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Research paper

Formulation development of freeze-dried oligonucleotide-loaded gelatin nanoparticles

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ABSTRACT

The freeze-drying properties of gelatin nanoparticles were investigated with the goal of providing practicable nanoparticle formulations for *in vitro* applications or clinical studies. Various excipients and rehydration protocols were assessed, and gelatin nanoparticles loaded with oligonucleotides were successfully freeze-dried and rehydrated. An NF-κB decoy oligonucleotide-loaded gelatin nanoparticle formulation was developed and applied in a drug targeting approach in an animal model. The high concentrations of nanoparticles achieved after rehydration with reduced volumes proved to be critical for the *in vivo* effect. Finally, short term storage stability under accelerated conditions was assessed for dried gelatin nanoparticles formulated in sucrose, trehalose, mannitol, or a mannitol/sucrose mixture. Size, size distribution, and residual moisture content were investigated. Sucrose- and trehalose-containing formulations exhibited the greatest stability, but mannitol-containing formulations also showed notable stabilization despite their crystalline nature.

1. Introduction

The application of nanoparticles in targeted drug delivery was reviewed more than 20 years ago [1,2]. Since that time, enormous progress has been made in the development of nanoparticulate drug carrier systems, prepared from a broad variety of starting materials. Despite intensive research work on a variety of nanoparticulate systems, liposomes are still the most prevalent colloidal drug delivery system in terms of marketed pharmaceuticals [3,4]. During drug development, shelf-life and stabilization of the potential products are important factors that must be considered. In this context, lyophilization was proposed in 1978 to improve long term stability of liposomes [5], which led to the market authorization for Ambisome™ as the first freeze-dried liposomal drug formulation in 1992. Freeze-drying has been used since the 1940s [6], and is widely applied for stabilizing labile pharmaceuticals, e.g., proteins [7]. The freeze-drying of liposomes has been intensively investigated, and was recently summarized by Van Winden [8]. In contrast, nanoparticles are still the subject of basic research and only little is known regarding their freeze-drying properties. Studies commonly report that freeze-drying was used during manufacturing, but typically very little information is provided about the applied freeze-drying cycle or particle characteristics before and after lyophilization. More detailed information is available on the freeze-drying of chitosan, poly(lactide) (PLA)/poly(lactide-co-glycolide) (PLGA), and solid lipid nanoparticles (SLN), as well as for nanoparticulate lipid- or polycation–DNA complexes.

As shown in Table 1, a wide variety of materials are being considered in the development of nanoparticles for drug delivery. While each material offers different advantages, recent work on the delivery of oligonucleotides and plasmid DNA has shown that gelatin nanoparticles are particularly promising [37,38]. Furthermore, gelatin offers the advantages of being biodegradable, cheap and readily available. In addition, gelatin contains functional groups that are accessible for various chemical modifications, which may be especially useful in developing targeted drug delivery vehicles. However, development of gelatin nanoparticles as commercial products will require formulations that are stable during shipping and storage. As is the case with many particulate systems, liquid formulations allow settling over time such that the stability of the delivery system and its cargo are compromised. Therefore, it would be of interest to determine whether freeze-drying could be used to obtain a stable, dehydrated formulation.

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Table 1
Freeze-dried nanoparticle preparations that have been described in literature

Nanoparticle preparation	Reference
Crosslinked acrylamido-2-desoxy-glucose (AADG) nanoparticles	[9][a]
Chitosan–DNA nanoparticles	[10][a]
Core-shell type lecithin/Pluronic® nanoparticles	[11]
Core-shell type poly(γ-glutamic acid) (γ-PGA)/L-phenylalanineethylester (L-PAE) nanoparticles	[12]
	[13][a]
	[14][a]
Gelatin nanoparticles	[15][a]
	[16][a]
Glucomannan-coated chitosan nanoparticles	[17]
	[18]
Lipid–DNA complexes	[19]
	[20]
	[21]
Polyethylenimine (PEI)–DNA complexes (Polyplexes)	[22]
	[23]
	[24]
Poly-ε-caprolactone nanospheres/nanocapsules	[25]
	[26]
Poly(isobutylcyanoacrylate) (PIBCA) nanoparticles	[27]
	[28]
Poly(L-lactic acid) (PLA) nanoparticles	[29][b]
	[30]
	[31]
Poly(lactide-co-glycolide) (PLGA)	[32]
	[33]
Poly(methylidene malonate 2.1.2) nanoparticles	[34]
Solid lipid nanoparticles (SLN)	[35][b]
	[36]

[a] Data without further information about the freeze-drying cycle or particle characteristics before and after lyophilization.
[b] Review articles.

The present work investigates the ability of several commonly used excipients (e.g., trehalose, sucrose, mannitol) to maintain the size and size distribution of gelatin nanoparticles during freeze-drying and rehydration [39,40]. Because moisture content plays a critical role in storage stability, the tendency of different formulations to absorb moisture during storage was also evaluated. In addition, we demonstrate the advantages of concentrating nanoparticle formulations by rehydrating freeze-dried preparations with reduced volumes. Finally, the potential of rehydrated, concentrated gelatin nanoparticle systems to deliver functional oligonucleotides is assessed in an animal model.

