A Protocol To Characterize Peptide-Based Drug Delivery Systems for miRNAs

Anna-Laurence Schachner-Nedherer, Oliver Werzer,* and Andreas Zimmer*©

Department of Pharmaceutical Technology and Biopharmacy, Institute of Pharmaceutical Sciences, University of Graz, 8010 Graz, Austria

ABSTRACT: Micro RNA (miRNA)-based medicines have attracted attention as new therapeutic strategies to treat genetic diseases and metabolic and immunological disorders. MiRNAs have emerged as key mediators of metabolic processes fulfilling regulatory functions in maintaining physiological conditions, while altered miRNA expression profiles are often associated with genetic diseases. However, naked miRNAs exhibit poor enzymatic stability, biomembrane permeation, and cellular uptake. To overcome these limitations, the development of appropriate drug delivery systems (DDS) is necessary. Herein, a DDS is characterized being assembled from miRNA-27a (negative regulator in fat metabolism) and the amphipathic N-TER peptide. Dynamic light scattering (DLS), electrophoretic light scattering, and atomic force microscopy (AFM) are used to investigate physicochemical properties (i.e., size, shape, and charge) of the DDS. Although surface charges should provide decent stabilization, the AFM results confirm a state of agglomeration, which is also suggested by DLS. Furthermore, AFM studies reveal adhesion on hydrophilic as well as hydrophobic substrates, which is related to the amphipathic properties of the N-TER peptide. Physicochemical properties of DDS are important parameters, which have an impact on cell internalization/uptake and have to be taken into account for in vitro studies to develop a successful peptide-based DDS for miRNA replacement therapy in metabolic diseases, such as obesity and others.

INTRODUCTION

Micro RNA (miRNA)-based medicines have attracted attention as promising tools for developing new therapeutic strategies to treat genetic diseases and metabolic and immunological disorders. In general, miRNAs are endogenous noncoding RNAs, which regulate posttranscriptional regulation of gene expression by degradation or translational repression, and thus, they are often involved in the onset and progression of various genetic diseases.1 These small nucleic acids fulfill important regulatory functions in diverse biological processes including cell proliferation, cell differentiation, apoptosis, and metabolism.2−4 However, abnormal miRNA expression, diverging from physiological levels, can be associated with disease development5 resulting in cancer,6−8 metabolic disorders,9,10 or autoimmune diseases.11,12 In particular, increasing prevalence of overweight and obesity has emerged as a global health problem and is often associated with obesity-related chronic conditions, such as diabetes, dyslipidemia, several cancers, and/or cardiovascular diseases.13,14 In this regard, antiadipogenic miRNAs, such as miRNA-27a, might act as a new therapeutic approach as miRNA mimic replacement therapy to prevent or treat obesity by inhibiting adipocyte differentiation through downregulation of adipogenic marker genes (i.e., PPARγ).14−16

Although nucleic acid-based therapies show great potential to treat several genetic diseases, application of naked miRNAs faces some additional challenges. On the one hand, unprotected nucleic acid molecules might be rapidly degraded, and on the other hand, the hydrophilic molecules often show low biomembrane permeability and cellular uptake, which is related to the anionic phosphodiester backbone resulting in electrostatic repulsion from the anionic cell membrane surface.5,17,18 Therefore, the development of suitable drug delivery systems (DDS) is of paramount importance to turn miRNAs into medicine. Appropriate DDS for miRNAs have to fulfill some requirements, which provide protection against enzymatic degradation, cell membrane interaction, cell uptake, and intracellular release and distribution. Promising candidates to develop delivery tools for nucleic acids comprise the family of cell penetrating peptides (CPP). CPP are a class of small cationic peptides, consisting of less than 30 amino acids and are designed from sequences of membrane-interacting proteins, which successfully enable the delivery of anionic molecules across cell membranes into intracellular compartments.18−21

The spontaneously induced self-assembly complexation between the cationic peptides and anionic nucleic acids, based on electrostatic interactions, represents a technically simple process to form a DDS. It has already been described by using different peptides of the CPP family, which includes cationic peptides, such as protamine,22,23 or amphipathic peptides, such as MPG24,25 standing for May, Pierre, and Gilles, the names of

