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Effect of Adjuvant Release Rate on the Immunogenicity of Nanoparticle-Based Vaccines: A Case Study with a Nanoparticle-Based Nicotine Vaccine

Zongmin Zhao[†], Yun Hu[†], Theresa Harmon[‡], Paul Pentel[‡], Marion Ehrich[§], Chenming Zhang^{*,†}

[†]Department of Biological Systems Engineering, Virginia Tech, Blacksburg, Virginia 24061, United States

[§]Department of Biomedical Sciences and Pathobiology, Virginia Tech, Blacksburg, Virginia 24061, United States

[‡]Minneapolis Medical Research Foundation, Minneapolis, Minnesota 55404, United States

Abstract

Adjuvants are a critical component for vaccines, especially for a poorly immunogenic antigen, such as nicotine. However, the impact of adjuvant release rate from a vaccine formulation on its immunogenicity has not been well illustrated. In this study, we fabricated a series of hybrid-nanoparticle-based nicotine vaccines to study the impact of adjuvant release rate on their immunological efficacy. It was found that the nanovaccine with a medium or slow adjuvant release rate induced a significantly higher anti-nicotine antibody titer than that with a fast release rate. Furthermore, the medium and slow adjuvant release rates resulted in a significantly lower brain nicotine concentration than the fast release rate after nicotine challenge. All findings suggest that adjuvant release rate affects the immunological efficacy of nanoparticle-based nicotine vaccines, providing a potential strategy to rationally designing vaccine formulations against psychoactive drugs or even other antigens. The hybrid-nanoparticle-based nicotine vaccine with an optimized adjuvant release rate can be a promising next-generation immunotherapeutic candidate against nicotine.

Graphical Abstract

*Corresponding Author: Mailing Address: 210 Seitz Hall, Department of Biological Systems Engineering, Virginia Tech, Blacksburg, VA 24061, United States; Voicemail: +1-(540)231-7601; Fax: +1-(540)231-3199; chzhang2@vt.edu. (C.Z.).

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Keywords

nicotine addiction; PLGA; nicotine vaccine; adjuvant release rate; inherent viscosity; anti-nicotine antibody

INTRODUCTION

Tobacco smoking has been a serious public health concern for many years. It remains the leading cause of preventable diseases and premature deaths worldwide.^{1,2} In the United States alone, tobacco smoking causes over 480 000 deaths and a \$300 billion economic loss every year.^{1,3} Nicotine, the major addictive substance in cigarettes, is such a small molecule that it can cross the blood–brain barrier to interact with nicotinic receptors to induce dopamine release.⁴ The rewarding effect that is caused by stimulation of the mesolimbic reward system by dopamine leads to nicotine addiction.⁵ Theoretically, blocking the access of nicotine to nicotinic receptors is a feasible approach to addressing nicotine addiction. Therefore, nicotine vaccines that can elicit the production of anti-nicotine antibodies to block the transportation of nicotine across the blood–brain barrier have been proposed as a promising immunotherapeutic strategy for treating nicotine addiction.⁶

Conjugate nicotine vaccines (CNVs) are the most-studied nicotine vaccines in the past two decades.^{6,7} To date, there have been five CNVs entered into clinical trials, including TANIC, NicQb, NicVax, Niccine, and NIC7.^{8,9} Unfortunately, none of these CNVs, except NIC7, which is still ongoing in a phase I clinical trial, achieved an enhanced overall smoking cessation rate.⁹ Despite this failure, these clinical trials offered two valuable pieces of information to researchers. First, the basic concept of using immunotherapy for treating nicotine addiction is fundamentally sound, as subjects with the highest anti-nicotine antibody titers showed an enhanced smoking cessation rate.^{10,11} Second, a successful nicotine vaccine needs to have a significantly better ability than current CNVs to induce a sufficiently strong immune response to ensure the vaccination efficacy.⁶

Many strategies have been developed to improve the immunogenicity of CNVs, such as a rational hapten design,¹² utilizing enantiopure haptens,^{13,14} applying hapten clustering,¹⁵ screening carrier proteins,¹⁶ using novel adjuvants,¹⁷ optimizing administration routes,¹⁸ developing multivalent vaccines,¹⁹ and designing peptide-free synthetic vaccines.²⁰ Although these strategies could considerably improve the immunogenicity of CNVs, they failed to overcome the intrinsic shortcomings of CNVs, such as poor recognition and capture by immune cells and difficulty integrating with molecular adjuvants.²¹ Therefore, it is necessary to explore completely new paradigms for nicotine vaccine development. The use of nanoparticles rather than proteins as hapten carriers is such a new paradigm that it has potential to overcome some of the innate drawbacks of CNVs.⁹ Particularly, as nanoparticles can be engineered to have large cargo space,²² nicotine vaccine components (hapten, T helper protein, and adjuvant) can be easily incorporated with a high loading capacity. In addition, because of the particulate nature of nanoparticles,²³ nicotine vaccine components can be better recognized and captured by immune cells.

