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Preparation and characterization of poly(DL-lactide-co-glycolide) nanoparticles for siRNA delivery

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ABSTRACT

Synthetic short interfering RNA (siRNA) is promising for specific and efficient knockdown of disease-related genes. However, *in vivo* application of siRNA requires an effective delivery system. Commonly used siRNA carriers are based on polycations, which form electrostatic complexes with siRNA. Such poly- or lipoplexes are of limited use *in vivo* due to severe problems associated with toxicity, serum instability and non-specific immune-responses. The aim of the present study was to prepare uniformly sized nanoparticles (NPs) with a high load of siRNA by use of the safe and biodegradable poly-(DL-lactide-co-glycolide) (PLGA) polymer without including polycations. The siRNA was encapsulated in the core of NPs by the double emulsion solvent evaporation method. To optimize the NP formulation, the effects of important formulation and processing parameters were investigated systematically. Generally, spherical siRNA-loaded NPs (<300 nm, PDI < 0.2, zeta potential −40 mV) were obtained. An encapsulation efficiency of up to 57% was achieved by adjusting the inner water phase volume, the PLGA concentration, the first emulsification sonication time, and stabilization of the water–oil interface with serum albumin. The integrity of siRNA was preserved during the preparation. Preparation of core-loaded siRNA-NPs based on PLGA and no cationic excipient seems possible and promising for delivery of siRNA.

1. Introduction

Targeted post-transcriptional silencing of disease-associated genes using exogenous short interfering RNA (siRNA) appears to be a promising strategy to treat various human diseases (Huang et al., 2008; Singh, 2008). However, the clinical application of siRNA has been hampered by its rapid degradation, non-specific distribution, poor cellular uptake and low endosomal escape efficiency (Xie et al., 2006). Therefore, delivery systems capable of protecting and transporting siRNA through extracellular and intracellular barriers to reach the site of action in the cytosol are required for successful development of siRNA-based therapeutics (de Fougerolles, 2008). Various non-viral siRNA delivery systems such as cationic lipids, cationic polymers and cell-penetrating peptides have been studied intensively, but toxicity, low serum stability, non-specific immune-stimulating effects and lack of biodegradability are critical issues that severely limits the use of cationic vectors for clinic applications (Akhtar and Benter, 2007).

Nanoparticles (NPs) based on poly(DL-lactide-co-glycolide) (PLGA) have been widely investigated as carriers for DNA and oligonucleotides due to their small particle size, favourable safety profile and sustained-release characteristics (Luten et al., 2008). Recently, siRNA-loaded PLGA NPs have shown gene silencing *in vitro* (Yuan et al., 2006). Generally, two different approaches are used in order to load nucleic acids into PLGA NPs: (1) encapsulation into the core of NPs and (2) adsorption onto the surface of modified, cationic PLGA NPs via electrostatic interactions. By optimizing the encapsulation procedures, drug delivery systems with sufficient loading, protection and controlled release profile of the nucleic acid drug can be prepared. In fact, biodegradable PLGA microparticles encapsulating pDNA have been tested in phase 2 clinical trials (Klencke et al., 2002; Garcia et al., 2004). The advantages of protection and long-term sustained-release are of particular interest for siRNA therapeutics, since the RNA backbone is more susceptible to serum nuclease hydrolysis than DNA, and the silencing effects of siRNA are transient (Gary et al., 2007).

With regards to formulation development, it is very challenging to efficiently encapsulate high amounts of hydrophilic macromolecular compounds like siRNA into uniform, nano-sized PLGA particles, mainly due to the hydrophobic nature of the PLGA and the absence of electrostatic interactions between siRNA and PLGA. One way to improve encapsulation of nucleic acids is to add a cationic excipient to the PLGA matrix (Tahara et al., 2008), but this approach may also raise safety concerns with respect to clinical applications. Therefore, the hypothesis of the current study was that the siRNA encapsulation efficiency could be improved by systematically and carefully exploiting the opportunities in varying the parameters

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used in the procedure for PLGA NP preparation. To our knowledge, no systematic study has so far been reported, in order to relate the effects of processing and formulation parameters to the encapsulation efficiency of siRNA in PLGA NPs in the absence of cationic excipients.