2. Materials and methods

2.1. Materials

Acetone and HCl were purchased from VWR International GmbH (Ismaning, Germany). Dextran 3000 was purchased from Spectrum Chemical Corp. (New Brunswick, NJ, USA). Gelatin Type A with Bloom 175, 1-ethyl-3-dimethyl aminopropylcarbodiimide (EDC), 2-aminoethyl-trimethylammoniumchlodride hydrochloride (cholamine), and glutaraldehyde were purchased from Sigma–Aldrich (Taufkirchen, Germany). D-Mannitol, sucrose, and trehalose were purchased from Ferro Pfanstiehl Lab., Inc. (Waukegan, IL, USA). Tween® 80 was purchased from Merck KGaA (Darmstadt, Germany). The phosphorothioate NF-κB decoy oligonucleotide (ODN) was purchased from biomers.net GmbH (Ulm, Germany).

2.2. Preparation and surface modification of gelatin nanoparticles

Gelatin nanoparticles were prepared by the two-step desolvation method [37]. Briefly, gelatin is dissolved in water (5% [w/w]) under heating and fractionated by a first desolvation with acetone. After adjusting the pH, the remaining sediment containing the high molecular weight fraction of gelatin is transferred into nanoparticles during a second desolvation step. Finally, the particles formed *in situ* are stabilized by crosslinking with glutaraldehyde. Nanoparticles were purified four times by centrifugation for 20 min at 20,000g (SIGMA 4K15, SIGMA Laborzentrifugen GmbH, Osterode, Germany) and redispersion in highly purified water. Surface modification (cationization) of gelatin nanoparticles was performed with the quaternary amine cholamine in a modified procedure based on the method previously described by Coester [38]. After preparation and purification, the nanoparticles were suspended in highly purified water followed by dissolving cholamine in the resulting suspension. After 5 min of stirring, EDC was added to the reaction vessel in order to activate the free carboxyl groups on the surface of the unmodified nanoparticles for the coupling with cholamine. After 3 h, the reaction was abandoned and the nanoparticles were purified as described above. Finally, the concentration of the nanoparticle dispersions was determined gravimetrically by drying three aliquots to constant weight.

2.3. Oligonucleotide loading of gelatin nanoparticles

Oligonucleotide loading was accomplished in highly purified water. An aliquot (47.3 μL) of an aqueous nanoparticle dispersion containing 1700 μg surface modified gelatin nanoparticles was incubated with 85 μg or 170 μg of the respective oligonucleotide in aqueous solution (i.e., 5% or 10% [w/w] oligonucleotide loading) and mixed with excipient solutions (1200 μL final volume) for 2 h at 22 °C and 800 rpm under constant shaking (Thermomixer Comfort, Eppendorf AG, Hamburg, Germany).

To determine the amount of oligonucleotide bound to the surface of the gelatin nanoparticle, nanoparticles are redispersed in purified water, centrifuged and the supernatant is monitored for the presence of oligonucleotide with UV-spectroscopy. The amount of oligonucleotide in the supernatant is then subtracted from that used for the preparation to obtain the bound fraction. In the present study, the applied amount of 5% or 10% [w/w] oligonucleotide was determined to be completely bound onto the surface of the gelatin nanoparticles. Thus, removing excess oligonucleotide upon the drug loading procedure was not required.

2.4. Freeze-drying of empty and oligonucleotide-loaded gelatin nanoparticles

Empty gelatin nanoparticles and gelatin nanoparticle formulations loaded with 5% and 10% [w/w] oligonucleotide containing 1.4 μg/ml nanoparticles were prepared in solutions containing different concentrations of mannitol, mannitol/sucrose (weight ratio = 4:1), sucrose, and trehalose. The excipient-to-ODN weight ratio was varied from 47:1 to 1 to 1000:1, and this value is used to identify different formulations, e.g., "T200" is a formulation possessing a trehalose-to-ODN weight ratio of 200. This range of excipient levels corresponds to a total amount of 0.3% up to 7% [w/v] excipient in the final preparations that were freeze-dried. Experiments involving empty nanoparticles employed the same excipient-to-nanoparticle weight ratio as that used in the experiments with ODN-loaded nanoparticles. After preparation, nanoparticle formulations were transferred to glass vials for freeze-drying. During initial formulation experiments 300 μL per vial were filled in 1 ml glass vials (West Pharmaceutical Services, Inc., Lionville, PA, USA), later samples prepared for storage stability and animal studies contained 300 μL and 1200 μL per vial, respectively, in 2 ml glass vials (Schott AG, Mainz, Germany) prior to freeze-drying. Freeze-drying in the laboratory of Prof. Thomas J. Anchordoquy was conducted in a Dura-Stop™ lyophilizer (FTS Systems, Inc., Stone Ridge, NY, USA). Samples prepared for storage stability and

animal studies in the laboratory of the Ludwig-Maximilians-University Munich, Germany were freeze-dried in an EPSILON 2-6D pilot scale freeze dryer and an EPSILON 2-12D freeze dryer, respectively (Martin Christ Freeze Dryers GmbH, Osterode, Germany). Nanoparticle formulations containing mannitol–sucrose, sucrose, and trehalose were dried as follows: after freezing samples at −40 °C for 3 h, the chamber pressure was reduced to 0.05 mbar. Primary drying included a temperature ramp increasing from −40 to −20 °C during 5 h and drying at −20 °C for another 5 h. Secondary drying was conducted at 20 °C for 8 h. After freezing at −40 °C, mannitol-containing samples were dried at increased temperature during primary drying (−5 °C) and decreased pressure (0.1 mbar). Secondary drying was conducted at 20 °C for 8 h. Upon completion of the respective cycles, the chamber was vented with nitrogen, samples were stoppered under slight vacuum at 800 mbar, and the sealed vials were stored at 20 °C. Rehydration was conducted with different volumes of highly purified water ranging from 1/30th of the original volume to the original volume. All samples were prepared and lyophilized in triplicate.

