SAXS Study of Sterically Stabilized Lipid Nanocarriers Functionalized by DNA

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Abstract. The structure of novel spontaneously self-assembled plasmid DNA/lipid complexes is investigated by means of synchrotron radiation small-angle X-ray scattering (SAXS) and Cryo-TEM imaging. Liquid crystalline (LC) hydrated lipid systems are prepared using the non-ionic lipids monoolein and DOPE-PEG2000 and the cationic amphiphile CTAB. The employed plasmid DNA (pDNA) is encoding for the human protein brain-derived neurotrophic factor (BDNF). A coexistence of nanoparticulate objects with different LC inner organizations is established. A transition from bicontinuous membrane sponges, cubosome intermediates and unilamelar liposomes to multilamellar vesicles, functionalized by pDNA, is favoured upon binding and compaction of pBDNF onto the cationic PEGylated lipid nanocarriers. The obtained sterically stabilized multicompartment nanoobjects, with confined supercoiled plasmid DNA (pBDNF), are important in the context of multicompartment lipid nanocarriers of interest for gene therapy of neurodegenerative diseases.

1. Introduction
Small-angle X-ray scattering (SAXS) and cryogenic transmission electron microscopy (cryo-TEM) are powerful structural methods [1-20] for assessment of the changes in the inner and overall supramolecular organizations of self-assembled cationic lipid nanoparticles (NPs) upon complexation with plasmid DNA (pDNA) in non-viral gene delivery vehicles [21-30]. The ionic interaction between positively charged lipid membranes and nucleic acids, to generate lipoplexes, may cause dramatic structural transformations in the initially formed lipid NPs. Depending on the degree of DNA compaction and uploading, the lipid phase may self-organize into periodic multilamellar, cubic or inverted hexagonal (Hh) structures as well as into non-periodic bilayer tubules or intermediate type aggregates. Studies have reported that nonbilayer type liquid crystalline vehicles, such as Hh-phase lipoplexes, display higher transfection activity [23]. Thus, investigations have been initiated to employ nonlamellar "helper" lipids for non-bilayer lipoplex preparation [2,3]. PEGylation of the lipid nanovehicles has been shown to provide longer circulation times in the biological fluid before gene
delivery [26-29]. The understanding of the interaction of non-viral self-assembled nanocarriers with the cellular barriers and their cellular internalization pathways, as well as of the nucleic acid release mechanisms from the pDNA/lipid nanoparticles, requires more detailed knowledge about the inner structural organization of the nanocarriers as determined by the involved compaction mechanism.

In this paper, we report SAXS and Cryo-TEM structural studies that elucidate the structure of novel pDNA/lipid nanoparticles designed for application in gene therapy of neurodegeneration diseases. Monoolein-based positively charged PEGylated lipid nanocarriers of liquid crystalline nature are prepared by self-assembly, and subjected to complexation with plasmid DNA encoding for human brain-derived neurotrophic factor (BDNF). The protein BDNF is a potential therapeutic agent for treatment of neurodegenerative diseases such as multiple sclerosis (MS), Alzheimer’s, Parkinson’s and Huntington’s diseases, and amyotrophic lateral sclerosis (ALS) [31]. The deficiency of the neuroprotector BDNF is expected to be compensated by gene transfection strategies [32].

We aim at preparation of advanced LC nanovehicles for delivery of the BDNF gene (pBDNF). The cationic amphiphile, cetyltrimethylammonium bromide (CTAB), is used to supply positive charges in the generated liquid crystalline lipid NPs serving for compaction of the plasmid DNA. In excess aqueous medium, the nonionic lipid monoolein (MO) self-assembles into a bicontinuous cubic liquid crystalline phase [33-39], which can be dispersed into nanoparticles upon inclusion of PEGylated amphiphiles or polymer surfactants of the Pluronic type [19,20,33,35]. By changing the molar ratio between the neutral lipid (MO) and the cationic (CTAB) component in the self-assembled lipid mixture we vary the quantity of the positive charges in the lipid membrane available for complexation with pBDNF. The obtained results indicate successful formation of sterically stabilized gene delivery vehicles and a rich variety of self-assembled nanoparticulate structures as interesting potential candidates for neuroregenerative gene therapy studies.

