Pharmaceutical Evaluation of Gas-Filled Microparticles as Gene Delivery System

Stefanie Seemann,[1] Peter Hauff,[1,3] Marcus Schultze-Mosgau,[1] Cathleen Lehmann,[2] and Regina Reszka[2]

Received October 24, 2001; accepted November 30, 2001

Purpose. To produce and characterize a nonviral ultrasound-controlled release system of plasmid DNA (pDNA) encapsulated in gas-filled poly(D,L-lactide-co-glycolide) microparticles (PLGA-MPs).

Methods. Different cationic polymers were used to form pDNA/polymer complexes to enhance the stability of pDNA during microparticle preparation. The physico-acoustical properties of the microparticles, particle size, pDNA integrity, encapsulation efficiency and pDNA release behavior were studied *in vitro*.

Results. The microparticles had an average particle size of around 5 μm. More than 50% of all microparticles contained a gas core, and when exposed to pulsed ultrasound as used for color Doppler imaging create a signal that yields typical color patterns (stimulated acoustic emission) as a result of the ultrasound-induced destruction of the microparticles. Thirty percent of the pDNA used was successfully encapsulated and approximately 10% of the encapsulated pDNA was released by ultrasound within 10 min.

Conclusions. Plasmid DNA can be encapsulated in biodegradable gas-filled PLGA-MPs without hints for a structural disintegration. A pDNA release by ultrasound-induced microparticle-destruction could be shown *in vitro*.

KEY WORDS: controlled release; DNA; gene therapy; microparticles; poly(D,L-lactide-co-glycolide); ultrasound.

INTRODUCTION

In gene therapy, efficient and target-site-specific *in vivo* gene delivery is a major challenge. Free DNA without any delivery system has been found to be highly susceptible to nuclease degradation (1) and is rapidly cleared from the plasma when injected intravenously (*i.v.*) (2). Although viral-based delivery systems such as adenoviruses (3) and retroviruses (4), efficiently introduce genes, they do suffer from immunogenicity and toxicity. The immunogenicity of viral vectors limits repeated use of the delivery system. Therefore nonviral gene delivery systems such as cationic lipids, liposomes, and polymeric microspheres (5–8), have been increasingly investigated as alternatives to viral vectors due to their potential advantages, such as ease of preparation and scale-up, as well as their relative safety and lack of immunogenicity. Their disadvantages may be the lower transfection efficiency and the transient nature of transfection compared to that of viral vectors. Currently, one of the most important obstacles to viral and nonviral gene delivery systems is the lack of organ and cell specificity.

Given the need for new, effective gene delivery systems, ultrasound contrast media (USCM) could be one of these promising nonviral gene delivery vehicles. USCM are agents, which increase the backscatter due to acoustic differences compared to the surrounding tissue, and it is well known that microbubbles are the most efficient scatterers. The microbubbles of the known USCM are protected against dissolution by a shell made of lipids, proteins, or polymers (9). In addition, USCM research has shown that diagnostic ultrasound of suitable frequency and intensity can destroy the shell of stabilized microbubbles resulting in the release of the encapsulated gas (10–12). The acoustically induced destruction of stabilized microbubbles, known as stimulated acoustic emission (SAEM), create a mosaic-like color pattern on the monitor of the ultrasound device using the color Doppler mode (13). Allowing the additional inclusion of genetic material (GM), the ultrasound-mediated destruction of stabilized microbubbles could be used as one way in using USCM for site-specific delivery of GM. Unger *et al.* developed an USCM for gene delivery, where the GM is entrapped in the center of the microbubbles (14). These microbubbles are stabilized by a so-called soft-shell consisting of a lipid bilayer. First *in vivo* studies have shown that the ultrasound treatment of the heart of rabbits led to gene expression after *i.v.* administration of encapsulated pDNA containing a marker gene (15). A second way is the coating of stabilized microbubbles with GM, which could be shown by Shohet *et al.* (16). They demonstrated that albumin stabilized microbubbles coated additionally with adenoviruses directs transgene expression to the myocardium of rats after their treatment with diagnostic ultrasound. Another observation is, that diagnostic ultrasound can even promote and enhance released plasmid DNA (pDNA) uptake by surrounding cells (sonoporation) (17–18). Overall, the major advantages seen in USCM as gene delivery systems are: (I) the protection of the GM in the blood stream against nuclease degradation, (II) the site-specific and spatio-temporally controlled delivery of GM, (III) the monitoring of the release procedure (SAEM) and (IV) the use of the ultrasound mediated sonoporation effect.

The propose of this study was to evaluate the suitability of pDNA encapsulation in polymer stabilized (hard-shell) microbubbles to determine both, the encapsulation and ultrasound mediated release efficiency of pDNA using poly(D,L-lactide-co-glycolide) (PLGA). The advantage in the use of these hard-shell stabilized microbubbles compared to soft-shell stabilized microbubbles is seen in their longer blood stability, resulting in a longer time-window for the site-specific ultrasound-induced pDNA release.

MATERIALS AND METHODS

Materials

Poly(D,L-lactide-co-glycolide) (PLGA) with a monomer ratio of 50:50 and inherent viscosity of 0.4dl/g (Resomer®

[1] Schering AG, Research Laboratories, 13342 Berlin, Germany.
[2] Max-Delbrueck-Center for Molecular Medicine, AG Drug Targeting, Robert-Roessle-Strasse 10, 13092 Berlin, Germany.
[3] To whom correspondence should be addressed. (e-mail: peter.hauff@schering.de)

ABBREVIATIONS: GM, genetic material; i.v., intravenously; pDNA, plasmid DNA; PEI, polyethylenimine; PLGA, poly(D,L-lactide-co-glycolide) acid; PLGA-MPs, poly(D,L-lactide-co-glycolide) microparticles; PLL, poly-l-lysine; PVA, polyvinylalkohol; SAEM, stimulated acoustic emission; USCM, ultrasound contrast media.

