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Functionalized bioengineered spider silk spheres improve nuclease resistance and activity of oligonucleotide therapeutics providing a strategy for cancer treatment

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Abstract

Cell-selective delivery and sensitivity to serum nucleases remain major hurdles to the clinical application of RNA-based oligonucleotide therapeutics, such as siRNA. Spider silk shows great potential as a biomaterial due to its biocompatibility and biodegradability. Self-assembling properties of silk proteins allow for processing into several different morphologies such as fibers, scaffolds, films, hydrogels, capsules and spheres. Moreover, bioengineering of spider silk protein sequences can functionalize silk by adding peptide moieties with specific features including binding or cell recognition domains.

We demonstrated that modification of silk protein by adding the nucleic acid binding domain enabled the development of a novel oligonucleotide delivery system that can be utilized to improve pharmacokinetics of RNA-based therapeutics, such as CpG-siRNA. The MS2 bioengineered silk was functionalized with poly-lysine domain (KN) to generate hybrid silk MS2KN. CpG-siRNA efficiently bound to MS2KN in contrary to control MS2. Both MS2KN complexes and spheres protected CpG-siRNA from degradation by serum nucleases. CpG-siRNA molecules encapsulated into MS2KN spheres were efficiently internalized and processed by TLR9-positive macrophages. Importantly, CpG-*STAT3*siRNA loaded in silk spheres showed delayed and extended target gene silencing compared to naked oligonucleotides. The prolonged *Stat3* silencing resulted in the more pronounced downregulation of interleukin 6 (IL-6), a proinflammatory cytokine and upstream activator of STAT3, which limits the efficacy of TLR9 immunostimulation.

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Conflict of Interest Statement

The authors declare no conflicts of interest.

Our results demonstrate the feasibility of using spider silk spheres as a carrier of therapeutic nucleic acids. Moreover, the modified kinetic and activity of the CpG-*STAT3* siRNA embedded into silk spheres is likely to improve immunotherapeutic effects *in vivo*.

Graphical abstract

Keywords

bioengineered spider silk spheres; CpG-siRNA; *STAT3*; targeted delivery; cancer immunotherapy

1. Introduction

The present day conventional cancer therapies comprise surgery, radiation or chemotherapy. However, besides serious adverse effects of those therapies, partially due to their lack of specificity, cured tumors often relapse. As tumor cells together with tumor-associated immune cells are able to inhibit an immune surveillance and develop a resistant niche through complex signaling network, the specific, efficient and non-toxic anti-cancer therapy remains an elusive goal. Recently, much attention is brought to immunotherapeutic approaches that target tumor-infiltrating immune cells and destroy tumor via modification of its microenvironment [1, 2].

The activation of Signal Transducer and Activator of Transcription 3 (*STAT3*) is prevalent in a wide variety of cancers and is known to mediate tumor proliferation, survival, metastasis, and immune evasion [3–6]. In normal cells, and under physiological conditions, negative regulators tightly control IL-6-induced JAK-*STAT3* activation, and the expression of *STAT3* is transient. In cancer, common immunosuppressive factors including IL-6, IL-10 or VEGF persistently activate *STAT3*, and this activation is spread from tumor to tumor stroma creating feed-forward loops [3]. The presence of chronically activated *STAT3* in both tumor and non-malignant infiltrating cells makes this molecule a desirable target for cancer therapy. However, inhibiting the transcription factor that lacks enzymatic activity remains challenging. First small molecule drugs directly inhibiting *STAT3* showed so far poor efficacy in initial clinical trials, partly due to ineffective pharmacokinetic profiles [7]. The inhibitors of Janus kinases, upstream from *STAT3*, have been tested in multiple clinical trials but due to unexpected toxicities (ADZ1480, SAR302503), their application is limited to myeloproliferative disorders.

The discovery of RNA interference (RNAi) phenomenon has allowed for a new therapeutic opportunity to specifically knock down any target gene in various species. 21–27-mer small interfering RNAs (siRNAs) have become a very attractive alternative for specific and

nontoxic inhibition of undruggable proteins, such as STAT3 transcription factor [4]. We have previously shown the application of STAT3siRNA for cancer immunotherapy [6, 8, 9]. We indicated that immune cells are themselves essential targets in cancer therapy [3, 5], and CpG oligodeoxynucleotides (ODN) - ligands for intracellular receptors Toll-like receptor 9 (TLR9) can be used as a functional component for specific delivery of siRNA into murine and human TLR9-positive cells [6, 8, 9]. Linking siRNA to CpG ODN led to the generation of CpG-siRNA chimeric constructs that targets murine dendritic cells (DC), macrophages and B cells - key components of tumor microenvironment [6]. CpG-siRNAs were quickly internalized into endosomes, underwent cleavage, and due to TLR9 activation uncoupled siRNAs were efficiently transported into the endoplasmic reticulum [10]. We demonstrated that simultaneous TLR9 triggering and blocking of STAT3 by CpG-*STAT3*siRNA constructs disrupted STAT3 signaling in tumor stroma, generated anti-tumor immune responses and led to tumor eradication in mice [6, 11]. The combined STAT3 targeting and TLR9 activation was shown to augment maturation of human antigen presenting cells similar to the effects observed in *Stat3*-deficient mouse model [8, 12].

The major hurdles in clinical application are specific and efficient delivery of siRNAs to the cytoplasm of a target cell and also a response of the immune system to nucleic acids [13, 14]. siRNAs which are a 13kDa molecules, with a negatively charged backbone are incapable of entering most cell types without assistance. Furthermore, exogenous siRNAs are very short-lived in the host organism and loose silencing capability due to degradation by RNases present in serum [15]. To address these challenges various carriers have been tested to improve targeted delivery of intact siRNA including antibody-protamine fusion molecules, liposome encapsulation, or nanoparticle formation with cationic lipids or polymers [16, 17]. In addition to a protective function, an ideal carrier has to be biocompatible, biodegradable, nontoxic, and have proper particle size and stability. It should also not cause any undesired immunogenicity at any destination site.

Spider silk has been considered as a material with a great potential in biomedical applications including cancer therapies [18, 19]. Due to limited availability of natural spider silk proteins, the technology of recombinant proteins has been developed [20]. Synthetic genes encoding bioengineered silk proteins that are based on consensus motifs of spidroins sequences can be cloned, expressed in various host, and purified, allowing for a large scale production of controlled high-quality protein polymer [20]. Recent approaches in bioengineered silk technology encompass further modification of silk molecules by adding sequences encoding the functional domains such as cell binding motifs, tumor-homing peptides, cell-penetrating or destabilizing peptides, antimicrobial peptides, or poly-lysine domains [21–25]. The hybrid silk protein is built of the bioengineered silk core sequence, which is responsible for the biomaterial structure (e.g., spheres) and the polypeptide sequence which allows functionalization of the resulting silk biomaterial. Hybrid silks containing ligands for proteins overexpressed by tumor can serve as specific drug vehicles in cancer therapy [23, 25]. The presence of poly-lysine domain allows for binding of plasmids or other types of nucleic acids [22, 25, 26].

