Published in final edited form as:
J Control Release. 2010 August 17; 146(1): 136–143. doi:10.1016/j.jconrel.2010.05.006.

Gene Delivery Mediated by Recombinant Silk Proteins Containing Cationic and Cell Binding Motifs

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Abstract

Silk proteins are biodegradable and biocompatible, and can also be tailored to contain additional features via genetic engineering, suggesting utility for gene delivery. In the present study, novel silk-based block copolymers were bioengineered both with poly(L-lysine) domains to interact with plasmid DNA (pDNA) and RGD, to enhance cell-binding and transfection efficiency. Ionic complexes of these silk-polylysine-RGD block copolymers with pDNA were prepared, characterized and utilized for gene delivery to HeLa cells and human embryonic kidney (HEK) cells. The material systems were characterized by agarose gel electrophoresis, zeta-potentialmeter, atomic force microscopy, and dynamic light scattering. Sizes and charges of the pDNA complexes were regulated by the polymer/nucleotide molar ratio. Samples with 30-lysine residues and 11 RGD sequences, prepared at the ratio of number of amines/phosphates from pDNA (N/P) of 2, had an average solution diameter of 186 nm and showed the highest transfection efficiency. The intracellular distribution of complexes of Cy5-labeled pDNA was investigated by confocal laser scanning microscopy. The Cy5-labeled pDNA was distributed near the cell membrane and around the nuclei, indicating that the pDNA was transferred near the nucleus. The results demonstrated the potential of bioengineered silk proteins with additional functional features as a new family of highly tailored gene delivery systems.

Keywords

RGD cell-binding motif; gene delivery; nanoparticle; recombinant silk

1. Introduction

Gene delivery, which is the introduction of genes into the cells of a tissue to treat disease, is a key aspect of gene therapy. Recently, RNA interference or gene silencing has provided a new option for the selective degradation of RNA to treat various diseases. However, the most common use of gene delivery has been with plasmid DNA (pDNA) transfer into cancer cells, where the supplied genes activate tumor suppressor control genes in the cell and decrease the activity of oncogenes. Although over 1,400 gene therapy clinical trials have

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Supplementary data
Supplementary data associated with this article can be found in the online version.

Competing financial interests
The authors declare that they have no competing financial interests.

been conducted since 1989 [1], there are no Food and Drug Administration (FDA)-approved gene therapies, in part because viruses are commonly used as gene carriers. Viruses present a number of potential problems, including toxicity, immune response and targeting issues [2].

Viral vectors including adenovirus and adeno-associated virus have been used in gene transfer, because of the relatively high efficiency of transfection and potential long term effects through integration into the host genome [2]. However, concerns remain about inducing immune responses by the introduction of viruses as the carriers. Nonviral vectors such as cationic polymer-based vectors have therefore been extensively studied as alternatives to viral carriers. Nonviral pDNA delivery systems using cationic liposomes or chemically synthesized polymers have been improved in effectiveness and in terms of biocompatible delivery over the last decade. Polyethyleneimine (PEI) has become the gold standard in many *in vitro* and *in vivo* applications for pDNA delivery with respect to transfection efficiency, pDNA protection, cell-binding, and endosomal release [3–5]. Nevertheless, improvements in synthetic polymer-based pDNA delivery systems are still needed to reduce cytotoxicity and to improve specific-delivery properties and targeting. A nonviral gene vector should be biocompatible, biodegradable, efficient, exhibit low toxicity, and able to be targeted to a specific cell type. These are challenging design goals to meet with liposomes or synthetic polymers.

Silk proteins have been used successfully in the biomedical field as sutures for decades because of their excellent mechanical properties and biocompatibility [6]. Silk proteins have also been explored as biomaterials for cell culture and tissue engineering and achieved FDA approval for such expanded utility [7,8]. Also, cell binding motifs (RGD), cell penetrating peptides [9–11], signal peptides of virus [12], and/or tumor-homing peptides [13–17] can be added through recombinant DNA techniques, which is an important advantage of recombinant silk proteins over liposomes and synthetic polymers as gene delivery systems. Thus, bioengineered silk proteins offer an approach to tailor chemistry, molecular weight and targeting based on system design. Silk-based polymers are therefore potentially useful candidates for nonviral gene vector. Silk-elastin-like proteins (SELP) prepared by recombinant DNA techniques have been utilized as gene delivery systems, by forming into hydrogels to release adenovirus containing reporter genes [18–20]. Enhanced gene expression was reported in target cells up to 10 fold when compared to viral injection without the SELP, demonstrating utility of this protein delivery system for head and neck solid tumors [19,20].

In our previous study, a silk-based block copolymer was generated by combining spider silk consensus repeats with poly(L-lysine) [21]. The silk-based block copolymers formed ion complexes with pDNA, and the sizes were controllable based on the polymer/DNA ratio or molecular weight of poly(lysine) bioengineered into the designs. Also, the pDNA complexes of silk-based block copolymers with less than 30 lysines showed no cytotoxicity toward human embryonic kidney (HEK) cells. This prior study demonstrated the feasibility of bioengineering highly designed silk-based pDNA delivery systems; however, the transfection efficiency was too low to be useful for gene vectors.

