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Paradox of PEGylation in fabricating hybrid nanoparticle-based nicotine vaccines

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Abstract

Polyethylene glycol (PEG) has long been used in the nanoparticle-based drug or vaccine delivery platforms. In this study nano-nicotine vaccines (NanoNicVac) were PEGylated to different degrees to investigate the impact of PEG on the immunological efficacy of the vaccine. Hybrid nanoparticles with various degrees of PEGylation (2.5%–30%) were assembled. It was found that 30% PEGylation resulted in a hybrid nanoparticle of a compromised core-shell structure. A higher concentration of PEG also led to a slower cellular uptake of hybrid nanoparticles by dendritic cells. However, increasing the quantity of the PEG could effectively reduce nanoparticle aggregation during storage and improve the stability of the hybrid nanoparticles. Subsequently, nicotine vaccines were synthesized by conjugating nicotine haptens to the differently PEGylated hybrid nanoparticles. In both *in vitro* and *in vivo* studies, it was found that a nicotine vaccine with 20% PEGylation (NanoNicVac 20.0) was significantly more stable than the vaccines with lower PEGylation. In addition, NanoNicVac 20.0 induced a significantly higher anti-nicotine antibody titer of $3.7 \pm 0.6 \times 10^4$ in mice than the other NanoNicVacs with lower concentrations of PEG. In a subsequent pharmacokinetic study, the lowest brain nicotine concentration of 34 ± 11 ng/g was detected in mice that were immunized with NanoNicVac 20.0. In addition, no apparent adverse events were observed in mice immunized with NanoNicVac. In summary, 20% PEGylation confers NanoNicVac with desirable safety, the highest stability, and the best immunological efficacy in mice.

Graphic abstract

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Conflict of interest

The authors declare no competing financial interests.

Keywords

Hybrid nanoparticle; Nicotine vaccine; Immunotherapy; PEGylation; Stability; Smoking cessation

1. Introduction

As the leading cause of preventable death in the United States, tobacco use results in tremendous social and economic problems [1]. These include 480,000 deaths per year, more than \$170 billion in direct medical care, and more than \$156 billion in lost productivity due to premature death [2]. Because of the highly addictive nature of nicotine, smoking cessation without medical interventions is a difficult if not impossible mission for smokers [3]. Even with the assistance from the currently available therapies, the long-term smoking abstinence rate is unacceptably low [4]. Therefore, there is an urgent need for developing novel and more effective treatments for tobacco addiction.

Among the new approaches, nicotine vaccine, which can induce the production of nicotine-specific antibodies, has proven promising in treating smoking addiction [5–11]. However, the conventional protein-nicotine conjugate vaccines are associated with some innate drawbacks, including low immunogenicity, low specificity, and short immune persistence [12, 13]. To overcome these disadvantages, a novel nanoparticle (NP) based-nicotine vaccine (NanoNicVac), was developed in our group [14]. Structurally, NanoNicVac is composed of a poly(lactic-co-glycolic acid) (PLGA) core and a lipid bilayer. One of the features of this vaccine is that the surface of the NP is coated with polyethylene glycol (PEG). PEGylation has been widely used in FDA-approved NP formulations [15]. In NanoNicVac, the terminal reactive groups on the PEG molecules, such as amine group and carboxylic group, can serve as linking sites for nicotine haptens [14]. PEG coating on the vaccine particle may also shield the surface from aggregation, opsonization, and phagocytosis, prolonging systemic circulation time [16, 17].

Immunogenicity is one of the most critical factors that govern the efficacy of a nicotine vaccine [18]. The immunogenicity of a nicotine vaccine can be determined by measuring the concentrations of the nicotine-specific antibody in the animals immunized with the vaccine [19]. In both preclinical trials in animals and clinical trials in humans, it was found that a higher nicotine antibody titer in serum was associated with a better immunological outcome [20, 21]. Although, PEGylation within certain degrees may improve the stability of NPs, high concentrations of the PEG molecules were found to cause instability in liposomes, which may undermine the stability of NanoNicVac NPs [22, 23]. In addition, high levels of PEGylation may impede the uptake of the vaccine particles by immune cells, resulting in a

suboptimal outcome of the immunization [17]. Therefore, it is necessary to produce a NanoNicVac with an optimal level of PEGylation, at which the vaccine can elicit a maximized immune response.

In this study, we assembled lipid-PLGA hybrid NPs containing varying concentrations of DSPEPEG(2000)COOH. The structural integrity of these hybrid NPs was examined. It was found that liposome containing 2.5%, 5%, 12.5%, and 20% DSPE-PEG(2000)COOH formed stable hybrid structure with the PLGA NPs. In contrast, liposome with 30% DSPE-PEG(2000)COOH failed to form a stable hybrid NP of an integral core-shell structure. Subsequently, nicotine haptens were conjugated to the stable hybrid NPs to synthesize NanoNicVacs. The results showed that NanoNicVac with 20% DSPE-PEG(2000)COOH (NanoNicVac 20.0) in the lipid layer achieved the highest anti-nicotine antibody titer in mice. Consistent with its immunogenicity, nicotine pharmacokinetics study in mice demonstrated that NanoNicVac 20.0 could reduce the entry of nicotine into brain more effectively than the other vaccines.