To overcome the sensitivity of siRNA to degradation in serum, we report for the first time the design of bioengineered spider silk spheres suitable for targeted delivery of the

therapeutic CpG-*STAT3*siRNA to tumor-associated immune cells in the presence of serum nucleases. Previously we constructed, expressed and purified a bioengineered silk MS2 that was based on MaSp2 protein from *N. clavipes* spider [27]. For this study we functionalized MS2 protein with the poly-lysine domain (KN) for binding of nucleic acids. A resulting hybrid variant of MS2 silk was named MS2KN, accordingly. Spherical particles loaded with CpG-siRNA were obtained by mixing MS2KN silk protein and nucleic acid with potassium phosphate. Here, we showed that silk spheres stabilized in serum and delivered a functional CpG-*STAT3*siRNA into immune cells. The kinetics of CpG-*STAT3*siRNA activity was different depending on the way of delivery: direct as naked molecules or enclosed into silk spheres. Spheres enabled delivery of CpG-*STAT3*siRNA more efficiently and with the slower kinetics of processing and thus silencing activity comparing with naked CpG-*STAT3*siRNA. Therefore, it might be a very promising strategy for delivery of therapeutic nucleic acid for cancer immunotherapy.

2. Materials and Methods

2.1. Oligonucleotide design and synthesis

The sequences of mouse cell-specific CpG1668-*STAT3*siRNA (CpG-*STAT3*siRNA) and CpG1668-*scrambled*RNA (CpG-*scr*RNA) have been described previously [6]. Briefly, the antisense strand of the *STAT3*siRNA (27-mer) was coupled to a TLR9 agonist, a CpG1668 oligonucleotide (20-mer), using a carbon chain linker and hybridized to (25-mer) sense strand of siRNA that was specific for mouse *STAT3* gene [6, 10]. CpG-linked double stranded non-coding RNA (CpG-*scr*RNA) was used as control construct with no silencing capability as it was determined previously [6]. For uptake studies, CpG-siRNAs were labeled using fluorescein (FITC)- CpG-*STAT3*siRNA^{5'}SS-FITC (Lumiprobe, Hallandale Beach, FL). We have previously confirmed that modifications of CpG-siRNA conjugates on the 3' end of the passenger strand with various fluorochromes did not alter their internalization by target cells or the gene silencing effect [10].

2.2. Construction of expression plasmid pETNX-MS2KN

Previously we constructed a pETNX-MS2(9x) plasmid carrying synthetic gene MS2 comprised of 9 repeats of the MaSp2 consensus sequence from *Nephila clavipes* [27]. In this study we used a 15-mer of MS2 that was obtained by ligation of 9- and 6-mer of the MS2 using a method described previously [27]. The hybrid construct was obtained by cloning sequence for poly-lysine nucleic acid binding domain (KN) into the *SpeI* restriction site for the 3'-terminal functionalization of the MS2 construct (pETNX-MS2KN). The sequences of the synthetic oligonucleotides encoding the KN domain were as follows: KN.F:5'-CTAGCAAAAAGAAAAAAGAAAAAGAAAAAAGAAAAAGAAAAAGAAAA, KN.R: 3'-GTTTTTCTTTTTTCTTTTTCTTTTTTCTTTTTCTTTTTCTTTGACT. KN.F and KN.R were annealed to a double-stranded DNA fragments with cohesive ends complementary to those generated by *NheI* and *SpeI*. After ligation into pETNX-MS2, the sequences of the resulting plasmids were confirmed by sequencing at the Adam Mickiewicz University Core Facility in Poznan. Enzymes for the digestion and ligation were supplied by Fermentas (Thermo Fisher Scientific Inc., Waltham, MA) and New England Biolabs, Inc. (Ipswich, MA), respectively.

2.3. Expression and purification of bioengineered spider silk

The plasmids pETNX-MS2 and pETNX-MS2KN were used to transform *E. coli* strain BLR (DE3; Novagen, Madison, WI). Bioflo 3000 (New Brunswick Scientific, Edison, NJ) fermentor was used for large-scale expression, according to previous protocol. Silk variants were purified by using the thermal method, as previously described [28, 29]. Briefly, bacteria were added to 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.5, and 100 mM NaCl, and the mixture was supplemented with Protease Inhibitor Cocktail (2 mM AEBSF, 1 mM phosphoramidon, 130 mM bestatin, 14 mM E-64, 1 mM leupeptin, 0.2 mM aprotinin, and 10 mM pepstatin A; Sigma, St. Louis, MO) and lysed with 0.2 mg/mL lysozyme (Thermo Fisher Scientific, Inc., Waltham, MA). Next, the lysate was sonicated, treated with DNaseI (Sigma, St. Louis, MO; 0.1 mg/mL) in the presence of 3 mM MgCl₂ (Sigma, St. Louis, MO), and it was then centrifuged at 50 000 × g for 30 min at 4 °C. Soluble bacterial proteins were precipitated by heat denaturation (80 °C for 20 min), and removed by sedimentation at 50 000 × g for 30 min at 4 °C. The soluble silk proteins were precipitated with 20% ammonium sulfate (ICN Biomedicals Inc., Aurora, OH), centrifuged at 10 000 × g for 15 min, rinsed with 20% ammonium sulfate, and then dissolved in 6 M guanidinium thiocyanate (ICN Biomedicals Inc., Aurora, OH). The resulting proteins were dialyzed against 50 mM sodium borate, pH 8.5 (Sigma, St. Louis, MO). ZelluTrans Regenerated Cellulose Dialysis Tubing with an MWCO of 12 000–14 000 Da (Carl Roth, Karlsruhe, Germany) was used for the dialysis. The protein concentration was determined by UV spectroscopy at 280 nm with molar extinction coefficients of 46720, 48830 M⁻¹ cm⁻¹ for MS2 and MS2KN proteins, respectively. SDS-PAGE gel electrophoresis was performed using 12.5% gel and the proteins were stained with colloidal blue (Roti-Blue; Carl Roth, Karlsruhe, Germany).

2.4. Nucleic acid binding assay

To test the binding capability of silk variants, 1 µL of 10 µM CpG-*scr*RNA was mixed at 1:1, 1:10, 1:100, 1:500 molar ratios with MS2 or MS2KN silk solution in 200 µL of 10 mM Tris-HCl pH 7.5. After 5 min of incubation at room temperature, the formation of resulting nucleic acid/protein complexes was analyzed by electrophoresis in 2% agarose gel and TAE-ethidium bromide staining (Sigma, St. Louis, MO). Complexes were observed using a G-box transilluminator (Syngene, Frederick, MD), and analyzed using GeneTools software (Syngene, Frederick, MD).

