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Programmable Self-Assembly of Antibody–Oligonucleotide Conjugates as Small Molecule and Protein Carriers

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Supporting Information

ABSTRACT: Dihydrofolate reductase single-chain variable fragment (scFv) fusion proteins can be used for the targeted cellular delivery of oligonucleotides, conjugated small molecules, and proteins via labeling of oligonucleotides by bis-methotrexate.

The advent and growth of antibody–drug conjugates (ADCs) has demonstrated the significant potential afforded by targeted drug delivery.[1] Drugs that have previously been abandoned from development pipelines due to extreme toxicities or undesirable pharmacokinetic properties have been resurrected for use as antibody conjugates, with targeted delivery bypassing their previous off target toxicities. Outside of the traditional medicinal chemistry development of therapeutic small molecule drugs, there has been continued interest in the therapeutic use of toxic proteins and oligonucleotides. These too have been developed as antibody conjugates, usually as fusion proteins or chemical conjugates, again allowing for their targeted delivery to cells of interest.[2] Small molecules and oligonucleotides have also been conjugated to the catalytic antibody 38C2 to create chemically programmable antibodies.[3] Changing the cytotoxic effector being delivered or the diversity of the targeting elements being utilized with these approaches requires re-engineering and development. Hence, it would be beneficial to develop a simple system that would require minimal redesign to attain robust targeted cellular and tissue drug delivery.

Previously, we have reported the formation of chemically self-assembled antibody nanorings (CSANs) by oligomerizing antiCD3 single-chain variable fragment (scFv) containing dimeric dihydrofolate reductase fusion proteins (**DHFR^{2}antiCD3**) with a bis-methotrexate (**bis-MTX**) ligand.[4] The antiCD3 CSANs interact with CD3+ T cells in a tissue specific manner similar to that of the parental antiCD3 monoclonal antibody (mAB). We hypothesized that **DHFR^{2}antiCD3** proteins could be used to carry single stranded oligonucleotides and DNA duplexes, with attached cargoes, inside cells via modification of **bis-MTX** (Figures 1a and 2a).

We have prepared a bis-MTX molecule with a third arm containing a maleimide for reaction with thiol functionalized oligonucleotides (Supporting Information (SI), Figures S1 and S2). In order to study the uptake of bis-MTX oligo conjugates by cells we prepared a bis-MTX oligo conjugate labeled with fluorescein (**bis-MTX oligo FITC**; Figure 1a). Bis-MTX oligo conjugates were analyzed and characterized by liquid chromatography mass spectrometry (SI, Figures S3 and S4).

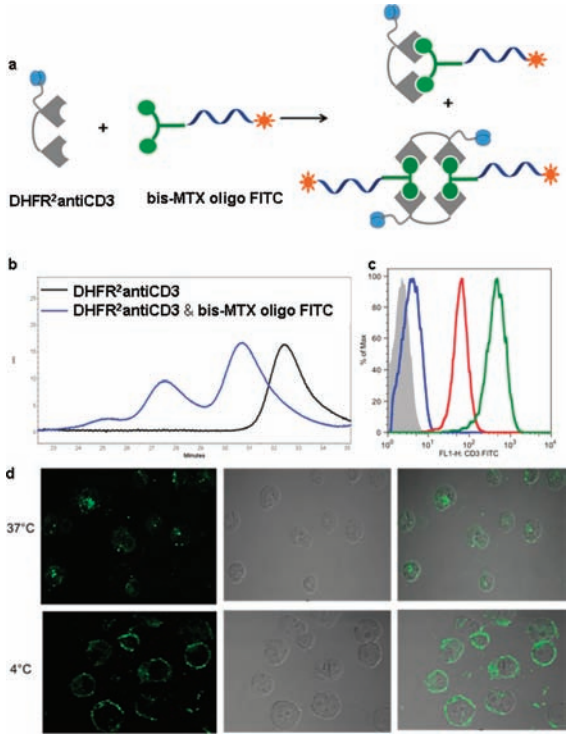

Figure 1. (a) Self-assembly of monomeric and dimeric antiCD3-oligonucleotide conjugates through DHFR-MTX binding. **DHFR^{2}antiCD3** contains two DHFR proteins (gray) and an antiCD3 scFv (blue). **Bis-MTX oligo FITC** has bisMTX, shown in green, attached to the oligo (blue) which is labeled with fluorescein isothiocyanate (FITC, orange). (b) Size exclusion chromatography analysis: **DHFR^{2}antiCD3** alone (black line) and after incubation with **bis-MTX oligo FITC** (blue line). (c) Flow cytometry data for binding of antiCD3 oligo-FITC CSANs (red line), oligo-FITC alone (blue line) and mAb antiCD3 FITC (green line) with unstained HPB-MLT cells (shaded gray). (d) Fluorescence confocal (first column) and differential interference contrast (second column) and overlay (third column) images of FITC-labeled oligonucleotides delivered to HPB-MLT cells via antiCD3 scFv at 37 and 4 °C.