2. Experimental section

2.1. PLGA NP fabrication

PLGA NPs containing immunological effectors, including keyhole limpet hemocyanin (KLH) and CpG ODN 1826, were formed via a method described in previous studies with modifications [14, 24, 25]. Briefly, PLGA (30 mg) dissolved in dichloromethane (1 mL) was emulsified with KLH (1.2 mg) and CpG ODN 1826 (0.6 mg) in phosphate-buffered saline (PBS) buffer (100 μ L, 10 mM, pH 7.4) using a Branson B1510DTH Ultrasonic Cleaner (Branson, Danbury, CT) for 10 min. This primary emulsion was added drop-wise into PVA (100 mL, 0.5% (w/v)), followed by stirring at 500 rpm for 10 min. The above mixture was further sonicated using a sonic dismembrator (Model 500; Fisher Scientific, Pittsburg, PA) at 70% amplitude for 30 s. The secondary emulsion was stirred overnight to allow evaporation of dichloromethane. Large particles precipitated and were removed after the mixture was sat at room temperature for 30 min. NPs in the suspension were recovered by centrifugation at 10,000 *g*, 4 °C for 60 min using an Eppendorf centrifuge (Eppendorf, Hauppauge, NY). The collected NPs were suspended in 10 mL PBS buffer (pH 7.4) and stored at 4 °C for later use.

2.2. Liposome formation

Liposomes with different concentrations of DSPE-PEG(2000)COOH were formed via a lipid film rehydration and sonication technique [14, 24]. Briefly, lipid films containing MPLA (0.2 mg), and other lipids (6 mg), including DOTAP, DSPE-PEG(2000)COOH, and cholesterol, with molar ratios of 92.5:2.5:5.0 (Liposome 2.5), 90:5.0:5.0 (Liposome 5.0), 82.5:12.5:5.0 (Liposome 12.5), 75.0:20.0:5.0 (Liposome 20.0), and 65.0:30.0:5.0 (Liposome 30.0) were hydrated with 1 mL 55 °C pre-warmed PBS buffer (pH 7.4). The lipid mixture was vortexed for 2 min, followed by 5 min sonication using a Branson B1510DTH Ultrasonic Cleaner (Branson, Danbury, CT) to form liposomes with differing degrees of PEGylation. The formed liposomes were stored at 4 °C for later use.

2.3. Lipid-PLGA hybrid NP assembly and NanoNicVac synthesis

Lipid-PLGA hybrid NPs with degrees of PEGylation of 2.5% (Hybrid 2.5), 5.0% (Hybrid 5.0), 12.5% (Hybrid 12.5), 20.0% (Hybrid 20.0), and 30.0% (Hybrid 30.0) were assembled via a sonication-aided fusion technique described before [14, 24]. The PLGA NPs and the liposomes prepared above were mixed and pre-homogenized for 15 min using a Branson B1510DTH Ultrasonic Cleaner, followed by 5 min sonication in an ice bath using a sonic dismembrator at 15% amplitude (pulse on 20 s, pulse off 50 s). Rac-trans 3'-aminomethyl nicotine (Nic) was conjugated to Hybrid 2.5, Hybrid 5.0, Hybrid 12.5, and Hybrid 20.0 to form NanoNicVac 2.5, NanoNicVac 5.0, NanoNicVac 12.5, and NanoNicVac 20.0, respectively. NanoNicVac was synthesized using a previously described method with modifications [14]. Briefly, the hybrid NPs were dialyzed against activation buffer (500 mL, 0.1M MES, 0.5M NaCl, pH 6.0) for 2 h. EDC (6.3 mg) and sulfo-NHS (17.3 mg) were added into the hybrid NP suspension and reacted for 20 min at room temperature. The hybrid NPs in the activation buffer were dialyzed against coupling buffer (1000 mL, 100 mM sodium phosphate, 150 mM NaCl; pH 7.2) for 30 min. Nic (6.3 mg) was reacted with the activated hybrid NPs in the coupling buffer for 4 h. Impurities were removed by dialysis against PBS buffer (pH 7.4) for 12 h. The assembled NanoNicVac was stored at 4 °C for future use.

2.4. Assembly of fluorescently-labeled NPs

The assembly process of fluorescently-labeled hybrid NPs was similar to that for regular hybrid NPs as described above. KLH in PLGA NP was labeled with Alexa Fluor® 647 (Alexa 647) or Alexa Fluor® 750 (Alexa 750) and the lipid layer was labeled with NBD PE. KLH was labeled with Alexa 647 or Alexa 750 using a method described in a previous study [14]. These fluorescently-marked vaccine particles did not contain either CpG ODN 1826 or MPLA.

2.5. Measuring the association rate of the lipids and the PLGA NPs

To calculate the association rate of lipids and PLGA in the hybrid NPs, the NBD intensity in the liposome, and Alexa 647 intensity in the PLGA NP were measured prior to the hybrid NP assembly. After hybrid NP assembly and purification via centrifugation, intensities of both NBD in the lipid layer and Alexa 647 in the PLGA core were recorded. The relative intensity ratios of NBD to Alexa 647 were calculated for hybrid NPs with varying degrees of PEGylation.

2.6. Characterization of physicochemical properties of NPs and NanoNicVac

Physicochemical properties, including surface charge, mean particle size, and size distribution of NPs and NanoNicVac were characterized using a Malvern Nano-ZS zetasizer (Malvern Instruments Ltd, Worcestershire, United Kingdom).

2.7. Morphological study of NPs and NanoNicVacs using a transmission electrical microscopy (TEM)

TEM images of liposome, PLGA NP, hybrid NPs, and NanoNicVacs were acquired using a method described in previous studies with proper modifications[24–26]. Briefly, particles in

PBS buffer 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).

