that immunized with the traditional nicotine-KLH (Nic-KLH) vaccine. In addition, NanoNiccine achieved up to a 400% higher titer of anti-nicotine IgG than the positive control, Nic-KLH. Additionally, the Th1/Th2 index of NanoNiccine suggested that the immune response induced by NanoNiccine was antibody response dominant. Furthermore, NanoNiccine was found to be safe in mice.

Keywords

Nicotine vaccine; hybrid nanoparticle; antibody; PLGA; KLH; dendritic cell

1. Introduction

Tobacco use continues to be the leading cause of preventable death worldwide, resulting in more than 6 million deaths and immeasurable economic loss each year [1]. It has been widely recognized that nicotine is the major component that is responsible for tobacco addiction [2]. Although, conventional pharmacotherapies [3] including nicotine replacement

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Conflict of interest

The authors declare no competing financial interests.

therapy, varenicline, and bupropion prove to be effective in treating nicotine addiction, the overall abstinence rate is highly limited and these therapies are more or less accompanied with adverse effects [4–6]. Therefore, there is an urgent need for a more effective and safer treatment method for nicotine addiction. In recent years, nicotine vaccines, which can induce the production of nicotine-specific antibodies and prevent nicotine entry into the brain, have exhibited great potential as a new-generation therapy to help people quit smoking [7]. Nicotine is a small compound and cannot induce immune response on its own; and thus it has to be associated with bigger molecules, such as proteins, for it to be immunogenic [8]. Following the above rationale, traditional nicotine vaccines share a common trait, in that nicotine haptens are covalently conjugated to proteins [9]. These vaccines prove to be effective in producing nicotine specific antibodies, and some of them have even advanced into clinical trials [10, 11]. However, such a nicotine-protein conjugate design has some drawbacks, which may limit the treatment efficacy of the resulting vaccines. Firstly, antigen presenting cells (APCs), such as dendritic cell (DC), macrophage, and B cell, prefer to capture and internalize particulate antigens [12], including virus, bacteria, and nanoparticles, instead of soluble protein antigens; secondly, if not impossible, nicotine-protein conjugate vaccines can hardly co-deliver antigens and adjuvant molecules to target immune cells, in contrast, nanoparticles-based vaccine can easily achieve such a task [13]; and lastly, carrier proteins themselves are immunogenic, which may result in wastage of the nicotine-protein conjugate vaccine for eliciting antibodies against the protein rather than nicotine.

In order to overcome the above shortcomings of the traditional nicotine-protein conjugate vaccines, in this study, we designed a novel lipid-PLGA hybrid nanoparticle-based nicotine vaccine (NanoNiccine). The major components of this vaccine are a PLGA core, a lipid surface layer, keyhole limpet hemocyanin (KLH) in the core, monophosphoryl lipid A (MPLA) as a molecular adjuvant in the lipid layer, and nicotine haptens covalently linked to the outer surface of the lipid layer. Different from the traditional nicotine-protein conjugate vaccine [14–16], KLH in the PLGA core of NanoNiccine solely served as a supplier of T cell antigens, instead of a carrier protein. This may reduce the possibility of generating antibodies against KLH. Another advantage of this design is that molecular adjuvants, such as MPLA [17], and CpG oligodeoxynucleotides (CpG ODNs) [18] can be co-delivered with antigens to immune cells, which may increase the magnitude of immune response. The immunogenicity of NanoNiccine and the traditional nicotine vaccine using KLH as a carrier protein (i.e. positive control) was studied in mice. The results showed that NanoNiccine generated a much higher titer of antibodies against nicotine than the traditional Nic-KLH conjugate vaccine.

2. Experimental section

2.1 Materials

Lactel® 50:50 PLGA was purchased from Durect Corporation (Cupertino, CA). Fetal bovine serum (FBS), granulocyte macrophage-colony stimulating factor (GM-CSF) recombinant mouse protein, alpha minimum essential medium, trypsin/EDTA, and Alexa Fluor® 647 hydrazide were purchased from Life Technologies Corporation (Grand Island, NY). The anti-mouse IgG from goat, anti-mouse IgG₁, IgG_{2a}, IgG_{2b}, IgG₃ HRP, and anti-goat IgG-

HRP were procured from Alpha Diagnostic Intl (San Antonio, TX). TMB one component microwell substrate was procured from SouthernBiotech (Birmingham, AL). Lipids, including 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000] (ammonium salt) ((DSPE-PEG2000) carboxylic acid), cholesterol, MPLA and 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt) (NBD PE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Poly(vinyl alcohol) (PVA, MW 89,000–98,000), dichloromethane (DCM), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Inc. (Saint Louis, MO). Alexa Fluor® 647 Hydrazide, KLH, Imject™ Alum Adjuvant (Alum), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), and sulfo-NHS were purchased from Thermo Fisher Scientific Inc. (Rockford, IL). JAWSII (ATCC® CRL-11904™) immature dendritic cells were purchased from ATCC (Manassas, VA). (San Diego, CA). Rac-trans 3'-aminomethyl nicotine was purchased from Toronto Research Chemicals Inc. (Toronto, Canada). All other chemicals were of analytical grade.

2.2. Synthesis of KLH-containing PLGA nanoparticles

PLGA nanoparticles were prepared using a reported double emulsion solvent evaporation method with modifications [19–21]. Briefly, PLGA (30 mg) was dissolved in DCM (1 mL), followed by mixing with 100 µL of KLH (20 mg/mL) for 2 min using a vortex mixer. The resultant mixture was emulsified in Branson B1510DTH Ultrasonic Cleaner (Branson, Danbury, CT) for 10 min. The primary emulsion was added drop-wise into 100 mL PVA (0.5% (w/v)), and continuously stirred for 10 min at 500 rpm. The above suspension was emulsified by sonication using a sonic dismembrator (Model 500; Fisher Scientific, Pittsburg, PA) at 50% amplitude for 120 s. The secondary emulsion was stirred overnight to allow DCM to evaporate. Large particles were removed after the mixture sat undisturbed at room temperature for 30 min. Nanoparticles in suspension were collected by centrifugation at 10,000 *g*, 4 °C for 60 min using an Eppendorf centrifuge (Eppendorf, Hauppauge, NY). The pellet was suspended in 10 mL phosphate buffered saline (PBS) buffer (pH 7.4) and stored at 2 °C until future use.

