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Brief Article

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Targeted Delivery of Antisense Oligonucleotides by Chemically Self-Assembled Nanostructures

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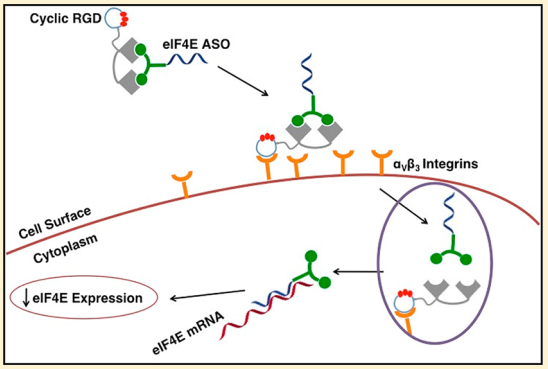
Supporting Information

ABSTRACT: Synthetic nucleic acids have shown great potential in the treatment of various diseases. Nevertheless, the selective delivery to a target tissue has proved challenging. The coupling of nucleic acids to targeting peptides, proteins, and antibodies has been explored as an approach for their selective tissue delivery. Nevertheless, the preparation of covalently coupled peptides and proteins that can also undergo intracellular release as well as deliver more than one copy of the nucleic acid has proved challenging. Recently, we have developed a novel method for the rapid noncovalent conjugation of nucleic acids to targeting single chain antibodies (scFv) using chemically self-assembled nanostructures (CSANs). CSANs have been prepared by the self-assembly of two dihydrofolate reductase molecules ($DHFR^2$) and a targeting scFv in the presence of bis-methotrexate (bis-MTX). The valency of the nanorings can be tuned from one to eight subunits, depending on the length and composition of the linker between the dihydrofolate reductase molecules. To explore their potential for the therapeutic delivery of nucleic acids as well as the ability to expand the capabilities of CSANs by incorporating smaller cyclic targeting peptides, we prepared $DHFR^2$ proteins fused through a flexible peptide linker to cyclic-RGD, which targets $\alpha v\beta 3$ integrins, and a bis-MTX chemical dimerizer linked to an antisense oligonucleotide (bis-MTX-ASO) that has been shown to silence expression of eukaryotic translation initiation factor 4E (eIF4E). Monomeric and multimeric cRGD-CSANs were then prepared with bis-MTX-ASO and shown to undergo endocytosis in the breast cancer cell line, MDA-MB-231, which overexpresses $\alpha v\beta 3$. The bis-MTX-ASO was shown to undergo endosomal escape resulting in the knock down of eIF4E with at least the same efficiency as ASO delivered by oligofectamine. The modularity, flexibility, and common method of conjugation may prove to be a useful general approach for the targeted delivery of ASOs, as well as other nucleic acids to cells.



KEYWORDS: *antisense, delivery, nucleic acid, nanotechnology*

Synthetic nucleic acids such as antisense oligonucleotides (ASOs), siRNA, and shRNA are being widely studied for the treatment of various diseases such as hepatitis B, hypercholesterolemia, and cancer.[1,2] Currently about 22 oligonucleotide-based drugs are in various stages of clinical trials, and many more are in preclinical development.[3,4] A major hurdle associated with the development of nucleic acid, including oligonucleotide-based drugs, is their delivery and cellular uptake by the target tissue. The inherent hydrophilicity and charged nature of oligonucleotides are significant barriers to their transport across the hydrophobic cell membranes to their site of action, i.e., cytoplasm or nucleus. This limitation was addressed with a number of molecular designs that involve the direct conjugation of oligonucleotides to various cell surface receptor targeted antibodies, peptides, small molecules, or aptamers enabling the receptor mediated endocytosis of the oligonucleotides.[5−9]