2.8. Study of cellular uptake of lipid-PLGA hybrid NPs by flow cytometry

Dendritic cells (DCs) were cultured in CytoOne(R) 35 × 10 mm TC dish (USA Scientific Inc, Ocala, FL) using the same method reported before [14]. For study on uptake of newly made hybrid particles with various degrees of PEGylation, 2×10⁶ DCs were incubated with fluorescently-labeled hybrid NPs (500 µg) for 30 min, 60 min, and 120 min, respectively. For study on uptake of NPs stored at 4 °C in PBS buffer for 30 days, 2×10⁶ DCs were incubated with fluorescently-labeled hybrid NPs (500 µg) for 120 min. After incubation, sample processing was similar to the method described before [14]. Briefly, the medium was immediately removed and cells were washed 5 times with deionized water. Cells were detached from culture plate using 1 mL trypsin/EDTA solution. Cell samples were immediately analyzed by a BD FACSARIA flow cytometer (BD, Franklin Lakes, NJ) and a Amnis® ImageStream® X Mark II flow cytometer (MilliporeSigma, Billerica, MA), respectively.

2.9. Study of KLH *in vitro* release from NanoNicVac

Two milligrams of NanoNicVac particles (KLH in the PLGA core was labeled with Alexa 647) in 1 mL human serum (5% v/v, pH 7.4) was placed in a Spectra/Por Float-A-Lyzer G2 dialysis device. The NPs were dialyzed against 30 mL human serum (5% v/v, pH 7.4) at 37 °C in darkness. At given time points, the fluorescence intensity of the NPs was recorded at excitation wavelength of 620 nm and emission wavelength of 680 nm using a Synergy HTX Plate Reader (BioTek, Winooski, VT). After each measurement, NPs was dialyzed against fresh human serum. The percentage of released KLH was defined as 100*(1-fluorescence intensity at a given time point / fluorescence intensity at 0 h). The fluorescence in the NPs after dialysis against human serum was also visualized with an IVIS SpectrumCT (PerkinElmer, Waltham, MA) at 0, 12, 24, 72, and 240 h, respectively.

2.10. Monitoring *in vivo* degradation of NanoNicVac in mice

Two milligrams of Alexa 750-labeled NanoNicVac particles was subcutaneously injected into female BALB/c mice (8–10 weeks). The degradation of the vaccine particles in the mice was monitored with an IVIS SpectrumCT (PerkinElmer, Waltham, MA). The *in vivo* fluorescence image was taken 0, 4, 12, 24 h post vaccine injection and fluorescence intensity (represented by radiation efficiency) from the region of interest (ROI) was defined manually.

2.11. Immunizing mice with NanoNicVac

All animal studies were carried out following the National Institutes of Health 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. Groups of n = 5 female BALB/c mice (8–10 weeks) were immunized by subcutaneous (s.c.) injection on

day 0 (primary injection) and day 14 (booster injection) with PBS buffer (10 mM, pH 7.4) (negatively control), NanoNicVac 2.5, NanoNicVac 5.0, NanoNicVac 12.5, and NanoNicVac 20.0, respectively. All vaccine constructs contained a total of 25 µg KLH and 10 µg of MPLA. Blood samples (~200 µL) were collected on days -2, 13, 28, and 35 via retroorbital puncture from each mouse. Sera centrifuged from blood were stored at -80 °C before analyses.

2.12. 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 modifications [8, 14]. Briefly, Nicotine- bovine serum albumin (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. Nic-BSA was synthesized using protocols described in a previous study [14]. MICROLON® 96 well plates (Greiner BioOne, Longwood, FL) were coated with Nic-BSA conjugate or KLH (10 µg/mL in carbonate buffer, 0.05 M, pH 9.6, 100 µL/well) and incubated at 25 °C for 5 h. The plates were washed with PBSTween (0.1%) for 3 times and distilled water for 3 times, followed by blocking with 300 µL Pierce® protein-free T20 blocking buffer for 12 h. After washing, each dilution (1:25, 1:125, 1:625, 1:3125, 1:15625, 1:78125, and 1:390625) of serum (100 µL) from each mouse was incubated in plates at 25 °C for 2 h. The plates were washed again, and incubated with anti-mouse IgG (100 µL) from goat (1:5000) from Alpha Diagnostic Intl (San Antonio, TX) for 1 h. The plates were washed as before, and incubated with 100 µL Anti-Goat IgG-HRP (1:5000) (Alpha Diagnostic Intl, San Antonio, TX) for 1 h. After washing as before, TMB One Component Microwell Substrate (SouthernBiotech, Birmingham, AL) (100 µL) was added into each well and incubated for 10 min, and the reaction was stopped by H₂SO₄ (100 µL, 0.5% (v/v)). The absorbance for each well at 450 nm was recorded. Titer was defined as the dilution factor at which OD₄₅₀ fell to half of the maximal.

2.13. Evaluation of the pharmacokinetic efficacy of NanoNicVac in mice

On day 37, both the mice immunized with NanoNicVacs and the mice in the negative control group were administered with nicotine (0.06 mg/kg) subcutaneously. The mice were sacrificed 4 min post nicotine challenge, and brain tissues were collected. Nicotine contents in the brain tissues were analyzed by gas chromatography/mass spectrometry according to a method reported previously [11, 27].

2.14. Data analysis

Data were compared among groups using one way ANOVA and comparisons among paired groups were analyzed with Tukey's HSD. The difference is considered as significant when P-value is less than 0.05. Each measurement was carried out at least three times, and the results were expressed as mean ± standard deviation.

3. RESULTS AND DISCUSSION

3.1. Characterization of physicochemical properties of hybrid NPs

In a previous study, a hybrid NP-based nicotine vaccine was reported to induce a strong and highly nicotine-specific antibody response [14]. In this study, the influence of PEG on the stability as well as the immunological efficacy of the vaccine were studied.

