Published in final edited form as:
Biomacromolecules. 2010 November 8; 11(11): 3189–3195. doi:10.1021/bm101055m.

Silk-Based Gene Carriers with Cell Membrane-Destabilizing Peptides

Keiji Numata and **David L Kaplan***
Department of Biomedical Engineering, Tufts University, 4 Colby Street, Medford, Massachusetts 02155, USA.

Abstract

Complexes of recombinant silk-polylysine molecules with ppTG1 peptide, a lysine-rich cell membrane-destabilizing peptide to bind plasmid DNA (pDNA), are designed as less-cytotoxic and highly efficient gene carriers. The peptide destabilizes the cell membrane and promotes gene transfer. Our particular interest is in how ppTG1 enhances transfection efficiency of the silk-based delivery system into human cells. Genetically engineered silk proteins containing polylysine and the monomeric and dimeric ppTG1 sequences are synthesized in *Escherichia coli*, followed by transfection experiments. The pDNA complexes of Silk-polylysine-ppTG1 dimer recombinant proteins prepared at an N/P 2 (the ratio of number of amines/ phosphates from pDNA) shows the highest transfection efficiency into human embryonic kidney (HEK) cells, the level of which is comparable to the transfection reagent Lipofectamine 2000. The assemblies show a globular morphology with an average hydrodynamic diameter of 99 nm and almost no beta-sheet structure. Additionally, the silk-based pDNA complexes demonstrate excellent DNase resistance as well as efficient release of the pDNA by enzymes that degrade silk proteins. Also, comparison with beta-sheet induced silk-based pDNA complexes indicates that the beta-sheet structure content of the silk sequence of the pDNA complexes controls the enzymatic degradation rate of the complexes, and hence can regulate the release profile of genes from the complexes. The bioengineered silk-based gene delivery vehicles containing cell membrane-destabilizing peptides are therefore concluded to have potential for a less-toxic and controlled-release gene delivery system.

Keywords

cell-membrane destabilizing peptides; gene delivery; nanoparticle; recombinant silk

Introduction

Gene therapy requires efficient and safe carriers to transfer genes into target cells. There are currently no Food and Drug Administration (FDA)-approved gene therapies, even though over 1,400 gene therapy clinical trials have been conducted since 1989.1 Viral vectors, including adenovirus and adeno-associated virus, have been used in gene delivery due to their relatively high efficiency of transfection and potential long term effects.2 However, safety concerns remain about immune responses by the introduction of viruses as carriers. Also, one of the problems of gene therapy using retroviruses is that the genes of the virus can be inserted into any arbitrary position in the genome of the host, which can lead to complications such as leukemia.3

CORRESPONDING AUTHOR FOOTNOTE, Phone: +1-617-627-3251, Fax : +1-617-627-3231, david.kaplan@tufts.edu.

Silk proteins have been used successfully in the biomedical field as sutures for decades, and also explored as biomaterials for cell culture and tissue engineering, achieving FDA approval for such expanded utility because of excellent mechanical properties, versatility in processing and biocompatibility.4–6 Further, the degradation products of silk proteins with beta-sheet structures, when exposed to alpha-chymotrypsin, have recently been reported and show no cytotoxicity to in vitro neuron cells.7–13 Therefore, silk-based polymers, which can have added functions through bioengineering, offer an efficient biomaterial platform with which to tailor chemistry, molecular weight, and targeting based on specific designs. This makes these protein systems useful candidates as non-viral gene carriers.

In our previous study, silk-based block copolymers were generated by combining spider silk consensus repeats with polylysine domains for gene carriers.14 The silk-based block copolymers formed ion complexes with plasmid DNA (pDNA), with sizes of the charge complexes controllable based on the polymer/DNA ratio or the molecular weight of the polylysine domain bioengineered into the designs. The pDNA complexes with the silk-based block copolymers with less than 30 lysines showed no cytotoxicity toward human embryonic kidney (HEK) cells.14 The prior study demonstrated the feasibility of bioengineering highly designed silk-based pDNA delivery systems; however, the transfection efficiency remained lower than commercial available transfection agents such as liposome and polyethyleneimine systems.15,16

Cell-penetrating and cell membrane-destabilizing peptides (CPPs) are defined as short peptides that efficiently penetrate cellular lipid bilayers or destabilize cellular membranes. Therefore, CPPs are useful candidates for new nonviral gene vectors. The CPP internalization mechanism was reported as a caveolae, clathrin-dependent endocytosis and macropinocytosis.17–21 There are reports that indicate cellular uptake is independent of endocytotic pathways and occurs through transient pore formation.22–26 Additionally, it has been suggested that CPPs simultaneously utilize different mechanisms of endocytosis and uptake occurs by an additional rapid translocation process.27 Thus, significant improvements are anticipated in the efficiency of gene delivery systems upon the addition of CPPs; however, to our knowledge, recombinant proteins that combine CPPs and the other functional sequences have not been investigated for non-viral gene delivery.

The aim of the present study was to enhance the transfection efficiency of silk-based cationic block copolymer systems as gene delivery vectors by adding CPPs to the recombinant silk-polylysine systems. The CPP used in the present study was ppTG1, which shows one of the highest transfection efficiencies of pDNA complexes with CPPs.28 However, DNase resistance and stability of the pDNA complexes with ppTG1 peptides have not been investigated, likely because the peptides contain no functional sequence, like a sequence of silk, to protect their incorporated genes from gene-degrading enzymes. The silk sequence was expected to play a role of coating around pDNA complexes and disturb enzymatic reaction between DNase and pDNA. The goals of the present study were centered about understanding design issues related to the efficiency of gene delivery and to DNase resistance and stability of these recombinant protein-based systems using ppTG1, polylysine, and spider silk sequences. Complexes of these silk-based block copolymers with pDNA were prepared for in vitro gene delivery to HEK and MDA-MB-435 cells, and characterized by agarose gel electrophoresis, zeta-potential meter, atomic force microscopy (AFM), and dynamic light scattering (DLS). The polymer properties of silks in terms of self-assembly, robust mechanical properties and controllable rates of degradation, in combination with tailored ionic complexation with pDNA and the cell-penetrating function reported here, provide a new family of vehicles for the systematic studies of gene delivery and optimization.

Materials and Methods

Design and Cloning of Silk Sequence

The spider silk repeat unit was selected based on the consensus repeat (SGRGGLGGQGAGAAAAAGGAGQGGYGGLGSQGT) derived from the native sequence of the dragline protein MaSp1 sequence from the spider *Nephila clavipes* (Accession P19837). The Silk6mer-30lys containing six contiguous copies of this repeat and 30 lysines was developed through the transfer of cloned inserts to pET-30a, according to our previously published procedures.14,29,30 Briefly, the 6mer containing six contiguous copies of this repeat was developed through the transfer of cloned inserts to pET-30a, which had been modified with a linker carrying the restriction sites *Nhe*I and *Spe*I. The sequences of the synthetic oligonucleotides encoding 15 lysine residues were as follows: Lys-a: 5´-*CTAGC*AAGAAAAAGAAAAAAAAGAAAAAAAAGAAAAAGAAAAAAAAGAAAA-3´, Lys-b: 5´-*CTAGT*TTTCTTTTTTTTCTTTTTCTTTTTTTTCTTTTTTTTCTTTTTCTTG-3´. The restriction sites for *Nhe*I and *Spe*I are italicized. Lys-a and Lys-b are complementary oligonucleotides which were annealed to form double stranded DNA. The newly formed double stranded DNA was then ligated and multimerized to form the dimer (30 lysines). The double stranded DNAs of polylysine sequences were ligated into pET30-6mer to generate pET30-6mer-polylysine by DNA ligase (New England Biolabs Inc, Ipswich, MA). The sequences of the synthetic oligonucleotides encoding ppTG1 residues were as follows: ppTG1-a: 5´-*CTAGC*GGCCTGTTTAAAGCGCTGCTGAAACTGCTGAAAAGCCTGTGGAAACTGCTGCTGAAAGCG*A*-3´, ppTG1-b: 5´-*G*CCGGACAAATTTCGCGACGACTTTGACGACTTTTCGGACACCTTTGACGACGACTTTCGC*TGATC*-3´.28 The restriction sites for *Spe*I are italicized. ppTG1-a and ppTG1-b are complementary oligonucleotides which were annealed to form double stranded DNA. The double stranded DNAs of ppTG1 sequences were ligated into pET30-Silk6mer-30lys to generate pET30-Silk6mer-30lys-ppTG1(s), as shown in Figure 1A and B, by DNA ligase (New England Biolabs Inc, Ipswich, MA).