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the people who discovered the peptide. Among the CPP, MPG belongs to the primary amphipathic peptides and consists of 27 amino acids arranged in a sequential assembly of hydrophobic and hydrophilic residues. The hydrophobic domain, derived from HIV-1 gp41 (GALFLGFLGAAGSTMGAWSQPKKKRKV), enhances interactions with the hydrophobic lipid moieties on the cell surface, whereas the hydrophilic domain, derived from the nuclear localization sequence (NLS) of SV40 large T antigen (PKKKRKV), promotes electrostatic interactions with negatively charged nucleic acid molecules as well as with glycosaminoglycans on the cell membrane surface. The hydrophobic and the hydrophilic domains of MPG, hereinafter referred to the trading name ‘N-TER peptide’, are connected with the linker sequence WSQ.18,25,26

The abilities of CPP, in particular of amphipathic N-TER peptide, to rapidly condense negatively charged miRNAs and to translocate their cargos into cells make these peptides promising candidates for drug delivery applications. To overcome biological barriers, however, physicochemical characterization is very important to understand the interactions between the therapeutic molecule (miRNA-27a) and the peptide (N-TER peptide), forming the DDS. Physicochemical parameters including size, shape, and charge often influence cellular internalization and uptake and provide essential information to design an appropriate DDS with optimal effectiveness for miRNAs in vitro.3,13,27,28 In addition, it must be taken into account that biological media, which often resemble both in vitro and in vivo conditions, highly influence and might alter physicochemical characteristics, particularly stability. Therefore, physicochemical properties need to be investigated extensively to provide new opportunities for developing miRNA-based therapeutics.

Herein, it is demonstrated that the N-TER peptide successfully forms complexes with miRNA-27a. Furthermore, physicochemical characterization using dynamic light scattering (DLS), electrophoretic light scattering (ELS), and atomic force microscopy (AFM) are used to investigate complexation behavior. Important key parameters of this peptide-based DDS for miRNAs, such as the particle size, geometry, zeta potential, and stability, will be presented and discussed in this paper.

## RESULTS AND DISCUSSION

### Topography of Particles

To gain knowledge about the complexation behavior of amphipathic N-TER peptides with nucleic acids, a gradual addition of different components to the peptide was performed. The investigations of various stages were analyzed using AFM height images (see Figure 1), whereby N-TER peptides mixed with RNase-free water (Figure 1a); RNase-free water and siRNA dilution buffer (Figure 1b); or RNase-free water, siRNA dilution buffer, and fluorescent-labelled nucleic acid control (FluoNTC; Figure 1c) were investigated.

Starting with the N-TER peptide alone, that is, only mixed with RNase-free water, reveals the formation of agglomerates (see the left hand side of Figure 1a). The AFM results show occasionally formed N-TER peptide complexes being located at the sample surface with a size of 200–300 nm and of rather spherical appearance. Further, a thin film of probably unbound peptide material is located in its surrounding. The presence of N-TER peptide complexes is interesting, whereby this self-assembly of this pure material might be explained by peptide–peptide interactions because of the hydrophobic domain of amphipathic peptides, which seems to favor agglomera-

![Figure 1. AFM height images of the N-TER peptide in RNase-free water (a), additionally mixed with siRNA dilution buffer (b), and finally complexed with FluoNTC (650 nM, c1). Images of the standard N-TER peptide–FluoNTC complexes (650 nM) are captured by combining fluorescence and DIC microscopy (c2), showing the same sample area as presented in c1. The scale is 0.5 μm for the N-TER peptide alone (a,b) and 2 μm for N-TER peptide–FluoNTC complexes (c1).](Image 315x497 to 555x740)
presenting the DDS. For this purpose, AFM investigations are combined with optical methods including fluorescence and differential interference contrast (DIC) microscopy. For complexation with the N-TER peptide, a FluoNTC is used in the presence of RNase-free water and siRNA dilution buffer. In Figure 1c1, the complexes are visualized by AFM imaging at a glass surface. Their size is 200–300 nm, which is about the same with respect to the pure peptide. Our special AFM-microscopy setup allows the determination of fluorescence at the very same position (see Figure 1c2), showing a clear fluorescent signal of the successfully complexed FluoNTC with the N-TER peptide (Figure 1c2, white circles). In contrast to the observations obtained from fluorescence microscopy, some complexes are detected by DIC microscopy and AFM without exhibiting any fluorescent signal (Figure 1c2, white squares). Based on the results in Figure 1a demonstrating peptide-based self-assembly, it is therefore assumed that the nonfluorescent complexes are only composed of the N-TER peptide, without FluoNTC. Such a result might be easily understood by the fact that adding an excess amount of the N-TER peptide is absolutely critical to form a highly stable DDS for nucleic acids. As this excess is prone to self-assembly, this means that two types of particles can be found in such an experiment.

