Plasmid DNA Encapsulation within Cationic Diblock Copolymer Vesicles for Gene Delivery

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We report the design and structural characterization of cationic diblock copolymer vesicles loaded with plasmid DNA based on a single emulsion technique. For this purpose, a DNA solution was emulsified in an organic solvent and stabilized by an amphiphilic diblock copolymer. The neutral block forms an interfacial brush, whereas the cationic attachment complexes with DNA. A subsequent change of the quality of the organic solvent results in the collapse of the brush and the formation of a capsule. The capsules are subsequently dispersed in aqueous medium to form vesicles and stabilized with an osmotic agent in the external phase. Inside the vesicles, the plasmid is compacted in a liquid−crystalline fashion as shown by the appearance of birefringent textures under crossed polarizers and the increase in fluorescence intensity of labeled DNA. The compaction efficiency and the size distribution of the vesicles were determined by light and electron microscopy, and the integrity of the DNA after encapsulation and subsequent release was confirmed by gel electrophoresis. We demonstrate reverse transfection of in vitro cultured HeLa cancer cells growing on plasmid−copolymer vesicles deposited on a glass substrate.

Introduction

Micro- and nanosized polymeric vesicles have vast industrial, medical, and pharmaceutical potential due to their capacity to encapsulate a reagent and to protect it against a possibly hostile medium.[1,2] The nowadays most common preparation procedure involves the adsorption of alternating layers of oppositely charged polyelectrolytes on a template particle to form a multilayer shell.[3,4] The template particle is subsequently destroyed, after which the resulting empty capsule is loaded with a drug or reagent. A drawback of this method is that the reagent is usually inserted after the preparation of the capsules, and hence, the loading efficiency depends on the permeability of the membrane. Recently, we proposed an alternative preparation procedure to encapsulate water-soluble macromolecules based on a single emulsion technique.[5] In this procedure, an aqueous solution containing the reagent (e.g., DNA) was emulsified in an organic solvent and stabilized by an amphiphilic diblock copolymer. The neutral block of the copolymer formed an interfacial brush, whereas the polyelectrolyte attachment formed a complex with the reagent at the copolymer/water interface. A subsequent change of the quality of the organic solvent results in a collapse of the brush and the formation of a capsule. The capsules can subsequently be transferred into aqueous medium to form vesicles, provided that they are stabilized with an osmotic agent (poly(ethylene glycol), PEG) in the external phase. In principle, there are no restrictions to the molecular weight of the encapsulated material because the reagent was introduced in the emulsion droplets before the membrane had been formed. In our previous work, we have accordingly prepared cationic diblock copolymer vesicles encapsulating a 50 nm fragment of DNA as well as a pUC18 plasmid (2686 base pairs).

Our encapsulation procedure is based on the formation of a complex between DNA and the cationic attachment of the diblock copolymer. Complexes of DNA and cationic polymer (polyplexes) are used for targeting DNA into cells.[6−8] A common feature of the previously investigated systems is the micellelike structure of the complex coarcevate in which all DNA is complexed.[9] The micelles contain a domain with DNA and the neutralizing polymer, possibly surrounded by a coronal layer composed of the neutral attachment of the copolymer. Our carrier system consists of a vesicular membrane; inside the vesicles, the DNA is retained without coadsorbed copolymer. As we will see in the present paper, once released from the vesicles, the plasmid is truly free, not complexed with cationic copolymer, and retains its biological functionality. In this paper, we further explore the design and structural characterization of synthetic vesicles made of cationic diblock copolymers loaded with plasmid DNA. In particular, we focus on the preparation of submicrometer-sized vesicles for gene delivery. We will show that these vesicles are easily deposited on a glass substrate and that they can be used for reverse transfection. In a reverse transfection experiment, the cells grow on top of the expression vector on a glass slide rather than the conventional method in which the DNA is added to the adherent cells.[10,11] It is our contention that the easy and efficient deposition of vesicles loaded with cloning vector DNA on a substrate, control of release, biocompatibility, and functionality of the carrier system have potential in the further development of transfected cell arraying techniques for functional genomics studies.

The organization of this paper is as follows. First, we will describe the production of submicrometer-sized vesicles loaded with plasmid DNA. This process is followed by polarized light, scanning electron microscopy, as well as (confocal) fluorescence microscopy of labeled DNA. Then, we move on to the determination of the stability of the vesicles against osmotic stress, the extent to which the plasmid is compacted, and the density distribution of the plasmid inside the vesicles. The

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integrity of encapsulated and subsequently released DNA is checked with gel electrophoresis. We have also investigated the permeability of the membrane for small, low molecular weight molecules by following the time dependence of the staining of the encapsulated DNA with a fluorescence dye. Most of these encapsulation experiments were done with the pUC18 plasmid. We then grow HeLa cancer cells on top of the encapsulated pEGFP-N1 plasmid (4733 base pairs) deposited on a glass slide. pEGFP-N1 encodes a red shifted variant of the wild-type green fluorescent protein (GFP), which has been optimized for brighter fluorescence and higher expression in mammalian cells. Finally, we will demonstrate reverse transfection by the fluorescence of the expressed GFP protein in the cultured cells.

