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A Wireframe DNA Cube – Antibody Conjugate for Targeted Delivery of Multiple Copies of Monomethyl Auristatin E

*Anders Märcher, Minke A. D. Nijenhuis, Kurt V. Gothelf**

Abstract: *In recent years, several antibody drug conjugates (ADC) have been accepted by the FDA as therapeutics against cancer. It is well-known that control of drug-to-antibody ratio (DAR) is vital for the success of an ADC, which inspires the advancement of better and simpler methods for tight control of DAR. We present the development of an antibody DNA wireframe cube conjugate for precise control of DAR. The DNA wireframe cube consists of four single strands, which when folded present eight single stranded domains. One domain is bound to a monofunctionalized antibody DNA conjugate, and the seven others are attached to DNA functionalized with the potent tubulin inhibitor MMAE, thereby preparing an ADC with a DAR of precisely seven. The formation of the ADC is investigated by gel electrophoresis and atomic force microscopy. Lastly, the developed MMAE loaded ADC was used for targeted drug delivery in vitro.*

The emergence of standardized techniques for development of humanized monoclonal antibodies (mAbs) that recognize specific targets, has revolutionized cancer treatment.[1–3] Numerous mAbs have been developed for tumor surface antigens, and have found usage as therapeutics.[4] In recent years drug developers have sought to improve the therapeutic activity of mAbs by preparing antibody drug conjugates (ADCs).[5,6] In these ADCs, cytotoxic drugs are conjugated to mAbs, to combine the therapeutic effect of the drug and the cancer specificity of the mAbs. They are designed to have a wider therapeutic index, than the unconjugated drug. Since the mAbs preferentially bind to cancer cells, the adverse effect of the free drug is diminished. Since 2011 three ADCs have entered the market, and more than 20 ADCs are currently undergoing phase I and II clinical trials.[7]
Typically, extremely potent drugs are used in ADCs, primarily tubulin inhibitors or DNA alkylating agents.[7] The drugs are often conjugated to the antibodies in one of two ways. By reduction of accessible disulfides and later functionalization of the sulfhydryl with maleimides,[8] or by reaction with lysine residues on the surface of the protein with activated esters, such as the well-known *N*-hydroxysuccinimide (NHS) esters.[9–11] Both methods produce nonhomogeneous conjugates, as neither the drug-to-antibody ratio (DAR) or the site-specificity are controlled using these methods. Studies have shown that controlling especially DAR is detrimental to the *in vivo* efficacy of the antibody drug conjugates.[12–14] Methods for controlling DAR more tightly are available, and especially the use of site-directed mutagenesis is important, allowing for insertion of cysteine residues or peptide tags that can later be modified through enzymatic or chemical reactions.[12,15] These methods can provide homogeneous ADCs, but they are labor intensive, often low yielding, and require different mutated mAbs to investigate different DAR constructs.

An alternative option for precise control of DAR is the use of oligonucleotide nanotechnology. In its core, the programmability of oligonucleotides allows for precise design of constructs, where chosen modifiers, such as drugs, targeting units and imaging agents, can be precisely placed throughout the structure in a predefined ratio.[16] Multiple different systems have been developed, but currently most of these systems are used for either imaging or delivery of less potent drugs such as the DNA intercalator doxorubicin.[17–21] We envisioned that by combining the use of a DNA nanostructure and an antibody, we would be able to prepare an ADC with high loading, good control of DAR, and use highly potent drugs. Only few antibody oligonucleotide constructs have been used as drug delivery vehicles, and in these cases the DNA structures used have only carried either a single highly potent drug or multiple drugs of much lower potency.[22,23] In both cases a DNA duplex has been used, and we anticipated that the use of a more complex DNA nanostructure would allow for high control of loading of more potent drugs. It should be mentioned that drug delivery involving DNA, has the obvious downside that the serum stability of DNA structures is low because of serum nucleases,[24] and there might be leakage from the structure as a result of this. Hence the method presented here is for proof of concept, and for *in vivo* applications it should be stabilized with modifications such as PEG chains[25] or nucleotide analogues,[17,26] to avoid low serum half-life.