In principle, the complex formation of the N-TER peptide with nucleic acids is explained by a noncovalent mechanism, which initially occurs through electrostatic interactions between both components. The electrostatic interactions involve the positively charged hydrophilic lysine-rich NLS domain of the peptide and the negatively charged phosphate backbone of nucleic acids, followed by peptide–peptide interactions involving the hydrophobic domains. This phenomenon of spontaneously induced self-assembly was described for positively charged CPP to condense polyamionic compounds, such as nucleic acids or oligonucleotides. Further hypothesized that additional interactions take place between the hydrophobic peptide moieties and most likely result in a peptide “cage” around the nucleic acids, which can successfully protect them from enzymatic degradation to form a stable DDS.

In Situ Size and Stability. As already shown in the AFM images, N-TER peptide-based systems tend to self-assemble by itself, which is mainly influenced by their hydrophobic amino acid composition. Nevertheless, on drying of the samples, these assembling might result as strong changes in the environment during solvent evaporation. To check particle size progression of N-TER peptide–nucleic acid complexes as a function of time and concentration, measurements using DLS are performed. The time-dependent DLS investigations are carried out (immediately, 1, 3, and 5 h after preparation) using standard N-TER peptide complexes containing 650 nM of FluoNTC, non-targeting control (NTC), or miRNA-27a, respectively, in the final dispersion (Figure 2). The DLS data in Figure 2 (on top) display a certain size range for the main particle fraction between 200 and 300 nm directly after preparation. This matches perfectly for all three nucleic acids used for complexation with the N-TER peptide. The continuous observations show that after 1 h, the initially observed particle size slightly increased. This increase continues with time, and after 3 and 5 h, still an increase of the particles size is observed, meaning that the agglomeration is continuing over the course of the experiment. This event is even further enhanced after 24 h, which results in continued agglomeration and increase of polydispersity, indicating that this DDS is not appropriate for long-term storage (unpublished experiments).

The increase in size is according to its statistical nature not well defined, so that the polydispersity index (PdI) increases over time. In detail, the PdI-values started at 0.2 immediately after preparation and increase up to 0.6 after 5 h (Figure 2, below). The PdI generally provides important information about mono- or polydispersive properties of DDS. The classification of PdI-values indicates that values ≤0.1 are considered to be highly monodisperse while values of 0.1–0.4 or ≥0.4 are considered to be moderately or highly polydisperse, respectively.

The DLS findings allow the following conclusions to be drawn: first, the standard N-TER peptide–nucleic acid complexes (650 nM) demonstrate that very moderate polydispersity or close to monodisperse properties are present immediately after preparation. Second, the time-dependent agglomeration tendency of the DDS might be explained, on the one hand, by the self-assembly properties of amphipathic peptides due to hydrophobic amino acids and, on the other hand, by a concentration-dependent effect due to a higher probability of particle interactions with increasing concentrations. Third, the three nucleic acids used for complexation with the N-TER peptide, namely FluoNTC, NTC, and miRNA-27a, show similar complexation possibilities. With regard to further studies, this comparable complexation behavior of the applied nucleic acids with the N-TER peptide is supported by exhibiting similar chain lengths (20–24 nucleotides) and molecular weights (about 14 000 g/mol).

Stability Depending on Environmental Changes. To identify the effect of the dilution medium on the particle stability, the behavior of N-TER peptide–nucleic acid complexes in RNase-free water, zeta water, or serum-free low glucose Dulbecco’s modified Eagle medium (DMEM) is evaluated using DLS. For this purpose, standard N-TER peptide–nucleic acid complexes (650 nM) are diluted...
immediately after preparation to obtain nucleic acid concentrations of 100 nM FluoNTC, NTC, or miRNA-27a, respectively. The selected concentration range is a common concentration applied in cell culture in vitro studies. DLS measurements are then performed (immediately, 1, 3, and 5 h after dilution) to monitor particle size progression considering time and dilution medium. The data of these experiments are summarized and displayed in Figure 3.