The classic double emulsion solvent evaporation method (DESE) enables relatively high encapsulation efficiencies for water-soluble drugs compared to other methods like the emulsion solvent diffusion method. However, the stability of siRNA during processing might be of major concern due to exposure to organic solvent, sonication and mechanical stress. Sonication is widely used to fabricate PLGA microparticles and NPs, but can introduce high pressure, temperature gradients, shear forces and free radicals (Krishnamurthy et al., 2000). In addition, labile compounds like siRNA are even more prone to destabilization during processing of NPs than microparticles, since substantially more energy is added to the system in order to decrease the particle size. There is little information available in the literature regarding the effect of these destructive factors on the stability of siRNA during NP preparation.

The aims of this study were (i) to prepare siRNA-loaded PLGA NPs without the addition of any cationic excipient to the system or compromising the siRNA integrity and (ii) to investigate the effects of important process and formulation parameters on the encapsulation efficiency, particle size and uniformity.

2. Materials and methods

2.1. Materials

A Dicer substrate siRNA targeting enhanced green fluorescent protein (EGFP) was provided by Integrated DNA Technologies, BVBA (Leuven, Belgium) in desalted and lyophilized form. The duplex was obtained by annealing the HPLC-purified sense and antisense strands whose sequences were as follows: sense 5′-pACCCUGAAGUUCAUCUGCACCACcg-3′, antisense 5′-CGGUGGUGCAGAUGAACUUCAGGGUCA-3′ (Rose et al., 2005). Capital letters are ribonucleotides, lower case letters are 2′-deoxyribonucleotides and underlined capital letters are 2′-O-methylribonucleotides. PLGA (lactide:glycolide molar ratio: 75:25, Mw: 20 kDa) was purchased from Wake Pure Chemical Industries, Ltd. (Osaka, Japan). Polyvinylalcohol (PVA403) with 80.0% of degree of hydrolysis was provided by Kuraray (Osaka, Japan). Acetylated bovine serum albumin (BSA) was obtained from Sigma Chemical Co. (St. Louis, MO). Quant-iT™ RiboGreen® RNA Reagent and SYBR® Green II RNA gel stain were purchased from Molecular Probes Ltd., Invitrogen (Paisley, UK). All other chemicals and reagents were obtained commercially at analytical grade.

2.2. Preparation of siRNA-loaded PLGA NPs

siRNA-loaded PLGA NPs were prepared by the water-in-oil-in-water ($w_1/o/w_2$) double emulsion solvent evaporation (DESE) method. In brief, siRNA solubilized in 50, 100 or 200 μl of Tris–EDTA buffer (10 mM Tris, 1 mM EDTA, pH 7.5) (TE buffer) with or without acetylated BSA was mixed with 500 μl dichloromethane (DCM) containing various amounts of PLGA, and the mixture was emulsified by sonication using a UP100H ultrasonic processor (Hielscher Ultrasonics GmbH, Teltow, Germany) into a primary w_1/o emulsion. Two ml of 2% (w/v) PVA in diethyl pyrocarbonate (DEPC)-treated water were poured directly into the primary emulsion and further emulsified by sonication for another 60 s to form a $w_1/o/w_2$ double emulsion. The resulting emulsion was diluted with 6 ml of 2% (w/v) PVA and stirred magnetically for 3 h at room temperature to evaporate DCM. The PLGA NPs were collected by ultracentrifugation (Optima™ Max Ultracentrifuge, Beckman Coulter, CA, USA) at 38,203 × *g* for 10 min at 4 °C, washed twice with 6 ml of DEPC-treated water, re-suspended in DEPC-treated water, and freeze-dried for 48 h without use of a cryoprotectant (Epsilon 2-4 LSC freeze dryer, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). For freeze-drying, the samples were frozen to −40 °C over 2 h on a temperature controlled shelf. The primary drying was done at −40 °C and 1.030 mBar for 30 h, the shelf temperature was gradually increased to −10 °C over 10 h, and the final drying was done at −10 °C and 0.0010 mBar for 10 h, the temperature increased to +10 °C over 1 h and drying was continued for another 16 h.