2.5. CpG-*scr*RNA and CpG-*STAT3*siRNA loading into silk spheres

Spheres loaded with nucleic acid were formed by mixing the complexes of silk and nucleic acid with potassium phosphate solution. First, 100 µL of 10 µM MS2 or MS2KN and 100 µL of 10 µM CpG-*scr*RNA or CpG-*STAT3*siRNA were mixed, incubated at room temperature for approximately 5 min, and then sphere nucleation and particle growth were induced by adding 1000 µL of 2 M potassium phosphate, pH 8.0. (Sigma, St. Louis, MO) and mixing using a pipette. Control spheres (MS2KN) were formed using similar protocol but without addition of nucleic acid. The resulting particles were incubated at room temperature overnight. After dialyzing against ultrapure water, the spheres were centrifuged at 14 000 × g for 15 min and then redispersed in 100 µL of ultrapure water. Spectrophotometric

measurements of the supernatants were performed to determine the amount of CpG-*scr*RNA or CpG-*STAT3*siRNA incorporated into the spheres. The absorbance of nucleic acid was measured at a wavelength of 260 nm using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). The loading was presented as % of the initial amount of CpG-*scr*RNA or CpG-*STAT3*siRNA. The loading efficiencies were measured to quantify the amount of spheres needed for each of the experiments in order to obtain the indicated amount of CpG-siRNA molecules. For the cellular uptake assays analyzed by flow cytometry and fluorescent confocal microscopy, FITC labeled CpG-*STAT3*siRNA was used and the bioengineered silk was first conjugated with the ATTO-647N fluorophore (Sigma, St. Louis, MO) as described before [23]. The sphere concentration was determined gravimetrically.

2.6. Characterization of silk spheres by scanning electron microscopy (SEM)

Silk spheres in water suspension were applied to coverslips (Thermo Scientific/Nunc, Langensfeld, Germany) and allowed to air-dry. After being sputtered with gold under a vacuum in a Quorum Sputter Coater Q150T ES (Quorum Technologies, Ringmer, UK), the samples were analyzed with a SEM (JEOL JSM-7001F, JEOL Ltd., Peabody, USA) at 10 kV of accelerating voltage. The particle sizes were determined with ImageJ 1.46r software.

2.7. Zeta potential (ZP) analysis of CpG-siRNA/silk spheres

Zeta potential evaluation was performed by using a Zetasizer Nano XS (Malvern Instruments, Ltd, Worcestershire, UK). Spheres were diluted directly before analysis to a concentration of 0.01 mg/mL in water and sonicated for 5 min in a sonic water bath. The experiment was performed three times in triplicate at 25 °C.

2.8. Serum Stability Assay

Complexes and spheres made of CpG-*scr*RNA and MS2KN spider silk or CpG-*scr*RNA alone were incubated in presence of 30% mouse serum for 0, 1 and 3 h at 37 °C. After incubation, the DNA Loading Dye (Thermo Scientific, Wilmington, DE) supplemented with 1% SDS was added to CpG-*scr*RNA and the complexes. The sphere samples were centrifuged for 30 min at $14\,000 \times g$, dissolved in buffer containing 100 mM NaH_2PO_4 , 10 mM Tris-HCl, pH 8.0, and 8 M Urea for 3 h with shaking at 37 °C, and then DNA loading Dye was added. Pre-run of the 15% Mini-PROTEAN® TBE-Urea Gel (Bio-Rad, Irvine, CA) was performed to remove urea traces from wells, then the samples were loaded, and gel electrophoresis was carried out for 15 and 90 min, at 250 and 200 Volts for pre-run and run, respectively. CpG-*scr*RNA was stained with TBE-ethidium bromide solution for 30 min at room temperature, visualized using G-box transilluminator in a presence of UV light, and analyzed by GeneTools software. In preliminary studies, we have not seen the differences in the rate of degradation of CpG-conjugates with various siRNA or double-stranded scrambled sequences. Due to lack of the sequence-specific effects in the serum stability studies, we decided to use in this study the CpG-*scr*RNA conjugate that was available for us in larger quantities.

2.9. Cells

Semi-adherent J774 murine macrophages and NIH/3T3 murine fibroblasts were used. Both cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM; PAA Laboratories GmbH, Pasching, Austria), supplemented with 10% FBS (PAA Laboratories GmbH, Pasching, Austria) or as indicated, and 80 µg/mL gentamicin (KRKA, Novo Mesto, Slovenia). Cells were grown at 37° C in a humidified atmosphere containing 5% CO₂.

2.10. Cytotoxicity study

A total of 2.5×10^4 of NIH/3T3 cells per well were seeded in a 96-well plate and incubated overnight. The next day, different concentrations of silk spheres loaded with CpG-*scrRNA* or control spheres without construct were added to the cell cultures. The untreated cells were used as a negative control. Particles were removed from the medium after 3 h and cells were cultured for additional 72 h, and the mitochondrial activity of cells was assessed using MTT assay as described previously [23]. The relative cell viability (%) related to the negative control were calculated by test sample/negative control $\times 100\%$. The experiment was repeated three times in triplicate.

2.11. Cellular uptake of CpG^{FITC}-STAT3siRNA/silk spheres analysis by flow cytometry

A total of 2×10^5 of J774 cells were cultured in DMEM with 10 % FBS on each well of 24-well plate for 24 h before the experiment (Corning, Corning, NY). Culture medium was replaced by DMEM with 2% FBS 3 h before the experiment. Next, the fluorescently labeled probes were added as indicated and incubated for 1 h. Fluorescent data was collected in FL1 and FL4 channels on BD FACSCanto flow cytometer (BD Pharmingen, San Jose, CA), and analyzed using FlowJo software (Tree Star, Ashland, OR). Three independent experiments were performed.

2.12. Cellular uptake of CpG^{FITC}-STAT3siRNA/silk spheres analysis by confocal microscopy

A total of 3×10^4 of J774 cells were grown for 24 h in 50 µL of DMEM with 10 % FBS on each well of 10-well Multitest slides (MP Biomedicals, Santa Ana, CA). 3 h before the experiment, the culture medium was replaced by DMEM with 2% FBS. Subsequently, J774 cells were incubated with 100 nM FITC conjugated CpG-*STAT3*siRNA, 100 nM ATTO-647N labeled MS2KN spheres loaded with 100nM CpG^{FITC}-*STAT3*siRNA, or 100nM control ATTO-647N conjugated MS2KN spheres for 30 min and 3 h or particles were removed from medium after 3 h and cells were cultured for additional 24 or 48 h in 2% FBS culture medium, in 37 °C. After incubation cells were prepared for analysis as described previously [23]. The experiment was repeated three times.

2.13. Real-time quantitative PCR

A total of 5×10^5 J774 cells were grown for 24 h in DMEM with 10% FBS on each well of 12-well plate. 3 h before the experiment the culture medium was replaced by DMEM with 2% FBS. Subsequently, cells were stimulated for 24, 48 or 72 h with naked 250 nM CpG-*scrRNA*, CpG-*STAT3*siRNA or 250 nM MS2KN spheres loaded with adequate siRNAs, both at a concentration of 250 nM. Untreated J744 cells or cells incubated with plain

MS2KN spheres served as controls. For quantitative PCR (qPCR), total RNA was extracted from cultured cells using TRI-Reagent (Sigma, Saint Luis, MO), RNA was quantified using NanoDrop (Thermo Scientific, Wilmington, DE), and 1 µg of total RNA was used for cDNA synthesis with iScript reaction mix (Bio-Rad, Irvine, CA). QPCR mouse specific primers and UPL Probes for *Stat3* (STAT3), *Il6* (Interleukin 6) and *Tubb* (beta-tubulin) were designed using UPL Assay Design Center (Roche, Basel, Switzerland) and are shown in the Table 1. Sequence-specific amplification with Real Time PCR Probe Master (Roche, Basel, Switzerland) was analyzed on the Light Cyclers 480 (Roche, Basel, Switzerland). Data was normalized to *Tubb* expression and the relative expression levels were calculated using the 2^{-C_t} method.