In the first experiment, N-TER peptide–nucleic acid complexes are diluted with RNase-free water, which is used as a reference to minimize the influence of additional ionic ambient conditions (Figure 3, left). It has to be noted that RNase-free water is always used for preparation of the standard N-TER peptide–nucleic acid complexes (650 nM). Despite dilution, Figure 3 (top left) demonstrates that the main particle fraction of 200–300 nm being also present initially in the standard N-TER peptide–nucleic acid complexes (shown in Figure 2) is still preserved. However, other than the experiments at high concentration, the intensity-based particle sizes of the diluted DDS do not change significantly over time. This more stable behavior might be attributed to a concentration-dependent effect by reducing the probability of particle interactions at lower concentrations.\(^\text{39,43}\) PdI values vary between 0.2 and 0.4 strongly indicating some polydisperse properties, which is additionally confirmed by relatively broad standard deviations (SD; Figure 3, bottom left).

For the second dilution test, N-TER peptide–nucleic acid complexes are diluted with zeta water, which contains a defined ion concentration (0.5 mM) of NaCl (Figure 3, middle). Again, the intensity-based results indicate a size range of 200–300 nm independent of the progressed time. Such particles represent the main particle fraction (Figure 3, middle above), which is also present in the standard (Figure 2) and RNase-free water-diluted (Figure 3, top left) samples. Therefore, the obtained results suggest that the ionic strength in the tested concentration range does not affect the size of the main particle fraction over time. The PdI values show, similar to the dilution with RNase-free water, no time dependence, that is, a rather stable situation. However, unlike before, the PdI values are slightly larger in the range of 0.3–0.7, showing a slightly stronger polydispersity developing (Figure 3, middle bottom).

In the third and last dilution step, N-TER peptide–nucleic acid complexes are diluted with serum-free low glucose DMEM, which is a commonly used cell culture medium and as such interesting for potential in vitro studies to expose the peptide-based DDS in cell-incubating conditions. In general, serum-free low glucose DMEM contains amino acids, vitamins, and inorganic salts, which are expected to affect the particle size and stability of the exposed N-TER peptide–nucleic acid complexes, and in fact, this can be seen in Figure 3 (right). In contrast to the previously shown DLS data regarding standard N-TER peptide–nucleic acid complexes (Figure 2) and RNase-free water or zeta water diluted samples (Figure 3 left/middle), the results using DMEM as the dilution medium clearly demonstrate significant differences in monitoring the particle size and PdI over time (Figure 3, right). Particle sizes of about 600–800 nm are detected immediately after dilution with further increase up to 1 μm (Figure 3, top right). After 5 h, however, the particle sizes of diluted N-TER peptide–nucleic acid complexes seem to decrease under DMEM conditions. While the increase might be understood in a time-dependent growth of the agglomerated on account of statistic processes, the decrease at the end is unexpected. There might be two possible explanations for this phenomenon: On the one hand, particle agglomerates could achieve a maximum size and start to decay. On the other hand, sedimentation of the bigger agglomerates might have started while smaller agglomerates are still influenced more by the Brownian motion, thus floating similar to before. This leads to a different settling behavior, which should be tested and compared to either Stokes’ equation or hindered Stokes’ equation to clarify this. PdI values clearly show the agglomeration tendency of the DMEM-diluted DDS and considerable increase of polydispersity up to 0.7 after continuous observations over 5 h (Figure 3, bottom right).
To assess whether the chosen dilution medium (RNase-free water, zeta water, or serum-free low glucose DMEM) influence the zeta potential of N-TER peptide–nucleic acid complexes as well as the particle size, ELS measurements are performed (Figure 4). In short, the zeta potential is a hypothetical plane within the diffuse layer, which acts as the interface between the charged particles moving in the electric field and the layer of the dispersant around them. The results allow for the conclusion on electrostatic stabilization to be made.43 The samples are diluted standard N-TER peptide–nucleic acid complexes with various media resulting in 80 nM solutions. The data of the results are summarized in Figure 4. The RNase-free water dilution serves again as a reference to investigate the zeta potential of the diluted samples without additional changes. The ELS results in Figure 4 (most left columns) for RNase-diluted N-TER peptide–nucleic acid complexes exhibit positively charged zeta potential values of about +25 to +30 mV. There is nearly no difference for all the different nucleic acid systems, reflecting again the similarity of different particle types.