2.5. Storage conditions during stability studies

Samples were stored under accelerated conditions in sealed vials at 30 and 40 °C as well as in open vials at 30 °C/30% relative humidity (RH) and 30 °C/60% RH. Storage was performed for 4 weeks and 10 weeks in drying chambers, where defined relative humidity was adjusted in closed containers by saturated solutions of magnesium chloride (maintaining 30% RH at 30 °C) and ammonium nitrate (maintaining 60% RH at 30 °C).

2.6. Characterization of gelatin nanoparticles

Size determination of gelatin nanoparticle batches was conducted by dynamic light scattering (DLS) using either a Nicomp 380 Particle Sizer (Particle Sizing Systems, Santa Barbara, CA, USA) or a Nanosizer ZS (Malvern Instruments, Worcestershire, UK). In contrast to the polydispersity index (PDI) calculated by the Malvern software, the Particle Sizing Systems instrument expresses the width of the size distribution by a coefficient of variation. Because the results of the initial experiments were acquired with the Nicomp 380 Particle Sizer, their quality is estimated by means of this coefficient. Prior to measurements samples were diluted 10× in highly purified water. All samples were prepared and measured in triplicate.

2.7. Karl–Fischer titration

Residual moisture content of samples was determined by coulometric Karl–Fischer titration with an Aqua 40.00 titrator comprising a headspace module (Analytik Jena AG, Halle, Germany). After placing into the headspace module, samples were heated up to 80 °C and the evaporated water was transferred to the titration solution (HYDRANAL®-Coulomat AG, Riedel-de Haën, Sigma–Aldrich GmbH, Seelze, Germany), where its amount was determined. Blank values were obtained from empty vials treated identically to vials containing samples throughout preparation and storage. All samples were prepared and measured in triplicate.

2.8. Differential scanning calorimetry (DSC)

DSC was used to study the glass transition of the maximally freeze-concentrated solution (T_g') of gelatin nanoparticle formulations. Around 20 mg of liquid samples were analyzed in crimped aluminum crucibles with a Perkin-Elmer Diamond DSC (Perkin-Elmer, Inc., Wellesley, MA, USA). Samples were heated from −50 to 25 °C with a heating rate of 10 K/min. All samples were prepared and measured in triplicate.

2.9. In vivo hepatic lipopolysaccharide (LPS) (sepsis) rat model and electrophoretic mobility shift assay (EMSA)

To evaluate the biological function of a nanoparticulate bound NF-κB decoy oligonucleotide after storage, formulations were tested in an *in vivo* LPS rat model. During the experiments, lipopolysaccharides (LPS) were used as exogenous stimulus for the NF-κB activation within rat liver. The NF-κB decoy oligonucleotide delivered on the surface of the gelatin nanoparticles binds to the activated transcription factor which inhibits the *in vivo* expression of NF-κB protein as detected by the electrophoretic mobility shift assay (EMSA). A successful inhibition of NF-κB *in vivo* is evident by diminished or even absent NF-κB bands on the electrophoresis gel of the shift assay.

Experiments were conducted in 6 week old male Sprague–Dawley rats weighing 190–220 g that were purchased from Charles-River-laboratories (Sulzfeld, Germany). All animals were housed in a 12 h/12 h day/night cycle with free access to food and tap water. All animals received human care in compliance with the "Principles of Laboratory Animal Care". Studies were registered and approved by the government authorities.

Rats were anesthetized, the abdomen opened, and the portal triad prepared. Freeze-dried NF-κB decoy oligonucleotide-loaded gelatin nanoparticles (sucrose-to-ODN ratio = 200) were rehydrated in purified water (resulting in a concentration of 10% sucrose [w/v]) and incubated with a Tween® 80 solution in a 1:1 nanoparticle-to-Tween® 80 (w/w) ratio. Four of these samples were combined and adjusted with a 10% [w/v] sucrose solution to a total volume of 1.2 ml containing 20 nmol/ml NF-κB decoy ODN. A 1-ml aliquot of the rehydrated NF-κB decoy ODN-loaded gelatin nanoparticle suspension was injected into the portal vein with a 1-ml syringe over a period of 5 min. Control experiments were conducted with nanoparticle- and ODN-free solvent containing water, sucrose and Tween® 80. After 15 min, 10 μg LPS (c = 50 μg/ml in PBS, *Escherichia coli* serotype 055:B05, Sigma–Aldrich GmbH, Taufkirchen, Germany) were injected into the portal vein, leading to sublethal sepsis. Experiments were terminated after an additional 30 min. Rats were sacrificed and blood was rinsed out. Liver was resected, frozen in liquid nitrogen and stored at −80 °C.