0724-8741/02/0300-0250/0 © 2002 Plenum Publishing Corporation

RG503) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Gelatin (from porcine skin) with a medium gel strength of 180 g Bloom was purchased from Fluka (Neu-Ulm, Germany). Polyvinylalcohol (PVA, molecular weight 9000–10,000) and DNA complexing agents such as poly-l-lysine (PLL, 9.5 kDa), protamin sulfate (PS, grade x from salmon) and polyethylenimine (PEI, 2 kDa) were obtained from Sigma (Deisenhofen, Germany). DAC-30™-liposomes were purchased from Eurogentec (Seraing, Belgium). All the other chemicals and reagents used were of analytical quality. The model plasmid pUT651™ containing the *E.coli* LacZ gene for β-galactosidase flanked upstream by the human cytomegalovirus promoter, and downstream by the transcriptional termination and polyadenylation signals from the SV40 was obtained from Cayla (Toulouse, France). The plasmid was propagated in *E.coli* DH5α and purified by a Qiagen kit (Hilden, Germany).

Preparation of Gas-Filled Microparticles

Gas-filled microparticles consisting of PLGA were prepared by a modified water-in-oil-in-water double-emulsion solvent evaporation method (w1/o/w2), developed at the Schering AG research laboratories, Germany. Briefly, an inner water phase (w1) consisting of 3% NaCl was prepared. 500 mg of PLGA was dissolved into 7 ml of CH_2Cl_2, forming the organic phase (o). 700 μl of the w1 phase was emulsified into the organic phase using a microtip probe sonicator (Branson Sonifier B15, G. Heinemann, Germany) for 30 s at level 7. The resulting primary emulsion was immediately added to 20 ml of an outer water phase (w2) (containing 2.5% gelatin or 1.5% PVA) stirred with a Dispermat F1 (VMA-Getzmann, Germany) at 10,000 rpm for 30 min. The resulting double-emulsion was stirred further using a magnetic stirrer for 1 h, completely removing dichloromethane. The microparticle solution was separated into aliquots, (2 g, filled in small glass vials), frozen in liquid nitrogen, stored overnight at –80°C and then freeze-dried for 2 days. After preparation, the freeze-dried microparticles were resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and gas-filled microparticles were separated from solid microparticles and polymer debris by centrifuging at 5000 x g for 1 min. Gas and pUT651™-pDNA containing PLGA-MPs were prepared either by dissolving a maximum of 520 μg native pDNA or different pDNA/cationic polymer complexes into the w1 phase of the resulting double-emulsion. Plasmid DNA/cationic polymer complexes were prepared by rapidly mixing the pDNA with the cationic polymer. The pDNA/polymer ratio was 1:5 for DAC 30™-liposomes, 1:1 for protamin sulfate, 5:1 for PEI and 2:1 for PLL, respectively. Complex formation was allowed to proceed spontaneously at room temperature for 20 min.

Microparticle Characterization

Particle Size Distribution

Lyophilized microparticles were redispersed in distilled water and particle size distribution was determined by laser diffractometry using Mastersizer S (Malvern Instruments Ltd., Malvern, United Kingdom). Particle size was expressed as the volume mean diameter in micrometers (μm) with standard deviations from three measurements per batch. The SPAN, which describes the polydispersity of suspensions was calculated using the following formula:

$$\left(\mathrm{SPAN} = \frac{d90\% - d10\%}{d50\%}\right).$$

Acoustical Efficacy

The ability of gas-filled PLGA-MPs to regenerate an ultrasound contrast was determined by the frequency-dependent ultrasound transmission attenuation *in vitro* (19). This method was used to characterize gas-filled microparticle formulations for their ultrasound contrast efficacy *in vitro*, and to determine the stability of a freshly prepared suspension of gas-filled microparticles over the time. For the measurement a respective suspension has been diluted 1:1000 and treated with ultrasound frequencies from 0.5 to 30 MHz. The measured attenuation of the gas-filled PLGA-MPs was expressed in decibel per centimeter (dB/cm) over the frequency range. The higher the attenuation signal is, the higher the ultrasound contrast efficacy of gas-filled PLGA-MPs. The acoustical efficacy of the batches tested was determined immediately 30 min and 60 min after the preparation of the suspension.

Electron Microscopy

The surface morphology of the respective gas-filled PLGA-MPs was examined by scanning electron microscopy (JSM-25SIII, Jeol, Tokyo, Japan). The gas-filled PLGA-MPs were attached to a Thermanox grid. After 20 min incubation, the microparticles were washed twice with distilled water and then air dried. For transmission electron microscopy using a JEM-100-CX (Jeol, Tokyo, Japan) the gas-filled PLGA-MPs were embedded in epoxy resin and cross-sections of 70-100 nm were prepared. To determine the localization of the encapsulated pDNA, pDNA labeled with cationic gold (PLL/gold) was used. For scanning electron microscopy, the gold label was enhanced by silver coating.

Integrity of Encapsulated pDNA

Plasmid DNA was extracted from PLGA-MPs for agarose gel electrophoresis by a dichloromethane/water extraction method. A total of 100 mg of lyophilized PLGA-MPs containing 50μg pDNA was dissolved in 50 ml dichloromethane followed by addition of 20 ml sterile TE buffer. Native pDNA was transferred into the aqueous solution by shaking. 10% (w/v) SDS solution was added to the TE buffer to separate pDNA complexes before precipitation. The two phases were separated by centrifuging for 10 min at 15,000 x g. The upper aqueous layer was separated from the organic phase. The extraction was repeated twice. The combined aqueous phase was mixed with 2.5 volumes ethanol, then 10% (v/v) 3 M sodium acetate was added to precipitate the pDNA. This mixture was incubated for 2 h at –20°C and thereafter the pDNA was collected by centrifuging for 30 min at 15,000 x g at 4°C. The pDNA pellet was washed once with 70% ethanol and was finally diluted in TE buffer containing 10% (w/v) SDS. Samples of control and recovered pDNA were separated on 0.8% (w/v) agarose gel.

Quantification of Encapsulated pDNA

To determine the encapsulation efficiency, pDNA was labeled with radioactivity by nick translation (Nick Translation Kit, Promega, United Kingdom) using 32-P-dATP (Amersham, Brunswick, Germany) before encapsulation. Nick translation was performed as per manufacturer's instructions. A total amount of 100 μg (4.8 μg pDNA/ml suspension = 198ng pDNA/mg PLGA) PLL-complexed pDNA (1 μg 32P-labeled) was used. For measurement purposes, four samples (2 g lyophilized microparticles/sample), of one batch of pDNA-loaded PLGA-MPs were resuspended in TE buffer. The gas-filled PLGA-MPs were separated from solid microparticles and fragments by centrifuging (1 min, 5000 x g). The pDNA in each sample was detected using a scintillation counter (Wallac 1409, Wallac, Freiburg, Germany).