N-TER peptide–nucleic acid complexes diluted in zeta water exhibit zeta potential values of about +25 mV as shown in Figure 4 (middle columns), which is only slightly smaller compared to RNase-diluted samples. As a consequence, it can be assumed that the conductivity of zeta water, which is set to 50 μS/cm by adding NaCl, is not strongly affecting the diffuse layer and changing the initial surface characteristics of the sample.44

By using serum-free low glucose DMEM for dilution, the ELS experiment shows a strong change. A decrease of zeta potential from about +25 mV from the other down to +10 mV is observed (Figure 4, most right columns), which can be explained by a higher conductivity of the cell culture medium DMEM. This is related to an increase of ionic strength regarding DMEM (135 mM) in comparison to zeta water (0.5 mM) because of the additionally charged components (e.g., amino acids, vitamins, and inorganic salts), which lead to compression of the diffuse layer, and as a consequence, the zeta potential of N-TER peptide–nucleic acid complexes decreases.40,43,45

To summarize the stability considerations followed from DLS and ELS for diluted N-TER peptide–nucleic acid complexes (100 nM), the recorded data of PdI-values clearly indicate polydisperse properties of the samples, independent of the dilution medium (RNase-free water, zeta water, or serum-free low glucose DMEM). However, the particle size characterization highlights the influence of the chosen dispersant on the detected main particle fraction. Dilution with dispersants exhibiting very low ionic strength, such as RNase-free water or zeta water, do not influence the particle size of the main particle fraction, which is already present in the standard N-TER peptide–nucleic acid complexes (650 nM). In addition, the

Figure 4. ELS measurements of diluted N-TER peptide complexes containing FluoNTC (white bars), NTC (weakly dashed bars), or miRNA-27a (strongly dashed bars) characterized as zeta potential. Final nucleic acid concentrations are 80 nM, respectively. As dilution medium, RNase-free water, zeta water, or serum-free low glucose DMEM are used.

Figure 5. AFM height and corresponding phase images with size cross section profiles of N-TER peptide–NTC complexes on silicon wafers with different chemical modifications. The scale is 0.5 μm.
particle sizes of the main particle fraction remain largely unchanged over time. On the contrary, DMEM-diluted samples show a significant increase of the particle size as well as agglomeration tendency over time, which is related to higher electrolyte concentrations in the dilution medium and thus, an increase of ionic strength. The discussed findings of DLS measurements are in accordance with the literature, which emphasize that agglomeration is very prominent for DDS in biological media. Enhanced electrostatic interactions and electrolyte adsorptions result in increased particle size diameters and degree of polydispersity.35,45,46

Furthermore, ELS measurements have contributed to obtain important information about zeta potential properties and colloidal stability of N-TER peptide—nucleic acid complexes, depending on the applied dilution medium ( RNase-free water, zeta water, or serum-free low glucose DMEM). The recorded data show positively charged zeta potential values, which can be attributed to the positively charged lysine residues in the hydrophilic N-TER peptide sequence, thus promoting electrostatic interactions with negatively charged nucleic acids and beyond that with negatively charged cell membranes. Guidelines for classifying and predicting colloidal stability of nanoparticulate DDS are available considering ±0 to 10 mV as highly unstable while ±10 to 20, ±20 to 30, and ±30 mV are classified as relatively, moderately, and highly stable, respectively.35,45,47 After dilution with RNase-free water and zeta water, the obtained zeta potential values (+25 to +30 mV) suggest a DDS with moderately stable colloidal stability, which is in contrast to the DLS measurements suggesting a polydisperse DDS. While zeta potential provides information about electrostatic repulsive forces, colloidal stability is also influenced by attractive interaction forces including hydrogen bonds and hydrophobic and van der Waals forces.36,43,48 Surface properties including surface charge and hydrophobicity can strongly affect stability and provide an explanation for the agglomeration properties of N-TER peptide-based DDS.49,50