Materials and Methods

Plasmid Isolation. pUC18 was isolated from *Escherichia coli* DH5α.[12] A colony was transformed with pUC18 and grown on a LB agar plate with ampicillin (100 μg/mL). A single colony was taken to grow a culture in terrific broth (TB) medium (12 g of tryptone, 24 g of yeast extract, 4 mL of glycerol, 2.3 g of KH_2PO_4, and 12.5 g of K_2HPO_4 per dm^3) and ampicillin at 37 °C. After 7 h, this culture was put into a fermentor, which contained 30 dm^3 TB medium, ampicillin, and an antifoam agent. The bacteria were cultured for 17 h at 37 °C under continuous shaking and aeration and, subsequently, were harvested and stored at −20 °C. The cells were suspended in TEG buffer (25 mM Tris, 10 mM EDTA, 50 mM glucose, pH 8) and lysed with an alkaline solution (0.2 M NaOH, 1% SDS) at room temperature. Bacterial genomic DNA, cellular debris, and proteins were precipitated by the addition of 3 M potassium acetate at 4 °C. After centrifugation, the supernatant was treated with 5 M ammonium acetate to precipitate any residual contaminants. RNA and protein were removed with RNase (20 μg/mL, 37 °C, 1 h) and proteinase K (100 μg/mL, 55 °C, 1 h) treatments, respectively. After precipitation with cold 2-propanol, the DNA pellet was dried for a short period and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) for storage at 4 °C.

The integrity of the plasmid before and after encapsulation was checked by 1% agarose gel electrophoresis in Tris-acetate buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.3) at 75 V for 1 h.[13] Less than 10% of the plasmid was nicked and open circular; the remaining fraction was supercoiled. The ratio of the optical absorption $A_{260}/A_{280} = 1.83$ indicated that the material was also free of protein. The hypochromic effect at 260 nm (>35%) confirms the integrity of the duplex. The pUC18 concentration in the stock solution (TE buffer) was 12 g/dm^3. pEGFP-N1 (Clontech, Palo Alto, CA) was isolated according to the same protocol as for pUC18. The pEGFP-−N1 concentration in the TE buffer stock solution was 3.8 g/dm^3.

Copolymer and Osmotic Agent. Poly(butadiene-*b*-*N*-methyl-4-vinyl-pyridinium iodide) [PBd-*b*-P4VPQI] diblock copolymer was purchased from Polymer Source Inc., Dorval, Canada. According to the manufacturer, the number-average molecular weights M_n of the PBd and P4VPQI blocks were 120 and 28.2 kg/mol, which corresponds with a degree of polymerization DP = 2220 and 115, respectively. The molecular weight polydispersity M_w/M_n ratio of the copolymer was 1.05. Solutions were prepared by dissolving the copolymer in toluene. The copolymer concentrations were determined by weight and had a value of 4 g/dm^3. Poly(ethylene glycol) (PEG) was purchased from Merck. Osmotic stress solutions were prepared by dissolving the PEG in TE buffer. For the pUC18 encapsulation experiments, the PEG concentration was 50 wt % with molecular weight 3 kg/mol. In the case of the transfection experiment with the pEGFP-N1 plasmid, we have used PEG with a molecular weight of 10 kg/mol and concentrations of 10 and 27 wt %.

Growth and Transfection Media. HeLa cells were transfected with the copolymer vesicles in phosphate buffered saline (PBS, 1.09 g of Na_2HPO_4, 0.32 g of NaH_2PO_4, and 9 g of NaCl per dm^3, pH 7.2) medium with 10 or 27 wt % PEG. After 2 h incubation with the DNA−copolymer vesicles, the cells were extensively rinsed with PBS and subsequently cultured for 48 h at 37 °C at 5% CO_2 pressure in Dulbecco's Modified Eagle Medium (DMEM) with 10% bovine serum.

