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Targeted Delivery of siRNA into Breast Cancer Cells via Phage Fusion Proteins

Deepa Bedi, MD, PhD^a, James W Gillespie, BS, Vasily A. Petrenko Jr.^a, Andreas Ebner^b, Michael Leitner^b, Peter Hinterdorfer, PhD^b, and Valery A. Petrenko, PhD, D.Sci^{a,*}

^aDepartment of Pathobiology, College of Veterinary Medicine, Auburn University, Auburn, Alabama, USA.

^bJohannes Kepler University Linz, Altenbergerstr. 69, A-4040 Linz, Austria

Abstract

Nucleic acids including antisense oligonucleotides, small interfering RNA (siRNA), aptamers and ribozymes, emerged as versatile therapeutics due to their ability to interfere in a well-planned manner with the flow of genetic information from DNA to protein. However, a systemic use of NAs is hindered by their instability in physiological liquids and inability of intracellular accumulation in the site of action. We first evaluated the potential of cancer specific phage fusion proteins as targeting ligands that provide encapsulation, protection, and navigation of siRNA to the target cell. The tumor-specific proteins were isolated from phages that were affinity selected from a landscape phage library against target breast cancer cells. It was found that fusion phage coat protein fpVIII displaying cancer-targeting peptides can effectively encapsulate siRNAs and deliver them into the cells leading to specific silencing of the model gene *GAPDH*. Complexes of siRNA and phage protein form nanoparticles (nanophages), that were characterized by atomic force microscopy and ELISA, and their stability was demonstrated by resistance of encapsulated siRNA to degradation by serum nucleases. The phage protein/siRNA complexes can make a new type of highly selective, stable, active and physiologically acceptable cancer nanomedicines.

Graphical Abstract

Keywords

Tumor; landscape phage; cancer-targeting peptides; siRNA; nanoparticles

*Corresponding author: Phone: 334-844-2897; Fax: 334-844-2652; petreva@auburn.edu.

Introduction

During the last two decades, nucleic acids emerged as versatile therapeutics due to their ability to interfere in a well-planned manner with the flow of genetic information from DNA to protein ¹. The most prospective nucleic acids, including antisense oligonucleotides, small interfering RNA (siRNA), aptamers and ribozymes demonstrate high diversity of structures and capability of expressing many enzymatic activities required for interfering with cell metabolism ². However, a systemic use of nucleic acids is hindered by their instability in physiological liquids, insufficient tissue bioavailability and inability of intracellular accumulation in the site of action ³. Various nano corpuscular vehicles, such as liposomes, polymers, and cell penetrating peptides were proposed to deliver nucleic acids into target cells ⁴. However, their lack of cell specificity and inability to control drug release remains the major drawback for their clinical use. Thus, along with necessary protection of nucleic acids, their delivery to specific cell types and control of drug release is a key challenge for turning nucleic acids into therapeutics. A number of cell specific ligands including antibodies, antibody fragments, and peptides have been explored to achieve targeted delivery of nucleic acids ⁵.

The availability of combinatorial peptide libraries adds another availing dimension to the concept of peptide-based antitumor pharmaceuticals ⁶. Phage display peptide libraries have emerged as a rich source for selecting of targeting ligands ⁷. Phage technique provided a new way of identification of peptides targeted to specific tissue, cell, receptor or cellular compartments in a high throughput fashion. Increasing number of cell-targeting peptides have been identified for various cancer cells, organs, proteins, receptors, and tumor vasculature ⁸⁻⁹. However, incorporation of the discovered targeting peptide ligands into nucleic acids - encapsulating nanoparticles requires expensive and time consuming methods of synthesis and chemical conjugation that complicates production and analysis of nucleic acid nanomedicines and can alter the specificity of targeting peptides. Selection of peptides fused to all copies of major coat protein in type 8 phage display system (landscape phages ¹⁰) allows to solve this problem. In this phage display format, a sequence encoding a foreign peptide is spliced in-frame into gene *gpVIII* encoding the major coat protein pVIII, leading to the landscape fashion of the phage display. In landscape phages thousands copies of the guest peptide fused to the phage coat protein cover the entire virion surface. The tumor-specific landscape phages can be affinity selected from multibillion clone libraries by their ability to interact very specifically with cancer-cell surface receptors and/or penetrate into the cells ¹¹⁻¹⁴. Fusion phage proteins isolated from selected landscape phages have been used successfully as targeting ligands for liposomal- and micelle-mediated delivery of doxorubicin, paclitaxel and siRNAs ¹⁵⁻¹⁸.