Protein Expression and Purification

The constructs pET30-Silk6mer-30lys-ppTG1 monomer and pET30-Silk6mer-30lys-ppTG1 dimer were used to transform *E. coli* strain RY-3041, a mutant strain defective in the production of the SlyD protein, and protein expression carried out by methods reported previously. 14,31,32 Briefly, cells were cultivated in LB broth containing kanamycin (50 mg/mL) at 37°C. Protein expression was induced by the addition of 0.5 mM IPTG (Sigma–Aldrich, St. Louis, MO) when the OD600nm reached 0.6. After approximately 4 h of protein expression, cells were harvested by centrifugation at 13000g. The cell pellets were resuspended in denaturing buffer (100 mM NaH_2PO_4, 10mM Tris–HCl, 8 M urea, pH 8.0) and lysed by stirring for 12 h followed by centrifugation at 13,000g at 4°C for 30 min. His-tag purification of the proteins was performed by addition of Ni–NTA agarose resin (Qiagen, Valencia, CA) and 20 mM imidazole to the supernatant (batch purification) under denaturing conditions. After washing the column with denaturing buffer at pH 6.3, the proteins were eluted with denaturing buffer at pH 4.5 (without imidazole). SDS-polyacrylamide gel electrophoresis (PAGE) was performed using 4–12% precast NuPage Bis-Tris gels (Invitrogen, Carlsbad, CA). The gel was stained with Colloidal blue (Invitrogen, Carlsbad, CA). Purified samples were extensively dialyzed against Milli-Q water. For dialysis, Spectra/Por Biotech Cellulose Ester Dialysis Membranes with MWCO of 100–500 Da (Spectrum Laboratories Inc, Rancho Dominguez, CA) were used. The recombinant proteins were further characterized to confirm sequence and molecular weight

at the Tufts University Core Facility by Matrix Assisted Laser Desorption /Ionization- Time of Flight (MALDI-TOF) mass spectrometry.

Preparation and Characterization of the Complex

Two types of pDNAs encoding Green Fluorescence Protein, (GFP, 7650 bp) or Firefly Luciferase (Luc, 7041 bp) were amplified in competent DH5α *E. coli* (Invitrogen) and purified using EndoFree Plasmid Maxi Kits (Qiagen, Hilden, Germany). The DNA concentration was determined by absorbance at 260 nm. To prepare the complexes of the recombinant silk proteins with pDNA, a solution containing the silk protein (0.1 mg/mL) was mixed with the pDNA solution (370 μg/mL) at various N/P ratios. Here, N/P ratio refers to the ratio of number of amines/ phosphates from pDNA. The mixture of recombinant silk and pDNA was incubated at room temperature (~20°C) overnight prior to characterization. To induce more beta-sheet structure, the pDNA complexes were collected by centrifugation (approximately 4600 g), the supernatant was removed, and then methanol-treated pDNA complexes were obtained after incubation of the pDNA complexes in 50% methanol solution for 24 h. The methanol-treated complexes were vortexed and used in the following experiments. The pDNA complexes were characterized by zeta-potential meter (Zetasizer Nano-ZS, Malvern Instruments Ltd, Worcestershire, UK), AFM (Dimension V, Veeco Instruments Inc, Plainview, NY), and FTIR-ATR (JASCO FT/IR-6200) equipped with a multiple-reflection, horizontal MIRacle ATR attachment (using a Ge crystal, from Pike Tech, Madison WI). The pDNA complex solution (around 70 μL) was added to ultra pure water (450 μL, Invitrogen) and then used as a sample for zeta potential and size measurement. Zeta potential and zeta deviation of samples were measured three times by a zeta-potential meter, and the average data were obtained using Dispersion Technology Software version 5.03 (Malvern Instruments Ltd). For AFM imaging, the pDNA complex solution was cast on cleaved mica, and observed in air at room temperature using a 200–250 μm long silicon cantilever with a spring constant of 2.8 N/m in tapping mode AFM. Calibration of the cantilever tip-convolution effect was carried out to obtain the true dimensions of objects by previously reported methods.33

DNase Resistance

The pDNA complexes were incubated with 100 μL of PBS containing 1 unit of DNase I (Sigma-Aldrich, St. Louis, MO) at 37°C for 1 h. The digestion reactions were stopped by addition of 20 μL of 0.5 M EDTA at 20°C. The pDNA complexes were also treated with protease XIV or alpha-chymotrypsin (150 μg/mL) at 37°C for 2 h. For agarose gel electrophoresis of the degradation products, 20 μL of each sample was mixed with loading buffer and analyzed on 1% agarose gel containing ethidium bromide (TAE buffer, 100V, 60 min).

Cell Culture, Transfection, and Viability

Human embryonic kidney (HEK) cells (293FT), which have been extensively used as an expression tool for recombinant proteins, were used as a model cell line.34 The MDA-MB-435 melanoma cell line was also used to compare with HEK cells. Cultures were grown to confluence using media consisting of Dulbecco's Modified Eagle Medium (DMEM), 10% FBS, 1% glutamine, 1% Non-Essential Amino Acid (NEAA). The cultures were detached from their substrates using 0.25% trypsin (Invitrogen), and then replated in the 24-multiwell plate at a density of 70,000 cells/well. pDNA (1.2 μg) and recombinant silk (appropriate amount) complexes were added into each well. After incubation of the cells for 6 h at 37°C, the media was exchanged to the media without pDNA complexes. After another incubation for 72 h fluorescence images were obtained by fluorescence microscopy (Leica Microsystems, Wetzlar, Germany) to evaluate GFP plasmid transfections. To evaluate

luciferase gene expression quantitatively, a Luciferase assay (Promega, Madison, WI) was performed (n=4). The amount of protein in each well was determined using a BCA protein assay (Pierce Biotechnology, Rockford, IL), and then the relative light units (output) / weight of protein (RLU/mg) was obtained. Lipofectamine 2000 (Invitrogen) was used as a positive control vector. For cell viability, HEK cells (5000 cells/well) were seeded into the 96-wells plates containing the pDNA complexes and cultured for 48 h in the media (100 μL) used in the transfection experiment. Cytotoxicity to HEK cells of the pDNA complexes was characterized by standard 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega, Madison, WI) according to the manufacturer's instructions (n=8). The pDNA complexes with an N/P ratio of 2 for Silk-polylysine-ppTG1 monomer and dimer at a concentration of approximately 100 μg/mL were used for the cell viability assay.

Statistical Analysis

The particle sizes on mica substrates were measured by AFM using a Research Nanoscope software version 7.30 (Veeco Instruments Inc). The average value of 30 measurements was used. Statistical differences in particle sizes by AFM, cell transfection efficiency, and cell viability were determined by unpaired *t*-test with a two-tailed distribution and differences were considered statistically significant at $p<0.05$. The data in the AFM, cell transfection efficiency, and cell viability experiments are expressed as means ± standard deviation.

Results

Preparation of Recombinant Silk Proteins

The recombinant silk proteins containing polylysine and ppTG1 sequences were expressed in *Escherichia coli* and purified with Ni-NTA chromatography. The domain structure and amino acid sequence of the spider silk variants generated with polylysine and the cell membrane destabilizing peptide (ppTG1) are shown in Figure 1A and B. Yields of the recombinant silk proteins were approximately 0.7 mg/L after purification and dialysis. The proteins after purification by Ni-NTA chromatography and dialysis were analyzed by SDS-PAGE and stained with Colloidal blue to evaluate purity. Silk-polylysine-ppTG1 monomer and dimer showed a major band corresponding to a molecular weight of approximately 33 and 34 kDa, respectively (Figure 1C), higher than the theoretical molecular weights (monoisotopic mass) of 27,602.29 and 30,067.87 Da, respectively. However, the results from MALDI-TOF mass spectrometry found 27,602.29 and 30,067.87 Da, respectively, for Silk-polylysine-ppTG1 monomer and dimer, confirming that the bioengineered proteins were correct. Generally, silk-based polymers do not run true to size on SDS-PAGE gels, due to the hydrophobic nature of the protein, but such gels are still useful to assess purity.14,29 The smear of the samples in SDS-PAGE was due to degradation products of the recombinant silk proteins during protein-expression by proteases in the bacteria, as well as translational pauses and truncation products, all of which were low in content relative to the main protein. The effect of these products on gene transfection was not considered significant according to previous studies.14,35 The Silk-polylysine-ppTG1 monomer and dimer were soluble in water (approximately 2.0 mg/mL) at room temperature.