Imaging. For light microscopy, a droplet of the emulsion was deposited on a microscope slide and sealed with a cover slip. DNA capsules were deposited on a microscope slide by the procedure as described next. The capsules were either directly observed in air or they were first immersed in PEG solution and sealed with a cover slip. Polarized light microscopy was done with a Leica DMR microscope with 10×, 63×, and 100× (oil immersion) objectives at ambient temperature. The magnification was calibrated with the help of a ruler. Images were collected with a Ricoh 35 mm photo camera. Phase contrast and fluorescence imaging were done with an Olympus BX-60 microscope equipped with a 100 W mercury lamp and a UV filter set (U-MWU/narrow band cube; excitation at 330−385 nm and with an emission filter at 420 nm). The exposure time of the DAPI fluorescence label was controlled by a UV light shutter. Images were collected with a charge coupled device (CCD) camera and analyzed with the public domain software Object-Image 2.06 (http://simon.bio.uva.nl/object-image.html). The radial fluorescence intensity of the emulsion droplets, capsules, or vesicles was measured with a 100× (oil immersion) objective, and the background was subtracted. The resolution of the microscope was 0.23 μm, which is more than an order of magnitude smaller than the size of the smallest imaged vesicle with a diameter of around 4 μm. We have checked by deconvolution with a Gaussian and an empirical point spread function acquired from a fluorescent 200 nm colloidal bead that deblurring has no significant effect on the fluorescence profiles and derived parameters. We have hence refrained from digital filtering, in particular because deconvolution easily introduces spurious signals such as ringing. Confocal fluorescence images were captured with an inverted Leica DMIRBE two-photon laser scanning microscope. The excitation was done with a MIRA laser operating at 800 nm, whereas the images were collected in the spectral range between 405 and 609 nm. Finally, the in situ staining of pUC18 with Hoechst dye as well as the transfection of HeLa cells with copolymer-pEGFP-N1 vesicles were monitored with a Zeiss Axiovert 135 fluorescence microscope equipped with Nikon Coolpix 950 CCD camera.

For scanning electron microscopy, capsules were deposited on a microscope slide and dried at 40 °C and 0.75 atm for 1 week. The specimens were subsequently sputter coated with gold under vacuum and imaged with a JEOL SEM 6400 microscope.

Results and Discussion

Production of the Vesicles. We have described the procedure for the preparation of the cationic diblock copolymer vesicles loaded with short fragment DNA (150 base pairs) in a previous publication. The same three-step procedure can be applied for the encapsulation of plasmid DNA. The first step involves the preparation of two solutions: one solution is made of the cationic diblock PBd-*b*-P4VPQI copolymer in toluene, and the second one is a solution made of either pUC18 (12 g/dm^3) or

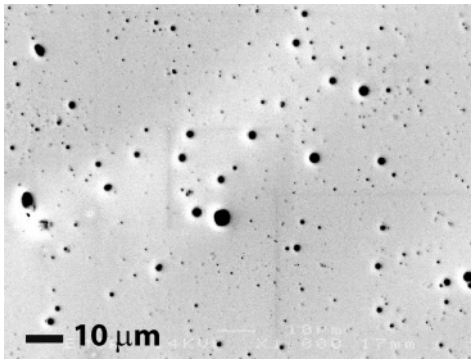

Figure 1. Scanning electron microscopy images of capsules loaded with pUC18 plasmid. The emulsion was sonicated with a power of 25 W applied for 5 min prior to the solvent quality induced collapse of the copolymer layer.

pEGFP-N1 (3.8 g/dm^3) plasmid suspended in TE buffer. The polybutadiene block is sufficiently large to ensure good solubility of the copolymer in toluene, despite the presence of the cationic block. Besides TE buffer, the plasmid solutions were prepared without the addition of salt. The aqueous plasmid solutions were subsequently mixed with the copolymer in a toluene solution in volume ratio of 1:9, respectively, and stirred for several hours to form an emulsion (some samples are sonicated to produce submicrometer-sized vesicles, see following discussion). The cationic block forms a bilayer complex with DNA at the copolymer−water interface, and the hydrophobic attachment prevents the droplets from coalescence. As in the case of the encapsulation of short fragment DNA, the emulsion was observed to be stable over months.

In the second step of the preparation procedure, the emulsion was transferred into ethyl acetate, which is a nonsolvent for polybutadiene. Under this condition, the polybutadiene attachment of the copolymer collapses and forms a shell-like membrane around the emulsion droplet. Besides the collapse of the hydrophobic block, during the transfer into ethyl acetate, water is extracted from the capsule and the DNA becomes compacted. The compaction is facilitated by the good miscibility of water and ethyl acetate and the permeability of the membrane for small molecules. As shown by the scanning electron and polarized light microscopies in Figures 1 and 2a, respectively, the integrity of the capsules loaded with pUC18 is preserved after evaporation of the volatile ethyl acetate. The polarized light micrograph shows that the capsules are birefringent, which indicates that the plasmid solution inside the capsules has become liquid−crystalline.

In the third and final step, vesicles are produced by taking up the dry capsules in an aqueous medium. Any possibly coadsorbed ethyl acetate will dissolve in the water phase and be removed from the vesicles. As in the case of short DNA fragments, the vesicles loaded with plasmid are not stable in pure water, and they need to be stabilized with an osmotic agent in the external phase. Figure 2b displays a polarized light micrograph of the vesicles with pUC18 suspended in 50 wt % PEG solution. The vesicles remain weakly birefringent, in accordance with a liquid crystalline molecular arrangement. The initial pUC18 concentration inside the emulsion droplets is 13 g/dm^3. As we will see next with fluorescence microscopy, the pUC18 concentration inside the vesicles is 62 g/dm^3. Since the critical boundaries pertaining to the first appearance of the anisotropic phase and disappearance of the isotropic phase of pUC18 dispersed in water or salt solution are around 5 and 15 g/dm^3, respectively, the concentration of the DNA inside the vesicles is in the liquid−crystalline regime.[14]