Quantification of *in Vitro* Released pDNA

Lyophilized gas-filled PLGA-MPs containing pDNA (1.8 μg/ml) were suspended in sterile phosphate buffered saline and separated from solid microparticles and fragments by centrifuging. Aliquots of 150 μl gas-filled PLGA-MPs suspensions were treated with diagnostic ultrasound (Ultramark 9, ATL, Bothell, USA, Scanner L10-5, transmission frequency 3.5 MHz, MI 1.0) at 37°C at different points of time (three samples / point of time). The amount of pDNA released from the destroyed microparticles was determined with PicoGreen® (Molecular Probes, Leiden, The Netherlands) fluorescence labeling following the manufacturer's instructions.

RESULTS

Integrity of Encapsulated pDNA

Figure 1 shows that non-encapsulated native pUT651™ pDNA was predominantly supercoiled, although a small amount of open circle pDNA was visible (lane 7). No supercoiled pDNA was observed after encapsulation and extraction of native pDNA. The pDNA largely converted to a linear and open circle form (Fig. 1; lane 1–6). In contrast, the pDNA that had been complexed with different cationic polymers such as poly-l-lysine, protamin sulfate, polyethylenimine and liposomes before encapsulation remained to a large extent supercoiled pDNA after encapsulation in gas-filled PLGA-MPs (Fig. 2). As seen from lanes 2 and 3, linearized pDNA was also present after encapsulation, but to a significantly smaller extent when compared to the encapsulated native pDNA. The occurrence of large amounts of open circle pDNA could not be prevented. For all further studies the pDNA was complexed with PLL prior encapsulation into gas-filled PLGA-MPs.

Particle Size Distribution

Table I shows the particle size distribution of 12 representative batches with (n = 6) or without (n = 6) encapsulated pDNA which were used for further experiments. The mean diameters (d50%) of the microparticles of these batches ranged between 3.3 μm and 7.4 μm. In all microparticle suspensions at least 90% of the microparticles were less than 14 μm in diameter, whereas 99% were less than 20 μm in diameter. The width of particle size distribution (SPAN) was around 1.1. No significant differences were given between both, the batches with and without encapsulated pDNA.

Acoustical Efficacy

The gas-filled PLGA-MPs were further characterized by measurements of the frequency-dependent ultrasound transmission attenuation *in vitro*. Concerning the frequency-dependent ultrasound transmission attenuation all gas-filled PLGA microparticle batches show the same characteristics. Gas-filled PLGA-MPs had an average attenuation maximum between 8–10 decibel per centimeters (dB/cm) at a frequency of around 4 MHz, which usually decreased to 6–8 dB/cm within 60 min. Figure 3 shows a representative attenuation

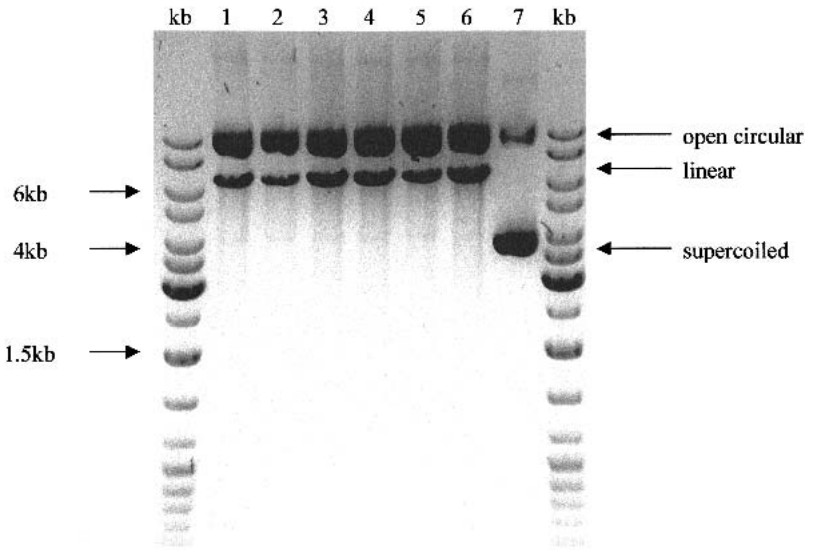

Fig. 1. Agarose gel electrophoresis of native pDNA extracted from gas-filled PLGA-MPs by a dichloromethane/water extraction method. ~10% of the recovered pDNA were seperated on a 0.8% agarose gel. Lanes 1–6: pDNA after encapsulation; lane 7: initial pDNA; kb = 1kb ladder.

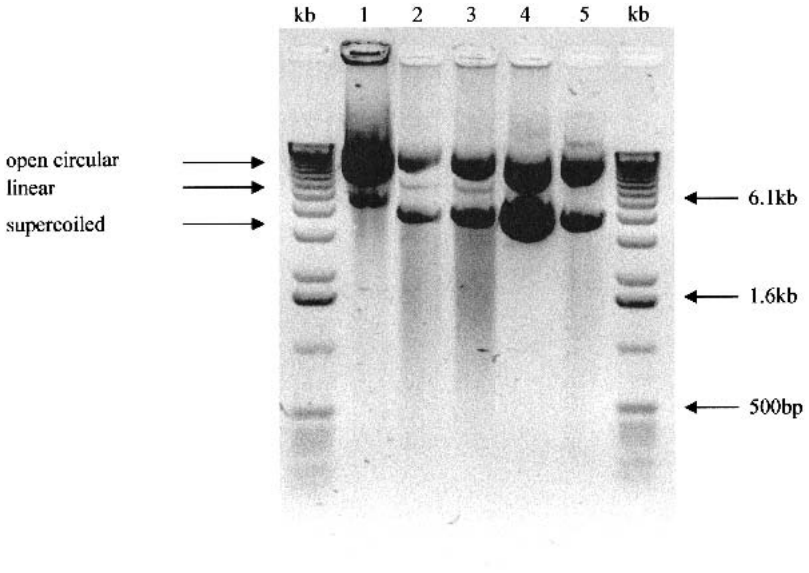

Fig. 2. Agarose gel electrophoresis of complexed pDNA extracted from gas-filled PLGA-MPs by a dichloromethane/water extraction method. ~10% of the recovered pDNA were separated on a 0.8% agarose gel. Lane 1: native pDNA; pDNA complexed with polyethylenimine (lane 2), protaminsulphate (lane 3), DAC-30 liposomes (lane 4), poly-l-lysine (lane 5). The complexes were separated with SDS before extraction and electrophoresis.

profile of pDNA containing gas-filled PLGA-MPs. The freshly resuspended microparticle suspension, was diluted to 1:1000 before the first attenuation measurement (t = 0 min). After 30 min and 60 min, respectively, the microparticle suspension was diluted (1:1000), again and the attenuation was measured. The microparticles had an attenuation maximum of about 9.5 decibel per centimeters (dB/cm) at a frequency of around 4 MHz (t = 0 min), which decreased to 8.0 dB/cm within 30 min and to 7.0 dB/cm within 60 min, respectively.