Characterization of pDNA Complexes

Ionic complexes formation with pDNA encoding luciferase and the recombinant silk proteins, Silk-polylysine-ppTG1 monomer and dimer, were characterized with different N/P ratios (the ratio of number of amines/ phosphates from pDNA) by DLS, zeta-potential meter and AFM. The hydrodynamic diameter and zeta potential of the pDNA complexes of the recombinant silks were measured by zeta-potential meter (Table 1). The average diameters of the Silk-polylysine-ppTG1 monomer and dimer at the concentration of 0.1 mg/mL

without pDNA were 169 and 163 nm, respectively. The average diameters of the complexes decreased with an increase in N/P ratio. The pDNA complexes prepared at an N/P 5 demonstrated a bimodal distribution of sizes, which denotes that excess silk molecules were aggregated and detected as larger complexes.14 The zeta potential of pDNA complexes increased slightly with an increase in N/P ratio. The pDNA complexes with monomeric ppTG1 showed a lower zeta potential than that with dimeric ppTG1, because of more negative charge of monomeric ppTG1 sequence. Based on the sizes and zeta potentials, the most suitable pDNA complexes for *in-vitro* transfection were those prepared at an N/P 2 for Silk-polylysine-ppTG1 monomer and dimmer. These were 108 and 99 nm in size and −37.5 ± 7.1 and −26.2 ± 6.3 mV in zeta potential, respectively. The pDNA complexes of both the recombinant silk proteins (N/P 2) after methanol treatment for 24 h to induce a protein transition to beta sheet for the silk domains demonstrated slightly increased dimensions and polydispersity indexes (PDIs), but the same zeta potentials as before methanol treatment (Table 1). The pDNA complexes of Silk-polylysine-ppTG1 dimer before and after the methanol treatment were also characterized by FTIR-ATR (Figure 2), resulting in a peak at 1625 cm^{-1} in the amide I region after the methanol treatment. This peak is indicative of beta-sheet structures (crystallization) in the silk sequences in the pDNA complexes.36 To determine the fraction of secondary structure of the crystals, FTIR spectra were deconvoluted in the amide I region, resulting that the Silk-polylysine-ppTG1 dimer before and after methanol treatment contained 26 and 37% beta-sheet, respectively, based on previous assignments.37,38

The pDNA complexes of Silk-polylysine-ppTG1 dimer prepared at an N/P 2 were cast on mica and imaged by AFM (Figure 3). All of the complexes prepared at an N/P 2 formed homogeneous globular complexes. Based on the AFM observations, the pDNA complexes of Silk-polylysine-ppTG1 dimer at N/P 2 demonstrated an average diameter and height of 185 ± 43 nm and 3.6 ± 1.1 nm, respectively (n=30). In the case of AFM observation in air, wet globular samples were observed like squashed on substrate because of drying of the samples, according to the previous report.33 On the basis of the volume of the particles, however, the dimensions determined by DLS and AFM showed reasonable agreements.33

DNase Resistance and Gene Release Behavior

The stability of pDNA incorporated with the recombinant silk protein, Silk-polylysine-ppTG1 dimer, against DNase was characterized using DNase I treatment and agarose gel electrophoresis (Figure 4). In comparison with free pDNA (Figure 4, lane 1), free pDNA degraded rapidly after enzymatic treatment for 1 h (Figure 4, lane 2), while no degradation was evident by protease XIV and alpha-chymotrypsin for 2 h (Figure 4, lanes 3 and 4). pDNA complexes after the DNase I treatment still showed pDNA in the well (Figure 4, lane 5). Subsequent to the DNase I treatment, the pDNA in the silk-polylysine-ppTGI dimmer was released from the complex after enzymatic treatment by protease XIV (Figure 4, lane 6). Alpha-chymotrypsin and protease XIV, hydrolases that digest silk proteins, released pDNA from the complexes (Figure 4, lanes 7 and 8).13,36,39 The methanol-treated pDNA complexes also protected the incorporated pDNA from DNase I treatment for 1 h (Figure 4, lane 9). The methanol-treated pDNA complexes after enzymatic treatment by alpha-chymotrypsin released less pDNA when compared with the treatment by protease XIV (Figure 4, lanes 11 and 12). This would be expected, as the crystallized silk is less susceptible to this protease than noncrystallized (non beta sheet) containing protein.13,39,40

Gene Transfection to Cells

In vitro transfection experiments were performed with HEK and MDA-MB-435 cells in order to evaluate the feasibility of the pDNA complexes in combination with the cationic recombinant silks with the ppTG1 cell membrane destabilizing peptides for gene delivery.

To determine the most efficient N/P ratio of the pDNA complexes, HEK cells were transfected via the Silk-polylysine-ppTG1 dimer with luciferase pDNA as a reporter gene. Figure 5A shows the transfection efficiencies to HEK cells for pDNA complexes of Silk-polylysine-ppTG1 dimer with N/P ratios ranging from 0.1 to 5 based on the luciferase assays (n=4). pDNA complexes of Silk-polylysine-ppTG1 dimer prepared at N/P 2 demonstrated that the highest transfection efficiency among the different N/P ratios, followed by a steep decrease in efficacy, presumably due to excess recombinant silk, which was detected as larger aggregation than pDNA complexes by zeta-potential meter (Table 1). Figure 5B shows the transfection efficiencies to HEK and MDA-MB-435 cells for pDNA complexes of both the recombinant silk proteins (N/P 2), as well as the transfection regent Lipofectamine 2000, as a control. The pDNA complexes of Silk-polylysine-ppTG1 dimer exhibited the comparable transfection efficiency to HEK cells as Lipofectamine 2000 and also showed significantly higher transfection efficiency to both cells in comparison to the Silk-polylysine-ppTG1 monomer at N/P 2. The transfection experiments with a GFP reporter gene to HEK and MDA-MB-435 cells were also performed (Figure 5C and D), and indicated that the transfection of pDNA complexes and their degradation products inside the cells did not significantly influence cell morphology.

In vitro cytotoxicities of the pDNA complexes with an N/P ratio of 2 for Silk-polylysine-ppTG1 monomer and dimer, which demonstrated the highest transfection efficiency between different N/P ratios, were determined using standard MTS assay. The pDNA complexes of Silk-polylysine-ppTG1 monomer and dimer at a concentration of 100 μg/mL showed 75 ± 3 and 69 ± 8 % of cell viabilities, respectively.

In vitro transfection behaviors for 144 h using the pDNA complexes of Silk-polylysine-ppTG1 dimer (N/P 2) before and after methanol treatment were also characterized by luciferase assay (Figure 6). Although the transfection efficiency was much lower for the methanol-treated Silk-polylysine-ppTG1 dimer than the same complexes without methanol treatment, the methanol-treated pDNA complexes demonstrated slow and constant release of pDNA for at least 144h (6 days).

Discussion

The novelty of this study was the tailored design of complexes of recombinant silk proteins with CPPs for gene delivery, with particular interest in how CPPs enhanced transfection efficiency. The recombinant silk proteins, Silk-polylysine-ppTG1 monomer and dimer, were prepared using *E. coli*, and then formed in complexes with pDNA (Table 1). The sizes of the pDNA complexes were as designed, and appropriate for gene delivery, according to the literature.41,42 The pDNA complexes prepared at an N/P 5 demonstrated useful transfection efficiency, however, the PDI of particle sizes was too wide. This is because the excess silk molecules were aggregated and detected as larger complexes, as reported prevously.14 We also consider that the excess silk proteins adsorbed on the cell surfaces and disturbed the pDNA complexes to be transfected into cells. The pDNA complexes before and after methanol treatment were capable of protecting the incorporated pDNA from DNase I (Figure 4), which implies that the silk sequence is protective or on the outside surface of the complexes and prevents DNase from access to the pDNA. The cell viability of HEK cells with Lipofectamine 2000 (Lipofectamine 2000 / pDNA: 7/1 vol/wt) was reported to be 67%.43 The cell viabilities of the pDNA complexes with the recombinant silks to contain ppTG1 were 75 ± 3 and 69 ± 8 % and comparable to Lipofectamine 2000, which is one of the golden standard gene vectors. These data including the enzymatic degradation experiment (Figure 4) denote that the pDNA complexes of the recombinant silk are biodegradable, low-cytotoxic, and also provide resistance to DNase, an advantage for a non-viral gene delivery carrier.

