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Development of a Recombinant Multifunctional Biomacromolecule for Targeted Gene Transfer to Prostate Cancer Cells

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Abstract

The objective of this study was to genetically engineer a fully functional single chain fusion peptide composed of motifs from diverse biological and synthetic origins that can perform multiple tasks including DNA condensation, cell targeting, cell transfection, particle shielding from immune system and effective gene transfer to prostate tumors. To achieve the objective, a single chain biomacromolecule (vector) consisted of four repeatative units of histone H2A peptide, fusogenic peptide GALA, short elastin-like peptide and PC-3 cell targeting peptide was designed. To examine the functionality of each motif in the vector sequence, it was characterized in terms of size and zeta potential by Zetasizer, PC-3 cell targeting and transfection by flowcytometry, IgG induction by immunogenicity assay and PC-3 tumor transfection by quantitative live animal imaging. Overall, the results of this study showed the possibility of using genetic engineering techniques to program various functionalities into one single chain vector and create a multifunctional non-immunogenic biomacromolecule for targeted gene transfer to prostate cancer cells. This proof-of-concept study is a significant step forward towards creating a library of vectors for targeted gene transfer to any cancer cell type at both in vitro and in vivo levels.

Keywords

gene delivery; recombinant vector; PC-3 cells; cancer gene therapy; biomimetic vector; fusion peptide

Introduction

The majority of human prostate cancer cell lines including the classical cell line PC-3 are reported to be androgen independent¹. This cell line represents metastatic prostate cancer cells that are characterized to be CAR⁻/HER2⁻. As a result, this type of prostate cancer may

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Supporting Information

Supporting Figures 1, 2 and 3. This material is available free of charge via the Internet at <http://pubs.acs.org>.

not be treated either by anti-HER2 immunotherapy or adenoviral gene therapy. Consequently, development of a therapeutic modality for this form of prostate cancer would be valuable to those patients who are suffering from this type of disease. Among the new emerging strategies for cancer therapy, gene directed enzyme prodrug therapy (GDEPT) is an attractive method in which the enzyme-coding gene is selectively delivered to cancer cells followed by the systemic administration of a prodrug^{2, 3}. The accumulation of the prodrug's toxic metabolites then causes target-specific death in the enzyme-bearing cancer cells and the surrounding ones through a phenomenon known as bystander effect⁴. This phenomenon can explain the observations that transduction of even less than 10% of tumor cells could be sufficient to eradicate the whole cancer cell population^{5, 6}. Unfortunately, one critical problem that currently restricts progress in cancer suicide gene therapy is poor gene transfer efficiency of the vectors, resulting in an inefficient drug delivery to the tumor environment. To overcome this obstacle, various research groups are working to develop highly efficient gene delivery systems using viral, non-viral, cell-based and bacterial-based vectors for cancer gene therapy⁷⁻⁹.

In parallel to the work of others and in an attempt to create a simple but efficient gene delivery system, our lab in the past decade has utilized genetic engineering techniques to develop single chain multifunctional biomacromolecules for targeted gene transfer to mammalian cells¹⁰⁻¹⁵. Using this approach, the *objective* of this study was to create a single chain multifunctional vector that could be used for targeted gene transfer to CAR⁻/HER2⁻ PC-3 prostate cancer cells in vitro and in vivo. To achieve the objective, we designed and genetically engineered a single chain peptide composed of the following segments: 1) PC-3 cell targeting peptide (Tp), 2) four repetitive sequences of histone H2A (H4) to enable pDNA condensation into nanosize particles, 3) fusogenic peptide known as GALA (G) to facilitate endosomal escape, and 4) (VPGSG)₅ low-immunogenic elastin-like peptide to minimize recognition of the peptide by the immune system and generation of neutralizing IgG antibodies. Each segment in the vector structure is rationally designed to achieve a certain pre-determined function. For example, the core structure of this vector is based on four repeating sequences of histone H2A which we have shown to not only condense pDNA, but also bears an inherent nuclear localization signal¹⁵. Among the most widely studied cationic and anionic pH-responsive fusogenic peptides; i.e., KALA, GALA, RALA, INF7 and H5WYG, we selected GALA in the design of the vector structure because our previous studies demonstrated its superior safety and activity¹⁶. To tailor make this vector for targeted gene transfer to PC-3 prostate cancer cells, we took advantage of a peptide sequence (CPGDRGQRRFLFSKIEGPC) that has been reported to bind to PC-3 cancer cells specifically but not normal human prostate cells¹⁷. This targeting peptide is reported to have no sequence similarity to bombesin, LHRH and prostate-specific antigen as confirmed by a search through Swiss-Prot databases and European Molecular Biology Laboratory¹⁷. This indicates that this peptide could be binding to an antigen that is specifically expressed on PC-3 cells but has not been exploited for targeted therapy before. Since one of the main obstacles facing nanotechnology-based drug delivery systems is their recognition by the immune system and secretion of neutralizing IgG antibodies after repetitive administrations, an elastin-like peptide (E) was also designed in the vector structure to reduce the potential of IgG response. Elastin is a protein that is expressed extracellularly and composed of few