Previously, we have reported the targeted cellular delivery of methotrexate-functionalized oligonucleotides to CD3+ T-leukemia cells, through the noncovalent binding of DHFR-antiCD3 scFv fusion proteins.[10] In addition, we have also observed that incubation of a bis-methotrexate ligand (bis-MTX) with a fusion protein composed of two copies of dihydrofolate reductase and an antiCD3 single chain variable fragment (scFv) ($DHFR^2$antiCD3) results in the formation of chemically self-assembled antibody nanorings (CSANs) displaying multiple copies of the antiCD3 scFv.[11,12] The size of the nanorings formed can be controlled by varying the size of

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the linker between the two DHFR molecules. If the linker is 13 amino acids long (13DHFR^2), monomeric and dimeric nanorings are formed, whereas if the linker is 1 amino acid, glycine (1DHFR^2), predominantly octameric nanorings are formed. The antiCD3 CSANs selectively bind to CD3 receptors on the membranes of T-leukemia cells and are endocytosed by a clathrin dependent process.[12] Further, development of the bis-MTX ligand resulted in bis-MTX ligands with a third arm which can be functionalized with oligonucleotides (bis-MTX-Oligo).[10] Incubation of bis-MTX-Oligo with DHFR^{2}antiCD3 resulted in oligomerization to form higher order species. We have shown that this oligonucleotide containing CSANs binds specifically to CD3 cell surface receptors on CD3+ T-leukemia cells and undergoes internalization. The novelty of this approach is the elimination of the need to covalently attach the oligonucleotides to the target ligands and the ability to display multiple copies of oligonucleotide on a single construct. Although this method shows great promise, the ability of oligonucleotides, such as ASOs, to be delivered by CSANs and undergo endosomal escape needs to be evaluated.

Eukaryotic translation initiation factor 4E (eIF4E) is the key rate-limiting component in cap-dependent translation: the major mechanism for protein synthesis within cells.[13,14] In solid tumors, eIF4E is overexpressed or hyperactivated feeding the oncoprotein addiction of the malignant cells.[13,15] Graff and others have shown that knocking down eIF4E in cancer cells decreases cell growth, induces apoptosis, and increases cell sensitivity toward common chemotherapeutic agents and radiation.[16−18] ASOs targeting eIF4E are currently in clinical development.

RGD4C is a cyclic Arg-Gly-Asp peptide, containing 4 cysteine residues, which has been shown to bind to cell surface integrins, with some specificity for $\alpha v\beta 3$. Integrins such as $\alpha v\beta 3$ are overexpressed on various solid tumors, including head and neck and breast tumors, as well as during formation of nascent tumor blood vessels by angiogenesis.[19,20] The peptide RGD (and analogues) have been used for targeting numerous tumor types, including breast cancer tumors.[21,22] We hypothesized that chemically induced self-assembly of RGD4C-DHFR nanostructures could be used to display multiple copies of the RGD4C peptide, thus targeting breast cancer cells. These peptide functionalized nanostructures could therefore be used for the specific delivery of ASOs targeting eIF4E causing selective reduction in the expression of eIF4E protein and a general suppression of cap-dependent translation.[16]

13DHFR^{2}RGD4C and 1DHFR^{2}RGD4C proteins were expressed in *Escherichia coli* cells as soluble proteins with the yield of about 20 mg of pure protein per liter culture.[23] Graff and co-workers have previously reported a 20-mer phosphorothioated oligonucleotide containing 2-methoxyethyl modified bases can act as an eIF4E ASO.[16] We obtained a simplified 20-mer phosphorothioated oligonucleotide containing the same sequence and a free 3′-thiol. The 3′-thiol of the oligonucleotide was coupled to the previously reported bis-MTX maleimide, and the resulting bis-MTX-ASO was used in the present study (Figure S1 in the Supporting Information).[10] 13DHFR^{2}RGD4C and 1DHFR^{2}RGD4C were mixed with bis-MTX-ASO to form monomeric and multimeric nanostructures respectively (Figure 1a). Analysis of the nanostructures by size exclusion chromatography (SEC) revealed that 13DHFR^{2}RGD4C (Figure 1b, black line) and the mixture of bis-MTX-ASO with 13DHFR^{2}RGD4C (Figure 1b, red line)