Alpha-chymotrypsin hydrolyzes non-crystalline silk fibroins, whereas protease XIV digests not only non-crystalline but also beta-sheet (crystalline) silk domains, according to the literatures.13,38,39 The methanol-treated pDNA complexes after the enzymatic treatment by alpha-chymotrypsin released smaller amounts of free pDNA in comparison with treatment by protease XIV (Figure 4, lanes 11,12). This result in addition to the beta-sheet structure contents calculated from FTIR-ATR measurements (Figure 2) indicate that the beta-sheet structure of silk sequences induced by the methanol treatment decreases the enzymatic degradation rate of the complexes. Further, the methanol-treated pDNA complexes, which contain more beta-sheet structures, showed a different transfection behavior from the complexes of Silk-polylysine-ppTG1 dimer without methanol treatment (Figure 6). Comparison of the enzymatic degradations of the pDNA complexes between before and after MeOH treatment shows that degradation rates of the MeOH-treated complexes with more beta-sheet contents were slower by protease and alpha-chymotrypsin. The previous study suggests that silk molecules of pDNA complexes are degraded and releasing pDNA by lysosome proteolytic activity in the cytosol, based on confocal laser scanning microscopy observations of HeLa cells incubated with silk-based complexes with Cy5-labeled pDNA.35 The pDNA can therefore be released from the complexes after partial degradation of the recombinant silk proteins by lysosome proteolytic activity in the cytosol. 35 Based on these data, slower transfection behavior of the MeOH-treated complexes (Figure 6) was considered due to slower enzymatic degradation behavior of the silk molecules with higher beta-sheet content. This result also indicated a way to achieve more sustained or constant release without burst of pDNA from the methanol-treated silk-based complexes vs the non-methanol treated system. Therefore, the secondary structure of the silk sequence, such as beta-sheet structure content, can be used to control the release profile of pDNA from these complexes due to different enzymatic degradation rates.

Figure 4 shows various pDNA and pDNA complexes of the silk molecules. Free pDNA shows two main bands, which are plasmids with different morphologies such as supercoiled and open circular structures. (lane 1). The bands in lane 3 and 4 were slightly different from the free pDNA in lane 1, maybe due to interactions between pDNA and positive charges at the surface of enzymes. The pDNA complex of Silk-polylysine-ppTG1 dimer was stacked in the well of lane 5. After the DNase treatment with the pDNA complexes, the enzymatic degradation of the complex by protease released pDNA from the complex as shown in lane 6, indicating that the complex of silk molecules protected pDNA from DNase treatment. Alpha-chymotrypsin and protease degraded and released pDNA from the complex without the methanol treatment (lane 7 and 8), denoting that pDNA was released by the enzymatic degradation of silk molecules. Lane 9 shows the pDNA complexes stacked in the well after the methanol treatment for 24 h. The methanol-treated complex after the DNase treatment for 1 h released pDNA by enzymatic degradation in presence of protease, suggesting that the methanol-treated silk molecules also protected pDNA from DNase (lane 10). However, slight pDNA was released from the complex by alpha-chymotrypsin treatment as well (lane 11), which means alpha-chymotrypsin cannot significantly digest methanol-treated silk molecules with beta-sheet structures, contrary to the protease treatment (lane 12). The biodegradability of the pDNA complexes of silk molecules before and after the methanol treatments differed between enzymes. This property of silk molecules showed potential of silk-based pDNA complexes to be used as switching of pDNA release by certain enzymatic reactions. Cationic compounds like polylysine are known to induce condensation of DNA with ordered morphologies such as toroidal and rod-like structures.44–46 These reports suggested that the pDNA in the silk-based complexes was condensed and supercoiled. On the basis of the present results, the condensed pDNA could be gradually relaxed during the enzymatic treatment of the pDNA complexes by alpha-chymotrypsin and protease, since enzymatic hydrolysis reactions enhance molecular mobility of polymeric substrates after

their reactions.31 Therefore, the supercoiled pDNA might be less observed than the open circular DNA after the enzymatic treatments (Figure 4, lanes 6–8 and 10–12).

Components of the recombinant silks, that is, 6-histidine, spider silk, poly(L-lysine), and ppTG1 sequences, have been reported as a part of gene-transferring carriers.47–51 Histidine contains an imidazole ring which increases proton buffering capacity in endosomes and lysosomes, and is known to act as endogenous buffers in skeletal muscle.47 Histidine is therefore widely used as a functional group to improve the endosomal escaping rate of several nonviral gene carriers.48–50 Poly(L-lysine) is known to assemble with pDNA and has been used as gene vectors by many groups.51 Longer-chain poly(L-lysine) demonstrates higher positive charges and *in vitro* and *in vivo* cytotoxicity to cells, though shows higher gene transfection efficiency.52 Cytotoxicity of silk and poly(L-lysine) (n = 45 a.a.) block copolymers was reported 88 ± 11% cell viability of HEK cells, and silk and poly(L-lysine) (n = 15 and 30 a.a.) block copolymers showed no significant cytotoxicities.14 Also, the recombinant silk block copolymer to contain 30 lysines showed higher transfection efficiency in comparison to the copolymers containing 15 and 45 lysines. Silk-based pDNA complexes have been recently reported as gene carriers to transfer plasmids into cells.43 Confocal laser scanning microscopy observations of HeLa cells incubated with silk-based complexes with Cy5-labeled pDNA indicates pDNA can be delivered to near the nucleus with recombinant silk proteins.43 The pDNA can therefore be released from the complexes after partial degradation of the recombinant silk proteins by lysosome proteolytic activity in the cytosol. ppTG1 cell membrane-destabilizing and penetrating peptide, which shows higher transfection efficiency at low pDNA doses in comparison with polyethylenimine has been investigated as *in vitro* and *in vivo* gene transfer vector.28

Although the Silk-polylysine-ppTG1 monomer did not provide useful transfection efficiency to two types of cells, the Silk-polylysine-ppTG1 dimer demonstrated 25-fold higher transfection efficiency than the monomeric version and a similar level of efficiency to HEK cells as Lipofectamine 2000 (Figure 5B). This enhancement of transfection efficiency by the addition of dimeric ppTG1 sequence vs. the monomeric version, demonstrates the importance of this peptide in terms of cell access. We are not aware of prior studies on the function of dimeric ppTG1. As mentioned above, ppTG1 peptide was reported to have functions to bind pDNA as well as to destabilize cell membranes.28 Also, ppTG1 was reported to show the highest *in-vitro* transfection efficiency, approximately 45-fold higher in comparison to the pDNA complex of polyethyleneimine, at low N/P ratio and low concentration (125 ng/mL), different from the other CPPs.28 Transfection experiments in the presence of Bafilomycin A, which is a specific inhibitor of the vacuolar proton pump,53 suggested that cellular uptake of pDNA complexes of the ppTG1 peptides is through the cytoplasmic membrane or via endocytosis.28 Also, molecular modeling of ppTG1 demonstrated that all lysines segregate on the same side when the structure is an alpha-helix, thus the helix is amphipathic. The lysine residues could then interact with the negative charges of DNA, because the mean distance between two amine groups is similar to the mean distance between two phosphates on the DNA strands, which is different from other CPPs.25–28 In the system reported here, the polylysine as well as ppTG1 sequences interact with pDNA and the complexes are transferred into cells through the cell membrane. Based on the present results, we suggest the Silk-polylysine-ppTG1 dimer formed the expected secondary structure to destabilize the cell membrane via ppTG1 sequences and showed much higher transfection efficiency (Figure 5B). This was not the case with the Silk-polylysine-ppTG1 monomer, where the polylysine sequence is proximal to the ppTG1 sequence (Figure 1A and B).

Conclusions

Recombinant silks modified to contain polylysine and ppTG1 sequences were prepared and used to form globular complexes with pDNA. Gene transfection experiments revealed that the pDNA complexes of Silk-polylysine-ppTG1 dimer prepared at N/P 2, with a globular morphology and approximately 99 nm in diameter, showed the highest efficiency to HEK and MDA-MB-435 cells. The dimeric sequence of ppTG1 significantly enhances transfection efficiency. In addition, the secondary structure of the silk sequence of the pDNA complexes is capable of controlling enzymatic degradation rates of the complexes, and hence can regulate release profiles of genes from the complexes. The recombinant silks containing polylysine sequences and CPPs have useful transfection efficiency, comparable to Lipofectamine 2000, but also the option to control gene release behavior. Thus, these new bioengineered silk delivery systems can serve as a versatile and useful new platform polymer for non-viral gene delivery systems.

Acknowledgments

This work has been supported by grants from the NIH (P41 Tissue Engineering Resource Center, EB002520) and the NSF (DK). KN was supported by a JSPS Postdoctoral Fellowship for Research Abroad.