repetitive amino acid residues. This includes the repetitive V-P-G-X-G amino acid sequence¹⁸. The type of X amino acid in the sequence of elastin can be changed to impart a variety of hydrophilicity or hydrophobicity properties¹⁹. Since we have previously demonstrated that an elastin-like peptide based on short repeating units of VPGSG (abbreviated as E_S) does not induce IgG production²⁰, we incorporated this sequence in the vector structure to make our multifunctional targeted fusion vector, namely TpE_SH4G. As a positive immunogenic peptide control, we genetically engineered TpE_EH4G where the X guest residue in the elastin sequence is glutamic acid (E)²⁰. All multifunctional fusion vectors were biosynthesized in *E. coli*, purified and then examined to validate the functionality of each motif. The vectors' ability to condense plasmid DNA (pDNA) into nanosize particles were studied by Malvern Zetasizer. The transfection efficiency of each vector was evaluated by flowcytometry and from that the most efficient construct was selected. The ability to target PC-3 prostate cancer cells but not normal prostate cells was also investigated. The suitability of the selected vector to efficiently deliver suicide genes to PC-3 cancer cells and resulting in significant cytotoxicity was evaluated. Ultimately, the potential immunogenicity of the vector in immunocompetent mice was studied by immunogenicity assay and the ability to deliver pDNA and transfect PC-3 tumors by quantitative live animal imaging.

Materials and Methods

Cloning, expression and purification of the recombinant vectors

The genes coding for recombinant vectors H4G (non-targeted), TpH4G (PC-3 targeted vector without elastin), TpE_SH4G (PC-3 targeted vector with elastin E_S), TpE_EH4G (PC-3 targeted vector with elastin E_E) were synthesized by Integrated DNA Technologies (Coralville, IA) (Table 1). The synthesized genes were then cloned into a pET21b expression vector (Novagen, MA) using standard cloning techniques as described previously by our group^{12, 15}. After verifying the sequences, each plasmid was transformed into *E. coli* BL21(DE3) pLysS (Novagen, MA) expression host. To prepare a starter culture, a single colony of BL21(DE3) pLysS was picked to inoculate 5 mL of Ciclegrow® media (MP Biomedicals) supplemented with 50 µg/ml carbenicillin and incubated overnight at 30°C. The next day, the whole starter culture was transferred into 500 mL of Ciclegrow® media supplemented with carbenicillin (50 µg/mL) and shaken at 30°C to reach OD₆₀₀ ~0.5. Then, 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was used to induce gene expression at 30°C. The cells were collected after 6 hours by centrifugation at 5000 g for 20 min and stored at -80°C freezer for further use.

All recombinant vectors were purified by utilizing Nickle column affinity chromatography. To purify, For each 1 gram of cell pellet, 20 ml lysis buffer (5M urea, 100mM NaH₂PO₄, 10mM Tris, 1M NaCl, 1% Triton, 10mM imidazole, pH 8) was added and the suspension was mixed for 1 hour. The lysate was centrifuged at 20,000 rpm for 1 hour at 4 °C to pellet the insoluble fraction. The supernatant was collected and transferred to a different tube containing 1ml of Ni-NTA agarose (Qiagen) equilibrated with lysis buffer. The slurry was shaken on ice for one hour followed by loading onto the column. First, the column was washed with 100 ml of lysis buffer followed by 40 ml of wash buffer composed of 5M urea,

100mM NaH₂PO₄, 10 mM Tris, 1 M NaCl, imidazole 40 mM, pH 8. The peptide was eluted by elution buffer composed of 3M urea, 100 mM NaH₂PO₄, 0.5 M NaCl, 10 mM Tris, 200 mM imidazole, pH 8) and stored at -20 °C.