2. Experimental

2.1 Materials

Liquid crystalline nanoparticles were prepared by the hydration of a lyophilized mixed lipid film followed by vortex shaking, agitation by sonication, and filtering through a 0.2 µm filter (Minisart High Flow, Sartorius). The mixed film consisted of a nonlamellar monoglyceride lipid 1-oleoyl-rac-glycerol (MO) (Mw 356.55, purity 99.5%, Sigma), a cationic amphiphile hexadecyltrimethylammonium bromide (CTAB) (Mw 364.46, BioUltra, ≥99.0%, Fluka), and a PEGylated lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(methoxy(polyethylene glycol)-2000) ammonium salt (DOPE-PEG2000) (Mw 2801.51, Avanti Polar Lipids). The PEGylated lipid was incorporated at a molar ratio MO/DOPE-PEG2000 98/2 (mol/mol). The molar ratios between the nonionic lipid (MO) and the cationic amphiphile (CTAB), MO/CTAB, were varied in the sequence 90/10, 85/15, 80/20 and 75/25 mol/mol. The hydration and dispersion of the self-assembled lipid phase in excess phosphate buffer solution (NaH2PO4/Na2HPO4, 1.10-2M, pH 7, p.a. grade, Merck) was performed at ambient temperature. An ultrasonication ice bath (Branson 2510 ultrasonic bath, "set sonics" mode) was applied during 2-4 minutes in order to get homogeneous dispersions of cationic nanoparticles. Endotoxin-free plasmid DNA (pBDNF), encoding for human BDNF, was prepared by custom gene synthesis, subcloning and purification (GenScript Co.). Sterically stabilized lipid/pBDNF nanocomplexes were assembled after dilution of the pBDNF solution in a phosphate buffer and subsequent incubation with a dispersion of cationic lipid nanocarriers MO/CTAB/DOPE-PEG2000. The concentration of the positive charges in the NPs systems was varied through the MO/CTAB molar ratio, while keeping the pBDNF concentration constant. The negative charges of pDNA were maintained in deficiency with respect to the cationic charges in order to avoid the overall precipitation of the generated pBDNF/lipid complexes.

2.2 SAXS

Small angle X-ray scattering measurements with diluted lipid and pDNA/lipid samples were performed at the I22 beamline of the Diamond Light Source (Didcot, U.K). The liquid solutions of nanoparticles were filled in borosilicate glass capillaries (Hilgenberg Gmbh, Germany, diameter 1.5 mm, 10 µm wall thickness). The samples were mounted in a holder in air, without a special vacuum chamber. SAXS measurements were performed at room temperature (21 °C). The detector at the I22
station was a gas wire RAPID 2D detector [40], the area of which was divided into 512x512 pixels. The wavelength of the incident X-ray beam was 0.155 nm during the experiments and the beam size at the samples was \(\sim 350 \times 250 \mu m^2\). The accessible \(q\)-range (magnitude of the scattering vector) was from 0.09 nm\(^{-1}\) to 4.17 nm\(^{-1}\). A metal attenuator was installed in order to prevent the liquid crystalline nanoparticle samples from X-ray damages. The exposure time was about 1 sec. The incident and transmitted beam intensities were simultaneously recorded. Silver behenate was used for the \(q\)-range calibration and glassy carbon for the intensity normalization. After the data acquisition, the obtained 2D images were integrated into 1D scattering curves by means of the \textit{Fit2D} software (www.esrf.eu/computing/scientific/FIT2D). The backgrounds, coming from the glass capillary and the solvent, were measured and subtracted using conventional procedures. One and the same capillary was used during the measurements with and without solvent. This allowed for very precise subtraction of the background.

The fitting of the experimental data was based on the multilamellar vesicle model [41]. The scattering intensity was modeled as a sum of the scattering from individual spherical shells:

\[
I(q) = \left( \sum_{i=0}^{n-1} \left[ K(q, R_c + i t_{sh} + i t_{sol}, \Delta \eta) - K(q, R_c + (i+1)t_{sh} + i t_{sol}, \Delta \eta) \right] \right)^2,
\]

where

\[
K(q, R, \Delta \eta) = \frac{4}{3} \pi R^3 \Delta \eta 3 \frac{\sin(qR) - qR \cos(qR)}{(qR)^3}
\]

is the scattering from a spherical shell, \(R\) is the radius of the shell, \(\Delta \eta\) is the difference between the scattering length density of the surfactant and the solvent. The number of surfactant layers is denoted by \(n\), and \(t_{sh}\) and \(t_{sol}\) are the thicknesses of the surfactant and the solvent layers, respectively.