In this study, we propose the use of landscape phage fusion proteins—easy to produce “substitute antibodies” ¹⁹—as targeting ligands for efficient protection and delivery of siRNA to cancer cells. The proposed new strategy of siRNA delivery to breast cancer cells explores unique propensity of phage proteins – their ability to self-assemble in the presence of nucleic acids, and on the contrary—to release the nucleic acid cargo during interaction with cellular membrane. siRNA and phage protein form particles, called here “phage-like nanoparticles”, or “nanophages”. The self-assembled siRNA-nanophages fulfill the major

requirements for the proficient nucleic acid delivery system: they 1) protect siRNA from serum endonucleases, 2) target siRNA to the specific cell type, and 3) deliver siRNA to cytoplasm of the target cells. These remarkable properties are inherited by nanophages from specifically selected phages able to bind very specifically to cancer cells, penetrate into the cells or accumulate in the tumor-surrounding vasculature¹¹⁻¹⁴. The unique properties of phage proteins as the efficient natural delivery system allowed us to propose the idea of using the phage-like platform as a nucleic acid delivery vehicle. In particular, as shown in this report, the phage protein/siRNA complexes can make a new type of highly selective, stable, active and physiologically acceptable cancer nanomedicines.

Methods and Materials

Cells

Cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cell line MCF-7 (ATCC, HTB 22TM) was derived from plural effusion of human breast adenocarcinoma. Human hepatocellular carcinoma HepG2 (ATCC, CRL-2235TM) cells and human non-tumorigenic mammary epithelial cells MCF-10A (ATCC, CRL-10317TM) were used as controls in selectivity assay. Cells were grown as recommended by ATCC and incubated at 37°C, 5% CO₂.

Breast cancer cell-specific phage

Breast cancer-specific phages have been selected from the 1.4×10⁹-clone type 8 landscape phage library f8/9, as described^{14, 20}. In this library, foreign nonamer peptides are displayed as an extension of each major coat protein unit due to an in frame random oligonucleotide insertion in the gene *gpVIII* resulting in the display of ~4000 guest peptide units on the surface of a phage particle. All general methods of handling phage, including propagation, purification, titrating, production of pure phage clone, and isolation of phage DNA have been previously described²¹. *Escherichia coli* (*E. coli*) strain K91BlueKan (Kan^r) {Hfr C thi lacZ M15 lac Y::mkh lacI^Q} used for phage titrating and propagation was kindly provided by George Smith (University of Columbia-Missouri).

The phage clone VEEGGYIAA (designated by the sequence of inserted foreign peptide) was retrieved from eluate (acid treated) fraction while the phage DWRGDSMDS was isolated from post elution (PEW) fraction. Binding specificity and selectivity of the phages VEEGGYIAA and DWRGDSMDS were determined in a phage capture assay²¹ adapted for 96-well culture plate format, as described¹⁴⁻¹⁶. To estimate a role of cellular metabolism in phage binding, association of phage with cells was studied under different temperature conditions, with or without adding of stimulating components of serum, as described¹⁴.

Phage fusion coat proteins

Breast cancer cell-targeting phage fusion 55-mer coat proteins a), b) and streptavidin-binding phage protein c):

- a) aVEEGGYIAApakaafdslqasateyigyawamvvvivgatigiklfkkftskas,
- b) aDWRGDSMDSpakaafdslqasateyigyawamvvvivgatigiklfkkftskas

c) **aVPEGAFSSdpakaafdsfqasateyigyawamvvvivgatigiklffkftskas**

(foreign peptide shown with bold font) were prepared by stripping phages VEEGGYIAA, DWRGDSMDS and VPEGAFGS in cholate buffer ^{11, 14, 22}. Briefly, the mixture of 350 UI phage (~1 mg/ml) in the TBS buffer, 700 µl of 120 mM cholate in 10 mM Tris-HCl, 0.2 mM EDTA, pH 8.0 and 27 µl of chloroform was incubated overnight at 37°C with rotation. The fusion protein was purified from the viral DNA and traces of bacterial proteins by size-exclusion chromatography using a Sepharose 6B-CL (Amersham Biosciences) column (1 cm × 45 cm), which was eluted with 100 mM cholate in 10 mM Tris-HCl, 0.2 mM EDTA pH 8.0. The chromatographic profile was monitored by the Econo UV monitor (Bio-Rad, CA); 5 ml per fraction were collected and stored at 4°C. Position of the major coat protein peak in comparison with the protein standards corresponded to a tetrameric form of the proteins.