Morphology and Adhesion Analysis. DLS and ELS are widely used techniques for physicochemical characterization to determine the particle size, polydispersity, and zeta potential. However, DDS, such as N-TER peptide—nucleic acid complexes, are often polydisperse and for this purpose, it is advisable to apply additional methods for the particle size and morphology analysis.28,46 The AFM technique is therefore used here to clarify the morphology of the structures made from N-TER peptide—nucleic acid complexes. In addition, adhesion behavior of the complexes is estimated for chemically modified substrates (Si-wafers) using bovine serum albumin (BSA) or polystyrol (PS) coatings, which exhibit hydrophilic or hydrophobic surface properties, respectively. The exemplary height images in Figure S reveal the presence of particles but most often agglomerates are found on all investigated substrates including uncoated, BSA- or PS-coated Si-wafers. The results clarify that smaller particles of about 100–300 nm are present on all substrates (marked as size profiles 1 in Figure S), which can be related to be the primary particles also noted in the DLS measurements right after fabrication. These particles represent the smallest building unit, which later form toward much larger size agglomerates even exceeding μm (marked as size profiles 2 in Figure S). These findings clearly prove that N-TER peptide-based DDS have pronounced propensity to agglomerate. The described phenomenon of particle agglomeration has already been discussed for other CPP by Lochmann et al.39 Regarding the particle shape, it can be demonstrated that the primary particles exhibit slightly elongated and very flat disk-shaped geometries based upon aspect ratios (AR, height-to-width relationship), which are assessed from the size cross section profiles 1 in Figure S.49,51,52 Remarkable is the fact that the primary particles only achieve very low heights (30–50 nm) compared to very broad corresponding widths (100–300 nm), which can be explained by a strong effort to bind as much as possible of the surface of the particles to the substrates. The driving forces for this phenomenon are probably electrostatic interactions between the negative surface charge of uncoated or BSA-coated Si-wafers and partially positively charged N-TER peptide—nucleic acid complexes. In comparison with uncoated Si-wafers, BSA coating leads to a slight improvement of AR from 0.1 to 0.2, which is related to a reduced negative charge density and thus reduced electrostatic interactions of the samples with the substrate.33–35 Although BSA exhibits a negative net charge depending on the current pH conditions (pH 5–7), some amino acids of the protein sequence still exhibit positive charges.53,54

On the one hand, electrostatic interactions are promoted with the negatively charged Si-wafer to enable BSA coating, but on the other hand, the negative charge density of the Si-wafer after BSA coating is reduced, resulting in an improvement of the height-to-width relationship. If the interaction forces between the sample and the substrate surface are too strong, the morphology of the sample might be changed, thus affecting the AR. On the contrary, the electrostatic interactions have to be strong enough to prevent detachment of the sample during the scanning process.28,54

Furthermore, the influence of different chemical pretreatments on adhesion behavior of the N-TER peptide—nucleic acid complexes can be estimated. Surface properties of the substrates can have an important impact on the extent of adhesion.57 As demonstrated in the exemplary height images of Figure S, N-TER peptide—NTC complexes adhere to all used silicon substrates, independent of hydrophilic (uncoated, BSA-coated) or hydrophobic (PS-coated) surface properties. In contrast to hydrophilic surfaces, the aqueous sample solution has spread evenly across the hydrophilic surfaces, showing good wettability. Additionally, the interaction forces between the sample and the hydrophilic/hydrophobic substrates must have been strong enough to withstand the forces occurring during the washing with RNase-free water after incubation, which is necessary to remove the siRNA dilution buffer. Interestingly, the molecular structure of the amphipathic N-TER peptide, containing hydrophilic and hydrophobic amino acids, seems to be responsible for the adhesion on hydrophilic as well as hydrophobic substrate surfaces.34,58 The hydrophilic amino acid sequence is composed of glycine (G), alanine (A), leucine (L), phenylalanine (F), methionine (M), tryptophan (W), proline (P), and valine (V), whereas the hydrophilic amino acids of the peptide are divided, on the one hand, into a polar class containing serine (S), threonine T, and glutamine Q and, on the other hand, into a charged class containing lysine (K) and arginine (R).59,60 Adhesion of the N-TER peptide—nucleic acid complexes on the hydrophilic substrates suggests electrostatic interactions predominantly driven by the positively charged lysine and arginine residues of the peptide. Conversely, the hydrophobic amino acids allow interactions with the hydrophobic PS-coated surface. This adhesion phenomenon of amphipathic peptides on hydrophilic silicon substrates or hydrophobic PS is in agreement with the literature, which supports the different bonding mechanisms (electrostatic or hydrophobic interactions).35,58
In addition to morphology and adhesion studies, phase imaging is used as an additional tool for characterizing surface properties and distinguishing between different materials. In the case of BSA, the coated silicon substrate reveals an irregular surface while the PS coating results in porous surface properties. The phase contrast results are used to distinguish between hard (dark) and soft (bright) surface areas. The recorded results in Figure 5 clearly reveal that N-TER peptide–NTC complexes are softer compared to the used substrate materials including uncoated, BSA-, or PS-coated silicon.