2.3. Particle size and zeta potential

Freeze-dried NPs were re-dispersed in DEPC-treated water (0.2 mg/ml) by a short period of sonication (<1 min). The mean particle diameter (*Z*-average) and polydispersity index (PDI) of NPs were determined by dynamic light scattering using the photon correlation spectroscopy (PCS) technique, and the zeta potential of NPs was measured by the laser Doppler electrophoresis technique. The measurements were performed on undiluted samples ($n = 3$) in DEPC-treated water at 25 °C using a Malvern NanoZS (Malvern Instruments Ltd., Worcestershire, UK) equipped with a 633 nm laser and 173° detection optics. The voltage used for the zeta potential measurements was selected automatically based on the measured conductivity of the sample. The Helmholtz–Smoluchowski equation was used to convert the electrophoretic mobility to the zeta potential. Malvern DTS v.5.00 software (Malvern Instruments Ltd., Worcestershire, UK) was used for data acquisition and analysis. A polystyrene size standard (220 ± 6 nm, Duke Scientific Corp., NC, USA) and zeta potential transfer standard (−50 ± 5 mV, Malvern Instruments Ltd., Worcestershire, UK) were used to verify the performance of the instrument.

2.4. Morphology

The morphology of the NPs was determined by transmission electron microscopy (TEM). The NPs were suspended in water (5 mg/ml) and the particles were visualized by a Morgagni 268D TEM instrument (FEI Inc., Hillsboro, OR, USA). Photographs were recorded using a Megaview III digital camera (Soft Imaging System) after negative staining of NPs with 2% (w/v) uranyl acetate in water.

2.5. siRNA encapsulation efficiency

The siRNA encapsulation efficiency was determined by measuring the amount of extractable siRNA in the freeze-dried PLGA NPs. For each sample, 2 mg of freeze-dried NPs were dissolved in 200 μl of chloroform, and 500 μl of TE buffer was added. The mixture was rotated end-over-end for 90 min at room temperature to facilitate extraction of siRNA from the chloroform phase into the aqueous phase. The aqueous and organic phases were separated by centrifugation for 20 min at 18,000 × *g* at 4 °C (refrigerated centrifuge SIGMA 1-15PK, Osterode am Harz, Germany). The supernatant was collected and incubated at 37 °C for 5 min to remove residual chloroform. The samples were then further diluted with TE buffer and the siRNA was quantified by the RiboGreen® RNA reagent according to the manufacturer's instructions using a FLUOstar OPTIMA plate reader (BMG Labtech GmbH, Offenburg, Germany) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Each sample was assayed in triplicate. The loading percentage (w/w, ng siRNA per mg dry NPs) and encapsulation efficiency were calculated

using the following Eqs. (1) and (2):

$$\text{siRNA loading} = \frac{\text{the weight of drug in microspheres (ng)}}{\text{the weight of microspheres (mg)}} \quad (1)$$

$$\text{encapsulation effciency} = \frac{\text{actual siRNA loading}}{\text{theoretical siRNA loading}} \times 100\% \quad (2)$$

2.6. siRNA stability

The stability of siRNA under various conditions during the emulsification process was investigated. First, the effect of sonication time was examined by sonicating a siRNA solution (0.36 mg/ml at 60 W) for 0, 30, 60, 90 and 120 s. At each time point, 2 μl aliquots of siRNA solution were withdrawn and analyzed. Second, the effects of PLGA on the stability of siRNA during emulsification were studied by emulsifying 100 μl siRNA solution (0.36 mg/ml) into 250 μl of DCM containing 15 mg PLGA by sonication at 60 W for 0, 30, 60, 90 and 120 s to form an emulsion. Then, the emulsion was centrifuged at 18,000 × *g*, 4 °C for 10 min, and the supernatant was collected. Third, the integrity of siRNA extracted as described above from freeze-dried PLGA NPs was investigated. The aqueous phase from the extraction was collected, and the siRNA was concentrated by precipitation with a mixture of 99.9% (v/v) ethanol and 5 M NaCl (25:1, v/v) at −20 °C.