Targeted phage protein-siRNA complexes (siRNA-nanophages)

A portion (1 µg) of GAPDH siRNA, scrambled siRNA, or Alexa fluor 488 negative control siRNAs (Qiagen) were mixed with increasing concentrations of phage proteins, giving rise to phage protein/siRNA molar ratios ranging from 1:1 to 80:1. The mixture was centrifuged and concentrated in 30 Kda amicon centrifugal units at 3900 rpm (Alegre 2R centrifuge) for 15 min to remove excess of the detergent. Remaining ~ 10 µl retentate was diluted to 4 ml with 10 mM Tris-HCl, 0.2 mM EDTA pH 8.0 and centrifuged for 15 min at the same speed. The retentate was suspended in 100 µl of 10 mM Tris-HCl, pH 8.0. The complex was characterized by UV spectroscopy. The concentration of the phage proteins was determined using the formulae: $1 A_{280} = 0.60 \text{ mg/ml}$ (for VEEGGYIAA- protein) and $1 A_{280} = 0.42 \text{ mg/ml}$ (for DWRGDSMDS-protein) using the program protean (Lasergene Core Suite, DNASTAR, Madison, WI). The purity of the siRNA, phage protein and siRNA-nanophages were determined by measuring the ratio A_{260}/A_{280} that was in the range 2.0, 0.57 and 1.2, respectively. Proteinase K (12 µl, 5 mg/ml, Sigma) was added to 10 µl of the complex and incubated 24 hrs at 37°C allowing the digestion of phage protein. The proteinase K treated and untreated complex were analyzed by agarose gel electrophoresis (1%) with SYBR-green staining.

Serum nucleases protection assay

Naked siRNA (1 µg) or siRNA-nanophage prepared as described earlier were incubated at 37 °C in 100% fetal bovine serum, (final concentration 50%). After 24 hours, the reaction was stopped by adding Proteinase K (12 µl, 5 mg/ml, Sigma) along with 2 µl (2 units) of Rnase Inhibitor (Qiagen) was added for 24 hour at 37 °C. The treated and untreated samples were analyzed on a 1% agarose gel with SYBR-green staining.

ELISA assay

Wells of polystyrene ELISA plate (Corning Incorporated) were coated with 45 µl of streptavidin-binding phage protein 7b1-siRNA complex, 7b1 protein (20 µg/ml), siRNA (1 µg/ µL) in 10 mM Tris-HCl, pH 8.0, and 7b1 phage (5×10^{11} vir/ml) in TBS overnight at 4°C. The wells were blocked with 0.1% BSA for 1 hr at 20°C and washed five times with 10 mM Tris-HCl-Tween (0.1%) using Bio-Tek ELx405 auto plate washer (Bio-Tek Instruments

Inc.). Forty-five-microliter samples of alkaline phosphatase conjugated-streptavidin (AP-SA) at concentrations ranging from 0.0001 to 3.125 µg/ml in AP-SA diluent (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% Tween 20), were incubated on a rocker at room temperature for 1 h, washed 5 times with TBS-Tween and filled with 90 µl of 0.5 mg/ml *p*-nitrophenyl phosphate (NPP) in 1 mM MgCl₂, 1 M diethanolamine-HCl, pH 9.8. The difference between the optical density (OD) at 405 and 490 nm was registered at 3 min intervals over 60 min period on a kinetic plate reader (Synergy H1 hybrid reader, Biotek) in order to obtain a slope (mOD/min) for each well.

Atomic force Microscopy

The complex of phage protein-siRNA was prepared as described above. A sample (2 µl) was incubated for 10 min on aminopropyl-triethoxysilane (APTES) functionalized mica²³, treated with 50 µl Tris-Buffer (100 mM) for 10 minutes and carefully rinsed with Tris buffer (10 mM). This concentration procedure resulted in a complete coverage of the surface with the phage protein-siRNA complex. For imaging of single phage protein-siRNA complexes, the Ni²⁺ method for sample preparation was used. Therefore, 4 µl of the sample (1.3 mg/ml) was incubated on mica with a mixture of 200 µl Tris (100 mM) and 200 µl Ni²⁺ (100 mM). After 20 minutes of incubation, the sample was carefully rinsed and imaged in Tris Ni²⁺ Buffer (10 mM, 1 mM) using atomic force microscope.