Electromobility shift assays were performed as described previously [41]. In brief, tissue was homogenized (Potter S, B. Braun Biotech) in Buffer A (10 mM Hepes pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM PMSF) centrifuged and further incubated (4 °C, 10 min) in Buffer A containing the detergent NP-40 10%. Samples were centrifuged, the supernatant discarded and the remaining pellet was suspended in Buffer B (20 mM Hepes pH 7.9; 0.4 mM NaCl; 1 mM EDTA; 1 mM EGTA; 20% glycerol; 1 mM DTT; 0.1 mM PMSF). Samples were kept at 4 °C for 30 min under continuous shaking. After centrifugation, supernatants were frozen at −80 °C. Protein contents in the nuclear extracts were analyzed by the Bradford method [42]. The consensus binding-sequence used for NF-κB was 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ (Promega, Mannheim, Germany). Specificity of the protein–DNA–complex was confirmed by adding a 100-fold excess of unlabeled NF-κB or AP-1 (5′-CGC TTG ATG AGT CAG CCG GAA-3′) binding-sequences (data not shown).

All animal studies and electrophoretic mobility shift assays were conducted by Florian Hoffmann at the department of pharmaceutical biology of the Ludwig-Maximilians-University Munich, Germany. Experiments were conducted twice for each nanoparticle formulation.

3. Results and discussion

3.1. Applicability of freeze-drying for gelatin nanoparticle suspensions

3.1.1. Characterization of nanoparticles

During the initial experiments, a Nicomp 380 Particle Sizer was available for particle size determination. The coefficient of variation calculated by the Nicomp software to describe the particle size distribution varies within broader ranges than the polydispersity index determined with the particle sizer from Malvern Instruments. To compare the coefficient of variation data and polydispersity indices, identically prepared samples were analyzed with a Nanosizer ZS. Interestingly, even high coefficients of variation represent homogenously distributed nanoparticle populations with polydispersity indices clearly below 0.100. Particle sizes slightly vary with a trend towards larger sizes determined with the Nanosizer ZS.

3.1.2. Initial freeze-drying experiments

Results from initial freeze-drying experiments of empty gelatin nanoparticle formulated in sucrose are displayed in Fig. 1. None of the formulations showed increased particle sizes or particle aggregation, and a slightly reduced particle size was obtained after rehydration. The smaller particle sizes may be explained by an incomplete recovery of the swollen state of the gelatin nanoparticles after freeze-drying. This suggestion is consistent with the work of Kang who monitored the porosity of freeze-dried crosslinked gelatin scaffolds [43] and observed shrinkage that may not be completely reversible. It should be noted that freeze-drying of gelatin nanoparticle formulations lacking sucrose resulted in completely aggregated samples that could not be redispersed for particle size analysis.

In addition to sucrose (S), three other excipient systems were chosen for evaluation of their stabilizing capacity for gelatin nanoparticles: trehalose (T), mannitol (M), and the combination of mannitol and sucrose in the ratio 4:1 (MS); the latter combines the good bulking properties of mannitol with the lyoprotection of sucrose as reported for protein stabilization [44]. Trehalose was chosen as it is one of the most widely used freeze-drying excipients for biomaterials [45] and is thought to be superior to sucrose because of its high T_g and temperature of "zero" mobility (T_0), respectively [46]. Mannitol readily crystallizes during freeze-drying which greatly compromises its ability to act as a lyoprotectant [47]. The ability of these excipients to preserve particle size and polydispersity after acute freeze-drying and rehydration is summarized in Table 2. For all formulations PDIs below 0.100 were determined prior to freeze-drying reflecting the very good quality of all samples applied for storage stability testing. Freeze-drying of formulations 100–800 exhibited slightly reduced particle sizes and maintained sample quality with PDIs still below 0.100. In contrast, formulation 47 exhibited increased PDIs for all freeze-drying excipients.

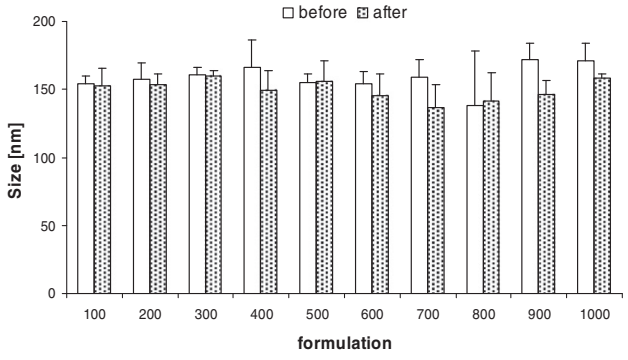

Fig. 1. Size of empty gelatin nanoparticles formulated in sucrose before and immediately after freeze-drying and rehydration.