**CONCLUSIONS**

The physicochemical investigations using DLS, ELS, and AFM techniques are very important to develop a fundamental understanding about complexation behavior between the amphipathic peptides and nucleic acids. Even though zeta potential values should provide decent stabilization, AFM investigations in the tapping mode qualitatively confirm a state of agglomeration of N-TER peptide-based DDS, which is already suggested by DLS data. Furthermore, AFM enables to estimate adhesion behavior of our N-TER peptide–nucleic acid complexes on different chemically modified substrates, exhibiting hydrophilic or hydrophobic surface properties. AFM phase contrast images confirm soft sample properties of the N-TER peptide–nucleic acid complexes on the used substrate materials. It is further demonstrated that all three nucleic acids, namely, miRNA-27a, NTC, and FluorNTC, exhibit similar complexation possibilities with the N-TER peptide. With respect to successful miRNA-based medicines, physicochemical properties of peptide-based DDS for nucleic acids might influence the therapeutic efficiency of miRNAs on the cellular level by promoting protection against enzymatic degradation, cell membrane interaction, cell uptake, and intracellular release. Therefore, future studies are planned in an in vitro cell culture model to study the anti-adipogenic effect of miRNA-27a on adipogenesis by applying our N-TER peptide-based DDS for nucleic acids.

**EXPERIMENTAL SECTION**

**Materials.** The N-TER Nanoparticle siRNA Transfection System from Sigma-Aldrich (Vienna, Austria) contains siRNA dilution buffer and the N-TER peptide with the sequence GALFLGFLGAAGSTMGAWSQPKRKKW (MW 1345 g/mol) to mimic the function of endogenous miRNAs.

- miRNA mimic negative control containing the sequence UCACAACCCUCUAAGAAGAGUAGA (MW 14 074.31 g/mol) to act as a NTC in miRNA mimic experiments
- miRNA mimic transfection control with Dy547 (FluorNTC) containing the sequence CUCUUUCUAG-GAGGUUGUGA 5’ Dy547 (MW 14 566.9 g/mol) to perform fluorescence experiments in vitro

RNase-free water (VWR, Vienna, Austria) was utilized to prepare N-TER peptide complexes. For dilutions, RNase-free water, zeta water, and DMEM containing 1 g/L d-glucose, referred to as “low-glucose DMEM” (Thermo Fisher Scientific, Vienna, Austria), were used. Zeta water was prepared by using distilled water adjusted with sodium chloride (NaCl) to a conductivity of 50 μS/cm (0.5 mM NaCl) and a pH of 5.5–6.0. The complex preparations were carried out under aseptic conditions using a laminar flow box (HeraSafe KS, Thermo Fisher Scientific). The RNase AWAY (Sigma-Aldrich) decon- tamination reagent was used for surface disinfection.

**Sample Preparation.** Stock solutions of miRNA-27a, NTC, or FluorNTC were prepared in RNase-free water and stored at −80 °C. After thawing, the stock solutions were diluted with RNase-free water to 5 μM solutions followed by further dilution with the siRNA dilution buffer to obtain 1.3 μM solutions. The N-TER peptide was diluted with RNase-free water. The working solutions of the N-TER peptide and miRNA-27a, NTC, or FluorNTC were intermixed using equal volumes. The standard N-TER peptide–nucleic acid complexes were incubated at ambient temperatures (about 23 °C) for 15–20 min containing finally a nucleic acid concentration of 650 nM. This was followed by 5 min of sonication (Emmi-D100, EMAG Technologies, Mörfelden-Wallldorf, Germany). To obtain samples suitable for the particle size (100 nM) and zeta potential (80 nM) measurements, the standard N-TER peptide–nucleic acid complexes (650 nM) were diluted either with RNase-free water, zeta water, or low-glucose DMEM.

