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14. ABSTRACT
The purpose of this research was to develop a gene delivery system that can target breast cancer cells specifically and transfect them efficiently. A biomimetic vector was genetically engineered to contain at precise locations: a) an adenovirus μ peptide to condense pDNA into nanosize particles, b) a synthetic peptide to target breast cancer cells, c) a pH-responsive synthetic fusogenic peptide to disrupt endosome membranes and facilitate escape of the nanoparticles into the cytosol, and d) a nuclear localization signal from human immuno-deficiency virus to transfer the genetic material to the nucleus. The vector was cloned and expressed in E.coli expression system followed by purification. The purified vector was able to condense plasmid DNA (pDNA) into nanosize carriers (~60nm) and protect pDNA from serum endonucleases. The vector could target ZR-75-1 breast cancer cells and internalize, efficiently disrupt endosome membranes and escape into cytosol, and mediate efficient gene transfer. The vector did not show significant binding to normal mammary cells. The vector did not show any significant cell toxicity. When complexed with plasmid DNA encoding TRAIL gene, the vector was able to kill ca. 60% of the ZR-75-1 cancer cells.

15. SUBJECT TERMS

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Introduction

Prologue: An annual progress report was submitted covering the period of 1/9/2007 to 31/8/2008. Although approved, the reviewers cited two important shortcomings:

1- The PI did not cite specific figures in the appended manuscript to support the experimental findings.

2- Acronyms were not defined upon first use.

As a result, the reviewers tagged Tasks 2 and 3 as "In progress". Task 1 was found complete.

In this final report, I would like to address the shortcomings by providing a list of abbreviations and citing specific figures in the appended manuscript. I hope that you find my report satisfactory.

List of Abbreviations:

CCS: Cathepsin D Cleavage Site
DCM: DNA Condensing Motif
EDM: Endosome Disrupting Motif
pDNA: plasmid DNA
NLS: Nuclear Localization Signal
TP: Targeting Peptide

The **overall purpose** of this project was to develop a new generation of non-viral vectors that can target and transfect breast cancer cells efficiently. **The rationale** is that a multi-domain recombinant vector based on a One-Domain-One-Function architecture can effectively perform an array of functions including a) self assembly into vector/DNA nanoparticles i.e., packaging the therapeutic gene into right sized packets, b) self-guidance, i.e. identification and targeting the breast cancer cells for specificity, c) self-preservation, i.e., escaping the endosomal compartment and nucleases inside the cells and, d) assured-delivery, i.e., successful localization into the nucleus for the expression of therapeutic gene. Two Specific Aims were proposed:

a) Synthesize four designer vectors (DCM, NLS-DCM, NLS-DCM-EDM, and NLS-DCM-EDM-CCS-TP).

b) Complex the vectors with pDNA to form nanoparticles and characterize the complexes using physicochemical and biological assays.

Body

Task 1- Synthesize/Biosynthesize DCM, NLS-DCM, NLS-DCM-EDM and NLS-DCM-EDM-CCS-TP.

This task was found complete by the reviewers of our annual report.

Task 2- Characterize the vectors using physicochemical and biological assays.

To perform the task:

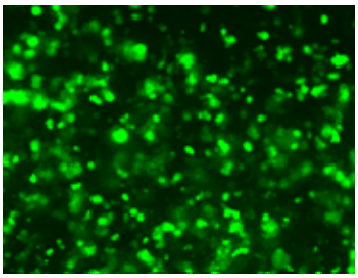
2.1. The following five vectors were complexed with pDNA encoding Green Fluorescent Protein (pEGFP): DCM, NLS-DCM, NLS-DCM-EDM, DCM-EDM-CCS-TP, and NLS-DCM-EDM-CCS-TP. This was to evaluate their ability to condense pEGFP into nanosize particles and mediate gene transfer.

They were all able to condense plasmid DNA into particles with sizes below 100nm. However, DCM, NLS-DCM and NLS-DCM-EDM were not able to mediate efficient gene transfer (<1%). Therefore, they were eliminated from the studies. This was attributed to the lack of targeting motif in their structure. The DNA condensation data for the complete vector (NLS-DCM-EDM-CCS-TP) is shown in **Figure 3a and b** of the attached manuscript (Appendix A)**.** The vector was able to mediate efficient gene transfer in both monomer and dimer configuration **(Figure 4a)**. The vector without NLS (DCM-EDM-CCS-TP) was able to condense pEGFP into nanoparticles with 70±5nm (mean±SEM) in diameter. However, the comparison of transfection efficiency between DCM-EDM-CCS-TP and NLS-DCM-EDM-CCS-TP revealed that the vector with NLS is more efficient (**Figure 6b)**.

2.2. The pH dependent hemolytic activity of NLS-DCM-EDM-CCS-TP was studied at pH 5.0, 7.0 and 7.4. The vector showed significant hemolytic activity at pH 5.0 but not at 7.4. Please see **Figure 2b** in the attached manuscript.

2.3. The vector/pEGFP complexes were examined in terms of protection of plasmid DNA from serum endonucleases **(Figure 4b)**, receptor mediated endocytosis of the nanoparticles **(Figure 5a)**, ZR-75-1 breast cancer cell targeting **(Figure 5b)**, and cell toxicity and therapeutic potential **(Figure 7)**. Cell transfection studies were performed using breast cancer (ZR-75-1) and normal human mammary cells (MCF-10A) **(Figure 5b)**. Cells were also transfected with the vector/pEGFP complexes in the presence of chloroquine, bafilomycine and nocodazole. The transfection efficiency was measured using a flow-cytometer **(Figure 6a)**. Presence of chloroquine had no significant effect on transfection efficiency indicating that the fusion peptide EDM was efficient in endosome membrane disruption and release of the trapped nanoparticles. The microtubule mediated localization of nanoparticles into the nucleus was disrupted upon addition of nocodazole proving that the translocation to nucleus was mediated by NLS. Transfection efficiency of 16% was obtained at a vector to DNA ratio of 10 as detected by flowcytometry **(Figure 5b)**. This level of transfection efficiency was sufficient to kill up to 62% of breast cancer cells with administration of one dose pTRAIL. pTRAIL encodes tumor related apoptosis inducing ligand. This molecule has a bystander effect. This has been discussed in more details on page 23 of the manuscript.

Note: The adenovirus encoding GFP (Ad.GFP) was purchased and used to transfect ZR-75-1 cells. However, the data was not used for comparison studies. This was due to the fact that adenovirus uses coxsackie adenovirus receptor (CAR) to enter the ZR-75-1 cells while the proposed vector in this study uses a different receptor. Therefore, their internalization pathways are different. Since the number of receptors on the surface of the cells determines the number of particles that can be internalized, such comparative studies between the two vectors that use different receptors may not be valid. The transfection efficiency of Ad.GFP (adenovirus carrying GFP gene) was approximately 58%.



ZR-75-1 breast cancer cells transfected with 10^{10} Ad.GFP particles at the multiplicity of infection of 100.

Task 3- Writing manuscripts, final report and dissemination of the data in conferences.

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1 manuscript is submitted for publication and is currently in revision. One reviewer has asked for animal data. An animal protocol is prepared and approved by IACUC. The animal studies will be started soon.

Key Research Accomplishments (Months 0-16)

- **a)** Developed reproducible expression and purification protocols for the production of highly basic vectors in E.coli system.
- **b)** Engineered a vector composed of five independent functional domains of diverse origin into a complex designer vector while preserving the functionality of each motif under one-domain-one-function concept.
- **c)** Proposed a model for the intracellular trafficking of the vector/pDNA nanocarriers and validation.
- **d)** Full vector characterization and evaluation demonstrating the vector's ZR-75-1 breast cancer cell specificity and high efficiency with no significant toxicity.
- **e)** Demonstrated the potential of vector to efficiently kill ZR-75-1 breast cancer cells.

Reportable Outcome

A) Manuscripts: A manuscript has been prepared and submitted for publication. This manuscript is under revision (Please see appendix A).

B) Presentations: One abstract has been published and one poster presented. (Please see appendix B)

C) Grant application: None

D) Training: None

E) Patent application: A provisional patent application is filed. Please see appendix C.