A nanoparticle-based nicotine nanovaccine (NanoNicVac) using lipid–polymeric hybrid nanoparticles as vehicles for efficient delivery of nicotine vaccine components was developed in our previous study.²¹ NanoNicVac was composed of four major components: a lipid–polymeric hybrid nanoparticle, a nicotine hapten (B cell epitope), a carrier protein (T cell epitope), and molecular adjuvants. NanoNicVac exhibited a significantly higher cellular internalization efficiency and immunological efficacy than the conjugate vaccine.²¹ In addition, we demonstrated that by modulating multiple factors, such as nanoparticle size,²¹ hapten density,²⁴ hapten localization,²⁵ carrier protein,²⁶ and molecular adjuvants,²⁷ the immunogenicity of NanoNicVac could be improved.

For a nanoparticle-based vaccine, the release rate of antigens or adjuvants from nanoparticles potentially affects the strength and persistence of stimulation to immune cells, thus influencing the immunological outcome of the vaccine.^{28,29} In addictive-drug vaccine development, extensive work has been done to screen immunostimulatory adjuvants.^{18,30,31} However, few studies have been conducted to explore the influence of adjuvant release rate on the immunological efficacy of vaccines. In this study, we aimed to engineer NanoNicVac to have a tunable adjuvant release rate and to investigate the impact of adjuvant release rate on its immunogenicity and pharmacokinetic efficacy. Three NanoNicVac nanoparticles, from which a molecular adjuvant was designed to be released at a fast, medium, or slow rate, were fabricated and characterized. The adjuvant release rate from NanoNicVac was analyzed. The immunogenicity and pharmacokinetic efficacy of NanoNicVac with different adjuvant release rates were studied in mice.

MATERIALS AND METHODS

Materials.

Lactel 50:50 ester-terminated poly(lactic-*co*-glycolic acid) (PLGA) (inherent viscosity = 0.20, 0.59, or 1.15 dL/g) was purchased from Durect Corporation (Cupertino, CA). Cholesterol (CHOL), 1,2-diphytanoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt) (NBD-PE), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-

[amine(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG2000-amine), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[maleimide-(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG2000-maleimide), and monophosphoryl lipid A (MPLA) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Resiquimod (R848) VaccineGrade was purchased from InvivoGen (San Diego, CA). *O*-Succinyl-3'-hydroxymethyl-(±)-nicotine (Nic) was purchased from Toronto Research Chemicals (North York, ON, Canada). 1-Ethyl-3-[3-(dimethylamino)propyl] carbodiimide hydrochloride (EDC), *N*-hydroxysulfosuccinimide (Sulfo-NHS), and coumarin-6 (CM-6) were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Tetanus toxoid (TT) was purchased from Statens Serum Institut (Copenhagen, Denmark). All other chemicals were of analytical grade.

Fabrication of NanoNicVac Nanoparticles.

R848-loaded PLGA nanoparticles were fabricated using a previously developed double-emulsion method.²⁷ In brief, 30 mg of PLGA with different inherent viscosities (0.20, 0.59, or 1.15 dL/g) was dissolved in 2 mL of dichloromethane (organic phase). These three inherent viscosities were selected based on the consideration that they are significantly different and thus can represent a low, medium, and high value of inherent viscosities. Subsequently, 1.44 mg of R848 in 300 μ L of DI water/DMSO (9:1) was added to the organic phase. The mixture was emulsified by sonication in ice bath using a Branson M2800H Ultrasonic Bath Sonicator (Danbury, CT). The emulsion was added to 11 mL of 0.5% w/v poly(vinyl alcohol) solution under continuous stirring. The mixture was emulsified by sonication using a sonic dismembrator (Model 500, Fisher Scientific, Pittsburgh, PA) at an amplitude of 70% for 40 s. The emulsion was continuously stirred in a hood for 12 h to evaporate dichloromethane completely.

PLGA nanoparticles were collected by centrifugation at 10 000*g*, 4 °C, for 30 min (Beckman Coulter Avanti J-251, Brea, CA, USA) and stored at 4 °C for later use. Unencapsulated R848 in the supernatant was quantified by reverse phase HPLC using a Luna C18 (2) column at 220 nm. The amount of encapsulated R848 was determined by subtracting the amount of unencapsulated R848 from the total added R848.

