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Rational Design of a Stable, Freeze-Dried Virus-Like Particle-Based Vaccine Formulation

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Virus-like particles (VLPs) have been extensively explored as vaccine candidates since the mid-1980s. Numerous VLPs have been designed as vaccines for prevention of virus-induced infectious diseases and for the therapeutical treatment of chronic diseases and drug addiction. Recently, a vaccine against nicotine addiction, which is based on VLPs of the RNA phage Qb to which nicotine haptens are covalently coupled via succinimate linkers (NicQb), has attracted a great deal of interest. Phase II clinical trials with this vaccine have shown that it is efficacious for smoking cessation in humans when antinicotine antibody levels are sufficiently high. For commercialization, the development of stable formulations enabling storage for prolonged periods is required. Hereby, lyophilization, a well-established method leading to stable and dry formulations, is often applied. In this study, we investigated the influence of different pH values and various excipients such as surfactants, polyols, sugars, and salts on the stability of NicQb in liquid formulations, during freeze thawing, freeze drying, and finally upon storage of the dried product. Lyophilized NicQb formulations were developed which were stable over 6 months at ambient temperature with fully retained biological activity. Hereby, it was found that a combination of the surfactant polysorbate 20 and the disaccharide trehalose was capable to prevent NicQb aggregation and to preserve its integrity (nicotine binding and integrity of VLP shell). Furthermore, asymmetrical flow field-flow fractionation (AF4), a new, promising analytical tool, was established for the investigation of VLP stability.

Keywords virus-like particles; vaccines; freeze thawing; freeze drying; lyophilization; storage stability; formulation; excipients; asymmetrical flow field-flow fractionation

INTRODUCTION

Most vaccines used in the past consisted of inactivated or attenuated forms of whole pathogens (Russo, Turin, Zanella, Ponti, & Poli, 1997). As there is always a slight risk of reversion to aggressive phenotypes, a lot of efforts have been taken to develop similarly potent but safer vaccines. One class of vaccines of growing interest are virus-like particles (VLPs), self assembled capsids of viruses which do not contain any infectious genetic information. VLPs can either be used for the prevention of virus-induced infectious diseases (Block et al., 2006) or for the therapeutical treatment of chronic diseases (Ambuehl et al., 2007) and drug addiction (Maurer & Bachmann, 2006). For the latter purposes, the VLPs are used as carriers that present antigens in a highly ordered repetitive array to the immune system (Bachmann et al., 1993). Thus, strong antibody responses can be induced even against small, low molecular weight antigens (Lechner et al., 2002).

A VLP-based vaccine against nicotine addiction (NicQb) was developed by Cytos Biotechnology AG (Schlieren, Switzerland) on a VLP platform. Nicotine was covalently coupled to the VLP of an RNA phage Qb via a succinimate linker (Maurer et al., 2005). Vaccination with NicQb leads to the induction of nicotine-specific antibodies. The antibodies bind nicotine (from inhaled tobacco smoke) in the blood and inhibit its passage to the brain, as antibodies normally cannot pass the blood–brain barrier. Phase II clinical trials with NicQb have shown that this vaccine is efficacious for smoking cessation in humans when antinicotine antibody levels are sufficiently high (Maurer & Bachmann, 2006).

The requirement of vaccine compositions to be stable and to minimize or avoid chemical and/or physical degradation implies the need of development of formulations satisfying such requirements (Brandau, Jones, Wiethoff, Rexroad, & Middaugh, 2003; Rexroad, Wiethoff, Jones, & Middaugh, 2002). Consequently, the development of a freeze-dried stable formulation is highly desirable. For pharmaceutical applicability, the freeze-dried formulation should meet several criteria: (a) The drug should be stable during manufacture and storage, ideally even at ambient temperature; (b) it should be composed

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of FDA-approved excipients; (c) it should have an acceptable and reproducible appearance; and (d) the preparation of the isotonic or nearly isotonic liquid formulation to be applied should be easy and fast.

Freeze drying is an established process to improve the stability of labile drugs, especially proteins (Wang, 2000) and complex vehicles like virus vaccines (Abdul-Fattah et al., 2007; Adebayo, Sim-Brandenburg, Emmel, Olaleye, & Niedrig, 1998; Sarkar, Sreenivasa, Singh, Dhar, & Bandyopadhyay, 2003; Scott & Woodside, 1976; Zhai et al., 2004), viral vectors (Cruz et al., 2006; Evans et al., 2004; Talsma et al., 1997), liposomes (Engel et al., 1994; Hinrichs, Sanders, De Smedt, Demeester, & Frijlink, 2005; Zingel, Sachse, Roessling, & Mueller, 1996), and lipid–DNA complexes (Allison & Anchordoquy, 2000; Molina et al., 2004). However, during freezing and subsequent drying, the drug is exposed to diverse stress factors which can cause significant loss of activity. During freezing, drug stability can be influenced by exposure to ice–water interfaces (Chang, Kendrick, & Carpenter, 1996), salt and drug concentration effects (Pikal, 2004), pH shifts due to selective crystallization of buffer species (Anchordoquy & Carpenter, 1996; Sarciaux, Mansour, Hageman, & Nail, 1999), and mechanical damage by growing ice crystals (Zhai et al., 2004). During drying, removal of stabilizing hydration shells (Prestrelski, Tedeschi, Arakawa, & Carpenter, 1993) can influence the stability of the drug, just to mention the most common known degradation causes.

Thus, to ensure the stability of the drug during freeze drying and subsequent storage, stabilizing excipients have to be employed. Surfactants, such as polysorbates, are to be added to prevent aggregation of the protein in the liquid formulation, during freezing and reconstitution (Chang et al., 1996; Jones et al., 2001; Shi et al., 2005). Cryoprotectants such as salts, polyols, and sugars are used to stabilize the therapeutic in solution and during freeze thawing (Arakawa, Kita, & Carpenter, 1991; Arakawa & Timasheff, 1982). Lyoprotectants, especially the disaccharides sucrose and trehalose, are utilized to stabilize the drug during drying (Crowe, Carpenter, & Crowe, 1998; Crowe, Crowe, & Carpenter, 1993a, 1993b). Additionally, bulking agents like mannitol can be added to enable faster drying times and to maintain lyophilisates with an attractive appearance (Johnson, Kirchhoff, & Gaud, 2002; Liao, Krishnamurthy, & Suryanarayanan, 2005). For a comprehensive description of stabilization mechanisms of the above-mentioned excipients, the authors refer the reader to more detailed reviews (Schwegman, Hardwick, & Akers, 2005; Wang, 2000).

For a fast progress of formulation development, it is essential to define an adequate analytical setup. Due to the specific properties of NicQb, such as its large size and complex composition, the choice of applicable analytical methods is challenging. Size exclusion-high performance liquid chromatography (SE-HPLC) is the standard analytical tool for detecting fragmentation products as well as soluble aggregate impurities up to several MDa in protein pharmaceuticals. As the molecular weight of a single VLP is already in this size range (Beckett, Wu, & Uhlenbeck, 1988), SE-HPLC can only reveal fragmentation properly in this specific case. Hence, dynamic light scattering (DLS) is commonly used as an analytical tool to investigate aggregated species of VLPs (Shi et al., 2005). As DLS is not capable of resolving different VLP fractions, the method only renders semiquantitative insight into sample composition (Philo, 2006; Santos & Castanho, 1996). A new sensitive and reliable analytical tool for the detection and quantification of VLP aggregates had to be developed. Asymmetrical flow field-flow fractionation (AF4) is described as a versatile separation technique for macromolecules from the size of insulin to colloidal particles of up to the micrometer range (Chuan, Fan, Lua, & Middelberg, 2008; Gabrielson et al., 2006; Giddings, 2000; Reschiglian, Zattoni, Roda, Michelini, & Roda, 2005). When coupling AF4 with multiangle light scattering (MALS) and UV detectors, even the molecular weight of specific fractions can be determined (Fraunhofer & Winter, 2004). Thus, AF4 was investigated for the determination and quantification of VLP aggregates.

Here we describe the rational, fast development of dried, stable NicQb formulations meeting all requirements necessary for large-scale clinical study and commercial use. Furthermore, we present AF4 as a new, highly sensitive, efficient tool for the analysis of VLP formulations which has enabled the fast progress of the formulation development studies.