The RGD sequence, arginyl-glycyl-aspartic acid, is known to selectively recognize and bind $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins that are expressed on cell surfaces of certain cell types such as endothelial cells, osteoclasts, macrophages, platelets, and melanomas [22–25]. The integrins are considered to be a class of transmembrane glycoproteins that interact with the extracellular matrix, and are exploited for cell-binding and entry by receptor-mediated endocytosis, which is a representative pathway for gene delivery systems [25]. RGD sequences are therefore a useful candidate as a ligand for gene vectors used for pDNA and

siRNA delivery, and have been added into several vectors to enhance target functions and transfection efficiency [26–30]. Poly(L-lysine) is a cationic polymer that interacts with DNA through electrostatic interactions to assemble into polyelectrolyte complexes. Poly(L-lysine) is degraded in cells and has been used as an alternative to recombinant viruses for the delivery of pDNA into cells [31–34].

The aim of the present study was to enhance transfection efficiency of silk-based gene vectors. The goal was to continue to exploit the biocompatible, biodegradable, and non-toxic cationic polymers but with the addition of the cell-binding motif, RGD. In addition, one of the goals of the present work was to systematically assess the influence of the position of RGD sequences in the bioengineered block copolymer systems in terms of transfection efficiency. Thus, the goals centered around understanding design issues by utilizing the tailorable approaches via genetic engineering of the vectors, as well as improving overall efficiency of the silk block copolymer systems. Complexes of these new silk-based block copolymers with pDNA were prepared for *in vitro* gene delivery to HeLa and HEK cells, which respectively express the integrins and do not express (Figure 1), and characterized by agarose gel electrophoresis, zeta potentialmeter, atomic force microscopy (AFM), and dynamic light scattering (DLS). The novel 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 cell-binding function reported here, a new family of vehicles for studies of gene delivery is described.

2. Materials and 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 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 [35,36]. The sequences of the synthetic oligonucleotides encoding RGD residues were as follows: RGD-a: 5′-*CTAGC*CGAGGCGAC*A*-3′, RGD-b: 5′-*CTAGT*GTCGCCTCG*G*-3′. The restriction sites for *Nhe*I and *Spe*I are italicized. RGD-a and RGD-b are complementary oligonucleotides which were annealed to form double stranded DNA. The double stranded DNAs of RGD sequences were ligated into pET30-6mer-polylysine to generate 5 types of pET30-6mer-polylysine-RGDs, as shown in Figure 2, by DNA ligase (New England Biolabs Inc, Ipswich, MA).

2.2. Protein expression and purification

The constructs pET30-RGD-6mer-30lysines, pET30-RGD-6mer-30lysines-RGD, pET30-6mer-30lysines-RGD, pET30-6mer-30lysines-2×RGD, and pET30-11×RGD-6mer-30lysines 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 [37]. Briefly, cells were cultivated in LB broth containing kanamycin (50 μg/ml) at 37°C. Protein expression was induced by the addition of 1.0 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, 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 MALDI-TOF.

2.3. Preparation and characterization of the complex

pDNA encoding Firefly Luciferase (7,041bp) 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, a solution containing silk protein (0.1 mg/mL) was mixed with the pDNA solution (370 μg/mL) at various N/P ratios (0.1 to 10). 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 to make sizes of the complexes homogeneous prior to characterization. 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). The pDNA complexes were also characterized by zeta nanosizer (Zetasizer Nano-ZS, Malvern Instruments Ltd, Worcestershire, UK) and AFM (Dimension V, Veeco Instruments Inc, Plainview, NY). Zeta potential and zeta deviation of samples were measured three times by zeta nanosizer, and the average data were obtained using Dispersion Technology Software version 5.03 (Malvern Instruments Ltd). DLS was performed with the zeta nanosizer using a 633 nm He-Ne laser at 25°C with a scattering angle of 173°, and the particle size and distribution (PDI) were determined using Dispersion Technology Software version 5.03 (Malvern Instruments Ltd). 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 [38].

2.4. Cell culture, transfection, and viability

HeLa cells, which have been reported to express $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, and human embryonic kidney (HEK) cells (293FT), which have been extensively used as a gene expression tool and reported to possess no $\alpha_v\beta_3$ and a few $\alpha_v\beta_5$ integrins, were used as a model cell line [22,39–41]. Cultures were grown to confluence using media consisting of Dulbecco's Modified Eagle Medium (DMEM), 10% FBS, 5% glutamine, 5% Non-Essential Amino Acid (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 7,000 cells/well. Media for transfection to HeLa and HEK cells were DMEM containing 10% FBS. 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 48 h, to evaluate the luciferase gene expression quantitatively, the Luciferase assay (Promega, Madison, WI) was performed (n=4), according to the manufactuer's protocol. Briefly, the transfected cells were washed with PBS (Invitrogen) and lysed with Luciferase Cell Culture Lysis Regent (Promega). The lysate was mixed with Luciferase Assay Substrate and Luciferase Assay Buffer (Promega), and then the luciferase gene expression was evaluated based on the intensity of photoluminescence (the relative light unit) using luminescence microplate reader (Spectra MAX Gemini EM, Molecular Devices Corporation, Sunnyvale, CA). The amount

of protein in each well was determined using BCA protein asay (Pierce Biotechnology, Rockford, IL), and then the relative light unit/weight of protein (RLU/mg) was obtained. Lipofectamine 2000 (Invitrogen) was used as a positive control vector in this experiment. For cell viability, HEK cells (5,000 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) according to the manufacturer's instructions (n=8).

2.5. Confocal laser scanning microscopy (CLSM)

pDNA was labeled with Cy5 using a Label IT Nucleic Acid Labeling Kit (Mirus, Madison, WI), according to the manufacture procedure. HeLa cells were seeded on Glass Bottom Culture Dishes (MatTeK Corporation, Ashland, MA) and incubated overnight in 2 mL of DMEM. Complexes of the labeled pDNA (2.4 μg) with 11RS protein (N/P 2) were added into the wells. After 6 h incubation, the medium was replaced with fresh medium. After another 48 h incubation, the cells were washed with PBS twice and incubated with 300 nM 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen) PBS solution for 10 min. The intracellular distributions of the pDNA complex labeled by Cy5 and the nuclei stained with DAPI were observed by confocal laser scanning microscopy (CLSM, Leica Microsystems) at a excitation wavelength of 488 nm (Ar laser), 633 nm (He-Ne laser), and 710 nm (Mai Tai laser).