2.3. Assembly of NanoNiccine

Lipid-PLGA nanoparticles were assembled using a method as described in previous reports. [20, 22] The lipid film containing 0.25 mg MPLA, 2.83 mg DOTAP, 3.08 mg (DSPE-PEG2000) carboxylic acid, and 0.1 mg cholesterol was hydrated with 1 mL of 55 °C pre-warmed PBS buffer. The resulting liposome suspension was vigorously mixed using a vortex mixer for 2 min, followed by sonication for 5 min, using a Branson B1510DTH Ultrasonic Cleaner (Branson, Danbury, CT) and then cooled to room temperature. The prepared liposome was added into the above prepared KLH-containing PLGA nanoparticles and pre-homogenized for 15 min using a Branson B1510DTH Ultrasonic Cleaner, followed by sonication for 5 min in an ice bath using a sonic dismembrator at 15% amplitude (pulse on 20 s, pulse off 50 s). The acquired lipid-PLGA nanoparticles were dialyzed against 500 mL activation buffer (0.1 M MES, 0.5 M NaCl, pH 6.0) for 2 h. EDC (4.1 mg) and sulfo-NHS (11.3 mg) were added into the hybrid nanoparticle suspension and allowed to react for 20 min at room temperature. The activated nanoparticles were dialyzed against 1000 mL PBS

buffer (100 mM sodium phosphate, 150 mM NaCl; pH 7.2) for 30 min. After dialysis, 4.1 mg rac-trans 3'-aminomethyl nicotine was incubated with the above nanoparticle suspension at room temperature for 4 h. The remaining impurities were removed by dialysis against PBS buffer (pH 7.4) for 12 h. The assembled NanoNiccine was stored at 4 °C until future use.

2.4. Synthesis of nicotine-KLH conjugate vaccine

KLH (4 mg) dissolved in 2 mL activation buffer (0.1 M MES, 0.5 M NaCl, pH 6.0) was incubated with 1 mg EDC and 2.8 mg sulfo-NHS for 20 min. The activated KLH was transferred to an Amicon Ultra 15 mL centrifugal filter unit (NMWL, 50 KDa), and purified by centrifugation at 5000 *g* for 20 min. The purified KLH was suspended in 2 mL PBS buffer (100 mM sodium phosphate, 150 mM NaCl; pH 7.2) and reacted with 1 mg rac-trans 3'-aminomethyl nicotine at room temperature for 4 h. The resultant mixture was then transferred to the centrifugal filter unit mentioned above and centrifuged at 5000 *g* for 20 min to remove free nicotine. The purified nicotine-KLH conjugate was suspended in 2 mL PBS buffer (pH 7.4) and stored at 4 °C until future use.

2.5. Synthesis of nicotine-bovine serum albumin (Nic-BSA) conjugate

Bovine serum albumin (BSA) (10 mg) dissolved in 5 mL activation buffer (0.1 M MES, 0.5 M NaCl, pH 6.0) was incubated with 2 mg EDC and 5.6 mg sulfo-NHS for 20 min. The activated BSA was transferred to an Amicon Ultra-15 Centrifugal Filter Unit (NMWL, 30 KDa), and purified by centrifugation at 5000 *g* for 20 min. The purified BSA was suspended in 5 mL PBS buffer (100 mM sodium phosphate, 150 mM NaCl; pH 7.2) and reacted with 2 mg rac-trans 3'-aminomethyl nicotine at room temperature for 4 h. The resultant mixture was then transferred to the centrifugal filter unit mentioned above and centrifuged at 5000 *g* for 20 min to remove free nicotine. The purified nicotine-KLH conjugate was suspended in 5 mL PBS buffer (pH 7.4) and stored at 4 °C until future use.

2.6. Synthesis of Alexa 647 labeled KLH

KLH (4 mg) dissolved in 2 mL activation buffer (0.1 M MES, 0.5 M NaCl, pH 6.0) was incubated with 1 mg EDC and 2.8 mg sulfo-NHS for 20 min. The activated KLH was transferred to an Amicon Ultra 15 mL centrifugal filter unit (NMWL, 50 KDa), and purified by centrifugation at 5000 *g* for 20 min. The purified KLH was suspended in 2 mL PBS buffer (100 mM sodium phosphate, 150 mM NaCl; pH 7.2) and reacted with 0.1 mg Alexa Fluor® 647 Hydrazide at room temperature for 4 h. The resultant mixture was then transferred to the centrifugal filter unit mentioned above and centrifuged similarly in order to remove the excess Alexa Fluor® 647 hydrazide. The purified Alexa 647-KLH conjugate was suspended in 2 mL PBS buffer (pH 7.4), lyophilized, and stored at 4 °C until future use.

2.7. Characterization of physicochemical properties of nanoparticles

The nanoparticles assembled above were diluted ten times in PBS buffer (pH 7.0). The physicochemical properties including particle size (diameter, nm) and surface charge (zeta potential, mV) were measured at room temperature using a Malvern Nano-ZS zetasizer (Malvern Instruments Ltd, Worcestershire, United Kingdom).

2.8. Imaging hybrid nanoparticles using confocal laser scanning microscopy (CLSM)

A Zeiss LSM 510 Laser Scanning Microscope (Carl Zeiss, German) was used to image NanoNiccine containing Alexa Fluor® 647 hydrazide-labeled KLH and NBD PE-labeled lipid shells. Fluorescently labeled NanoNiccine was formed using the same method for regular NanoNiccine, except that KLH was replaced with Alexa 647-KLH, and 0.1 mg NBD PE was added to the existing lipids.

2.9. Imaging nanoparticles using transmission electrical microscopy (TEM)

Nanoparticle suspensions (0.5 mg/mL), including KLH-containing PLGA nanoparticles, MPLA-containing liposomes, and NanoNiccine nanoparticles, were dropped onto a 300-mesh Formvar-coated copper grid. After standing for 10 min, the remaining suspension was carefully removed with wipes, and the samples were negatively stained using fresh 1% phosphotungstic acid for 20 s, and washed with ultrapure water twice. The dried samples were imaged on a JEOL JEM 1400 transmission electron microscope (JEOL Ltd., Tokyo, Japan).