The lipid-PLGA hybrid NP in this study was assembled by sonication-aided fusion of liposome with PLGA NPs. As shown in Fig. 1A, the mean sizes of liposomes with 30% (Liposome 30.0), 20% (Liposome 20.0), 12.5% (Liposome 12.5), 5% (Liposome 5.0), and 2.5% (Liposome 2.5) DSPE-PEG(2000)COOH were 287.1 ± 4.0 nm, 288.3 ± 1.6 nm, 292.8 ± 7.5 nm, 288.0 ± 3.7 nm, and 294.9 ± 10.5 nm, respectively. As shown in Fig. 1B, the surface charges represented by zeta potential of Liposome 30.0, Liposome 20.0, Liposome 12.5, Liposome 5.0, and Liposome 2.5 were -22.77 ± 0.65 mV, -17.60 ± 0.44 mV, -13.73 ± 0.35 mV, -8.43 ± 0.25 mV, and -6.83 ± 0.35 mV, respectively. In the meantime, the PLGA NP was fabricated via double emulsion and solvent evaporation [14]. As shown in Fig. 1A and Fig. 1B, the mean size of the PLGA NP was 229.6 ± 4.5 nm and its mean surface charge was -25.88 ± 0.42 mV. The physicochemical properties of the lipid-PLGA hybrid NPs were also characterized. Also illustrated in Fig. 1A, the hybrid NPs with 30% (Hybrid 30.0), 20% (Hybrid 20.0), 12.5% (Hybrid 12.5), 5% (Hybrid 5.0), and 2.5% (Hybrid 2.5) PEGylation had a mean size of 245.6 ± 2.6 nm, 236.1 ± 2.3 nm, 233.7 ± 1.3 nm, 235.8 ± 3.0 nm, and 237.7 ± 1.1 nm, respectively. As shown in Fig. 1B, the surface charges of Hybrid 30.0, Hybrid 20.0, Hybrid 12.5, Hybrid 5.0, and Hybrid 2.5 were -23.30 ± 1.02 mV, -8.39 ± 0.24 mV, -6.59 ± 0.21 mV, -5.80 ± 0.20 mV, and -3.99 ± 0.22 mV, respectively.

3.2. Morphologies of hybrid NPs

Consistent with the size results, the TEM micrographs (Fig. 2A–Fig. 2C) showed that the particles, including the liposome, the PLGA NP, and the hybrid NPs, had a diameter at around 200 nm. In Fig. 2A, a distinct bilayer structure with a thickness of 10 nm was observed in the liposomes. In agreement with previous findings, the PLGA NPs exhibited a solid and spherical structure with a narrow size distribution (Fig. 2B) [24, 25]. After sonication, liposomes with different degrees of PEGylation were coated onto the PLGA NPs. As also indicated in previous studies, the size of the hybrid NP was largely dictated by that of the PLGA NP [24, 28]. All hybrid NPs in this study had a size distribution similar to that of the PLGA NPs. This was also supported by the similar mean particle sizes between the two types of particles (Fig. 1A). As shown in Fig. 2C1–Fig. 2C5, a lipid layer (the black ring on the surface of the hybrid NPs) was observed in all lipid-PLGA hybrid NPs with the exception of Hybrid 30.0. The lipid layer of Hybrid 30.0 (Fig. 2C5) looked markedly different from those of the other hybrid NPs (Fig. 2C1–C4). The color of the lipid ring in Hybrid 30.0 was apparently lighter than those of the other hybrid NPs, which might be caused by the relatively lower quantity of lipids in the lipid layer. In addition, the confocal images of the hybrid NPs supported the speculation that Hybrid 30.0 had a lower quantity of lipids in the lipid layer than the others'. As described in a previous studies, labeled with NBD in the lipid layer and Alexa Fluor® 647 (Alexa 647) in the PLGA core, hybrid NPs displayed as yellow dots under a confocal microscope [14, 26]. In this study, the confocal

images of the hybrid NPs from Fig. 2D1 to Fig. 2D4 exhibited yellow color, which resulted from the combination of the green color (NBD) emitted from the lipid layer and the red color (Alexa 647) emitted from the PLGA core. In contrast, only red color was detected in most of the Hybrid 30.0 particles in Fig. 2D5. Moreover, the mean size and surface charge of Hybrid 30.0 did not follow the patterns of the other hybrid NPs (Fig. 1A and Fig. 1B). Hybrid 30.0 with a mean size at 245 nm was significantly bigger than those of the other hybrid NPs, which shared a particle size at around 235 nm. The surface charge of Hybrid 30.0 was much lower than that of the others'. To explain these differences between Hybrid 30.0 and the other hybrid NPs, these NPs were labeled with Alexa 647 in the PLGA core, and NBD in the lipid layer, and the NBD/Alexa 647 fluorescence ratio was calculated before and after the formation of the hybrid NPs. As shown in Fig. 1C, NBD/Alexa 647 ratio in Hybrid 20.0, Hybrid 12.5, Hybrid 5.0, and Hybrid 2.5 was not significantly different from that before hybrid NP assembly. In contrast, Hybrid 30.0 had a significantly lower NBD/Alexa 647 ratio than that before its assembly, revealing that significantly lower quantity of lipids was coated onto the PLGA core in Hybrid 30.0. Due to its compromised structure, Hybrid 30.0 was not used for subsequent studies.