MATERIALS AND METHODS

Materials

Chemicals

Trehalose-dihydrate was purchased from Ferro Pfanstiehl Laboratories (Waukegan, IL, USA). Sodium chloride, di-sodium hydrogenphosphate dihydrate, sodium dihydrogenphosphate dihydrate, potassium dihydrogenphosphate, and polysorbate 20 were obtained from Sigma Aldrich Chemie GmbH (Buchs, Switzerland). Mannitol was obtained from Sigma-Aldrich Laborchemicalien GmbH (Seelze, Germany), sucrose and di-potassium hydrogenphosphate trihydrate were purchased from Merck KGaA (Darmstadt, Germany). All other chemicals were of analytical grade and purchased from commercial suppliers.

Virus-Like Particles

Several NicQb batches, manufactured as described by Maurer et al. (2005), were obtained from Cytos Biotechnology AG as liquid, frozen and stored at −80°C until use.

Methods

pH Stability Studies

NicQb solutions in a pH range of 4.6–8.2 were manufactured by adjusting the pH of the bulk material either with 0.1 N NaOH or 0.1 N H_3PO_4 and subsequent dilution of the solutions to 1 mg/mL NicQb. The samples were stored at room temperature

up to 14 days. After storage, samples were analyzed via DLS, SE-HPLC, and reversed phase-HPLC (RP-HPLC) at least in duplicate.

Freeze-Thaw Studies

One milliliter of NicQb solutions, with a drug concentration of 0.2 mg/mL, with 30, 60, 90, or 150 mM sodium chloride, 10% trehalose, with or without addition of 0.005% polysorbate 20 buffered with 20 mM sodium phosphate buffer at a pH of 6.4, were filled into 1.5-mL Eppendorf Tubes (Eppendorf AG, Hamburg, Germany). Additionally, samples without trehalose, 0.005% polysorbate 20, and 30 mM NaCl were prepared. The samples were frozen by placing the tubes in a refrigerator at −80°C. After a minimum of 3 h, the samples were thawed at ambient temperature. Freeze-thaw cycles were repeated for five times. Subsequently, the samples were analyzed via DLS at least in duplicate.

Freeze-Drying Studies

NicQb formulations, composed according to Table 1, were prepared by mixing NicQb bulk material with excipient stock solutions. After preparation, generally aliquots of 600 µL were transferred into 2R glass vials (Schott AG, Mainz, Germany) for freeze drying. The samples were freeze dried either in an EPSILON 2–6D pilot scale freeze dryer or an EPSILON 2–12D freeze dryer (Martin Christ Freeze Dryers GmbH, Osterode, Germany) using three different protocols. The samples were frozen to −40°C with a cooling rate of 1°C/min and held below −40°C for a minimum of 3 h. Protocol 1 comprises two primary drying steps, about 25 h at −35°C and 10 h at −20°C, and a 10-h secondary drying step at 20°C at a pressure of 0.045 mbar. In protocol 2, primary drying was conducted at −15°C for 20 h at 0.045 mbar and secondary drying at 40°C for 10 h at 0.007 mbar. Protocol 3 was alike protocol 2 with a 2-h annealing step at −15°C. After annealing, the samples were again frozen to −40°C with a cooling rate of 1°C/min and held below −40°C for further 3 h before primary drying. VLP formulations containing trehalose were dried according to freeze-drying protocol 1. The mannitol- and trehalose-containing formulations were dried according to protocol 2 and 3, and the mannitol-based formulation was dried applying protocol 3. After freeze drying, the chamber was vented with nitrogen and the vials were stoppered with Polydimethylsiloxane and Ethylenetetrafluoroethylen-coated lyophilization stoppers (West Pharmaceutical Services, Inc., Lionville, PA, USA) under vacuum at 800 mbar. The samples were rehydrated with highly purified water to a volume being equivalent to the volume prior to lyophilization. Subsequently, the samples were analyzed via AF4, SE-HPLC, differential scanning calorimetry (DSC), and Karl Fischer (KF) titration at least in duplicate.

Storage Stability Studies

Dried samples of formulations A03, A13, A14, and A15 were stored in stoppered lyophilization vials sealed with aluminium seals in incubators at 2–8, 25, and 40°C for up to 25 weeks and analyzed at multiple time points (0, 6, optionally 15 and 25 weeks). The samples were analyzed via AF4, RP-HPLC, SE-HPLC, DSC, X-ray powder diffraction (XRD), and KF titration at least in duplicate.

VLP Integrity: SE-HPLC

VLP degradation and aggregation was determined by SE-HPLC on a Summit HPLC System (Dionex GmbH, Idstein, Germany) using a TSKgel G5000PW$_{XL}$, 7.8 × 300 mm, 10-µm

TABLE 1
Compositions of NicQb Formulations

Code	Trehalose % (wt/vol)	Mannitol % (wt/vol)	Polysorbate 20% (wt/vol)	Sodium Chloride (mM)	Sodium Phosphate (mM)	Potassium Phosphate (mM)	pH
A01	10	—	—	—	20	—	6.2
A02	10	—	0.0025	—	20	—	6.2
A03	10	—	0.005	—	20	—	6.2
A04	10	—	0.0075	—	20	—	6.2
A05	10	—	0.01	—	20	—	6.2
A06	10	—	0.005	—	—	20	6.2
A07	10	—	0.005	30	—	20	6.2
A08	10	—	0.005	60	—	20	6.2
A09	10	—	0.005	90	—	20	6.2
A10	10	—	0.005	150	—	20	6.2
A11	5	—	0.005	—	—-	20	6.2
A12	7.5	—	0.005	—	—	20	6.2
A13	1.1	4.4	0.005	—	20	—	6.2
A14	—	5.0	0.005	—	20	—	6.2
A15	10	—	0.005	—	20	—	5.8

SE-HPLC column (Tosoh Biosience GmbH, Stuttgart, Germany). The running buffer was composed of 20 mM sodium phosphate and 150 mM sodium chloride (pH 7.2). The analytics were performed at a flow rate of 0.8 mL/min with UV detection at 260 nm.

Determination of Free Nicotine Derivatives by RP-HPLC

The free nicotine derivatives hydroxymethyl-nicotine and succinyl-hydroxy-methyl-nicotine were separated from NicQb by filtration at 14,000 rcf (relative centrifugal force) in Nanosep 3K Omega spin filters (3-kDa cut-off) from PALL Corporation (Dreieich, Germany). The flow through was analyzed by RP-HPLC on a Summit HPLC System (Dionex GmbH) using a Hypersil BDS-C18, 4.0 × 125 mm, 5-µm column (Agilent Technologies Deutschland GmbH, Böblingen, Germany). A flow rate of 1.0 mL/min with UV detection at 260 nm was applied. An elution gradient was used, introducing acetonitrile and a sodium dihydrogen phosphate/triethylamine buffer (pH 7.0) as eluents. The concentration of the nicotine derivatives was calculated from the regression of a nicotine standard curve. The values for free nicotine derivatives were given as percentage of total nicotine.

Determination of Total Nicotine by RP-HPLC

The nicotine moiety covalently linked to Qb-VLP was quantitatively cleaved during the 3-h incubation at 40°C and pH > 11. Subsequently, proteins were precipitated with hydrochloric acid and removed by centrifugation. The concentration of the hydrolysis product hydroxymethyl-nicotine in the supernatant was determined as described in "Determination of free nicotine derivatives by RP-HPLC."

Dynamic Light Scattering

The DLS measurements were performed using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). The polydispersity index (PI) and the hydrodynamic diameter were determined by using a NNLS (non-negatively constrained least squares) fitting algorithm. The size distributions by intensity and volume were calculated form the correlation function by using the multiple narrow mode of the Dispersion Technology Software version 4.00 (Malvern Instruments Ltd.).