2.10. Flow cytometry (FACS) measurement of the uptake of lipid-PLGA hybrid NPs by DCs

JAWSII (ATCC® CRL-11904™) immature DCs from ATCC were cultured with alpha minimum essential medium (80% v/v) including ribonucleosides, deoxyribonucleosides, 4 mM L-glutamine, 1 mM sodium pyruvate and 5 ng/mL murine GM-CSF, along with fetal bovine serum (20% v/v) at 37 °C, 5% CO₂ in CytoOne(R) 35 × 10 mm TC dish (USA Scientific Inc., Ocala, FL). Alexa 647 and NBD PE labeled NanoNiccine (100 µg) was added into each dish containing 2×10⁶ cells, and incubated for 5, 30, 60, and 120 min, respectively. After incubation, the medium was immediately removed and cells were washed five times with PBS buffer (pH 7.4). Cells were detached from the culture plate using trypsin/EDTA solution and centrifuged at 200 g for 10 min, and cell pellets were suspended in 2 mL PBS buffer (pH 7.4). Cell samples were immediately analyzed by flow cytometer (BD FACSAria I, BD, Franklin Lakes, NJ).

2.11. Imaging uptake of lipid-PLGA hybrid NPs by DCs using CLSM

Cells were cultured in a 2 well chamber slide (Thermo Fisher Scientific Inc., Rd, Rockford, IL) using the same method described above. To investigate the uptake of hybrid NPs by DCs, 100 µg of freshly prepared NanoNiccine (labeled with Alexa Fluor® 647 Hydrazide and NBD PE) was incubated with 4×10⁵ cells for 5, 30, 60, and 120 min, respectively. After incubation, the medium was immediately removed and cells were washed five times with PBS buffer (pH 7.4). Freshly prepared 4% (w/v) paraformaldehyde (2 mL) was added into each well, and cells were fixed for 15 min. This was followed by washing three times with PBS buffer (pH 7.4). Fixed cells were labeled with DAPI Fluoromount-G® (SouthernBiotech, Birmingham, AL). Cell samples were covered with a glass cover. Images were acquired using a Zeiss LSM 880 Laser Scanning Microscope (Carl Zeiss, Germany).

2.12. Active immunization of mice with nicotine vaccines

All animal studies were carried out following the National Institutes of Health (NIH) guidelines for animal care and use. Animal protocols were approved by the Institutional

Animal Care and Use Committee (IACUC) at Virginia Polytechnic Institute and State University (VT). Groups of $n = 8$ female BALB/c mice (6–7 weeks, 16–20 g) were immunized by subcutaneous (s.c.) injections on days 0 (primary injection), 14 (1st booster), and 28 (2nd booster) with PBS (pH 7.4), Nic-KLH conjugate vaccine (with 4 mg Alum), NanoNiccine without nicotine hapten (with 4 mg Alum), NanoNiccine with MPLA (without Alum), NanoNiccine containing no MPLA but adjuvanted with 4 mg Alum, and NanoNiccine containing MPLA and adjuvanted with 4 mg Alum (all the vaccine constructs contained a total amount of 40 μ g KLH). Following vaccine administration, blood samples (~ 200 μ L) were collected on days -2, 13, 27, 35, and 55 via retro orbital puncture from each mouse. Sera were collected from blood by centrifugation and stored at -80 $^{\circ}$ C.

2.13. Measurement of specific anti-Nicotine IgG and anti-KLH IgG antibodies using enzyme-linked immunosorbent assay (ELISA)

Mice sera were analyzed according to the ELISA procedure described in previous publications with proper modifications [23]. Briefly, Nic-BSA was used as the coating material for anti-Nic IgG measurement, and KLH was used as the coating material for anti-KLH measurement. MICROLON[®] 96 well plates (Greiner BioOne, Longwood, FL) were coated with Nic-BSA conjugate or KLH (10 μ g/mL in carbonate buffer, 0.05 M, pH 9.6, 100 μ L/well) and incubated at 25 $^{\circ}$ C for 5 h. The plates were washed with PBS-Tween (0.1%) and distilled water for three times, followed by blocking with 300 μ L Pierce[®] protein-free T20 blocking buffer for 12 h. After washing, 100 μ L of each dilution (1:25, 1:125, 1:625, 1:3125, 1:15625, 1:78125, and 1:390625) of serum from each mouse was incubated in plates at 25 $^{\circ}$ C for 2 h. The plates were washed again, and incubated for 1 h with 100 μ L anti-mouse IgG. The plates were washed as before, and incubated with 100 μ L Anti-Goat IgG-HRP (1:5000) (Alpha Diagnostic Intl, San Antonio, TX) for 1 h. After washing as before, 100 μ L of TMB one component microwell substrate was added into each well and incubated for 10 min, and the reaction was stopped by adding 100 μ L of 0.5% (v/v) H₂SO₄. The absorbance for each well was recorded at 450 nm. Titer was defined as the dilution factor at which OD₄₅₀ fell to half of the maximal.

2.14. Measurement of specific anti-nicotine IgG subtype antibodies using ELISA

Anti-Nic IgG antibodies, of different subtypes, including IgG₁, IgG_{2a}, IgG_{2b}, and IgG₃ from the 55th day sera were measured using ELISA. The ELISA protocol for anti-nicotine IgG subtypes measurement was the same as above, except that 100 μ L (1:10000 diluted) anti-Mouse IgG₁ HRP, Anti-Mouse IgG_{2a} HRP, Anti-Mouse IgG_{2b} HRP, and Anti-Mouse IgG₃ HRP were directly applied after coating with Nic-BSA for 2h. After reacting with 100 μ L TMB one component microwell substrate for 10 min, the reaction was stopped by the addition of 100 μ L of 0.5% (v/v) H₂SO₄. The absorbance for each well was recorded at 450 nm. Titer was defined as the dilution factor at which OD₄₅₀ fell to half of the maximal.

2.15. Th1/Th2 index calculation

As described in a previous work [8], Th1:Th2 index was calculated as $([IgG_{2a} + IgG_3]/2)/(IgG_1)$ for each immunization groups. According to such calculations, an index value less than one represents a Th2 polarization; and a value greater than one represents a Th1 polarization.