Incubation of **bis-MTX oligo FITC** with **DHFR^{2}antiCD3**, which has a 13 amino acid linker between the DHFR proteins, results in a mixture of dimeric and internal monomeric antiCD3

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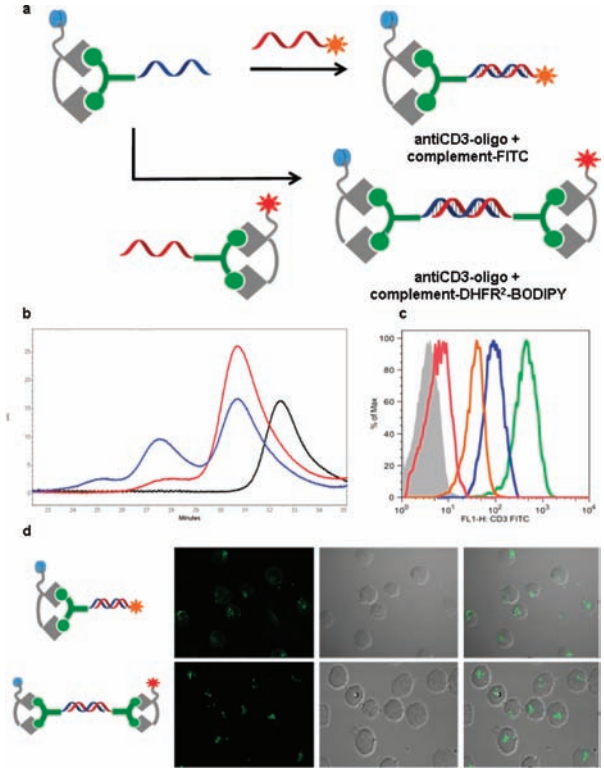

Figure 2. (a) Schematic showing self-assembly of **DHFR²antiCD3**-oligonucleotide duplex conjugates to carry small molecules or proteins inside cells. **DHFR²antiCD3**-oligo is formed from **DHFR²antiCD3** and **bis-MTX oligo**. Incubation with either complement-FITC (top arrow) or complement-**DHFR²-BODIPY** (bottom arrow, DHFR in gray and BODIPY in red) results in duplex formation between the complementary oligos yielding the final constructs. (b) SEC showing **DHFR²antiCD3** (black line), **DHFR²antiCD3** and **bis-MTX oligo** (blue line) and the mixture of species obtained when **DHFR²antiCD3** is preincubated with NADPH prior to addition of **bis-MTX oligo** (red line). (c) Flow cytometry data for binding of **DHFR²antiCD3**-oligo + complement-FITC (blue line), **DHFR²antiCD3**-oligo + complement-**DHFR²-BODIPY** (orange line), mAb antiCD3 FITC (green line), **DHFR²-BODIPY** (red line) with unstained HPB-MLT cells (shaded gray). (d) Fluorescence confocal (first column) and differential interference contrast (second column) and overlay (third column) images of complement-FITC (top row) and complement-**DHFR²-BODIPY** (bottom row) delivered to HPB-MLT cells via duplex formation with **DHFR²antiCD3**-oligo at 37 °C.

nanorings as analyzed by size exclusion chromatography (SEC; Figure 1b). The black line reveals that **DHFR²antiCD3** (68 kDa) elutes as a single peak with a retention time of 32.7 min. Upon incubation with **bis-MTX oligo FITC**, this peak disappears and two new prominent peaks appear, at 27.5 and 30.6 min. Both of these peaks show absorbance at 494 nm, revealing the presence of FITC labeled oligonucleotide in the eluted species. The elution profile is similar to that obtained when **bis-MTX** is incubated with **DHFR²antiCD3**,[4a] although there appears to be a shift toward more internal monomer when **bis-MTX oligo FITC** is used for the dimerization. When **DHFR²antiCD3** is incubated with **bis-MTX** the internally cyclized **DHFR²antiCD3** peak elutes slightly later than **DHFR²antiCD3**, due to the decreased hydrodynamic radius of the species.[4a] Here the internal monomeric species is larger than **DHFR²antiCD3** alone as **bis-MTX oligo FITC** is of higher molecular weight and will have a greater hydrodynamic radius as compared to **bis-MTX**.