The integrity of siRNA in the samples was analyzed on a 15% polyacrylamide gel containing 7 M urea and TBE buffer (0.089 M Tris base, 0.089 M boric acid, and 2 mM sodium EDTA, pH 8.3). Electrophoresis was carried out with 1× TBE buffer at a constant voltage of 200 V for 1 h. siRNA was visualized using an image station (Kodak Image station 1000, Eastman Kodak Company, OR, USA) after staining for 40 min with a 1:10,000 dilution of SYBR® Green II RNA gel stain in DEPC-water.

2.7. Statistics

The experiments were performed in triplicate, unless otherwise stated. Values are given as means ± S.D. The statistical significance of the results was determined using a Student's *t*-test where $P < 0.05$ was considered significant.

3. Results and discussion

3.1. Characterization of siRNA-loaded PLGA NPs

siRNA was successfully encapsulated into PLGA NPs using the classic DESE method. The morphology of siRNA-loaded PLGA NPs was visualized by TEM, and a representative formulation is shown in Fig. 1. The main information to be drawn from the micrograph is that the NPs were spherical with a smooth surface. Particles with sizes between approximately 50 and 400 nm were observed in the sample indicating a relatively broad size distribution. However, for that specific sample the mean particle diameter measured by PCS was 224.0 nm and the size distribution was rather monodisperse (PDI 0.096) (Table 1). Generally, prepared PLGA particles with an acceptable PDI (<0.2) were in the nanometer range (220–260 nm) and the size was controlled by varying formulation parameters (see Section 3.2). The zeta potential of all NPs prepared in this study was approximately −40 mV (results not shown).

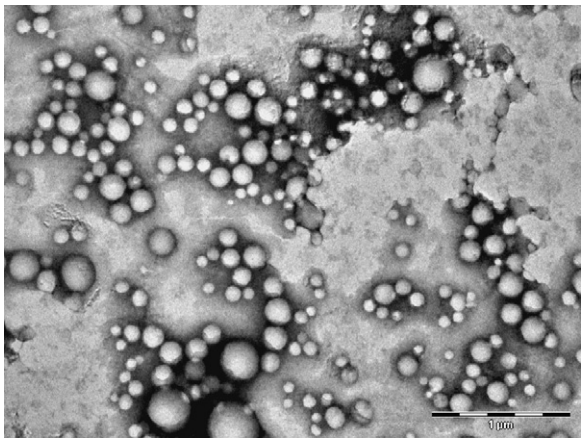

Fig. 1. Transmission electron micrograph of siRNA-loaded PLGA NPs prepared using 2.4 μg siRNA, a w_1/o ratio 0.1, a PLGA concentration of 30 mg/ml, 60 s sonication and no BSA. The size bar represents 1 μm.

3.2. Formulation and process parameters determine the encapsulation efficiency and the mean particle size

Various formulation and process parameters were studied systematically to produce siRNA-loaded PLGA NPs with the desired properties such as a mean particle size below 300 nm, a monodisperse size distribution (PDI lower than 0.2) and a high encapsulation efficiency without the use of cationic complexing excipients. The parameters included (i) the volume ratio of the inner water phase to the oil phase (0.1–0.4), (ii) the PLGA concentration (30–200 mg/ml), (iii) the sonication time for the first emulsification (30–90 s) and (iv) the siRNA concentration (2.4–6.0 μg/mg PLGA) and (v) the BSA content (0–360 μg). The parameter ranges were chosen based on initial pilot experiments in order to provide the largest possible design space without compromising the feasibility of the process.