2.16. Histopathological examination

Mice immunized with PBS, Nic-KLH, NanoNiccine with MPLA, with Alum, and with both MPLA and Alum were scarified, and their tissues, including heart, lung, kidney, spleen, liver, and stomach were harvested and fixed in 10% buffered formalin. Haemotoxylin and eosin (H&E) staining was carried out within two weeks after organ harvest according to the method described before [8]. Sections were examined by light microscopy on an Olympus CKX41 inverted microscope and images were captured using an INFINITY 1 camera.

2.17. Data analysis

Antibody titers were compared among groups using one way ANOVA and comparisons among paired groups were analyzed with Tukey's honest significant difference (HSD). The difference is considered as significant when a P-value is less than 0.05. Each measurement was carried out at least thrice, and the results were expressed as mean $\pm$ standard deviation.

3. Results

3.1. Morphological and structural study of NanoNiccine by CLSM and TEM

As illustrated in Scheme 1, NanoNiccine was assembled by conjugating nicotine haptens to the surface of previously well characterized lipid-PLGA hybrid nanoparticles [20]. The morphology and structure of NanoNiccine were investigated by CLSM and TEM. For the CLSM study, structural components of NanoNiccine, including KLH in the PLGA core and the lipid layer, were labeled with Alexa 647 (red color) and NBD PE (green color), respectively. As shown in Fig. 1, both Alexa 647 and NBD were visible on almost all NanoNiccine particles, indicating that a hybrid core-shell structure was formed for the majority of NanoNiccine particles. In addition, the size of most NanoNiccine particles was in nano-range, reflecting the structural uniformity of the vaccine produced by the protocol described in this study. To study the structural details of NanoNiccine, nanoparticles, including PLGA nanoparticles, liposomes, and NanoNiccine were negatively stained and examined by TEM. PLGA nanoparticles displayed a spherical structure with a mean size of around 250 nm in diameter (Fig. 2A). Similar to PLGA nanoparticles, liposomes were also spherically shaped with a diameter of around 300 nm (Fig. 2B). As shown in Fig. 2C, NanoNiccine particles also displayed a spherical morphology and their sizes were close to that of both the PLGA nanoparticles and the liposomes. However, the difference between the NanoNiccine particles and the PLGA nanoparticles or liposomes, was that the NanoNiccine particles clearly exhibited a hybrid structure, in which a white solid core was surrounded by a thin layer of gray membrane. This suggested that the PLGA nanoparticles and liposomes were successfully hybridized via sonication to form the NanoNiccine particles.

3.2. Characterization of physicochemical properties of NanoNiccine particles

Physicochemical properties, such as mean particle size, size distribution, and surface charge (represented by zeta potential) were characterized for NanoNiccine particles without nicotine hapten (i.e. blank NanoNiccine), with MPLA, without MPLA. As shown in Fig. 3, blank NanoNiccine, NanoNiccine without MPLA, and NanoNiccine with MPLA have average sizes of 260.4 ± 4.9 nm, 232.3 ± 6.9 nm, and 238.1 ± 11.5 nm, respectively.

Consistent with the results acquired by CLSM and TEM, the size distributions of all the three particles were in a narrow range with a center at around 150 nm, demonstrating that the majority of the NanoNiccine particles were of a uniform size. Zeta potentials of blank NanoNiccine, NanoNiccine without MPLA, and NanoNiccine with MPLA were -4.14 ± 0.25 mV, -10.80 ± 0.57 mV, and -11.30 ± 0.59 mV, respectively, indicating that all the three particles carried a net negative charge on their surface. The difference in surface charges between blank NanoNiccine and the other two particles might be due to the presence of nicotine haptens on the other two.

3.3. Uptake of NanoNiccine particles by DCs

To elicit an immune response, antigens have to be internalized and processed by APCs [24]. Therefore, the uptake of NanoNiccine by APCs is of great importance to its immunological outcome. In this study, the uptake of NanoNiccine by DCs was investigated by flow cytometry (FACS). Mouse DCs (2×10^6) in a culture dish were treated with 100 μ g NanoNiccine (particles were fluorescently labeled with both Alexa 647 and NBD). The percentage of cells that internalized NanoNiccine as well as the relative amount of NanoNiccine taken up by the DCs were then monitored. As shown in the top panel of Fig. 4, the uptake of NanoNiccine by the DCs was time-dependent; the percentages of cells that internalized NanoNiccine particles were 1.83%, 57.3%, 93.9%, and 96.3% at 5, 30, 60, and 120 min, respectively. Both Alexa 647 and NBD were detected in NanoNiccine treated DCs, indicating that NanoNiccine hybrid particles as a whole were internalized by the DCs. The relative amount of NanoNiccine internalized by DCs was also recorded by measuring the fluorescence intensity of both Alexa 647 and NBD in DCs. As shown in the bottom panel of Fig. 4, the fluorescence intensity of both Alexa 647 and NBD increased with time, in which the NBD median intensity increased from 108 at 5 min to 3236 at 120 min, and that of Alexa 647 increased from 35 at 5 min to 1140 at 120 min. Within 115 min, median intensities of both Alexa 647 and NBD increased by about 30 times. The percentages of DCs that emitted NBD and Alexa 647 after 120 min treatment were as high as 96.2% and 98.8%, respectively.

The *in vitro* cellular uptake of NanoNiccine was also studied using confocal microscopy. DCs (4×10^5) placed in a cell chamber were incubated with 100 μ g fluorescently marked NanoNiccine particles (KLH was labeled with Alexa 647 and the lipid layer was labeled with NBD) for 5, 30, 60, and 120 min, respectively. As shown in Fig. 5, in concordance with the results from the FACS study, the number of cells that internalized NanoNiccine and the amount internalized were both found to increase with time. In addition, NanoNiccine particles with a hybrid structure were internalized as a whole entity. After a treatment period of 5 min, NanoNiccine was detected in few cells, and its amount in each cell was quite limited, which was reflected by the dim fluorescence in both NBD and Alexa647 channels. In contrast, after 60 min treatment, NanoNiccine was observed in most of the DCs, and the quantity was found to increase considerably. In addition, we found that the degradation of NanoNiccine in the DCs might occur in a step-wise and time-dependent manner. At 30 min, the lipid layer was removed from NanoNiccine, which was reflected by the wide dispersion of NBD. For KLH in the PLGA core, in the first 60 min, the red fluorescence was confined within the vesicles, indicating that the major portion of PLGA core stayed intact. However,

by 120 min, large portion of the PLGA particles was degraded and Alexa 647-labeled KLH was released, leading to a wide distribution of red fluorescence in the DCs.