References

1. Gene Therapy Clinical Trials Worldwide. Provided by the Journal of Gene Medicine. Mar 2009. Updated.
2. Lundstrom K. Trends Biotechnol. 2003; 21:117–122. [PubMed: 12628368]
3. Edelstein ML, Abedi MR, Wixon J, Edelstein RM. J. Gene. Med. 2004; 6:597–602. [PubMed: 15170730]
4. Kaplan, DL.; Adams, WW.; Farmer, B.; Viney, C., editors. ACS Symp. Ser. 1994. Silk polymers: materials science and biotechnology; p. 544
5. Altman GH, Diaz F, Jakuba C, Calabro T, Horan R, Chen J, Lu H, Richmond J, Kaplan DL. Biomaterials. 2003; 24:401–416. [PubMed: 12423595]
6. Wang Y, Kim H-J, Vunjak-Novakovic G, Kaplan DL. Biomaterials. 2006; 27:6064–6082. [PubMed: 16890988]
7. Hollander DH. Med. Hypotheses. 1994; 43:155–156. [PubMed: 7815968]
8. Wen CM, Ye ST, Zhou LX, Yu Y. Ann. Allergy. 1990; 65:375–378. [PubMed: 2244708]
9. Kurosaki S, Otsuka H, Kunitomo M, Koyama M, Pawankar R, Matumoto K. Nippon Ika Daigaku Zasshi. 1999; 66:41–44. [PubMed: 10097590]
10. Rossitch E Jr, Bullard DE, Oakes WJ. Childs Nerv. Syst. 1987; 3:375–378. [PubMed: 3329961]
11. Dewair M, Baur X, Ziegler K. J. Allergy Clin. Immunol. 1985; 76:537–542. [PubMed: 4056241]
12. Zaoming W, Codina R, Fernández-Caldas E, Lockey RF. J. Investig. Allergol. Clin. Immunol. 1996; 6:237–241.
13. Numata K, Cebe P, Kaplan DL. Biomaterials. 2010; 31:2926–2933. [PubMed: 20044136]
14. Numata K, Subramanian B, Currie HA, Kaplan DL. Biomaterials. 2009; 30:5775–5784. [PubMed: 19577803]
15. Ross PC, Hui SW. Gene Ther. 1999; 6:651–659. [PubMed: 10476225]
16. Fischer D, Bieber T, Li Y, Elsässer HP, Kissel T. Pharm. Res. 1999; 16:1273–1279. [PubMed: 10468031]
17. Järver P, Langel K, El-Andaloussi S, Langel U. Biochem. Soc. Trans. 2007; 35:770–774. [PubMed: 17635145]
18. Richard JP, Melikov K, Vives E, Ramos C, Verbeure B, Gait MJ, Chernomordik LV, Lebleu B. J. Biol. Chem. 2003; 278:585–590. [PubMed: 12411431]
19. Ferrari A, Pellegrini V, Arcangeli C, Fittipaldi A, Giacca M, Beltram F. Mol. Ther. 2003; 8:284–294. [PubMed: 12907151]

20. Holm T, Johansson H, Lundberg P, Pooga M, Lindgren M, Langel U. Nat. Protoc. 2006; 1:1001–1005. [PubMed: 17406337]
21. Lundberg P, Langel U. Int. J. Pept. Res. Ther. 2006; 12:105–114.
22. Vivès E, Schmidt J, Pèlegrin A. Biochim. Biophys. Acta. 2008; 1786:126–138. [PubMed: 18440319]
23. Deshayes S, Gerbal-Chaloin S, Morris MC, Aldrian-Herrada G, Charnet P, Divita G, Heitz F. Biochim. Biophys. Acta. 2004; 1667:141–147. [PubMed: 15581849]
24. Deshayes S, Heitz A, Morris MC, Charnet P, Divita G, Heitz F. Biochemistry. 2004; 43:1449–1457. [PubMed: 14769021]
25. El-Andaloussi S, Johansson HJ, Lundberg P, Langel U. J. Gene. Med. 2006; 8:1262–1273. [PubMed: 16900561]
26. Abes S, Moulton H, Turner J, Clair P, Richard JP, Iversen P, Gait MJ, Lebleu B. Biochem. Soc. Trans. 2007; 35(Pt1):53–55. [PubMed: 17233600]
27. Duchardt F, Fotin-Mleczek M, Schwarz H, Fischer R, Brock R. Traffic. 2007; 8:848–866. [PubMed: 17587406]
28. Rittner K, Benavente A, Bompard-Sorlet A, Heitz F, Divita G, Brasseur R, Jacobs E. Mol. Ther. 2002; 5:104–114. [PubMed: 11829517]
29. Prince JT, McGrath KP, DiGirolamo CM, Kaplan DL. Biochemistry. 1995; 34:10879–10885. [PubMed: 7662669]
30. Rabotyagova OS, Cebe P, Kaplan DL. Macromol. Biosci. 2010; 10:49–59. [PubMed: 19890885]
31. Numata K, Kikkawa Y, Tsuge T, Iwata T, Doi Y, Abe H. Macromol. Biosci. 2006; 6:41–50. [PubMed: 16374769]
32. Arai T, Freddi G, Innocenti R, Tsukada M. J. Appl. Polym. Sci. 2004; 91:2383–2390.
33. Li M, Ogiso M, Minoura N. Biomaterials. 2003; 24:357–365. [PubMed: 12419638]
34. Ross PC, Hui SW. Gene Ther. 1999; 6:651–659. [PubMed: 10476225]
35. Numata K, Hamasaki J, Subramanian B, Kaplan DL. J. Control Release. 2010; 146:136–143. [PubMed: 20457191]
36. Almofti MR, Harashima H, Shinohara Y, Almofti A, Li W, Kiwada H. Mol. Membr. Biol. 2003; 20:35–43. [PubMed: 12745924]
37. Almofti MR, Harashima H, Shinohara Y, Almofti A, Li W, Kiwada H. Mol. Membr. Biol. 2003; 20:35–43. [PubMed: 12745924]
38. Tretinnikov ON, Tamada Y. Langmuir. 2001; 17:7406–7413.
39. Chen X, Shao Z, Knight DP, Vollrath F. Proteins. 2007; 68:223–231. [PubMed: 17436322]
40. Bowman EJ, Siebers A, Altendorf K. Proc. Natl. Acad. Sci. U. S. A. 1988; 85:7972–7976. [PubMed: 2973058]
41. Huang J, Valluzzi R, Bini E, Vernaglia B, Kaplan DL. J. Biol. Chem. 2003; 278:46117–46123. [PubMed: 12963711]
42. Yan SZ, Beeler JA, Chen Y, Shelton RK, Tang WJ. J. Biol. Chem. 2001; 276:8500–8506. [PubMed: 11113152]
43. Thomas P, Smart TG. J. Pharmacol. Toxicol. Methods. 2005; 51:187–200. [PubMed: 15862464]
44. Maurisse R, De Semir D, Emamekhoo H, Bedayat B, Abdolmohammadi A, Parsi H, Gruenert DC. BMC Biotechnol. 2010; 10:9. [PubMed: 20144189]
45. Miyata K, Kakizawa Y, Nishiyama N, Harada A, Yamasaki Y, Koyama H, Kataoka K. J. Am. Chem. Soc. 2004; 126:2355–2361. [PubMed: 14982439]
46. Laemmli UK. Proc. Natl. Acad. Sci. USA. 1975; 72:4288–4292. [PubMed: 1060108]
47. Osada K, Yamasaki Y, Katayose S, Kataoka K. Angew. Chem. Int. Ed. 2005; 44:3544–3548.
48. Abe H. Biochemistry (Moscow). 2000; 65:757–765. [PubMed: 10951092]
49. Midoux P, Monsigny M. Bioconjugate Chem. 1999; 10:406–411.
50. Yang SR, Lee HJ, Kim J-D. J. Controlled Release. 2006; 114:60–68.
51. Pichon C, Gonalves C, Midoux P. Adv. Drug Delivery Rev. 2001; 53:75–94.