2.14. Western Blotting

A total of 1×10^6 J774 cells were grown in DMEM with 10% FBS on each well of 6-well plate 24 h before the experiment. Culture medium was replaced by DMEM with 2% FBS 3 hours before the experiment. Subsequently, cells were stimulated for 24 or 48 h with spheres loaded with CpG-*scr*RNA or CpG-STAT3siRNA. To measure silencing effect of constructs on total STAT3 protein level Western blotting was performed as previously described [5]. Briefly, total cellular lysates were prepared using RIPA buffer (Sigma-Aldrich, Saint Louis, MO) supplemented with a Protease Inhibitor Cocktail (Sigma-Aldrich, Saint Louis, MO) and Phosphatase Inhibitor Cocktail (Sigma-Aldrich, Saint Louis, MO). Total protein amount was measured employing BCA protein assay (Thermo Scientific, Wilmington, DE), and 40 µg of protein was loaded into 4–15% Mini-PROTEAN®TGX™ gel (Bio-Rad, Irvine, CA). The gel was blotted against PVDF membrane (Bio-Rad, Irvine, CA) using semi-dry Trans-Blot® Turbo™ Transfer System (Bio-Rad, Irvine, CA). Membrane was blocked for 1 h in 5% BSA in TBS buffer with 0.1% of Tween-20 (Sigma Aldrich, Saint Louis, MO), and analyzed using antibodies specific to total Stat3 C-20 (Santa Cruz Biotechnology, Dallas, TX) and β-actin (Santa Cruz Biotechnology, Dallas, TX) according to manufacturer's protocol. Chemiluminescent bands corresponding to detected proteins were visualized using an enhanced chemiluminescence detection kit ECL-Plus (Amersham Biosciences, Piscataway, NJ, USA) and analyzed using a G:BOX F3 Gel Documentation System (Syngene, Frederick, MD) and GeneTools software (Syngene, Frederick, MD).

2.15. Statistics

To analyze the significant differences between MS2, MS2KN and MS2KN-CpG-siRNA treated groups, one-way and two-way ANOVA were used. In the case of significance ANOVA ($p < 0.05$) post hoc tests with Bonferroni correction were performed. The differences were considered significant when $p < 0.001$ (***), $p < 0.01$ (**), $p < 0.05$ (*). The data presented are expressed as mean ± standard error of the mean (SEM). The statistical differences were calculated with GraphPad Prism 8 software.

3. Results

3.1. Expression and purification of bioengineered spider silk variants and CpG-siRNAs construction

Amino acid sequences of silk spider variants and nucleic acid sequences of two CpG-siRNA-based constructs used in this study are shown in Figure 1A. Bioengineered spider silk MS2 was functionalized by adding poly-lysine ($15 \times K$) nucleic acid binding domain (KN) to develop the nucleic acid delivery system. The average yield for MS2 silk protein after purification was 1.209 mg while for MS2KN was – 0.647 mg per 1 g of bacterial pellets. An SDS-PAGE analysis of MS2 and MS2KN revealed bands corresponding to molecular weights of approximately above 60000 Da for MS2 and MS2KN (Fig. 1B). Observed molecular weights were higher than theoretical molecular weights calculated for MS2 and MS2KN, 46720 Da and 48643 Da, respectively. Our previous data concerning the shorter version of MS2, i.e. 9-mer of MS2 silk (MS2(9x)), indicated that although the SDS-PAGE analysis showed also a higher than expected molecular weight of silk, the MALDI-TOF results were in agreement with predicted value [27]. Similarly, the migration of the 15-mer version of MS2 in the SDS-PAGE gel was impaired. Moreover, neither samples degradation nor impurities were observed in SDS-PAGE gel. We decided to test the feasibility of using MS2KN proteins to generate a nanoparticle-based vehicle for the delivery of CpG-siRNA oligonucleotides. Molecular weight, cellular uptake and immunostimulatory properties of naked CpG-siRNAs together with CpG-*STAT3*siRNA silencing function and its therapeutic outcome were described previously [6, 10].

3.2. Nucleic acid binding domain of functionalized spider silk protein binds CpG-siRNA

Agarose gel electrophoresis experiments were performed to investigate the interaction properties and electrolytic stabilities of the complexes of soluble silk variants and CpG-siRNA mixed at different molar ratios. MS2 silk variant did not form any complexes with CpG-*scrRNA* at lower ratios and slightly retarded migration of small amount of nucleic acid at higher silk:nucleic acid ratios (100:1 and 200:1). Stable complexes of CpG-*scrRNA* and soluble MS2KN were formed at and above 1:10 ratio and they did not migrate from the agarose well (Fig. 2).

3.3. Functionalized spider silk forms spheres that can be efficiently and stably loaded with CpG-siRNA oligonucleotides

Functionalized spider silk MS2KN alone and in the presence of CpG-siRNA construct formed spheres (Fig. 3A and B). The average sphere size for plain functionalized MS2KN spheres was approximately 182.7 nm, with a size distribution of ± 6.4 nm. The average size of MS2KN spheres loaded with CpG-siRNA was 201.8 nm, with a size distribution of ± 14.8 nm. The size difference was not statistically significant and morphology of functionalized spheres was similar after loading with the nucleic acid construct (Fig. 3A).

CpG-siRNA was efficiently loaded into MS2KN spheres (Figure 3B, C). The absorbance analysis of supernatant after sphere formation indicated that approximately 95% of nucleic acid constructs were entangled into spheres (data not shown). The loading efficiency was also confirmed by confocal microscopy of FITC-labeled CpG-*STAT3*siRNA (Fig. 3B).

Moreover, the zeta potential of the formed spheres was measured. It was observed that negatively charged nucleic acid lowered the zeta potential of MS2KN spheres (ZP of MS2KN/CpG-*scrRNA* and MS2KN were $-34.7 (\pm 1)$ mV and $-9.4 (\pm 0.5)$ mV, respectively).

3.4. Spider silk protects CpG-siRNA from degradation by serum nucleases

As degradation of naked CpG-siRNA by serum nucleases has been described [6], the ability of functionalized silk protein to serve as a protective carrier for CpG-siRNA was tested using mouse serum degradation assay. First, soluble MS2KN silk was mixed with CpG-*scrRNA*, and the resulting complexes were incubated in the presence or absence of 30% mouse serum for indicated time points. Naked CpG-*scrRNA* incubated under the same conditions served as a control. Untreated CpG-*scrRNA* construct alone remained stable throughout 3 h incubation period, however, its degradation process started immediately after the addition of mouse serum, and approximately half of the construct was degraded after 3 h (Figure 4). MS2KN silk protected CpG-*scrRNA* from degradation, and negligible degradation could be observed after 3 h of incubation in the presence of serum (Fig. 4A). Moreover, similar protection effect was observed when CpG-*scrRNA* was loaded into MS2KN spheres (Fig. 4B); intact full-length construct was observed even after 3 h of incubation in mouse serum. Contrary to the complexes, the spheres did not release CpG-siRNA constructs into DNA Loading Dye with 1% SDS, thus spheres had to be dissolved before final gel analysis. Despite the application of the dissolving procedure of spheres, a substantial amount of construct probably remained bound to the spheres, and thus only a small portion of the construct could migrate freely in the gel during electrophoresis. In order to better explain the observed smaller amount of released CpG-siRNA, we included the additional control sample that was composed of spheres loaded with CpG-siRNA, however it was not-treated with serum. As indicated in the Fig. 4B, the amount of intact full-length construct was comparable in the sphere samples incubated with or without serum and no degradation could be seen in both cases.