Herein, we present the use of a drug-functionalized DNA wireframe cube conjugated to an antibody for targeted drug delivery. The DNA antibody construct is illustrated in Figure 1a. We were inspired by the elegant DNA wireframe cube originally designed by the group of Sleiman, a structure that has been applied in several studies during the last decade.[27,28] In 2018 they showed the preparation of nanoparticles within the cube, and showed that the particles could be modified with specified DNA sequences.[29] Later they used the cube to investigate the cellular fate of fluorescently labeled DNA nanostructures.[30]

The cube is relative small with a diameter of approximately 15 nm and as such comparable to the height of an immunoglobulin G1.[31] In IgG1s the most remote parts are approximately 15 nm apart.[29] The cube entail, in the core structure, four single strands of 90 bases (CbS1, CbS2, CbS3, CbS4). The cube is constructed of eight single stranded domains, and we envisioned that seven of these could bind complementary strands conjugated to the highly cytotoxic drug monomethyl auristatin E (MMAE).[7] The last single stranded domain will be used for connection to an antibody, thereby preparing an ADC with a DAR of seven. In earlier studies (data not shown), we have attempted the preparation of a 22-mer DNA strand with multiple copies of MMAE. We find that preparing

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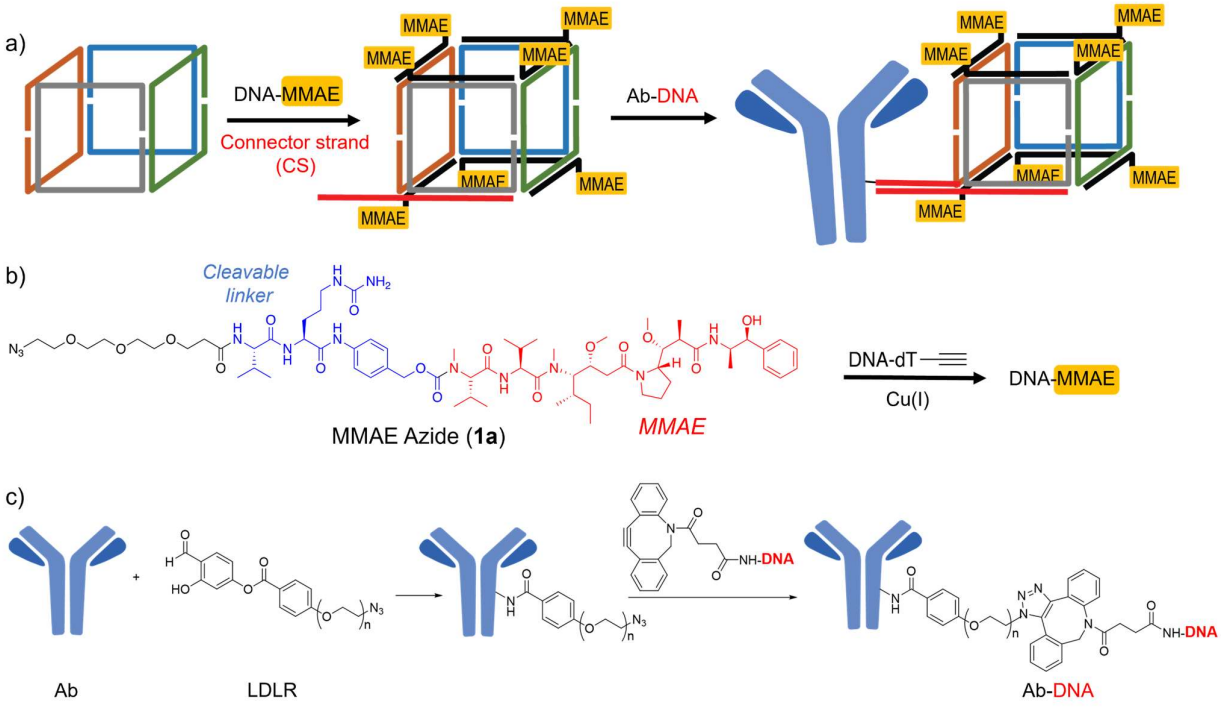

Figure 1. a) Illustration of the formation of the Cube-C_{MMAE}-Ab along with the required components. b) Visualization of linker **1a,** which is used for the formation of the C_{MMAE} strand. The linker consists of the black part with an azide, which is used to attach the rest of the linker to DNA, the blue part, which is a cleavable spacer, that upon cleavage self-immolates and releases the red part, which is the tubulin inhibitor MMAE. c) Illustration of the use of the LDLR to prepare a single modified DNA antibody conjugate.

strands with more than two copies of MMAE, results in a sharp decline in yield. This is likely because of the hydrophobicity of MMAE, as this parameter is problematic for most drugs used in ADCs.[32,33] We hypothesized that the use of the DNA wireframe cube, would allow for the assembly of a DNA construct with multiple MMAE copies, because of the control of MMAE positioning in the three dimensional DNA structure.