Electron Microscopy

Electron microscopic investigations of gas-filled PLGA-MPs containing pDNA showed that the pDNA was localized inside the gas-filled core of the microparticles as well as on the microparticles surface and of those dissociated in the solvent. Figure 4A shows the scanning electron micrograph of the surface of PLGA-MPs containing cationic gold-labeled pDNA. The pDNA could be detected as white spheres on the particle surfaces following silver enhancement of the cationic gold. In contrast, the surface of pDNA free particles remained smooth after silver enhancement (Fig. 4B). Transmission electron analysis of thin microparticle sections recovered the cationic gold-labeled pDNA as small black spheres inside the microparticles as well as bound to their surface (Fig. 5A). In addition, pDNA was also found dissociated from microparticles in the aqueous solvent (Fig. 5B).

Table I. The Particle Size Distribution of Gas-Filled PLGA Microparticles.

PLGA microparticles Batch	Size distribution			
	d50%	d90%	d90%	SPAN*
without pDNA				
1	5.5 ± 0.3	9.9 ± 1.5	14.4 ± 3.0	1.3
2	3.3 ± 0.0	5.1 ± 0.2	6.7 ± 0.2	1.0
3	5.1 ± 0.2	8.6 ± 0.9	12.3 ± 2.2	1.1
4	5.9 ± 0.2	10.0 ± 0.8	14.0 ± 1.8	1.2
5	6.1 ± 0.0	9.0 ± 0.0	11.7 ± 0.0	0.9
6	6.7 ± 0.1	10.8 ± 0.5	14.1 ± 0.7	1.0
with pDNA				
7	4.7 ± 0.2	8.3 ± 0.9	12.0 ± 2.2	1.2
8	5.6 ± 0.3	9.1 ± 1.5	12.1 ± 2.6	1.0
9	7.4 ± 0.4	13.1 ± 1.7	18.5 ± 3.3	1.3
10	4.4 ± 0.1	7.0 ± 0.7	19.0 ± 1.6	1.0
11	4.1 ± 0.0	6.4 ± 0.1	8.3 ± 0.2	1.0
12	4.6 ± 0.1	7.8 ± 0.8	12.5 ± 3.0	1.2

* SPAN = $d90\% - d10\%/d50\%$ = width of particle size distribution.

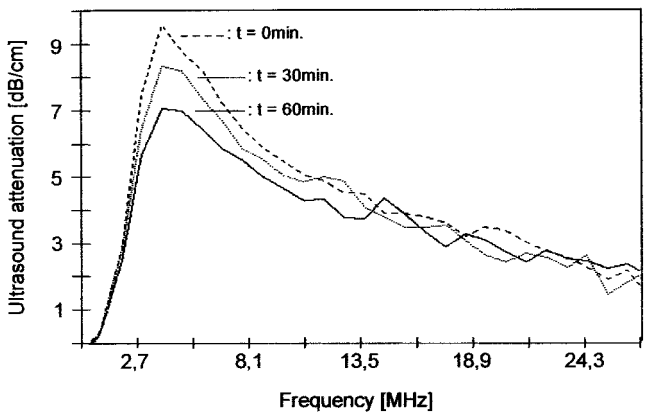

Fig. 3. Representative *in vitro* ultrasound transmission attenuation profile of p DNA containing gas-filled PLGA-MPs expressed in Decibel per centimeter (dD/cm) over the frequency. the decrease in particle stability is seen as decrease in the attenuation over the time. Over the frequency of around 4 MHz the PLGA-MPs had an attenuation maximum of about 9.5 Decibel per centimeters (dB/cm) at the beginning of the measurement (t = 0 min) and to 7.0 dB/cm within 60 min (t = 60 min), respectively.

Quantification of Encapsulated pDNA

Using 32-P labeled pDNA, an average amount of 1.45 μg pDNA per milliliter microparticle suspension, or an average amount of 106 ng pDNA per milligram lyophilized microparticles, respectively, (30% of total pDNA used) was recovered from gas-filled PLGA-microparticles following separation from solid particles, fragments and solvent (Fig. 6). Four different particle batches were analyzed, each having a total pDNA amount of 4.8 μg/ml. Fifty percent of total pDNA was bound to solid particles and fragments, while approximately 20% was solubilized in the aqueous solvent, completely dissociated from the microparticles.

Quantification of pDNA Release

The progress of the *in vitro* release of pDNA from gas-filled PLGA-MPs during ultrasound treatment is shown in Fig. 7A. In detail, the acoustically induced disruption (SAEM) of PLGA-MPs (visible as a mosaic-like color Doppler enhancement, Fig. 8A) was visible on the monitor of the ultrasound device during the first 10 min of ultrasound treatment. No further signals of disrupting PLGA-MPs were observed later. Before the ultrasound treatment, an average amount of 376 ng pDNA per milliliter suspension was detected in the supernatant. A total of 125 ng pDNA per milliliter suspension was released during the first 10 min. Following another period of ultrasound treatment (another 80 min), additional 84 ng/ml pDNA was released into the supernatant, but no further stimulated acoustic emission signals were observed (Fig. 8B). Without ultrasound treatment, there was no significant increase in the pDNA amount in the supernatant from the microparticles over a period of 180 min. During incubation an average amount of ~350 ng/ml pDNA was detected in the supernatant all over the time (Fig.7B). As seen in the agarose gel electrophoresis (Fig. 7C), there was no damage in pDNA structure observed after the period of ultrasound treatment. The released pDNA was predominantly supercoiled, only a small amount of open circle pDNA was visible after ultrasound-induced microparticle destruction (lanes 3–6).