3.5. Functionalized MS2KN spheres loaded with CpG-siRNA are not cytotoxic

Plain spheres made of MS2 silk were not cytotoxic when examined at a wide range of concentration (Fig. 5). The functionalization of spheres by adding poly-lysine domain significantly caused toxicity towards NIH/3T3 cells, however, this effect was not observed when spheres were loaded with nucleic acid construct (Fig. 5). There was no significant difference between MS2 and MS2KN/CpG-*STAT3*siRNA spheres in terms of cytotoxicity.

3.6. CpG-siRNA encapsulated into functionalized MS2KN spheres is efficiently internalized by macrophages

To determine the uptake of functionalized spheres loaded with CpG-siRNA construct by TLR9-positive cells, we incubated J774 murine macrophages with fluorescently-labeled MS2KN-ATTO-647N spheres loaded with CpG^{FITC}-*STAT3*siRNA for 1 h, in the absence of transfection reagents. Naked CpG^{FITC}-*STAT3*siRNA construct served as positive control. Flow cytometric analysis indicated efficient cellular uptake of MS2KN/CpG-siRNA spheres (approximately 80-100% of cells) (Fig. 6). The uptake of CpG^{FITC}-*STAT3*siRNA loaded into silk spheres was even higher than the uptake of naked CpG^{FITC}-*STAT3*siRNA (Fig. 6).

The confocal microscopy further confirmed cellular internalization of silk spheres loaded with CpG-siRNA (Fig. 7). The uptake of MS2KN/CpG-siRNA spheres was detected as early as 30 min and remained present in the cytoplasmic localization still after 3 h and 24 h. At 48 h, singular spheres and CpG-*STAT3*siRNA were present in the cytoplasm (Fig. 7A). In contrast, the naked and unformulated CpG-siRNA oligonucleotides were efficiently internalized into J774 cells but were not detectable after 48 h of incubation (Fig. 7C). Importantly, our results indicated that CpG-siRNA oligonucleotides (green) co-localize with silk spheres (red) in the cytoplasm of J774 cells during the first 3 h of incubation (Fig. 7B). However, both components separated at 24-48 h indicating the efficient although delayed release of CpG-siRNA from silk spheres.

3.7. CpG-*STAT3*siRNA loading into silk spheres leads to prolonged silencing effect on *Stat3* and *IL-6* mRNA in macrophages

Gene silencing effects of CpG-*STAT3*siRNA (250 nM) loaded into functionalized spheres in J774 cells were determined by quantitative real-time PCR analysis of *Stat3* and *Il6* mRNA, with normalization to *Tubb* (beta-tubulin) as a reference. Naked CpG-*STAT3*siRNA silencing potential was described in various TLR9 positive cells previously, and therefore the construct alone served as a positive control [6, 9, 11]. Plain MS2KN spheres did not significantly affect *STAT3* expression (Fig. 8A).

We compared the silencing effect of the CpG-*STAT3*siRNA and control CpG-*scrRNA* when used as naked or when encapsulated into silk spheres (Fig. 8B and C). As expected, in the presence of serum-containing media and at the concentration of 250 nM the naked CpG-*STAT3*siRNA failed to induce significant target gene silencing for up to 72 h of incubation. In contrast, cells incubated with CpG-*STAT3*siRNA loaded into MS2KN spheres showed downregulation of *Stat3* expression after 48 h, which continued for up to 72 h after single treatment (Fig. 8B). Furthermore, mRNA level of *Stat3* was significantly lower in cells incubated for 72 h with CpG-*STAT3*siRNA-loaded spheres compared with cells treated with naked CpG-*STAT3*siRNA (Fig. 8B).

The time-dependent *STAT3* silencing in J774 treated with CpG-*STAT3*siRNA encapsulated into silk was further confirmed at protein level. The concentration-dependent decrease in *STAT3* levels was observed in cells incubated for 24 h with 250 and 500 nM CpG-*STAT3*siRNA loaded spheres, respectively (Fig. 8D). The 48 h incubation led to further dose-dependent downregulation of *STAT3* protein in comparison with spheres loaded with CpG-*scrRNA* (Fig. 8D).

The immunostimulatory effect of CpG ODNs and TLR9 activation are limited by the release of IL-6 cytokine, which acts as a negative feedback regulator through induction of *STAT3* signaling [6, 11]. In cells that received naked CpG-*STAT3*siRNA, a significant decrease in *Il6* mRNA level was observed after 24 h of incubation in comparison with cells treated with CpG-*scrRNA* (Fig. 8C). The downstream effect of naked CpG-*STAT3*siRNA was gone after 48 and 72 h. For CpG-*STAT3*siRNA delivered into spheres a trend in reducing *Il6* mRNA levels in J774 cells after 24 and 48 h, whereas a significant drop in *Il6* mRNA were observed after 72 h as compared with cells incubated with CpG-*scrRNA*-loaded spheres (Fig. 8C). The *Il6* levels in J774 cells incubated for 48 h with CpG-*STAT3*siRNA-loaded spheres were

significantly lower than in cells treated with naked CpG-*STAT3*siRNA. A similar trend could be observed after 72 h incubation, although it was not significant (Fig. 8C). The *Il6* compared with *Stat3* mRNA showed greater reduction of expression at earlier times. Our results were presented as fold inductions in relation to control that was set as 1. Thus, the observed effect of *Il6* was likely a result of the impaired up regulation of *Il6* and not the reduction of basal *Il6* levels as it is in case of *Stat3*. IL-6 expression by TLR9 signaling requires cooperation of STAT3 and NF- κ B.

At the protein level, the incubation with 500 nM CpG-*STAT3*siRNA loaded into MS2KN resulted in the more pronounced effect than 250 nM CpG-*STAT3*siRNA into MS2KN (Fig. 8D). However, the analysis of the *Il6* mRNA level that was observed after application 250 nM CpG-*STAT3*siRNA delivered by MS2KN spheres indicated that this concentration was effective to trigger the modulation of the STAT signaling crosstalk. These results suggest that the encapsulation of CpG-*STAT3*siRNA in silk spheres alleviated the IL-6 mediated negative feedback regulation and therefore can further enhance immunostimulatory potential of TLR9 triggering.