Asymmetrical Flow Field-Flow Fractionation

AF4 measurements were performed using a Wyatt separation channel equipped with a 350-µm spacer, an Eclipse2 separation system (Wyatt Technology Europe GmbH, Dernbach, Germany), Agilent 1100 HPLC series isocratic pump, autosampler, degasser, UV detector (Agilent Technologies, Palo Alto, CA, USA), and Wyatt DAWN EOS MALS detector. Regenerated cellulose, cut-off of 10 kDa, was used as ultrafiltration membrane. About 20 µL of NicQb solutions (1 mg/mL) were injected for fractionation. Running buffer was sodium phosphate buffer pH 7.0 with 150 mM sodium chloride. The channel flow was 1.5 mL/min. The cross flow was 2.0 mL/min for 18 min, subsequently reduced to 0.15 mL/min in 15 min and held for 5 min. In a final step, the cross flow was 0 mL/min for 5 min. The VLP concentration was determined at 260 nm via UV detection. The MALS detector was used for the determination of the average molecular weight of the VLP fractions.

Transmission Electron Microscopy

The VLPs were analyzed in a transmission electron microscope CM12 (Philips, Eindhoven, the Netherlands) at 100 kV. Therefore, parlodion films mounted on 300 mesh/inch were carbon coated by electron gun evaporation at 10^{-5} mbar in a vacuum unit BAE 121 (BalTec Maschinenbau AG, Pfäfficon, Switzerland). The VLPs were adsorbed onto these coated films and negatively stained with uranylacetate (Sigma Aldrich Chemie GmbH). Images were taken using a CCD low scan camera (Gatan Inc., Pleasanton, CA, USA).

Determination of Glass Transition of Lyophilizates by Differential Scanning Calorimetry

Thermal analysis of dried VLP formulations was performed using a Netzsch 204 Phoenix® DSC (Netzsch-Gerätebau GmbH, Selb, Germany). The lyophilizate was removed from the lyophilization vial, homogenized in an agate mortar, and 2–15 mg of the powder were cold sealed in aluminium crucibles. Preparation was performed in a dry-nitrogen-purged glove box to prevent moisture uptake from the ambient atmosphere. In a first heating scan, samples were heated from −20°C to 90°C at 10°C/min, cooled to −20°C at 10°C/min and again heated in a second heating scan from −20°C to 170°C. The glass transition (Tg) temperature Tg (point of inflection) of the disaccharides and the melting temperature (peak) of the mannitol in the formulations were determined from heating scans.

Determination of Residual Water Content

Residual moisture contents of the lyophilizates were determined by coulometric KF titration using an Aqua 40.00 titrator with a headspace module (Analytic Jena AG, Halle, Germany). For the measurements, the samples were heated in 2R glass vials to 80°C for at least 5 min. The vaporized water was transferred with nitrogen into the titration solution and the amount of water was determined.

X-Ray Powder Diffraction

The morphology of the lyophilized products was analyzed by XRD from 5–40°2-θ, with steps of 0.05°2-θ (2 s per step) on an XRD 3000TT X-ray diffractometer (Seiffert, Ahrenburg, Germany), equipped with a copper anode (40 kV, 30 mA, wavelength 154.17 pm).

Determination of In-Vivo Antigenicity

A bioactivity assay was performed with the lyophilized formulation A03 stored at 2–8, 25, and 40°C for 41 weeks.

Additionally, NicQb bulk and samples of formulation A03, frozen at −20°C at the beginning of the stability study, were analyzed. Groups of 10 female balb/c mice were implicated for any of the five formulations at a dose level of 100 μg. The drug was applied subcutaneously. Mice were boosted after 7 days with the same amount of drug. Sera were collected after another 7 days and analyzed by enzyme-linked immunosorbent assay (ELISA). ELISA analysis was carried out by measuring the specific antinicotine IgG titers of individual mice on RNAseA-nicotine conjugate. A pooled standard mouse serum was used to establish a nicotine standard curve. Data fitting of the standard curve was performed using a four-parameter Marquardt fit. Titers of individual mice were determined from triplicated analysis of different dilutions. Individual measurements within the linear range of the ELISA curves were used for calculations.

RESULTS AND DISCUSSION

Effect of pH on Stability of NicQb

The stability of vaccines in solutions is strongly influenced by solution conditions like pH, ionic strength, osmolarity, and the presence of excipients. Among these parameters, formulation pH is described as one of the most critical variables concerning the chemical and physical stability of a drug (Brandau et al., 2003). As the drug has first to be formulated in a liquid formulation prior to freeze drying and has to be handled for several hours in the liquid state (especially with regard to large scale manufacture), the influence of the pH has to be properly investigated. Furthermore, the formulation pH can also affect the stability of dried products during long-term storage (Wang, 2000).

Therefore, the stability of NicQb was first investigated in dependence of the pH in the range of 4.6–8.2 in a liquid formulation. The cleavage of nicotine from the VLP as well as the degradation and aggregation of the VLP was analyzed.

As determined by RP-HPLC, it was found that the content of free nicotine derivatives increased with increasing pH values (Figure 1A). Up to a pH of 6.6, the amount of free nicotine derivatives was below 5% even after storage for 14 days at ambient temperature. Between pH 6.6 and 8.2, the amount of cleaved nicotine increased remarkably with increasing pH values upon time. After 2 weeks, almost 50% of total nicotine was cleaved at a pH of 8.2.

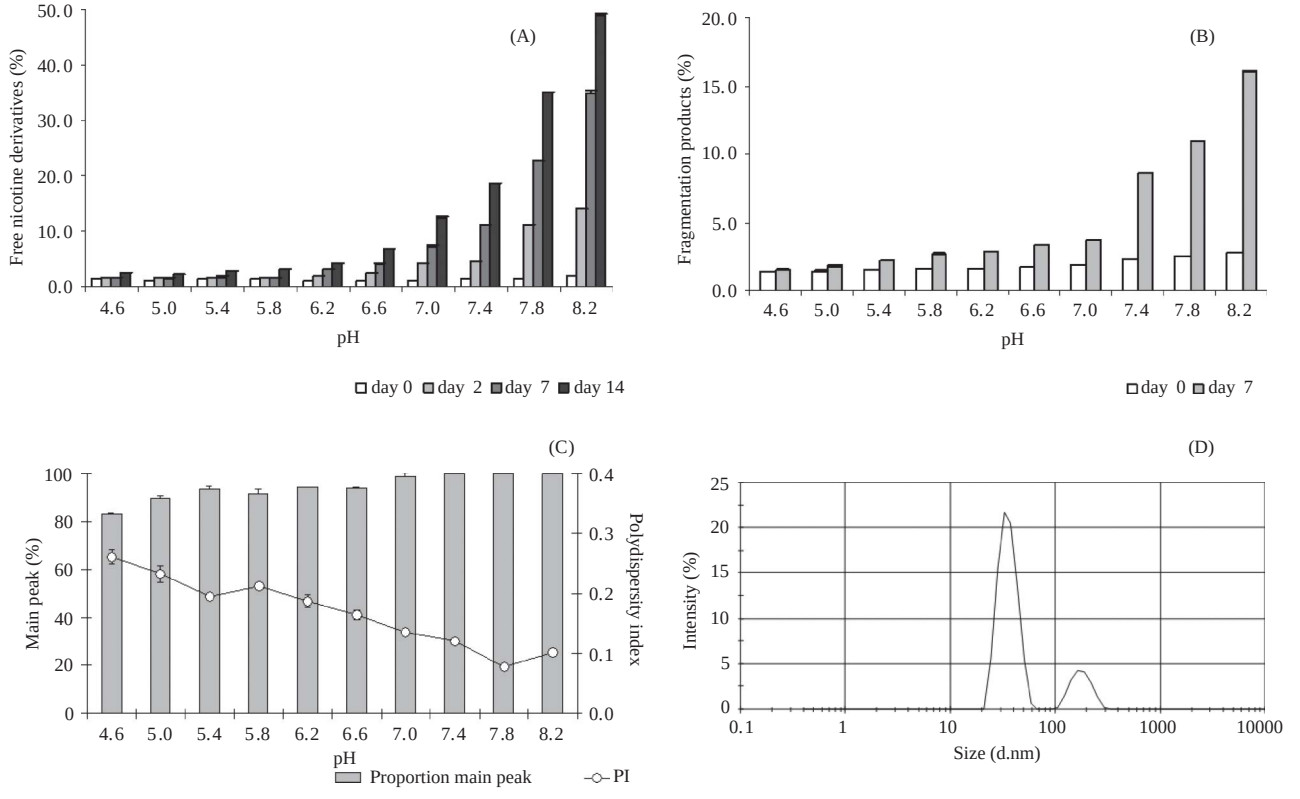

FIGURE 1. pH stability of NicQb (1.0 mg/mL) at ambient temperature. Amount of free nicotine derivatives as determined by reversed phase-high-performance liquid chromatography (RP-HPLC) right after manufacture and after 2, 7, and 14 days (A). Amount of fragmentation products as determined by size exclusion-high performance liquid chromatography (SE-HPLC) right after manufacture and after 7 days (B). Proportion of main NicQb peak and polydispersity index (PI) as determined by dynamic light scattering (DLS) after 14 days by applying the intensity conversion model (C). Size distribution as determined by DLS for NicQb sample at a pH of 4.6 stored for 14 days (D).