DISCUSSION

Stability of pDNA

The conversion of pDNA from a supercoiled to a circular structure has only a minor effect on gene transfer efficiency (20). Subsequent conversion to linear pDNA reduces gene expression by 90% whereas transformation to oligonucleotide fragments lacks gene expression potency (21). However, the amount of supercoiled pDNA is still an important stability indicator of progressive pDNA damage. Therefore, the development of pDNA containing gas-filled microparticles for gene therapy requires a better understanding of the factors influencing pDNA stability during the manufacture of such microparticles. Our results demonstrate that pDNA stabilized as a complex with cationic polymers can be encapsulated into gas-filled PLGA-MPs, and released by ultrasound treatment without a significant loss of integrity. The double-emulsion method used to produce the gas-filled PLGA-MPs involved sonication and homogenization. Mechanical forces such as cavitation and shearing effects can adversely affect pDNA

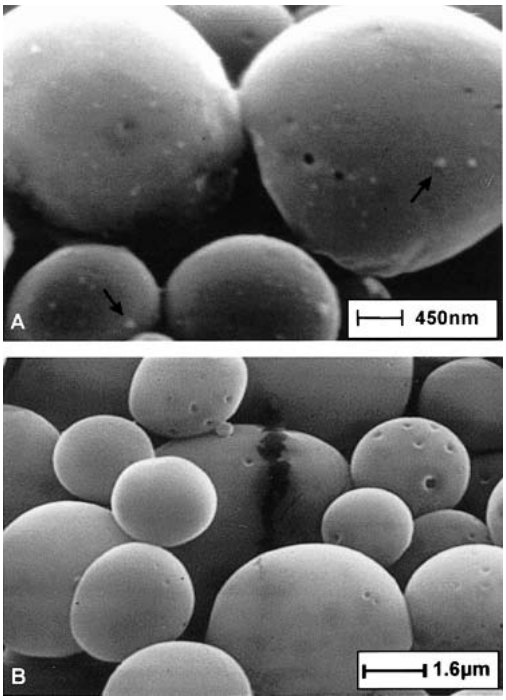

Fig. 4. Scanning electron micrographs showing the surface of PLGA-MPs after encapsulation of pDNA. A small amount of cationic gold labeled pDNA was found on the surface of the MPs (small white spheres) after silver enhancement (A). Without encapsulated cationic gold labeled pDNA the surface of the particles remained smooth after silver enhancement (B).

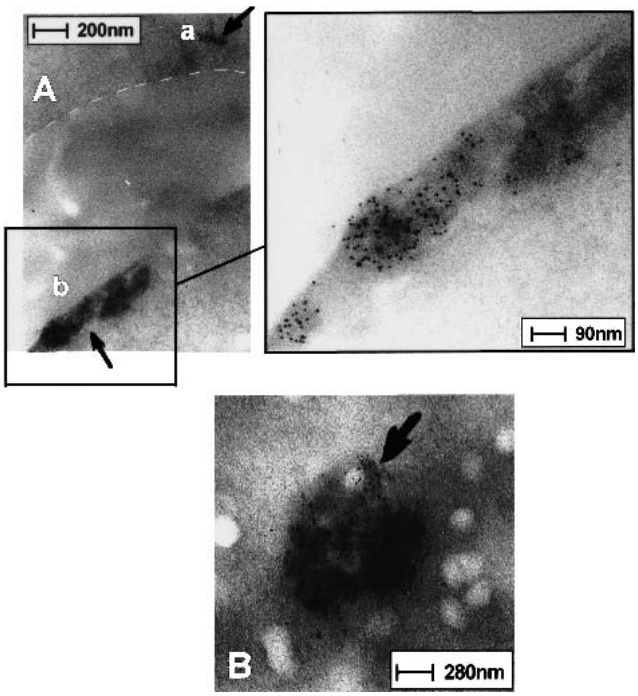

Fig. 5. Transmission electron micrographs showing a thin section of a pDNA containing PLGA-MP (A). The outline of the microparticle is signed by a white dashed line. The cationic gold labeled pDNA (small black spheres) is localized on the surface of the microparticle (a) as well as inside the gas-filled core (b) of the PLGA-MP. B) unbounded cationic gold labeled pDNA solubilized in the solvent.

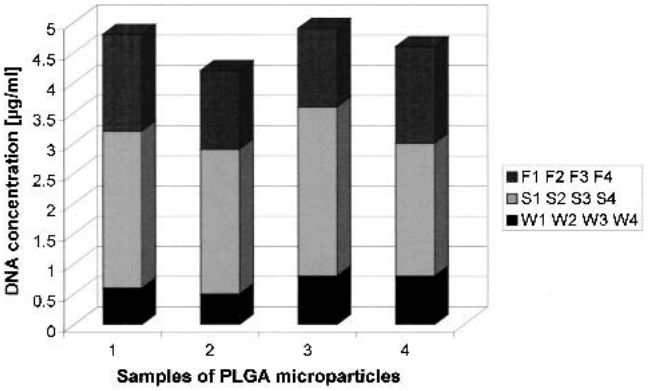

Fig. 6. The amount of pDNA [µg/ml] encapsulated in gas-filled PLGA-MPs (F1–4), associated with solid particles and fragments. (S1–4) and unbounded in the suspension (W1–4). To determine the encapsulation efficiency. pDNA was labeled with radioactivity by nick translation using 32-P-dATP before to encapsulation. A total amount of 100 µg (4.8 µg/ml) PLL-complexed pDNA (1µg 32P-labeled) was used.

integrity. Studies from Walter *et al.* (22) showed that the formation of a primary w/o emulsion by sonication has a detrimental effect on pDNA, resulting in complete degradation of supercoiled pDNA. In contrast, Wang *et al.* (23) previously reported that PLGA by itself protects pDNA from the degradation caused by shear forces during solid microsphere preparation. However, our microparticle preparation did not allow the encapsulation of native pDNA without conversion from supercoiled pDNA to the linearized form. Conditions exerting minimal effects on the supercoiled pDNA topology such as reduced sonication or homogenization power which were found in earlier experiments (not published), diminished the yield of gas-filled acoustic microparticles. Therefore, it was necessary to form pDNA complexes with cationic polymers before their encapsulation in gas-filled PLGA-MPs that were more resistant to pDNA damage. All the cationic polymers and liposomes used prevented pDNA damage during the encapsulation procedure, but this protection was not complete. Nevertheless, we speculated that complexing of pDNA with cationic polymers in combination with reduced stirring speed during particle preparation may protect from pDNA damage. According to Capan *et al.* (24), who recently observed that the amount of supercoiled pDNA in solid PLGA microspheres rises from 16% to 85% when the pDNA was complexed to PLL before encapsulation, we used for all further investigations pDNA/PLL complexes for the preparation of gas-filled microparticles.