It is interesting that mannitol is able to maintain nanoparticle integrity considering that it is known to crystallize during freeze-drying. Similar results have been previously reported by Allison et al. for lipid-based nanoparticles [18]. These authors explained their results by the particle isolation mechanism whereby virtually any excipient applied in sufficient amounts provides a spatial separation of particles within the unfrozen fraction, and thereby reduces aggregation. This is consistent with the inability of very low levels of any excipient (i.e., formulation 47) to maintain sizes and polydispersity indices comparable to untreated samples (Table 2). Our data suggest that at sufficient levels, the abilities of sucrose, trehalose, and mannitol to protect nanoparticles are equivalent. Surprisingly, mannitol–sucrose formulations failed to stabilize gelatin nanoparticles in all investigated excipient/nanoparticle ratios despite combining two excipients with good stabilizing properties.

After successful freeze-drying of empty gelatin nanoparticles, lyophilization of oligonucleotide-loaded gelatin nanoparticles were investigated (Fig. 2). Our results indicate that only minor increases in particle size were observed at low excipient/ODN weight ratios (100, 200), and particle size was retained at higher excipient levels. Comparable results were reported by Brus for the stabilization of oligonucleotide–polyethylenimine (PEI) complexes during freeze-drying [22].

3.1.3. Concentrating suspensions of oligonucleotide-loaded gelatin nanoparticles by freeze-drying

It is important to point out that nonviral vectors are typically assembled by electrostatic interactions between the positively charged nanoparticles and negatively charged oligonucleotides. Due to this assembly process, preparation of loaded nanoparticles at the high concentrations that are advantageous for *in vivo* administration results in aggregation. However, previous studies have shown that nanoparticles can be concentrated by freeze-drying after the components have been assembled [21]. To this end, 5% and 10% [w/w] NF-κB decoy ODN-loaded gelatin nanoparticle

Table 2
Size and polydispersity indices of empty gelatin nanoparticles before freeze-drying and rehydrated right after freeze-drying

Formulation	Size [nm]				PDI			
	Before		After		Before		After	
	Mean	Stdv	Mean	Stdv	Mean	Stdv	Mean	Stdv
Sucrose								
47	177.0	2.0	183.7	1.2	0.041	0.016	0.117	0.011
100	177.0	1.0	174.3	1.2	0.073	0.014	0.048	0.006
200	176.0	1.0	171.3	1.2	0.050	0.005	0.046	0.006
400	176.0	1.0	171.7	1.5	0.059	0.016	0.051	0.011
800	179.0	1.7	171.0	1.0	0.092	0.012	0.035	0.005
Trehalose								
47	179.0	1.7	182.0	1.7	0.066	0.019	0.114	0.028
100	177.0	1.0	172.0	1.0	0.054	0.009	0.055	0.008
200	177.0	1.0	171.7	0.6	0.051	0.013	0.039	0.009
400	177.3	0.6	170.3	2.5	0.062	0.002	0.051	0.012
800	178.3	0.6	172.0	1.0	0.059	0.017	0.061	0.011
Mannitol								
47	175.3	0.6	175.7	1.5	0.039	0.008	0.082	0.008
100	175.3	1.5	170.0	1.7	0.059	0.002	0.082	0.012
200	173.7	1.2	170.0	1.0	0.063	0.014	0.065	0.011
400	174.3	2.1	169.7	2.1	0.054	0.006	0.047	0.025
800	175.0	1.0	174.3	6.8	0.068	0.011	0.051	0.005
Man-Suc								
47	208.0	8.7	198.0	1.7	0.085	0.024	0.123	0.019
100	210.3	2.1	188.0	1.0	0.094	0.006	0.075	0.013
200	210.0	1.0	187.3	2.5	0.076	0.019	0.069	0.022
400	203.7	7.6	186.7	1.5	0.080	0.006	0.072	0.009
800	210.3	0.6	187.3	1.5	0.077	0.016	0.062	0.007

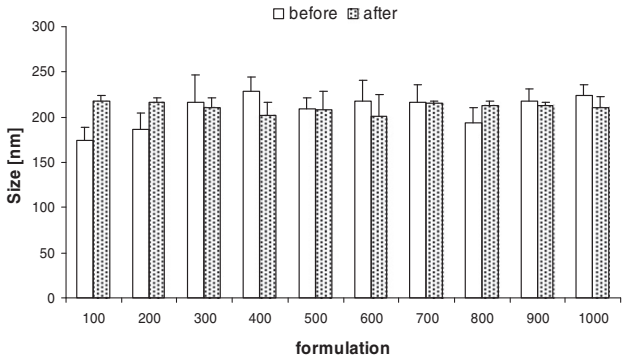

Fig. 2. Size of oligonucleotide-loaded gelatin nanoparticles formulated in sucrose before and immediately after freeze-drying and rehydration.

formulations were prepared with sucrose levels (24:1, 71:1, and 141:1 w/w) that resulted in isotonic solutions (10% sucrose w/v) upon rehydration in 1/10th, 1/20th, and 1/30th of the original volume (450 μL). Particle sizing was conducted before ("before") and after freeze-drying and rehydration with the original volume ("after 450"). In addition, the particle sizes of samples rehydrated with reduced volumes ("conc.") were also monitored (Fig. 3). The data indicate that particle sizes were slightly larger in formulations loaded with lower levels of ODN. In contrast, nanoparticles loaded with 10% ODN maintained particle sizes after rehydration with reduced volumes. In addition, the size distribution remained almost constant after freeze-drying and rehydration (data not shown). We conclude that oligonucleotide-loaded gelatin nanoparticles can be concentrated up to 30-fold by freeze-drying, and that this may be beneficial for *in vivo* applications.