3.2.1. Volume ratio of the inner water phase to the oil phase

The effects of the volume ratio of the inner water phase to the oil phase in the primary emulsion on the properties of NPs were first addressed. The following parameters were kept constant: (i) the siRNA theoretical loading (2.4 μg siRNA/mg PLGA), (ii) the polymer concentration (30 mg/ml) and (iii) the sonication time (60 s) for the formation of the primary emulsion. The results are summarized in Table 1. The mean particle size increased significantly by increasing the volume of the inner water phase above 100 μl. The encapsulation efficiency also increased significantly from 10.9% to 38.2% when the inner phase volume was increased from 50 to 200 μl. There was a direct correlation between the larger volume of the inner water phase and a higher siRNA loading and encapsulation efficiency. Similarly, the encapsulation efficiency of another large and hydrophilic molecule, fluorescein isothiocyanate-labeled bovine serum albumin, into PLGA NPs was increased with increas-

Table 1
The effects of the volume ratio of the inner water phase to the oil phase.

V_{w1} (μl)	V_{w1}/V_o	Diameter (nm)	PDI	siRNA loading (ng/mg NPs)	Encapsulation efficiency (%)
50	0.1	224.0 ± 4.4	0.096 ± 0.018	260.1 ± 11.9	10.9 ± 0.5
100	0.2	228.1 ± 1.6	0.128 ± 0.012	514.8 ± 11.1	21.5 ± 0.8
200	0.4	251.1 ± 1.6	0.130 ± 0.011	914.0 ± 30.9	38.2 ± 1.3

Values represent mean ± S.D., $n = 3$. Fixed values were: siRNA concentration 2.4 μg/mg PLGA, PLGA concentration 30 mg/ml, sonication time 60 s, BSA amount 0 μg.
V_{w1}: Volume of the inner water phase; V_o: volume of oil phase; NPs: nanoparticles; PDI: Polydispersity index.

Table 2
The effects of the PLGA concentration in the oil phase.

Conc. of PLGA (mg/ml)	Diameter (nm)	PDI	siRNA loading (ng/mg NPs)	Encapsulation efficiency (%)
30	228.1 ± 1.6	0.128 ± 0.012	514.8 ± 11.1	21.5 ± 0.8
60	242.6 ± 1.4	0.169 ± 0.018	346.8 ± 18.2	29.0 ± 1.5
200	422.7 ± 4.2	0.384 ± 0.028	107.8 ± 26.7	30.0 ± 7.4

Values represent mean ± S.D., $n = 3$. Fixed values were: siRNA concentration 2.4 μg/mg PLGA, w_1/o ratio 0.2, sonication 60 s, BSA amount 0 μg.
PDI: polydispersity index; NPs: nanoparticles.

Table 3
The effects of the sonication time.

Sonication time (s)	Diameter (nm)	PDI	siRNA loading (ng/mg NPs)	Encapsulation efficiency (%)
30	248.0 ± 4.1	0.166 ± 0.010	214.9 ± 20.1	18.0 ± 1.7
60	242.6 ± 1.4	0.169 ± 0.018	346.8 ± 18.2	29.0 ± 1.5
90	250.2 ± 1.1	0.122 ± 0.026	506.4 ± 21.6	42.3 ± 1.8

Values represent mean ± S.D., $n = 3$. Fixed values were: siRNA concentration 2.4 μg/mg PLGA, PLGA concentration 60 mg/ml, w_1/o ratio 0.2, BSA amount 0 μg.
PDI: polydispersity index; NPs: nanoparticles.

ing volume of the inner water phase (Bilati et al., 2005). For the same nominal siRNA loading, increasing the inner water phase volume will reduce the siRNA concentration gradient between the inner and outer water phase, and, consequently limit the diffusion of siRNA from the inner to the outer water phase and increase the encapsulation efficiency. However, the volume ratio of the inner water phase in the primary emulsion should be increased with care. If the phase volume ratio is too high, water droplets will likely coalesce and form a less stable emulsion due to the high surface energy of water (72.8 mJ/m^2). Consequently, the particle size will increase significantly, and the encapsulation efficiency may decrease, respectively.