Conclusions

We have successfully demonstrated that a multi-domain designer vector with complex chimeric architecture can retain individual functionality of its constituents. This vector is fully functional with ability to target breast cancer cells with high specificity, overcome the biological barriers associated with targeted gene transfer, and mediate efficient gene transfer. We have gained a valuable experience in development of complex designer vectors despite the lack of "rational-design" database for *de novo* protein architecture evolution. This would allow creation of efficient and targeted systems that can be fine tuned for various gene delivery needs. The reported designer biomimetic vector can be modified, equipped with a variety of targeting motifs, and programmed to transfer genes to various breast cancer cell types overexpressing different biomarkers. The ability of the developed vector to efficiently target and kill breast cancer cells *in vitro* is investigated with exciting results.

References

None

Appendices

A. Submission of a manuscript for publication.

B. Publication of an abstract and poster presentation. S.S. Mangipudi, Arash Hatefi, **Function-Based Combinatorial Vector Engineering for Gene Therapy Research.** 2nd International Conference on Biomolecular Engineering; Santa Barbara, CA, USA

C. Provisional patent application. **Arash Hatefi,** Biomimetic Vectors for Targeted Gene Transfer. Provisional Patent Application Number 61106484.

Development of a Designer Biomimetic Vector for Targeted Gene Transfer to Breast Cancer Cells

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Short title: A Biomimetic Vector for Targeted Gene Transfer

ABSTRACT

The *objective* of this research was to package multiple natural motifs with diverse functions into a single chain macromolecule while preserving the functionality of each. A designer biomimetic vector was engineered to contain at precise locations: a) an adenovirus μ peptide to condense pDNA into nanosize particles, b) a synthetic cyclic peptide to target breast cancer cells and enhance internalization of nanoparticles, c) a pH-responsive synthetic fusogenic peptide to disrupt endosome membranes and facilitate escape of the nanoparticles into the cytosol, and d) a nuclear localization signal from human immuno-deficiency virus for microtubule mediated transfer of genetic material to the nucleus. The vector was characterized using physicochemical and biological assays to demonstrate the functionality of each motif in the vector backbone. The results demonstrated that the vector is able to condense plasmid DNA into nanosize particles (<100nm), protect pDNA from serum endonucleases, target ZR-75-1 breast cancer cells and internalize, efficiently disrupt endosome membranes, utilize microtubules to reach nucleus and mediate gene expression. The therapeutic potential of the vector was evaluated by complexing with plasmid DNA encoding TRAIL (pTRAIL) and transfecting ZR-75-1 cells. The results demonstrated that up to 62% of the ZR-75-1 breast cancer cells can be killed after administration of pTRAIL in complex with the vector.

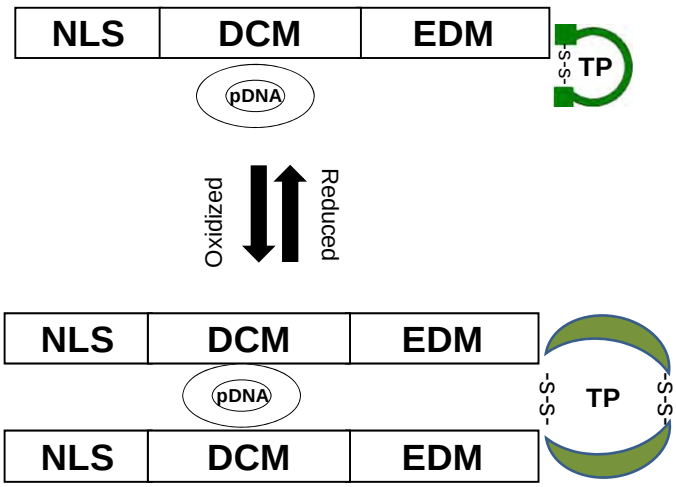
Key words: Biomimetic vector, Non-viral vector, Cancer gene therapy

1. INTRODUCTION

Gene therapy is perceived as a ground-breaking technology with the promise to cure almost any disease, provided that we understand its genetic basis. However, enthusiasm has rapidly abated as multiple clinical trials failed to show efficacy. The limiting factor seems to be the lack of a suitable delivery system to carry the therapeutic genes safely and efficiently to the target tissue[1]. Gene-transfer technology is still in a nascent stage owing to several inherent limitations in the existing delivery methods. Viral vectors are the vehicles of choice for cancer gene therapy particularly due to their ability to overcome the intracellular barriers and the enormous possibility for recombinant engineering. However, non-specific binding to all cells that over-express coxsackievirus and adenovirus receptor (CAR), potential immunogenicity, high costs of production, and the fact that the majority of cancer cells do not express CAR has limited their use for cancer gene therapy [2, 3]. To overcome these problems, non-viral vectors such as cationic lipids and polymers have been developed. While lipoplexes provide relatively high transfection efficiency, their large scale production, reproducibility and cytotoxicity remain major concerns [4]. On the other hand, cationic polyplexes are robust and relatively biocompatible but they are marred by poor gene-transfer efficiency [5]. What has been long sought after is a technology which combines biocompatibility, efficiency, and engineer-ability in a single effective gene-transfer technology platform. An alternative recombinant biomimetic non-viral approach is on the horizon but it is not without its own inherent challenges. Significant amounts of preliminary groundwork has been done addressing the feasibility concerns of this approach [6-10].

Herein, we report a fusion vector which is an ensemble of biological and combinatorially-screened motifs for targeted gene transfer. The first generation of this class of gene delivery

systems, namely Designer Biomimetic Vector (DBV), is based on a one-domain – one-function architecture concept. While viruses delegate different functions to multiple peptide subunits, the non-viral DBV reported here embeds several functional domains onto a single module (biomacromolecule). Using this approach, the functional propensity of the vector was scripted into the primary sequence in order to perform an array of self-guided functions. These include: a) efficient condensation of the plasmid DNA (pDNA) into deliverable nanoparticles by a DNA condensing motif (DCM) obtained from adenovirus μ peptide [11], b) cell-specific delivery of the nanoparticles using a combinatorially screened cyclic targeting peptide (TP) [12], c) endosomal disruption by an engineered pH-responsive endosome disrupting motif (EDM) mimicking influenza virus fusogenic peptide [13], and finally d) microtubule assisted transport of the genetic material towards the nucleus for efficient gene expression by a nuclear localization signal (NLS) obtained from human immuno-deficiency virus type 1 (HIV-1) [14]. Due to the cyclic nature of the targeting peptide, the DBV was engineered to stably exist as a monomer or dimer. The general structure and sequence of each domain in the vector structure is shown in **Figure 1**.



NLS sequence: RRNRRRRWRERQRQ
DCM Sequence: MRRAHHRRRRASHRRMRG
EDM sequence: GLFHAIAHFIHGGWHGLIHGWYG
TP sequence: FYCVIERLGVCLY

Figure 1

We hypothesize that the proposed DBV is able to condense pDNA into nano-size particles, protect pDNA from degradation by serum endonucleases, target model cancer cells and internalize via receptor-mediated endocytosis, disrupt endosome membranes to escape into the cell cytoplasm, exploit microtubules to facilitate translocation of the genetic material into the nucleus, and ultimately mediate gene transfer. In this study, the word “transfection efficiency” encompasses both percent transfected cells and total green fluorescent protein expression.

2. MATERIALS AND METHODS

2.1. Cloning, expression, and purification of DBV

The gene encoding DBV was designed in our lab, custom synthesized by Integrated DNA Technologies, Inc. and provided in pZErO plasmid. The resident DBV gene flanked on either side by NdeI and XhoI restriction sites was cloned into a pET21b expression vector under the control of a T7 promoter. The resultant expression construct pET21b:DBV was tested for its fidelity to the original design by DNA sequencing.

The pET21b:DBV expression vector was transformed into *E. coli* BL21(DE3) pLysS. Starter cultures, 5 ml, were then grown in LB media at 37°C overnight with 50 μg/ml carbenicillin. Circlegrow media (MB Biomedicals, Solon, OH), 500 ml, was inoculated with the starter culture and allowed to grow at 30°C until the absorbance reached 3.0 at 600nm. The culture was inducted with 0.4 mM IPTG for 2 hours. The culture broth was centrifuged at 6000 rpm for 7 minutes at 4°C and the pellet was frozen until further use.