### 2.3 CryoTEM

For cryo-TEM experiments, a drop of the NPs solution was placed on a pretreated copper grid, which was coated with a perforated polymer film. The excess solution was removed by blotting with a filter paper. The sample film preparation was done under controlled environment conditions at a constant temperature of 25°C. A chamber with a relative humidity of 98-99% was used in order to avoid evaporation of the liquid sample. Rapid vitrification of the thin film was achieved by plunging the grid into liquid ethane maintained just above its freezing point. The sample was then transferred to the electron microscope Zeiss 902A (Carl Zeiss NTS, Oberkochen, Germany). The instrument was operated at an accelerating voltage of 80 kV and in zero-loss bright-field mode. The temperature was kept below -165 °C. The sample was protected against atmospheric conditions during the entire procedure in order to prevent the formation of ice crystals. The resolution of the method was 3-5 nm. Digital images were acquired with a BioVision Pro-SM Slow Scan CCD camera (Proscan Electronische Systeme, GmbH, Germany). The image processing was done by means of the \textit{iTEM} software (Olympus Soft Imaging Solutions, GmbH, Germany).

### 3. Results

Figure 1 shows the scattering curves from the investigated NPs samples prepared with pBDNF (Fig.1a) and without plasmid DNA (Fig. 1b). The position of the characteristic 1st peak is located around \(q=0.04 \ \text{Å}^{-1}\). The corresponding repeat lamellar spacings, \(d\), nearly coincide in values, which are in the range 15.4 – 15.6 nm for three of the NPs samples (see the SAXS patterns 2, 3 and 4 in Fig.1b). For the first sample (SAXS pattern 1 in Fig. 1b), the first peak is very broad and is positioned at \(q=0.037 \ \text{Å}^{-1}\), which corresponds to a spacing of 16.8 nm. After the formation of pDNA/lipid complexes, the position of the characteristic Bragg peak shifts to \(q=0.106 \ \text{Å}^{-1}\) and the corresponding lamellar spacing becomes \(d = 5.9 \ \text{nm}\). This result demonstrates the dramatic condensation of the plasmid DNA in the compacted MO/CTAB/DOPE-PEG\(_{2000}\)/pBDNF lipoplexes.

The SAXS patterns (Fig. 1) and the cryo-TEM images (Fig. 2) suggest that the NPs system, obtained
at a neutral-to-charged lipid ratio MO/CTAB 90/10 (mol/mol), contains liquid crystalline objects of mainly spongosome and intermediate cubosome types. Multilamellar vesicles are not a representative feature at this molar ratio. The small portion of positive charges (only 10 mol% CTAB) appears to provide lower complexation for pBDNF (see the weak peak of the pDNA/lipid complex in Fig.1a). The broad SAXS peaks indicate sponge-like membrane organization and a very small fraction of periodic lamellar formations.

The obtained structural results indicate a coexistence of multicompartment liquid crystalline NPs and vesicles in the MO/CTAB/DOPE-PEG\textsubscript{2000} samples with MO/CTAB molar ratios 85/15 and 80/20 (mol/mol). They reveal that the increase of the number of positive charges, in the sequence from a molar ratio 85/15 to 80/20 (MO/CTAB, mol/mol), results in a corresponding augmentation of the percentage of the pBDNF complexation. Thus, the number of ordered multilamellar formations increases and the intensity of the characteristic SAXS peaks rises (Fig.1a).

The SAXS data and the cryo-TEM images evidence a structural transition (Fig. 3) from multicompartment, sponge-type NPs to multilamellar vesicular nanocarriers upon increasing pDNA complexation at rising positive charges. It is notable that the pBDNF/cationic lipid interaction leads to flattening of the lipid bilayer curvature in the sponge membranes and formation of multilamellar-type carriers. The SAXS peak of the multilamellar vesicles (MLV) becomes stronger and more narrow with the degree of the pBDNF complexation, indicating an enhanced multilamellar formation inside the generated lipoplex NPs at a MO/CTAB molar ratio 80/20.

The most positively charged sample, with a MO/CTAB ratio 75/25 (mol/mol), involves predominantly vesicular NPs (cryo-TEM images not shown here). The lower intensity of the SAXS patterns (Fig. 1a) suggests that the complexation of pBDNF with the closed bilayer vesicles seems to be weaker as compared to its complexation with the cationic liquid crystalline NPs involving flexible bilayers and an inner network of open channels. Another possible explanation for this observation is that the internal organisation of the NPs in sample 4 (MO/CTAB 75/25, mol/mol), is not so dense (the NPs interior is filled by aqueous phase) with regard to the sponge membrane NPs interior, which is built-up from a random lipid bilayer network forming channels (Fig. 3, left).