3.4. Nicotine-specific IgG antibody titer induced by nicotine vaccines

On days 0, 14, and 28, each group of eight mice was immunized with PBS (negative control), Nic-KLH (positive control), NanoNiccines without hapten, with MPLA, with Alum, and with MPLA and Alum, respectively. Anti-Nic IgG from sera on days 13, 27, 35, and 55 were measured. No anti-Nic antibody was detected in mice immunized with PBS at any of the time points. As shown in Fig. 6, 13 days after the primary injection, NanoNiccine with MPLA elicited antibody titer as high as $10.2 \pm 1.8 \times 10^3$, which was significantly higher than those in the other four groups. No anti-Nic antibody was detected in the mice injected with NanoNiccine without nicotine hapten.

The first booster injection enormously promoted nicotine antibody production among all nicotine vaccines, except NanoNiccine without hapten. Thirteen days after the first booster injection, the antibody titer reached $14.0 \pm 1.3 \times 10^3$, $65.0 \pm 11.8 \times 10^3$, $54.9 \pm 14.9 \times 10^3$, and $70.5 \pm 4.7 \times 10^3$ for Nic-KLH, NanoNiccines with MPLA, Alum, and with MPLA and Alum groups, respectively. No anti-Nic antibody was detected in the group without nicotine hapten. The fold increase in the antibody titer after the first booster was found to be 4, 5.3, 21.4, and 22.3, in the Nic-KLH group, the NanoNiccines with MPLA, with Alum, and with MPLA and Alum group, respectively. The immunogenicity of NanoNiccine with all the formulations, except in that without hapten, was stronger than the Nic-KLH conjugate vaccine. As compared to Nic-KLH, NanoNiccine with MPLA, with Alum, and with MPLA and Alum generated an anti-Nic antibody titer 4.6, 3.9, and 5 times higher, respectively.

Seven days after the second booster injection, the antibody titers of Nic-KH, NanoNiccines with MPLA, with Alum, and with MPLA and Alum, dropped to $6.4 \pm 1.4 \times 10^3$, $30.3 \pm 1.6 \times 10^3$, $41.7 \pm 8.1 \times 10^3$, and $32.3 \pm 3.1 \times 10^3$, respectively. However, anti-Nic antibody titers of NanoNiccine groups were still significantly higher than that of Nic-KLH.

On day 55, no significant changes in anti-Nic antibody titer were detected from those on day 35 among all vaccine groups. Titers in mice treated with Nic-KH, NanoNiccines with MPLA, with Alum, and with MPLA and Alum, were $6.7 \pm 0.7 \times 10^3$, $38.4 \pm 5.5 \times 10^3$, $43.5 \pm 8.5 \times 10^3$, and $37.8 \pm 7.4 \times 10^3$, respectively. NanoNiccine groups maintained a superiorly higher antibody titer than that of Nic-KLH. Within the NanoNiccine groups, the anti-Nic antibody titers did not significantly differ from one another.

3.5. KLH specific IgG antibody titer induced by nicotine vaccines

Anti-KLH antibody titers were measured using the same sera as for the anti-Nic antibody assay. No anti-KLH antibody was detected in the mice immunized with PBS at any time points. As shown in Fig. 7, on day 13, anti-KLH antibody titers of $10.1 \pm 0.8 \times 10^3$, $3.9 \pm 1.0 \times 10^3$, 462 ± 51 , 596 ± 111 , and $2.2 \pm 0.3 \times 10^3$ were found for Nic-KLH, NanoNiccines without hapten, with MPLA, with Alum, and with MPLA and Alum, respectively. Nic-KLH generated a significantly higher anti-KLH antibody titer as compared to all the NanoNiccine groups. On day 27, anti-KLH antibody titers increased to $151.3 \pm 41.5 \times 10^3$, $127.6 \pm 26.2 \times 10^3$, $16.0 \pm 6.9 \times 10^3$, $66.2 \pm 12.9 \times 10^3$, and $104.9 \pm 30.9 \times 10^3$ for Nic-KLH, NanoNiccines without

haptens, with MPLA, with Alum, and with MPLA and Alum, respectively. Despite the tremendous increase, anti-KLH antibody titer of NanoNiccine with MPLA was still significantly lower than all other vaccine formulations. On day 35, anti-KLH antibody titers of Nic-KLH, NanoNiccines without haptens, with Alum, and with MPLA and Alum group considerably decreased to $87.0 \pm 13.5 \times 10^3$, $64.4 \pm 9.7 \times 10^3$, $54.7 \pm 9.0 \times 10^3$, and $55.1 \pm 7.6 \times 10^3$, respectively. In contrast, the anti-KLH antibody titer of NanoNiccine with MPLA increased significantly to $44.0 \pm 4.3 \times 10^3$. The anti-KLH antibody titer of Nic-KLH was significantly higher than those in all the NanoNiccine groups. On day 55, similar to the anti-Nic antibody titer, anti-KLH titers stayed close to those on day 35, which were $73.9 \pm 8.9 \times 10^3$, $64.4 \pm 9.7 \times 10^3$, $44.0 \pm 4.3 \times 10^3$, $48.5 \pm 10.1 \times 10^3$, and $48.7 \pm 6.3 \times 10^3$, respectively. Nic-KLH maintained a significantly higher anti-KLH titer than the NanoNiccine groups. In addition, no significant difference in the anti-KLH antibody titer was detected among the NanoNiccine groups.