2.6. Statistical analysis

The particle sizes on mica substrates were measured by AFM using a Research Nanoscope software version 7.30 (Veeco). The average value of 30 measurements was used. Statistical differences in 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 five spider silk variants generated with polylysine and RGD cell-binding motifs (RS, RSR, SR, S2R, and 11RS) are shown in Figures 2 and S1. 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. RS, RSR, SR, S2R, and 11RS each showed a band corresponding to a molecular weight of approximately 33, 32, 30, 30, and 35 kDa, respectively (Figure 3), higher than the theoretical molecular weights (monoisotopic mass) of 26,068.1, 26,584.4, 25,565.9, 26,082.1, 31,669.86 Da, respectively. However, the results of MALDI-TOF showed the exact molecular weights, confirming that the bioengineered proteins were the expected recombinant silk proteins. Generally, silk-based polymers do not run true to size on SDS-PAGE gels, due to the hydrophobic nature of the protein, but gels are still useful to assess purity [35]. The recombinant proteins showed the theoretical pI of 10.6 and were soluble in water (5.0 mg/mL) at room temperature.

3.2. Characterization of pDNA complexes

DNA-Protein complex formation with pDNA encoding luciferase with the five types of recombinant silk proteins (RS, RSR, SR, S2R, and 11RS) was characterized by AFM, DLS, and zeta-potentialmeter.

The hydrodynamic diameters of pDNA complex of the recombinant silks were measured by DLS (Table 1). The average diameters of the complexes decreased with an increase in N/P ratio. The pDNA complexes prepared at too high N/P ratio such as N/P 10, however, demonstrated bimodal size distributions. The pDNA complexes of the recombinant silks prepared at N/P 2 were cast on mica and observed by AFM (Figure 4). All the complexes formed globular complexes as shown in Figures 4A. Based on the AFM observations, the pDNA complexes of 11RS at N/P 2 demonstrated the average diameter and height of 223 ± 32 nm and 30 ± 8 nm, respectively (n=30). On the basis of the volume of the particles, the dimensions determined by DLS and AFM showed reasonable agreements, according to the literature [38].

Agarose gel electrophoresis experiments were performed to investigate the interaction properties and electrolytic stabilities of the complexes of pDNA and recombinant silks. Figure 5A shows the migration of free pDNA and the pDNA complexes of 11RS with various N/P ratios ranging from 0.1 to 10 in 1% agarose gels. The pDNA complexes with 11RS at N/P 0.1 and 1 migrated to the same direction as free pDNA, whereas the complexes at N/P over 2 migrated in the opposite direction or did not migrate from the well. These results indicated that the pDNA complexes with 11RS at N/P below 1 were negatively charged, while the complexes at N/P over 2 were positively charged. The pDNA complexes of the other four recombinant silks prepared at N/P 2 were also characterized by agarose gel electrophoresis, and the all samples demonstrated positive charges (Figure 5B). To measure the values of the positive charge, the zeta potential of the pDNA complexes was determined. Figure 5C shows the zeta potential of pDNA complexes of 11RS with varying N/P ratios. The zeta potential increased with N/P ratio, and became positive at the N/P of 2. The zeta potential of the pDNA complexes of 11RS prepared at N/P 2 was 0.1 ± 4.5mV.

Cytotoxicity of pDNA complexes with the N/P ratio of more than 10 for RS, RSR, SR, S2R, and 11RS was measured using the MTT assay. Figure 6 shows that the complexes of all samples exhibited no cytotoxicity to HEK cells at higher concentration than that used in the transfection experiments (1.9 mg/mL).

3.3. pDNA transfection to HeLa and HEK cells

In vitro transfection experiments were performed with HeLa and HEK cells in order to evaluate the feasibility of the pDNA complexes with the cationic recombinant silks to contain RGD peptides for gene delivery via integrin-mediated endocytosis. For a comparison of pDNA transfection efficiency of various pDNA complexes with different N/P ratios, HeLa cells were transfected with luciferase pDNA as a reporter gene. Figure 7A shows the transfection efficiencies to HeLa cells for pDNA complexes of 11RS with N/P ratios ranging from 0.1 to 10 based on the luciferase assays (n=4). pDNA complexes of 11RS prepared at N/P 2 demonstrated the highest transfection efficiency among the different N/P ratios, followed by a steep decrease in efficacy, presumably due to excess recombinant silk interacting with the cells. Figures 7B and 7C show the transfection efficiencies to HeLa and HEK cells for pDNA complexes of different samples (N/P 2) as well as Silk6mer-30lys block copolymer (S in the figures) and Lipofectamine 2000 as controls. Silk6mer-30lys block copolymers, which contained no RGD sequence, did not show useful transfection to HeLa and HEK cells. The relative order of the transfection efficiency at N/P 2 decreased as follows: 11RS > RSR ≈ S2R > RS ≈ SR. Importantly, the pDNA complexes of 11RS

exhibited significantly higher transfection efficiency to HeLa cells in comparison to the other samples to contain a RGD or two at N/P 2 (Figure 7B). On the other hand, the pDNA complexes of 11RS did not show significantly higher transfection efficiency to HEK cells in comparison to RSR and SR2 (Figure 7C).