Fluorescence Microscopy

Phage protein-Alexa fluor 488 siRNA complexes were prepared as described above. MCF-7 or MCF-10A cells were grown on Lab-tek chamber slides (Thermo Fisher Scientific) and incubated with phage protein/siRNA complexes for 4 and 24 hours at 37 °C. Cells were then washed with PBS, covered with coverslips (Fisher), and visualized with a Cytoviva microscope system (Cytoviva, Auburn, Alabama).

Quantitative uptake of siRNA delivered by phage protein into MCF-7 cells

1 µg of Alexa fluor 488 siRNA (Invitrogen) was complexed with different concentrations of VEEGGYIAA protein (80:1, 40:1, 20:1 and 10:1 mol protein:mol siRNA) as described before or with lipofectamine RNAimax (Invitrogen, Carlsbad, CA) according to the manufacturers protocol. The complexes were laid on MCF-7 cells, grown in 96 well plate in complete MEM medium with 10% FBS, for 4 hrs. After treatment, the cells were washed three times with serum-free media and trypsinized for 10 min to remove extracellular peptide/siRNA complexes. The cells were centrifuged for 5 min at 1000 g, and the supernatant was removed and the cell pellets were lysed in 30 µl 0.1 M NaOH. The cell lysate was centrifuged for 10 min at 10,000 g to remove cell debris, and the supernatant was used to measure fluorescence 490/518 nm on a Synergy H1 hybrid spectrofluorometer reader, Biotek. The fluorescence was normalized to the amount of protein determined using a detergent-compatible DC Biorad protein assay.

Analysis of GAPDH protein expression in MCF-7 cells using Western-blot technique

To study inhibition of GAPDH synthesis by siRNA, 10⁵ MCF-7 or MCF-10A cells in 6-well culture plates were transfected with phage protein-GAPDH siRNA or phage protein-negative

siRNA (50 nM) complexes at 37°C for 24 h. After 24 h, media was aspirated and fresh complete L-15 medium was added. The plates were rocked gently for 5 min at room temperature and incubated at 37°C for 72 h. Media was changed every 24 h. After 72 h, cells were lysed with RIPA buffer (Sigma, St. Louis, Missouri) containing a protease inhibitor cocktail (Sigma, St. Louis, Missouri) and PMSF (2 mM final concentration). The protein concentration in the whole cell lysate was measured by the BioRad DC protein assay (Hercules, California). A portion (15 µg) of the whole cell extract was analyzed by electrophoresis in 4-20% polyacrylamide gradient Tris-HCl gels (Hercules, California) and transferred to PVDF membrane (Millipore, Billerica, Massachusetts). Proteins in the gel were transferred to an Immobilon-P PVDF membrane (Millipore) and resulting blots were probed with mouse polyclonal anti- GAPDH antibody (Abcam, Cambridge, MA) (1:2000 dilution) followed by incubation with peroxidase-conjugated Affinipure Goat Anti-mouse IgG (1:5000) (Jackson ImmunoResearch, West Grove, Pennsylvania) before being visualized using a chemiluminescent substrate solution (Pierce, Rockford, Illinois). Membranes were stripped using the Western blot stripping buffer (Thermo Scientific, Rockford, Illinois) for 10 min and probed with rabbit polyclonal anti- β -Actin antibody (Abcam) (1:2000) followed with incubation with peroxidase-conjugated Affinipure goat anti-rabbit IgG (1:5000) (Jackson ImmunoResearch) and visualized using chemiluminescent substrate solution.

Results

Phage selection and characterization

Phages VEEGGYIAA and DWRGDSMDS were previously selected from the phage displayed 9-mer peptide library f8/9²⁰ via biopanning against MCF-7 cells¹⁴. The phage clone VEEGGYIAA was retrieved from eluate (acid treated) fraction while phage DWRGDSMDS was isolated from post elution wash fraction. Selectivity of the phages was determined by measuring their binding to breast cancer cells in comparison with normal breast epithelial cells MCF-10A, hepatocellular carcinoma cells HepG2 and serum¹⁴. It demonstrated a high specificity of the selected phages towards the target breast cancer cells¹⁴.

