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Bioengineered Silk Protein-Based Gene Delivery Systems

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Abstract

Silk proteins self-assemble into mechanically robust material structures that are also biodegradable and non-cytotoxic, suggesting utility for gene delivery. Since silk proteins can also be tailored in terms of chemistry, molecular weight and other design features via genetic engineering, further control of this system for gene delivery can be considered. In the present study, silk-based block copolymers were bioengineered with poly(L-lysine) domains for gene delivery. Ionic complexes of these silk-polylysine based block copolymers with plasmid DNA (pDNA) were prepared for gene delivery to human embryonic kidney (HEK) cells. The material systems were characterized by agarose gel electrophoresis, atomic force microscopy, and dynamic light scattering. The polymers self-assembled in solution and complexed plasmid DNA through ionic interactions. The pDNA complexes with 30-lysine residues prepared at a polymer/nucleotide ratio of 10 and with a solution diameter of 380 nm, showed the highest efficiency for transfection. The pDNA complexes were also immobilized on silk films and demonstrated direct cell transfection from these surfaces. The results demonstrate the potential of bioengineered silk proteins as a new family of highly tailored gene delivery systems.

1. Introduction

Silk proteins are commonly produced by insects and spiders, form fibrous materials in nature, and have been used as medical sutures because of their excellent mechanical properties and biocompatibility [1]. Beyond their traditional use, silk fibroin has also been explored as a biomaterial for cell culture and tissue engineering and achieved FDA approval for such expanded utility [2,3]. Also the solubility and biodegradability of silk-based materials can be controlled by manipulating the secondary structure, which helps in the design of biomaterials with selective features [4,5].

Silk proteins modified by genetic engineering have are capable of displaying new features alongside the native properties [6-9]. Modified spider silks bioengineered to include RGD cell-binding domains to enhance cell adhesion have also been reported [10]. Furthermore, biomaterial scaffolds prepared from this modified silk protein displayed enhanced ability to differentiate human bone marrow derived mesenchymal stem cells with regard to osteogenic outcomes [10]. Many other examples of bioengineered silks can be described, from inclusion of molecular triggers to control of self-assembly [11,12], chimeric silk proteins for controlled mineralization [13,14], and recent all silk block copolymer designs [15].

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Cationic polymers and poly(amino acid)s can interact with DNA through electrostatic interactions to assemble into polyelectrolyte complexes, which have been proposed as an alternative to recombinant viruses for the delivery of plasmid DNA (pDNA) into cells [16-22]. pDNA delivery is an attractive approach for a variety of disease states because of the option of pDNA to generate bioactive proteins in the modified host cells. Poly(L-lysine), which is degraded by cells, has been used as a cationic polymer to form delivery vehicles (vectors) for small drugs [16]. The interaction of polylysine with DNA has been characterized in detail by agarose gel electrophoresis (charge and size), electron microscopy (shape and size), atomic force microscopy (AFM) (shape and size), and dynamic light scattering (DLS) (size and size distribution) [16]. However, positively charged complexes can potentially induce cytotoxicity and form aggregates in biological media containing plasma proteins, indicating that clinical applications of positively charged complexes may be restricted [17,18].

A useful nonviral gene vector is biodegradable, has low toxicity, and can be targetable to specific cell types. These are challenging design goals to meet with synthetic polymers. Different cationic block copolymers as gene vectors have been studied in recent years, including cationic liposomes, polylysine copolymers, polyethyleneimine (PEI) copolymers, and polysaccharides [16,19-22]. Natural biopolymers, in particular are increasingly attractive as nonviral vectors because of their non toxicity and biocompatibility.

We are interested in designing silk-based nonviral gene vectors which are non-cytotoxic, biodegradable, and utilize non-toxic cationic polymers. Silk-based polymers are potentially useful candidates for nonviral gene vector, because functions can be added through recombinant techniques, offering a highly efficient approach to tailor chemistry, molecular weight and targeting based on system design. In the present study, a silk-based block copolymer was designed, generated and characterized by combining spider silk and poly(L-lysine). Complexes of these silk-based block copolymers with pDNA were prepared for *in vitro* gene delivery to human embryonic kidney (HEK) cells (Figure 1), and characterized in terms of sizes and charges. Silk films containing the pDNA complexes were also prepared and cell transfection experiments were carried out on these films. When considering 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 plasmid DNA and options for cell targeting reported here, a new family of vehicles for studies of gene delivery is described.

2. Experimental methods

2.1. 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 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 according to our previously published procedures [23]. The sequences of the synthetic oligonucleotides encoding 15 lysine residues were as follows:

Lys-a: 5′-*CTAGC*AAGAAAAAGAAAAAAAAGAAAAAAAAGAAAAAGAAAAAAAAG AAA*A*-3′,

Lys-b: 5′-*CTAGT*TTTCTTTTTTTTCTTTTTCTTTTTTTTCTTTTTTTTCTTTTTCTT*G*-3′.

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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 monomer (15 lysines), dimer (30 lysines), and trimer (45 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).