3.3. *In vitro* uptake of newly-assembled hybrid NPs by DCs

Although, the PEG molecules in the hybrid NPs can function as a shield for the NPs from harsh physiological environment to minimize NP degradation during circulation, they may exert steric hindrance to the interaction between NPs and immune cells [24, 28–30]. The influence of PEGylation on the uptake of hybrid NP by DCs was investigated. Two million DCs were incubated with 500 μ g hybrid NPs with various degrees of PEGylation (the lipid layer was labeled with NBD, and keyhole limpet hemocyanin in the PLGA core was labeled with Alexa 647) for 30 min, 60 min, and 120 min, respectively. To quantitatively study their uptake, the fluorescence intensities of both NBD and Alexa 647 emitted from the hybrid NPs internalized into the DCs were recorded by a BD FACSARIA flow cytometer. As shown in Fig. 3A, over 90% of the cells internalized NPs regardless of their lipid composition. However, the number of NPs taken up by the DCs judging from the median intensities of NBD and Alexa 647 (shown in Fig. 3B and Fig. 3C, respectively) suggests that the uptake of the NPs was both time-dependent and composition-dependent. The quantities of the NPs captured by the DCs increased over time. From 30 min to 120 min, the Alexa 647 median intensities in the cells increased at least 400% for all hybrid NPs. In addition, the DCs internalized less hybrid NPs that contained more PEG in the lipid layer than those with less PEG. For instance, 80% more Hybrid 2.5 was taken up by the DCs than Hybrid 20.0 at 120 min. These results suggest that PEG in the lipid layer hindered cellular uptake of the NPs. Consistent with previous results, the DCs showed both green (NBD) and red (Alexa 647) fluorescent colors at any given time points, indicating that NPs with an intact core-layer hybrid structure was captured by the cells [14]. The cellular uptake of the hybrid NPs was also qualitatively studied using an ImageStream flow cytometer. Consistent with the results in Fig. 3B and Fig. 3C, the microscopic images in Fig. 3D show that the quantities of the captured NPs increased with time for all the hybrid NPs, which was represented by the brighter fluorescence at longer time points. In addition, brighter fluorescence were observed in the cells treated with the hybrid NPs of lower degrees of PEGylation at all the studied time points. These results indicate that PEG molecules in the hybrid NPs may exert steric

hindrance between the NPs and immune cells, leading to an impeded cellular uptake of the NPs.

3.4. Impact of PEGylation on the size of stored hybrid NPs

The standard of practice for vaccines is that, before use, a prepared vaccine will be stored for a certain period of time. Therefore, it is important to maintain the stability of vaccine particles during storage. In this study, the impact of long-term storage of hybrid NPs on their particle size were studied. Hybrid NPs (labeled with Alexa 647 in the PLGA core) were stored at 4 °C in PBS buffer for 30 days. The mean particle sizes of these NPs were recorded before and after storage. As shown in Fig. 1D, newly made hybrid NPs, including Hybrid 20.0, Hybrid 12.5, Hybrid 5.0, and Hybrid 2.5, had a mean particle size of 247.4 ± 1.9 nm, 246.1 ± 2.6 nm, 246.7 ± 2.0 nm, and 248.3 ± 4.0 nm, respectively. After storage, the meansize of Hybrid 12.5, Hybrid, 5.0, and Hybrid 2.5 significantly increased to 295.5 ± 5.2 nm, 356.2 ± 5.9 nm, and 466.7 ± 4.4 nm, respectively. In contrast, the mean size of Hybrid 20.0 only slightly increase to 251.5 ± 2.8 nm, which was not significantly different from that of the freshly prepared Hybrid 20.0. A previous research found that hybrid NPs could aggregate with each other to form bigger particles during storage [24]. The smaller increase in particle size among NPs with higher PEG densities indicates that PEGylation may reduce the chance of aggregation among the hybrid particles. This explanation is supported by other findings that PEG can increase the steric distance among NPs and decrease the surface energy of NPs and minimize van der Waals attraction, thereby reducing NPs aggregation [15, 31].

3.5. The influence of the change in size of the stored hybrid NPs on cellular uptake

Results from a previous study demonstrated that particle size of NanoNicVac could have significant impact on the uptake of vaccine particles by immune cells and immunogenicity of the vaccine [27]. Therefore, the influence of size change on the uptake of NanoNicVac by DCs was studied. Two million DCs were incubated with 500 µg of the stored hybrid NPs for 120 min. As shown in Fig. 4A, the increase in particle size had a significant impact on the uptake of NPs by the DCs. The increased size of hybrid NPs resulted in remarkably impeded cellular uptake. At 120 min, only 44.9% of the DCs internalized Hybrid 2.5 after storage, which was considerably lower than that of newly-formed Hybrid 2.5. Significantly higher percentages of the cells captured hybrid NPs with higher PEG densities than those with lower PEG densities. In addition, as shown in Fig. 4B, the quantity of the internalized NPs, represented by the Alexa 647 median intensity, was positively related to the degree of PEGylation. The DCs internalized 200% more Hybrid 20.0, which didn't undergo significant size increase after storage, than Hybrid 2.5. The uptake of the stored hybrid NPs was further qualitatively studied using an ImageStream flow cytometer. Consistent with the results in Fig. 4B, the microscopic images in Fig. 4C showed that Alexa 647 fluorescence is much dimer in the cells treated with NPs containing less PEG than those with more PEG. The slower uptake of NPs of bigger size by the DCs was expected and was consistent with findings from other studies [17, 32]. These results indicate that more PEG in the lipid layer could more effectively reduce the size increase of the hybrid NPs during storage, leading to improved cellular uptake of the stored hybrid NPs.

3.6. Characterization of the physicochemical properties and morphology of NanoNicVacs

NanoNicVacs were assembled by conjugating *rac-trans* 3'-aminomethyl nicotine onto the hybrid NPs with different degrees of PEGylation. Their physicochemical properties, including particle mean size, size distribution, and surface charge, were characterized. In addition, the TEM images of NanoNicVacs were captured to study the morphology of NanoNicVacs. As shown in Fig. 5, NanoNicVac 2.5, NanoNicVac 5.0, NanoNicVac 12.5, and NanoNicVac 20.0 had very similar mean sizes, which were 241 ± 1.3 nm, 240 ± 4.9 nm, 238.9 ± 4.3 nm, and 240.4 ± 6.4 nm, respectively. The zeta potentials of NanoNicVac 2.5, NanoNicVac 5.0, NanoNicVac 12.5, and NanoNicVac 20.0 were -4.51 ± 0.73 mV, -6.21 ± 0.97 mV, -7.38 ± 2.32 mV, and -10.10 ± 1.14 mV, respectively. All NanoNicVac particles in TEM micrographs (Fig. 5) displayed a core-shell structure with a particle size at around 200 nm. The similar sizes of the different NanoNicVacs suggests that the vaccine assembly process was robust and could produce uniform particles. As reported in the previous studies, vaccine particles with nano-ranged size and a core-shell structure were likely to elicit high magnitude of immune response [5, 9, 11, 14].