Complexes of phage proteins with siRNA (nanophages-siRNA)

Interaction of phage fusion proteins with nucleic acids was investigated using a negative control 21-mer siRNA duplex as a model. siRNA was incubated with excess of phage proteins in cholate buffer at different molar ratios, and freed from excess of cholate by ultrafiltration. siRNA and phage protein form particles, called here “phage-like nanoparticles”, or “nanophages” as shown in Fig 1. The formation of phage protein/siRNA complexes was analyzed by agarose gel-electrophoresis. As shown in Fig. 2B (*left*), formation of stable siRNA-nanophage complexes is initiated at a molar ratio of phage protein to siRNA 16:1, and is completed at a molar ratio of 80:1. The ratio of absorbance of the complex at 260/280 nm, as measured by UV spectroscopy, is 1.17 (Fig. 2A) which is greater than ratio of absorbance at 260/280 nm of protein itself (0.58) and less than ratio of siRNA alone (2.0), indicative of siRNA-nanophage complex with stoichiometry of protein and siRNA ~ 42:1⁴³. Digestion of the complex (80:1) with proteinase K led to a release of

the encapsulated siRNA (Fig. 2B, *right*) indicating that siRNAs retain their integrity when complexed to phage protein.

We investigated the effect of serum nucleases on the stability of siRNA-nanophage. The naked siRNA or siRNA-nanophage complex (80:1) were incubated with 50% serum for 24 hours and then treated with proteinase K to free the encapsulated siRNA by digestion of phage protein. The naked siRNAs were rapidly degraded by serum but in contrast, no significant degradation of siRNA was observed when siRNA was complexed with phage protein indicating that phage protein protects the siRNA from serum nucleases, as revealed by agarose gel electrophoresis (Fig. 2C).

Atomic force microscopy revealed the structure of the complex as small particles of diameter 11 nm (Fig. 2D, 2E). At the same time, much bigger particles with diameter 50 and 100 nm were revealed by ZetaSizer (Malvern), (data not shown). The difference in results can be attributed to the aggregation of small particles to bigger ones at higher concentration used for measuring the size of the particles using ZetaSizer.

To test the functional activity of fusion peptides in the nanophage- siRNA complexes, streptavidin binding phage (7b1) was used as a model in ELISA based test. Fig. 3 shows that nanophage- siRNA complex retains its specific binding activity towards streptavidin, demonstrating even higher binding than protein or phage alone. Control siRNA did not show any binding to streptavidin.

Cellular uptake of nanophage- siRNA by target cells

The ability of phage proteins to deliver siRNA into MCF-7 or MCF-10A living cells was evaluated by fluorescence microscopy. Substantial diffuse cytoplasmic fluorescence was observed in MCF-7 cells treated with VEEGGYIAA - nanophage Alexa-fluor 488-labeled siRNA after 4 h (Fig. 4A *Upper*) and 24 h (Fig. 4B *Upper*), while little fluorescence was observed in MCF-10A treated cells (Fig. 4C *Upper*). Similarly, treatment of the target cells MCF-7 with Alexa-fluor 488-labeled siRNA nanophage DWRGDSMDS resulted in punctuate granular fluorescence indicative of vesicular localization of siRNA after 4h (Fig. 4A *Lower*), changing to diffuse cytoplasmic fluorescence after 24 h (Fig. 4B *Lower*). Negligible fluorescence was observed in control MCF-10A cells (Fig. 4C *Lower*).

Nanophage mediated siRNA delivery increases with protein concentration

A quantitative study measuring the internalized siRNA was performed to determine whether the interaction of the phage proteins with siRNA, as shown in the gel shift assay, was able to increase siRNA uptake. As seen in **Fig. 5**, the intracellular fluorescence intensity increases with nanophage- Alexa-fluor 488-labeled siRNA ratio indicating that the cellular uptake of siRNA was proportional to the molar ratio of nanophage- Alexa-fluor 488-labeled siRNA complex with the highest uptake observed at 80/1 nanophage-siRNA molar ratio. Lipofectamine-siRNA complex also showed comparable intensity as 80/1 nanophage-siRNA.

Targeted knockdown of the genes by siRNA-nanophages

The ability of phage proteins to deliver active siRNA molecules into target breast cancer cells was investigated by monitoring inhibition of the model GAPDH protein synthesis. We tested the ability of the preselected proteins and their complexes with siRNA to specifically target the cancer cells MCF-7 in comparison with control cells MCF-10A. MCF-7 cells were treated with GAPDH siRNA-nanophage (50 nM final siRNA concentration) for a period of 72 h. Western blot analysis showed that, GAPDH siRNA-nanophages VEEGGYIAA and DWRGDMDS downregulated GAPDH protein synthesis in MCF-7 cells while produced no effect on GAPDH protein levels in MCF-10A cells (Fig. 6, 7, respectively).