2.2. Protein expression and purification

The constructs pET30-6mer (control), pET30-6mer-15lysines, pET30-6mer-30lysines, and pET30-6mer-45lysines were used to transform the *E. coli* strains RY-3041, a mutant strain defective in the production of the SlyD protein, and protein expression carried out by methods reported previously [24,25]. Briefly, cells were cultivated in LB broth containing kanamycin (50 μg/ml) at 37°C. Protein expression was induced by the addition of 0.5 mM IPTG (Sigma-Aldrich, St. Louis, MO) when the OD600 nm reached 0.6. After approximately 4 h of protein expression, cells were harvested by centrifugation at 13,000 *g*. The cell pellets were resuspended in denaturing buffer (100 mM NaH_2PO_4, 10 mM Tris HCl, 8 M urea, pH 8.0) and lysed by stirring for 12 h followed by centrifugation at 13,000 *g* 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, Slide-A-Lyzer Cassettes (Pierce, Rockford, IL) with MWCO of 3,500 were used. The dialyzed samples were dissolved in 1 mL of hexafluoroisopropanol (HFIP). The recombinant proteins were further characterized for sequence confirmation at the Tufts University Core Facility by LC/MS/MS analysis.

2.3. Preparation and characterization of the ion complex

pDNA encoding GFP (EGFP, 7,650 bp) was 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, an HFIP solution containing silk protein (10 mg/mL) was mixed with the pDNA solution (370 μg/mL) at various P/N ratios. Here, P/N ratio refers to the molar ratio of the recombinant silk to nucleotides in pDNA. The mixture of recombinant silk and pDNA was incubated at room temperature (~20°C) overnight prior to characterization. The pDNA complexes were characterized by agarose gel electrophoresis, dynamic light scattering (DLS, Brookhaven Instruments Corporation, Holtsville, NY) and atomic force microscope (AFM, Dimension V, Veeco Instruments Inc, Plainview, NY). For agarose gel electrophoresis, 10 μL of each sample was mixed with loading buffer and analyzed on 1% agarose gel containing ethidium bromide (TAE buffer, 100V, 60 min). DLS was performed using a 532 nm laser at 37°C with a scattering angle of 90°, and the particle size and its distribution were analyzed using Dynamic Light Scattering software (Brookhaven Instruments Corporation). The pDNA complex solution (around 70 μL) was added to ultra pure water (450 μL, Invitrogen) and then used as a sample for DLS measurement. AFM observations were performed 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 [26].

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2.4. Preparation of films containing pDNA complexes

Silk fibroin was extracted from the cocoons from *B. mori* silkworm (Tajima Shoji Co, Yokohama, Japan) and silk solution (5 wt%) was prepared as previously described [27]. The silk solution was cast in 24-multiwell and 96-multiwell plates, and silk films were obtained after evaporation of solvent, afterwards, the silk films were sterilized with ethanol solution (70%). To prepare the silk films containing the pDNA complexes, the pDNA complex solution (HFIP/water) was cast on the silk film and dried for at least 12 h at room temperature to remove the solvent (HFIP/water). The silk films were washed with ultra pure water (DNAse, RNAse free, Invitrogen) to remove free pDNA before their use in cell transfection experiments.

2.5. Cell culture and transfection

HEK cells (293FT), which have been extensively used as an expression tool for recombinant proteins, were used as a model cell line [28]. Cultures were grown to confluence using media consisting of DMEM, 10% FBS, 5% glutamine, 5% NEAA. The cultures were detached from their substrates using 0.25% trypsin (Invitrogen), and then replated on the films in the 24-multiwell plate at a density of 5,000 cells/cm^2 with lipofectamine (2.5 μL, Invitrogen). After incubation of the cells for 24 h at 37°C, fluorescence images were obtained by fluorescence microscope (Leica Microsystems, Wetzlar, Germany) to evaluate GFP plasmid transfection. Expression results (n=4) were represented as the percentage of positive cells for GFP fluorescence relative to total cells counted.

2.6. Cell viability

HEK cells (50,000 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 a standard 3-(4,5-dimethylythiazol-2-yl)2,5-diphenyltetrazolium bromide) (MTT) assay (Promega, Madison, WI) according to the manufacturer's instructions (n=8).

2.7. Statistical analysis

The particle sizes on the silicon wafers were measured by AFM using a Research Nanoscope software version 7.30 (Veeco). 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.

3. Results

3.1. Expression and purification of silk protein

The amino acid sequences of the four spider silk variants generated, with and without polylysine, are shown in Figure 2. Yields of the recombinant silk proteins were approximately 10 mg/L after purification and dialysis. The proteins before and after purification by Ni-NTA chromatography were analyzed by SDS-PAGE and stained with Colloidal blue to evaluate purity (Figure 3). The Silk6mer control showed a band corresponding to a molecular weight of approximately 27 kDa (Figure 3A and B, lane 1). The recombinant silk proteins containing the lysine sequences, Silk6mer-15lys, Silk6mer-30lys, and Silk6mer-45lys, also showed molecular weights of around 30 kDa (Figure 3A and B, lane 2, 3, 4), which was in accord with the theoretical molecular weights of 23, 25, and 27 kDa, respectively. The results of protein identification by LC/MS/MS using the gel bands confirmed that the bioengineered proteins were the expected recombinant silk proteins (data not shown). The recombinant proteins were partially soluble in water and also soluble in HFIP (10 mg/mL) at room temperature.

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3.2. Characterization of pDNA complexes

DNA-Protein complex formation with pDNA encoding GFP with the four types of recombinant silk proteins (Silk6mer, Silk6mer-15lys, Silk6mer-30lys, and Silk6mer-45lys) was characterized by AFM, DLS, and agarose gel electrophoresis.