The pellet was suspended in lysis buffer containing 100 mM NaH_2PO_4, 10 mM Tris-HCl (pH 8.0), and 6M guadinium hydrochloride and the lysate was centrifuged at 18000 rpm for 60 min. For purifying the dimer no reducing agent was used, whereas for the purification of the

monomer 12 mM 2-mercaptoethanol was used in all buffers. The soluble fraction was adjusted to 12 mM imidazole and 0.5 ml of Ni-NTA resin equilibrated in the same buffer was added. The expressed DBV was allowed to bind in a batch mode for 60 min at room temperature. The tubes were centrifuged at 1000g for 5 min and the supernatant discarded. The resin was loaded in a 0.8 x 4 ml BioRad PolyPrep chromatography column, washed with 25 ml of lysis buffer supplemented with 15 mM imidazole, and eluted with 250 mM imidazole. The DBV was stored in 50% glycerol at -20°C. The purity and expression of the DBV were determined by SDS-PAGE and western blot analysis (monoclonal anti-6XHis), respectively. The monomer and dimer were loaded onto the SDS-PAGE gel using a loading buffer without reducing agent. Using the same genetic engineering protocol mentioned above, the DBV without NLS was also expressed and purified.

2.2. Preparation of DBV stock solution

Before use, DBV was precipitated out of the storage buffer by adding saturated ammonium sulfate solution (4.2M) in small increments while incubating on ice. The salted out vector was collected in a microfuge tube by centrifugation at 5000 g and removal of supernatant. Subsequently, DBV was re-dissolved in 10mM bis-tris propane buffer (pH 7) to make ca. 2.0 mg/ml stock solution. This stock solution was used in ensuing studies.

2.3. Hemolysis assay

Two milliliters of sheep red blood cells (Innovative Research, Novi, MI) was washed several times with phosphate buffered saline (PBS). Cell numbers were adjusted to 3.2 x 10^8 cell/ml in Dulbecco's PBS at pH 7.4 and 7.0 or 50 mM acetate buffer at pH 5.0, adjusted to

physiological ionic strength with NaCl. The cell suspension in buffers was supplemented with 1, 10, 20 or 30 μg of the vector. The mixture was incubated at 37°C for 1h, centrifuged and the absorbance of the supernatant was measured at 541 nm. Triton X-100 (1%) was used as the positive and phosphate and acetate buffers as negative controls. The percentage of hemolysis for the test groups (dimer) is reported as relative to fully lysed cells by Triton X-100 (defined as 100%). Data is reported as mean ± s.d., n=3. The statistical significance was tested using t-tests ($p<0.05$).

2.4. Particle size analysis

Vectors were complexed with plasmid DNA encoding green fluorescent protein (pEGFP Clontech, CA, USA) at various N:P ratios (the molar ratio of positively charged nitrogen atoms to negatively charged phosphates in pDNA). In dimer, the total number of positively charged residues in NLS and DCM domains is 36 excluding histidines. Complexation was achieved by adding various amounts of vector solution in 10mM bis-tris propane buffer (pH 7) to an equal volume of buffer containing 1 μg pEGFP. For example, at N:P ratio of 10, twenty microgram of vector was complexed with 1 μg of pEGFP. The mean hydrodynamic particle size measurements for vector/pDNA complexes were performed using Dynamic Light Scattering (DLS) by a Malvern Nano ZS90 instrument and DTS software (Malvern Instruments, UK). The data is reported as mean ± SEM, n=3. Each mean is an average of 15 measurements and n represents the number of independent batches prepared for size measurements.

2.5. Particle stability in serum

The stability of the nanoparticles in the presence of serum was examined using gel retardation assay. In one set of tubes, 1µg pEGFP was complexed with the vector (dimer), incubated at room temperature for 15 minutes, and the mobility of pDNA was visualized by ethidium bromide staining and agarose gel electrophoresis. In the second set, particles were formed by complexing 1µg pEGFP with the vector followed by addition of fetal bovine serum (Invitrogen, CA, USA) at the final concentration of 10% (v/v). The complexes were incubated for 90 minutes at 37 ^{o}C in the presence of serum and then electrophoresed on a 1% agarose gel. The pDNA mobility was visualized by ethidium bromide staining.

To evaluate the ability of the vector (dimer) in protecting pDNA from endonucleases, complexes were formed at an optimal N:P 10 in a microfuge tube and incubated with serum for 90 minutes. Subsequently, sodium dodecyl sulfate was added (10%) to the tubes to release the pDNA from the vector. Samples were electrophoresed on agarose gel and visualized by ethidium bromide staining.

2.6. Transmission electron microscopy

The vector was complexed with pEGFP and nanoparticles were prepared at N:P 10 on Formvar coated grids and stained with uranyl acetate for 5 minutes. The nanoparticles were imaged using JEOL 1200 EX II (120 kV).

2.7. Cell culture and transfection

ZR-75-1 (ATCC, Manassas, VA) breast cancer cells were seeded in 96 well tissue culture plates in RPMI 1640 supplemented with 10% serum at a density of 5 x 10^4 cells per well. MCF-10A (ATCC, Manassas, VA) normal human mammary cells were seeded in 96 well tissue

culture plates in MEBM complete media at 3 x 10^3 cells per well. Cells were approximately 80% confluent at the time of transfection. Two hours before transfection, ZR-75 cells were conditioned in serum free media (RPMI-1640 supplemented with Insulin-Transferrin-Selenium), Dexamethasone, and fibronectin human plasma). A 100 µl aliquot of vector/pEGFP complex at various N:P ratios (1 to 16) containing 1 µg pEGFP was added to each well and the plates incubated for 2 h in CO_2 incubator at 37 °C. After 2 h, the media was removed and replaced with RPMI 1640 supplemented with 10% (v/v) serum. When used, chloroquine (100µM), bafilomycin A1 (10nM), or Nocodazole (10µM) were added. The expression of GFP was visualized after 48 hrs by an epifluorescent microscope (Zeiss Axio Observer Z1). The percent transfected cells as well as total green fluorescent protein expression were determined by flowcytometry (FacsCalibur, Becton Dickinson, San Jose, CA). Vector without NLS in complex with pEGFP was used as a control to examine the role of NLS. Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used as a positive control to validate the transfection process. Lipofectamine was complexed with pEGFP in Opti-MEM (Invitrogen) as per manufacturer's protocol and used to transfect cells. The data are presented as mean ± s.d., n=3. The statistical significance was evaluated using t-test ($p<0.05$).

2.8. Inhibition assay

Cells were seeded at a density of 5 x 10^4 cells per well in a 96 well plate. Aliquots of targeting peptide in serum free media were added at concentrations of 0, 0.07, 0.7, 1.4, 2.8, and 5.5 nM to the wells. The plates were placed at 37°C in CO_2 incubator for 1h with intermittent mixing at intervals of 15 min. Aliquots of dimer (100 µl) in complex with 1 µg pEGFP at N:P 10 were added to each of the wells. The control well with 0 µg/ml concentration received PBS. The

measured percent transfected cells for the test groups (pre-treated with targeting peptide) are expressed as percent of the control (untreated) defined as 100%. The targeting peptide was synthesized using solid phase peptide synthesis by Anaspec Inc. (San Jose, CA). The data are shown as mean ± s.d., n=3. The statistical significance was examined using t-test ($p<0.05$).