The data obtained from SE-HPLC analysis (Figure 1B) revealed that with increasing pH values, the amount of fragmentation products of NicQb increased clearly upon storage within 7 days. Up to a pH of 7.0, the amount of fragmentation products increased only up to 2% in comparison to the starting material, whereas at higher pH values, the amount of fragmentation products rose up to 15% at a pH of 8.2.

Thus, concerning the stability of the esterbond between the VLP-linker and nicotine and the integrity of the VLP, it can be stated that a pH value below 6.6 is desirable.

DLS data (Figure 1C) revealed that with decreasing pH values, the aggregation level of NicQb increased. The PI increased and the intensity of the main NicQb peak decreased, indicating an increasing polymodal size distribution. Below a pH of 6.2, the intensity of the main NicQb peak is less than 95%. At a pH of 4.6, not only a remarkable increase of the PI and decrease of the intensity of the main peak could be observed, but even a second peak at a size of several 100 nm appeared, proving the presence of VLP aggregates (Figure 1D).

Therefore, a compromise between the hydrolysis of the esterbond between VLP-linker and nicotine and the degradation of the VLP at high pH values, and the aggregation of the VLPs at low pH values had to be made. A pH range from 6.2 up to 6.6 appears to be optimal to assure the chemical and physical stability of NicQb in a liquid formulation.

Effect of Freeze-Thaw Cycling on the Stability of VLP

A freeze-drying process consists of two major steps: freezing of the drug solution and drying of the frozen solid under vacuum. Each step generates different kinds of stresses, and depending on the specific properties of a drug, its stability can be influenced by either one and/or both steps (Rexroad et al., 2002; Wang, 2000). Therefore, the particular influence of well-established excipients used for stabilization during freezing (cryoprotectants) and drying (lyoprotectants) had to be investigated. The effect of polysorbates, described as potent cryoprotectants for viruses (Evans et al., 2004), VLPs (Shi et al., 2005) and proteins (Chang et al., 1996; Kreilgaard et al., 1998; Sarciaux et al., 1999), and trehalose, described as efficient cryo- and lyoprotectant for virus vaccines (Gupta, Leszczynski, Gupta, & Siber, 1996; Sarkar et al., 2003; Worrall, Litamoi, Seck, & Ayelet, 2001), on the stability of NicQb during freeze thawing was assessed. Additionally, the effect of different concentrations of sodium chloride, often used to adjust the tonicity of liquid formulations, on the stability of NicQb was analyzed.

Samples with a NicQb concentration of 0.2 mg/mL, with 30 up to 150 mM sodium chloride, and 10% trehalose, with or without addition of 0.005% polysorbate 20, were prepared. Additionally, samples without trehalose, with or without 0.005% polysorbate 20 and 30 mM sodium chloride, were manufactured. Following the results from the pH stability study, the pH of the samples was kept at 6.4 throughout this study.

As aggregation of protein therapeutics induced by denaturation at ice–water interfaces is described as the major degradation pathway during freezing (Chang et al., 1996; Jones et al., 2001), the focus of this experiment was laid on the physical stability of NicQb.

DLS data revealed that with increasing concentrations of sodium chloride in NicQb formulations without polysorbate 20, the proportion of the main NicQb peak decreased, indicating an increase of the aggregation level. Furthermore, the PI of these formulations was higher than 0.4, referring to a polymodal size distribution. By contrast, in the presence of polysorbate 20, aggregation of NicQb could be prevented, even at high sodium chloride concentrations. However, the best results rendered the NicQb formulation with the lowest amount of sodium chloride (30 mM) and 0.005% polysorbate 20 with 99.9% proportion of the main NicQb peak and a PI of 0.20 (Figure 2).

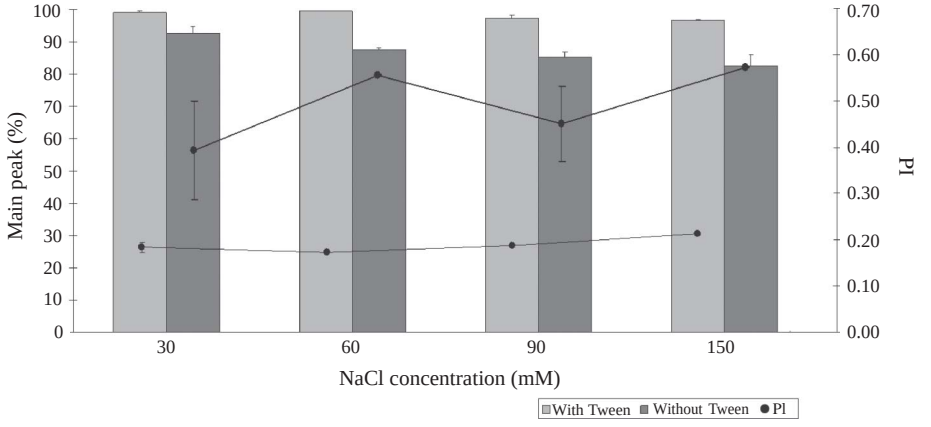

FIGURE 2. Stability of NicQb (0.2 mg/mL) in dependence of sodium chloride concentration (30, 60, 90, and 150 mM) and addition of polysorbate 20 (0 vs. 0.005%) after five freeze-thaw cycles. Polydispersity index (PI) and proportion of main NicQb peak, determined by dynamic light scattering (DLS) by applying a volume conversion model.

It was found that trehalose had no stabilizing or destabilizing effect on NicQb during freeze thawing. NicQb formulations with or without trehalose led to comparable results. Without the addition of polysorbate 20, the proportion of the main peak was in the range of 93–95% with a PI about 0.4, indicating aggregation of the VLPs, whereas NicQb formulations with 0.005% polysorbate rendered values for the main VLP peak higher than 99% and PIs about 0.2, independent of the presence of trehalose. The protective effect of polysorbate 20 might be explained by either binding to hydrophobic regions of the drug thereby preventing interaction with hydrophobic surfaces (Bam, Randolph, & Cleland, 1995) or by competing with the therapeutic for adsorption at liquid–surface interfaces (Shi et al., 2005). Thus, in summary, the addition of polysorbate 20 is beneficial to prevent aggregation of NicQb, the addition of sodium chloride should be avoided because it leads to aggregation of NicQb, and trehalose has no effect during freeze thawing.

AF4 as New Analytical Tool to Investigate the Stability of VLP

The results obtained from the freeze-thaw study proved that aggregation of the VLP is a critical factor concerning the stability of NicQb. The DLS measurements performed during this study showed a clear trend concerning the aggregation level of VLP formulations prepared without polysorbate 20 in dependence of the salt concentration. However, for the VLP formulations with polysorbate 20, only slight differences in the PI and the proportion of the main peak could be observed. Furthermore, DLS as a bulk method—without physical separation—has strong limitations in distinguishing and quantifying different VLP species. Thus, for further formulation development studies, there was the need for a more sensitive analytical tool that allows separation, characterization, and quantification of different VLP fractions. AF4 was discussed as promising analytical tool capable of separating and quantifying different species of proteins, protein aggregates, biopolymers, and nanoparticles in the size range from a few nanometers up to several hundred micrometers (Fraunhofer & Winter, 2004; Zillies, 2007). Therefore, we investigated whether AF4 can render a better insight into VLP compositions than DLS.