3.7. *In vitro* antigen release from NanoNicVac

To evaluate the stability of NanoNicVacs under physiological conditions, the release of KLH from NanoNicVacs during incubation with human serum (5% v/v, pH 7.4) was studied. As shown in Fig. 6A, all NanoNicVac particles displayed a burst release of KLH in the first 48 h. This is consistent with results from other studies on drug/antigen release from hybrid NPs [28, 33]. The burst release might be caused by bulk erosion of the PLGA core [34]. However, NanoNicVac 20.0 released significantly lower amount of KLH compared to the other NPs at any given time points. For instance, NanoNicVac 20.0 released $42.7\pm2.7\%$ of KLH after 48 h, while NanoNicVac 2.5, NanoNicVac 5.0, and NanoNicVac 12.5 released $83.2\pm0.4\%$, $78.1\pm0.6\%$, and $71.9\pm3.1\%$, respectively. In addition, the antigen release rate was negatively corrected with the concentration of PEG. This might be caused by the higher stability in the NanoNicVacs that had higher concentrations of PEG in the lipid layer [35]. These results were substantiated by the Alexa 647 fluorescence in NanoNicVacs visualized using an IVIS Spectrum CT. As shown in Fig. 6B, before the dialysis, all vaccine NPs showed similar fluorescence intensity. The NPs were losing fluorescence in human serum over time. In addition, NPs with less PEG lost the fluorescence faster than those with more PEG. At 240 h, very little Alexa 647 was detectable in NanoNicVac 2.5, while considerable amount of Alexa 647 was still in NanoNicVac 20.0, indicating NanoNicVac 20.0 had a remarkably higher stability than NanoNicVac 2.5. The high stability of NanoNicVac 20.0 may enable its extended circulation in the body and improve the bioavailability of the NP-based vaccine to the immune system, thereby promoting the immunological efficacy of vaccine [36].

3.8. *In vivo* degradation of NanoNicVac in mice

To study the *in vivo* degradation of NanoNicVac, mice were subcutaneously injected with Alexa 750-labeled NanoNicVac 20.0, NanoNicVac 12.5, NanoNicVac 5.0, and NanoNicVac 2.5, respectively. The degradation of the vaccine particles were monitored using an IVIS Spectrum CT. As shown in Fig. 7A, the degradation of all NanoNicVac was time-dependent,

and most of the vaccine particles regardless of the degree of PEGylation were degraded within 24 h. Consistent with the results from the *in vitro* antigen release (Fig. 6), NanoNicVacs with higher PEGylation degraded slower than those with lower PEGylation. The fluorescence intensity (represented by radiation efficiency) from the vaccine particles was also measured. As shown in Fig. 7B, significantly higher fluorescence intensities were detected in mice administered with NanoNicVac 20.0 than those administered with the other NanoNicVacs 4, 12, and 24 h post vaccine injection. These results show that NanoNicVac 20.0 is more stable *in vivo* than the other vaccine particles. The slow *in vivo* degradation of NanoNicVac 20.0 may enhance its immunogenicity in two different ways. On one hand, its high stability could maintain the integrity of the hybrid structure, which is essential for inducing a nicotine-specific antibody response [14]. On the other hand, prolonged presence of NanoNicVac 20.0 may provide extended contact with immune cells, leading to enhanced immune response [37].

3.9. Nicotine and KLH specific IgG antibody titers induced by NanoNicVacs

In a previous study, we found that a second booster shot of the hybrid NP-based nicotine vaccine did not increase the titers of anti-nicotine antibodies in the mice, therefore, the mice in this study were immunized with a primary shot and one booster shot [14]. Moreover, our previous results demonstrated that the nanoparticle-based nicotine vaccine is significantly better in inducing immune response against nicotine than nicotine-protein conjugate vaccine, [14] and therefore, no conjugate vaccines were used as a control for comparison. Each group of 5 mice were injected with PBS buffer (negative control), NanoNicVac 2.5, NanoNicVac 5.0, NanoNicVac 12.5, and NanoNicVac 20.0, respectively, on day 0 and day 14. As shown in Fig. 8A, different levels of anti-nicotine antibody titer were elicited by NanoNicVacs with varying degrees of PEGylation. On day 13, NanoNicVac 2.5, NanoNicVac 5.0, NanoNicVac 12.5, and NanoNicVac 20.0 induced anti-nicotine IgG titers of $0.9 \pm 0.2 \times 10^3$, $3.1 \pm 0.4 \times 10^3$, $2.9 \pm 0.1 \times 10^3$, and $3.2 \pm 0.4 \times 10^3$, respectively. Two weeks after the booster injection, NanoNicVac 20.0 achieved a titer of $2.5 \pm 0.3 \times 10^4$, which was significantly higher than $1.9 \pm 0.4 \times 10^3$, $4.9 \pm 1.3 \times 10^3$, and $3.9 \pm 0.9 \times 10^3$ that were induced by NanoNicVac 2.5, NanoNicVac 5.0, and NanoNicVac 12.5, respectively. On day 35, anti-nicotine IgG titers in the mice immunized with NanoNicVac 2.5, NanoNicVac 5.0, NanoNicVac 12.5, and NanoNicVac 20.0 rose to $7.5 \pm 1.2 \times 10^3$, $8.9 \pm 1.1 \times 10^3$, and $6.3 \pm 0.9 \times 10^3$, and $3.7 \pm 0.6 \times 10^4$, respectively. The antibody titers induced by NanoNicVacs demonstrate that NanoNicVac 20.0 with the highest degree of PEGylation was significantly more immunogenic than the other NanoNicVacs. It is well known that uptake of antigen by B cells is mediated by the cognate binding of the hapten on an antigen to the B cell receptors [38]. The nicotine hapten on NanoNicVacs was linked to the PEG molecules, and thus the nicotine hapten density was decided by the degree of PEGylation. The higher nicotine hapten density on NanoNicVac 20.0 may enable more efficient uptake by B cells than the other vaccines. In addition, part of the intracellular signals for B cell activation is generated by the crosslinking of B cell receptors with haptens of an antigen [39, 40]. A higher hapten density on NanoNicVac may allow more extensive and effective B cell receptor crosslinking to produce stronger signals for B cell activation. The stronger immunogenicity of NanoNicVac 20.0 may partially be caused by its higher stability, which may improve bioavailability of the vaccine particles to the immune cells [41]. PEG is usually not an essential part of nanoparticles but serves as an