Particle Size Distribution

Although the smallest capillary vessels in the blood-vessel system are around 6–7 µm in diameter, a fraction of particles with diameters of more than 7 µm is tolerated by the organism. Davis *et al.* (25) found that the incompatibility of a particular formulation intravenously administered increases with particle diameters, especially above 13 µm. However, they gave no information about the maximal tolerable number of microparticles above 13 µm. Our gas-filled PLGA-MP formulations have mean diameters at d50% of about 5.0 µm and at d99% of about 13 µm, with a polydispersity index around one, (Table I). No significant size differences were given between both, the batches with and without encapsulated pDNA. The gas-filled PLGA-MPs are in a size range similar e.g. to gas-filled lipid bilayers prepared as USCM (26).

Acoustical Efficacy

In vitro measurements of the ultrasound transmission attenuation suggest that gas-filled PLGA-MPs may be more

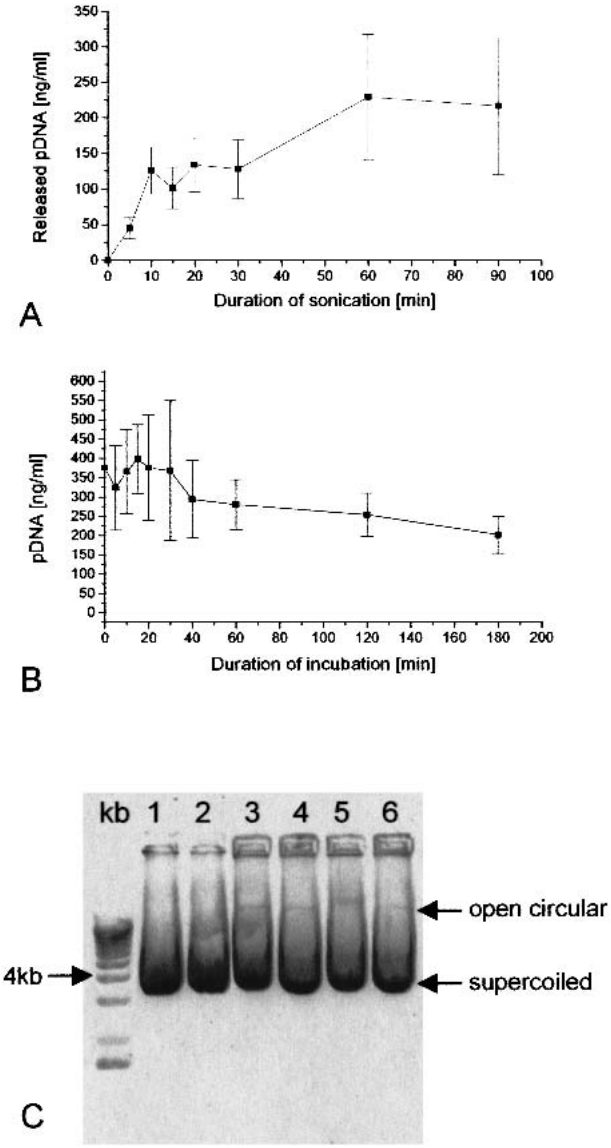

Fig. 7. *In vitro* release profiles of pDNA encapsulated into gas-filled PLGA-MPs. the pDNA amount released from gas-filled PLGA-MPs into the supernatant during ultrasound-induced particle destruction (A). In this graph the pDNA amount, which was determined in the supernatant before the ultrasound-induced destruction of gas-filled PLGA-MPs was set to zero for a better demonstration of the released pDNA amount. Plasmid DNA amount from gas-filled PLGA-MPs in the supernatant without ultrasound treatment (B). Agarose gel electrophoresis of PLL complexed pDNA released after ultrasound-induced microparticle destruction shows no structural alteration (C). kb = 1kb ladder; lane 1: native pDNA before encapsulation; lane 2; pDNA after encapsulation without ultrasound treatment; lane 3–6: released pDNA after ultrasound-induced microparticle destruction; (3 = 10 min, 4 = 20 min, 5 = 30 min, 6 = 60 min).

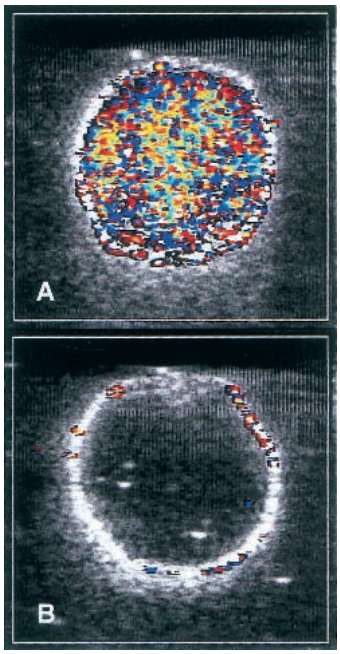

Fig. 8. The monitoring of the ultrasound induced *in vitro* destruction of gas-filled PLGA-MP in color Doppler mode during the pDNA release study. Mosaic-like color Doppler pixel are visible caused by the acoustically induced disruption of gas-filled microparticles during sonication (SAEM) (A). After 10min of sonication no more SAEM signals could be observed, due to complete destruction of gas-filled PLGA-MP (B).

stable than most soft shell USCM. Soft-shell stabilized microbubbles demonstrated *in vivo* acoustical efficacy up to 1 min (26), whereas hard-shell stabilized microbubbles showed acoustical efficacy for several minutes (27). The observed decrease in the ultrasound transmission attenuation of ~22% within 60 min was due to hydrolytic degradation. The *in vitro* measurements of the ultrasound transmission attenuation of gas-filled PLGA-MP suspensions showed a relatively high stability over the time (up to 60 min) compared to the *in vivo* stability of other hard-shell USCM. The reasons for the shorter verification of the ultrasound efficacy *in vivo* are the more complex situation in the bloodstream in contrast to the *in vitro* situation. One reason is the present of several gases with different properties and partial pressures in the blood, which is mostly different to that in a gas bubble leading to gas diffusions between the stabilized gas bubbles and the blood. This can result in such strong shrinking or dilating processes (depending on the type of gas) that the stabilized gas bubbles can loss their gas core and hence their acoustic efficacy (28). Other reasons are degradation processes, phagozytosis or the ultrasound-induced destruction of the gas-filled microparticles by permanent sonication during the ultrasound examination.

