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

Anna Karolina Kozlowska [a], Anna Florczak [a,b], Maciej Smialek [b,1], Ewelina Dondajewska [a], Andrzej Mackiewicz [a,b], Marcin Kortylewski [c], Hanna Dams-Kozlowska [a,b,*]

[a] Chair of Medical Biotechnology, Poznan University of Medical Sciences, 61-701 Poznan, Poland
[b] Department of Diagnostics and Cancer Immunology, Greater Poland Cancer Centre, 61-866 Poznan, Poland
[c] Department of Immuno-Oncology, Beckman Research Institute, City of Hope National Medical Center, Duarte, CA 91010, USA

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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.

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*.

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-*STAT3*siRNA therapeutics.

* Corresponding author at: Department of Cancer Diagnostics and Immunology, Greater Poland Cancer Center, 15 Garbary St., 61-866 Poznan, Poland.
E-mail address: hanna.dams-kozlowska@wco.pl (H. Dams-Kozlowska).
[1] Present address: Institute of Human Genetics, Polish Academy of Sciences, 60-479 Poznan, Poland.

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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 13 kDa 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$^{5'SS\text{-}FITC}$ (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