3.6. Titers of anti-nicotine antibody of different subtypes induced by nicotine vaccines

For all the nicotine vaccine groups, titers of anti-nicotine subtype antibodies from sera on day 55 were assayed. As shown in Fig. 8, no antibody titer of any subtype was detected in the NanoNiccine without haptens group, and all the other vaccines generated antibody subtypes at various levels. IgG₁ and IgG_{2b} were the most dominant and the least dominant antibody subtype, respectively, among all the vaccine groups. In agreement with the total IgG titer results, Nic-KLH generated significantly lower titers of all subtypes compared to those in most of the NanoNiccine groups. For IgG₁, Nic-KLH, NanoNiccines with MPLA, with Alum, and with MPLA and Alum, achieved titers of $8.0 \pm 0.9 \times 10^3$, $14.7 \pm 1.6 \times 10^3$, $12.3 \pm 2.3 \times 10^3$, and $12.4 \pm 2.1 \times 10^3$, respectively. For IgG_{2a}, Nic-KLH, NanoNiccines with MPLA, with Alum, and with MPLA and Alum, achieved anti-KLH antibody titer of $2.0 \pm 0.1 \times 10^3$, $11.5 \pm 1.1 \times 10^3$, $2.2 \pm 0.3 \times 10^3$, and $8.5 \pm 1.1 \times 10^3$, respectively. The titers of IgG_{2b} were $0.5 \pm 0.1 \times 10^3$, $2.0 \pm 0.3 \times 10^3$, $1.4 \pm 0.2 \times 10^3$, and $2.6 \pm 0.4 \times 10^3$ for Nic-KLH, NanoNiccines with MPLA, with Alum, and with MPLA and Alum, respectively. The four vaccines attained IgG₃ titers of $1.7 \pm 1.0 \times 10^3$, $10.0 \pm 1.3 \times 10^3$, $2.4 \pm 0.6 \times 10^3$, and $9.0 \pm 1.1 \times 10^3$, respectively. To evaluate the relative magnitude of antibody response and cell-mediated response, the Th1/Th2 index was also calculated based on the titers of the different subtype antibodies. It was found that the Th1/Th2 indices achieved by all the nicotine vaccines were less than one. Among these vaccines, NanoNiccine with Alum achieved the lowest Th1/Th2 index of 0.112, while that with MPLA achieved the highest index of 0.434.

3.7. *In vivo* toxicity of NanoNiccine

Mice injected with PBS and nicotine vaccines were sacrificed on day 57. Major organs from the mice, including heart, lung, kidney, spleen, stomach, and liver were stored in 10 % formalin. These organs were stained with H&E and examined under microscope within two weeks after harvest. As shown in Fig. 9, no significant difference was detected between the mice treated with PBS and those treated with nicotine vaccines, in all the examined organs, thus indicating the safety of NanoNiccines.

4. Discussion

Nicotine vaccines, exhibiting great potential as a future treatment against tobacco addiction, have been intensively investigated [25]. Previous studies on development of nicotine vaccine mainly focused on improving the nicotine epitope, screening carrier protein, selecting adjuvants, and optimizing injection routes [14, 26, 27]. Despite the differences in nicotine vaccine design among various research groups, they were structurally similar to one another, that is nicotine haptens were covalently conjugated to a carrier protein [28]. To a great extent, such a design was inspired by the idea that small molecules, like nicotine, heroin, and cocaine, are unable to elicit an immune response on their own, and have to be associated with larger and more complex molecules to be immunogenic [29]. In animal trials, some of the traditional nicotine-protein conjugate vaccines were discovered to be highly immunogenic and could effectively block the entry of nicotine into the brain [30, 31]. In addition, some of them achieved encouraging results in early stages of clinical trials [32]. However, these vaccines are associated with some innate shortcomings, which may limit their immunological efficacy and future improvement. The first problem of these vaccines is that there may exist immune response targets not only on the nicotine molecule, but also on amino acid sequences on the carrier protein. Given the much greater variations in structure and composition of a carrier protein as compared to those of the nicotine hapten, large quantities of polyclonal antibodies may be generated against the carrier protein. This may undermine the specificity of the nicotine vaccine, which is supposed to produce only nicotine specific antibodies. Moreover, vaccine conjugate may be drained to produce carrier protein specific antibodies, resulting in its lowered utilization efficiency. Thirdly, co-delivery of increasingly important molecular adjuvants by nicotine-protein conjugate vaccine is difficult [33, 34], thereby limiting the ability for further improving the immunogenicity of the vaccine.

To overcome the shortcomings of the traditional nicotine protein conjugate vaccines, in this study, a lipid-PLGA nanoparticle based nicotine vaccine—NanoNiccine was invented. Core-shell hybrid nanoparticles have been intensively studied as delivery systems for anti-cancer drugs and vaccines [35–38]. These hybrid nanoparticles proved highly biocompatible and biodegradable. As shown in Scheme 1, the nicotine haptens and protein (KLH) are no longer covalently conjugated; instead, nicotine haptens are linked to the outer surface of the hybrid nanoparticle and KLH is enclosed within the PLGA core. As shown in Scheme 2, NanoNiccine may minimize the exposure of the protein to immune cells, and effectively present nicotine haptens to the immune system, thereby improving the specificity of the vaccine. Different from the traditional nicotine vaccines, protein in NanoNiccine does not act as a carrier for nicotine, but serves solely as an antigenic peptide supplier to bridge the interaction between DC, B cell, and T cell [39].

The assembly of the hybrid nanoparticle based nicotine vaccine in this study primarily involved three steps: the first step was PLGA nanoparticle formation, followed by lipid-PLGA assembly, and the last step involved conjugating the nicotine epitope onto the hybrid nanoparticles. The whole process and components appeared to be complex, but each step was easy to perform. Moreover, according to previous studies [20, 40], the physicochemical properties of the vaccine particles were controllable. Lipid-PLGA hybrid nanoparticle has

proven to be an excellent delivery system for vaccines and anti-cancer drugs [41]. In addition, all the components of NanoNiccine in this study exhibited good safety for animals or human use [42–46].

The lipid layer of NanoNiccine was composed of three lipids, including DOTAP, DSPE-PEG(2000)COOH, and cholesterol. Each of the three lipids has its unique function. DOTAP [47], as a cationic lipid, may strengthen the association between the lipid layer and the negatively charged PLGA core via electrostatic attraction. The carboxylic acid groups on DSPE-PEG(2000)COOH serve as the ligand for conjugating nicotine epitope. Cholesterol acts as a stabilizer in the lipid layer to improve the stability of NanoNiccine [48]. As reported in our previous work [40], PEGylated lipid-PLGA hybrid nanoparticles are resistant to the harsh physiological environment. PEGylation may enable prolonged circulation of NanoNiccine and improve the bioavailability of the vaccine to immune cells. Since the adaptive immune system has evolved to recognize highly repetitive structures in antigens [49], the repetitive copies of the nicotine epitopes on the surface of NanoNiccine may allow its fast and effective recognition by immune cells, thereby leading to rapid development of immune response. Another important feature of NanoNiccine is that it can co-deliver molecular adjuvants [50], such as Toll-like receptor 9 (TLR 9) agonists (CpG ODNs), TLR 4 agonist (MPLA) and antigens. CpG ODNs can be easily enclosed within the PLGA core, and MPLA can be readily incorporated into the lipid layer. The incorporation of these molecular adjuvants may further improve the immunogenicity of NanoNiccine.