3.2.2. PLGA concentration in oil phase

The PLGA concentration is a critical factor influencing the characteristics and the encapsulation efficiency. As shown in Table 2, the encapsulation efficiency was increased from 21.5% to 29% when the PLGA concentration in oil phase was increased from 30 to 60 mg/ml. However, there was no significant increase in the encapsulation efficiency when the concentration was increased further to 200 mg/ml, probably due to saturation of the oil phase. On the other hand, the siRNA loading decreased with the increase in the PLGA concentration because of the reduced nominal siRNA loading. Also, for the high PLGA concentration, the mean size of the particles increased above 400 nm and the size distribution was rather polydisperse (PDI ~ 0.4). This can be explained by the increased viscosity of the oil phase, which hinders the diffusion of siRNA from the inner water phase. Therefore, less siRNA is leaking into the exterior water phase during the course of evaporation. However, a more viscous polymer solution is less likely to separate into smaller droplets at the same sonication power. As a result, the particle size increased significantly and the size distribution broadened when a higher concentration of PLGA was used to produce the NPs. Taking both the encapsulation efficiency and mean particle size into consideration, a PLGA concentration of 60 mg/ml seems optimal for siRNA loading.

3.2.3. Sonication time

The effect of sonication time during preparation of the primary emulsion was investigated. The critical step in the DESE process is the dispersion of the inner water phase in the organic solution containing the polymer, and the sonication time determines the size of the primary emulsion droplet. Previous studies report that higher encapsulation efficiencies are obtained with water-soluble drugs in PLGA microspheres when there is an increased size difference between the primary w_1/o emulsion and the secondary $w_1/o/w_2$ emulsion (Ito et al., 2008). As a result of a small difference in the droplet size between the two emulsions, a thin oil layer forms around the inner water droplet, and the drug in the inner water phase can thus easily leak through the oil phase. In order to avoid this, siRNA-loaded PLGA NPs were prepared by applying different periods of sonication to the primary emulsion. The effects on the mean particle size and encapsulation efficiency were investigated keeping the theoretical siRNA loading at 2.4 μg/mg PLGA, the polymer concentration at 60 mg/ml and the phase volume ratio at 0.2. As expected, longer sonication time increased the encapsulation efficiency significantly, however, the mean particle diameter did not change significantly with sonication for between 30 and 90 s (Table 3).

Increasing the sonication time during the primary emulsification can compromise the integrity of siRNA. Therefore, the stability of siRNA was examined by applying different periods of sonication to the primary emulsion followed by recovery of siRNA from the primary emulsion by centrifugation. The results are shown in Fig. 2. siRNA remained intact even after 120 s of sonication, regardless of the absence or presence of PLGA in the oil phase.

3.2.4. Theoretical loading of siRNA in the internal water phase

The theoretical loading of siRNA is defined as the ratio of the nominal amount of siRNA against the total amount of PLGA. The encapsulation efficiency approached 57.5% when the theoretical

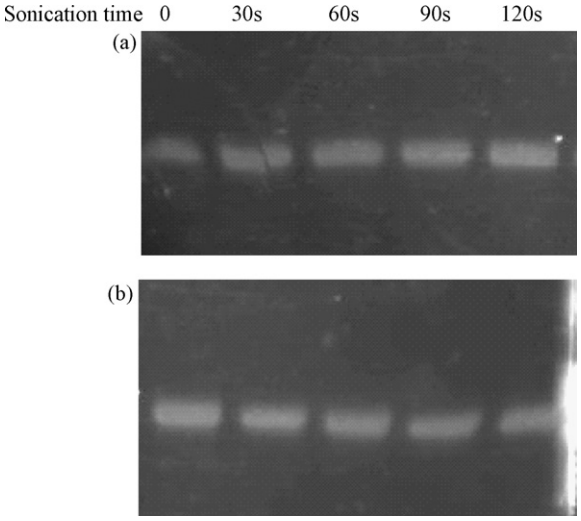

Fig. 2. Gel electrophoresis of siRNA recovered from the primary emulsion prepared using different sonication times (a) in the absence of PLGA in the oil phase and (b) in the presence of PLGA in the oil phase.