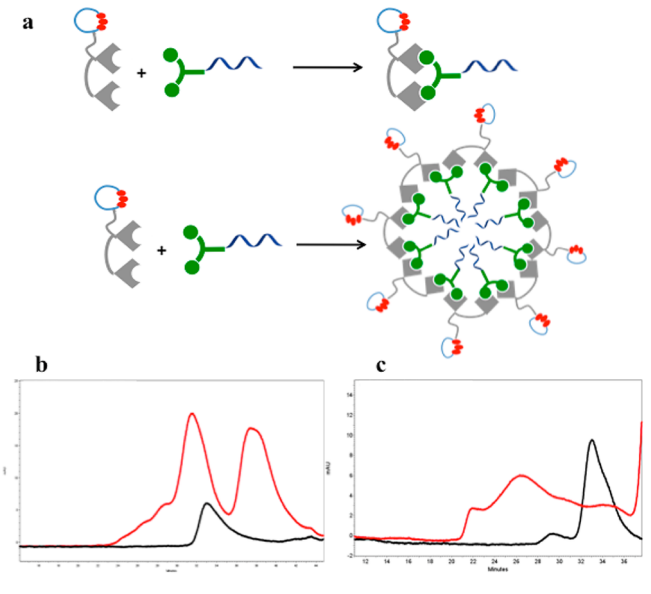

Figure 1. (a) Scheme for the formation of oligo functionalized monomeric (upper) and multimeric (lower) nanostructures. DHFR^{2}RGD4C contains two DHFR proteins (gray) and cRGD peptide (blue ring with 3 red dots). Bis-MTX-ASO has a bis-MTX, shown in green, attached to the ASO (blue). Size exclusion chromatography (SEC) trace of 13DHFR^{2}RGD4C (b) or 1DHFR^{2}RGD4C (c) alone (black) and after incubation with bis-MTX-ASO (red).

have similar retention times (approximately 31 min). This indicates that they have similar hydrodynamic radii, thus suggesting the presence of monomeric species. The incorporation of bis-MTX-ASO into the nanostructures (Figure 1b, red line) was further confirmed by the observation of MTX absorbance at 302 nm. Excess bis-MTX-ASO elutes at approximately 38 min. A number of higher order structures (trimers and dimers) are observed eluting at 24−28 min. In contrast, similar to previous observation for the oligomerization of 13DHFR^2 with bis-MTX, when 13DHFR^{2}RGD4C was incubated with bis-MTX, dimeric species were formed predominantly.[11] This presumably results from the steric bulk of the 20-mer oligonucleotide causing the formation of smaller rings. A similar change in the size of species formed is observed when 1DHFR^{2}RGD4C is incubated with bis-MTX-ASO and a number of higher order multimeric species ranging from pentamer to heptamer are formed (Figure 1c, red line). However, incubation of 1DHFR^{2}RGD4C with bis-MTX results in the formation of mostly octameric species.

In order to study the ability of CSANs-RGD4C nanostructures to deliver ASOs to the cells by confocal laser scanning microscopy, we have used 5′ FITC labeled bis-MTX-ASO (bis-MTX-ASO-FITC). The nanostructures were prepared with 13DHFR^{2}RGD4C or 1DHFR^{2}RGD4C by mixing with bis-MTX-ASO-FITC and incubated with $\alpha v\beta 3$ integrin positive MDA-MB-231 breast cancer cells at 37 °C. Green fluorescent punctates were observed inside the cells treated with either the monomer or multimer nanostructures indicating endocytosis (Figure 2a). Cells treated with bis-MTX-ASO-FITC in the absence of DHFR^{2}RGD4C proteins did not show internalized fluorescence (Figure S3 in the Supporting Information). To confirm that the internalization of monomer and multimeric nanostructures is mediated by $\alpha v\beta 3$ integrins, a competition experiment with excess commercially available cRGD peptide