52. Kwoh DY, Coffin CC, Lollo CP, Jovenal J, Banaszczyk MG, Mullen P, Phillips A, Amini A, Fabrycki J, Bartholomew RM, Brostoff SW, Carlo DJ. Biochim. Biophys. Acta. 1999; 1444:171–190. [PubMed: 10023051]

53. Wolfert MA, Seymour LW. Gene. Ther. 1996; 3:269–273. [PubMed: 8646559]

54. Sun YX, Zeng X, Meng QF, Zhang XZ, Cheng SX, Zhuo RX. Biomaterials. 2008; 29:4356–4365. [PubMed: 18718656]

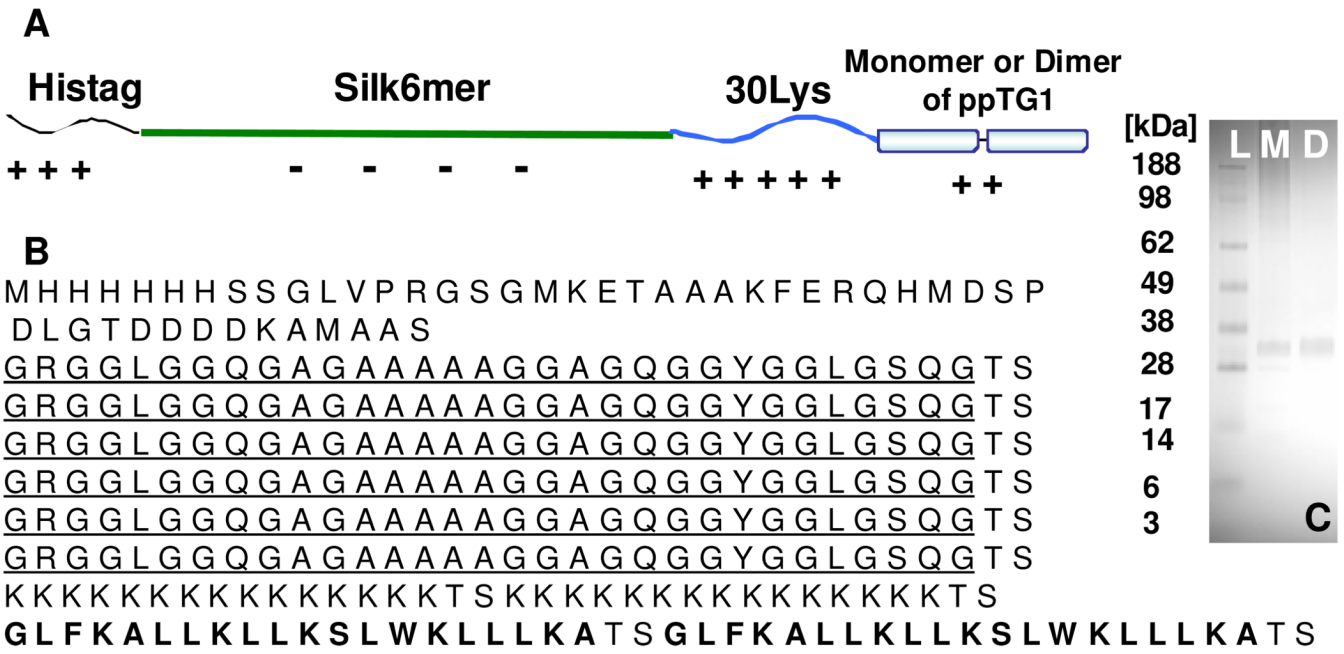

Figure 1.

Schematic of the recombinant silk protein sequence (A). Amino acid sequences of the recombinant spider silk proteins with poly-L-lysine and ppTG1 sequences (B). Underline: representative 6mer of spider silk sequence. Bold: ppTG1 sequence. SDS-PAGE of the recombinant silk proteins after purification by Ni-NTA chromatography (C). Molecular weight ladder (L), Silk-polylysine-ppTG1 monomer (M), and Silk-polylysine-ppTG1 dimer (D) are listed in each line.

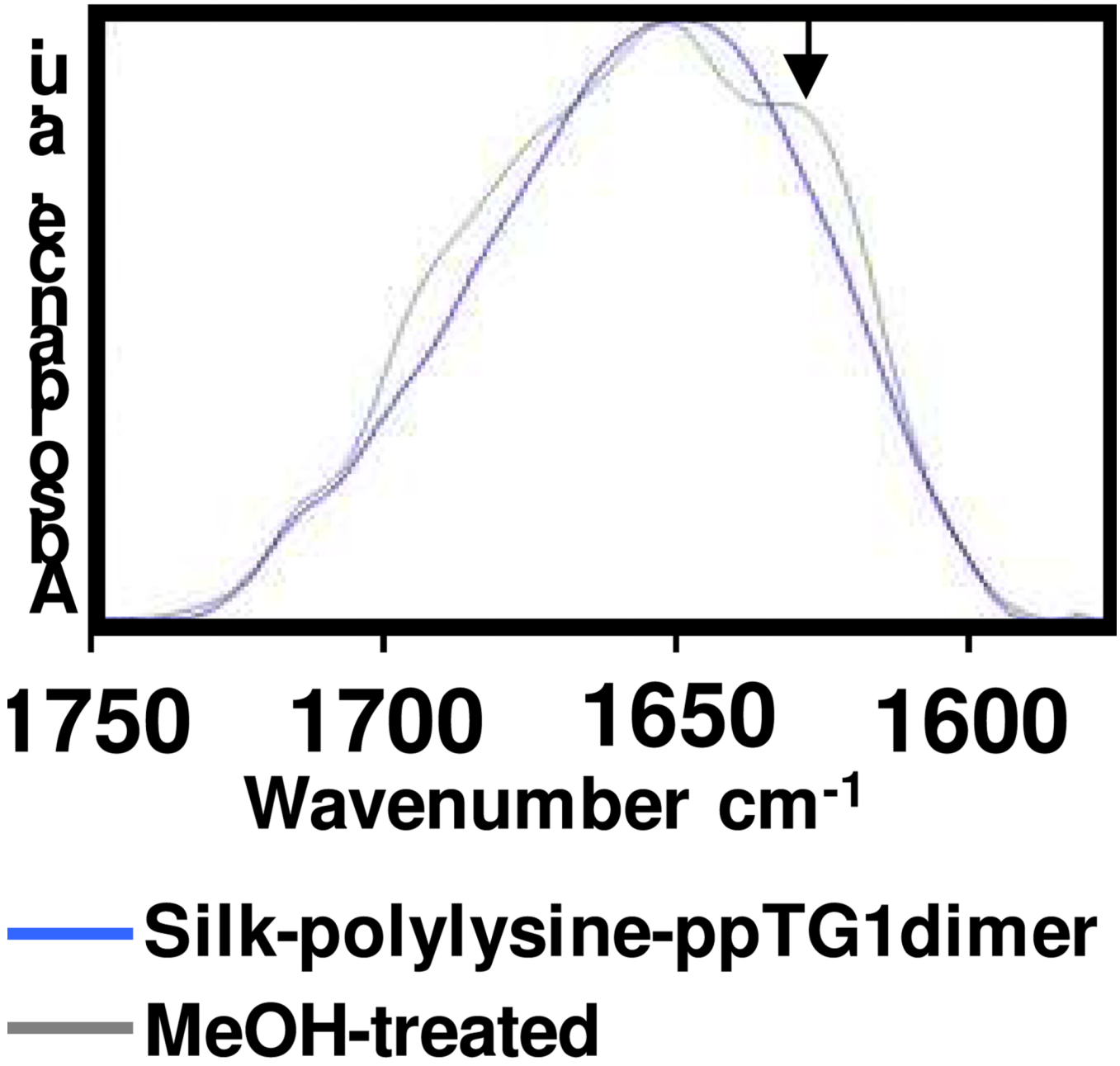

Figure 2.
FTIR-ATR spectra of Silk-polylysine-ppTG1 dimer before and after the methanol treatment for 24 h. An arrow indicates a peak at 1625 cm^{-1} originated from beta-sheet structure.