Table 4
The effects of the theoretical loading of siRNA in the internal water phase.

Theoretical loading of siRNA (μg/mg PLGA)	Diameter (nm)	PDI	siRNA loading (ng/mg NPs)	Encapsulation efficiency (%)
2.4	255.5 ± 1.9	0.178 ± 0.010	1379.6 ± 37.4	57.5 ± 1.6
6.0	258.1 ± 1.8	0.174 ± 0.014	1476.2 ± 243.4	24.6 ± 4.1

Values represent mean ± S.D., $n = 3$. Fixed values were: PLGA concentration 60 mg/ml, w_1/o ratio 0.4, sonication time 90 s, BSA amount 0 μg.
PDI: polydispersity index; NPs: nanoparticles.

Table 5
The effects of acetylated bovine serum albumin (BSA) in the internal water phase.

Amount of BSA (μg)	Ratio of BSA to siRNA	Diameter (nm)	PDI	siRNA loading (ng/mg NPs)	Encapsulation efficiency (%)
0	0	251.8 ± 1.7	0.171 ± 0.023	1133.1 ± 6.1	26.1 ± 0.1
180	2:1	254.5 ± 3.0	0.169 ± 0.015	1478.1 ± 35.8	34.0 ± 4.7
360	4:1	250.3 ± 1.9	0.152 ± 0.030	1997.8 ± 262.7	46.0 ± 8.7

Values represent mean ± S.D., $n = 3$. Fixed values were: siRNA concentration 2.4 μg/mg PLGA, PLGA concentration 60 mg/ml, w_1/o ratio 0.2, sonication time 60 s.
PDI: polydispersity index; NPs: nanoparticles

loading was kept constant at 2.4 μg/mg PLGA and the NPs were prepared by the optimal parameters determined by the previous studies, namely with a concentration of 60 mg/ml PLGA, a volume ratio w_1/o of 0.4, and applying 60 s of sonication for the first emulsification (Table 4). However, the encapsulation efficiency decreased to 24.6% when the theoretical loading was increased to 6.0 μg siRNA/mg PLGA although showing comparable actual siRNA loadings of the two formulations, even though much more siRNA was added into the first formulation. This is in line with the results obtained with the small peptide mellitin (Cui et al., 2005), and can be explained by that an increase in the concentration of the inner water phase will increase the siRNA concentration gradient between inner and outer water phases, which will enhance the diffusion from the inner to the outer water phase with subsequent loss in encapsulated siRNA.

3.2.5. Addition of acetylated BSA

Acetylated BSA was added to the internal water phase to increase the loading of siRNA in the NPs. The same protein has been used in the formulation of DNA-loaded PLGA NPs to accelerate the release of DNA. Also, acetylated BSA was used since it is available in nuclease-free quality (Prabha and Labhasetwar, 2004). The effects of adding BSA on the characteristics of siRNA-NPs were investigated by keeping the other parameters constant and systematically varying the amount of acetylated BSA in the inner water phase. Addition of acetylated BSA did not influence the mean particle size and size distribution significantly, but resulted in a significant increase in the encapsulation efficiency (Table 5). The encapsulation efficiency almost doubled when the amount of acetylated BSA was 4-fold higher than the amount of added siRNA, as compared to the formulation without acetylated BSA.

The stability of the primary w/o emulsion plays a crucial role for the drug loading and encapsulation efficiency of water-soluble drugs in PLGA particles manufactured by the DESE method. Stabilization of the emulsion can also reduce the leakage of water-soluble drugs from the inner water phase to the outer water phase (Ito et al., 2008). In the field of emulsion-based food production, proteins have been widely used as emulsifiers to retard the de-emulsification process because the protein molecules localize at the oil–water interface, and thereby facilitate the formation and improve the stability of the emulsions by lowering the interfacial tension and thus preventing the coalescence of oil droplets (Dalgleish, 1997). This explains the stabilization of the primary emulsion by acetylated BSA. The encapsulation efficiency of siRNA was increased significantly as a consequence of the stabilization. In addition, the increased viscosity of the inner water phase after adding BSA is also suggested to contribute to the enhanced stability of the emulsion and the increased encapsulation efficiency.