Discussion

The potential of nucleic acids including siRNAs, for cancer therapy was recognized soon after their discovery²⁴⁻²⁵. It was hypothesized that therapeutic nucleic acids can suppress the growth of pathological unregulated cancer cells promoting oncogenes while leaving normal genes undisturbed²⁶. Though nucleic acids therapy offers a great promise, their short half-life in blood and inability to reach and penetrate the target tissues still does not allow realizing their clinical potential.

Various strategies have been designed to improve the pharmacology of nucleic acids - based therapy. For example, nucleic acid modifications²⁷, fusogenic and non-fusogenic lipids, dendrimers²⁷⁻²⁸, liposomes^{18, 29}, cyclodextrin-based polycations²⁶, poly(ethylene glycol) protein based vectors³⁰⁻³¹ and targeting moieties have been used for protection and targeting of nucleic acids. Delivery of nucleic acids using nanoparticles is one of the most promising embodiments of nanotechnology. The ideal systemic delivery vehicle should be ~10-50 nm in size, stable, non-toxic, capable of efficient intracellular delivery, and should demonstrate specific targeting ability.

Targeting of nucleic acids-nanomedicines can further increase the therapeutic performance of encapsulated nucleic acids. For example, targeting to the diseased cell, tissue or organ not only enhance the silencing potency of siRNA nanoparticles³²⁻³³ but also prevent side effects by avoiding damaging of non-diseased cells. Expression of a variety of unique markers on the surfaces of the cancer cells, such as specific antigens and receptors provide valuable recognition sites for developing targeted nanomedicines. Antibodies, aptamers, small peptides and other ligands that bind to the signature molecules with high specificity and affinity have been studied extensively for their ability to stabilize siRNA⁴⁴ and guide it to the target tissues and cells³⁴⁻³⁵.

In this study, we first evaluated the potential of cancer specific phage fusion proteins as targeting ligands that not only navigate siRNA to its destination site in the target cell but also provides its encapsulation and protection. Our work demonstrated that fusion phage coat protein fpVIII displaying cancer-targeting peptides can effectively encapsulate siRNAs and deliver them into the target cells leading to gene silencing.

The tumor-specific proteins were isolated from the phages that were affinity selected from multibillion-clone landscape phage library f8/9 by their ability to bind very specifically

breast cancer cells. The phages VEEGGYIAA and DWRGDSMDS used in this work demonstrate high selectivity and specificity towards target cells versus control unrelated cells ¹⁴.

To obtain encapsulated model siRNAs, we used self-assembly of phage coat proteins in the presence of siRNAs – the process mimicking morphogenesis of filamentous phages in bacterial cells ⁴¹. During phage assembly, multiple copies of mature major coat protein accumulate in the internal bacterial membrane, which protects the protein against aggregation. In the presence of DNA, proteins aggregate around DNA forming a stable phage capsid. In our experiments the role of the stabilizer played cholate. We used 100 mM cholate during phage disintegration and protein chromatography, resulting in preparation of pure protein in the tetramer form, as evaluated by chromatography on calibrated column (not shown). Specific aggregation of proteins with siRNA was initiated by gradual decreasing of cholate concentration by ultrafiltration and dilution. The disappearance of siRNA encapsulated by pVIII phage coat protein on the gel stained with gel green stain and its appearance upon digestion of coat protein by proteinase K demonstrated formation of the siRNA-protein complex.

We hypothesized that the structure of the complex of siRNA with major coat protein is similar to the phage virion structure ⁴². In this complex, four positively charged lysine amino acid at the C-terminal end of the major coat protein interact with negatively charged phosphate groups of siRNA leading to the formation of very stable phage capsid, or siRNA-nanophage complex. The protein protects siRNA molecules in the same way as phage protects its DNA against degradation in unfavorable environmental conditions ³⁶ as demonstrated by the resistance of siRNA in the complex by serum nucleases. To check our hypothesis regarding the nanophage structure, we prepared the complex of siRNA with streptavidin-binding fpVIII protein and studied its interaction with streptavidin using ELISA. As shown in Fig. 3, complex of siRNA with fusion protein demonstrate slightly higher binding to streptavidin than corresponding parental phage 7b1. These results clearly show that the N-terminal binding peptide is exposed on the surface of the complex, while the C-terminus is probably bound to siRNA.