Peptide desalting and preparation of stock solution

For desalting the peptides, a disposable PD-10 desalting column with Sephadex G-25 resin (GE Health Care, MA, US) was equilibrated with 100mM HEPES buffer (pH 7.4)²⁰. Then each purified peptide fraction was loaded onto the column and eluted with additional 4mL of buffer driven by gravity. The concentration of each eluent was measured by Bradford protein assay kit and protocol (Hercules, CA). A standard curve was plotted using the provided bovine serum albumins and the absorbances of samples were measured at 595 nm.

Nanoparticle size and surface charge analysis

Nanoparticles were prepared and characterized as reported previously by our group²⁰. In brief, to prepare nanoparticles, total amount of vector required for a specific N:P (nitrogen to phosphate) ratio was determined. Then, vector in 50 µL of 100mM HEPES buffer (pH 7.4) was flash mixed with 1 µg of plasmid DNA (pEGFP) to prepare nanocomplexes at different N:P ratios in a total volume of 100µL. Flash mixing, also known as flash nanoprecipitation, means instant addition of vector solution to pEGFP solution. For instance, to prepare N:P ratio of 1, 1.45 µg of H4G, 1.60 µg TpE_SH4G, 1.81 µg of TpE_EH4G or 1.52 µg of TpH4G was used to complex with 1µg of pEGFP. Three batches of each sample were prepared independently and the mean hydrodynamic size and zeta potential of the nanoparticles determined by using NanoZS Zetasizer (Malvern Instruments, UK). The results are reported as mean ± s.d (n=3).

Cell transfection studies

For cell transfection and measurement of percent transfected cells by flow cytometry, we followed our previously published standard protocol¹⁶. In brief, PC-3, RWPE-1 and SKOV-3 cells were purchased from ATCC (Manassas, VA), seeded in 96-well plates at the density of 4×10^4 cells per well and incubated at 37°C in appropriate growth media as recommended by ATCC overnight to reach approximately 70% confluency. Immediately before transfection, the media was replaced with 200 µl of basal media supplemented with ovalbumin, human insulin, selenious acid, human transferrin, fibronectin and dexamethasone. Using 1 µg of pEGFP, nanocomplexes were prepared as mentioned above at N:P ratios of 8 to 12. Nanocomplexes were added into each well and incubated at 37°C for four hours. Then, the media was replaced with 300 µl of appropriate media for each cell line supplemented with 10% FBS.

To observe the expression of green fluorescent protein (GFP), a fluorescent microscope was used. To prepare cells for analysis by flow cytometer, they were first trypsinized and then collected by centrifugation at 300 g for 6 min. The collected cells were then analyzed by a flowcytometer to determine not only the percentage of transfected cells, but also GFP expression. For each sample, 5000 cells were counted and analyzed. The total green fluorescent intensity of transfected cells was normalized to the total fluorescent intensity of untransfected ones (background control). Then, the percentage of transfected cells was

quantified by using Kaluza software (Beckman Coulter). Total green fluorescent intensity (TFI), which is indicative of green fluorescent protein expression was also measured as reported previously¹⁶. The data are presented as mean $\pm$ s.d (n=3).

Cell viability studies

To examine vectors' toxicity to PC-3 cancer cells, a WST-1 cell proliferation assay (Roche, Nutley, NJ) was performed. Cells were seeded at the density of 4×10^4 cells per well in a 96-well plate. Nanocomplexes carrying 15 μ g of pEGFP were prepared and used to transfect cells as described above. After twenty four hours, WST-1 reagent was added into each well and cell toxicity was assessed by measuring the absorbance of media at 450 nm. The absorbance of wells that contained untransfected cells was considered as 100% viable. The results are presented as mean $\pm$ s.d (n=3).

For suicide gene therapy of PC-3 cancer cells with yeast cytosine deaminase: uracil phosphoribosyltransferase (yCD:UPRT), a pDNA was genetically engineered to express yCD:UPRT under EF1 α promoter as described previously²¹. The plasmid was complexed with the TpEsH4G to form nanoparticles and used to transfect 4×10^4 cells seeded in a 96-well plate. Twenty four hours post transfection, the cells were treated with various concentrations of prodrug 5-FC and incubated for 96 hours. The ability of the yCD:UPRT/5-FC enzyme/prodrug system to kill PC-3 cancer cells was measured by WST-1 assay as mentioned above.