Figure 4 shows a typical AFM height image of the pDNA complexes with the recombinant silks (P/N 10) cast on a silicon wafer. Silk6mer-15lys molecules without pDNA were linear (Figure 4A), whereas Silk6mer-15lys with pDNA formed globular complexes (Figure 4B). Further, globular complexes were also observed with the Silk6mer-30lys and Silk6mer-45lys (Figures 4C and 4D). The average diameter of the pDNA complexes for Silk6mer-15lys, Silk6mer-30lys, and Silk6mer-45lys were 335 ± 104 nm, 392 ± 77 nm, and 436 ± 91 nm, respectively (Table S1). On the other hand, Silk6mer molecules randomly aggregated with pDNA (Figure 4E), and the resulting features were not globular complexes but large aggregates with a diameter of 857 ± 290 nm. Also, the statistical analysis of the dimensions of pDNA complexes determined by AFM demonstrated significance differences between the complexes of Silk6mer and the other samples (Table S1).

The hydrodynamic diameter of the recombinant silks and their pDNA complex were measured by DLS. (Table 1 and Figure S1). The average diameters of Silk6mer without and with pDNA were 570 nm and around 550-790 nm, respectively. The other three types of recombinant silks containing polylysine showed an average diameter of around 210-270 nm without pDNA. The diameter of pDNA complexes of the recombinant silk with polylysine sequences increased with increase in polylysine sequence or P/N ratio. In the case of Silk6mer-30lys (P/N 25) and Silk6mer-45lys (P/N 10 and 25), the diameters were bimodal, indicating both small and large complexes. The pDNA complexes prepared at P/N 50 resulted in large precipitates and were not able to be characterized by DLS.

Agarose gel electrophoresis experiments were performed to investigate the interaction properties and electrolytic stabilities of the complexes of pDNA and recombinant silks (Figure 5). Figure 5A shows the migration of free pDNA (lane 1) and the pDNA complexes of the recombinant silks in 1% agarose gels (lanes 2-5). The migration of Silk6mer mixed with pDNA demonstrated that free pDNA was still present along with the Silk6mer molecules, whereas the recombinant silks containing polylysine sequences showed bands in the wells and migrated slower than free pDNA, indicating that the pDNA was partially bound on the recombinant silks; some release of pDNA may have occurred during electrophoresis. The mixtures of pDNA and Silk6mer-30lys with various P/N ratios were analyzed by agarose gel electrophoresis (Figure 5B). The P/N ratios ranging from 2.5 to 50 showed little variations in gel migration, indicating that the stability of the complexes were similar between these P/N ratios.

3.3. Preparation and characterization of silk films containing pDNA complexes

The complexes of pDNA and recombinant silks were deposited as cast silk films. After washing the silk film with water to remove free pDNA, the surface of the silk films containing pDNA complexes was examined by AFM to evaluate the integrity of the complexes. Figure 6 shows the AFM height image of the surface of the Silk6mer30-lys film containing pDNA complexes of Silk6mer-30lys. The particles were nearly identical in the size with the pDNA complex images acquired before casting on films (Figure 4C), confirming the integrity of the particles after being cast on the films. It is also evident from Figure 6 that the complexes were individually immobilized on the surface of silk film. As shown in Figure 6B, the pDNA complexes were adsorbed on the surface, and the height of the complexes was approximately 20 nm.

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3.4. pDNA transfection to HEK cells

To evaluate the feasibility of the pDNA complexes with the cationic recombinant silks for gene delivery, *in vitro* transfection experiments were carried out with HEK cells. For a comparison of pDNA transfection efficiency of various pDNA complexes with different P/N ratio, HEK cells were transfected with GFP pDNA as a reporter. Figure 7 shows fluorescence microscopy images of cells incubated on the silk films containing pDNA complexes of Silk6mer-30lys prepared at P/N 2.5 (A), 5 (B), 10 (C), 25 (D), and 50 (E). The transfection efficiencies for various P/N ratios are summarized based on the fluorescent cells in four independent field areas (Figure 7F). The transfection experiments with various P/N ratio resulted that pDNA complexes of Silk6mer-30lys (P/N=10) demonstrated a highest percentage (14 ± 3 %) of GFP-positive cells among the different complexes. The transfection efficiency based on the GFP-positive cells decreased in the following order: P/N=10, 25, 50, 5, and 2.5. Hence thereafter we limited further experiments to the pDNA complexes prepared at a P/N ratio of 10.

Figure 8 shows the fluorescence microscopy images of cells incubated on the silk film containing pDNA complexes with the P/N ratio of 10 for Silk6mer (A), Silk6mer-15lys (B), Silk6mer-30lys (C), and Silk6mer-45lys (D). Figure 8E shows the efficiency ratios of transfection determined as described above by counting GFP positive cells. The pDNA complexes of Silk6mer-30lys exhibited the highest transfection efficiency (14 ± 3 %) among the four samples, whereas the mixture of Silk6mer and pDNA failed to show effective transfection (0.4 ± 0.1 %). The relative order of the transfection efficiency decreased as follows: Silk6mer-30lys, Silk6mer-15lys, Silk6mer-45lys, and Silk6mer.

3.5. Cytotoxicity pDNA complexes to HEK cells

Cytotoxicity of pDNA complexes with the P/N ratio of 10 for Silk6mer, Silk6mer-15lys, Silk6mer-30lys, and Silk6mer-45lys was measured using the MTT assay. Figure 9 shows that the complexes of Silk6mer, Silk6mer-15lys, and Silk6mer-30lys exhibited no toxicity to HEK cells at the concentrations used in the transfection experiments (0.76 mg/ml). The pDNA complexes of Silk6mer-45lys showed 88 ± 11 % of cell viability, which was significantly different and lower in comparison with the other recombinant silk complexes.