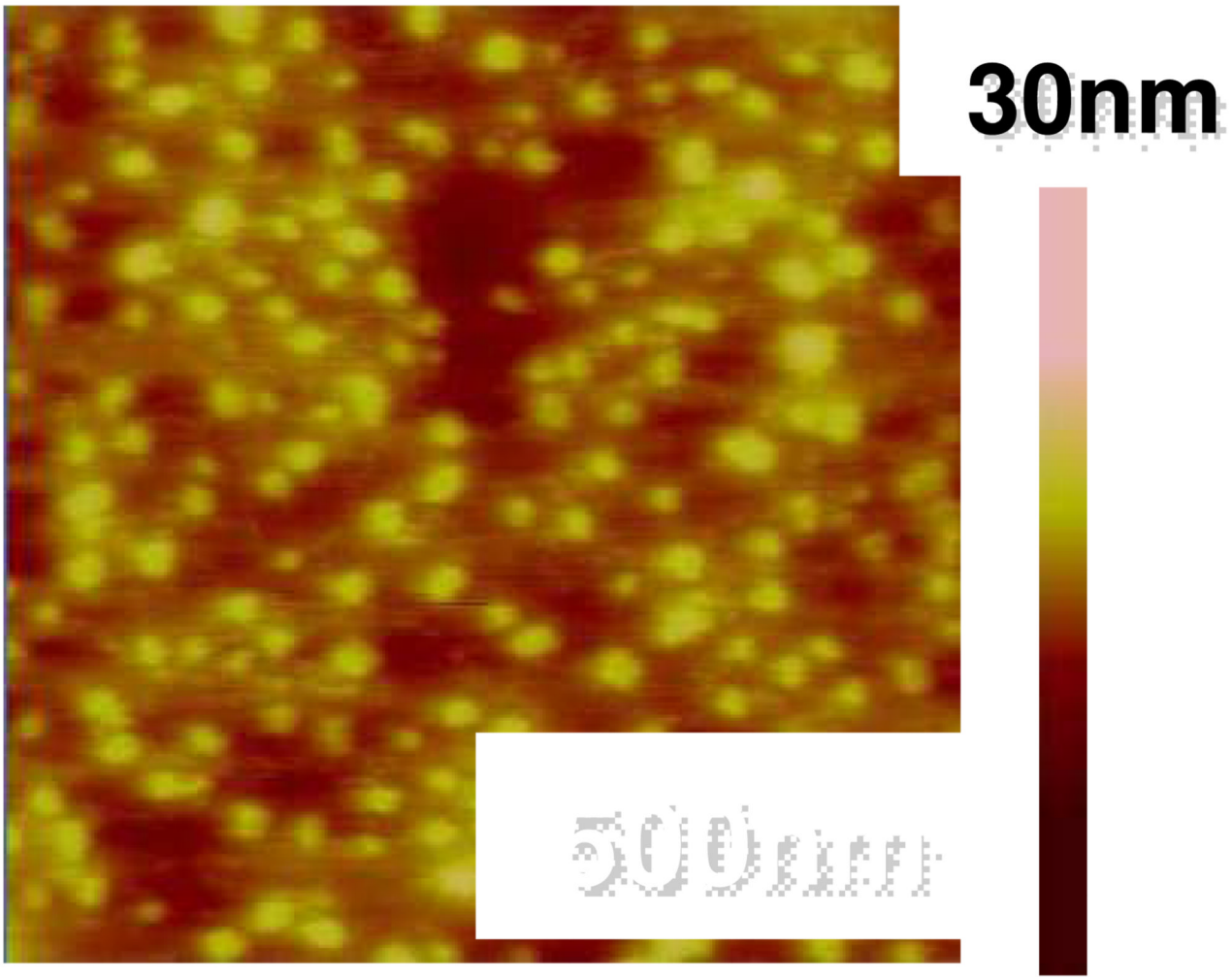

Figure 3.
AFM height images of pDNA complexes with Silk-polylysine-ppTG1 dimer prepared at N/P ratio of 2 on mica.

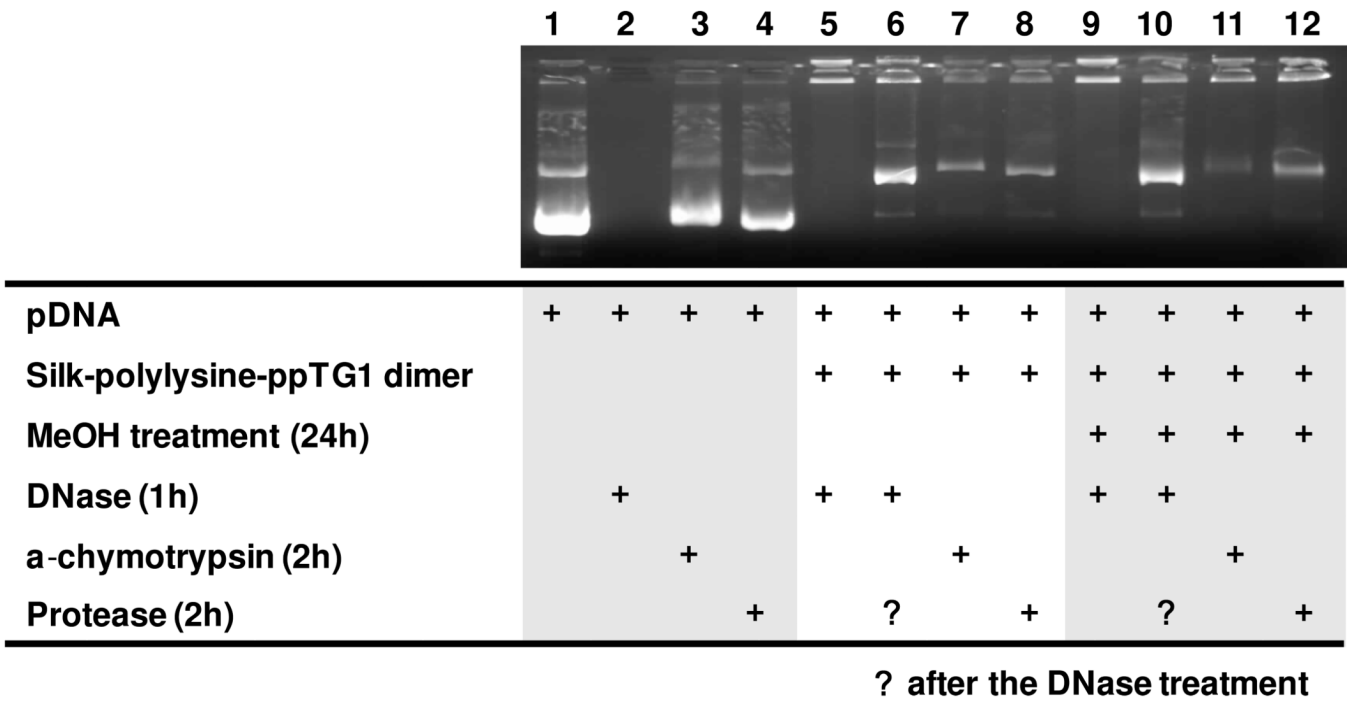

Figure 4.

pDNA protection from DNase I enzymes. Digestion of pDNA exposed to DNase I was measured for the pDNA complexes of Silk-polylysine-ppTG1 dimer with or without methanol (MeOH) treatment. (1) free pDNA only, (2) free pDNA and DNase, (3) free pDNA and alpha-chymotrypsin, (4) free pDNA and protease XIV, (5) pDNA complexes of Silk-polylysine-ppTG1 dimer and DNase, (6) pDNA complexes of Silk-polylysine-ppTG1 dimer and protease XIV after DNase treatment, (7) pDNA complexes of Silk-polylysine-ppTG1 dimer and alpha-chymotrypsin, (8) pDNA complexes of Silk-polylysine-ppTG1 dimer and protease XIV, (9) MeOH-treated pDNA complexes of Silk-polylysineppTG1 dimer and DNase, (10) MeOH-treated pDNA complexes of Silk-polylysine-ppTG1 dimer and protease XIV after DNase treatment, (11) MeOH-treated pDNA complexes of Silk-polylysine-ppTG1 dimer and alpha-chymotrypsin, and (12) MeOH-treated pDNA complexes of Silk-polylysine-ppTG1 dimer and protease XIV.