We started out preparing the MMAE strand. The drug should be released upon endosomal uptake of the ADC, and therefore we have linked MMAE in **1a** *via* the FDA approved Val-Cit-PAB-Drug linker (Figure 1b).[6] The Val-Cit-PAB linkers have been used for preparing ADCs previously, some of which are FDA approved anti-cancer therapeutics.[34,35] The linker works by a well-known and studied mechanism, which involves the cleavage of the N-terminal peptide bond of citrulline by lysosomal peptidases, followed by a 1,6-elimination and release of the chosen drug.[36,37]

The linker used here is prepared in eight steps and is designed to self-immolate and release MMAE upon endosomal uptake of the ADC and cleavage of the C-terminal peptide bond of citrulline by lysosomal peptidases. The MMAE derivative **1a** is prepared with an azide that is further conjugated to an alkyne modified DNA strand. The alkyne strand (C_{Alk}) is designed with a complementary region of fifteen bases to seven of the single stranded domains on the wireframe DNA cube, with the alkyne modification placed on the end of a five thymine overhang at the 5' end. Using the Cu(I)-catalyzed azide-alkyne cycloaddition reaction (CuAAC) the C_{Alk} strand is modified with **1a**, to prepare the MMAE-DNA conjugate (C_{MMAE}) (Figure 1c). We want to ensure that the C_{MMAE} DNA strand retains binding to the cube strands and therefore investigated the thermal denaturation of a duplex between C_{MMAE} and the complementary part of a cube strand. This was compared to the same duplex with the C_{Alk} strand. No difference in thermal stability is observed, as the melting temperature remains at 58 °C,

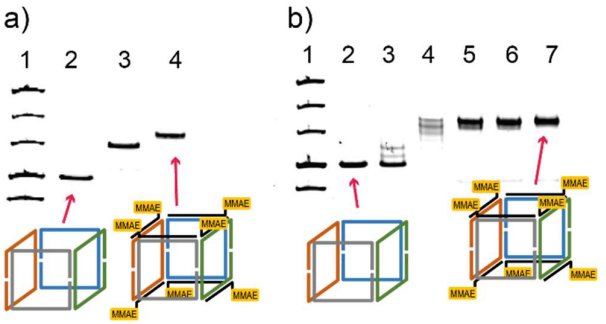

Figure 2. a) Investigation of the wireframe DNA cube, Cube-C_{Alk} and Cube-C_{MMAE} constructs with native TBE-PAGE gel (4-20%) analysis. Lane 1: DNA ladder, Lane 2: DNA wireframe Cube, Lane 3: Cube-C_{Alk}, Lane 4: Cube-C_{MMAE}. b) Investigation of the required equivalents of C_{MMAE} to form the Cube-C_{MMAE} analyzed with native TBE gel analysis. Lane 1: DNA ladder, Lane 2: DNA

as is also found for the alkyne modification (Supporting information Fig. S3-4).

Initial results of forming the wireframe DNA cube, without complementary strands showed, that the cube forms near quantitatively after following the temperature ramp for assembly of the cube reported by Sleiman and coworkers (Figure 2a).[29] Studies using C_{Alk} to investigate the formation of the wireframe DNA cube with complementary sequences also proved