The intracellular distribution of the complexes of 11RS with Cy5-labeled pDNA and the nuclei stained with DAPI were investigated by CLSM. Figure 8 shows typical CLSM images of HeLa cells incubated with the pDNA complexes. The Cy5-labeled pDNA (red) was distributed near the cell membrane as well as around the nuclei (blue), indicating that the pDNA was transferred near the nucleus via the 11RS recombinant proteins.

4. Discussion

The focus of this study was to design complexes of recombinant silk molecules with cell-binding motifs for pDNA gene delivery, with a particular interest in how location and content of the cell-binding domain impacted outcomes. Five types of recombinant silks, RS, RSR, SR, S2R, and 11RS were cloned, expressed, and purified from *E. coli*. Globular nano-sized ion complexes of silk molecules containing 30 lysines were prepared and then complexed with pDNA (Figure 4). pDNA complexes just after the preparation showed bimodal size-distribution and almost no transfection efficiency (data not shown). Therefore, pDNA complexes were incubated for 24 h before the characterization of pDNA complexes and transfection experiments to obtain homogeneous pDNA complexes in size, according to the literature [22]. The average sizes of pDNA complexes of RS, RSR, SR, S2R, and 11RS at N/P 2 were 382, 315, 565, 207, and 186 nm, respectively according to DLS measurements (Table 1). The diameter of the pDNA complexes decreased with an increase in the N/P ratio (Table 1), suggesting the sizes of the complexes can be controlled by the N/P ratio. Also, no pDNA was released from the complexes during electrophoresis (Figure 5A and B), indicating that pDNA was packed in the globular complexes, unlike our previous report where some of the pDNA was released during electrophoresis [21].

The transfection experiments into HeLa and HEK cells with the complexes revealed that the pDNA complex of 11RS prepared at N/P 2, which was slightly positively charged (0.1±4.5 mV) and 186 nm in diameter, was the most efficient complex of the recombinant silks prepared in the study (Figures 7). The pDNA complexes of 11RS prepared at N/P 10 showed lower transfection efficiency in comparison with N/P 5 and N/P 2, because of their bimodal size distribution as listed in Table 1. S2R and RSR showed almost the same transfection efficiency to HeLa cells. RS and SR, which contained only one RGD sequence, demonstrated slightly lower transfection efficiency in comparison to S2R and RSR, suggesting position of RGD motif, at the N-terminus or C-terminus, did not influence the transfection efficiency of the pDNA complexes with the recombinant silk block copolymers. In other words, the recombinant silk molecules in the pDNA complexes were considered to be randomly assembled with pDNA and RGD sequences on the surface of the complexes as shown in Figure 1. This finding also suggests that other functional peptides can be added into different positions in the designs of these delivery systems in order to construct additional novel protein vectors.

The RGD sequence has been used as a ligand to enhance cell-binding and cell transfection efficiency of gene vectors, because of selective recognition and binding $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, which have been reported to be expressed in HeLa cells [22–25,42–46]. The transfection efficiency of pDNA complexes is also dependent on type of cells. In the case of HEK cells, the transfection happens more easily than HeLa cells independent of type of pDNA complexes (e.g. lipofectamine 2000 and poly(ethylene glycol)-polylysine block copolymer), as reported previously [22]. Hence, the transfection efficiency of different gene

vectors needs to be compared in the same cell line to determine the effect of RGD sequences. The addition of 11 RGD sequence into the recombinant silk significantly enhanced transfection efficiency into HeLa cells, whereas the other recombinant silk did not enhance significantly transfection efficiency into HeLa cells (Figure 7B). On the other hand, the recombinant silk with 11 RGD (11RS) did not significantly enhance the transfection efficiency to HEK cells in comparison to the recombinant silks with two RGD sequences (RSR and SR2) (Figure 7C). Therefore, 11 RGD sequence was supposed to induce RGD-integrin mediated transfection, though the other RGD sequence, dimeric and monomeric RGD sequences, did not induce RGD-integrin mediated transfection. Also, CLSM observation of the HeLa cells incubated with the Cy5-labeled pDNA complexes indicated pDNA was delivered to near the nucleus with the 11RS recombinant silk proteins (Figure 8). Based on these results, pDNA could be transferred to the nucleus with the recombinant silk proteins, 11RS, via the integrin-mediated endocytosis. Also, a few RGD sequences were not enough to recognize the integrins and to be transferred into cells, whereas 11RGD seem to be sufficient for the integrin-mediated transfection. In consideration of the strength of ionic interactions between pDNA and polylysine sequences as shown in Figure 5, the ionic interactions seem enough strong to maintain the pDNA complexes even inside cells. The pDNA can therefore be released from the complexes after partial degradation of the recombinant silk proteins by lysosome proteolytic activity.

Complexes of PEI and RGD peptides were investigated and showed higher transfection efficiency to HEK cells in comparison with PEI molecules alone, however, the cytotoxicity of the complexes of PEI and RGD peptides was approximately 50% at the concentration of 400 μg/mL [42]. Poly(ethylene glycol) (PEG)-based vectors demonstrated almost no cytotoxicity to HEK cells, and also exhibited comparable transfection efficiency to PEI [43]. However, the addition of RGD into the platform of chemical synthesis of delivery polymers requires multi-reaction steps with yields around 57% [22]. In the present study, the recombinant silks are synthesized using genetic techniques, a one-step synthesis with monodisperse polymer chains as a result. This tight control of polymer features, both in the design and placement of key motifs as well as in control of molecular weight, should provide significant advantages in terms of controlling lifetimes, targeting and distribution *in vivo* and overall understanding of design rules to optimize specific needs in gene delivery.