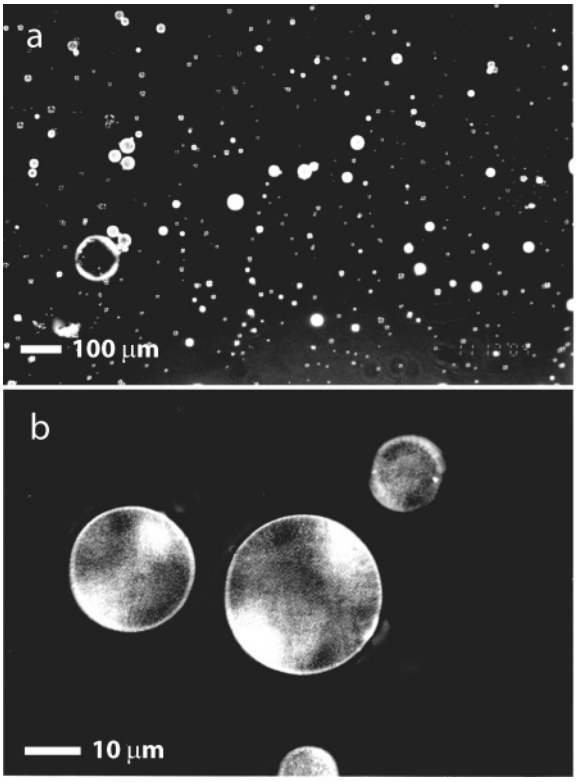

Figure 2. Polarized light microscopy of the encapsulation of pUC18: (a) capsules after evaporation of the volatile organic solvent in air and (b) vesicles immersed in aqueous PEG solution (C_{PEG} = 50 wt %). The specimens are observed through crossed polarizers.

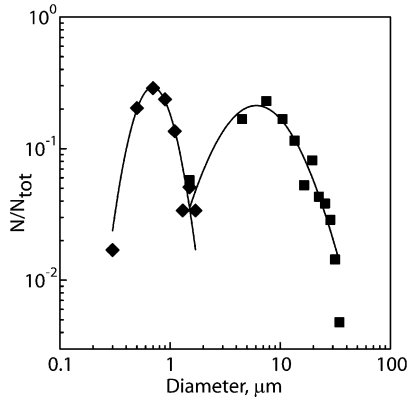

Figure 3. Size distribution of dry capsules loaded with pUC18 as obtained from scanning electron and light microscopy: without (squares) and with (diamonds) sonication of the emulsion prior to the solvent quality induced collapse of the copolymer layer. The solid lines represent log-normal distributions.

For growth of the mammalian cells on top of the encapsulated plasmid, the size of the vesicles is preferably smaller than the size of the cells. Since the dimensions of the cells are on the order of several tens of micrometers, it is necessary to prepare submicrometer-sized vesicles. For this purpose, we have sonicated the emulsion prior to the solvent quality induced collapse of the copolymer layer with a power of 25 W applied for 5 min. The size distributions of the dry capsules were measured with scanning electron and light microscopy and are shown in Figure 3. Without and with sonication, log-normal size distributions were obtained with average diameters of 10 ± 0.7 and 0.7 ± 0.2 μm, respectively. In the scanning electron micrograph (Figure 1), the minimum observable diameter is 0.3 μm. This value is well below the typical dimensions of the cells. However, before we can do the reverse transfection experiment, we need

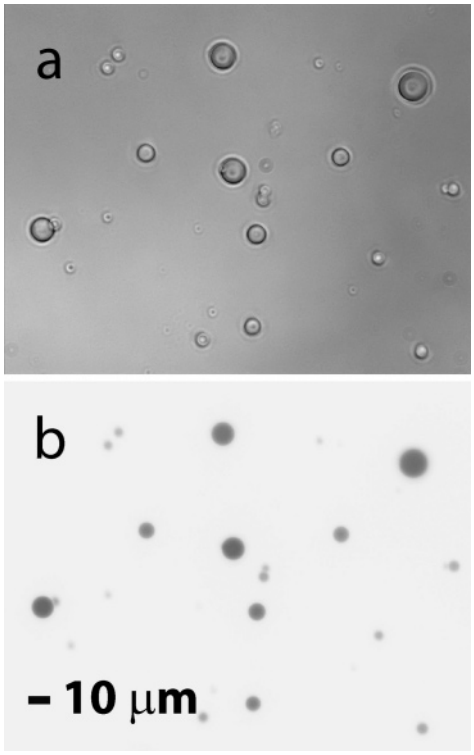

Figure 4. Phase contrast (a) and fluorescence (b) micrographs of the vesicles in 50 wt % PEG solution.

to explore the stability of the vesicles, the extent to which the plasmid is compacted, the integrity of the DNA after encapsulation and subsequent release, and the permeability of the membrane.