To be functional, the formation of core-shell hybrid structure is critical for NanoNiccine. Both CLSM and TEM images of NanoNiccine confirmed the formation of the hybrid structure. In our previous studies [20, 40], it was found that the hybrid structures of lipid-PLGA nanoparticles can be built via sonication mediated fusion, which was used in this study. Moreover, the hybrid nanoparticles proved to be highly stable under physiological conditions over time [40]. As illustrated in Scheme 2, to elicit immune response, NanoNiccine needs to be recognized through the cognate interaction between nicotine hapten on the lipid layer and B cell receptors [51], which is followed by cellular uptake of the vaccine particle. Therefore, the core-shell hybrid structure is of great importance to the immunological outcome of NanoNiccine. In this study, the prevalent existence of hybrid nanoparticles in both CLSM and TEM images demonstrated the high effectiveness and robustness of the hybrid nanoparticle assembly process.

For antibody response, vaccines need to be internalized and processed by APCs [39]. It has been discovered that APCs, especially DCs [52], preferably take up antigens with dimensions comparable to that of bacteria and viruses. Therefore, to facilitate the uptake of vaccine particles by immune cells, the size of NanoNiccine was designed to be within the nano-range. The results from the size distribution study confirmed that the three NanoNiccine particles, regardless of the formulation, had an average size of around 250 nm. Another advantage of a nano-sized vaccine is that the vaccine particles can freely drain from the site of injection into the lymph node [53], where they can extensively interact with the immune cells, thereby enhancing immune response. The FACS analysis showed that up to 96% of the DCs internalized NanoNiccine particles within 120 min, and there was a 30-fold increase in its uptake from 5 to 120 min, demonstrating that the physicochemical properties

of NanoNicotine were quite favorable for cellular uptake. Rapid internalization of NanoNicotine by the DCs may lead to faster antibody production and reduce its nonspecific clearance during circulation.

Another pivotal step of antibody response development is antigen processing by APCs [39]. As shown in Scheme 2, after uptake by APCs, the protein (KLH) enclosed inside the PLGA core needs to be released and processed into antigenic peptides before being presented to the T helper cells. Therefore, the time that taken for antigen processing may also influence the outcome of the immune response. Previous studies showed that considerable amount of antigens was released from hybrid nanoparticle within 24 h in PBS buffer or human serum [54]. The antigen release might be faster in DCs than that in buffers, because DCs have some efficient mechanisms for antigens processing [55]. Although, we do not have direct evidence to show the degradation of hybrid nanoparticles in DCs, KLH, stained with Alexa 647 (red color), was not likely to diffuse out of hybrid nanoparticles due to its big size [56]. Therefore, it is highly possible that the widely distributed KLH in DCs at 120 min shown in Fig. 5 was released from hybrid nanoparticles after their degradation. This indicates that the proteins in NanoNicotine can be rapidly released and processed, which may allow rapid development of immune response.

The potent immunogenicity of NanoNicotine with MPLA was reflected by the significantly higher anti-nicotine antibody titer than that elicited by Nic-KLH after primary injection. Based on the minimal anti-KLH antibody titer shown in Fig. 7, it is highly possible that such high anti-nicotine antibody titers could largely be accredited to the ability of NanoNicotine to stimulate the immune system in a highly specific way. Interestingly, NanoNicotine administered with Alum did not achieve an anti-Nic antibody titer as high as the one without Alum. This might be caused by the depot effect of Alum [57], which may slow the movement of NanoNicotine particles and limit the interaction of NanoNicotine with the immune cells. Alum has long been used as a vaccine adjuvant due to its ability to strongly promote immune response [58]. The potent adjuvanticity of Alum was demonstrated by the tremendous increase in the anti-Nic antibody titer after the first booster. Despite the lower anti-Nic antibody titer after the primary injection, NanoNiccines with Alum achieved a level of antibody comparable to that with only MPLA. However, the level of anti-KLH antibody also considerably increased after the second injection of NanoNicotine supplemented with Alum. It is possible that KLH was released from some NanoNicotine particles, which were degraded after being retained by Alum for a long time. In contrast, NanoNicotine without Alum still maintained a significantly lower level of anti-KLH antibody titer compared to other vaccine formulations.

Surprisingly, the second booster injection did not increase antibody level in any of the vaccine formulations. In contrast, anti-Nic antibody titers of all vaccine groups considerably dropped after the third vaccine injection. Although, the exact mechanism is unknown, it is possible that the anti-Nic antibody already exceeded the threshold level of the immune response after the first booster injection and the immune system was insensitive to the nicotine vaccine at the third injection. Meanwhile, the IgG antibody in mice has a half-life of around one week [59], and this can also partially explain the sharp decrease in anti-Nic antibody concentration. Similar to the anti-Nic antibody, a large decrease in anti-KLH

antibody was also detected in most vaccines after the second injection. However, anti-KLH antibody concentration significantly increased after the third injection of NanoNiccine with MPLA. The seemingly confusing results are in agreement with the above explanation that the immune system was tolerant to the NanoNiccine vaccines after high antibody levels were reached. It is possible the increase in anti-KLH antibody level after the third injection of NanoNiccine with MPLA is simply because the anti-KLH antibody level still did not reach the threshold level after the second injection.

As discussed above, NanoNiccine may have extended half-life after injection due to its ability to evade nonspecific clearance. Due to the short half-life of IgG, anti-Nic antibody level from the 55th day sera was supposed to be lower than that from the 35th day. On the contrary, anti-Nic antibody level from all NanoNiccine groups increased slightly in the final sera, indicating that the NanoNiccine particles could exist long enough to maintain a high level of anti-Nic antibody for a long term.