Encapsulation Efficiency

One of the adjuvants preferred for use in microparticle preparation is gelatin. In contrast to other adjuvants such as polyvinylalcohol, gelatin, dissolved in the outer water phase (w2) of the w/o/w double emulsion yields the best quality of gas-filled microparticles. For pDNA analysis, extraction was difficult because of strong pDNA/gelatin interaction (data not shown). To overcome this difficulty we prepared the PLGA-MPs with polyvinylalcohol in the w2 phase, especially for pDNA extraction. This alteration in particle formulation allowed us to extract the pDNA by the dichloromethane/water extraction method, but it resulted in a poorer quality of echogenic microparticles. Different analytical methods were tested to quantify the amount of encapsulated pDNA. The most commonly used technique for characterization of nucleic acid concentration is the measurement of absorbance at 260 nm. In general, the disadvantages of the absorbance method are the unspecific contribution of nucleotides and single-stranded nucleic acids to the signal, the inability to distinguish between DNA and RNA and the low sensitivity of the assay. In our case, the major disadvantage of the absorbance method was a strong interference at 260 nm caused by contaminants such as poly(D,L-lactide-co-glycolide) present in the dichloromethane/water extract. To overcome these limitations, we applied the PicoGreen® fluorescence label. PicoGreen® reagent is an ultrasensitive fluorescent nucleic acid stain used for the quantification of double-stranded DNA in solution. However, this quantitative method failed when used for analyzing pDNA extracted from microparticle formulations. Both the absorbance method and the fluorescent method presume DNA extraction before determination. There are only a small variety of organic solvents (dichloromethane, acetone/ dichloromethane mixtures or dimethylsulfoxide) in which gas-filled pDNA-containing PLGA-MPs could be dissolved. Acetone and dimethylsulfoxide hinder pDNA extraction in general, because of their miscibility with water. We found that dichloromethane residues in the pDNA extract influence the signal intensity of the PicoGreen® reagent in such a way that pDNA quantification with the fluorescent method was hampered (results from unpublished previous experiments). Radioactive labeling of the pDNA was regarded as an alternative to fluorescence-based quantification methods. The advantage of this method was the direct determination of the encapsulated pDNA in the gas-filled microparticles without any prior extraction step. Hsu *et al.* (29) reported on an encapsulation procedure that allows the encapsulation of pDNA in solid microparticles with efficiencies between 18% and 43%, using different PLGA polymer solutions. The higher the viscosity of the polymer solution, the higher the amount of pDNA encapsulated. We found an encapsulation efficiency of around 30%, which was similar to the findings of Hsu *et al.*

In Vitro Release Studies

In general, the release of drugs encapsulated in solid polymeric microparticles occurs by two different mechanisms. First, the release of encapsulated drugs may occur due to diffusion through pores or channels formed during microparticle preparation. Secondly, drug release may follow polymer degradation or solubilization. Both Wang *et al.* (23) and Capan *et al.* (30) reported that the incubation of solid PLGA microspheres containing pDNA resulted in a constant pDNA release over 20 days due to diffusion through the porous polymer matrix. In contrast to solid PLGA microspheres, which promote an *in vitro* release of ~30% of encapsulated pDNA within a relative long period of time (3 days) (30), the ultrasound-induced destruction of gas-filled PLGA-MPs promotes a controlled *in vitro* release of ~10% of encapsulated pDNA

within a short period of time (10 min). Our results indicate that gas-filled PLGA MPs have the potential for their use as a nonviral gene delivery system. Further optimization of the manufacturing conditions will probably increase the pDNA encapsulation and release efficiency.

CONCLUSION

We have shown that plasmid DNA can be encapsulated in biodegradable gas-filled PLGA-MPs without losing its structural integrity. Furthermore, the encapsulated pDNA can be released by ultrasound-induced microparticle-destruction *in vitro*. These results are promising in terms of the development of a nonviral target specific gene delivery system.

ACKNOWLEDGMENTS

The authors would like to thank Mrs. Violetta Sudmann, Mrs. Tanja Goehring and Mr. Michael Reinhardt for their excellent technical assistance.