Bis-MTX oligo FITC and **DHFR²antiCD3** were incubated with CD3+ HPB-MLT (T-leukemia) cells and after washing the cells were analyzed by flow cytometry (Figure 1c). Cells treated with antiCD3-oligo-FITC showed increased fluorescence intensity (red line), as compared to untreated cells (shaded gray), suggesting interaction between the FITC labeled oligo and CD3+ cells. Incubation with **bis-MTX oligo FITC**, in the absence of **DHFR²antiCD3**, showed only a small increase in fluorescence (blue line), presumably due to nonspecific binding, demonstrating the necessity of antiCD3 scFv for the interaction of the oligonucleotide with the cells. Preincubation of HPB-MLT cells with the parental antiCD3 mAb (UCHT-1) prevented binding of **Bis-MTX oligo FITC** and **DHFR²antiCD3**, as indicated by no increase in fluorescence, showing the specific interaction between the construct and cell-surface CD3 (SI, Figure S5). The cell-surface-specific nature of binding was further confirmed by incubation of HPB-MLT cells with varying concentrations of construct. The observed fluorescence, as measured by flow cytometry, decreased with decreasing concentration of fluorescently labeled species (SI, Figure S6). Incubation of **Bis-MTX oligo FITC** and **DHFR²antiCD3** with CD3 negative Raji cells (B cells) resulted in only minor nonspecific binding, showing cell specificity (SI, Figure S7).

To further probe the cellular interaction, **bis-MTX oligo FITC** and **DHFR²antiCD3** were incubated with HPB-MLT cells at either 4 or 37 °C, after which the cells were imaged by fluorescence confocal and differential inference contrast microscopy (Figure 1d). Green fluorescent punctates were observed inside cells treated with antiCD3-oligo-FITC at 37 °C, thus indicating that the **bis-MTX oligo FITC** has been endocytosed along with **DHFR²antiCD3** (Figure 1c, upper panels). A similar image with green fluorescent punctates is obtained upon incubation of FITC labeled UCHT-1 with HPB-MLT cells.[4] Cells treated at 4 °C (Figure 1c, lower panels) show green fluorescence on the cell surface suggesting that at this temperature **DHFR²antiCD3**, with **bis-MTX oligo FITC**, binds to the CD3 receptor on the cell membrane but is not internalized. The temperature dependence of internalization indicates that endocytosis occurs via an energy dependent mechanism, which is consistent with the internalization of CD3.[3b] Co-localization studies at 37 °C with transferrin, a marker of receptor-mediated endocytosis, suggested that **DHFR²antiCD3**, with **bis-MTX oligo FITC** localize to endosomes, in a similar manner to UCHT-1 (SI, Figure S8). This also suggests that the construct follows an internalization mechanism similar to transferrin, which relies on clathrin-dependent endocytosis.[5] Incubation of **bis-MTX oligo FITC** with HPB-MLT cells, in the absence of **DHFR²antiCD3**, results in no increased cellular fluorescence, thereby demonstrating the necessity of the scFv-receptor interaction for oligonucleotide binding and uptake (data not shown).

Having shown the ability of **DHFR²antiCD3** to carry bis-MTX functionalized single stranded oligonucleotides inside T-leukemia cells, we explored the capability of the antiCD3-oligo conjugates to deliver cargoes inside cells through the formation of double-stranded helices (Figure 2a). In order to reduce the heterogeneity of the species formed, we preincubated **DHFR²antiCD3** with NADPH prior to addition of the bis-MTX oligo. This results in formation of only the intramolecular dimer species as analyzed by SEC (Figure 2b). NADPH enhances the affinity of MTX for DHFR by at least 100-fold, thus favoring intramolecular dimerization.[6] This can be seen by

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the decrease in the absorbance of the peak eluting at 27.5 min and a concomitant increase in the peak eluting at 30.6 min (red trace), as compared to the elution profile when the species are mixed in the absence of NADPH (blue trace). Preincubation with NADPH has a similar affect on the elution profile when the protein is mixed with **bis-MTX** (data not shown).