By applying a proper separation protocol, we were able to resolve VLP compositions in VLP fragments, monomers, dimers, and oligomers/aggregates (Lang & Winter, 2006). Furthermore, by coupling the AF4 to UV and MALS detectors, quantification and determination of the Mw of the specific fractions was possible (Figure 3A). For VLP monomers, a Mw about 3.3 MDa could be determined. As the recombinant VLPs have a lower amount of host cell RNA (about 25%) (Bachmann, Maurer, Meijerink, Proba, & Schwarz, 2006) as compared to the native bacteriophage Qb (50% RNA content), the data obtained fit well with the data from literature appointing the Mw of the native phage to 4.2 MDa (Hohn & Hohn, 1970). The Mw of the VLP dimers could be assigned to 6.2 MDa. The presence of VLP monomers and dimers was confirmed by transmission electron microscopy (TEM) analysis (Figure 3B).

As the developed AF4 method was found to be able to determine even slight differences in varying VLP compositions,

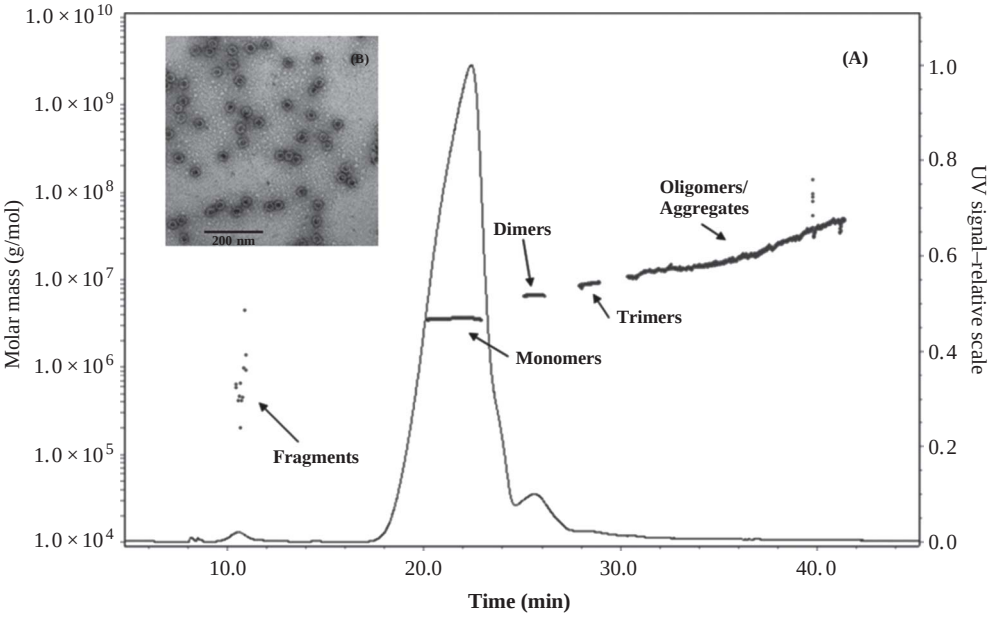

FIGURE 3. Fractionation of NicQb by asymmetrical flow field-flow fractionation (AF4). UV signal (continuous line) and molecular weight (dots) calculated from respective UV and multiangle light scattering (MALS) signal (A). Transmission electron microscopy (TEM) picture of NicQb (B).

it was used as the lead method during the following formulation development studies for the investigation of the physical stability of VLP.

Freeze-Drying Experiments

The effect of the freezing step during freeze drying had been investigated during freeze-thaw experiments. However, during the drying step further stresses, such as dehydration, are imposed. Dehydration during secondary drying leads to at least partial removal of the protecting hydration shell of proteins (Carpenter, Prestrelski, Anchordoguy, & Arakawa, 1994) and viruses which can lead to conformational changes and/or aggregation of the drug. Therefore, lyoprotectants, such as disaccharides, are often added to the formulations (Wang, 2000). The two main protective mechanisms proposed are the "vitrification" and the "water-replacement" hypotheses. "Vitrification" ascribes to the formation of an amorphous glass during lyophilization leading to increased drug stability by slowing down conformational changes of biomolecules (Chang et al., 2005; Crowe et al., 1998). The "water-replacement" hypothesis involves the formation of hydrogen bonds between the protein or virus and the excipients (Bieganski, Fowler, Morgan, & Toner, 1998; Crowe et al., 1993b; Crowe et al., 1998). In comparison to sucrose, a maximally freeze-concentrated trehalose solution has a Tg′ about 3°C higher (trehalose −29.5°C vs. sucrose −32°C) (Levine & Slade, 1988) enabling drying at higher temperatures and subsequently shorter freeze-drying times. Furthermore, dried trehalose has a higher Tg temperature as compared to sucrose (Crowe, Reid, & Crowe, 1996). The latter point is particularly important for the long-term stability at ambient temperature and accelerated storage conditions. Thus, in order to minimize the development time and to come up as soon as possible with a stable, freeze-dried formulation, trehalose was chosen as lyoprotectant. Furthermore, based on the instructions given by Tang and Pikal (Tang & Pikal, 2004) and Franks (Franks, 1998), a gentle but robust freeze-drying cycle was developed, which is on the one hand concerning the freezing rate, shelf temperatures, and pressure easily transferable to large-scale freeze dryers, and on the other hand short enough (2 days) to meet the criteria for economically acceptable processes.

In this study, the effect of different trehalose concentrations in the range of 5–10% (wt/vol), different buffer salts (potassium and sodium phosphate), different polysorbate 20 concentrations (0–0.01%), and sodium chloride concentrations (0–150 mM) were investigated. The pH of the NicQb formulations was adjusted to 6.2. In comparison to the formulations investigated in the freeze-thaw stability study, the pH was reduced from 6.4 to 6.2 with the intention to further increase the chemical stability of the drug (see pH stability study). This seemed to be reasonable as it was found from the freeze-thaw studies that the aggregation of the drug could be prevented by polysorbate 20.

After freeze drying, all formulations showed excellent appearance (i.e. did not collapse). Furthermore, the residual moisture content of all formulations was below 1.2%. The Tg temperatures of the formulations without sodium chloride were in the range of 80–85°C. Thus, it can be concluded that the process applied was adequate to obtain stable, amorphous lyophilizates with well-acceptable residual moisture contents (Rexroad et al., 2002) for all formulations tested.

The stability of NicQb was analyzed by AF4 and SE-HPLC. Concerning the effect of polysorbate, it was found that the addition of polysorbate is highly beneficial to prevent aggregation of VLP during freeze drying. Formulation A01, without polysorbate, showed a clear increase of the aggregation level from initially 3.9% aggregates (the term aggregate includes all VLP species larger than VLP dimers) to 7.9% after freeze drying. This indicates that the presence of trehalose alone is not sufficient to prevent aggregation of the VLP during freeze drying. By contrast, if polysorbate was added in a concentration of 0.0025%, aggregation of the VLPs could be prevented (Figure 4A). A further increase of the polysorbate 20 concentration showed no further positive effect on the stability of VLP during freeze drying.

AF4 measurements revealed that with increasing sodium chloride concentrations, the aggregation level of the VLP formulation was increased, confirming the results of the freeze-thaw experiments. The aggregation level remained stable upon

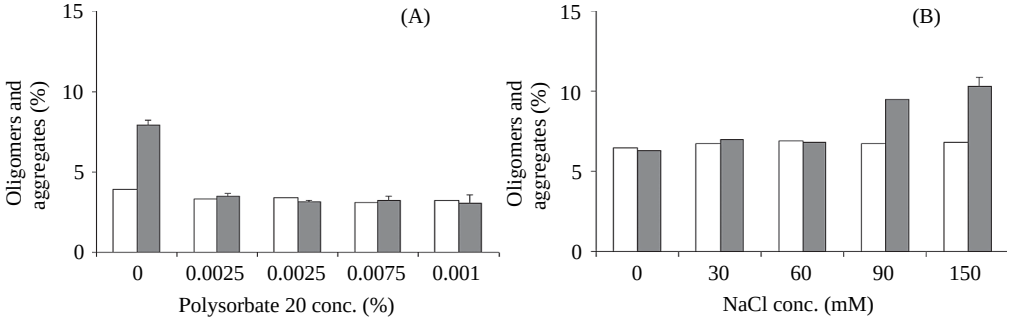

FIGURE 4. Stability of NicQb (1 mg/mL) in dependence of polysorbate 20 concentration (0, 0.0025, 0.005, 0.0075, and 0.01 %) (A) and addition of sodium chloride (0, 30, 60, 90, and 150 mM) (B). Aggregation level determined by asymmetrical flow field-flow fractionation (AF4) prior to freeze drying (white bars) and after freeze drying (grey bars).

freeze drying if no sodium chloride was present and increased from 6.5% prior freeze drying up to 10.3% in a formulation containing 150 mM sodium chloride even in the presence of 0.005% polysorbate 20 (Figure 4B).