Stability, Density, and Compaction. The mechanism for the confinement of the plasmid is the elasticity of the membrane formed by the complex of the collapsed copolymer and DNA. In our previous work on short DNA fragments, we have shown that the vesicles need to be stabilized with an osmotic agent in the external medium. If the osmotic pressure drops below a certain critical value, the membrane ruptures, and the DNA becomes released. To investigate the stability against osmotic stress and the extent to which the plasmid is compacted, we have performed (confocal) fluorescence microscopy with DAPI-labeled pUC18. Figure 4 displays the phase contrast and fluorescence micrographs of vesicles immersed in 50 wt % PEG solution. As shown by the confocal fluorescence microscopy image in Figure 5, the distribution of the plasmid inside the vesicles is uniform. As in the case of short fragment DNA, the plasmid is released from the vesicles once the PEG concentration drops below 5 wt %. The critical minimum PEG concentration depends on the size of the vesicles and the initial plasmid concentration. The larger vesicles are less stable against osmotic pressure; they eject their DNA at a higher external pressure. The inset of Figure 5 displays a confocal fluorescence micrograph of such a large, discharged vesicle with a ruptured membrane (with a piggy-back vesicle attached to it). Since PEG is an inert, water-soluble polymer, we do not expect any effect on the membrane stability besides osmotic effects.

Another characteristic property of the polyelectrolyte copolymer vesicles is the sensitivity to ionic strength. The stability and permeability of the membrane depend on the electrostatic interactions between ionic block of the copolymer and DNA. At very high salinity (1 M), the electrostatic interactions in the polyelectrolyte bilayer are effectively screened, and the membrane becomes permeable for the encapsulated material. Under

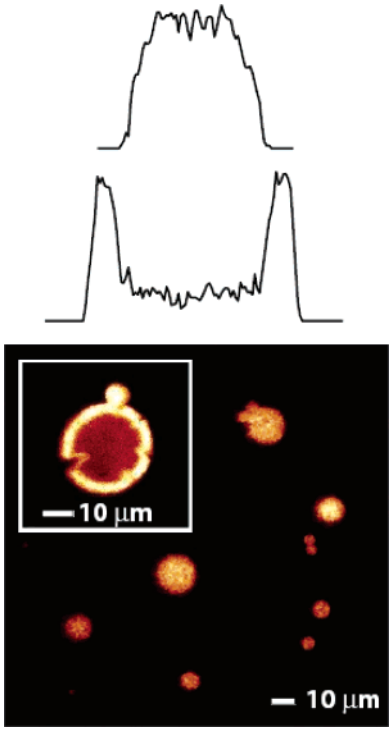

Figure 5. DAPI-labeled pUC18 fluorescence confocal microscopy of vesicles in 50 wt % PEG solution. The plasmid density inside the vesicles is uniform. The inset shows a discharged vesicle with a ruptured membrane and a small piggy-back vesicle. The cross-sectional profiles of a discharged (inset) and full vesicle (the one in the center of the image) are also displayed.

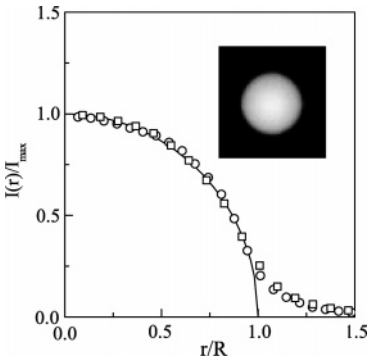

Figure 6. Normalized radial distribution of the fluorescence intensity of DAPI-labeled pUC18 inside an emulsion droplet in toluene (squares) and vesicles immersed in 50 wt % PEG solution (circles). The intensities are normalized to the maximum value, and the radial coordinate is scaled by the radius R. The solid curve represents a fit to a uniform profile with $R = 2.7$ and 3.7 μm in toluene and 50 wt % PEG solution, respectively. The inset shows the fluorescence micrograph of the vesicles in 50 wt % PEG solution. Notice that the data points are slightly over-sampled with a digital to optical resolution of 0.16−0.23 μm, respectively.

the conditions of the transfection experiment to be described next (10 wt % PEG, PBS medium), vesicles with a diameter smaller than 5 ìm are observed to be stable.

The radial dependencies of the azimuthally averaged fluorescence intensities in emulsion droplets in toluene and the vesicles suspended in 50 wt % PEG solution are displayed in Figure 6. Notice that the radial coordinate has been scaled by the maximum radius R and that the intensities are divided by the corresponding intensities measured at the center. The scaled intensities collapse to a single master curve, which shows that the density profiles are similar for pUC18 in an emulsion droplet or a vesicle immersed in aqueous medium. If the density is