Liposomes that were composed of DOTAP, DSPE-PEG2000-amine, DSPE-PEG2000-maleimide, CHOL, and MPLA were prepared using a previously reported lipid-film-hydration-sonication method.²¹ Lipid-polymeric hybrid nanoparticles were prepared using a previously reported sonication method.²⁴ Particularly, 2.5 mg of liposomes were coated to 25 mg of PLGA nanoparticles. Nic hapten was conjugated to the surface of lipid-polymeric hybrid nanoparticles using an EDC/NHS mediated method as reported previously by taking advantage of the reaction between amine groups on liposomes and carboxylic groups on the Nic hapten.²⁵ Nic-TT conjugate was prepared using a previously reported EDC/NHS mediated method.²⁴ The loading of Nic on TT was determined using a 2,4,6-trinitrobenzenesulfonic acid (TNBSA)-based method as reported previously.²⁶ After analysis, it was found that, on average, 5.6 Nic haptens were conjugated to each TT protein. Finally, Nic-TT conjugate was conjugated to an appropriate amount of Nic-lipid-polymeric hybrid nanoparticles to form NanoNicVac nanoparticles according to a previously reported

method.²¹ Specifically, 10 μ L of Traut's reagent (2 mg/mL in water) was added to the Nic–TT conjugate containing 1.0 mg of TT dissolved in PBS to form thiolated Nic–TT conjugate. The thiolated conjugate was mixed with 27.5 mg of lipid–polymeric nanoparticles, and the reaction was allowed to proceed for 2 h at room temperature. NanoNicVac nanoparticles were collected by centrifugation at 10 000g, 4 °C, for 30 min, and unconjugated Nic–TT conjugates in the supernatants were quantified by the bicinchoninic acid assay. The amount of immunoactive components of the nanovaccine formulations are listed in Table 1. Nanovaccine nanoparticles were stored at 4 °C for later use.

Characterization of Nanoparticles.

To measure the size, polydispersity index (PDI), and zeta potential of nanoparticles, nanoparticles were dissolved in 10 mM pH 7.4 phosphate buffer saline (PBS) and assayed on a Nano ZS Zetasizer (Malvern Instruments, Worcestershire, United Kingdom) at 25 °C. To characterize the morphology of nanoparticles, nanoparticle samples were negatively stained for 20 s using 1% phosphotungstic acid and imaged on a JEOL JEM 1400 transmission electron microscope (JEOL, Tokyo, Japan).

Measuring the Release Kinetics of R848 from Nanoparticles.

The release kinetics of R848 from nanoparticles was assayed using a dialysis method.³² In brief, 25 mg of nanoparticles was dissolved in 1 mL of 10 mM pH 7.4 PBS and added to ready-to-use dialysis tubes (MWCO 6000–8000) (Spectrum Laboratories, Inc., Rancho Dominguez, CA). The nanoparticle samples were dialyzed against 30 mL of 10 mM pH 7.4 PBS with 0.01% v/v Tween 20 supplemented. The release testing was conducted under continuous stirring at 37 °C. At predetermined time points, a 1 mL sample was taken out, and an equal volume of fresh buffer was added. The concentration of R848 was quantified by reverse phase HPLC. The HPLC analysis was conducted on an Agilent 1200 HPLC system equipped with a Luna 5 μ m C18 (2) column (250 $\times$ 4.6 mm). The flow rate was set as 1 mL/min, and the detection was performed using a UV detector at 220 nm. The mobile phase consisted of acetonitrile/TFA/water. Specifically, a linear gradient from 100% water containing 0.1% TFA to 100% acetonitrile containing 0.1% TFA was conducted over 40 min. The sample injection volume was 30 μ L.

Characterization of the Cellular Uptake of Nanoparticles by Dendritic Cells (DCs).

The cellular uptake efficiency of NanoNicVac nanoparticles by DCs was evaluated using flow cytometry. JAWSII (ATCC CRL-11904) immature DCs (2×10^6 /well) were seeded to and cultured in 35 mm Petri dishes. *N*-(7-Nitro-2-1,3-benzoxadiazol-4-yl) (NBD)-labeled NanoNicVac nanoparticles were fabricated using a similar method as described except that 5% w/w NBD was added to the lipid mixture for labeling. After being cultured for 24 h, the medium was replaced with 2 mL of fresh medium containing 20 μ g of NBD-labeled NanoNicVac nanoparticles. After incubating for 0.5 or 2 h, the medium was discarded, and cells were washed three times using 10 mM pH 7.4 PBS. After being detached from the Petri dish by trypsinization, cells were collected by centrifugation at 200g for 10 min. Cells were suspended in 10 mM pH 7.4 PBS and analyzed on a flow cytometer (FACSAria I, BD Biosciences, Franklin Lakes, NJ, USA).