4. Discussion

In this study, we presented an efficient, stable and cell-specific delivery of therapeutic siRNA molecules to cells using bioengineered silk spheres vehicles. The proof of concept and feasibility of the system were based on the following findings: (1) bioengineered, functionalized spider silk protein can be produced in a bacterial expression system and purified using methods based on the properties of natural spider silk without any additional Tag sequences, (2) the functional nucleic acid binding motif can be fused to the primary silk sequence without any additional peptide linkers and this domain does not impede the self-assembly property of the silk, (3) C-terminally functionalized silk allows for efficient binding of nucleic acids hybrid DNA/RNA constructs such as CpG-siRNA, (4) CpG-siRNA molecules can be loaded into silk spheres and they do not affect the silk spheres morphology, (5) half-life of therapeutic CpG-siRNA is prolonged in serum when using silk particles as vehicles, (6) silk spheres loaded with CpG-siRNA can be internalized and processed into TLR9-positive target cells and are not toxic, (7) CpG-siRNA molecules delivered in silk spheres persist inside the cells longer compared with naked nucleic acid construct, (8) the specific activity of CpG-siRNA is observed for longer time when delivered by silk spheres compared with the administration of naked constructs and results in a different kinetics of its activity, (9) simultaneous blocking of STAT3 expression and triggering TLR9 receptor signaling by CpG-*STAT3*siRNA loaded into spheres allows for the modulation of STAT3 signaling crosstalk.

Previously, we constructed, produced and purified two bioengineered silk variants: MS1 and MS2 [23, 27]. The bioengineered silk MS1 was based on the MaSp1 protein and the MS2 silk on MaSp2 from *N. clavipes* spider. We indicated that spheres made of MS2 silk demonstrated higher dispersion and had no tendency to aggregate comparing with spheres made of MS1 [23, 27]. Thus we selected MS2 protein for further study. Moreover, we previously showed that the thermal denaturation method resulted in purified, non-immunogenic, non-cytotoxic bioengineered spider silk protein [28]. Thus, the same

purification method was employed for MS2KN functionalized spider silk variants tested in this study. The MS2 was functionalized with nucleic acids binding poly-lysine motif (KN) that was fused to the C-terminus of MS2. Functionalization with poly-lysine binding domain could account for lower yield obtained for MS2KN silk variant compared with the yield for MS2. Indeed, it has been previously demonstrated that positively charged peptides might be toxic to bacterial hosts [30].

The question to be addressed was whether functionalized MaSp2-based silk would bind nucleic acids in a long-lasting manner. MS2KN formed stable complexes with CpG-siRNA whereas only a slight binding between MS2 lacking the poly-lysine domain and CpG-siRNA was observed in the presence of a high excess of MS2 that could not be applied *in vivo*. The poly-lysine domain was previously shown to functionalize bioengineered spider silk that was based on a different spidroin - MaSp1 from *N. clavipes* [22, 25, 26, 31]. The efficacy of binding was demonstrated using complexes of those variants of soluble silks with plasmid DNA. For our application, not only did we test the complexes, but also the silk spheres that were formed based on the assembly property of silk. It has been demonstrated that use of the high concentration of phosphate potassium allowed for efficient self-assembly of silk into spheres independent of the silk origin [23, 27, 32, 33], and in this study the same method was applied. Since the spheres were produced using functionalized silk variant (MS2KN), the poly-lysine domain did not impede the silk self-assembly properties. Moreover, MS2KN spheres made with or without CpG-siRNA displayed similar morphology, indicating that the presence of bound nucleic acid did not interfere with the process of particle formation. However, based on the ZP measurements, the bound nucleic acid molecules influenced the colloidal stability of MS2KN spheres. The higher ZP value of MS2KN/CpG-scrRNA compared with control MS2KN spheres indicated stronger repulsion between MS2KN/CpG-scrRNA particles. In general, particles displaying high ZP (positive or negative) are more stable and resist aggregation, whereas particles with low ZP (closer to zero) tend to aggregate due to less repulsion between particles.

A major hurdle in prolonged systemic drug circulation of siRNA in body fluids is its vulnerability to cleavage by serum RNAses [15]. Besides the fact that local intratumoral delivery into solid tumors [11] and intravenous treatment in leukemia model [9] caused a significant reduction in tumor burden, the half-life of the naked CpG-siRNA construct remained limited especially in the case of systemic delivery (M.K., data not published). Hauptenthal et al. demonstrated that the degradation of siRNAs occurred faster in human than in mouse serum [15], suggesting the shortened half-life and loss of silencing may pose a major challenge in a future therapeutic application of siRNAs in humans in comparison to mouse models. An important advantage of the silk sphere system over the complexes of a soluble silk and nucleic acid is the controlled release of substances during drug trafficking and at the destination site of a drug. Based on the ZP value of the MS2KN spheres loaded with nucleic acid, we can presume that at least parts of the constructs were present on the surface of silk spheres. However, in the presence of 1% SDS it was impossible to release CpG-siRNA from spheres. Moreover, after internalization into cells, the co-localized signals of silk spheres and CpG-siRNA were observed in the cytoplasm after 0.5, 3 and 24 hours of incubation and the separated signals later after 48 and 72 hours. Presented data indicated that nucleic acids were stably embedded into silk spheres. It is in agreement with our data that

functionalized silk spheres served as protective shells for siRNA against serum degradation. Furthermore, it is likely that the endosomal enzymatic degradation of spheres is necessary for the release of the free oligonucleotides. CpG-siRNA molecules are known to be internalized through scavenger receptor (SR)-mediated uptake into early endosomes, where they bind to TLR9 and thereby induce cytoplasmic release of the endosomal content [10]. Whether it also applies for CpG-siRNA constructs loaded into silk spheres requires further study.

We performed and compared two experiments using i) complexes and ii) spheres composed of MS2KN silk as materials that protect nucleic acid molecules from degradation. We showed that both silk structures protected CpG-siRNA from degradation in comparison with their respective non-serum treated controls. It is in agreement with a study by Numata et al. that indicated that silk-based plasmid DNA (pDNA) complexes demonstrated DNase resistance [22]. Moreover, the methanol-treated silk-pDNA complexes contained higher percentage of beta-sheet structures and were less susceptible to protein degradation and released less pDNA compared with the not-methanol-treated complexes [22]. We indicated previously, that application of the kosmotropic ions triggered the conformational change of the MS2 silk molecules that increased the beta-sheet structure formation [27]. That could partially explain why the spheres did not release CpG-siRNA constructs into DNA Loading Dye with 1% SDS, and thus the additional dissolving procedure had to be applied.