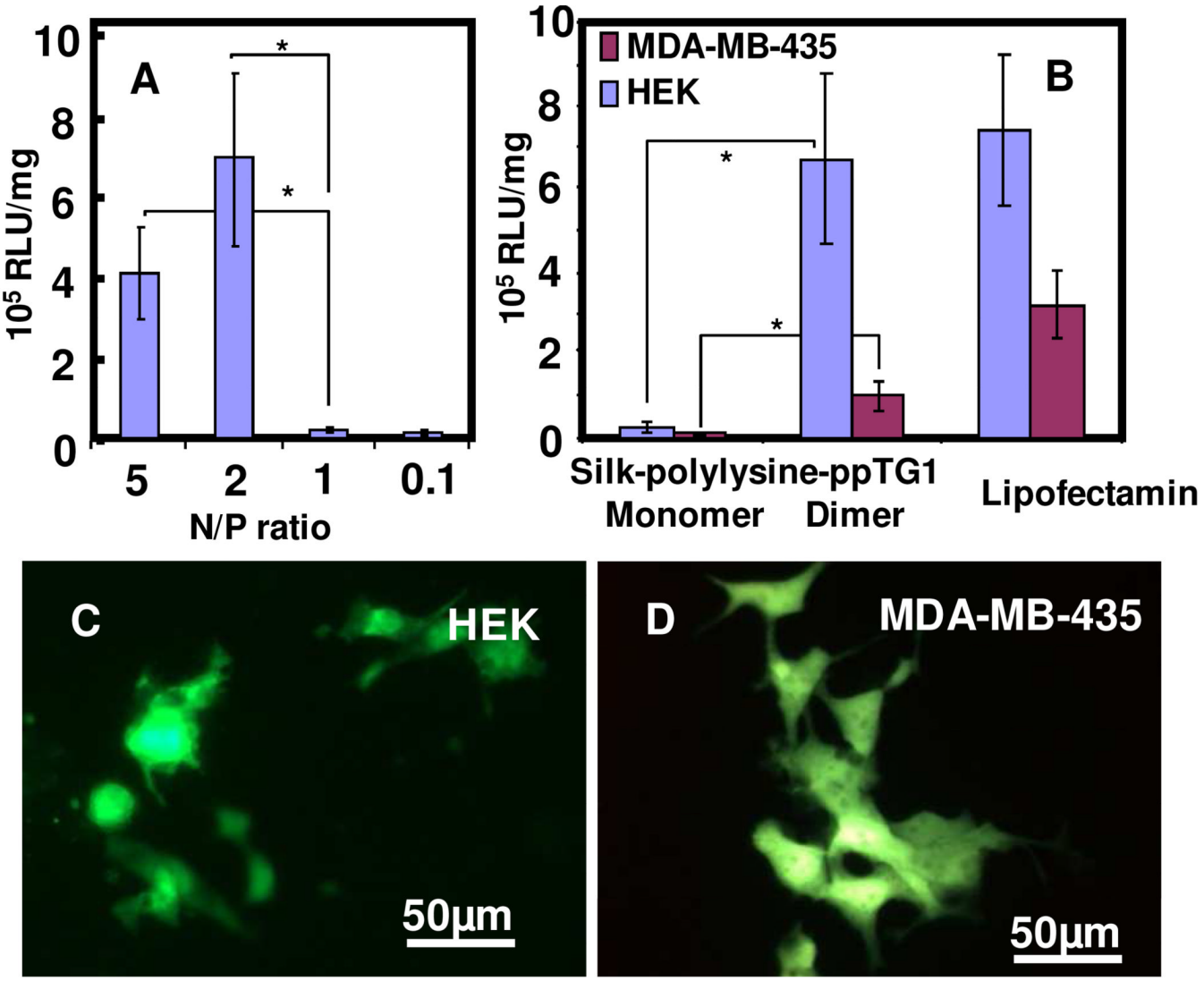

Figure 5.

Transfection results in loading pDNA complexes of the recombinant silk proteins in HEK and MDA-MB-435 cells. (A) Transfection results of Silk-polylysine-ppTG1 dimer with different N/P ratios in HEK cells. (B) Transfection results of Silk-polylysine-ppTG1 monomer and dimer prepared at N/P 2 in HEK and MDA-MB-435 cells. Lipofectamine 2000 were used as positive control samples. Data are shown as means ± standard deviation (n=4). *Significant difference between two groups at $p < 0.05$. GFP-transfected HEK (C) and MDA-MB-435 cells (D) with Silk-polylysine-ppTG1 dimer prepared at N/P 2. RLU means Relative light unit.

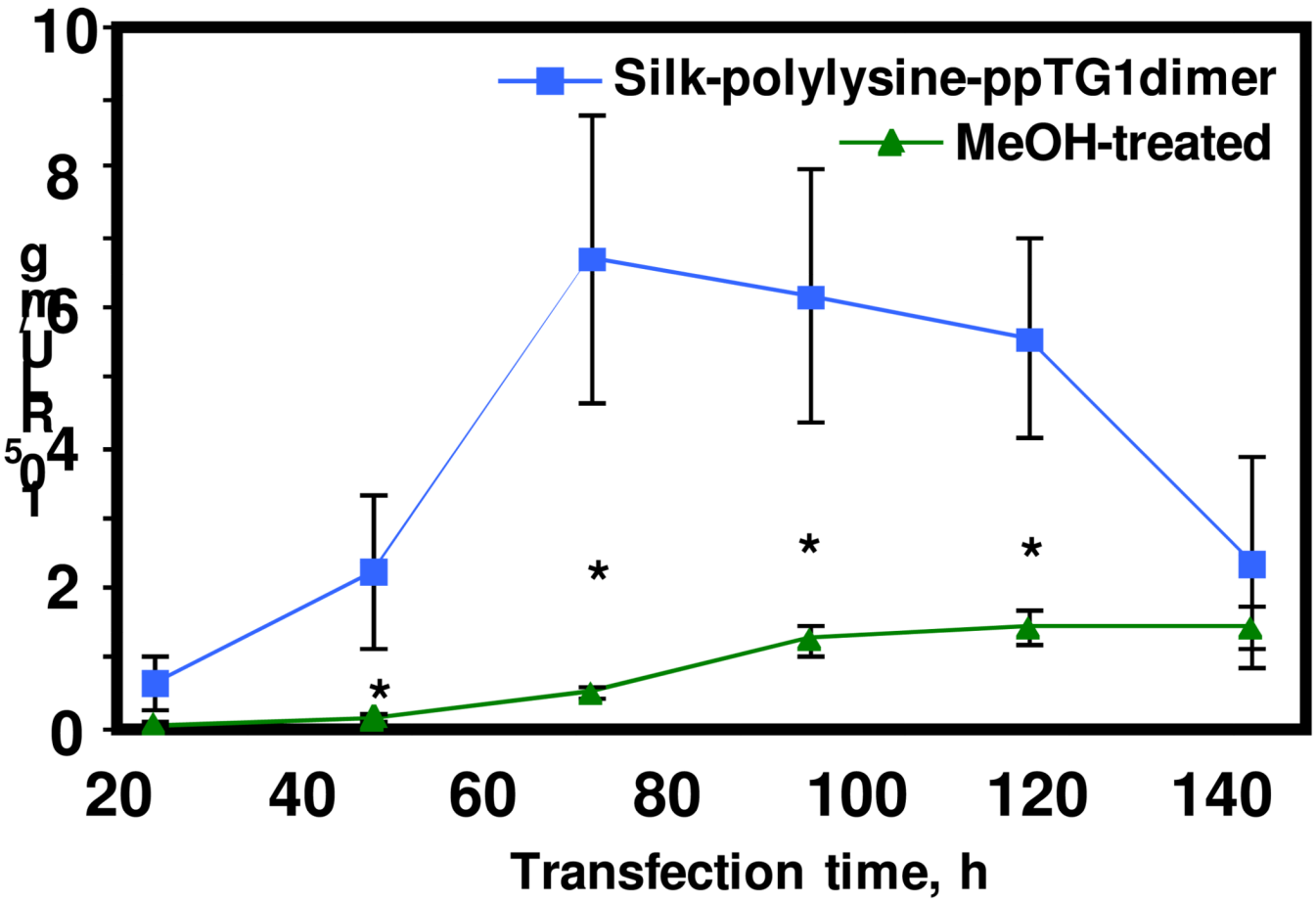

Figure 6.
Time course of transfection with the pDNA complexes of Silk-polylysine-ppTG1 dimer prepared at N/P 2 before and after MeOH treatment for 24 h. *Significant difference between two groups at $p < 0.05$. RLU means Relative light unit.

Table 1

Sizes and zeta potentials of the pDNA complexes prepared.

Samples	Sizes [nm] and PDI		Zeta potential [mV]	
	SL-Monomer[a]	SL-Dimer[a]	SL-Monomer[a]	SL-Dimer[a]
N/P 0.1	563 (0.679)	265 (0.856)	−43.1 ± 3.9	−33.4 ± 5.5
N/P 1	326 (0.862)	187 (0.720)	−37.9 ± 6.4	−29.8 ± 5.5
N/P 2	108 (0.564)	99 (0.524)	−37.5 ± 7.1	−26.2 ± 6.3
N/P 5	48.7, 698 (−)[b]	31.5, 575 (−)[b]	−36.9 ± 5.4	−22.7 ± 6.2
Silk proteins without pDNA	169 (0.453)	163 (0.821)	−30.5 ± 6.9	−13.9 ± 6.3
MeOH-treated (N/P 2)	146 (0.824)	130 (0.802)	−37.5 ± 5.1	−29.6 ± 5.7

[a] SL-Monmer: Silk-polylysine-ppTG1 monomer, SL-Dimer: Silk-polylysine-ppTG1 dimer.

[b] Bimodal size distribution and their PDI were not determined correctly.