agent that can improve the stability of nanoparticles and prolong their circulation time [16]. However, in this study, PEG is an indispensable component of NanoNicVac, because nicotine hapten is conjugated to the reactive groups on the terminal of PEG molecules. The degree of PEGylation in NanoNicVac does not only affect the circulation time of the vaccine, but also decide the density of nicotine haptens, thereby influencing the immunological performance of the vaccine.

In this study, KLH enclosed in the vaccine particles was intended to function as a source of T-cell antigens for activating helper T cells [42]. As discussed in a previous study, one of the important features of NanoNicVac was that it could minimize the production of antibody against T-cell antigen from KLH, improving its specificity [14]. In this study, to evaluate the specificity of the vaccines, the titers of anti-KLH IgG were measured. As shown in Fig. 8B, the titers of IgG against KLH were also measured. On day 13, the titers of anti-KLH IgG were $1.1 \pm 0.3 \times 10^3$, $1.0 \pm 0.2 \times 10^3$, $1.1 \pm 0.2 \times 10^3$, and $1.0 \pm 0.2 \times 10^3$ for NanoNicVac 2.5, NanoNicVac 5.0, NanoNicVac 12.5, and NanoNicVac 20.0, respectively. The levels of anti-KLH antibody generated by NanoNicVacs were minimal after the primary injection. However, 14 days after the booster injection (day 28), anti-KLH antibody titers remarkably increased to $4.5 \pm 0.6 \times 10^4$, $1.2 \pm 0.3 \times 10^4$, $1.5 \pm 0.2 \times 10^4$, and $1.0 \pm 0.3 \times 10^4$ in mice immunized with NanoNicVac 2.5, NanoNicVac 5.0, NanoNicVac 12.5, and NanoNicVac 20.0, respectively. Continued increase in anti-KLH antibody were observed on day 35 for all the vaccine groups, in which NanoNicVac 2.5 group increased to $6.9 \pm 0.5 \times 10^4$, NanoNicVac 5.0 group increased to $5.7 \pm 0.5 \times 10^4$, NanoNicVac 12.5 group increased to $10.0 \pm 0.5 \times 10^4$, and NanoNicVac 20.0 group increased to $8.0 \pm 2.0 \times 10^4$. The increase in the titer of anti-KLH antibody after the booster injection was expected. These anti-KLH IgG might be caused by the KLH released from NanoNicVac particles, which were degraded after long-term circulation in the body.

3.10. Brain nicotine concentrations in mice

Nicotine in tobacco is widely considered the primary substance that is responsible for smoking addiction [43]. The treatment efficacy of a nicotine vaccine is largely influenced by its ability to reduce the quantity of nicotine that enters into the brain [44]. As shown in Fig. 9, the negative control group had a brain nicotine concentration as high as 95 ± 15 ng/g. In contrast, the mice immunized with NanoNicVac 2.5, NanoNicVac 5.0, NanoNicVac 12.5, and NanoNicVac 20.0 had a brain nicotine concentration of 65 ± 8 ng/g, 55 ± 20 ng/g, 74 ± 10 ng/g, and 34 ± 11 ng/g, respectively. The significantly lower quantity of nicotine in the brain of the immunized mice as compared to that in the negative control demonstrates that all NanoNicVac, regardless of the degree of PEGylation, could effectively block the entry of nicotine into the brain. In addition, the ability of NanoNicVac to reduce the brain nicotine concentration was highly correlated with their capability in producing anti-nicotine antibodies. The mice with a higher titer of anti-nicotine antibody (Fig. 8A) had a lower nicotine concentration in the brain. Such a correlation between the antibody titer and the nicotine pharmacokinetics was also widely observed in other studies [45, 46]. As shown in Fig. 9, it appears that NanoNicVac with a denser PEG tends to result in a lower brain nicotine concentration. Overall, NanoNicVac 20.0 achieved considerably better immunological efficacy than the other NanoNicVacs. The superior performance of

NanoNicVac 20.0 might be attributed to its better ability to activate B cells and improved bioavailability to the immune system.