One of the most important features to be considered when designing nanoparticle-based carriers is their lack of cellular cytotoxicity and induction of inflammatory reactions. It has been demonstrated by numerous studies that various structures made of bioengineered spider silks were not cytotoxic including our MS2 spheres [21, 23, 27, 28, 31, 34, 35]. However, the polylysine peptides were shown in many studies to be cytotoxic and to mediate the enhanced cellular uptake via a non-specific adsorptive endocytosis mechanism [36, 37]. The cytotoxicity of polylysine constructs was dependent on the molecular weight and structure of these polypeptides [38, 39]. In our system, the addition of the poly-lysine domain into MS2 silk indeed induced significant toxic effect in fibroblasts. However, this effect was not observed for MS2KN spheres bound with the nucleic acids. As it was mentioned above, based on the ZP value of the MS2KN spheres loaded with nucleic acid, we can presume that at least part of the nucleic acid constructs was localized on the surface of silk spheres. The bound CpG-siRNA molecules could prevent the exposure of poly-lysine domains and thus minimize their toxic effect. In addition, no upregulation of *Stat3* mRNA was observed in J774 target macrophages after incubation with the plain silk carrier and silk carrier loaded with CpG-*scrRNA* (data not shown). In contrast, naked CpG-*scrRNA* upregulated STAT3 levels in target cells as expected due to IL-6 mediated negative feedback effect [6]. Thus, the tested silk spheres did not have an unspecific effect on target gene levels proving not only to be non-cytotoxic but also very neutral vehicles in terms of TLR9/STAT3 activation in macrophages.

Numata et al. first designed MaSp1-based-silk complexes of plasmid DNA that required transfection reagent for cell delivery, an approach that could not be applied in patients [31]. The initial concept was later refined by adding cell binding/tumor homing peptides to the existing silk/DNA ionic complexes that allowed for reagent-free gene delivery into the cells

with reasonably low toxicity [25, 26, 35]. Although the basic concept of using poly-lysine - functionalized silk protein was similar, we took advantage of the ligand properties of CpG that is linked to the therapeutic siRNA, and thus no additional homing polypeptide sequences were necessary. In our system, the presence of poly-lysine fused to the silk allowed for binding CpG-siRNA. CpG-ODN was shown to be a ligand for Macrophage Mannose receptor 1 (C-type mannose receptor 1 - MRC1, CD206) that mediates cellular uptake and endosomal delivery [40]. Upon delivery into the endolysosomes, CpG-ODN activates the DNA sensor i.e. TLR9 [41, 42]. MRC1 is primarily present on the surface of macrophages and immature dendritic cells [43–45], and TLR9 is expressed by numerous cells of the immune system such as dendritic cells, macrophages, B lymphocytes, monocytes [46], natural killer (NK) cells [47] that are the substantial components of the tumor microenvironment. Several TLR9-positive hematologic malignancies and solid tumors are also targets for CpG-ODN [8, 9, 48]. Thus the application of CpG-*STAT3*siRNA constructs provides targeted delivery into tumor microenvironment or directly into tumor cells and therefore the specificity of the therapy.

Subsequently, we compared the uptake efficiency and kinetics of constructs present in target cells depending on the way of the delivery: direct as naked molecules or embedded into silk spheres. According to flow cytometry analysis, the internalization of CpG-siRNA encapsulated into MS2KN spheres was slightly higher than the internalization of the naked CpG-siRNA molecules. One of the possible explanations for the observed results could be that MS2KN/CpG-siRNA spheres were internalized by macrophages using two independent pathways: i) by CpG and ii) by poly-lysine dependent mechanisms. As mentioned above, the bound CpG-siRNA molecules could prevent the exposure of poly-lysine domains and thus minimize their toxic effect. However, we cannot exclude that some of the poly-lysine domains can be still accessible on the sphere surface. The mechanism of the internalization of MS2KN/CpG-siRNA was not in the scope of this study, and it needs further examination.

Although, internalization of plain MS2KN spheres, spheres loaded with CpG-siRNA construct and naked CpG-siRNA occurred as early as 30 minutes after administration, silk spheres with or without CpG-siRNA persisted inside the cells for longer time period compared with naked CpG-siRNA suggesting delayed endosomal processing and removal. After 48 hours only CpG-siRNA constructs liberated from spheres could be detected in the cytoplasm, but not ones delivered as naked form. The presence of nucleic acid constructs inside the cells was associated with their activity. The activity trend of naked CpG-*STAT3*siRNA was observed after 24 hours in terms of STAT3 silencing causing significant downstream signaling processing. However, it should be noticed, that in our long-term study the naked form of CpG-*STAT3*siRNA was used at the concentration of 250 nM, while its most effective concentration was shown to be 500-1000 nM [6]. The 250 nM concentration of CpG-*STAT3*siRNA loaded into silk spheres led to prolonged (up to 72 hours) silencing effect on STAT3 mRNA in TLR9-positive cells observed as a trend in comparison with CpG-*scrRNA* delivered in spheres and a significant decrease compared with the activity of naked CpG-*STAT3*siRNA. Moreover, the activity of CpG-*STAT3*siRNA loaded into silk spheres caused stable and long-lasting effects on downstream signaling. Indeed, after 72 hours significantly decreased mRNA levels could be seen for IL-6 - a cytokine that expression level is regulated by STAT3 signaling.

CpG-*STAT3*siRNAs are in clinical translation for cancer immunotherapy of hematologic malignancies. Based on preclinical studies, local administration of CpG-*STAT3*siRNA using intratumoral/intranodal injections is sufficient for generation of systemic antitumor immune responses. However, the encapsulated MS2KN/CpG-*STAT3*siRNA generates an opportunity to adapt this strategy for systemic administration also to other types of disseminated human cancers.

5. Conclusions

In this study, we showed that silk particles made of functionalized bioengineered protein based on MaSp2 *N. clavipes* sequence could be loaded with CpG-siRNA constructs, internalized and processed by target macrophages in the absence of transfection reagents. However, the uptake, the kinetics of the presence in the cytoplasm and therefore the activity of the delivered nucleic acid constructs were different depending on the way of its delivery; the naked CpG-*STAT3*siRNA was delivered less in comparison with CpG-*STAT3*siRNA loaded into silk spheres, with the faster kinetics of processing and thus silencing activity. The application of silk spheres not only protected siRNA therapeutics from degradation in serum but also modified the kinetics of their action thus it can elicit a different effect *in vivo*. The biological characterization of the obtained delivery system indicated its applicability, however, a detailed physical and chemical characterization of MS2KN/CpG-siRNA spheres requires future investigation.

Since STAT3 and IL-6 drive growth and progression of various tumors including colorectal, pancreatic, lung, prostate and breast cancer [49–53], the application of CpG-*STAT3*siRNA loaded into MS2KN silk spheres may be of great importance for cancer immunotherapy. Moreover, the simplicity of the proposed system allows for further redesign in order to 1) silence other tumorigenic and/or immunosuppressive transcription factors, 2) link other nucleic acids used in experimental therapies such as aptamers or to 3) modify silk properties by adding peptides or 4) assembly various silk structures (such as a sphere, film, scaffold, etc.). Our findings demonstrated that the functionalized bioengineered spider silk spheres have a great potential as a delivery vehicle of therapeutic nucleic acids.

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Statement of Significance

We demonstrated that modification of silk protein by adding the nucleic acid binding domain enabled the development of a novel oligonucleotide delivery system that can be utilized to improve pharmacokinetics of RNA-based therapeutics. Although, the siRNA constructs have already given very promising results in the cancer therapy, the in vivo application of RNA-based oligonucleotide therapeutics still is limited due to their sensitivity to serum nucleases and some toxicity. We propose a carrier for RNA-based therapeutics that is made of bioengineered spider silk. We showed that functionalized bioengineered spider silk spheres not only protected RNA-based therapeutics from degradation by serum nucleases, but what is more important the embedding of siRNA into silk spheres delayed and extended target gene silencing compared with naked oligonucleotides. Moreover, we showed that plain silk spheres did not have unspecific effect on target gene levels proving not only to be non-cytotoxic but also very neutral vehicles in terms of TLR9/STAT3 activation in macrophages. We demonstrated advantages of novel delivery technology in safety and efficacy comparing with delivery of naked CpG-STAT3siRNA therapeutics.