4. Discussion

The primary focus of this study was to design complexes of recombinant silk molecules with pDNA for gene delivery. Four types of recombinant silks were cloned, expressed, and purified from *E. coli*. The pDNA complex of Silk6mer without the lysine sequence did not form globular particles based on AFM analysis (Figure 4E). This is likely due to interactions between the pDNA with the few positive charged amino acids in the silk sequence. Additionally, agarose gel electrophoresis showed free pDNA when mixed with the Silk6mer molecules (Figure 5A, lane2). On the other hand, according to the electrophoresis experiments (Figure 5A) and the AFM images (Figure 4), globular complexes of pDNA with recombinant silks containing polylysine were formed, suggesting that the polylysine sequence was necessary to form globular nano-sized ion complexes of silk molecules with pDNA. The diameter and size distribution of the pDNA complexes increased with an increase in the molecular weight of polylysine sequence and the P/N ratio (Table 1 and Figure S1). In the case of Silk6mer-lys45 or complexes prepared at P/N=25 or 50, relatively high positive charges of the recombinant silk produced larger and more widely-distributed complexes. Part of the pDNA was released from the complexes during electrophoresis (lanes 3, 4, 5 in Figure 5), implying that the pDNA might be packed in the interior of the globular complexes as previously reported [29,30], but also on the surface, although more studies are required to clarify this issue.

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Silk films containing the pDNA complexes on the surface was prepared (Figure 6A). Comparison of the height of complexes before and after deposition on the films (Figures 4C and 6B) supported that the pDNA complexes were half-buried and immobilized on the surface of silk film, likely in part due to the partial local solubilization of the surface by the HFIP prior to evaporation. Additionally, silk-silk (protein-protein) hydrophobic interactions between the silk in the pDNA complexes and the surfaces of silk biomaterial films supports the immobilization of the pDNA complexes.

The transfection experiments with the complexes containing the GFP gene into HEK cells revealed that the pDNA complex of Silk6mer-30lys prepared at P/N 10, which was 380 nm in diameter by DLS, was the most efficient complex of the Silk6mer and polylysine block copolymers (Figures 7 and 8). These findings with regard to the variations in efficiency might be related to particle size as described previously [31-33]. In particular, it has been reported the particles of less than 200 nm in diameter are almost exclusively internalized, while particles of 500 nm in diameter are not, suggesting the size of particles for gene delivery are critical [31-33]. Though it is speculative in this study, the diameters of pDNA complex of Silk6mer-45lys (P/N 10, 590 nm) and the complexes prepared at P/N>10 (more than 400 nm in diameter) might be too large to be transfected into the cells. Also, the relatively high positive charges of the complexes due to higher molecular weight of polylysine and P/N ratio might result in the formation of disordered aggregates in solution and induce cytotoxicity and reduce transfection efficiency according to the literature [17,18,34,35]. In this respect, the higher positive charge of the Silk6mer-45lys showed lower cell viability in comparison with the other complexes (Figure 9), and also reduced the transfection efficiency (Figure 8). We therefore concluded that the pDNA complex of Silk6mer-30lys prepared at P/N of 10 was the most feasible for gene delivery.

Chemical synthetic polymer-based nonviral pDNA delivery systems have been improved in effectiveness and in terms of non-cytotoxic delivery over the last decade. PEI has become the standard in many *in vitro* and *in vivo* applications for pDNA delivery with respect to transfection efficiency, pDNA protection, cell-binding, and endosomal release [36-40]. One of the highest transfection efficiencies of PEI/pDNA complexes to HEK cells was reported to be approximately 75%, obtained using the complex at a PEI/pDNA ratio of 5:1 (w/w) after incubation of 54 h [40]. Nevertheless, it is necessary to improve synthetic polymer-based pDNA delivery systems in cytotoxicity and specific-delivery properties and targeting [20]. Cell viability from PEI was reported to be below 50% at concentrations of 0.1 mg/mL for 24h [20], whereas the present bioengineered silk-based gene delivery system using the Silk6mer-30lys demonstrated no cytotoxicity for 48 h as shown in Figure 9. However, even the best transfection efficiency (14 ± 3 %) in this study was lower in comparison with the PEI system [20,36-40]. This is likely because the pDNA complex of Silk6mer-30lys needs to be smaller, viral size, and positively charged to interact readily with cell surfaces [16,29,37]. In order to enhance the effective and specific delivery, the silk and polylysine block copolymers prepared herein can be further modified with functional peptides, such as for cell-penetration, cell-binding, and tumor-homing, through the use of genetic engineering [41-44]. In particular, one of the highest transfection efficiencies of pDNA complexes with cell-penetrating peptides was reported to be approximately 45-fold higher in comparison to the pDNA complex of PEI at low DNA concentration (125 ng/mL) and without the specific penetrating peptide [42]. Thus, we anticipate significant improvements of our system upon addition of such peptides as an approach to enhance transfection efficiency. Thus, the recombinant silk modified to contain the polylysine sequence has the potential to be effective, specific, biodegradable, and completely non-cytotoxic gene delivery system, even though the present transfection efficiency of silk-based delivery system is lower than the other competitors.