The pDNA complexes from the recombinant silks showed no cytotoxicity to HEK cells at the highest concentrations used in the transfection experiments (1.9 mg/mL), while also exhibiting the integrin-mediated transfection by 11RGD sequences. Further, the recombinant silks can be designed to add any number of peptides in selected positions and numbers to the silk carrier molecules. In this respect, this recombinant silk-base gene delivery system offers some important benefits and options for general polymer-based gene delivery systems. In order to further enhance the efficiency and specificity of gene delivery, the recombinant silks prepared herein can be further modified with multi functional peptides, such as for cell-penetration and tumor-homing peptides [9–17]. 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 [10]. Thus, we anticipate further improvements with our system upon addition of such peptides as an approach to further enhance transfection efficiency and selectivity. Therefore, we suggest that the recombinant silks modified to contain polylysine charged complexes and cell targeting domains such as RGD, are a new platform polymer, like PEG, for gene delivery, but with tremendous versatility in design and function.

5. Conclusions

This is the first report of the transfection of pDNA in cells through biodegradable and biocompatible recombinant silks modified to contain RGD cell-binding motifs. Recombinant silks modified to contain polylysine and RGD sequences were prepared and used to form globular complexes with pDNA. Transfection of the pDNA complexes to HeLa and HEK cells was successfully carried out. The gene transfection experiments in HeLa cells revealed that the pDNA complexes of 11RS prepared at N/P 2, which were positively charged particles of approximately 186 nm in diameter, showed the highest efficiency to HeLa cells of the recombinant silks examined because of integrin-mediated transfection with 11 RGD sequences. On the other hand, the transfection efficiency to HEK cells was strongly dependent on the number of RGD cell-binding motifs. Further, the position of RGD motif, at the N- or C-terminus of the recombinant silks, did not influence the transfection efficiency of the pDNA complexes. The recombinant silks containing polylysine sequences have demonstrated feasibility to be a new platform polymer for non-viral gene delivery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Dr Robert Nicolosi, University of Massachusetts Lowell, for technical support. This work has been supported by grants from the NIH (Tissue Engineering Resource Center), the NSF and the AFOSR (D.K.). K.N. was supported by a Japan Society for the Promotion of Science Postdoctoral Fellowship for Research Abroad.

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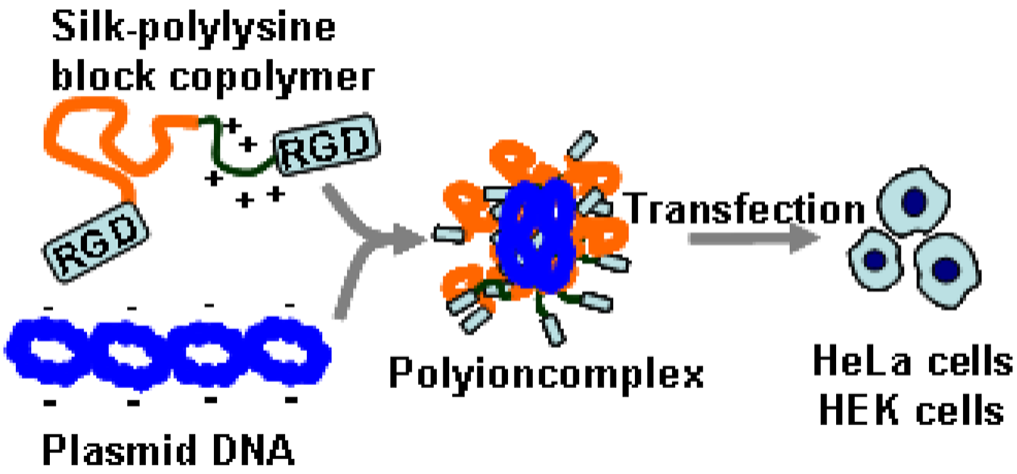

Figure 1.
Schematic of the strategy used showing pDNA complex formation with silk-polylysine-RGD block copolymers and cell transfection using the pDNA complex.