2.9. Cancer cell killing efficiency

This assay was performed in serum free media on ZR-75 cells for the vector (dimer), vector/pEGFP complexes (N:P 10), and vector/pTRAIL-GFP (N:P 10). ZR-75-1 cells were seeded at a density of 5 x 10^4 cells per well in 96 well plates. Cells were treated with serial dilutions of vector alone, vector in complex with pEGFP or vector/pTRAIL-GFP complexes for two hours. Plasmid DNA encoding tumor necrosis factor related apoptosis inducing ligand fused to GFP (pTRAIL-GFP) was kindly provided by Dr. Forrest at the University of Kansas. Subsequently, the media was removed and replaced with fresh RPMI supplemented with 10% serum followed by overnight incubation at 37°C in a humidified CO_2 atmosphere. The control well with 0 μg/ml concentration received PBS. After 48 hours, WST-1 reagent (Roche Applied Science, Indianapolis, IN) was added, incubated for 4 hours, and absorbance was measured at 440 nm. The measured absorbance for test groups is expressed as percent of the control where the control is defined as 100% viable. Lipofectamine in complex with pTRAIL-GFP was used as positive control. The expression of TRAIL-GFP was confirmed using an epifluorescent microscope. The data are reported as mean ± s.d., n=3. The statistical significance was determined using a t-test ($p<0.05$).

3. RESULTS

3.1. Cloning, expression, and purification of DBV

The gene encoding the vector, namely DBV, was cloned into a pET21b expression vector to make pET21b:DBV and the sequence of the gene was verified by DNA sequencing. The results showed no signs of deletion or mutation during the cloning process. The expression system was transformed into *E. coli* BL21 (DE3) plysS and DBV was expressed and purified at a 2 mg/liter yield. The general structure and sequence of the vector is shown in **Figure 1**.

The purity and expression of DBV in both monomer (molecular weight: 11.4 kDa) and dimer (molecular weight: 22.8 kDa) configurations with high purity were confirmed by SDS-PAGE and westernblot analysis, respectively **(Figure 2a)**. We have previously established and reported the technology to produce highly cationic vectors in *E.coli* under similar conditions [9, 10].

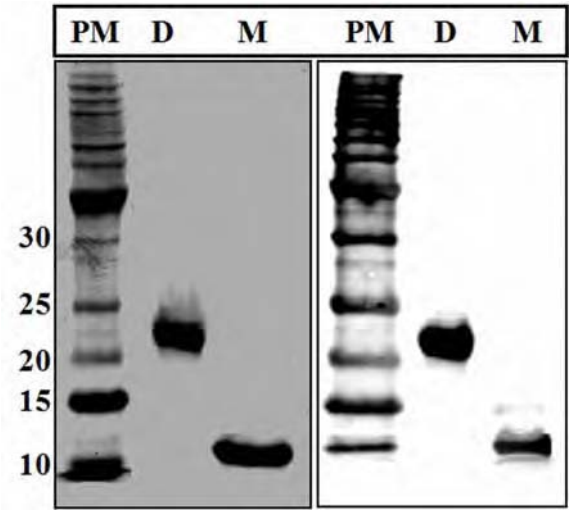


Figure 2a

3.2. Hemolysis assay

The results of hemolysis assay demonstrated that the vector does not induce significant membrane lysis at pH 7.4 in the range tested (1-30 µg) **(Figure 2b)**. At pH 7.0 and concentrations below 10 µg no significant hemolytic activity was observed in comparison to negative control. However, as the concentration increased, the hemolytic activity at pH 7.0 also

increased. In comparison to negative control, the vector demonstrated significant hemolytic activity at pH 5.0 and at concentrations ≥10 µg.

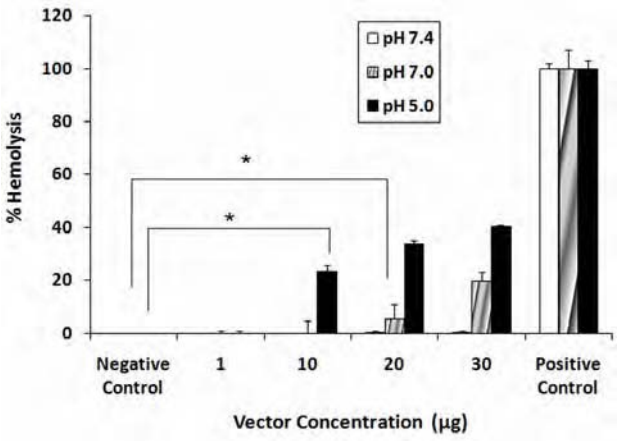


Figure 2b

3.3. Particle shape and size analysis

The TEM images show that the vector/pEGFP complexes are compact spherical nanoparticles with 60-70nm size **(only dimer is shown; Figure 3a).**

The results of particle size analysis showed that both dimer and monomer were able to efficiently condense pEGFP into nanoparticles with average sizes below 100nm at N:P ratios between 4 to 10 **(Figure 3b)**.

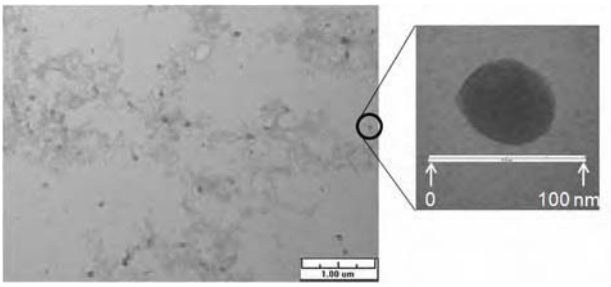


Figure 3a

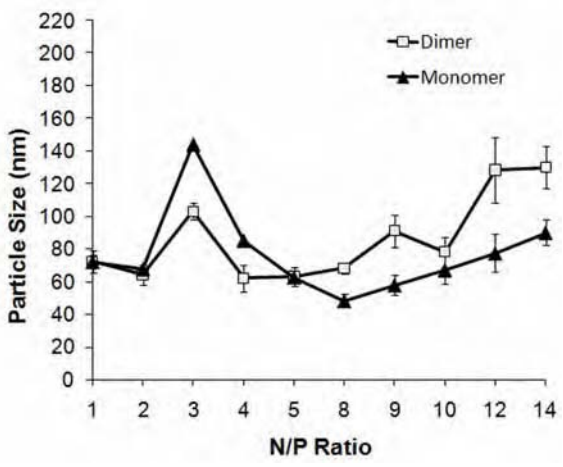


Figure 3b

3.4. Determination of optimum N:P ratio for transfection

ZR-75-1 cells were transfected with the vector/pEGFP complexes formed with monomer and dimer at various N:P ratios. While GFP expression was observed for both monomer and dimer at all N:P ratios (data not shown), the highest level of transfection efficiency was observed at N:P 10 **(Figure 4a)**. We did not observe any significant difference between the transfection efficiency of dimer versus monomer at this N:P ratio. Although the total green fluorescent expression with dimer seems slightly higher than lipofectamine, the difference is not statistically significant (p= 0.14) **(Figure 4a)**.

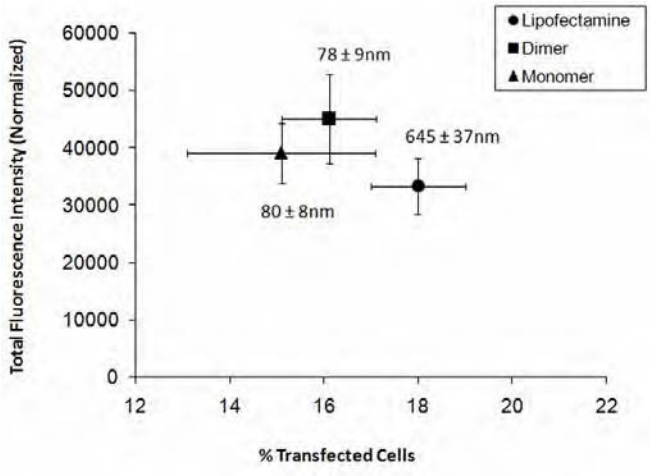


Figure 4a

3.5. Particle stability in serum

The vector (dimer) was complexed with pEGFP at N:P 10 and examined for its ability to protect pDNA from serum endonucleases. The results showed that these particles were stable in the absence and presence of 10% serum **(Figure 4b, lanes 2 and 3)** and effectively protected pDNA from degradation by the serum nucleases **(Figure 4b, lane 4)**.