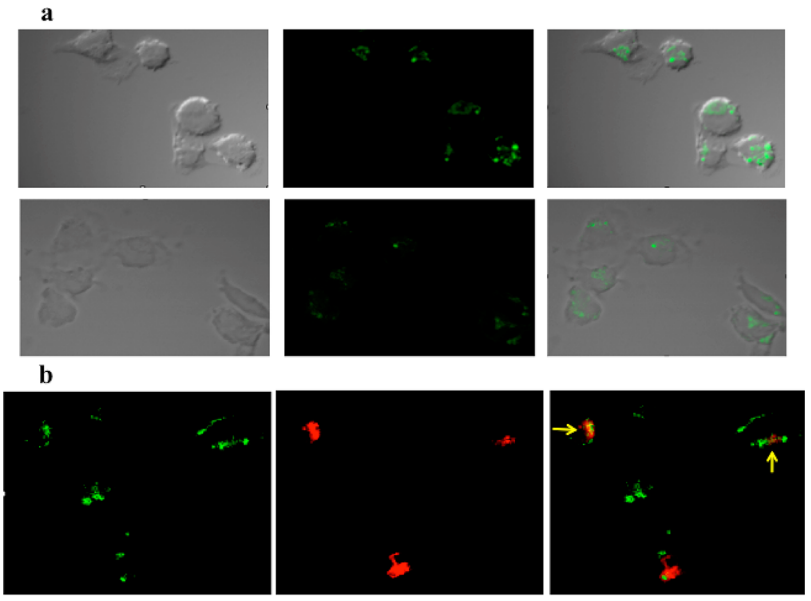

Figure 2. Subcellular localization by confocal microscopy. (a) MDA-MB-231 cells: images depicting differential interference contrast (first column), FITC fluorescence confocal channel (second column), and overlay of both channels (third column) for bis-MTX-ASO-FITC incubated with either 1DHFR^{2}RGD4C (upper panel) or 13DHFR^{2}RGD4C (lower panel) at 37 °C. (b) FITC fluorescence channel for 1DHFR^{2}RGD4C nanostructures (first column), cholera toxin Alexa Fluor 594 (second column), overlay of green and red channel (third column).

was performed. 1DHFR^{2}RGD4C was mixed with bis-MTX-ASO-FITC, treated with MDA-MB 231 cells in the presence of excess cRGD peptide, and analyzed by confocal microscopy. Incubation with cRGD completely blocked the uptake of nanostructures compared to the cells that were not treated with cRGD peptide (Figure S4 in the Supporting Information).

Subcellular colocalization of the oligonucleotide bearing nanostructures was studied by confocal microscopy. cRGD is known to undergo internalization after binding to $\alpha v\beta 3$ by caveolar/lipid raft mediated endocytosis.[24,25] 1DHFR^{2}RGD4C was mixed with bis-MTX-ASO-FITC and incubated with MDA-MB 231 cells for 4 and 24 h. During the last 30 min of incubation, cells were treated with Cholera Toxin Alexa Fluor 594 (marker for caveolin endocytosis). Consistent with caveolin-mediated endocytosis, confocal microscopy visualization at 24 h revealed substantial overlap between fluorescein labeled nanostructures (green) and Alexa Fluor 594 labeled cholera toxin (red) (Figure 2b, Figure S5 in the Supporting Information).