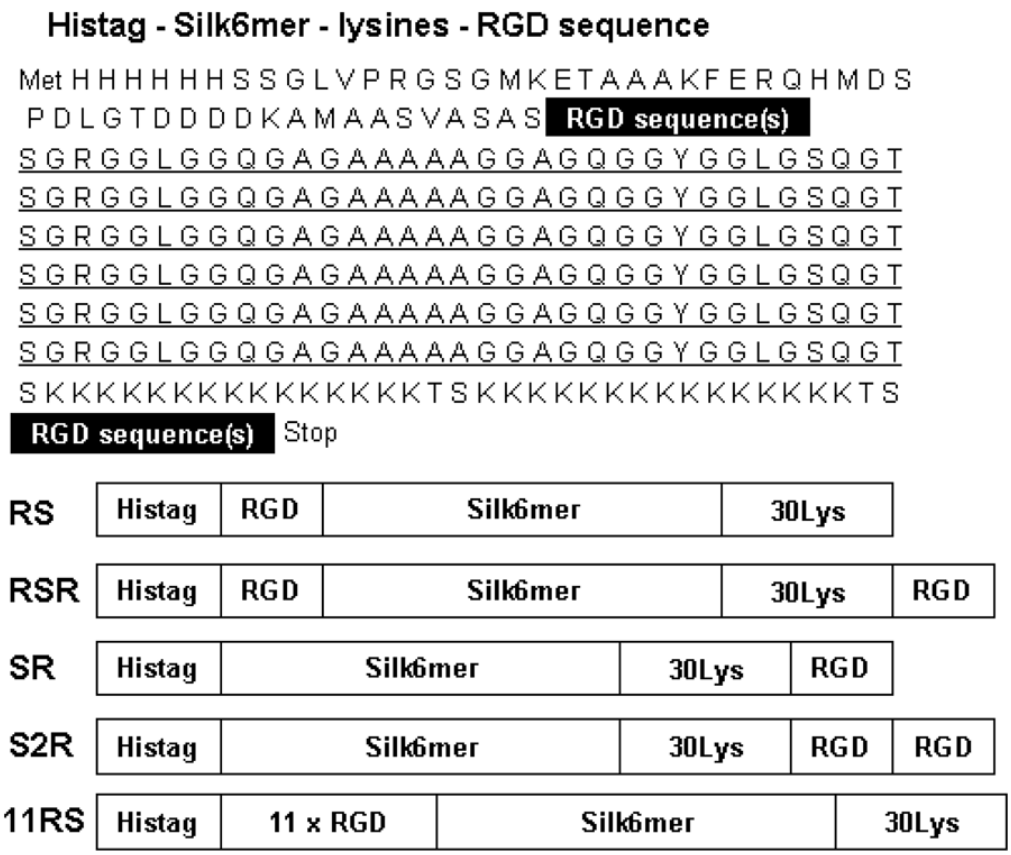

Figure 2.
Amino acid sequences of the recombinant spider silk proteins with poly-L-lysine and RGD sequences. Underline: representative 6mer of spider silk sequence.

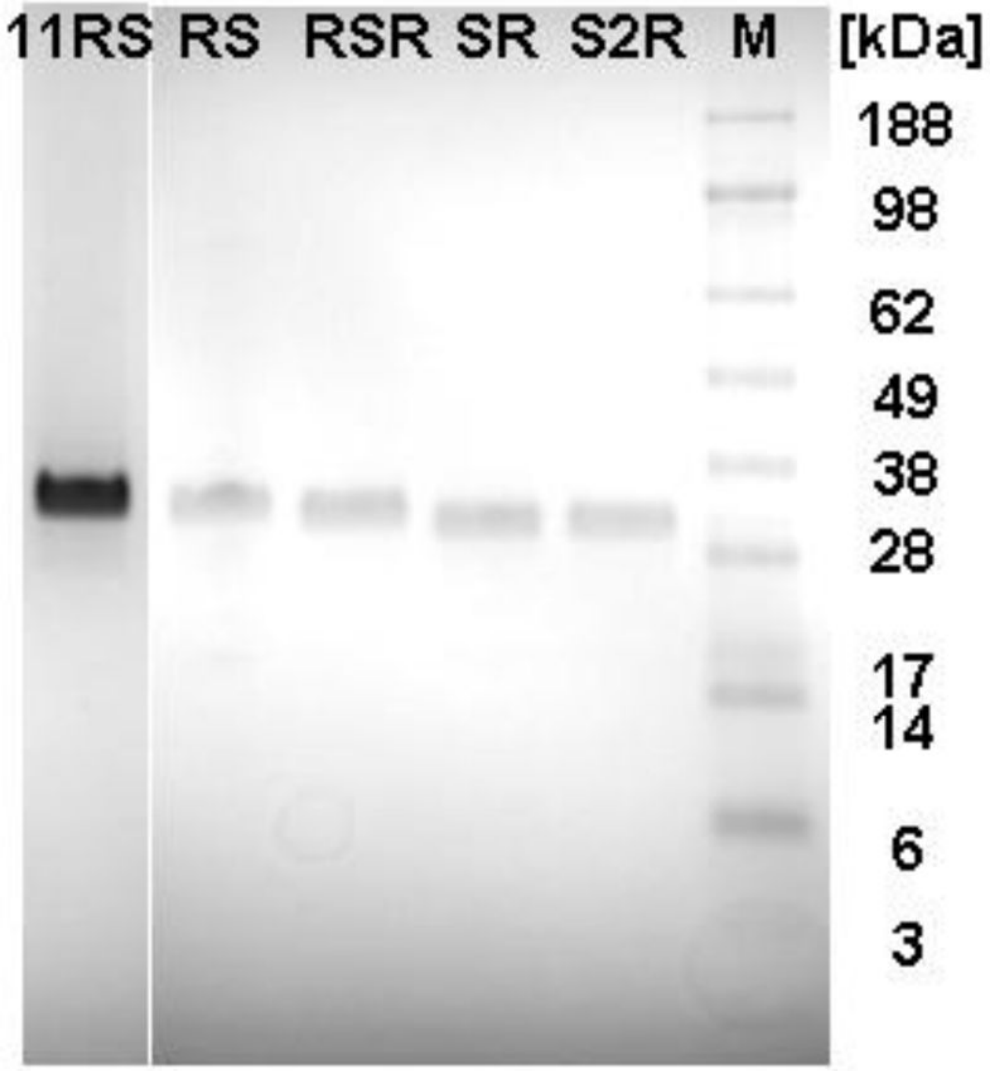

Figure 3.
SDS-PAGE of the recombinant silk proteins after purification by Ni-NTA chromatography. RS, RSR, SR, S2R, 11RS, and molecular weight markers (M) are listed in each line.