The cellular uptake of NanoNicVac nanoparticles was also visualized using confocal laser scanning microscopy (CLSM). CM-6-labeled nanoparticles were prepared according to a similar method described above except that CM-6 was loaded to the PLGA core. DCs (2×10^5 /chamber) were seeded to two-well chamber slides. After culturing for 24 h, the medium was replaced with 1.5 mL of fresh medium containing 10 μ g of CM-6-labeled nanoparticles. Cells were incubated with nanoparticles for 4 or 24 h. Subsequently, the medium was discarded, and cells were washed using 10 mM pH 7.4 PBS and fixed with 4% w/v paraformaldehyde. The membrane of cells was permeabilized by adding 0.5 mL of 0.1% (v/v) Triton X-100 for 10 min. Cell nuclei were stained by 4',6-diamidino-2-phenylindole (DAPI). The samples were visualized on a Zeiss LSM 510 laser scanning microscope (Carl Zeiss, Germany).

In Vivo Experimentation in Mice.

Animal studies were conducted following the National Institute of Health guidelines for animal care and use. Animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech. The immunogenicity and pharmacokinetic efficacy of NanoNicVac were studied in female Balb/c mice. The animal number was determined to be 5 per group to achieve a power of at least 0.8 (a common practice) based on the power analysis of our previous published data.^{21,24,25,27} Mice (6–7 weeks, 16–20 g, 5 per group) were immunized subcutaneously with NanoNicVac containing 40 μ g of TT, 34 μ g of R848, and 20 μ g of MPLA that was dissolved in 200 μ L of sterilized PBS on days 0, 14, and 28. The dose of MPLA was determined based on the assumption that 100% of MPLA in the lipid mixture was incorporated to the lipid layer of NanoNicVac nanoparticles. A blank control group was injected with 200 μ L of sterilized PBS. Blood was collected under isoflurane anesthesia on days 26 and 40. The titers of anti-nicotine antibodies in serum were assayed using an enzyme-linked immunosorbent assay (ELISA) as reported previously.²¹ Particularly, serum samples with dilution factors of 50, 250, 1250, 6250, 31 250, and 156 250 were used for the ELISA assay. Antibody titer was defined as the dilution factor at which the absorbance at 450 nm declined to half maximal.

On day 47, mice were subcutaneously administered 0.06 mg/kg nicotine. Mice were euthanized and decapitated 3 min post nicotine injection, and the blood and brain samples were collected. Brain samples were rinsed three times with water to remove excess blood. Nicotine levels in the brain and serum were analyzed using a previously reported GC/MS method.³³ After decapitation, major mouse organs, including heart, liver, spleen, lung, and kidney, were extracted and immersed in 10% formalin. Tissue blocks were stained with hematoxylin and eosin (H&E) according to a standard protocol and imaged on a Nikon Eclipse E600 light microscope. During the entire study, the body weight of mice was monitored and recorded.

Statistical Analyses.

Data are expressed as means $\pm$ standard error (SEM) unless specified. Comparisons between two groups and among multiple groups were conducted using Student's *t* test and one-way ANOVA followed by Tukey's HSD test, respectively. Differences were considered significant when *p*-values were less than 0.05.

RESULTS

Fabrication and Characterization of NanoNicVac.

The morphology of nanoparticles was characterized by TEM (Figure 1A). The PLGA nanoparticles with different inherent viscosities exhibited a similar spherical morphological feature. The corresponding lipid-PLGA hybrid and NanoNicVac nanoparticles, independent of the release rates, also shared comparable morphological characteristics. Particularly, a core-shell structure was observed on all lipid-PLGA nanoparticles, suggesting the formation of a hybrid structure. In addition, a “cloud” layer was coated on the core-shell structure on NanoNicVac nanoparticles, evidently indicating the efficient conjugation of protein antigens (Nic-TT). The physicochemical properties of nanoparticles, including size and zeta potential, were measured (Figure 1B,C). The diameter of NanoNicVac nanoparticles with a fast, medium, and slow adjuvant release rate was 128.5 ± 6.5 , 150.1 ± 18.3 , and 165.5 ± 7.5 nm, respectively. The PDI of the three NanoNicVac nanoparticles was 0.230 ± 0.018 , 0.246 ± 0.011 , and 0.228 ± 0.020 , respectively. All three NanoNicVac nanoparticles had an insignificantly different zeta potential, which was -8.2 ± 0.2 , -8.9 ± 1.4 , and -6.8 ± 0.6 mV, respectively. The amount of adjuvant loaded to NanoNicVac nanoparticles was also quantified (Figure 1D). The loading efficiency of adjuvant in the three NanoNicVac nanoparticles was 35.0 ± 4.3 , 29.7 ± 4.0 , and 30.7 ± 2.3 $\mu\text{g}/\text{mg}$, respectively. The NanoNicVac nanoparticle with a fast release rate exhibited a higher adjuvant loading efficiency than the other two. However, the difference was not significant.