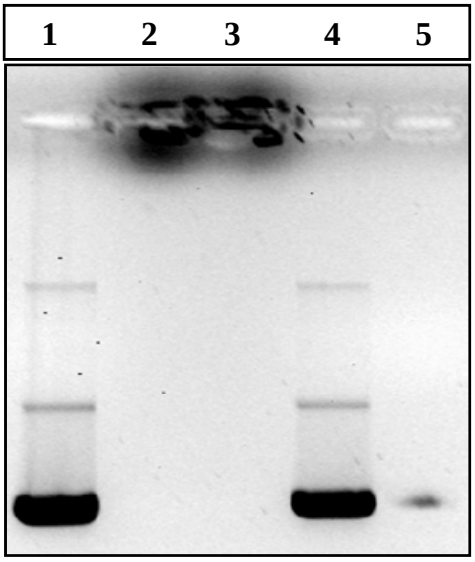


Figure 4b

3.6. Evaluation of the functionality of TP

An inhibition assay was performed to demonstrate internalization of the nanoparticles via the receptors that correspond to the targeting peptide. The results of this assay revealed that as the concentration of the targeting peptide (competitive inhibitor) increased, the levels of gene expression decreased **(Figure 5a)**. At 2.8 nM ligand concentration, almost complete inhibition of the transfection was achieved.

In addition, to demonstrate targeted delivery of the nanoparticles, the vector/pEGFP complexes at the optimum N:P ratio of 10 were used to transfect ZR-75-1 breast cancer cells and MCF-10A normal mammary cells. The results showed that 16±1 percent of the ZR-75-1 cells were transfected in comparison to 0.01±0.004 percent for MCF-10A cells **(Figure 5b)**.

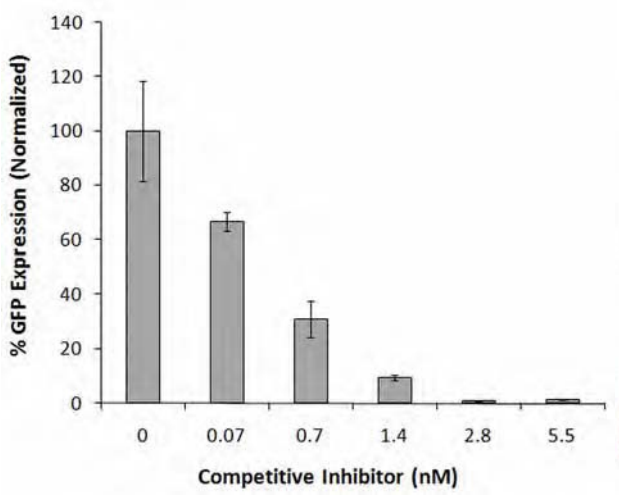


Figure 5a

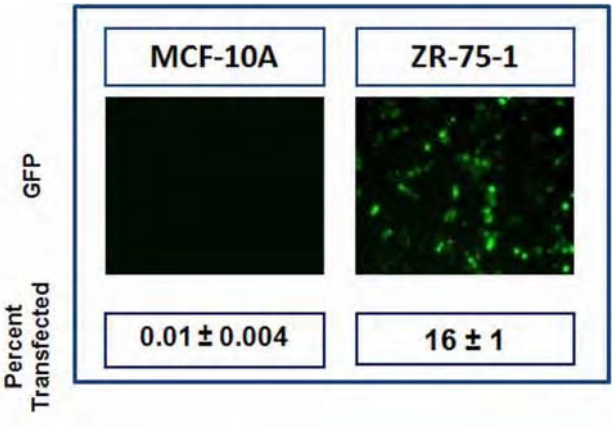


Figure 5b

3.7. Evaluation of the functionality of EDM and NLS

To study the ability of the vector to efficiently disrupt endosome membranes, ZR-75-1 cells were transfected in the presence of bafilomycin A1 and chloroquine. The results of the transfection studies in the presence and absence of bafilomycin A1 showed significant reduction in total green fluorescent protein expression from 45,000±7,800 to 4,100±2,700 AFU (arbitrary fluorescence units) **(Figure 6a)**. The percent transfected cells were also significantly reduced from 16±1 to 4±0.7 in the absence and presence of bafilomycin A1, respectively. In the presence of chloroquine, no significant increase in transfection efficiency was observed **(Figure 6a)**.

The role of microtubules in facilitating translocation of nanoparticles towards nucleus was evaluated by transfecting cells in the presence of nocodazole. The results revealed significant reduction in gene expression from 45,000±7,800 to 5,200±2,300 AFU when microtubule networks were disrupted by nocodazole. The percent transfected cell was also significantly reduced from 16±1 to 3±0.4 in the absence and presence of nocodazole, respectively **(Figure 6a)**.

The impact of NLS in the vector structure on transfection efficiency was further studied by transfecting ZR-75-1 cells with DBV (dimer) and DBV without NLS. The results demonstrated significant reduction in transfection efficiency from 45,000±7,800 to 11,500±2,300 AFU when vector without NLS was used **(Figure 6b)**.

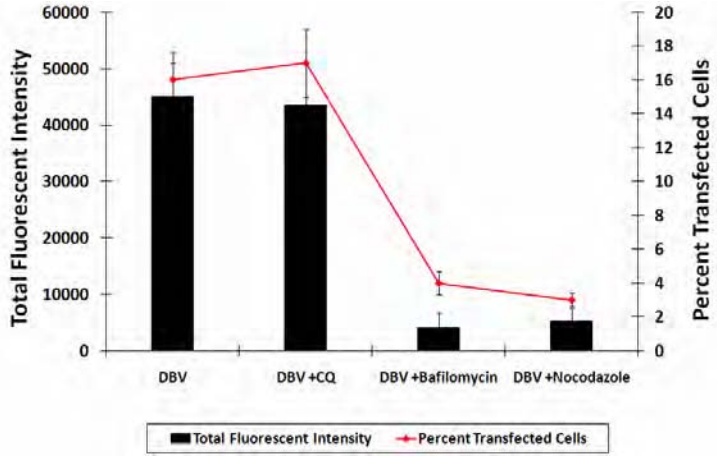


Figure 6a

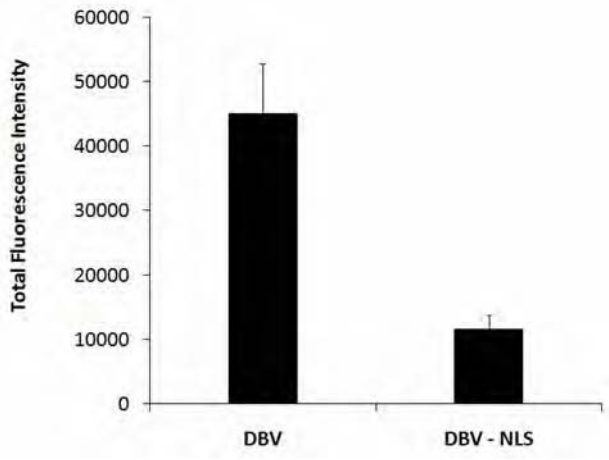


Figure 6b

3.8. Cancer cell killing efficiency

The vector related toxicity was measured by transfecting ZR-75-1 cells with vector only or vector in complex with pEGFP. The results exhibited no significant cell toxicity related to the vector or vector in complex with pEGFP at N:P ratio of 10 **(Figure 7)**. The potential of the vector in delivering plasmid DNA encoding TRAIL (tumor-related apoptosis inducing ligand) for cancer gene therapy was demonstrated by transfecting ZR-75-1 cells with the

vector/pTRAIL-GFP complexes. Up to 62% of the cells died when treated with one dose of vector/pTRAIL-GFP complexes. Lipofectamine in complex with pTRAIL-GFP was used as a control and resulted in killing up to 50% of the cancer cells which was significantly lower than vector/pTRAIL-GFP complexes **(Figure 7)**.