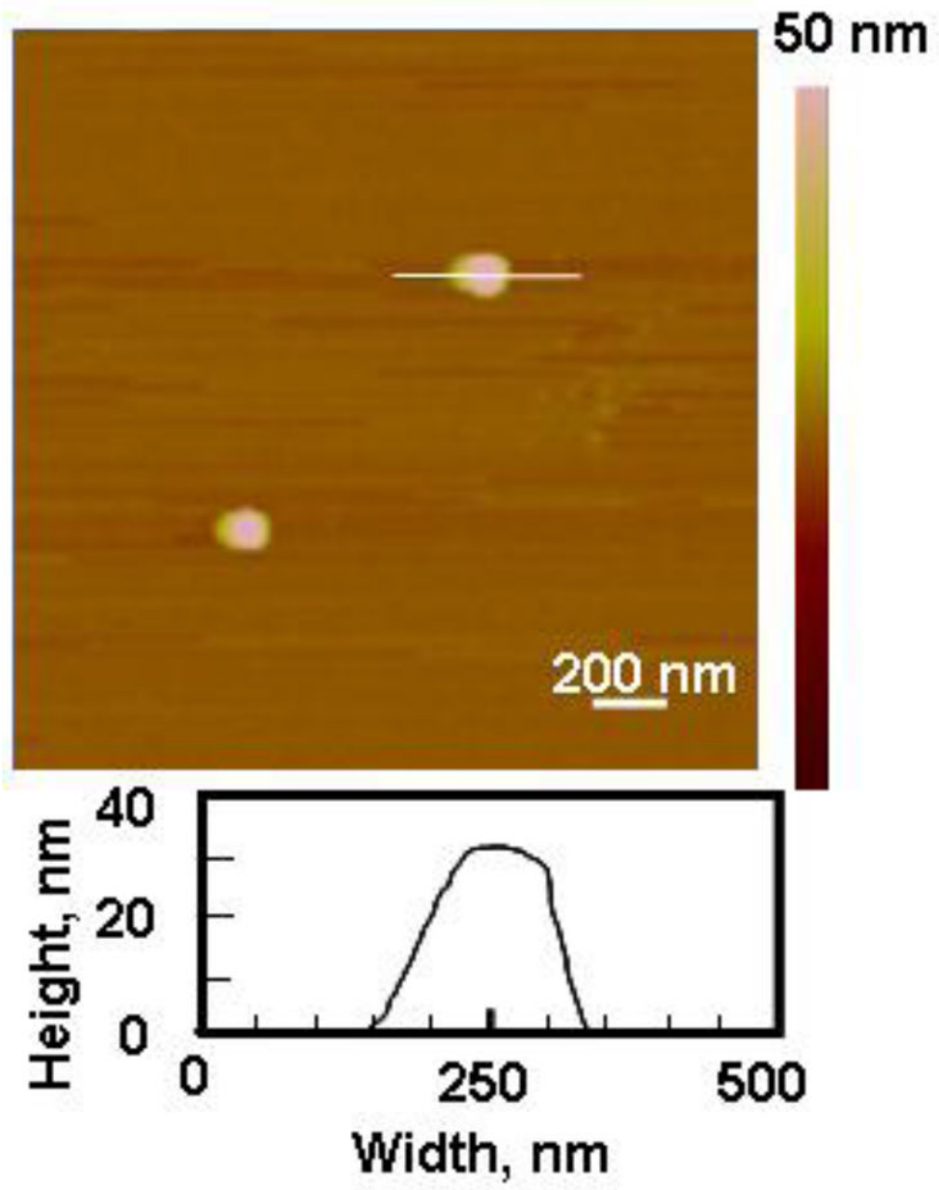

Figure 4.
AFM height images of pDNA complexes with 11RS prepared at N/P ratio of 2 on mica.

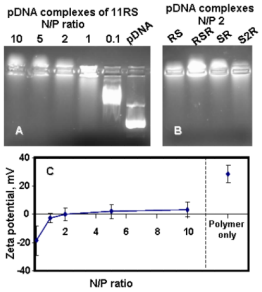

Figure 5.

Electric charges of the pDNA complexes with the recombinant silks. Agarose gel of pDNA and pDNA complexes of 11RS with different N/P ratios (A) and pDNA complexes with different recombinant silks prepared at a N/P of 2 (B). (C) Zeta potential of pDNA complexes of 11RS as a function of N/P ratio.

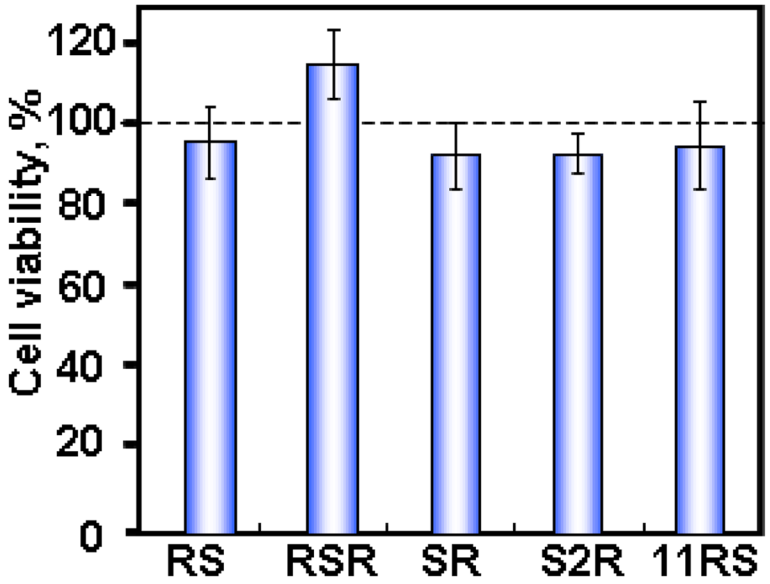

Figure 6.