Sodium phosphate and potassium phosphate were investigated as buffering agents. The rationale behind this selection was the often discussed pH shift to more acidic pH values in sodium phosphate-buffered systems, caused by an earlier crystallization of Na_2HPO_4 than NaH_2PO_4 (Wang, 2000) during the freezing step. For potassium phosphate-buffered systems, this pH shift is not that distinct (Sarciaux et al., 1999). Based on the data obtained during the pH stability study, we assumed that a pH drop might lead to aggregation of NicQb. However, as determined by AF4, the two buffer systems showed similar aggregation levels. Both formulations showed no increase of the aggregation level as compared to the liquid formulation prior to freeze drying (data not shown). The pH shift expected for sodium phosphate did not occur in the present formulation or at least not in a manner affecting the physical stability of the VLP. A reason might be the inhibition of buffer crystallization by trehalose as described by Chang and Randall (1992). Thus, in the compositions investigated, sodium phosphate and potassium phosphate are both applicable as buffering agents for NicQb during freeze drying.

In order to investigate the effect of trehalose on the stability of NicQb, formulations with sugar concentrations in the range of 5–10% (wt/vol) with resulting lyoprotectant/drug ratios in the range of 45:1–90:1 (wt/wt) were investigated. The AF4 measurements revealed that in this concentration range, all tested trehalose concentrations were capable of stabilizing NicQb. None of these formulations showed an increase of the aggregation level as compared to the liquid formulation prior to freeze drying (data not shown). As a consequence, a trehalose concentration of 9–10% is preferable as this composition is already isotonic with an osmolality of 300–330 mOsm/kg.

SE-HPLC results of all formulations tested during these freeze-drying experiments revealed that, with the exception of formulation A01, none of the tested formulations led to a clear increase (more than 0.5%) of the degradation levels of NicQb. However, for formulation A01, an increase of the degradation level from 1.9% prior to freeze drying to 3.0% after freeze drying was observed. Thus, it seems that polysorbate 20 not only prevents NicQb aggregation but also inhibits VLP degradation during freeze drying.

In summary, it can be stated that formulation A03 with trehalose as lyoprotectant and polysorbate 20 as cryoprotectant was the most promising candidate for stabilizing NicQb during freeze drying.

Storage Stability Study of Freeze-Dried NicQb Formulations

In order to define a formulation capable of stabilizing NicQb during storage, three formulations (A03, A13, and A15) were assessed during a storage stability study for 25 weeks at 2–8, 25, and 40°C. A03 was the lead formulation designated from the FD experiments. A15, a formulation similar to A03 except for a pH of 5.8, was included because of the higher chemical stability of the drug at lower pHs (refer to pH stability study). The aim was to investigate whether degradation of the VLP and the cleavage of nicotine upon storage are reduced at this pH. Based on the findings of the freeze-thaw and freeze-drying studies, it was expected that the theoretically more pronounced aggregation of the VLP at the lower pH value could be prevented by polysorbate 20. Additionally, formulation A13 composed of mannitol as bulking agent and trehalose as lyoprotectant in a weight ratio of 4:1 was included. The underlying rationale was that a combination of a crystalline bulking agent (mannitol) and a noncrystallizing disaccharide can offer a very robust crystalline matrix leading to a more elegant final product and enabling shorter drying times (primary drying can be conducted at higher temperatures due to the eutectic point of mannitol at −1.5°C) and protects the drug by the amorphous disaccharide (Johnson et al., 2002) part.

After freeze drying, all formulations showed excellent appearance which did not significantly alter upon storage. The reason for the good physical stability of the formulations A03 and A15 is the high Tg temperature of the trehalose matrix, higher than 90°C even after storage for 25 weeks at 40°C when moisture contents of the lyophilizates had increased slightly. XRD and DSC measurements revealed that both formulations were amorphous after freeze drying and that the glassy state was maintained upon storage for 25 weeks even at 40°C. The residual moisture levels of these two formulations increased slightly from 0.5% after freeze drying up to 1.6% (A03) and 1.4% (A15), respectively, after 25 weeks at 40°C (Table 2).

Concerning the physical state of the mannitol/trehalose-based formulation A13, the XRD measurements revealed that after freeze drying, the lyophilizates were partially crystalline/partially amorphous and that mannitol crystallized in an uncontrolled manner upon storage (Figure 6A). First heating DSC scans of the formulation stored for 6 weeks showed that mannitol crystallized completely upon storage at 40°C, whereas the samples stored at 2–8 and 25°C remained partially amorphous (Figure 6B). The residual moisture content after freeze drying was below 0.2% caused by the high secondary drying temperature at 40°C. Upon storage, the water content increased up to 0.9% after 15 weeks of storage at 40°C due to water uptake from the environment (Table 2).

Concerning the aggregation of NicQb, AF4 measurements indicated that all formulations were capable of stabilizing the drug during freeze drying. This can be related to the prevention of aggregation of the VLP at interfaces by polysorbate 20 and to the protection of the drug in the amorphous trehalose matrix. Upon storage, all formulations showed only a slight increase of the aggregation level, independent of the storage temperature (Figure 5A, Table 2). It was found that the theoretically more pronounced aggregation

TABLE 2
Results—Stability Study NicQb Lyophilizates

Code	Time Point (weeks)	Storage Temperature (°C)	Residual Moisture (%)	Degradation Products (%) Determined by SE-HPLC	Oligomers and Aggregates (%) Determined by AF4	Free Nicotine (% of Total Nicotine)
A03	T_0	—	0.5	2.3	5.1	<0.2[a]
	T_6	2–8	0.6	2.3	6.1	0.4
		25	0.6	2.3	6.3	0.4
		40	0.9	2.6	6.6	0.7
	T_{15}	2–8	0.6	2.4	7.1	0.3
		25	0.7	2.3	6.1	0.4
		40	1.2	2.6	6.6	0.9
	T_{25}	2–8	0.7	2.8	8.0	0.5
		25	0.8	2.4	7.8	0.6
		40	1.6	2.6	7.1	1.3
A13	T_0	—	0.2	2.7	5.5	<0.2[a]
	T_6	2–8	0.1	2.6	6.5	0.3
		25	0.2	2.4	5.9	0.4
		40	0.5	4.6	6.4	0.7
	T_{15}	2–8	0.1	3.0	6.9	0.3
		25	0.3	2.4	7.2	0.5
		40	0.9	4.9	6.2	1.4
A15	T_0	—	0.5	2.5	5.3	<0.2[a]
	T_6	2–8	0.6	2.5	6.9	0.3
		25	0.6	2.5	6.8	0.4
		40	0.8	2.8	7.0	0.8
	T_{15}	2–8	0.5	2.6	6.9	0.3
		25	0.6	2.5	7.1	0.4
		40	1.2	2.8	7.9	1.1
	T_{25}	2–8	0.5	2.4	8.4	0.4
		25	0.78	2.4	7.3	0.6
		40	1.4	2.7	7.8	1.3

[a]Amount of nicotine not detectable due to detection limit.

of the VLP at the lower pH value in formulation A15 was prevented by polysorbate 20.

Concerning the integrity of the VLP, determined by SE-HPLC, it was found that it was preserved in all formulations during freeze drying and subsequent storage at all temperatures tested (Figure 5B, Table 2).