Evaluation of IgG response against vectors

We have previously reported a method for the assessment of the IgG response against systemically administered nanoparticles²⁰. Following this method, immune-competent Balb/c mice with an average age of 6–8 weeks old (~25 g) were purchased from Jackson Labs (Maine, USA). All mice were cared for following the Rutgers's IACUC approved protocol. Mice were distributed into different groups (5mice/group) and injected with pDNA alone (5 μ g) or in complex with TpH4G, TpEsH4G or TpE_HH4G at N:P ratio of 10. Here, we used a pCpGfree pDNA (Invivogen) which is engineered to be free of any CpG Islands. For its propagation, a GT115 E. coli strain (Invivogen) was used. Using a 5 mm animal lancet (GoldenRod, NY), ~200 μ l of blood was collected via submandibular puncture from each mouse and transferred into a 1.5ml tube. To pellet the cells, the collected blood was centrifuged for 10 minutes at 15,000 rpm. The supernatant (plasma) was transferred into a new tube and stored at -80°C for future use. On days 7 and 21, mice received nanoparticle injections via the venous sinus (retroorbital) using a 27 G needle to immunize. In comparison to tail vein injection, the retroorbital route for systemic delivery is considered a more reliable method²². All mice were euthanized on day 28 and blood was collected via cardiac puncture using a 25G needle. The collected blood samples were transferred into 1.5 ml tubes and processed as above. Enzyme-Linked Immunosorbent Assay (Bethyl laboratories, TX) was performed to measure the concentration of IgG in plasma samples following manufacturer's kit and protocol.

In vivo tumor transfection studies

For quantitative in vivo transfection studies, we followed our previously published method²¹. In brief, male nude mice (4–6 weeks old) were purchased from Jackson laboratory (Bar Harbor, ME). All mice were cared for following the Rutgers's IACUC approved protocols. Mice were first anesthetized by isoflurane inhalation. Then, 5×10^6 PC-3 cells which were suspended in 100 μ l Dulbecco's PBS (50% Matrigel®) implanted subcutaneously in dorsal flanks to generate two tumors in each mouse. The tumor size change was measured by a caliper until reached 200–300 mm³. At this point, all mice were randomly divided into three groups each containing three mice. First group received retroorbital injection of TpH4G/pLuc complexes (equivalent to 15 μ g pDNA). Second group received retroorbital injection of TpEsH4G/pLuc complexes (equivalent to 15 μ g pDNA). Third group received retroorbital injection of H4G/pLuc (equivalent to 15 μ g pDNA). Twenty four hours post injection, D-luciferin was administered (150 mg/Kg) intraperitoneally and mice were imaged by IVIS Spectrum In Vivo imaging system (Perkin Elmer Inc. USA) to determine the total luciferase expression. The mice bioluminescent images are presented in “photon” mode; thereby, the signal intensity represents radiance (p/sec/cm²/sr). This indicates the number of photons per second that are leaving a square centimeter of tissue and radiating into a solid angle of one steradian (sr). The results are reported as mean $\pm$ s.d (n=6).

Results and Discussion

The concept of engineering recombinant fusion vectors for gene delivery dates back to late 1990s²³. However, due to significant technical difficulties related to fusion peptide production and formulation of stable and efficient nanoparticles, recombinant fusion vectors remained ineffective for more than a decade as reviewed and discussed previously²⁴. Since 2006, we have worked to overcome these challenges and through use of several innovative approaches have successfully created highly efficient receptor targeted fusion vectors for various gene delivery needs including targeting different cancer cell types or compartments inside the cells^{11, 13, 14, 25, 26}. To address the discussed deficiencies for prostate cancer cell targeting and overcome the obstacles, we used genetic engineering techniques to create a novel fusion vector for targeted gene delivery to PC-3 prostate cancer cells. First, we cloned the genes encoding H4G, TpH4G, TpEsH4G, TpE_HH4G fusion peptides into pET21b plasmid and verified the fidelity of the sequences to the original design by DNA sequencing. Then, the genes were transformed into an E. coli expression host and purified (Figure 1).