MPLA [60], as a molecular adjuvant, was incorporated into the lipid layer of NanoNiccine to promote the immune response. Although Alum has been conventionally used as a vaccine adjuvant for many years due to its strong adjuvanticity and acceptable safety, it has a couple of problems that have already been discussed in a previous study, including causing lesions at the site of injection, poorly defined adjuvant mechanism, and causing neurological complications [61]. In addition, as shown in the results, NanoNiccine with MPLA achieved a comparable level of anti-Nic antibody as NanoNiccine adjuvanted with Alum. Therefore, MPLA might be used as a candidate to replace Alum as an adjuvant for NanoNiccine. To study the polarity of the immune response induced by NanoNiccines, the Th1/Th2 index was calculated [62–64]. The low Th1/Th2 index in NanoNiccine supplemented with Alum substantiated that Alum is a potent adjuvant for antibody production [58], which was reflected by the lower Th1/Th2 indices in vaccines supplemented with Alum. As reported in previous studies, MPLA primarily promotes cell mediated immune response instead of a antibody response [65, 66]. It was found that NanoNiccine with MPLA as the sole adjuvant had a Th1/Th2 index of 0.434, indicating that the immune response induced by this vaccine was Th2 skewed (which means that the antibody response was dominant).

Safety is always the most important criterion taken into consideration while evaluating a vaccine. All the components in NanoNiccine, including KLH, nicotine hapten, MPLA, and lipid-PLGA hybrid nanoparticles have proved to be safe in previous studies [14, 67–69]. The histopathological examination on major organs of NanoNiccine immunized mice confirmed its safety.

5. Conclusion

In summary, we successfully constructed a lipid-PLGA hybrid nanoparticle based nicotine vaccine (NanoNiccine). NanoNiccine was designed to improve the specificity of the generated antibody and lengthen the immune response. The cellular uptake studies showed that NanoNiccine possessed physicochemical properties that enable a fast and efficient uptake by the DCs. Results from trials in mice showed that NanoNiccine exhibited superior immunogenicity compared to nicotine-protein conjugate vaccine. NanoNiccine could

effectively minimize the generation of antibodies against KLH and tremendously promote the production of anti-Nic antibodies. The low Th1/Th2 index of NanoNicotine indicated that it could dominantly induce antibody response. Lastly, the histopathological examination of the major organs of the vaccinated mice demonstrated that NanoNicotine possessed excellent safety. Based on all reported results, NanoNicotine holds great promise as a candidate vaccine against nicotine addiction.

Acknowledgments

This work was financially supported by National Institute on Drug Abuse (R21DA030083 and U01DA036850).

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Fig. 1. Confocal image of NanoNiccine particles, in which the lipid layer was labeled with NBD PE (green color) and PLGA core encapsulated Alexa 647-labeled KLH (red color). Red dots display PLGA core, which contains KLH, and green dots display lipid layer. The scale bars represent 10 μm.

Fig. 2.
TEM image of nanoparticles: (A) KLH containing PLGA nanoparticles; (B) liposomes; and (C) NanoNiccine particles. Freshly synthesized nanoparticles were negatively stained and images were acquired via JEOL JEM 1400 TEM.

Fig. 3. Zeta potential and size distributions of nanoparticles. Newly prepared nanoparticles, including NanoNiccines without nicotine haptan, without MPLA, and with MPLA, were suspended in PBS buffer (pH 7.0), and their physicochemical properties (zeta potential and particle size) were measured by Malvern Nano-ZS zetasizer.

Fig. 4.

Investigation of the internalization of lipid-PLGA nanoparticles by FACS. Alexa 647 and NBD labeled NanoNiccine (100 μ g) was added into culture dish containing 2×10^6 dendritic cells, and incubated for 5, 30, 60, and 120 min, respectively. The percentage of cells that internalized NanoNiccine and the relative quantity that was internalized were quantified using FACS.

Fig. 5. Study of NanoNiccine uptake and degradation by dendritic cells using CLSM. Dendritic cells (4×10^5) in a culture chamber were treated with 100 μg fluorescently labeled (lipid layer was marked by NBD PE and PLGA core contained Alexa 647-KLH) NanoNiccine for 5, 30, 60, and 120 min. Scale bars represent 10 μm.

Fig. 6. Time course of nicotine-specific antibodies titers elicited by Nic-KLH, NanoNiccines without hapten, with MPLA, with Alum, and with MPLA and Alum. Each group of eight mice was injected with vaccines containing 40 μ g KLH on days 0, 14, and 28. Nicotine specific antibodies in mice sera from days 13, 27, 35, and 55 were measured using ELISA. ** means P-value < 0.01.

Fig. 7. Time course of KLH specific antibodies elicited by Nic-KLH, NanoNiccines without hapten, with MPLA, with Alum, and with MPLA and Alum. Each group of eight mice was injected with vaccines containing 40 μ g KLH on days 0, 14, and 28. KLH specific antibodies in mice sera from days 13, 27, 35, and 55 were measured using ELISA. ** means P-value < 0.01.

Fig. 8. Titers of anti-Nic IgG₁, IgG_{2a}, IgG_{2b}, and IgG₃ from sera of day 55. Based on subtype antibody titer, the Th1/Th2 index, which indicates dominance of antibody response and cell mediated response, was calculated using equation, $\text{Th1/Th2 index} = ([\text{IgG}_{2a} + \text{IgG}_3]/2)/(\text{IgG}_1)$.

Fig. 9.

H&E staining of the sections of main organs including heart, lung, kidney, spleen, stomach and liver harvested from the mice immunized with different nicotine vaccines. Mice were sacrificed on day 57 and their major organs were stored in 10% formalin before H&E staining. Scale bars represent 200 μ m.

Scheme 1.

Schematic illustration of lipid-PLGA nanoparticle based nicotine vaccine—NanoNiccine. This nicotine vaccine is composed of KLH containing PLGA core, a lipid layer (formed by DOTAP, cholesterol, MPLA, and DSPE-PEG (2000) carboxylic acid), and rac-trans 3'-aminomethyl nicotine covalently linked to the outer terminal of DSPE-PEG (2000) carboxylic acid.

Scheme 2.
Schematic illustration of antibody production induced by NanoNicotine.