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There are a number of advantages of these cationic recombinant silks when compared with polylysine alone as gene delivery systems. Polylysine has been used to form pDNA complexes for gene delivery for over a decade and offer important features such as biodegradability, low-cytotoxicity, and flexibility regarding the size of the pDNA complex (15-150 nm in diameter) [16]. However, pDNA complexes with polylysine need improved *in-vivo* stability against enzymes that degrade the pDNA [16]. Further, polylysine heterogeneity with respect to molecular weight, leads to challenges in the preparation of homogeneously sized pDNA complexes. In contrast, the presence of the silk sequences enhance *in-vivo* stability of pDNA complexes [45,46]. Further, the homogeneous molecular weight of the recombinant silk and polylysine system described here provides monodisperse polymeric components that should provide improved control of the final pDNA complexes as we continue to refine the current system. The immobilization of pDNA complexes on the surface of films enhanced the internalization of pDNA by cells and promoted surface-mediated transfection [47-50].

In the present study, we have also succeeded in directly transfecting the pDNA complexes immobilized on the surface of silk films to HEK cells. The silk-based complexes with pDNA, which could be transfected in cells, were able to be adsorbed on the surface of the silk film, implying that this new gene delivery system can be applied not only to silk films but also to other silk-based biomaterials for pDNA delivery. The versatility in both design and application of these new bioengineered silk protein delivery systems for pDNA suggests future utility in many gene delivery applications.

5. Conclusions

The transfection of pDNA in cells through biodegradable and non-cytotoxic silk-based complexes was demonstrated. Recombinant silks modified to contain polylysine sequences were prepared and used to form globular complexes with pDNA. Silk films containing the pDNA complexes on their surface were also prepared, and direct transfection of the pDNA complexes immobilized on the surface of silk films to HEK cells was successfully carried out. The gene transfection experiments in HEK cells revealed that the pDNA complex of Silk6mer-30lys prepared at P/N 10, which were 380 nm in diameter by DLS, was the complex with the highest efficiency of all Silk6mer and polylysine block copolymers examined. Thus, recombinant silks containing polylysine sequences have demonstrated feasibility for application to silk-based materials for pDNA delivery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledegments

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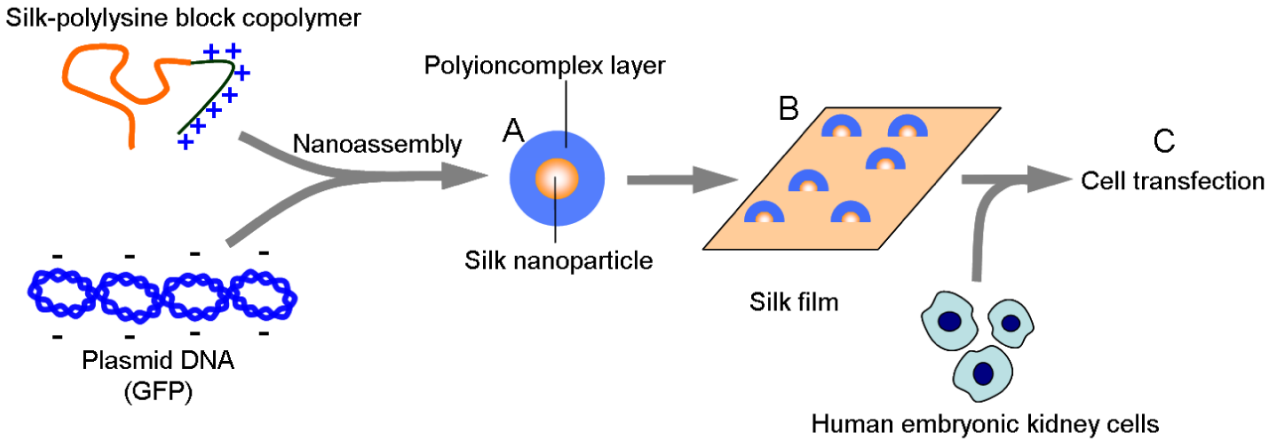

Figure 1.
Schematic presentation of the strategy used in this study. pDNA complex formation with silk-polylysine block copolymer (A). Preparation of a silk film containing pDNA complex (B). Cell transfection using the silk film containing pDNA complex (C).

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Silk6mer - lysine sequence

Met H H H H H H S S G L V P R G S G M K E T A A A K F E R Q H M D S P D L G T D D D D K A M A A
S G R G G L G G Q G A G A A A A A G G A G Q G G Y G G L G S Q G T
S G R G G L G G Q G A G A A A A A G G A G Q G G Y G G L G S Q G T
S G R G G L G G Q G A G A A A A A G G A G Q G G Y G G L G S Q G T
S G R G G L G G Q G A G A A A A A G G A G Q G G Y G G L G S Q G T
S G R G G L G G Q G A G A A A A A G G A G Q G G Y G G L G S Q G T
S G R G G L G G Q G A G A A A A A G G A G Q G G Y G G L G S Q G T S **Lysine Sequence** Stop

Lysine sequence

15lys K K K K K K K K K K K K K K T S
30lys K K K K K K K K K K K K K K T S K K K K K K K K K K K K K K T S
45lys K K K K K K K K K K K K K K T S K K K K K K K K K K K K K K T S K K K K K K K K K K K K K K T S

Figure 2.

Amino acid sequences of the silk6mer-lysine (recombinant spider silk protein) and poly-L-lysine sequences. Underline: representative monomeric spider silk unit.