Effective NA delivery to the tumor site is mainly dependent on the size of the delivery vehicle, particles ranging from 10-100 nm ³⁷⁻³⁸ or as large as 200 nm ³⁹ being the optimal size. They are large enough to be retained by the body yet small to access the cell surface receptors and pass through the cell membrane by receptor mediated endocytosis ³⁸. However, nanoparticles with greater than 100 nm diameters are recognized by the reticuloendothelial system (RES) and responsible for their short half-life ⁴⁰. Atomic force microscopy of the highly diluted siRNA-nanophage particles immobilized on APTES functionalized mica revealed the complex as spherical structure of 11 nm diameter making it ideal for stabilization of siRNA and its efficient delivery to cytoplasm.

The efficiency of the complex to enter target cancer cells was demonstrated by fluorescence microscopy and quantitative uptake of siRNA. It was observed that Alexa-fluor 488 siRNA-nanophage VEEGGYIAA readily enters target cells, as indicated by appearance of diffuse fluorescence in MCF-7 cells after 4 and 24 h but does not enter into control MCF-10A cells,

Thus, targeting specificity of the cancer-specific selected phage is translated to major coat protein-siRNA complex. The punctuate fluorescence observed with Alexa-fluor 488 siRNA-nanophage DWRGDSMDS indicated the vesicular localization of the complex after 4 h of treatment. After 24 h fluorescence became diffuse, that may correspond to the escape of the siRNA from vesicles. Using fluorescent siRNA, it is shown that siRNA uptake increases with increasing molar ration of phage protein.

The ability of the complex to enter cells and efficiently deliver siRNA to the cytoplasm was further confirmed by its capacity to knockdown target gene in the cancer cells. As shown by Western blot analysis, the GAPDH siRNA-nanophages VEEGGYIAA and DWRGDSMDS efficiently inhibited synthesis of GAPDH protein in MCF-7 cells but not in MCF-10A cells implying the specific cell-targeting role of the fusion proteins in mediating delivery of siRNA into the cells. It is interesting to note that there is a significant difference ($p < 0.05$) between the silencing and inhibition of *GAPDH* protein synthesis by GAPDH siRNA-nanophages VEEGGYIAA and DWRGDSMDS. This difference might be due to the different mechanism of penetration of VEEGGYIAA and DWRGDSMDS phage proteins. As shown in Fig. 4A, siRNA-VEEGGYIAA nanophage showed cytoplasmic intracellular staining by 4 h of treatment indicating release of siRNA into the cytoplasm as early as 4 h while staining for siRNA-DWRGDSMDS was still vesicular and became cytoplasmic only during 24 h as evident by Fig. 4B.

Suggested delivery of siRNA to target cells via preselected phage proteins exploits the ability of the phage coat protein to interact with siRNA and form a self-assembled complex, which circumvents any chemical modification technique. Moreover, rapid identification of target specific phages by screening of polyvalent phage display libraries provides an opportunity to obtain a diverse repertoire of ligands that can provide an efficient target-specific siRNA delivery and gene-silencing *in vivo*.

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Fig 1.

Phage like nanoparticles or nanophage formed by mixing of cholera solubilized phage fusion protein with siRNA with the gradual removal of cholera. N-terminus of phage protein is the targeting ligand whereas positively charged C-terminal end of the major coat protein interact with negatively charged phosphate groups of siRNA.

A

B

C

D

E

Fig 2.

Analysis of the complex of siRNA with proteins (nanophage). A. UV spectroscopy of the siRNA (*left*), phage protein (*center*) and siRNA-nanophages (*right*). B. Left: Gel-electrophoresis analysis of the complexes (1% agarose, Gel Green stain). 1: siRNA control; 2-5: phage protein-siRNA (molar ratio 16:1-80:1) *Right*. Proteinase K-mediated release of siRNA from phage protein-siRNA complex. 1: siRNA control; 2: phage protein-siRNA (molar ratio 80:1), 3: Proteinase K-mediated release of siRNA from siRNA-nanophage. C. siRNA protection assay in serum. Degradation of siRNA exposed to serum was measured for siRNA-nanophage compared with free siRNA. The siRNA-nanophage, or free siRNA, was incubated in 50% serum for 24 h hour. Undegraded, intact siRNA is detected after polyacrylamide gel electrophoresis and SYBR green staining. 1) siRNA (not treated with 50% FBS), 2) siRNA (treated with 50%FBS), and 3) siRNA-nanophage (treated with 50% FBS) for 24 hrs. D. The atomic force microscopy of the siRNA-nanophage complex shows (A) a dense layer of the complex. (B) Nanolithography experiments demonstrating presence of a dense layer (step height ~2nm). The holes on the surrounding area are defects in the APTES layer. E. Phage protein and siRNA complex on different scan sizes (A-3 μ m, B-500 nm, C-1.5 μ m, D-250 nm). The diameter of the single complex is larger then the determined values because of the tip broadening effect.