With respect to the stability of the esterbond linking nicotine to the VLP surface, RP-HPLC analyses revealed that it remained stable during freeze drying in all formulations tested (Table 2). Upon storage at 2–8 and 25°C, a marginal increase of the content of free nicotine derivatives was detected for all formulations which was obviously more pronounced after storage at 40°C (Table 2). The increased hydrolysis of the esterbond can be related to the increased water content and less stability of the esterbond at higher temperatures, illustrated exemplarily in Figure 5C for formulation A03 after 25 weeks storage time. However, even for these samples, the amount of free nicotine derivatives was clearly below 1% of total coupled nicotine.

As the formulations A03 and A15 showed almost similar results for the amounts of degradation products and free nicotine derivatives, it can be stated that for the dried formulation, the pH effect is not that distinctive as for liquid formulations. The study revealed that the lowering of the pH in formulation A15 (pH 5.8), as compared to the lead formulation A03 (pH 6.2), led to no further improvement of chemical stability.

In summary, it can be stated that all formulations were capable of stabilizing the drug during freeze drying and prolonged storage at 2–8 and 25°C. Nevertheless, the uncontrolled crystallization of mannitol upon storage, observed for formulation A13, must be prevented, for example, by applying an annealing step during the specific freeze-drying process.

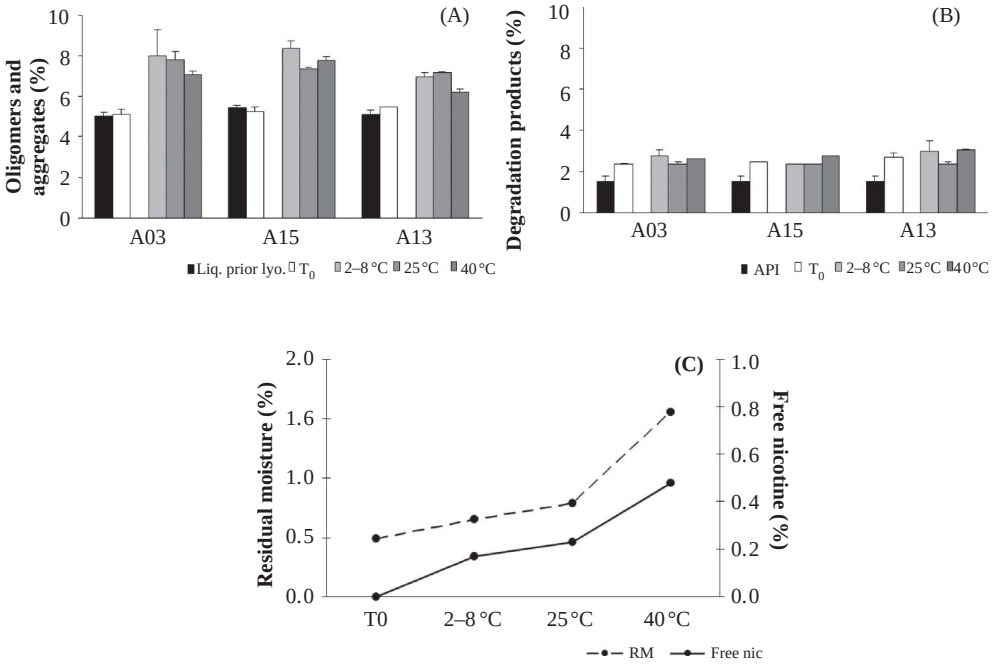

FIGURE 5. Stability of NicQb (1 mg/mL) in formulations A03, A15, and A13 during freeze drying and upon storage for 25 weeks (A03 and A15) and 15 weeks (A13). Aggregation level determined by asymmetrical flow field-flow fractionation (AF4) (A). Degradation level determined by size exclusion-high performance liquid chromatography (SE-HPLC) (B). Moisture content determined by Karl Fischer (KF) titration and amount of free nicotine derivatives determined by reversed phase-high-performance liquid chromatography (RP-HPLC) for samples of formulation A03 stored at 40°C for 25 weeks (C).

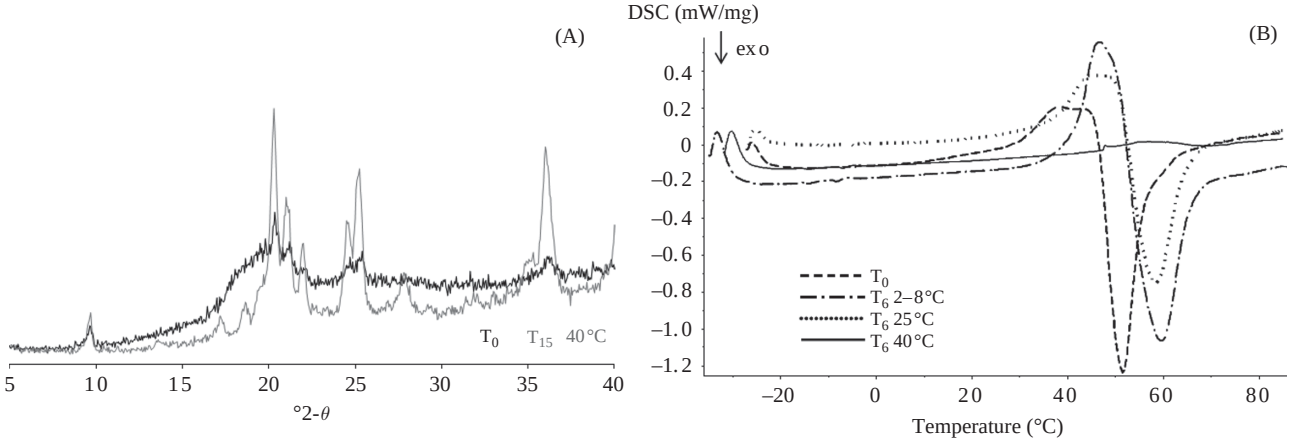

FIGURE 6. Physical state of formulation A13 as determined by X-ray powder diffraction (XRD) (A) and DSC (B) after freeze drying and storage.

Bioactivity Testing of NicQb Lyophilizates

As it was found that the formulations A03 and A15 were capable of stabilizing NicQb upon storage, retention of biological activity was verified. As formulation A15 displays no difference concerning the chemical stability of NicQb as compared to A03, only formulation A03 was assessed for bioactivity. The bulk material (used as internal standard, stored at –80°C) and lyophilized samples (frozen at –20°C right after manufacture) were compared to samples stored at 2–8, 25, and 40°C for 41 weeks. Upon immunization with 100 µg NicQb, none of the samples stored for 41 weeks, independent of storage temperature, showed loss of activity in comparison to the material right after manufacture (Figure 7). An unpaired two-tailed *t* test (confidence interval 95%) performed for the results of each preparation in comparison with the bulk material also showed no significant differences, except for A03 stored at 2–8°C, with a *p*-value only slightly below .05. Moreover, the mean titer of A03 (2–8°C) is higher than the mean titer of the bulk material

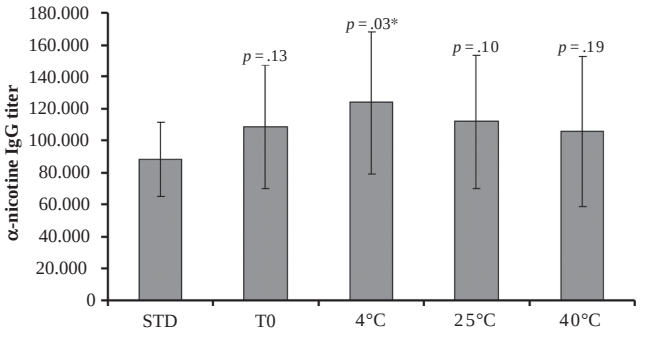

FIGURE 7. Bioassay analysis of formulation A03 upon immunization of mice with 100 µg of formulated product stored at different temperatures for 41 weeks. *p*-values obtained by comparison with the bulk material (defined as internal standard, STD) and formulated product stored at –20°C (T0) using an unpaired two-tailed *t* test with a confidence interval of 95% are shown on top of each bar. Differences identified as significant are marked by asterisk (*).

and the lyophilized starting material. Thus, it can be concluded that even long-term storage of formulation A03 at accelerated temperatures does not result in lower antibody titers compared with the bulk and the formulation right after freeze drying.