4. Conclusions

In summary, PEGylation presents a paradox in the development of nanovaccines against nicotine addiction. Higher degree of PEGylation tends to stabilize the nanovaccine particles, but it may hinder the uptake of the particles by the antigen presenting cells, such as DCs. Hybrid NP with 30% PEGylation failed to form a stable hybrid structure and was not suitable for vaccine assembly. Higher degree of PEGylation in the hybrid NPs resulted in slower uptake by DCs. However, hybrid NPs with more PEGs in the lipid layer were more stable and could more effectively reduce aggregation than those with less PEGs during storage, leading to facilitated cellular uptake. In both *in vitro* and *in vivo* studies, NanoNicVac 20.0 with the highest degree of PEGylation showed significantly higher stability than other NanoNicVacs. NanoNicVac 20.0 also achieved the highest anti-nicotine IgG titer as well as the lowest brain nicotine level in mice. These results suggest that a 20% PEGylation was optimal for the immunogenicity of NanoNicVac. Lastly, no safety issues were detected in the mice immunized with any of the NanoNicVac formulations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Characterization of physicochemical properties of NPs. (A) Mean diameter of NPs. (B) Surface charge of NPs. (C) NBD/Alexa 647 intensity ratios before and after assembly of hybrid NPs. For fluorescence labeling, the lipid layer was labeled with NBD and KLH in the PLGA core was labeled with Alexa 647. (D) Change in particle size of the hybrid NP after storage. Hybrid NPs including Hybrid 20.0, Hybrid 12.5, Hybrid 5.0, and Hybrid 2.5 were stored under 4 °C in PBS buffer for 30 days. The mean size of the hybrid particles were recorded before and after storage. ***denotes a P-value that is less than 0.01.

Fig. 2.

Morphology of NPs. TEM images of (A) liposome, (B) PLGA NP. TEM confocal images of (C1) Hybrid 2.5, (C2) Hybrid 5.0, (C3) Hybrid 12.5, (C4) Hybrid 20.0, and (C5) Hybrid 30.0. Confocal images of (D1) Hybrid 2.5, (D2) Hybrid 5.0, (D3) Hybrid 12.5, (D4) Hybrid 20.0, and (D5) Hybrid 30.0. In the confocal images, the lipid layer and the KLH in the PLGA core were labeled with NBD and Alexa 647, respectively. The scale bars represent 200 nm in the TEM images and 20 μ m in the confocal images.

Fig. 3.

Cellular uptake of newly-synthesized hybrid NPs. Two million DCs were treated with 500 μg hybrid NPs, which were labeled with NBD (green) in the lipid layer and Alexa 647 (red) in the PLGA core, for 30, 60, and 120 min, respectively. The cellular uptake of the NPs were quantitatively and qualitatively studied using a BD FACSARIA flow cytometer and an Amnis® ImageStream flow cytometer, respectively. (A) The percentage of cells that internalized NPs. (B) The NBD median intensity in the cells. (C) The Alexa 647 median intensity in the cells. (D) Representative microscopic images of cellular uptake of the hybrid NPs by the DCs.

Fig. 4.

Cellular uptake of stored hybrid NPs. Two million DCs were incubated with 500 μg hybrid NPs that underwent 30 days' storage at 4 °C in PBS buffer for 120 min. The cellular uptake of the NPs which were labeled with Alexa 647 were quantitatively and qualitatively studied using a BD FACSARIA flow cytometer and an Amnis® ImageStream flow cytometer, respectively. (A) Dots plot of cellular uptake of NPs. (B) The Alexa 647 median intensity in the DCs. (C) Representative microscopic images of cellular uptake of the hybrid NPs. *** in Fig. 4. A means the percentage of cells internalized NPs that contained more PEG was significantly higher than those with less PEG in the lipid layer. *** denotes a P-value that is less than 0.01.

Fig. 5. Characterization of the physicochemical properties and morphology of NanoNicVacs. NanoNicVacs with different densities of nicotine hapten were schematically illustrated. Their corresponding size distribution, mean particle size, surface charge, and TEM images were shown. (A) NanoNicVac 2.5. (B) NanoNicVac 5.0. (C) NanoNicVac 12.5. (D) NanoNicVac 20.0. The scale bars in the TEM images represent 200 nm.

Fig. 6.

KLH release from NanoNicVacs. Two mgs of NanoNicVac NPs (KLH as an antigen in the PLGA core was labeled with Alexa 647) in 1 mL human serum (5% v/v, pH 7.4) was placed in a Spectra/Por Float-ALyzer G2 dialysis device and dialyzed against 30 mL human serum (5% v/v, pH 7.4) at 37 °C. (A) Cumulative release of KLH from NanoNicVacs. (B) Images of Alexa 647 fluorescence in NanoNicVacs visualized by an IVIS SpectrumCT at 0, 12, 24, 72, and 240 h, respectively.*** denotes the amount of released KLH in NanoNicVac 20.0 was significantly less than those in the other NanoNicVacs.

Fig. 7.

In vivo degradation of NanoNicVacs in mice. KLH in NanoNicVac was labeled with Alexa 750. Mice were injected with NanoNicVacs and the degradation of the vaccine particles was studied with an IVIS SpectrumCT. (A) Images of NanoNicVac degradation in mice (from left to right, NanoNicVac 20.0, NanoNicVac 12.5, NanoNicVac 5.0, and NanoNicVac 2.5) at 0, 4, 12, and 24 h post vaccine injection. (B) Radiant efficiency of Alexa 750 in mice. *** denotes a P-value that is less than 0.01.

Fig. 8. Time course of anti-nicotine IgG titer and anti-KLH IgG titer in mice immunized with NanoNicVacs. Each group of 5 mice were injected with NanoNicVacs containing 25 μ g KLH on days 0 and 14. The titers of anti-nicotine IgG and anti-KLH IgG in mice sera from days 13, 28, and 35 were measured using ELISA. *** denotes a P-value that is less than 0.01.

Fig. 9.

Brain nicotine concentrations. The mice that received either PBS buffer or NanoNicVacs on day 0 and day 14 were subcutaneously injected with 0.06 mg/kg nicotine on day 37, and the brain nicotine concentrations were analyzed. ** denotes a P-value that is less than 0.05, and *** denotes a P-value that is less than 0.01.