4. Discussion

The obtained SAXS results provide evidence that the interplay between the membrane charges and curvature plays a key role in the determination of the structural organization of the pBDNF/lipid nanocarriers. The functionalization of the NPs by supercoiled pDNA appears to lead to a flattening of the curved lipid bilayer membranes constituting the monoolein bicontinuous cubic structure. The same tendency is produced also by the CTAB and DOPE-PEG\textsubscript{2000} co-lipids, which help to disperse the bicontinuous cubic phase of MO into nanoparticles. The cationic amphiphile CTAB has been known as an emulsifier providing high plasmid loading efficiency into particles. To ensure steric repulsion between the obtained nanocarriers, the investigated here pBDNF/lipid complexes were prepared in a deficiency of negatively charged pDNA with regard to the total number of cationic charges in the NPs system.

The SAXS results showed that, at increasing molar fractions of CTAB in the MO/CTAB/DOPE-PEG\textsubscript{2000} self-assembled mixtures, the initially formed liquid crystalline NPs, lacking inner multilamellar organization, are progressively transformed into bilayer-based nanoobjects with lower interfacial curvature of the lipid membrane. According to the molecular shape concept, the average critical packing parameter of the mixture of a cationic lipid (CTAB), a helper lipid (MO) and a co-lipid (DOPE-PEG\textsubscript{2000}) will be modified upon rising the CTAB molar fraction as well as upon interaction with pDNA. The reorganization of the lipid bilayer from a 3D sponge-type membrane with open channels (sample 1) to a lamellar-type nanocarrier, upon gene loading, is schematically presented in Figure 3. The SAXS results established the coexistence of cationic lipid NPs without associated DNA and lipid nanocarriers loaded with pBDNF. The latter involve periodically organized lipid/pBDNF complexes with more compact structures as evidenced by the strong Bragg diffraction peaks in the SAXS patterns (Fig. 1a).

The particles morphology, determined by cryo-TEM imaging, suggests that plasmid DNA can be associated on the nanocarrier surfaces but also entrapped between the lipid bilayer surfaces forming multicompartment nanocontainers (Figure 2). The performed EM imaging established a coexistence of
nanovehicles of a liquid crystalline type, displaying dense inner membrane organisation, and nanovehicles of a vesicular membrane type, involving lipid lamellae that surround an aqueous core. Figure 2 reveals that the complexation of the cationic lipid NPs with pBDNF facilitates the bridging between the bilayer surfaces, i.e. favours the tendency of increasing the number of lamellae in the generated lipoplex NPs.

The reported here structural findings prove to be useful for the design of novel lipid nanocarriers of the BDNF gene. The choice of the MO/CTAB molar ratio will allow to directly determining the amphiphilic MO/CTAB/DOPE-PEG\textsubscript{2000} compositions, which could compromise the fusogenic and release properties of the NPs. A recent work [8] has stressed that highly compacted multilamellar lipopexes appear to be rather stable topologically-closed particles, which do not easily release the entrapped pDNA from the closed multilamellar lipid containers. However, a small number of lamellas in the NPs would not hamper the pDNA release. The delivery of pDNA from self-assembled lipid carriers should be facilitated also by open-channel structures such as membrane sponges and tubular assemblies. Some of the advanced nanovector systems, prepared in this investigation, correspond to these criteria.

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**Figures**
Figure 1. Synchrotron radiation SAXS patterns obtained with diluted samples of PEGylated cationic nanoparticles (MO/CTAB/DOPE-PEG$_{2000}$) complexed with pBDNF (a) or lacking plasmid DNA (b). The neutral-to-cationic lipid molar ratio, MO/CTAB, in the self-assembled mixtures is: 90/10 (line 1, green), 85/15 (line 2, red), 80/20 (line 3, blue), and 75/25 (line 4, orange) mol/mol.
Figure 2. Cryo-TEM micrographs of coexisting self-assembled PEGylated lipid nanoparticles (MO/CTAB/DOPE-PEG$_{2000}$) interacting with negatively charged plasmid DNA encoding for human BDNF (pBDNF). The nonionic-to-cationic (MO/CTAB) amphiphile molar ratio in the nanocarriers is 80/20 mol/mol. The left column represents nanoobjects with a low degree of compaction, while the right column shows multicompartement nanoparticles with an extended multilamellar organization favoured by the pBDNF compexation. The scale bar corresponds to 200 nm in every image.
Figure 3. Structural transformation of the inner organisation of self-assembled liquid crystalline cationic lipid nanoparticles induced by the complexation with negatively charged plasmid DNA and revealing the morphological change from a sponge membrane type to a multilamellar vesicular type nanocarrier.