REFERENCES

1. K. Kawabata, Y. Takkura, and M. Hashida. The fate of plasmid DNA after intravenous injection in mice: Involvement of scavenger receptors in its hepatic uptake. *Pharm. Res* **12:**825–830 (1995).
2. M. Yoshida, R. I. Mahato, K. Kawabata, Y. Takakura, and M. Hashida. Disposition characteristics of plasmid DNA in the single-pass rat liver perfusion system. *Pharm. Res.* **13**:599–603 (1996).
3. T. Matsuse and S. Teramoto. Progress in adenovirus-mediated gene therapy for cystic fibrosis lung disease. *Curr. Ther. Res.-Clin. Exp* **61**:422–434 (2000).
4. K. M. Kurian, C. J. Watson, and A. H. Wyllie. Retroviral vectors. *J. Clin. Pathol. Mol. Pathol.* **53**:173–176 (2000).
5. S. L. Hart. Synthetic vectors for gene therapy. *Exp. Opin. Ther. Patents.* **10**:199–208 (2000).
6. P. Deléfine, C. Guillaume, V. Floch, S. Loisel, J.-J. Yaouanc, J.-C. Clément, H. Des Abbayes, and C. Férec. Cationic phosphonolipids as nonviral vectors: *in vitro* and *in vivo* applications. *J. Pharm. Sci.* **89**:629–638 (2000).
7. S. Simoes, V. Slepuskin, P. Pires, R. Gaspar, M. C. P. de Lima, and N. Duzgunes. Human serum albumin enhances DNA transfection by lipoplexes and confers resistance to inhibition by serum. *Biochim. Biophys. Acta-Biomem.* **1463**:459–469 (2000).
8. D. H. Jones, S. Corris, S. McDonald, J. C. S. Clegg, and G. H. Farrar. Poly(DL-lactide-co-glycolide)-encapsulated plasmid DNA elicits systemic and mucosal antibody responses to encoded protein after oral administration. *Vaccine* **15**:814–817 (1997).
9. D. O. Cosgrove and M. J. K. Blomley. Echo-enhancing ('contrast') agents for ultrasound. In D.O. Cosgrove, R.G. Grainger (eds.), Textbook of Contrast Media, Isis Medical Media, Oxford, United Kingdom, 1999, pp. 451–452.
10. D. M. Skyba, R. J. Price, A. Z. Linka, T. C. Skalak, and S. Kaul. Direct in vivo visualization of intravascular destruction of microbubbles by ultrasound and its local effects on tissue. *Circulation* **98**:290–293 (1998).
11. R. J. Price, D. M. Skyba, S. Kaul, and T. C. Skalak. Delivery of colloidal particles and red blood cells to tissue through microvessel ruptures created by targeted microbubble destruction with ultrasound. *Circulation* **98**:1264–1267 (1998).
12. M. L. Main and P. A. Grayburn. Clinical applications of transpulmonary contrast echocardiography. *Am. Heart J.* **137**:144–153 (1999).
13. P. Hauff, T. Fritzsch, M. Reinhardt, W. Weitschiess, F. Lueders, V. Uhlendorf, and D. Heldmann. Delineation of experimental liver tumors in rabbits by a new ultrasound contrast agent and stimulated acoustic emission. *Invest. Radiol.* **32**:94–99 (1997).
14. E. C. Unger, E. Hersh, M. Vannan, and T. McCreery. Gene delivery using ultrasound contrast agents. *Echocardiography* **18**: 355–361 (2001).
15. E. C. Unger, T. P. McCreery, and R. Schweitzer. Ultrasound enhancement of gene expression and gene delivery using acoustically active carriers. Abstract, *Macromolecular Drug Delivery Conference*, Breckenridge, Colorado, p.20 (1999)
16. R. V. Shohet. S.Chen, Y.-T. Zhou, Z. Wang, R.S. Meidell, R.H. Unger, and P.A. Grayburn. Echocardiographic destruction of albumin microbubbles directs gene delivery to the myocardium. *Circulation* **101**:2554–2556 (2000).
17. K. Anwer, G. Kao, B. Proctor, I. Anscombe, V. Florack, R. Earls, E. Wilson, T. McCreery, E. Unger, A. Rolland, and S. M. Sullivan. Ultrasound enhancement of cationic lipid-mediated gene transfer to primary tumors following systemic administration. *Gene Ther.* **7**:1833–1839 (2000).
18. S. Bao, B. D. Thrall, and D. L. Miller. Transfection of a reporter plasmid into cultured cells by sonoporation in vitro. *Ultrasound Med. Biol.* **23**:953–959 (1997).
19. U. Kaatze and K. Lautscham. High-frequency ultrasonic absorption spectroscopy on aqueous suspensions of phospholipid bilayer vesicles. *Biophys. Chem.* **32**:153–160 (1988).
20. D. Bergan, T. Galbraith, and D. L. Sloane. Gene transfer in vitro and in vivo by cationic lipids is not significantly affected by levels of supercoiling of a reporter plasmid. *Pharm. Res.* **17**:967–973 (2000).
21. R. C. Adami, W. T. Collard, S. A. Gupta, K. Y. Kwok, J. Bonadio, and K. G. Rice. Stability of peptide-condensed plasmid DNA formulations. *J. Pharm. Sci.* **87**:678–683 (1998).
22. E. Walter, K. Moelling, J. Pavlovic, and H. P. Merkle. Microencapsulation of DNA using poly(DL-lactide-co-glycolide): stability issues and release characteristics. *J. Control. Release* **61**:361–374 (1999).
23. D. Wang, D. R. Robinson, G. S. Kwon, and J. Samuel. Encapsulation of plasmid DNA in biodegradable poly(D,L-lactide-co-glycolic acid) microspheres as a novel approach for immunogene delivery. *J. Control. Release* **57**:9–18 (1999).
24. Y. Capan, B. H. Woo, S. Gebrekidan, S. Ahmed, and P. DeLuca. Preparation and characterization of poly (D,L-lactide-co-glycolide) microspheres for controlled release of poly (L-lysine) complexed plasmid DNA. *Pharm. Res.* **16**:509–513 (1999).
25. M. A. Davis and R. A. Taube. Pulmonary perfusion imaging: Acute toxicity and safety factors as a function of particle size. *J. Nucl. Med.* **19**:709–712 (1978).
26. E. Unger, T. Fritz, D.-K. Shen, P. Lund, D. Sahn, R. Ramaswami, T. Matsunaga, D. Yellowhair, and B. Kulik. Gas filled lipid bilayers as imaging contrast agents. *J. Liposome Res.* **4**:861–874 (1994).
27. A. Bauer, M. Mahler, A. Urbank, M. Zomack, R. Schlief, and H.-P. Niendorf. Microvascular imaging – results from phase I study of the novel polymeric ultrasound contrast agent SHU 563A. In N.C. Nanda, R. Schlief, and B.B. Goldberg (eds.), Advances in echo imaging using contrast enhancement, Kluwer Academic Publishers, Dordrecht, The Netherlands, 1997, pp.685-690.
28. D. Heldmann, T. Fritzsch, and P. Hauff. Microbubble pharmacokinetics. In D.O. Cosgrove, R.G. Grainger (eds.), Textbook of Contrast Media, Isis Medical Media, Oxford, United Kingdom, 1999, pp. 468-470.
29. Y.-Y. Hsu, T. Hao, and M. L. Hedley. Comparison of process parameters for microencapsulation of plasmid DNA in poly(D,L-lactic-co-glycolic) acid microspheres. *J. Drug Target.* **7**:313–323 (1999).
30. Y. Capan, B. H. Woo, S. Gebrekidan, S. Ahmed, and P. P. DeLuca. Influence of formulation parameters on the characteristics of poly(D,L-lactide-co-glycolide) microspheres containing poly(L-lysine) complexed plasmid DNA. *J. Control. Release* **60**: 279–286 (1999).