Biomaterials 182 (2018) 72–81

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Paradox of PEGylation in fabricating hybrid nanoparticle-based nicotine vaccines

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ARTICLE INFO

Keywords:
Hybrid nanoparticle
Nicotine vaccine
Immunotherapy
PEGylation
Stability
Smoking cessation

ABSTRACT

Polyethylene glycol (PEG) has long been used in 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.

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

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https://doi.org/10.1016/j.biomaterials.2018.08.015
Received 16 May 2018; Received in revised form 23 July 2018; Accepted 4 August 2018
Available online 07 August 2018

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 DSPE-PEG(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 structures 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 the 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 μL, 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, hybrid NPs were dialyzed against activation buffer (500 mL, 0.1 M MES, 0.5 M 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. 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 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 NPs 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 transmission electron microscopy (TEM)

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