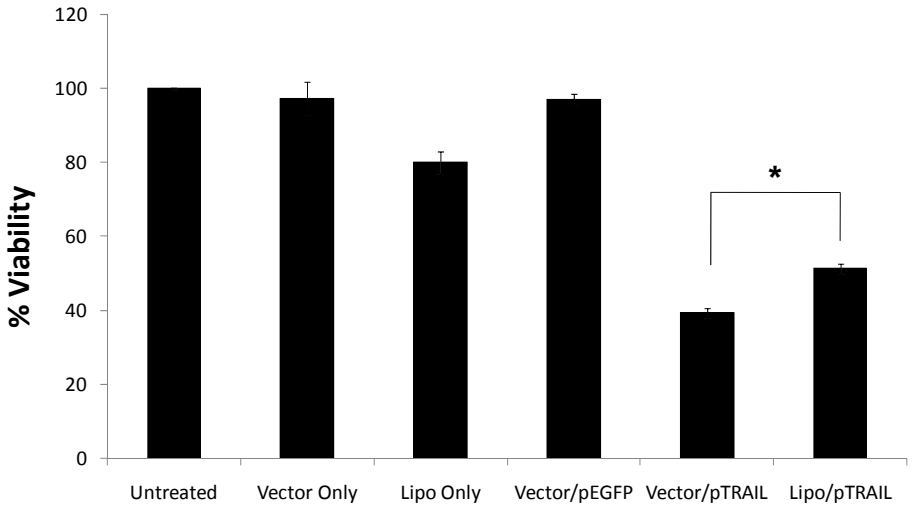


Figure 7

4. DISCUSSION

During the evolution process, nature has designed various motifs that can efficiently condense DNA (e.g., histones and virus DNA condensing peptides) [11, 15], disrupt endosomes (e.g., adenovirus fusogenic peptide and mellitin) [16, 17], or actively translocate genetic materials to the cell nucleus (e.g., adenovirus nuclear localization signal) [18]. The *objective* of this research was to examine the possibility of packaging multiple natural motifs with diverse functions into one vector while preserving the functionality of each.

Using genetic engineering techniques, a multi-domain biomimetic vector was designed, cloned, and expressed in both monomer and dimer configurations **(Figure 2a)**.

We characterized the vector in terms of its pH and concentration dependent membrane disrupting activity by incubating the vector with red blood cells. It was observed that the membrane disrupting activity of the vector significantly increased as pH decreased **(Figure 2b)**. The endosome disrupting motif used in this study was first developed by Monsigny's group [13], namely 5HWYG. It mimics the endosome disrupting activity of fusogenic peptide in influenza virus but at a pH close to 6.9. The pH changes causes the EDM to change conformation into α-helical structure, fuse with the early endosome membranes, and escape into the cytoplasm. At pH 7.0, the fusogenic peptide seems to have partially changed conformation into alpha-helical structure. As a result, at low concentrations the hemolytic activity is insignificant whereas it becomes prominent at higher concentrations. Therefore it can be construed that as the number of vector molecules used to complex with one pDNA molecule increases, the endosome disrupting efficiency of the nanoparticles could increase. These features of EDM, the pH and concentration dependence membrane disrupting activities are extremely important as it minimizes the possibility of causing unintended cell damage while in contact with normal cell membranes. So far, we have characterized the vector in terms of expression, purity and membrane disrupting activity. The next logical step is to examine the ability of the vector to complex with pDNA followed by physicochemical and biological characterization.

In the vector structure we have utilized arginine rich adenovirus μ peptide [19] to complex with pDNA and form condensed nanosize particles. To examine the extent of pDNA condensation, vectors were complexed with pEGFP and characterized in terms of size by dynamic light scattering technique and shape by transmission electron microscopy. The TEM images and particle size analysis results showed that the vectors were able to efficiently

condense pEGFP into nanoparticles in the size range that is suitable for receptor mediated endocytosis (i.e., <150nm) **(Figures 3a and 3b)** [20].

Nanoparticles were further characterized in terms of their ability to mediate gene transfer into ZR-75-1 model breast cancer cell line in order to identify the optimum N:P ratio for transfection. This cell line was selected because the model targeting peptide used in the vector structure was developed through combinatorial screening to specifically target this cell line with minimal binding affinity towards normal mammary cells [12]. The identity of the receptor to which the targeting peptide binds is unknown at this point but under investigation by others [12]. As a positive control we used lipofectamine to validate the transfection protocol. It is noteworthy that commercially available transfection reagents such as lipofectamine (non-targeted) are usually formulated to flocculate into large size particles so that they can precipitate readily onto the cells surface for maximum transfection efficiency. Such non-targeted systems may not be suitable for systemic gene delivery not only due to their non-specific binding to normal cells but their large size. For targeted nanoparticles, size plays an extremely important role in mediating efficient gene transfer because it has been shown that only particles with sizes less than 200nm can fit into clathrin-coated vesicles [20]. Since we did not observe any significant difference between the tranfsection efficiency of dimer versus monomer **(Figure 4a)**, we proceeded with the characterization of dimer which holds a more complex architecture.

To examine whether the vector is able to protect pDNA from serum endonucleases, nanoparticles were incubated with serum. The observations indicate that the vector has not only the potential to efficiently protect the pDNA from plasma endonucleases but could maintain its integrity until reaching the target cells **(Figure 4b)**.

We also performed an inhibition assay to evaluate the functionality of the targeting motif in the vector structure and the internalization of nanoparticles via receptor-mediated endocytosis. This was done by pre-treatment of ZR-75-1 cells with the targeting peptide to saturate the receptors followed by transfection of the cells with vector/pEGFP complexes. The inverse relationship between ligand concentration and total GFP expression indicates that addition of the targeting peptide to the media blocks internalization of the targeted nanoparticles resulting in lower levels of gene expression **(Figure 5a)**. This also means that the nanoparticles were internalized via the receptors that were blocked by the targeting peptide.

To demonstrate targeted delivery of the nanoparticles, the vector/pEGFP complexes at optimum N:P ratio of 10 were used to transfect ZR-75-1 breast cancer and MCF-10A normal mammary cells. The results showed that the percent transfected cells for MCF-10A cells were significantly lower than ZR-75-1 **(Figure 5b)**. This can be attributed to the absence of the necessary receptors on the surface of MCF-10A cells for the targeting peptide. It also suggests that the targeting peptide facilitated receptor-mediated internalization of the nanoparticles in ZR-75-1 cells with no significant internalization in normal mammary cells. This finding was expected as the targeting peptide in the vector structure was developed by a bacterial display system (combinatorial screening) to specifically target ZR-75-1 but not MCF-10A cells [12].

We then asked the question whether the EDM in the vector structure played a role and had a significant effect on the endosomal escape and subsequent transgene expression. The designed fusogenic peptide is expected to effectively increase the delivery of pDNA into the cytosol via membrane destabilization of acidic endocytotic vesicles containing vector/pDNA complexes. This was assessed by transfecting ZR-75-1 cells in the absence and presence of bafilomycin A1 and chloroquine. Choloroquine is a buffering agent known to disrupt the

endosomal membrane by increasing the pH of the endosome environment [21]. In contrast, bafilomycin A1 is an inhibitor of vacuolar ATPase endosomal proton pump which prevents the escape of the cargo into cytosol by inhibiting the acidification of the endosome environment [22]. The significant reduction in transfection efficiency in the presence of bafilomycin A1 highlights the fact that the acidification of the endosomal compartment is necessary for the escape of the nanoparticles into cytosol **(Figure 6a)**. These results in combination with the results obtained from the hemolysis assay suggest that EDM played a significant role in enhancing gene expression due to its pH-dependent fusogenic activity. Interestingly, in the presence of chloroquine, no significant increase in transfection efficiency was observed **(Figure 6a)**. This observation supports the hypothesis that the EDM motif preserves its functional integrity and assists the nanoparticles in efficient escape from the endosomes. It is plausible that this significant endosome disrupting activity is the result of additive or synergistic effects of fusogenic peptide and histidine residues in the vector structure. Because we have designed a histag in the vector sequence to facilitate purification of the vector from the *E.coli*, it could have contributed to the disruption of endosomes via the proton sponge effect [23, 24]. It is also noteworthy that EDM and DCM contain 5 and 3 histidine residues in their sequences, respectively. Therefore, the presence of 14 histidines out of 95 amino acid residues in the vector sequence along with the fusogenic peptide could be the reason for the efficient endosome membrane disruption. More in depth studies on this finding are needed to better understand this phenomenon.