Adjuvant Release from NanoNicVac Nanoparticles.

The release rate of payload could be correlated with the inherent viscosity of nanoparticles.²⁸ In this study, by modulating the inherent viscosity of PLGA core, molecular adjuvant is conceptualized to be released at different rates (Figure 2A). The release rate of adjuvant (R848) from NanoNicVac nanoparticles was analyzed using a dialysis method. As shown in the upper panel of Figure 2B, on day 2, 46.6, 27.9, and 17.7% of the total loaded adjuvant was released from NanoNicVac nanoparticles with a fast, medium, and slow release rate. As shown in the lower panel of Figure 2B, on day 4, 76.6, 47.3, and 25.4% of the loaded adjuvant was released from fast-, medium-, and slow-release NanoNicVac nanoparticles, respectively. After day 4, the adjuvant release from the fast-release NanoNicVac nanoparticle was almost saturated. In comparison, adjuvant continued to be released from the medium- and slow-release nanoparticles in a sustained manner after day 4. At the end of the release study on day 11, 84.4, 70.8, and 62.9% of loaded adjuvant was released from fast-, medium-, and slow-release NanoNicVac nanoparticles, respectively. The above adjuvant release results suggest that adjuvant (R848) could be released at different rates from NanoNicVac nanoparticles with different inherent viscosities.

Cellular Uptake of NanoNicVac Nanoparticles by Antigen Presenting Cells (APCs)

The cellular uptake of NanoNicVac nanoparticles by dendritic cells, which are professional antigen presenting cells, was investigated by flow cytometry (Figure 3). Dendritic cells were treated with NBD-labeled NanoNicVac nanoparticles with different adjuvant release rates, in which NBD was added to the lipid layer for labeling, for 0.5 or 2 h. As shown in Figure 3A, after 0.5 h of incubation with NanoNicVac nanoparticles, > 83% of the studied cells were

NBD positive, indicating a rapid uptake of nanoparticles by dendritic cells. Moreover, after 2 h of incubation, > 93% of cells were stained by NBD, further indicating the efficient cellular internalization of NanoNicVac nanoparticles. The relative uptake efficiency of NanoNicVac nanoparticles with different adjuvant release rates was investigated by comparing the percentage of NBD positive cells and the median fluorescence intensity (M.F.I.) of cells (Figure 3B,C). Evidently, the fast-, medium-, and slow-release NanoNicVac nanoparticles were taken up by dendritic cells with a similar efficiency, which may be attributed to their similar physicochemical properties (Figure 1).

The intracellular distribution of NanoNicVac nanoparticles was visualized using CLSM (Figure 4). A fluorescent dye (CM-6) was loaded to NanoNicVac nanoparticles for tracking the intracellular distribution of nanoparticles. After 0.5 h of incubation, a substantial amount of NanoNicVac nanoparticles were detected in cells, further suggesting a rapid and efficient uptake of nanoparticles by dendritic cells. Noticeably, the green fluorescence was shown as individual dots in all NanoNicVac groups. This result suggested that the payload had not been sufficiently released from NanoNicVac nanoparticles. After 12 h of incubation, the intracellular distribution of green fluorescence seemed to be dramatically different among NanoNicVac groups with different release rates. Specifically, in cells treated with the fast-release NanoNicVac nanoparticles, the green fluorescence was widely distributed throughout the entire cell, and very few individual dots were observed. However, in cells treated with medium- or slow-release NanoNicVac nanoparticles, although a portion of green fluorescence was distributed widely in cells, a substantial amount of green fluorescence was still displayed as individual dots. All these results suggested that the encapsulated payload could be released at different rates upon nanoparticles being internalized by dendritic cells.

Immunogenicity of NanoNicVac against Nicotine in Mice.