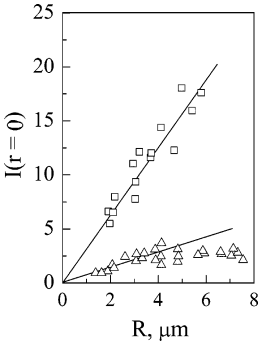

Figure 7. Fluorescence intensity of DAPI-labeled pUC18 at the center of the two-dimensional image of droplets in toluene (triangles) and vesicles in 50 wt % PEG solution (squares). The solid lines represent linear fits of eq 2 to the experimental data in the range $R = 1-6$ nm. The slopes α are 0.7 ± 0.2 and 3.2 ± 0.5 au, which corresponds with plasmid concentration $C_{DNA} = 13 \pm 3$ and 62 ± 9 g/dm^3 inside emulsion droplets and vesicles in PEG solution, respectively.

uniform, the azimuthally averaged profile of the two-dimensional projected image of a spherical object with maximum radius R takes the form

$$I(r) = \alpha R(1 - (r/R)^2)^{1/2} \quad (1)$$

where r is the distance away from the center and α denotes a constant that is proportional to the DNA concentration. As can be seen in Figure 6, the radial intensities satisfy eq 1, which shows that the DNA distribution inside the vesicles is indeed uniform. The deviations observed for $r/R > 1$ are related to the optical resolution of the microscope. These results confirm the observations made with the confocal microscope, where the uniform distribution of the plasmid in a cross-sectional slice is visualized (Figure 5).

The fluorescence intensity measurements can be used to determine the extent to which the plasmid is compacted. For each emulsion droplet in toluene or vesicle in aqueous medium, the radius R and the fluorescence intensity at the center $I(r = 0)$ were determined from a fit of eq 1 to the radial fluorescence intensity profile. The results are displayed in Figure 7, for a population in radii between, for example, 2 and 8 μm. For a uniformly filled spherical object and if the DNA concentration does not depend on the size, the fluorescence intensity at the center of the two-dimensional image is linear in the radius

$$I(r = 0) = \alpha R \quad (2)$$

As shown in Figure 7, for the vesicles, no systematic deviation from a linear dependence of $I(r = 0)$ versus R is observed. In the case of the larger emulsion droplets, the fluorescence intensities fall below the line. This is due to the finite depth of the focal plane, which becomes smaller than the object size for larger droplets. Accordingly, for larger droplets, only part of the emitted light is collected. As already indicated by the occurrence of birefringent textures, the concentration increases, and hence, the plasmid becomes compacted from the emulsion to the vesicles in aqueous medium. The concentrations can be derived from the slopes α, because α is proportional to the DNA concentration and the DNA concentration inside the emulsion droplets is known (13 g/dm^3 pUC18 in TE buffer). From the ratios of the slopes with respect to the one pertaining to the emulsion (compaction factors), we obtain 62 ± 9 g/dm^3 for the pUC18 concentration inside the vesicles in aqueous medium. Our encapsulation procedure has resulted in a 5-fold compaction of DNA. In the case of short fragment DNA, we have reported a 10-fold compaction factor. The smaller compaction factor for

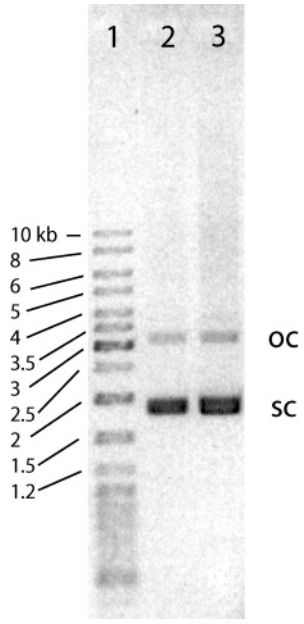

Figure 8. Analysis of the pUC18 plasmid by agarose gel electrophoresis. Lane 1: linear marker; lane 2: after encapsulation and subsequent release; and lane 3: isolated batch prior to encapsulation. The chromatogram shows less than 10% nicked, open circular (oc) DNA, irrespective of the encapsulation history.

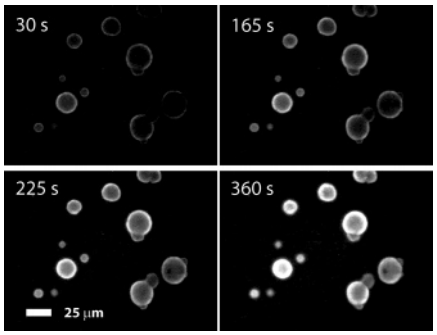

Figure 9. In situ staining of vesicles loaded with pUC18 plasmid. The fluorescence micrographs were taken at the indicated times after transferring the vesicles in 10 wt % PEG solution with 0.008 g/dm^3 staining agent Hoechst 33342.

the plasmid might be due to the differences in molecular weight and topology.