Fig 3.

Analysis of activity of siRNA nanophage by ELISA. Phage 7b1 protein-siRNA complex, phage 7b1 protein (20 µg/ml), phage 7b1 (5×10^{11} vir), siRNA (10 µg/ml) and BSA (0.1%) were immobilized onto the wells of ELISA and reacted with graded concentrations of streptavidin labeled with alkaline phosphatase (AP-SA). Antigen remaining bound after washing was quantified as described in Materials and methods.

A**MCF-7 cells**

VEEGGYIAA

DWRGDSMDS

FITC Fluorescence

No Fluorescence

B**MCF-7 Cells**

VEEGGYIAA

DWRGDSMDS

FITC Fluorescence

No Fluorescence

C**MCF-10A cells** **Fig 4.**

A. Binding of Phage protein/siRNA complex (80:1) to (A) MCF-7 or (B) MCF-10A cells. A. MCF-7 cells were incubated with Alexa fluor 488 siRNA- VEEGGYIAA nano phage (*Upper*) or Alexa fluor 488 siRNA-DWRGDSMD nano phage (*Lower*) for 4 h. After 4 h, cells were washed with PBS twice, covered with coverslip and viewed under cyto viva fluorescence microscope. B. MCF-7 cells were incubated with Alexa fluor 488 siRNA- VEEGGYIAA nano phage (*Upper*) or Alexa fluor 488 siRNA -DWRGDSMD nano phage (*Lower*) for 24 hrs. After 24 hrs, cells were washed with PBS twice, covered with cover slip and viewed under cyto viva fluorescence microscope. C. MCF-10A cells were incubated with Alexa fluor 488 siRNA-VEEGGYIAA nano phage (*Upper*) or Alexa fluor 488 siRNA -DWRGDSMD nano phage (*Lower*) for 4 h. After 4 hrs, cells were washed with PBS twice, covered with coverslip and viewed under cyto viva fluorescence microscope.

Fig 5. siRNA delivery increases with peptide concentration. 0.5 μ g of alexa fluor 488 siRNA was complexed with different concentration of VEEGGYIAA protein (80:1, 40:1, 20:1 and 10:1) (mol protein:mol siRNA) or with lipofectamine. The complexes were laid on MCF-7 cells for 4 hrs, wells were washed with PBS 3 times, after which they were lysed, and then fluorescence was measured and normalized to protein content. Phage protein increased siRNA uptake in a concentration-dependent manner. Control – cells not treated, Lipofectamine-siRNA- cells treated with lipofectamine-siRNA complex.

Fig 6.

Inhibition of GAPDH protein synthesis by Western blot. MCF-7 or MCF-10A cells were treated with GAPDH siRNA-VEEGGYIAA nano phage (80/1) (50 nM final siRNA concentration) or negative siRNA-VEEGGYIAA nano phage (50 nM) and incubated for 72 h. A. Relative level of protein synthesis in cells treated with: 1. VEEGGYIAA nanophage MCF-7-targeted GAPDH siRNA, 2. VEEGGYIAA nanophage MCF-7-targeted negative siRNA, 3. Control non-treated MCF7-cells, 4. VEEGGYIAA nanophage - MCF-10A targeted GAPDH siRNA, 5. Control non-treated MCF10A-cells. B. Western blot band intensities quantified using actin as a control for quantification by Image J software (NIH). All data represent the mean $\pm$ S.D. * $p < 0.05$, student-t-test.

Fig 7. Inhibition of GAPDH protein synthesis by Western blot. MCF-7 or MCF-10A cells were treated with GAPDH siRNA- DWRGDSMDS nanophage (80/1) (50 nM final siRNA concentration) or negative siRNA -DWRGDSMDS nano phage (50 nM) and incubated for 72 h. A. Relative level of protein synthesis in cells treated with: 1. DWRGDSMDS nanophage MCF-7-targeted GAPDH siRNA, 2. DWRGDSMDS nanophage MCF-7-targeted negative siRNA, 3. Control non-treated MCF7-cells, 4. DWRGDSMDS nanophage protein-MCF-10A targeted GAPDH siRNA, 5. Control non-treated MCF10A-cells. B. Western blot band intensities quantified using actin as a control for quantification by Image J software (NIH). All data represent the mean $\pm$ S.D. * $p < 0.05$, student-t-test.