The purified vectors were complexed with pEGFP to form nanoparticles and characterized in terms of size and surface charge. The results of this study revealed that all four vectors could condense pEGFP into nanosize particles at N:P ratios above 4 (Figure 2). For all four vectors, the nanoparticle sizes appeared to stabilize and remain statistically the same at N:P ratios of 8, 10 and 12 ($p > 0.05$). The statistical analysis of surface charge revealed that TpEsH4G and TpE_HH4G had statistically similar surface charges at N:P ratios of 8, 10 and 12 ($p > 0.05$). However, the TpH4G had statistically higher surface charge in comparison to the other two targeted vectors and at all three N:P ratios ($p < 0.05$). In addition, we performed the same size/charge analyses on the control vector E_SH4G and the results revealed formation of condensed nanoparticles with surface charges close to zero; suggesting the

presence of ELP motif on the surface (Supporting Figure 1). These results indicate that the elastin motif in the vector sequence did not interfere with the pDNA condensation process but contributed to reducing the surface charge of the nanoparticles. These observations were in line with our previous studies corroborating that the H4 core was highly efficient in pDNA condensation and also presence of short repeating units of elastin-like peptide did not interfere with the nanoparticle formation process^{12, 20}. The nanoparticles formed with H4G (non-targeted vector) carried significantly higher surface charges in comparison to the other three targeted vectors. In summary, we observed the following order of surface charges among the above mentioned vectors: H4G>TpH4G>TpE_SH4G=TpE_EH4G>E_SH4G. It is noteworthy that the targeting peptide has four positively charged residues in its sequence which may have contributed to increasing the surface charges of the TpE_SH4G nanoparticles to slightly above zero. These observations altogether point out that the presence of elastin motif and targeting peptide could effectively mask the high positive surface charges of nanoparticles and also indirectly indicate the targeting peptide in the targeted vectors (i.e., TpE_SH4G, TpE_EH4G and TpH4G) was most likely exposed on the surface.

To examine the functionality of the targeting peptide, we selected TpH4G targeted vector as an example, complexed with pEGFP and used to transfect PC-3 prostate cancer cells as well as RWPE-1 normal prostate cells and SKOV-3 ovarian cancer cells. The H4G vector which is non-targeted was used as control. The results of this study showed TpH4G could effectively transfect PC-3 cells, but not RWPE-1 and SKOV-3 cell lines (Figure 3). In contrast, the non-targeted H4G vector transfected all three cell lines efficiently. These results indicated that the targeting peptide could facilitate internalization of TpH4G into PC-3 cells but not the other two cell lines. A complementary inhibition assay also confirmed the ability of the TpH4G vector to target receptors on the surface of PC-3 cells (Supporting Figure 3). Considering the low surface charge of the TpH4G nanoparticles, the non-specific cell transfection rates in RWPE-1 and SKOV-3 cells were low. In our previous studies where the H4G vector was equipped with a HER2 targeting peptide, the vector could efficiently transfect HER2 overexpressing SKOV-3 cells¹⁵. It is interesting to see that a simple replacement of one targeting peptide with another the ability of the vector in cell transfection can be easily modulated. We believe this is significant as it could open the door for the production of a library of H4G-based vectors tailor-made for transfection of various cell lines.

In the next step, we investigated the impact of elastin-like peptide in the vector structure on gene transfer efficiency. For this purpose, all three targeted vectors (i.e., TpH4G, TpE_EH4G and TpE_SH4G) were complexed with pEGFP to form nanoparticles and used to transfect PC-3 cancer cells at N:P ratios of 8, 10 and 12. We only looked at these three N:P ratios because the particle size data showed the highest rate of nanoparticle size stability at these ratios. The results of this study showed TpE_SH4G at N:P 10 had the highest transfection efficiency (Figure 4). This was an interesting observation because despite the lower surface charge of the TpE_SH4G nanoparticles in comparison to TpH4G, the former could induce a higher rate of gene transfer. Since the E_SH4G control vector almost failed to transfect PC-3 cells (Supporting Figure 2), one possible reason for higher efficiency of TpE_SH4G over TpH4G is that the presence of elastin motif may have helped the targeting peptide to

protrude with more flexibility and fit better into the receptor cavity facilitating more effective internalization.