The immunogenicity of NanoNicVac with different adjuvant release rates was studied in female Balb/c mice. As shown in Figure 5A, mice were immunized following a prime-boost procedure. Specifically, mice received subcutaneously administered NanoNicVac on days 0, 14, and 28. According to our previous study, mice generated a significantly strong anti-nicotine immune response after booster injections.^{21,24,27} Therefore, in this study, blood was collected from mice on days 26 and 40 to monitor the induced anti-nicotine antibodies in serum. The time-course results of anti-nicotine antibody titers are shown in Figure 5B. The titers of anti-nicotine antibodies induced by the fast-, medium-, and slow-release NanoNicVac were $20\ 100 \pm 4600$, $36\ 400 \pm 10\ 100$, and $31\ 900 \pm 9600$, respectively, 12 days after the first booster immunization (on day 26). For all three NanoNicVac, the anti-nicotine antibody titers on day 40 were significantly higher than those on day 26. This result suggests that the second booster immunization (on day 28) significantly boosted the immune response, which was consistent with our previous reports.²⁵ As shown in Figure 5C, on day 40, the fast-, medium-, and slow-release NanoNicVac elicited an anti-nicotine antibody titer of $41\ 600 \pm 4500$, $71\ 200 \pm 10\ 900$, and $60\ 500 \pm 9200$, respectively. Statistical analysis suggested that the titers induced by the slow- and medium-release NanoNicVac were significantly higher than that resulted from the fast-release counterpart.

Ability of NanoNicVac To Reduce Nicotine from Entering the Brain.

The pharmacokinetic efficacy of NanoNicVac with different adjuvant release rates, which was defined as the ability to sequester nicotine in serum and thus reduce it from entering the brain, was studied. As shown in Figure 5A, on day 47, mice were challenged subcutaneously with 0.06 mg/kg nicotine for 3 min, and the concentrations of nicotine in the serum and brain were measured (Figure 6). As shown in Figure 6A, the control (PBS) group had an average serum nicotine concentration of 11.7 ng/mL. The serum nicotine concentrations in the fast-, medium-, and slow-release NanoNicVac groups were 5.5-, 10.2-, and 9.3-fold higher than that of the control (PBS) group, respectively. Evidently, the medium- and slow-release NanoNicVac exhibited a considerably better ability to sequester nicotine in serum than the fast-release NanoNicVac. Figure 6B shows the brain nicotine concentrations after nicotine challenge. The control (PBS) group has an average brain nicotine concentration of 93.7 ng/g. In contrast, compared to that of the control group, the brain nicotine concentrations in the fast-, medium-, and slow-release NanoNicVac were reduced by 48.3, 66.1, and 71.4%, respectively. Statistical analysis indicated that the slow-release NanoNicVac resulted in a significantly lower brain nicotine concentration than the fast-release NanoNicVac. In addition, the brain nicotine concentration resulted from the medium-release NanoNicVac was much lower than that caused by the fast-release NanoNicVac, although the difference was not statistically significant. The brain nicotine concentration results suggest that the medium- and slow-release NanoNicVac, especially the slow-release NanoNicVac, exhibited a better ability to reduce nicotine from entering the brain than the fast-release counterpart.

Preliminary Safety of NanoNicVac in Mice.

The safety of NanoNicVac with different adjuvant release rates was evaluated by monitoring local inflammatory reactions, body weight change, and histopathological examination. During the entire study, we did not detect obvious local inflammation reactions at the injection site for all groups, including the PBS and NanoNicVac groups. In addition, histopathological examination was performed on major organs of mice, including kidney, liver, spleen, lung, and heart, to study any potential lesions caused by administration of NanoNicVac (Figure 7A). All the examined organs of mice that were immunized with NanoNicVac, regardless of the adjuvant release rate, exhibited similar characteristics compared to those of mice injected with PBS. Meanwhile, no obvious lesions were observed on any of the examined organs in both the PBS and NanoNicVac groups. The body weight of mice during the study was monitored and recorded (Figure 7B). No body weight loss was detected in any of the groups in which PBS or NanoNicVac was administered. Moreover, there is no notable difference of body weight between the PBS and NanoNicVac groups. All the above results suggest that the NanoNicVac, regardless of the adjuvant release rate, was safe for mice.

DISCUSSION

By inducing nicotine specific antibodies and thus blocking the access of nicotine to acetylcholine nicotinic receptors in the brain, nicotine vaccines have potential to be an alternative or additive to current pharmacological medications for treating nicotine

addiction. Currently, the mostly-studied conjugate nicotine vaccines using proteins as hapten carriers have not shown enhanced smoking cessation efficiency,⁶ primarily because of their poor immunogenicity caused by their poor recognition and internalization by immune cells.²¹ In our previous studies, we explored the possibility of developing a next-generation nanoparticle-based nicotine vaccine using lipid–polymeric hybrid nanoparticles as vaccine component delivery vehicles.²¹ By enhancing its immunogenicity by modulating multiple factors, such as nanoparticle size,²¹ hapten density,²⁴ hapten localization,²⁵ carrier protein,²⁶ and molecular adjuvants,²⁷ the hybrid-nanoparticle-based nicotine nanovaccine (NanoNicVac) exhibited great promise as a next-generation nanoparticle-based immunotherapeutic strategy against nicotine addiction. In this present study, we further investigated the impact of adjuvant release rate on the immunological efficacy of NanoNicVac. Results indicated that the slow- and medium-release NanoNicVac had a better efficacy in resulting in higher titers of anti-nicotine antibodies and reducing more nicotine from entering the brain than the fast-release counterpart. Specifically, the optimized NanoNicVac resulted in a 71% brain nicotine reduction in mice. In comparison, two clinically tested conjugate nicotine vaccines, NicQb and NIC7, were reported to reduce 61 and ~60% nicotine from entering the brain of mice, respectively.^{34,35} Thus, NanoNicVac seems to have a better immunological efficacy than current conjugate nicotine vaccines, although experiments that directly compare these vaccines are needed.