Integrity and Permeability. We have checked the integrity of the plasmid after encapsulation and subsequent release with the help of gel electrophoresis. For this purpose, a 19 g/dm^3 solution of pUC18 in TE buffer was emulsified in toluene and stabilized with PBd-*b*-P4VPQI copolymer. This emulsion was kept for 2 months at 4 °C, after which capsules were produced according to the procedure described previously. The capsules were dried and stored for another week. Prior to the gel electrophoresis experiment, the encapsulated plasmid was released by transferring the capsules into TE buffer. Because of the absence of an osmotic agent, the plasmid was released from the vesicles and was collected and put on agarose gel. The result of the gel electrophoresis experiment is displayed in Figure 8. The chromatogram of the encapsulated and subsequently released plasmid is the same as the one pertaining to the control experiment with plasmid that has not been encapsulated. Besides the supercoiled plasmid, the chromatograms show a small amount of open circular DNA but no linear plasmid (the marker in lane 1 represents linear DNA). Further-

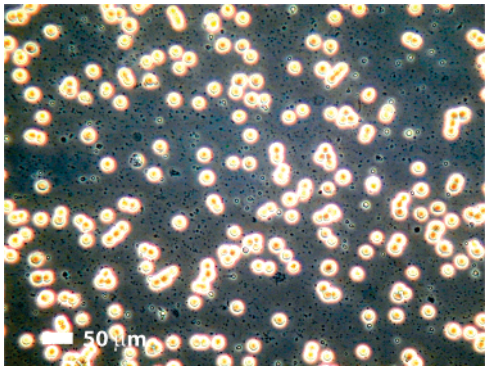

Figure 10. Phase contrast light micrograph of the HeLa cells in the reverse transfection experiment. The microscopy slide was first covered with vesicles, after which the cells in PBS medium with 10 wt % PEG were deposited. Notice that the vesicles are one order of magnitude smaller than the cells.

more, we observe no band smear, which shows that the released plasmid is truly free and not complexed with copolymer.

Although the vesicles retain the high molecular weight DNA, the vesicular membrane should be permeable for water molecules to facilitate the compaction. Here, we demonstrate the permeability of the membrane for small molecules with an in situ staining experiment. For this purpose, vesicles loaded with pUC18 plasmid were deposited on a glass slide and immersed in 10 wt % PEG solution containing a fluorescent dye (Hoechst 33342). The staining of the plasmid inside the vesicles was followed in time, and the results are displayed in Figure 9. Within seconds, the staining agent penetrated the membrane. The vesicles became fully and uniformly stained after 5−10 min with a rate proportional to the surface area of the vesicles, which shows that the staining is limited by the diffusion of the dye through the porous membrane.

Reverse Transfection. We will now show that the copolymer vesicles can be used for reverse transfection of in vitro cultured HeLa cells. According to the procedure described previously, we first covered a glass slide with vesicles containing pEGFP-N1 plasmid. pEGFP-N1 encodes a red shifted variant of the wild-type green fluorescent protein (GFP), which was optimized for brighter fluorescence and higher expression in mammalian cells. The concentration of the plasmid in the initial emulsion droplets was 3.8 g/dm^3. Because of sonication of the emulsion prior to the solvent quality induced collapse of the copolymer layer, the deposited capsules had a log-normal size distribution with a most probable diameter of 0.9 μm. To remove the volatile organic solvent, the slides were allowed to dry for a week at ambient temperature.

The necessity of using PEG as an osmotic agent to stabilize the copolymer vesicles in combination with living cells presents some concerns. At low concentrations (0−15 wt %), PEG with a molecular weight in the range of 8−10 kg/mol tends to deplete from cell surfaces, creating an osmotic gradient that brings cells together. At higher concentrations (15−45 wt %), the high osmotic pressure eventually leads to cell fusion and/or dehydration of the cells.[15] On the other hand, the addition of 2−6% PEG to the transfection media enhances the association between liposome−DNA complexes and targeted cell membranes and results in up to 100 times increase in transfection.[16] We have done reverse transfection experiments with 10 and 27 wt % PEG added to the PBS medium. In the case of 27 wt % PEG, the cells did not survive following a 2 h immersion in the transfection medium, which is likely due to dehydration and/or disruption of the cell membrane. The addition of 10 wt % PEG with a molecular weight of 10 kg/mol was a good compromise; the cells survived the incubation medium, although they aggregated to some extent. We checked by confocal fluorescence microscopy that the vesicles with a diameter smaller than 5 μm 10 wt % PEG in PBS were stable and retained their pEGFP-N1 plasmid (results not shown). Figure 10 displays an optical micrograph of HeLa cells (in PBS medium with 10 wt % PEG) growing on top of the vesicles. The image was taken 2 h after the deposition of the cells. Notice that the size of vesicles is 1 order of magnitude smaller than the cells.

The microscopy slides with HeLa cells and copolymer vesicles were incubated for 2 h in PBS medium with 10 wt % PEG at 37 °C. During this period, the cells attached to the slide on top of the vesicles. Transfection occured either by cytosis or by fusion of the vesicles and the cellular membrane, and there was subsequent release of the plasmid into the cytoplasm.