To determine whether any of the vectors were toxic to the cells that could negatively impact transfection efficiency, a WST-1 cell toxicity assay was carried out. As shown in Figure 5A, none of the vectors were toxic in the range tested. Learning that the vectors are not toxic at N:P 10, we then examined whether the level of gene expression in transfected cells is high enough to produce a meaningful therapeutic response. For this, we transfected PC-3 cells with TpE_SH4G carrying plasmid DNA that encoded yCD:UPRT followed by the administration of increasing amounts of 5-FC prodrug. The rationale is that the transfected PC-3 cells will produce a sufficiently high concentration of yCD:UPRT to convert non-toxic 5-FC into cytotoxic 5-FU resulting in the death of transfected cells. Among various enzyme/prodrug systems that exists⁴, we chose yCD:UPRT/5-FC system because we have shown that this system is far more effective than thymidine kinase/ganciclovir or nitroreductase/CB1954 systems²¹. The results of this study showed approximately 50% of the PC-3 cells could be killed when transfected with TpE_SH4G in complex with 0.5µg of yCD:UPRT and in combination with 25 µM 5-FC (Figure 5B). This suggests that TpE_SH4G was able to transfect not only high numbers of PC-3 cells but also generate high amounts of enzymes in each transfected cell.

So far, the obtained data demonstrate that the H4G and Tp motifs in TpE_SH4G structure are active and can help target and efficiently transfect PC-3 cancer cells. The level of gene expression is also high enough to induce significant cell death when suicide gene therapy is applied. To examine whether the elastin motif is also functional and can minimize the induction of IgG production, we performed an immunogenicity assay. In the past, we have shown that presence of short repeating units of VPGSG in the peptide/pDNA nanocomplexes mitigates IgG response whereas VPGEG and VPGKG enhance IgG response²⁰. Using the same approach, we injected immuno-competent mice with TpE_SH4G in complex with pCpGfree plasmid as per protocol shown in Figure 6A. Then, the IgG response which is indicative of long-term high affinity immune response against immunogens was evaluated. Here, we used a pCpG free plasmid because it is well-understood that CpG islands in plasmids are potent inducers of IgG response^{27, 28}. Our previous studies have also shown that the pCpGfree plasmid that we used in this study is non-immunogenic²⁰. The results of this study showed that TpE_SH4G nanoparticles did not induce IgG response but TpE_FH4G nanoparticles did which substantiated our previous observations²⁰. We also noticed that TpH4G on average increased the IgG production although statistically speaking it was not significant. While various factors influence IgG response against foreign materials including physico-chemical properties of immunogens (e.g., foreignness, size, complexity of structure, physical form and degradability), one important element is antigen dose 29, 30. Therefore, there is a high probability that TpH4G could be immunogenic if higher doses were used (e.g., >5µg). Here we focused on IgG response only (long-term immune response) because it is one of the major contributing factors to the clearance of nanoparticles. In addition, we were very much interested to know whether VPGSG sequence in the structure of the H4G-based vector could mitigate IgG response similar to what we observed for vectors with significantly different sequences²⁰. Since our observations in this study are in agreement with our previous immunogenicity

studies on VPGSG- and VPGEg-based elastin-like peptides²⁰, we believe that further in depth look at the potential use of elastin based peptides for nanoparticle shielding is warranted.

The presented data thus far demonstrate that each motif in the TpE₅H4G structure is active and functional. In the next and final step, we evaluated the ability of the TpE₅H4G to remain stable in the blood stream in order to reach and transfect the PC-3 tumors. For this purpose, we used TpE₅H4G to complex with pLuc (encodes luciferase) and injected systemically in nude mice bearing subcutaneous PC-3 tumors on their flanks. As controls, TpH4G and H4G vectors were also used. The quantitative imaging data showed a significantly high gene expression levels in PC-3 tumors that were transfected with TpE₅H4G and almost no gene expression with H4G transfected tumors (Figure 7). Considering the high positive surface charge of the H4G nanoparticles, lack of gene expression in this control group was understandable as the majority of them were most likely cleared from the blood stream through opsonization followed by macrophage phagocytosis. It is well-understood that highly positively charged nanoparticles may interact electrostatically with negatively charged albumin in the blood and cleared^{31, 32}. The TpH4G targeted vector could transfect the tumors but with significantly less efficiency than TpE₅H4G. This observation points to the positive effect of elastin motif in the vector structure on transfection efficiency either by keeping the nanoparticles more stable or prolonging the circulation time of the nanoparticles. It is important to mention that TpE₅H4G vector may have transfected other non-targeted organs (e.g., liver, spleen, etc.) but the level of transfection were so low that the imaging system could not differentiate from the background tissue auto-bioluminescence. At this stage, we did not intend to perform a comprehensive biodistribution study as the evaluation of the pharmacokinetics of nanoparticles was outside the scope of this research. However, before considering the suitability of the developed vector for clinical translation, more in depth immunogenicity studies (acute and chronic) as well as biodistribution and therapeutic efficacy studies are needed. Nonetheless, these results show the merit of the approach in designing multifunctional vectors for targeted cancer gene therapy.