3.2. Storage stability tests of freeze-dried gelatin nanoparticles

It should be recognized that prevention of aggregation during storage requires that nanoparticles remain spatially separated in the dried cake [18]. It follows that mobility must also be severely restricted, and the role of water in increasing molecular mobility within dried cakes is well established [48]. Accordingly, our initial storage experiments were aimed at assessing the ability of different excipients to maintain low residual moisture contents. For these experiments, empty nanoparticles were incorporated into formulations containing excipient levels consistent with our previous experiments on acute freeze-drying stress. Different excipient formulations were subjected to accelerated storage conditions for

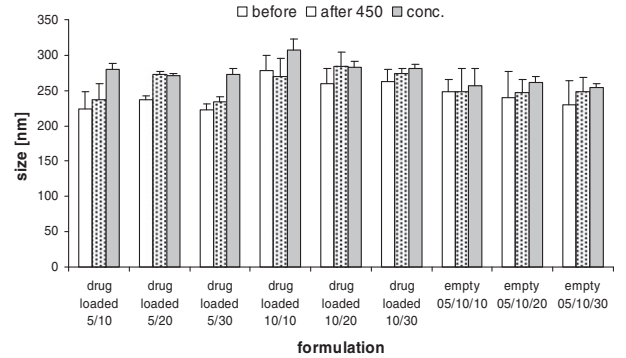

Fig. 3. Size of oligonucleotide-loaded (5% and 10% [w/w]) ("5/X" and "10/X") and empty ("5/10/X") gelatin nanoparticles formulated in sucrose before and immediately after freeze-drying and rehydration in the original volume ("after 450") and in 1/10th, 1/20th, and 1/30th ("X/10", "X/20", and "X/30") of the original volume ("conc.").

closed samples (30 and 40 °C) and open samples (30 °C/30% RH and 30 °C/60% RH) for a period of up to 10 weeks. Open storage was used as a worst-case scenario to assess the robustness of the formulations towards incomplete drying and suboptimal container closure systems.

Residual moisture was initially determined to be below 1% except for sucrose formulation 47 (Fig. 4a) and mannitol formulation 200 (Fig. 5a). After 4 weeks of storage in closed vials, sucrose and trehalose formulations absorbed considerable quantities of water, especially at the lower sugar levels (Fig. 4). Previous studies have demonstrated the potential for water transfer from the stopper to the dried cake, which can be detrimental for storage stability [49]. Mannitol and mannitol–sucrose formulations absorbed much less water (Fig. 5), and formulations with high levels of pure mannitol maintained residual moisture contents below 1% for 10 weeks. These findings are consistent with studies by Fakes et al. who systematically investigated the sorption behavior of bulking agents used in lyophilized products [50]. In general, water sorption was complete after 4 weeks of storage in sealed vials, with no further increases at 10 weeks (Figs. 4 and 5). In contrast, sucrose and trehalose formulations stored in open vials at 30 °C/60% RH continued to absorb water at later timepoints (Fig. 6).

3.2.1. Oligonucleotide-loaded gelatin nanoparticles

Storage stability of oligonucleotide-loaded gelatin nanoparticles was investigated with the major emphasis placed on the assessment of oligonucleotide function in an LPS (sepsis) rat model. In addition, particle sizing data were gained to estimate the respective formulation's quality prior to animal studies. The data in Fig. 7 reveal a monomodal size distribution suggesting the absence of larger aggregates, which was macroscopically confirmed. Interestingly, the PDI of all investigated nanoparticle excipient combinations significantly decreased as a result of freeze-drying. Sucrose and trehalose provided good stabilization in closed vials, whereas mannitol and mannitol–sucrose failed to stabilize the

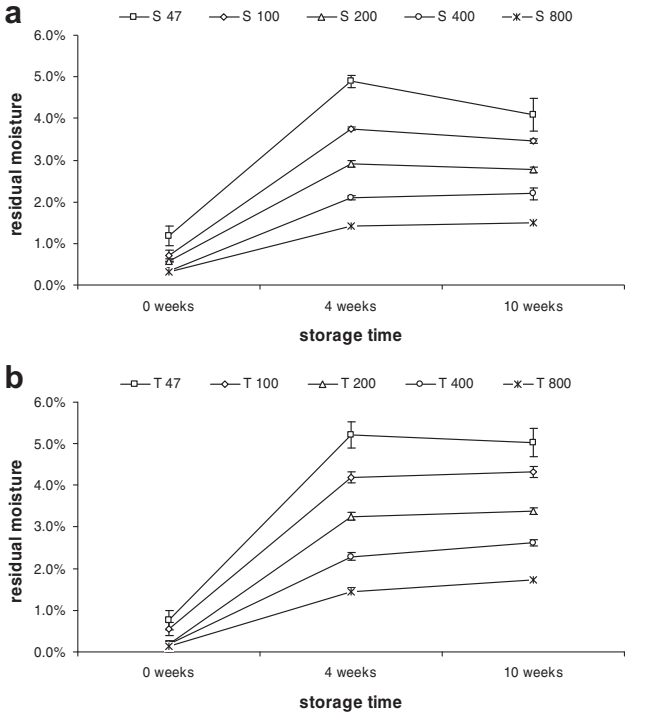

Fig. 4. Residual moisture content of freeze-dried gelatin nanoparticle formulations containing (a) sucrose and (b) trehalose immediately after drying, and after 4 and 10 weeks closed storage at 30 °C.