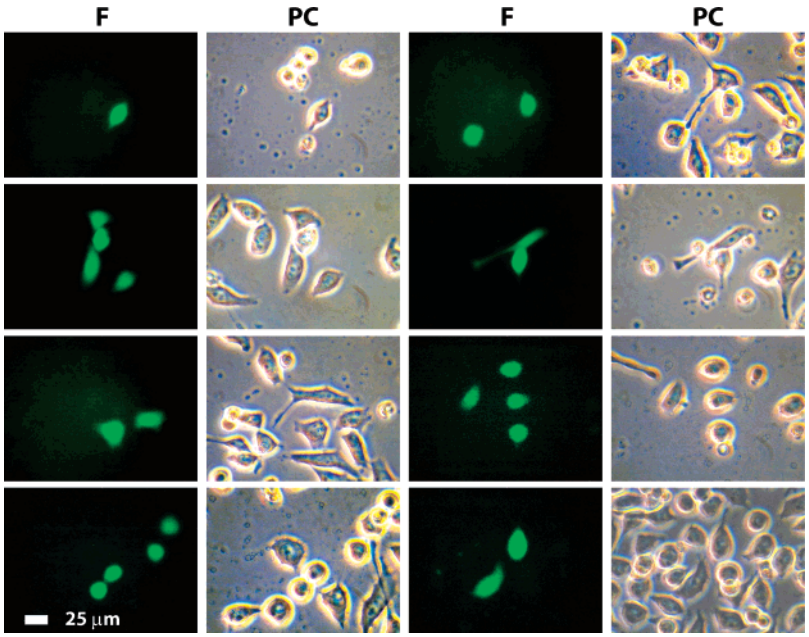

Figure 11. Fluorescence (F) and phase contrast (PC) light micrographs of in vitro cultured HeLa cells growing on top of pEGFP-N1 plasmid−copolymer vesicles in a reverse transfection experiment. The micrographs were recorded 48 h after incubation with the vesicles.

After the incubation period, the osmotic agent, excess unattached cells, possibly released plasmid, and copolymer debris were flushed by extensive rinsing of the slides with PBS. The HeLa cells were subsequently cultured in DMEM medium with 10% bovine serum at 37 °C in a 5% CO_2 atmosphere. After 24 and 48 h, the cells were screened for the presence of expressed GFP inside the cytoplasm. Fluorescence and phase contrast micrographs of the HeLa cells recorded 48 h after incubation with the plasmid−copolymer vesicles are displayed in Figure 11. It is clear from the observed green fluorescence that transfection has occurred; a significant fraction of the cells has expressed the GFP protein encoded by the pEGFP-N1 plasmid. We have checked that cells that were deposited on a slide without copolymer vesicles did not show any green fluorescence.

Conclusion

We have demonstrated that we can encapsulate plasmid DNA in cationic diblock copolymer vesicles with a single emulsion technique. Advantages of our methodology are that there are no restrictions to the molecular weight and that the size of the vesicles can be controlled by the emulsification procedure. To illustrate these features, we have encapsulated supercoiled pUC18 and pEGFP-N1 plasmids in submicrometer-sized vesicles. Inside the vesicles, the plasmid is compacted in a liquid−crystalline fashion as shown by the appearance of birefringent textures under crossed polarizers and the increase in the fluorescence intensity of labeled DNA. The mechanism for confinement is the elasticity of the membrane formed by the complex of the collapsed copolymer layer and DNA. Long time stability of the vesicles can be achieved by using an osmotic agent in the supporting medium. The plasmid can be released from the vesicles by at least two different but related mechanisms. If the osmotic pressure of the supporting medium drops below a certain critical value, the membrane ruptures, and the plasmid is no longer retained. At very high salinity (around 1 M), the electrostatic interactions in the polyelectrolyte bilayer composed of the ionic block of the copolymer and DNA are effectively screened, and the membrane becomes permeable for the encapsulated material. We have checked by gel electrophoresis that the released plasmid is truly free and not complexed with copolymer. This shows that there is no coadsorbed copolymer inside the vesicles. In this respect, our carrier system is different from the classical preparation of nonviral gene vectors, where all DNA is complexed. Furthermore, we observed no changes in the topology of the plasmid induced by the encapsulation procedure (i.e., no detectable linearization of the plasmid or change in the ratio of supercoiled vs open circular, nicked DNA).

An advantage of our preparation procedure is that the vesicles can easily be deposited on a glass substrate. With fluorescence staining experiments, we have shown that the copolymer membrane is highly permeable for small molecules such as fluorescence dyes. This is also supported by the permeability of the membrane for small ions, which results in release of the DNA from the vesicles in very high salt concentrations. We have demonstrated that our model carrier system can be used for the delivery of the cloning vector DNA into in vitro cultured HeLa cancer cells in a reverse transfection experiment. Potential advantages of the DNA/vesicle system are higher DNA coverage, control of release, protection of the DNA against nucleases produced by the cells, and possibilities for specific targeting by grafting of functional groups to the exterior of the vesicles. However, the biocompatibility, stability, and functionality of the vesicles need further optimization to increase efficiency.

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