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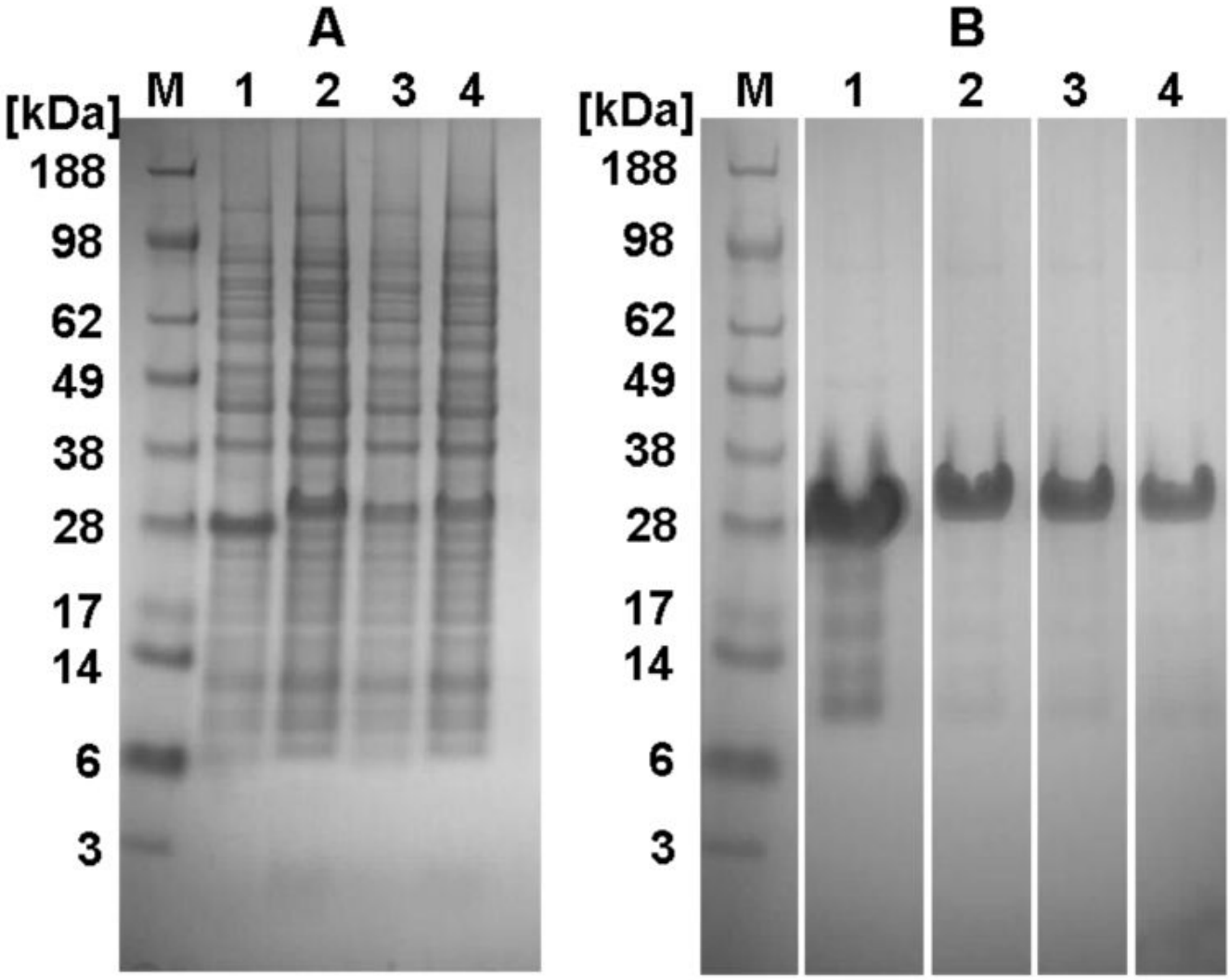

Figure 3.
SDS-PAGE of the recombinant silk protein before (A) and after purification by Ni-NTA chromatography (B), where lane 1: Silk6mer, lane 2: Silk6mer-15lys, lane 3: Silk6mer-30lys, and lane 4: Silk6mer-45lys. M: molecular weight markers.

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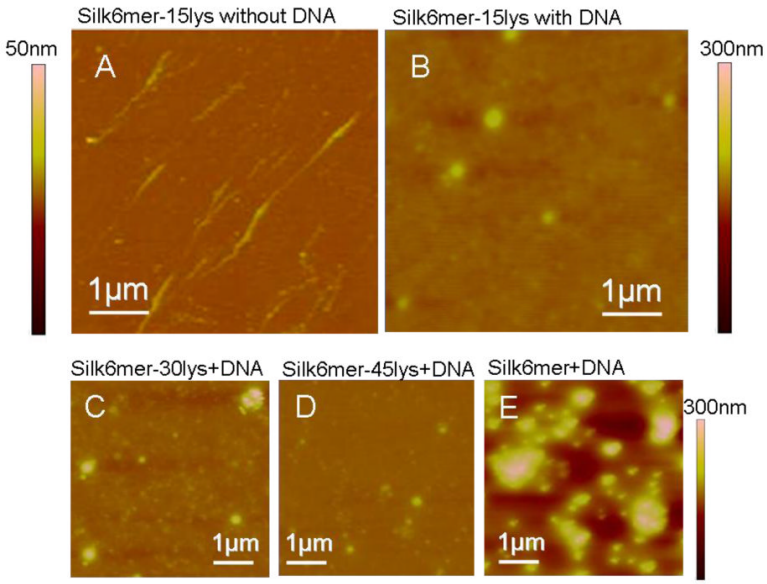

Figure 4.