Although there is limited understanding of the cellular and molecular mechanisms involved with synthetic vector mediated gene transfer, transfection efficiency seems to be fundamentally limited by inefficient trafficking of DNA from the cytosol to the site of gene

transcription in the nucleus [25]. To overcome this obstacle, we utilized the NLS from the *Rev* protein (residues 35-51) of Human Immunodeficiency Virus (HIV) and engineered the signal sequence at the vector's N-terminal to promote the transport of nanoparticles via microtubules across the cytoplasm towards the nucleus. Presence of the NLS in the vector structure could reduce the cytosolic residence time and increase the probability of accumulation of nanoparticles inside the nucleus through either the nuclear pore complex or during the mitosis phase of cell cycle. For nanoparticles with diameter smaller than 30nm the possibility of entering the nucleus via the nuclear pore complex exists, however bigger particles are more likely to enter the nucleus during the mitosis phase when nuclear membrane dissolves. To investigate the effect of microtubules on the translocation of pDNA to the nucleus, ZR-75-1 cells were transfected in the presence and absence of nocodazole, a reagent known to depolymerize microtubule structures [26]. The results revealed significant reduction in gene expression when the microtubule network was disrupted **(Figure 6a)**. Therefore, it can be deduced that the nanoparticles exploited microtubules to reach the nucleus. Furthermore, we examined whether the presence of NLS in the vector structure had any significant impact on enhancing gene transfer efficiency. This was achieved by transfecting ZR-75-1 cells with the DBV that did not have NLS in its structure. In comparison to DBV, there was an approximately 75% reduction in the expression of GFP in the DBV lacking NLS **(Figure 6b)**. This indicates that presence of the NLS had a significant role in the nanoparticles reaching the nucleus.

So far, we have examined each motif in the vector structure and have shown that by correct positioning in the vector backbone, the functionality of each motif can be preserved. This can be explained by the fact that during the pDNA condensation process, hundreds of vector molecules participate to condense one molecule of pDNA. The total number of positively

charged residues in the vector structure that participate in pDNA condensation is calculated based on the total number of arginines and lysine in the vector sequence minus the arginine residue in the targeting peptide (Figure 1). The arginine residue in the targeting peptide sequence is excluded from the calculations due to its proximity to the neighboring glutamic acid residue (negatively charged). For example, at an N:P ratio of 10, approximately 2600 vector molecules are employed to condense one pEGFP molecule (9462 negative charges). Therefore, it is probable to have a fraction of fully functional targeting motifs, NLSs, and EDMs in the vector/pDNA complex architecture. Thus, the nanocomplex is rendered ready to bind to the receptors, fuse with the endosome membranes, and utilize the microtubules for active translocation of genetic material to the cell nucleus.

One important question that we asked was whether this level of transfection efficiency is sufficient to mediate significant therapeutic outcome. We have deliberately chosen a highly specific targeting peptide which is customized for ZR-75-1 cells. In **Figure 6a** we showed that approximately 16 percent of the cells can be transfected with high levels of gene expression. Based on this observation, we selected a therapeutic molecule named TRAIL (tumor necrosis factor related apoptosis inducing ligand) as a model drug. TRAIL was selected because of its bystander effect as well as relative sensitivity of ZR-75-1 cells to this molecule [27, 28]. To examine the therapeutic potential of the vector in delivering TRAIL, ZR-75-1 cells were incubated with the vector alone, vector in complex with pEGFP and vector in complex with pTRAIL-GFP. The results exhibited no significant cell toxicity related to the vector or vector in complex with pEGFP **(Figure 7)**. However, up to 62% of the cells died when treated with vector/pTRAIL-GFP. This means that for every single cell that was killed after transfection with pTRAIL, three additional neighboring cells died as a result of the bystander effect. Application of other

therapeutic molecules which possess higher levels of bystander effect could result in more significant cell death. Thus, achievement of significant therapeutic outcomes is based not only on percent transfected cells, but on the level of gene expression and proper selection of therapeutic molecules.

One last question that we asked was why all the cells were not transfected, even though the vector seems to have overcome the major known cellular barriers. Beside the administered pDNA dose, the answer could be as simple as the fact that the abundance of entry gates (i.e., receptors) on the surface of cancer cells dictates the number of particles that can be internalized. Viruses such as adenovirus are a good example as they are able to transfect various cell lines with different transfection efficiencies depending on the number of coxsackie adenovirus receptor present on the surface of the cells [29]. In addition, not all ZR-75-1 cancer cells over-express the receptors required for the internalization of the nanoparticles because cancer cell populations are usually heterogeneous. Nonetheless, other more complex reasons may be involved which calls for more mechanistic studies to unravel the mysteries of efficient gene transfer.

5. CONCLUSION

We have demonstrated that a multi-domain designer vector with a complex chimeric architecture can retain individual functionality of its constituents. There is an increasing body of evidence that proteins are more flexible than previously thought and can retain functional integrity in multiple conformations; thus, boosting the possibility of successful *in vitro* evolution of complex designer macromolecules [30]. This would allow creation of efficient and targeted systems that can be fine tuned for various gene delivery needs. Such systems can be equipped

with a variety of cell specific targeting motifs and used to transfer genes to various cell types with potential applications in gene therapy for cancer, cardiovascular disease, wound healing, and many others.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1: Schematic representation of the designer biomimetic vectors. The vector (monomer) is composed of a nuclear localization signal (NLS), a DNA condensing motif (DCM), an endosome disrupting motif (EDM), and a cyclic targeting peptide (TP). The dimer is created through the linkage of cysteine residues in targeting peptide via disulfide bonds.

Figure 2: Expression and characterization of the DBV. **a)** SDS-PAGE (left panel) and western blot analysis (right panel) of purified vector. PM stands for protein marker, M is monomer, and D is dimer. **b)** hemolytic activity of the dimer with various concentrations at pH 7.4, 7.0, and 5.0.

Figure 3: Nanoparticle shape and size analysis. **a)** The TEM picture of the spherical nanoparticles formed at N:P 10. **b)** The particle size analysis of vector/pEGFP complexes formed with monomer and dimer at various N:P ratios.

Figure 4: Nanoparticle characterization in terms of mediating gene transfer and stability. **a)** Gene transfection efficiency of monomer (N:P 10), dimer (N:P 10), and lipofectamine. pEGFP was used as a model reporter gene. The corresponding nanoparticle size is also reported. **b)** Gel retardation assay of pDNA and vector/pDNA complexes at N:P 10. Lane 1: pDNA in the absence of serum. Lane 2: vector/pDNA complexes in the absence of serum. Lane 3: vector/pDNA complexes incubated with serum. Lane 4: released pDNA from the vector/pDNA complexes after incubation with serum. Lane 5: pDNA incubated with 10% serum for 30 minutes.

Figure 5: Evaluation of the functionality of targeting peptide in dimer. **a)** Internalization of nanoparticles via receptor-mediated endocytosis is demonstrated by an inhibition assay. Targeting peptide at various concentrations was used as a competitive inhibitor. **b)** Epifluorescent images and percentage of the MCF-10A and ZR-75-1 cells transfected with vector/pEGFP complexes at N:P ratio of 10. The percentage of transfected cells was measured by flowcytometry.

Figure 6: Evaluation of the functionality of EDM and NLS. **a)** Quantitative measurements of the total gene expression as well as percent trasfected cells for the ZR-75-1 in the presence and absence of chloroquine (CQ), bafilomycin A1 and nocodazole. B) Measurement of total green fluorescent protein expression for ZR-75-1 cells transfected with the DBV and DBV without NLS.

Figure 7: Evaluation of the cancer cell killing efficiency of the vector/pTRAIL-GFP complexes using WST-1 cell toxicity assay. Vector alone, vector/pEGFP complexes and lipofectamine/pTRAIL-GFP complexes were used as controls.