To evaluate the use of duplex formation for the delivery of small molecules (e.g., drugs, dyes, etc), we decided to again use a FITC labeled oligonucleotide to allow the use of flow cytometry and confocal microscopy to visualize delivery. However, this time we conjugated bis-MTX to an oligonucleotide that is complementary to the FITC labeled oligonucleotide. The bis-MTX oligo was incubated with **DHFR^{2}antiCD3**, in the presence of NADPH, to form the intramolecular species (Figure 2a, top left). To this mixture was added the complementary FITC labeled oligonucleotide, thus allowing sequence specific DNA duplex formation (Figure 2a, top half). For the delivery of proteins, we conjugated both complementary strands at the 3′ end with bis-MTX. One strand was then conjugated to **DHFR^{2}antiCD3** in the presence of NADPH (*vide supra*). In a similar manner, the other strand was incubated in the presence of NADPH with a **DHFR^2** quadruple mutant (C85A/C152S/C257A/C324S) containing a C-terminal Gly-Gly-Cys tag that has been site specifically labeled with the maleimide BODIPY-FL fluorescent dye (**DHFR^2-BODIPY**). The two intramolecular dimers, with complementary oligos, were incubated at 4 °C overnight allowing for duplex formation (Figure 2a, bottom half). The **DHFR^{2}antiCD3**-duplex FITC and **DHFR^{2}antiCD3**-duplex **DHFR^2-BODIPY** conjugates were then incubated with HPB-MLT cells and analyzed by flow cytometry (Figure 2c) and confocal microscopy (Figure 2d).

Flow cytometry analysis of HPB-MLT cells incubated with **DHFR^{2}antiCD3**-duplex FITC revealed an increase in fluorescence as compared to the unlabeled cells (blue line and shaded gray respectively; Figure 2c). Similarly, cells treated with the **DHFR^{2}antiCD3**-duplex **DHFR^2-BODIPY** showed an increase in fluorescence (orange line), while only a small increase in fluorescence (red line) and hence only minimal nonspecific interactions with the HPB-MLT cells were observed for cells treated with **DHFR^2-BODIPY** alone. Neither of the constructs showed specific binding to the CD3 negative Raji cells (SI, Figure S7). Unlabeled UCHT-1 (antiCD3 mAb) was able to prevent binding of both fluorescently labeled constructs, as observed by minimal increases in fluorescence as compared to the unstained cells (SI, Figures S9 and S10). Both constructs also show concentration dependence of the observed fluorescence when incubated with HPB-MLT cells, in a similar manner to **DHFR^{2}antiCD3** with **bis-MTX oligo FITC** (SI, Figures S11 and S12).

Fluorescence confocal microscopy demonstrated that cells treated with **DHFR^{2}antiCD3**-oligo + complement-FITC (Figure 2d, upper panels) and **DHFR^{2}antiCD3**-oligo + complement **DHFR^2-BODIPY** at 37 °C (Figure 2d, lower panels) have internalized green punctates indicating binding and uptake of the labeled oligonucleotide and protein. Again, only cell surface binding was observed for cells treated with the conjugates at 4 °C (SI, Figure S13). Thus, the DHFR^2-BisMTX based chemically self-assembled antibody−oligonucleotide conjugates affords a unique system for the programmable assembly and cellular delivery of oligonucleotides, drug conjugated oligonucleotides or DHFR^2 fusion proteins.

Herein, we have reported on the use of **DHFR^{2}antiCD3** scFv fusion proteins for the targeted cellular delivery of single stranded oligonucleotides through attachment to bis-MTX, a ligand for **DHFR^2** proteins. We have also shown that oligonucleotide conjugated small molecules and proteins can be delivered to cells through formation of double stranded helices. The modular nature of this system should be useful for the delivery of a variety of cargoes (nucleic acids, small molecules, proteins) to cells for which an internalizing scFv or peptide is known. Compared to other oligonucleotide conjugation approaches, the ease of the production of DHFR^2 fusion proteins, the robustness of the binding of DHFR^2 to bis-MTX and the control over binding stoichiometry are attractive features of this system. Currently, we are exploring the delivery of functional nucleic acids, DNA nanostructures and proteins to cells and tissues by this methodology; the results will be reported in due course.

■ 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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