AFM height images of Silk6mer-15lys proteins without (A) or with pDNA (B) on silicon wafer substrates. (C): pDNA complexes with Silk6mer-30lys, (D): pDNA complexes with Silk6mer-45lys, and (E): pDNA complexes with Silk6mer. The pDNA complexes in this figure were prepared at P/N ratio of 10.

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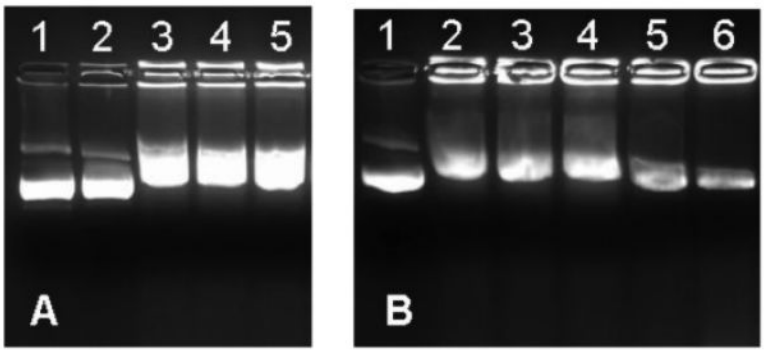

Figure 5.

Agarose gel of pDNA and pDNA complexes with different molecular weights of lysine sequence (A) and different P/N ratio (B). A1 and B1: pDNA (control), A2: Silk6mer and pDNA (P/N 10), A3: Silk6mer-lys15 and pDNA (P/N 10), A4: Silk6mer-lys30 and pDNA (P/N 10), A5: Silk6mer-lys45 and pDNAm (P/N 10), B2: Silk6mer-lys30 and pDNA (P/N 2.5), B3: Silk6mer-lys30 and pDNA (P/N 5), B4: Silk6mer-lys30 and pDNA (P/N 10),), and B5: Silk6mer-lys30 and pDNA (P/N 25), B6: Silk6mer-lys30 and pDNA (P/N 50).

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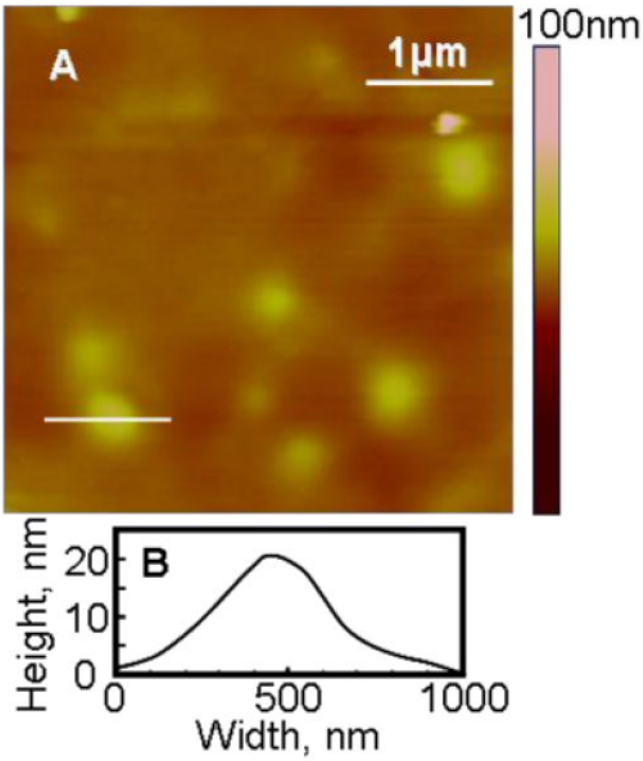

Figure 6.
AFM height image of the surface of the silk film containing pDNA complexed with Silk6mer-30lys (A). (B) Line profile data of the white line in Figure 6A.

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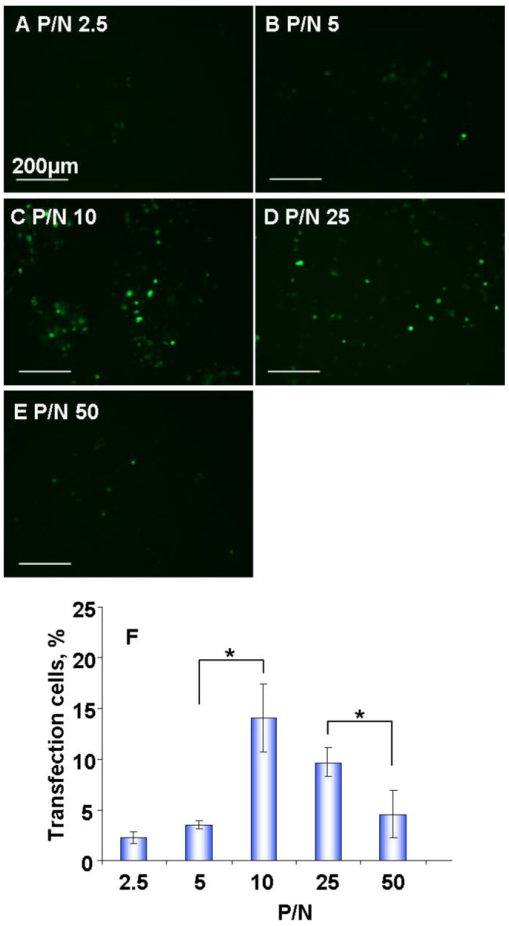

Figure 7.