NanoNicVac nanoparticles with different adjuvant release rates were prepared by modulating the inherent viscosity of the PLGA core. The fast-, medium-, and slow-release NanoNicVac possessed a low, medium, and high viscosity, respectively. The dynamic light scattering results revealed that the slow-release NanoNicVac nanoparticle exhibited a larger size than the medium- and fast-release nanoparticles. In terms of average, these sizes are significantly different. However, considering their relatively large PDI, these particles are not remarkably different in terms of size distribution. The TEM results revealed that nicotine–protein antigens were successfully conjugated to the surface of “core-shell” lipid–polymeric hybrid nanoparticles. The efficient antigen conjugation is critical for the hybrid-nanoparticle-based vaccine design. First, the stable lipid–polymeric nanoparticle can provide the conjugated antigen with a particulate nature,^{36–38} leading to an improved recognition and capture by antigen presenting cells, as the immune system prefers to recognize particulate antigens rather than soluble protein antigens.^{23,39,40} Second, the antigens (conjugated to nanoparticle surface) and molecular adjuvants (encapsulated within nanoparticles) could be codelivered to the same antigen presenting cells. The co-localization of antigens and adjuvants in the same antigen presenting cells could augment antigen presentation and T help cell activation,⁴¹ thus facilitating B cell activation and maturation.

In vitro cellular study results revealed that the fast-, medium-, and slow-release NanoNicVac nanoparticles were taken up by dendritic cells with a similar efficiency. However, the CLSM results revealed that the model adjuvant was released at a different rate from the NanoNicVac nanoparticles with different inherent viscosities. This result was consistent with the results of the in-buffer release assay. However, the model adjuvant seemed to be released faster in cells than in PBS. This result was expected. There are many hydrolases and other enzymes inside cells, especially in endosome/lysosome compartments, which would destruct NanoNicVac nanoparticles to lead to a faster adjuvant release.³²

The data of the anti-nicotine antibody titers and pharmacokinetic efficacy revealed that the immune response induced by the hybrid-nanoparticle-based nicotine vaccine is correlated with the release rate of adjuvant. Specifically, a sustained adjuvant release was beneficial for eliciting a stronger immune response against nicotine than a fast adjuvant release. Although we do not have direct evidence to show the exact mechanism, the following may explain the phenomenon. First, upon being subcutaneously administered, NanoNicVac nanoparticles would accumulate around the injection site. Before being captured by antigen presenting cells, a portion of adjuvant would be prematurely released from nanoparticles. For the fast-release NanoNicVac, a substantial amount of adjuvant would be released. However, for the sustained-release NanoNicVac, less adjuvant could be prematurely released. As a result, more adjuvant would be available to antigen presenting cells. Second, upon cellular internalization, adjuvant would be released from nanoparticles to interact with Toll-like receptors inside antigen presenting cells.^{42,43} A sustained adjuvant release would lead to a continuous interaction with Toll-like receptors, possibly leading to a persistent stimulation of antigen presenting cells, facilitating cytokine release, cell maturation, and antibody secretion.^{44,45} However, interestingly, we did not detect significant differences in immunological efficacy between the NanoNicVac with a medium adjuvant release rate and the one with a slow release rate. To induce a strong immune response, two pivotal processes may be required: fast activation of immune cells to initiate an immune response and prolonged stimulation of immune cells to facilitate the immune response. Compared to the medium release rate, the slow release rate has a less initial release and a more sustained release. Therefore, it may cause a weaker initiation but a stronger prolonged stimulation of the immune response than the medium adjuvant release. As a result, the slow adjuvant release resulted in a comparable immunological efficacy to the medium adjuvant release. The immunological effect of adjuvant release rates observed in this study may be applicable to other small molecule or biomolecule adjuvants. However, further studies need to be conducted to investigate the applicability.