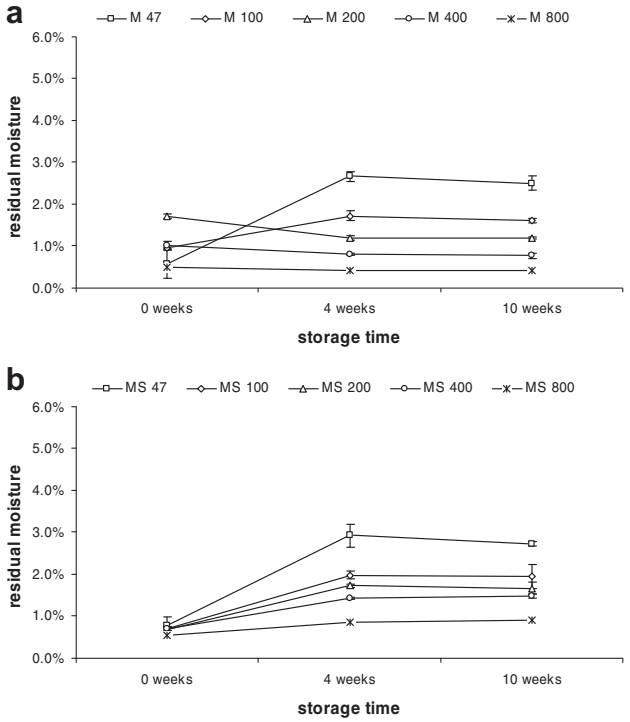

Fig. 5. Residual moisture content of freeze-dried gelatin nanoparticle formulations containing (a) mannitol and (b) mannitol–sucrose immediately after drying, and after 4 and 10 weeks closed storage at 30 °C.

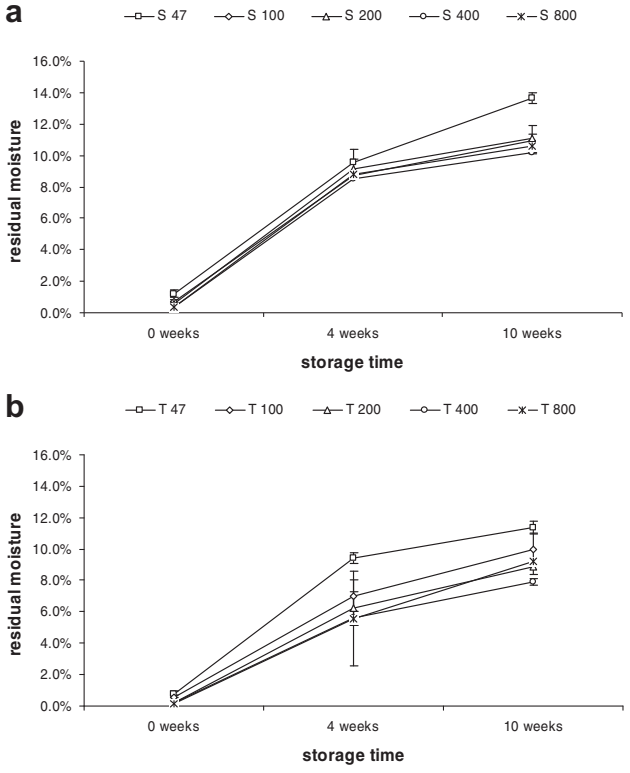

Fig. 6. Residual moisture content of freeze-dried gelatin nanoparticle formulations containing (a) sucrose and (b) trehalose immediately after drying, and after 4 and 10 weeks open storage at 30 °C/60% RH.

ODN-loaded gelatin nanoparticles under all storage conditions. None of the samples stored at 30 °C/60% RH were recovered with acceptable size or size distribution (data not shown).

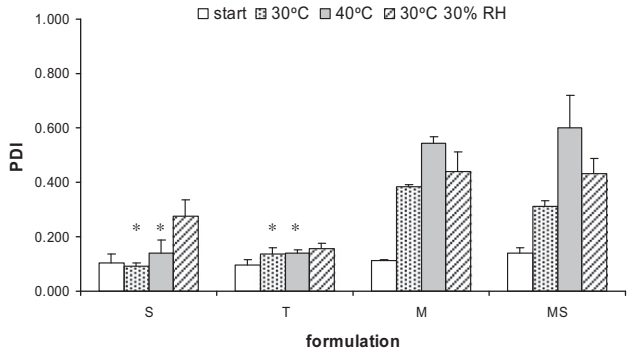

Fig. 7. Polydispersity indices of NF-κB decoy ODN-loaded gelatin nanoparticles formulated (formulation 200) with sucrose (S), trehalose (T), mannitol (M), and mannitol–sucrose (MS) rehydrated immediately after freeze-drying, after 4 weeks closed storage at 30 and 40 °C, and after 4 weeks open storage at 30 °C/30% RH. *Formulations that were further investigated *in vivo* within the LPS rat model.