Having ascertained the ability of the CSANs-RGD4C nanostructures to deliver oligonucleotides to MDA-MB-231 cells, we probed the ability of the oligonucleotides to escape the endosome and produce an antisense effect within the cells. We evaluated the efficacy of antisense suppression of eIF4E expression by two different methods: a cellular translational reporter assay and Western blot analysis. In this system, cells are transfected with a plasmid that expresses mRNA for the reporter enzyme, *Renilla reniformis* luciferase (RLUC). The translation of RLUC is strictly cap-dependent[26] and thus, reflects on the abundance/activity of eIF4E.[27] MDA-MB 231 cells, which transiently expressed luciferase reporter plasmid, were treated for 48 h with CSANs-RGD4C nanostructures containing bis-MTX-ASO (or controls). The cells were lysed after the treatment, and luciferase activity was measured by luminometry. Luminescence in lysates from untreated cells (neutral control) was set at 100% (Figure 3). Cells treated with 1 μM 13DHFR^{2}RGD4C, 1DHFR^{2}RGD4C, or bis-MTX-ASO

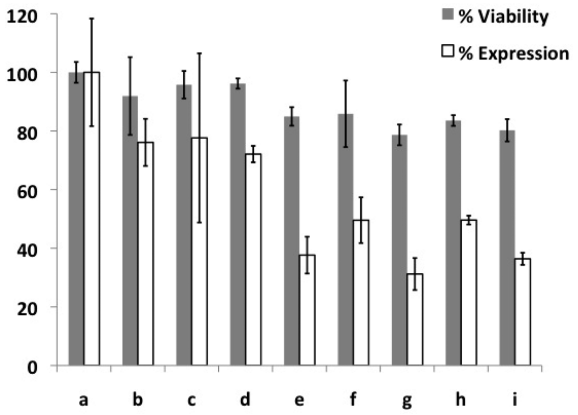

Figure 3. Translational reporter assay data with cell viability (gray bars) and % expression (white bars) of luciferase in MDA-MB 231 cells at 48 h, no treatment a; 1DHFR^{2}RGD4C, b; 13DHFR^{2}RGD4C, c; Bis-MTX-ASO, d; ASO with Oligofectamine, e; 0.5 μM multimer, f; 1.0 μM multimer, g; 0.5 μM monomer, h; 1.0 μM monomer, i.

alone (negative controls) showed an approximately 20% decrease in luminescence levels. Transfection of the cells with 1 μM ASO with Oligofectamine (positive control) resulted in a 60% knock down in luciferase expression. By comparison, delivery of 1 μM bis-MTX-ASO by either 13DHFR^{2}RGD4C or 1DHFR^{2}RGD4C reduced the luciferase expression by nearly 65%. Dose dependence could be observed for the effect of ASO, since treatment of the MDA-MB-231 cells with the bis-MTX-ASO and either 13DHFR^{2}RGD4C or 1DHFR^{2}RGD4C at the lower concentration of 0.5 μM monomer resulted in only about 55% luminescence in both cases. Dose dependent decrease in luminescence was also observed when 1DHFR^{2}RGD4C was mixed with bis-MTX-ASO and treated with MDA-MB 231 cells at concentration raging from 0.1 μM to 1 μM (Figure S6 in the Supporting Information). Viability of the cells was determined by quantifying the total amount of protein in the cell lysate by Bradford's protein assay. Control

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cell viability was found to be greater than 90% after 48 h, while 75−80% of the cells treated with the CSANs-ASO remained viable over the same time period. The minimal reduction in viability shows that the decrease in luminescence is a result of activity by the ASO and not due to a reduction of viability resulting from inherent nanostructure toxicity.