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

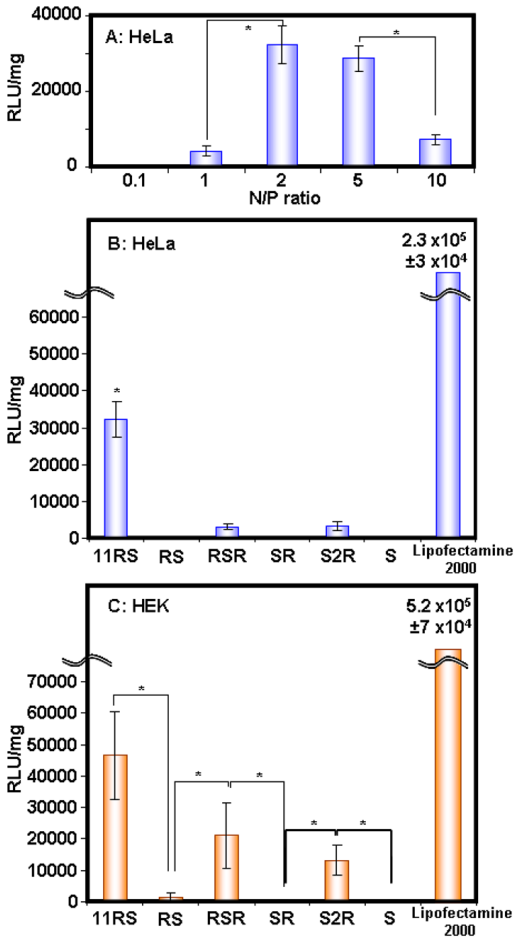

Figure 7.

Transfection results in loading pDNA complexes of 11RS with different N/P ratios in HeLa cells (A) and of the recombinant proteins prepared at N/P 2 in HeLa (B) and HEK cells (C). Silk6mer-30lys block copolymer (S) and Lipofectamine 2000 were used as control samples. Data are shown as means ± standard deviation (n=4). *Significant difference between two groups at $p < 0.05$.

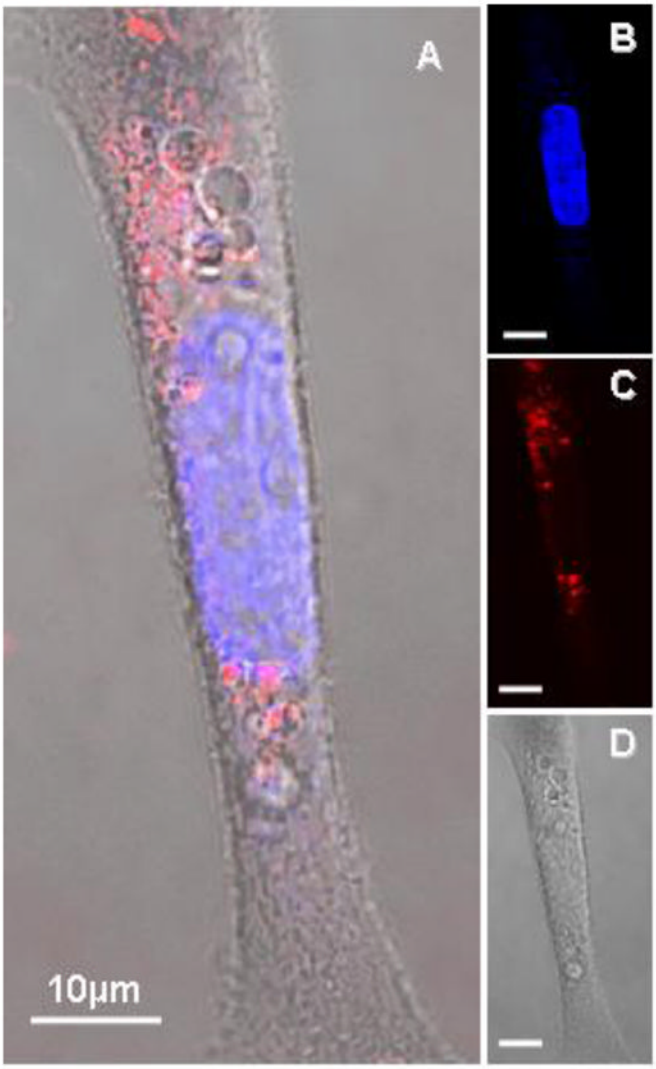

Figure 8.
Intracellular distribution of pDNA complexes with the recombinant silk (11RS) in HeLa cells. (A) Overlay of the three images (B–D), (B) DAPI, (C) Cy5, and (D) phase contrast. The CLSM observation was carried out using a 63x objective. pDNA was labeled with Cy5 (red), and the nuclei were stained with DAPI (blue). Each scale bar represents 10 μm.

Table 1

Sizes (nm) and their distribution (PDI) of pDNA complexes of the recombinant silks determined by DLS.

N/P	RS	RSR	SR	S2R	11RS
0.1	2030 (0.456)	1230 (0.275)	2650 (0.675)	2490 (0.352)	2670 (0.362)
1	676 (0.360)	567 (0.260)	948 (0.601)	498 (0.879)	693 (0.564)
2	382 (0.475)	273 (0.387)	565 (0.533)	207 (0.357)	186 (0.415)
5	360 (0.132)	305 (0.385)	419 (0.672)	181 (0.523)	190 (0.659)
10	428, 2070 (−)[a]	226, 681 (−)[a]	400, 1530 (−)[a]	201 (0.814)	172, 861 (−)[a]
Polymer only	464 (0.692)	380 (0.600)	437 (0.658)	284 (0.453)	162 (0.416)

[a]PDIs were not determined precisely because of their bimodal distribution.