Fig. 1.

CpG-RNAs and spider silks sequences and SDS-PAGE analysis of silks. **A)** Amino acid sequences of control bioengineered spider silk (MS2), functionalized silk (MS2KN) with polylysine nucleic acid binding domain and nucleic acid sequences of CpG-*STAT3* siRNA and CpG-*scr* RNA are shown. **B)** Control and hybrid proteins were analyzed on 12.5% SDS-PAGE gel. 1. Protein molecular weight marker (PageRuler, Thermo Scientific), 2. Control spider silk MS2, 3. MS2KN functionalized spider silk.

Fig. 2. Binding efficacy of spider silk protein with CpG-*scr*RNA. CpG-*scr*siRNA and bioengineered silks (control MS2 and functionalized MS2KN) were mixed at different molecular ratios and then co-incubated for 5 min at room temperature. Binding of nucleic acid to silk proteins was examined by agarose gel electrophoresis. The molecular ratios of silks and siRNA construct are shown in the table under the gel image.

Fig. 3.

Loading of silk spheres with CpG-*STAT3* siRNA. **A)** The morphology of MS2KN and CpG-*STAT3* siRNA loaded MS2KN spheres were analyzed by scanning electron microscopy (SEM) (scale bar - 1 μm). **B)** Spider silk spheres were loaded with labeled CpG^{FITC}-*STAT3* siRNA and analyzed by confocal laser scanning microscopy (CLSM) (scale bar - 10 μm). To visualize the construct, excitation wavelength 488 nm and emission filters 495-525 nm were applied. **C)** Zeta potentials of control MS2KN and CpG-scrRNA loaded MS2KN spheres were measured at 25 °C in ddH₂O at the sphere concentration of 0.01 mg/mL. The means and standard deviations of three independent experiments are shown.

Fig. 4.

Analysis of CpG-scrRNA stability when loaded into silk complexes and spheres during incubation in serum. The CpG-scrRNA molecules were **A)** mixed with MS2KN to form complexes or **B)** loaded into silk spheres and then the obtained samples were incubated with 30% mouse serum for 0, 1 and 3 h in 37 °C. The samples were analyzed in 15% Urea-PAGE gel. Naked CpG-scrRNA served as control. After incubation, gels were stained with TBE-ethidium bromide solution for 30 min at room temperature, and nucleic acid was visualized in a presence of UV light.

Fig. 5. Cytotoxicity analysis of silk spheres and silk spheres loaded with CpG-scrRNA. NIH/3T3 cells were cultured in the presence of the silk spheres loaded with CpG-siRNA (MS2KN/CpG-siRNA) and control silk spheres (MS2, MS2KN). Cytotoxicity was studied using MTT assay. The % of the MTT reduction was calculated in reference to non-treated control cells. Results are expressed as means of three independent experiments and error bars show the standard error of the mean (SEM). (**) represents the statistical significance $p < 0.01$, and (*) $p < 0.05$.

Fig. 6.

Flow cytometry analysis of the cellular binding of spheres loaded with CpG^{FITC}-*STAT3*siRNA. J774 murine macrophages were incubated for 1 h with **B**) naked CpG^{FITC}-*STAT3*siRNA (250 nM) or **C**) ATTO-647 labeled MS2KN silk spheres loaded with CpG^{FITC}-*STAT3*siRNA (250 nM) and compared with **A**) non-treated cells. Percentage of FITC-, ATTO 647- or double-positive cells were assessed by flow cytometric analysis. The picture shows one representative analysis out of three independent experiments.

Fig. 7. Confocal microscopy analysis of the cellular uptake of MS2KN silk spheres, CpG^{FITC}-*STAT3* siRNA loaded into MS2KN and naked CpG^{FITC}-*STAT3* siRNA. **A)** control ATTO-647-MS2KN spheres, **B)** ATTO-647-MS2KN spheres loaded with CpG^{FITC}-*STAT3* siRNA at 1:1 ratio and **C)** naked CpG^{FITC}-*STAT3* siRNA were incubated with J774 murine macrophages for 30 min, 3, 24 or 48 h. Green - indicates CpG^{FITC}-*STAT3* siRNA; red - ATTO-647-MS2KN, and blue - nuclear staining with DAPI (scale bar - 10 μ m).

Figure 8. Analysis of STAT3 mRNA and protein levels after treatment with naked CpG-STAT3siRNA or CpG-STAT3siRNA loaded into MS2KN spheres

J774 murine macrophages were incubated for 24, 48 or 72 h with naked CpG-scrsiRNA (250nM), CpG-STAT3siRNA (250nM) or CpG-scrsiRNA (250nM) or CpG-STAT3siRNA (250 nM) loaded into MS2KN spheres and then quantitative real-time PCR analysis was performed. A) *Stat3* mRNA level was normalized to tubulin expression and the fold change of the *Stat3* mRNA in J774 cells treated with plain MS2KN spheres was calculated in reference to non-treated control cells. B) *Stat3* and C) *Il6* mRNA levels were normalized to tubulin expression and the fold change of the *Stat3* and *Il6*mRNA, respectively, in J774 cells

treated with naked CpG-*STAT3*siRNA or CpG-*STAT3*siRNA loaded into MS2KN silk spheres were calculated in reference to cells treated with naked CpG-*scrsiRNA* or CpG-*scrsiRNA* loaded MS2KN silk spheres, respectively. *Stat3* and *Il6* levels in cells treated with naked CpG-*scrsiRNA* and CpG-*scrsiRNA* loaded into MS2KN spheres were normalized to 1 at all tested time points. Results are expressed as means of three independent experiments and error bars show the standard variation of the mean (SEM). (*) represents the statistical significance $p < 0.05$. D) The J774 murine macrophages were incubated for 24 or 48 h with 100, 250 or 500 nM of CpG-*scrsiRNA* or CpG-*STAT3*siRNA loaded into MS2KN silk spheres and then the protein level of STAT3 in the cells were assessed by using Western blotting. β -actin was used as a loading control. Representative results of three independent experiments are shown.

Table 1

Real-time quantitative PCR primer sequences and UPL probe numbers.

Gene	Primer sequence	Probe
<i>Tubb</i>	F: GCTGGACCGAATCTCTGTGT	#95
	R: GACCTGAGCGAACGGAGTC	
<i>Il6</i>	F: GCTACCAAACTGGATATAATCAGGA	#6
	R: CCAGGTAGCTATGGTACTCCAGAA	
<i>Stat3</i>	F: GTTCCTGGCACCTTGGATT	#71
	R: CAACGTGGCATGTGACTCTT	

Abbreviations: *Tubb* – β tubulin, *Il6* – Interleukin 6, *Stat3* - STAT3, F-forward primer, R-reverse primer.