CONCLUSION

In summary, by modulating the inherent viscosity of PLGA polymers, a series of lipid–polymeric hybrid-nanoparticle-based nicotine nanovaccines, from which a molecular adjuvant could be released at different rates, were fabricated. It was found that the molecular adjuvant could be released from nanoparticles in a fast, medium, or slow release rate both in buffer and in cells. In vivo study in mice suggested that a sustained (medium and slow) adjuvant release resulted in a significantly stronger immune response against nicotine than a fast adjuvant release. This study illustrated the necessity in tuning adjuvant release rates for nanoparticle-based nicotine vaccine design. The findings in this study can potentially be applied to other nanoparticle-based vaccines. Moreover, the hybrid-nanoparticle-based nicotine nanovaccine with an optimal adjuvant release rate can be a promising candidate as the next-generation nanoparticle-based immunotherapy against nicotine addiction.

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Figure 1. Characterization of NanoNicVac nanoparticles. (A) TEM images of PLGA nanoparticles, lipid-PLGA hybrid nanoparticles, and NanoNicVac nanoparticles. Scale bars represent 200 nm. (B) Size and PDI of nanoparticles. (C) Zeta potential of nanoparticles. (D) Adjuvant loading efficiency in NanoNicVac nanoparticles. Significantly different: * $p < 0.05$, ** $p < 0.01$. N.S.: not significantly different. Data shown in (B–D) are results of three independent lots of nanoparticles.

Figure 2.

Adjuvant release from NanoNicVac nanoparticles. (A) Schematic illustration of adjuvant release from NanoNicVac nanoparticles at different rates. (B) In vitro release profiles of adjuvant from NanoNicVac nanoparticles in 0.01 M pH 7.4 PBS supplemented with 0.01% Tween 20. Percentage of cumulatively released adjuvant in short term (2 days) and long term (11 days) is shown in the upper and lower panels, respectively. Data is shown as means $\pm$ standard deviation from three independent lots of nanoparticles. Significantly different compared to the medium release: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Significantly different compared to the slow release: # $p < 0.05$, ## $p < 0.01$.

Figure 3. Cellular uptake efficiency of NanoNicVac nanoparticles by dendritic cells. (A) Flow cytometry assay showing the recorded events of dendritic cells after being treated with NBD-labeled NanoNicVac nanoparticles with different adjuvant release rates for 0.5 or 2 h. (B) The percentage of NBD positive cells after being treated with NanoNicVac nanoparticles. (C) NBD median fluorescence intensity (M.F.I.) in dendritic cells after incubation with NanoNicVac nanoparticles. No significant differences were observed among

the three NanoNicVac nanoparticles for both percentage of NBD positive cells and NBD M.F.I..

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Figure 4.

Intracellular distribution of NanoNicVac nanoparticles by dendritic cells. Cells were treated with NanoNicVac nanoparticles, in which a fluorescent dye (CM-6) were encapsulated, for 0.5 or 12 h. Cell nuclei were stained by DAPI. Scale bars represent 10 μm .

Figure 5. Effect of adjuvant release rate on the immunogenicity of NanoNicVac in mice. (A) Schematic illustration of the immunization and bleeding regimen used in the mouse trial. (B) Time-course of anti-nicotine antibody titers in response to NanoNicVac with different adjuvant release rates. (C) Statistical comparison of anti-nicotine antibody titers on day 40. Each circle represents the anti-nicotine antibody titer of each mouse ($n = 5$). Significantly different: * $p < 0.05$, ** $p < 0.01$.

Figure 6. Influence of adjuvant release rate on the pharmacokinetic efficacy of NanoNicVac in mice ($n = 5$). (A) Nicotine concentration in serum after nicotine challenge. (B) Nicotine concentration in the brain after nicotine challenge. Mice were challenged with 0.06 mg/kg nicotine on day 47. Significantly different compared to the PBS group: # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$. Significantly different: * $p < 0.05$.

Figure 7. Safety of NanoNicVac with different adjuvant release rates in mice. (A) Histopathological analysis showing representative H&E staining images of tissues from major organs of mice, including kidney, liver, spleen, lung, and heart. (B) Body weight change of mice during the study period ($n = 5$).

Table 1.

Amount of Immunoactive Components in Different Nanovaccine Formulations

Nanovaccines	TT ($\mu\text{g}/\text{mg}$)	Nic ($\mu\text{g}/\text{mg}$)	MPLA ($\mu\text{g}/\text{mg}$)	R848 ($\mu\text{g}/\text{mg}$)	Nic/TT molar ratio
Fast release	66.2 ± 1.7	0.73 ± 0.02	17.85	35.0 ± 4.3	5.6
Medium release	65.5 ± 2.5	0.71 ± 0.03	17.85	29.7 ± 4.0	5.6
Slow release	63.5 ± 1.3	0.69 ± 0.01	17.85	30.7 ± 2.3	5.6