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Function-Based Combinatorial Vector Engineering for Gene Therapy Research

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Introduction: In an effort to develop a biomimetic vector for efficient and targeted gene transfer, we have engineered a multi-domain biomacromolecule. Using a novel technique, namely Function-based Combinatorial Vector Engineering, we have screened and identified the structure of a biomacromolecule where the functional propensity is scripted into the primary sequence structure to perform an array of self-guided functions. These include: a) efficient condensation of the plasmid DNA into deliverable nanoparticles by adenovirus mu peptide, b) delivery of the nanoparticles to model breast cancer cells using a combinatorially screened cyclic targeting peptide (TP) [1], c) endosomal disruption using a synthetic fusogenic peptide (FP) facilitating escape of the cargo into cytosol, and finally d) localization of the gene in the nucleus by HIV nuclear localization signal (NLS). By shuffling the order of the functional domains, a library of the DNA transporters was genetically engineered to screen the active candidate resulting in the optimum sequence shown in (**Figure 1**).

Methods:

Cloning and expression of the vectors: The genes encoding various motifs were synthesized and cloned into a pET21b expression vector. The expression vector was transformed into E. coli BL21(DE3) and induced with IPTG. The expressed vectors were purified to homogeneity on a Ni-NTA column.

Transmission Electron Microscopy: The vector/pDNA nano-complexes were prepared, stained with uranyl acetate for 5 min, and visualized.

Particle characterization: Vector/pDNA complexes were prepared at different N/P ratios by adding various amounts of the vector into 1 µg of pDNA (pEGFP). The mean hydrodynamic size of the complexes was measured by Photon Correlation Spectroscopy (PCS). Results are reported as Mean ± SEM.

Hemolysis assay: Thoroughly washed sheep red blood cells were reconstituted to 3.2 x 108 cells/ml in phosphate buffered saline pH 7.4, 7.0 and 5.0 supplemented with various amounts of the vector. The lysis mixture was incubated at 37°C for 1h, centrifuged and the supernatant was read at 541 nm. Triton X-100 was used as positive control, whereas buffer only used as negative control.

Cell culture and transfection: ZR-75-1 breast cancer and MCF-10A normal human mammary cells were seeded in 96-well tissue culture plates. Cells were approximately 70% confluent at the time of transfection. pEGFP was mixed with various ratios of the vector to form complexes followed by addition to the growth media. When used, chloroquine (100µM), bafilomycin (10nM), Nocodazole (10µM) were added to assist the particles escape into cytoplasm. The expression of GFP and the percent transfected cells was determined by flowcytometry.

Inhibition assay: Cells were seeded at a density of 5 x 104 cells per well in a 96 well plate. Aliquots of targeting peptide in serum free media were added at concentrations of 0, 0.07, 0.7, 1.4, 2.8, and 5.5 nM to the wells. Aliquots of vector in complex with 1 μg pEGFP at N:P 10 were added to each of the wells. The control well with 0 μg/ml concentration received PBS. The measured percent transfected cells for the test groups (pre-treated with targeting peptide) are expressed as percent of the control defined as 100%. The data are shown as mean ± s.d., n=3.

Results:

As a result of the Function-Based Combinatorial Vector Engineering technique, the structure of a single chain biomacromolecule was identified that was able to: a) condense plasmid DNA into spherical and compact nanoparticles with 60 ± 5nm size, b) lyse the red blood cells efficiently at pH values less than 7.0, c) target ZR-75-1 breast cancer cells specifically with no significant effect on human normal mammary cells, d) disrupt endosome membranes efficiently, e) utilize microtubules to translocate the genetic material towards the nucleus, and f) mediate efficient gene transfer.

Conclusion:

This study demonstrates that by using Function-based Combinatorial Vector Engineering a multifunctional biomacromolecule can be engineered that is able to mimic virus characteristics and efficiently transfer genetic materials to the target cells.

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November 11, 2008

Sita S. Pappu, Ph.D.
Senior Technology Manager
WSU Office of Intellectual Property and
WSU Research Foundation
1610 NE Eastgate Boulevard
Pullman, WA 99163

Re: U.S. Provisional Application No. 61/106,484
BIOMIMETIC VECTOR FOR TARGETED GENE TRANSFER
Your Reference: 1016
Our Reference: 67901-120

Dear Sita:

Pursusant to your instructions, we filed a provisional application for this matter with the U.S. Patent Office on October 17, 2008. Enclosed for your files is a copy of the application as filed. The application is number 61/106,484 and has an official filing date of October 17, 2008. Please note that the filing of this application does not constitute a publication. Therefore, it is a confidential document, and must be handled accordingly. We will forward a copy of the filing receipt to you when it is received from the Patent Office.

You may now use, in connection with those products which are the subject of this application, the terms "patent pending," "patent applied for," or words to the same effect for the entire period during which this application is pending.

In order to secure patent protection for the invention described in the provisional application it will be necessary to file a nonprovisional application claiming the benefit of the provisional application within twelve months from the provisional filing date. Furthermore, if you are interested in protecting this invention in foreign countries, it is necessary to apply for and obtain a patent in each country where protection is desired. In most instances, it is necessary to file foreign applications within one year of the provisional filing date or the possibility of obtaining patent protection may be lost forever.

The final, inextensible date by which a nonprovisional application and any corresponding foreign applications must be filed in this case is October 17, 2009.

We will contact you several months in advance of these due dates to discuss the best course of action.

Please be aware that priority based on the provisional application is dependent on the completeness of the provisional application. To the extent that the invention is well described in the provisional application, there will be no problems in claiming priority both for a U.S. nonprovisional application and foreign applications. However, aspects of the invention that are inadequately described or not described at all will not benefit from the filing date of the provisional application. In such a situation, developments by competitors or your own activities prior to filing a nonprovisional or foreign applications (*e.g.*, sales of more than one year prior to filing a U.S. nonprovisional application or public disclosures of the invention before filing a foreign application) may affect your ability to obtain U.S. and foreign patents, depending on the nature and timing of the events that occurred.

We have also enclosed an Assignment from the inventor to WSU and ask that you have the inventor sign it before a notary public. Once that is done, please return it to us for filing with the Patent Office.

If you have any questions or comments, please do not hesitate to contact us.

Very truly yours,

Davis Wright Tremaine LLP

Barry L. Davison

BLD:jr

Enclosures: Application As Filed
Assignment

cc: Mary J. Edwards, Program Administrative Manager (w/o enclosures)
Attorney General of Washington, WSU (w/enclosures)

Electronic Acknowledgement Receipt

EFS ID:	4136209
Application Number:	61106484
International Application Number:	
Confirmation Number:	1205
Title of Invention:	BIOMIMETIC VECTORS FOR TARGETED GENE TRANSFER
First Named Inventor/Applicant Name:	Arash Hatefi
Customer Number:	22504
Filer:	Barry Davison/Kay Bulen
Filer Authorized By:	Barry Davison
Attorney Docket Number:	67901-120
Receipt Date:	17-OCT-2008
Filing Date:	
Time Stamp:	19:32:38
Application Type:	Provisional

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$110
RAM confirmation Number	4512
Deposit Account	040258
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

Charge any Additional Fees required under 37 C.F.R. Section 1.19 (Document supply fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)

APPLICATION DATA SHEET

Application Information

Application number::	
Filing Date::	October 17, 2008
Application Type::	Provisional
Subject Matter::	Utility
Suggested classification::	
Suggested Group Art Unit::	
CD-ROM or CD-R?::	None
Number of CD disks::	
Number of copies of CDs::	
Sequence submission?::	No
Computer Readable Form (CRF)?::	
Number of copies of CRF::	
Title ::	BIOMIMETIC VECTORS FOR TARGETED GENE TRANSFER
Attorney Docket Number::	67901-120
Request for Early Publication?::	No
Request for Non-Publication?::	No
Suggested Drawing Figure::	
Total Drawing Sheets::	21
Small Entity?::	Yes
Petition included?::	No
Petition Type::	
Licensed U.S. Gov't Agency::	Yes
Contract or Grant No::	Department of Defense/Army Contract #W81XWH-07-1-0533

Secrecy Order in Parent Appl.?:: No

First Applicant Information

Applicant Authority Type::	Inventor
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Status::	Full Capacity
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Representative Information

Representative Customer Number::		**22504**

Domestic Priority Information

Application ::	Continuity Type::	Parent Application::	Parent Filing Date::

Foreign Priority Information

Country::	Application number::	Filing Date::	Priority Claimed::

Assignee Information

Assignee name::	Washington State University
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