Particle sizing data obtained from sucrose and trehalose formulations stored for 4 weeks in closed vials at 30 and 40 °C showed that particle size was maintained (data not shown). As described above, lyophilized samples were rehydrated with reduced volumes, and concentrated formulations were used to address the *in vivo* delivery of an NF-κB decoy oligonucleotide loaded onto gelatin nanoparticles. A sucrose-to-ODN ratio of 200:1 was chosen for freeze-drying of 10% oligonucleotide-loaded gelatin nanoparticles. The dried formulations were rehydrated with 141.5 μL highly purified water, resulting in a 7-fold concentration of the original preparation. Four of these samples were combined, and isotonic sucrose was added to achieve a total volume of 1 ml. The dilution to a total volume of 1 ml was accomplished for technical reasons only and can be abandoned for future studies.

Upon exposure to plasma, the nanoparticles were observed to aggregate immediately. This effect can be explained by the elevated ionic strength of the plasma that reduces the electrostatic repulsion/stabilization among the cationic nanoparticles. Similar results have been reported by Goetting for oligonucleotide loading of cationic polystyrene nanoparticles. To overcome this problem the authors successfully utilized a surfactant coating with poloxamer 388 that stabilized the ODN-loaded nanoparticles against aggregation [51]. With our gelatin-based system, we could show that our nanoparticles were macroscopically stable in plasma after incubating ODN-loaded nanoparticles for 1 h with Tween® 80. The Tween® 80 coating of the nanoparticles was conducted in a 1:1 [w/w] Tween® 80:nanoparticle ratio subsequent to rehydration and before dilution to the final volume. This approach was then utilized to investigate the biological activity of the NF-κB decoy oligonucleotide loaded onto gelatin nanoparticles in an *in vivo* sepsis rat model.

The *in vivo* model assesses the ability of the delivered oligonucleotide to act as an NF-κB decoy and reduce the expression of NF-κB protein in response to a dose of lipopolysaccharide (LPS). The resulting electrophoresis gel demonstrates that the NF-κB band is dramatically diminished compared to that in untreated animals, indicating that the biological activity of loaded gelatin nanoparticles is maintained under these storage conditions (Fig. 8). In addition, the fact that all formulations possessed potent biological activity indicates that the nature of the respective cake, i.e., amorphous for sucrose and trehalose or crystalline for mannitol and mannitol–sucrose formulations, does not affect the biological activity of the oligonucleotide.

Previous work with lipid/DNA complexes formulated with glucose, sucrose, and trehalose has demonstrated a reduction in biological activity after only 10 weeks, and suggested that reactive oxygen species in the lyophilized cake may be responsible for

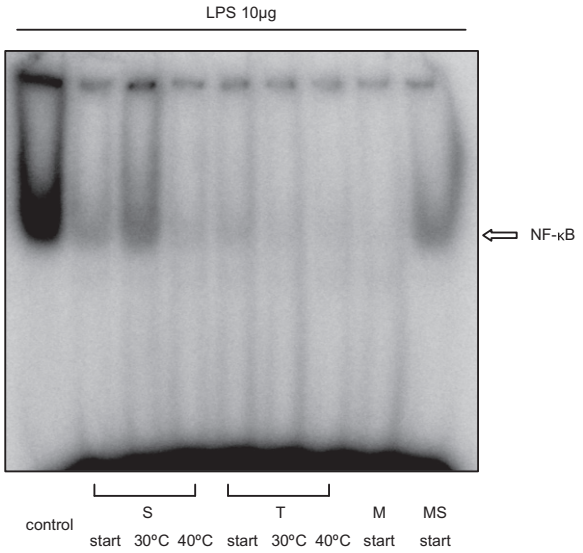

Fig. 8. EMSA of hepatic NF-κB activation after stimulation with 10 µg LPS of animals treated with 20 nmol NF-κB decoy oligonucleotide-loaded gelatin nanoparticles and of untreated animals; nanoparticle formulations (formulation 200) containing sucrose (S) and trehalose (T) were investigated immediately after freeze-drying, and after 4 weeks closed storage at 30 and 40 °C. Mannitol (M) and mannitol–sucrose (MS) containing formulations (formulation 200) are only investigated immediately after freeze-drying. Each lane represents one animal.

degradation [19]. More recent work by these authors has implicated the lipid component as a major source for the reactive oxygen species responsible for degradation [52], and thus it might be expected that nanoparticles lacking lipid (e.g., gelatin nanoparticles) could possess greater shelf-lives; consistent with our findings.

4. Conclusion

Freeze drying of gelatin nanoparticles was shown to be a convenient and robust method that provides excellent stabilization. In addition, we demonstrate that gelatin nanoparticles could be concentrated 30-fold by rehydrating with reduced volumes, resulting in a highly concentrated formulation that possesses potent biological activity *in vivo*. Furthermore, the storage stability studies revealed the need for a minimal amount of excipients to obtain sufficient stabilization, and trehalose proved to be superior due to its high glass transition temperature and its amorphous nature in the dried state. Taken together, these results clearly show that freeze-dried formulations of gelatin nanoparticles retain biological activity *in vivo* after 4 weeks storage at 40 °C, and suggest that this oligonucleotide delivery system has tremendous potential for commercial development.

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