Optimization of Freeze-Drying Process for Mannitol/Trehalose-Based Formulation and Study on the Effect of a Pure Crystalline Formulation on the Stability of NicQb

Data obtained during the stability study for formulation A13 with mannitol as bulking agent and trehalose as lyoprotectant revealed that this composition is capable of stabilizing NicQb during freeze drying and storage at 2–8°C and 25°C over 15 weeks. However, the uncontrolled crystallization of mannitol upon storage at 40°C is undesirable.

Thus, the aim was to optimize the freeze-drying process to ensure complete mannitol crystallization in a controlled way during the freezing step. Therefore, an annealing step is performed (Izutsu & Kojima, 2002). Hence, formulation A13 was freeze dried according to two freeze-drying protocols, with (FD protocol 3) and without (FD protocol 2) applying an annealing step. For a better understanding of the effect of an amorphous matrix in comparison to a crystalline or partially crystalline matrix formulation on the stability of NicQb, a further formulation (A14), based solely on crystalline mannitol, was investigated as well. The stability of the drug and the physical state of all formulations were investigated right after manufacture and after storage for 6 weeks at 2–8, 25, and 40°C.

XRD measurements of the lyophilizates showed for all formulations a typical peak pattern for crystalline mannitol. For formulation A14 peaks of all mannitol modifications (α-, β-, and δ-mannitol) were detected, whereas both A13 formulations, independent of implementation of an annealing step, showed solely the typical peaks for δ-mannitol (Hawe & Friess, 2006) (Figure 8A). However, the DSC measurements revealed that the mannitol in formulation A13 was only partially crystalline when freeze dried without annealing, whereas it was completely crystalline when an annealing step was applied. The first DSC heating scan of the nonannealed material showed an exothermal event indicating the crystallization of amorphous mannitol (Figure 8B). This crystallization peak of mannitol could not be detected for the lyophilizates of the formulations A13 and A14 which were produced according to FD protocol 3 including an annealing step (Figure 8B). Thus, it can be concluded that applying an annealing step is essential to achieve complete mannitol crystallization during freeze drying and to prevent uncontrolled crystallization of mannitol upon storage.

Concerning the stability of NicQb, it was found that independent from applying an annealing step formulation, A13 was capable of stabilizing the drug during freeze drying and upon storage for 6 weeks at 2–8, 25, and 40°C. AF4 results revealed only a negligible increase of the aggregation level during freeze drying (Figure 8C). Additionally, the integrity of the VLP was preserved as determined by SE-HPLC. Only a marginal increase of degradation products could be detected (Figure 8D) during freeze drying and upon storage at 2–8, 25, and 40°C.

In contrast, AF4 and SE-HPLC data obtained for the purely crystalline formulation A14, solely composed of mannitol as bulking agent, showed a clear increase of both the aggregation level and the degradation products during freeze drying and in an even more pronounced manner upon storage at 25 and 40°C (Figure 8C and D). Thus, it can be stated that a completely crystalline matrix is not capable of stabilizing the VLP during freeze drying and storage, and implementation of an amorphous lyoprotectant is indispensable.

It can be summarized that formulation A13, freeze dried according to FD protocol 3, is capable of stabilizing NicQb during freeze drying and storage. The combination of crystalline mannitol as bulking agent, leading to physically remarkably stable lyophilizates with excellent appearance, and amorphous trehalose, which stabilizes the drug, is a further promising formulation for NicQb.

CONCLUSION

In conclusion, a grid of parameters important for stabilizing the VLP-based vaccine NicQb during freeze drying and storage at 2–8°C and ambient temperature was determined. We have found that an amorphous disaccharide matrix is suitable for the stabilization of the drug. Furthermore, the addition of polysorbate 20 was a key parameter for preventing aggregation of the VLP during freeze drying. All dried formulations showed excellent appearance and were easily reconstituted to isotonic liquids. Due to the well-structured study setup from pH, freeze thaw, freeze drying, and finally long-term stability studies a very fast progress towards a commercializable product was feasible. This was definitely promoted by the early establishment of AF4 as a powerful, sensitive analytical

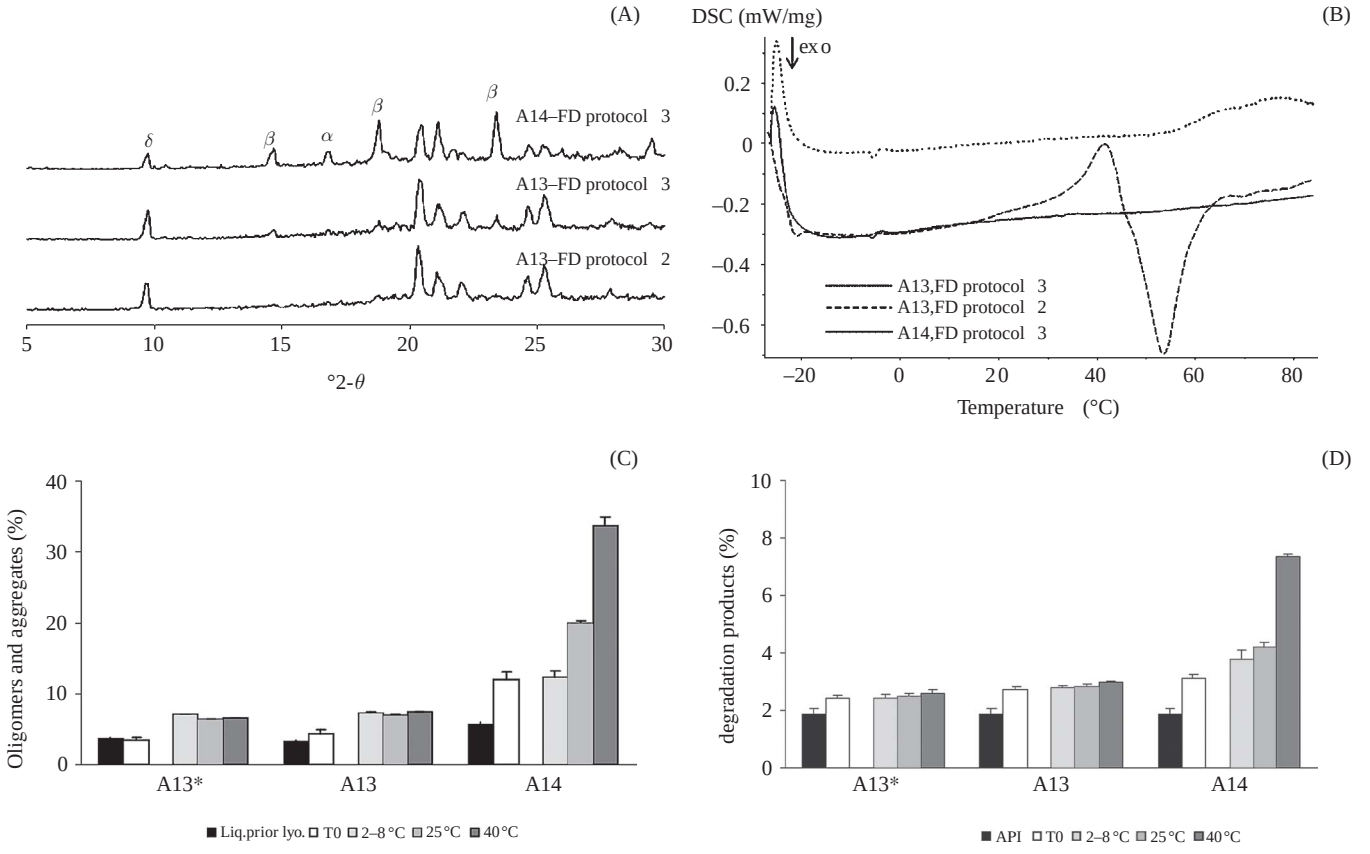

FIGURE 8. Physical state of formulations A13 (annealed and non-annealed) and A14 (annealed) after freeze drying as determined by X-ray powder diffraction (XRD) (A) and DSC (B). Aggregation level of NicQb as determined by asymmetrical flow field-flow fractionation (AF4) (C) and degradation level determined by size exclusion-high performance liquid chromatography (SE-HPLC) (D).

tool for the investigation of the physical stability of VLP, enabling the detection of even slight differences between the formulations tested. Finally, full retention of biological activity of VLP lyophilizates, even after long-term storage, was demonstrated.

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