Efficient and reproducible loading of siRNA into NPs is a prerequisite for their use as a siRNA delivery system. It is very difficult to obtain a high siRNA encapsulation efficiency in PLGA due to the hydrophilic nature and relatively small molecular size of siRNA. Therefore, siRNA molecules easily escape from the PLGA phase and diffuse to the outer water phase, which results in siRNA loss during the preparation process. The cationic polymer polyethyleneimine (Patil and Panyam, 2009) and the cationic lipid 1, 2-dioleoyl-3-trimethylammoniumpropane (Tahara et al., 2008) have been used to pre-complex siRNA prior to entrapment in PLGA nanoparticles. However, since cationic compounds are known to be cytotoxic (Lv et al., 2006), the addition of cationic agents to the formulation will increase the safety concerns connected with future clinic application. Furthermore, the complex formation might delay the release of siRNA significantly (Patil and Panyam, 2009; Tahara et al., 2008). Therefore, it is desirable to increase the encapsulation efficiency by formulation and process optimization instead of adding cationic excipients. The current results consolidate that the encapsulation efficiency can be increased significantly simply by optimizing the formulation and process parameters. Apart from a high encapsulation efficiency and proper physical characteristics of the NP, it is crucial to preserve the integrity of the siRNA upon encapsulation, as well as to ensure that it is released from the NPs upon administration.

3.3. Integrity of siRNA is preserved

The integrity of siRNA in the final product was evaluated by extracting siRNA from dissolved PLGA NPs, followed by gel elec-

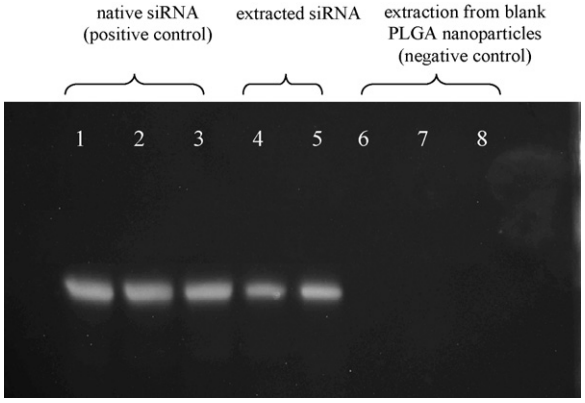

Fig. 3. Gel electrophoresis of siRNA extracted from the nanoparticles. Lanes 1–3: native siRNA (positive control); lanes 4 and 5: extracted siRNA; lanes 6–8: extraction from blank PLGA nanoparticles (negative control).

trophoresis (Fig. 3). siRNA appeared intact during the production process, and critical steps such as sonication and freeze-drying had no influence on the integrity of the siRNA.

4. Conclusion

The results of the present study show that siRNA can be incorporated successfully, i.e. with high encapsulation efficiency, into biodegradable PLGA NPs using the DESE method. The obtained siRNA-loaded PLGA NPs were spherical in shape, had a smooth surface and a net negative surface charge. The particle size and the encapsulation efficiency in particularly were influenced by various formulation and processing parameters. By optimizing those parameters, NPs with siRNA encapsulation efficiency up to 57% and particle diameter around 250 nm could be produced without the aid of cationic excipients. Optimal parameters were 2.4 μg siRNA/mg PLGA, 60 mg/ml PLGA, w_1/o volume ratio of 0.4, and 90 s of sonication time for the first emulsification. In addition, the presence of BSA increases the encapsulation efficiency in the NP. We are currently determining the siRNA release profiles and silencing effects *in vitro*.

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