As a secondary measure of antisense activity, Western blotting was performed to evaluate the knock down of eIF4E expression in MDA-MB-231 cells (Figure 4). Results were

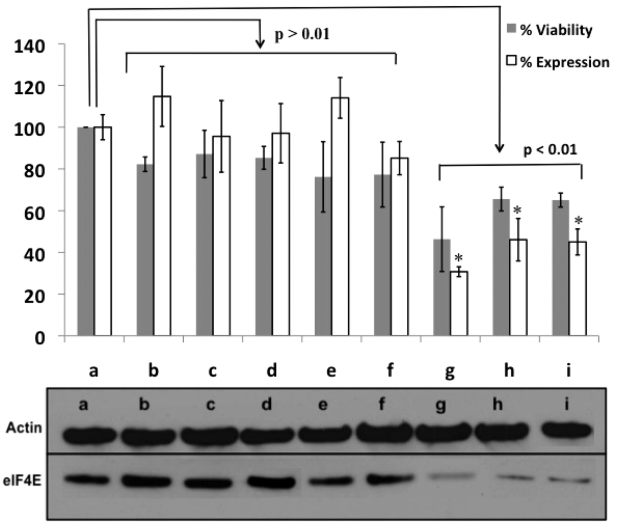

Figure 4. Western blotting data with cell viability (gray bars) and % expression of eIF4E/actin (white bars) of MDA-MB-231 cells at 48 h, a; 1DHFR^{2}RGD4C, b; 13DHFR^{2}RGD4C, c; Bis-MTX-ASO, d; 1.0 μM multimer scrambled, e; 1.0 μM monomer scrambled, f; 1.0 μM ASO with Oligofectamine, g; 1.0 μM multimer, h; 1.0 μM monomer, i. (* $p > 0.01$.)

normalized to untreated MDA-MB-231 cells. Treatment with 1 μM 13DHFR^{2}RGD4C, 1DHFR^{2}RGD4C, or bis-MTX-ASO alone showed no significant decrease in eIF4E expression levels (two sample t test, $p > 0.01$). Delivery of an oligonucleotide by either 13DHFR^{2}RGD4C or 1DHFR^{2}RGD4C, in which the oligonucleotide sequence was scrambled, did not significantly affect eIF4E expression (two sample t test, $p > 0.01$), whereas CSAN nanostructure (monomer and multimer) directed delivery of ASO resulted in a greater than 50% reduction in eIF4E expression at a concentration of 1 μM, which is comparable to the 70% reduction in eIF4E expression observed for ASO delivered with the Oligofectamine reagent (two sample t test, $p > 0.01$). Further, CSANs without RGD4C peptide did not knock down eIF4E expression in MDA-MB 231 cells (data not shown). Viability of the cells was determined by manually counting the cells. Even though the viability of the cells was slightly lower as compared to luciferase assay, the general trend was similar in both assays.

We have shown that chemically self-assembled nanostructures can be modified to display cell targeting peptides, specifically RGD4C, a cyclic peptide targeting integrins found on the surface of solid tumors and neovasculature. Modification of the bis-MTX ligand allows for attachment of oligonucleotides which can be delivered to breast cancer cells which overexpress $\alpha V\beta 3$ integrins. The benefit of using RGD4C as a targeting element is that it consists of natural amino acids and as such can be expressed as part of the DHFR fusion protein in *E. coli* cells. The results of the luciferase reporter assay and Western blot analysis of eIF4E levels are consistent with the delivery and endosomal escape of ASO by CSANs-RGD4C nanostructures. The monomeric and multimeric nanostructures showed similar knockdown. This indicates that, with the help of CSANs, if we keep the concentration of ASO constant, we are able to see a similar antisense effect irrespective of whether we use monomeric or multimeric CSANs. There are number of examples in the literature in which the cellular delivery of drugs, oligonucleotides, and nanoparticles has been facilitated by conjugation to cyclic RGD peptides or analogues. For example, Juliano and co-workers have recently shown that nucleic acids can be delivered selectively to cancerous cells by conjugation to a semisynthetic RGD based peptide.[24] We are also exploring the use of CSANs for the delivery of siRNA, shRNA, and miRNA species. In addition, the flexibility and modular nature of CSANs will allow the potential for nucleic acid delivery by scFvs and peptides that target a wide range of receptors to be investigated.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthetic methods, characterization, other experimental details, and additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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