Transfection results in loading pDNA complexes with different P/N ratio in HEK cells. Fluorescence microscopy images of cells incubated on the silk films containing pDNA complexes of Silk6mer30lys. (A) P/N 2.5, (B) P/N 5, (C) P/N 10, (D) P/N 25, and (E) P/N 50. The green in the images represents successfully transfected cells. (F) Plot of transfection efficiency loading pDNA complexes in HEK cells according to fluorescence images, and data are shown as means ± standard deviation (n=4). *Significant difference between two groups at $p < 0.05$.

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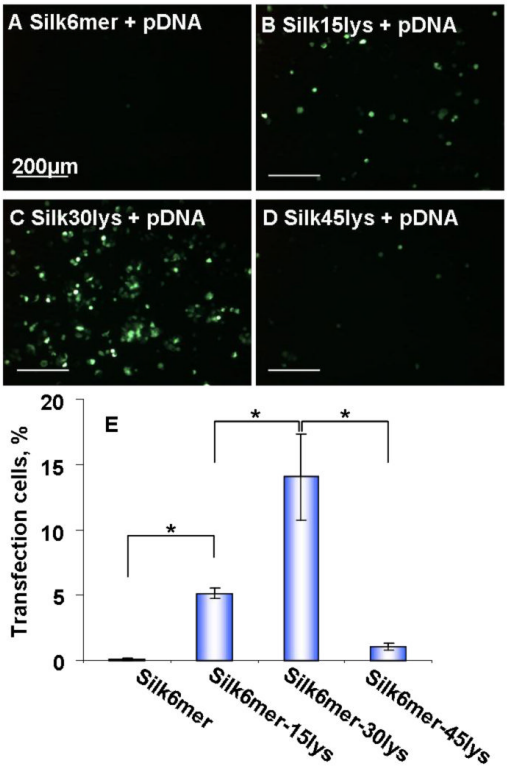

Figure 8.

Transfection results in loading pDNA complexes with different polylysine sequences in HEK cells. Fluorescence microscopy images of cells incubated on the silk films containing pDNA complexes of Silk6mer (A), Silk6mer-15lys (B), Silk6mer-30lys (C), and Silk6mer-45lys (D). The green in the images represents successfully transfected cells. Figure (E) shows plot of transfection efficiency from the fluorescence images, and data are shown as means ± standard deviation (n=4). *Significant difference between two groups at $p < 0.05$.

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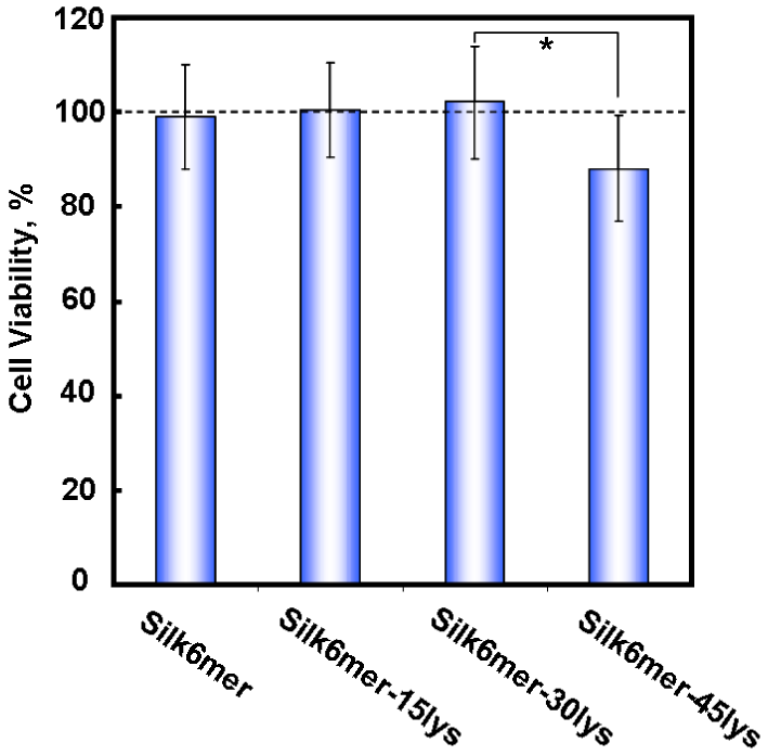

Figure 9.
Cell viability after treatment of HEK cells with pDNA complexes (P/N=10) with different lysine sequences. Data are shown as means ± standard deviation (n=8). *Significant difference between two groups at $p < 0.05$.

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Table 1

Average diameters of the recombinant silks and their complexes determined by DLS

Polymer	P/N	Diameter, nm
Silk6mer	-[a]	570
	2.5	550
	5	790
	10	730
	25	750
	50	780
Silk6mer-15lys	-[a]	270
	2.5	320
	5	280
	10	310
	10	310
	25	380
	50	-[b]
Silk6mer-30lys	-[a]	210
	2.5	120
	5	330
	10	380
	25	400, 1150
	50	-[b]
Silk6mer-45lys	-[a]	210
	2.5	80
	5	370
	10	140, 590
	25	350, 1320
	50	-[b]

[a]The recombinant silk molecules without pDNA.

[b]There were too much precipitation for analysis by DLS.