Conclusion

Using genetic engineering techniques, we created a single chain fusion peptide that could condense pDNA into nanosize particles with very low positive surface charge, efficiently transfect PC-3 cells, minimize the possibility of IgG immune response and effectively transfect xenograft PC-3 tumors. The presented data demonstrate that by using our approach multiple functional domains can be fused together without the loss of functionality given that thousands of vector molecules come together to condense one plasmid DNA into a nanosize particle. Considering that the genetic engineering approach provides this opportunity for the vector developers to design targeted gene carriers in one single step without the need for using multiple chemical conjugation steps, one may envision facile creation of a large library of vectors equipped with various targeting moieties for targeted gene transfer to any cancer cell type.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. SDS-PAGE analysis of the purified fusion peptides. The theoretical molecular weights of H4G, TpH4G, TpEsH4G and TpEH4G are 19,752 Da, 23,109 Da, 24,352 Da and 24,562 Da, respectively.

Figure 2.

The analysis of particle size and charge for TpH4G (A), TpE_HH4G (B), TpE_sH4G (C) and H4G (D) in complex with pEGFP at various N:P ratios.

Figure 3.

Qualitative (fluorescent microscopy) and quantitative (flow cytometry) demonstration of the targeted gene transfer to PC-3 cells with TpH4G/pEGFP complexes (N:P 10) and non-targeted gene transfer by H4G/pEGFP (N:P 10).

Figure 4.

Evaluation of the transfection efficiency of T_pH4G , T_pE_H4G and T_pE_S4G vectors in complexation with pEGFP at N:P ratios of 8, 10, and 12. **A)** Flow cytometry histograms displaying the percentage of GFP positive cells. Each display is an overlay of three independent histograms. **B)** Fluorescent microscopy images of the transfected PC3-3 cells. **C)** A bar chart that summarizes the percent transfection and total green fluorescent protein expression in each group. The results are presented as mean $\pm$ s.d (n = 3).

Figure 5. WST-1 cell toxicity assay. **A)** Percentage of viable PC-3 cells after transfection with TpH4G, TpE_HH4G and TpE_SH4G vectors in complexation with 1 μg pEGFP. **B)** Evaluation of cell killing efficiency of TpE_SH4G vector in complexation with plasmid encoding yCD:UPRT and in combination with various concentrations of 5-FC. * indicates statistical significance.

Figure 6.

Evaluation of IgG response against the vector/pDNA nanocomplexes. A) The dosing schedule that was used to immunize mice and induce IgG response. B) Measurement of fold increase in IgG response against vector/pCpGfree nanocomplexes and control groups. Statistical significance is shown by * ($p < 0.05$).

Figure 7.

Measurement of luciferase expression in transfected PC-3 tumors. A-C) Bioluminescence intensity of PC-3 tumors transfected with H4G/pLuc, TpH4G/pLuc and TpEsH4G/pLuc nanoparticles. D) Quantitative analysis of gene expression in transfected tumors. The gene expression in tumors transfected with H4G was not quantified as they did not show any detectable bioluminescence. The results are shown as mean $\pm$ s.d. (n=6). * indicates statistical significance.

Table 1

The amino acid sequences of the designed recombinant vectors

Name	Targeting Peptide	ELP Sequence	DNA Condensing Motif	Fusogenic Peptide
H4G	N/A	N/A	(SGRGKQGGKKARAKAKTRSSRAGLQFPVGRVHRLLRK)4	WEAALAEALAEALAEHLAEALAEALAEALAA
TpH4G	CPGDRGQRRRLFSKIEGPC	N/A	(SGRGKQGGKKARAKAKTRSSRAGLQFPVGRVHRLLRK)4	WEAALAEALAEALAEHLAEALAEALAEALAA
TpEsH4G	CPGDRGQRRRLFSKIEGPC	(VPGSG)5	(SGRGKQGGKKARAKAKTRSSRAGLQFPVGRVHRLLRK)4	WEAALAEALAEALAEHLAEALAEALAEALAA
TpE _F H4G	CPGDRGQRRRLFSKIEGPC	(VPGE _F G)5	(SGRGKQGGKKARAKAKTRSSRAGLQFPVGRVHRLLRK)4	WEAALAEALAEALAEHLAEALAEALAEALAA