**Measurement Techniques.** A Zetasizer Nano ZS from Malvern Instruments (Malvern, UK) was employed to determine the particle size by using DLS (intensity-based size distribution) and the zeta potential using ELS technologies, respectively. The setup is equipped with a green laser (532 nm). Particle size measurements were performed using UVette cuvettes (Eppendorf, Hamburg, Germany) at 25 °C with back-scattering detection at 173°. The data processing model used for analysis was general. In addition, the polydispersity was estimated using the Pdl implemented in the Malvern evaluation software. The zeta potential was evaluated by using the Smoluchowsky approximation at 25 °C. Clear folded capillary cells from Malvern Instruments were used. For the DLS and the ELS, each sample condition was prepared in triplicates as well as each measurement. All results are presented as mean values ± SD.

Using AFM, the topographies of particles were evaluated. The investigations were performed with a FlexAFM atomic force microscope, equipped with an EasyScan 2 controller (Nanosurf, Switzerland). Height and phase images were recorded in the tapping mode using Tap300 Al-G cantilevers (Budgetsensors, Sofia, Bulgaria). All recorded data were processed and analyzed with the software package Gwyddion. All AFM measurements were performed under dry conditions, that is, the samples were prepared onto either silicon wafers containing a native oxide layer (about 1 nm) or glass substrates. To clean, the substrates were treated with piranha solution (3:1 concentrated H2SO4/30% H2O2), soaked for 20 min, and rinsed thoroughly with deionized water making it highly hydrophilic. Three different nucleic acids from Dharmacon (GE Healthcare, Vienna, Austria) were purchased and used as delivered to prepare nucleic acid complexes on the used substrate materials. To clean, the substrates were treated with piranha solution (3:1 concentrated H2SO4/30% H2O2), soaked for 20 min, and rinsed thoroughly with deionized water making it highly hydrophilic. Additionally, chemical modifications of some of these substrates were achieved by coating the surfaces with BSA or PS. To coat the substrates with BSA, a phosphate-buffered saline solution containing 1 mg/mL BSA was applied and incubated for 15 min. The substrates were then rinsed with RNase-free water and dried in air overnight. A film of PS was obtained by spin-coating using tetrahydrofuran (THF) solution containing 5% (v/v) PS. The samples of the N-TER peptide alone or complexed with nucleic acids were applied by drop-casting immediately after preparation on the substrates and incubated for 20 min. Afterward, the samples were rinsed with RNase-free water to...
remove dried up siRNA dilution buffer residues and unbound/excess complexes, followed by drying in air overnight.

The N-TER peptides complexed with a FluoNTC were further evaluated using fluorescence imaging and oil DIC optics (63×/1.4), both present in the Zeiss Axios Observer (Göttingen, Germany). Filter settings were chosen according to the absorption/emission spectra (557 nm/570 nm) of the fluorescence marker Dy547. Because of the transmission microscopy, glass substrates were utilized. Mounting the AFM on top of the specimen holder of the Zeiss Axios Observer, this set up further allows simultaneous imaging by AFM together with the fluorescent microscope at the very same position, making combined investigations possible.

**AUTHOR INFORMATION**

**Corresponding Author**
E-mail: andreas.zimmer@uni-graz.at.

**ORCID**
Oliver Werzer: 0000-0003-0732-4422
Andreas Zimmer: 0000-0003-3127-287X

**Notes**
The authors declare no competing financial interest.

**ABBREVIATIONS**
AFM, atomic force microscopy; BSA, bovine serum albumin; AR, aspect ratio; CPP, cell-penetrating peptides; DDS, drug delivery systems; DLS, dynamic light scattering; ELS, electro-phoretic light scattering; miRNA, micro RNA; NLS, nuclear localization sequence; PS, polystyrol; THF, tetrahydrofuran

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