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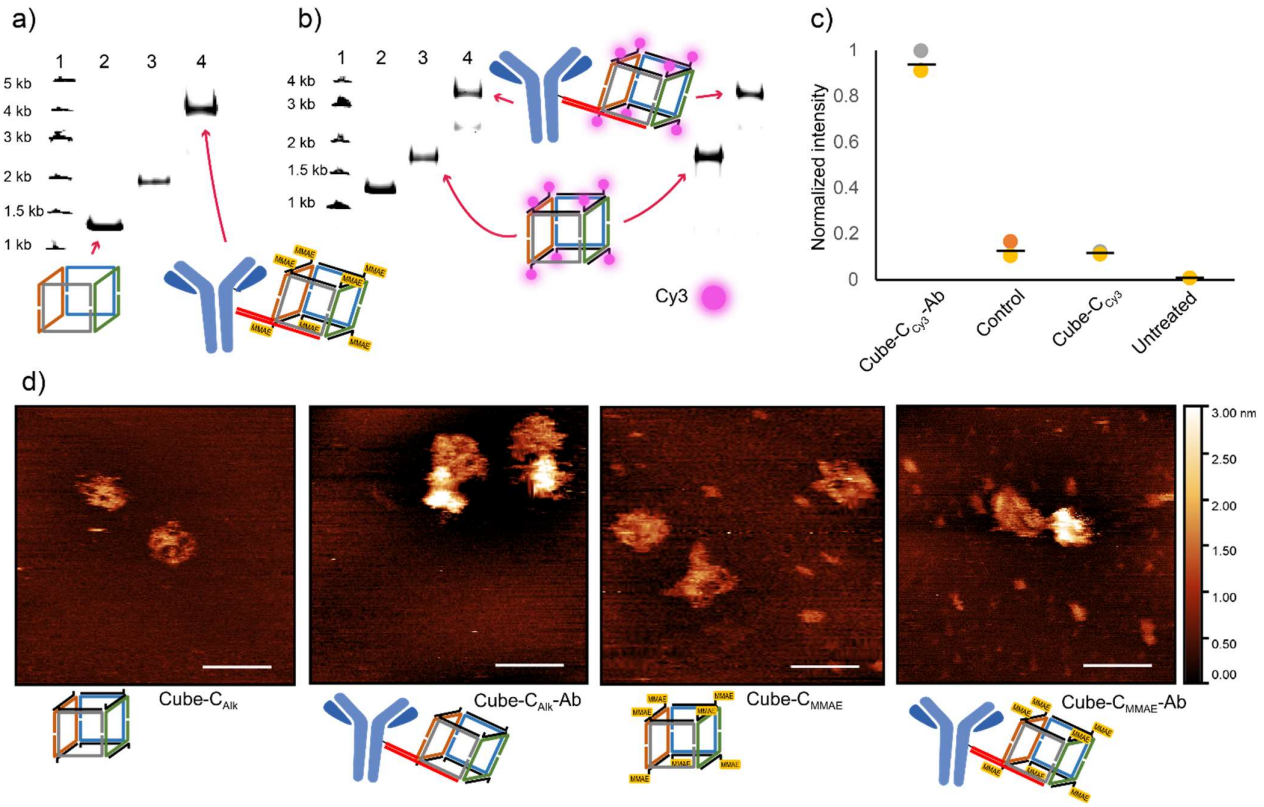

Figure 3. a) The formation of Cube-C_{MMAE}-Ab investigated with native TBE-PAGE gel (4-20%) analysis. Lane 1: DNA ladder, Lane 2: DNA wireframe cube, Lane 3: Cube-C_{MMAE}, Lane 4: Cube-C_{MMAE}-Ab. b) The formation of Cube-C_{Cy3}-Ab investigated with native TBE-PAGE gel (4-20%) analysis. Lane 1: DNA ladder, Lane 2: DNA wireframe cube, Lane 3: Cube-C_{Cy3}, Lane 4: Cube-C_{Cy3}-Ab. Left: SybrGold stain, right: Cy3 fluorescent scan. c) Flow cytometry on SKBR3 (HER2+) using different Cy3 Cube constructs. The control sample is a pretreatment of the cells with native unconjugated antibody. d) AFM images of different Cube constructs. Height scale applicable for all images is shown on the left. Scale bar is 30 nm. Additional AFM images are available in the supporting information Fig. S15-18. For full gel images see Fig. S9-S10 in the Supporting information.

successful, using only nine eq. of the C_{Alk} strand (Supporting information Fig. S8). We then attempted formation of the DNA cube with all seven C_{MMAE} strands. Initial attempts of adding the C_{MMAE} strands during the temperature ramp with the rest of the cube strands showed no formation of the MMAE loaded cube on native gel electrophoresis. Eventually we found that preparing the cube with the four cube strands using the normal temperature ramp, followed by addition of the C_{MMAE} strand at room temperature overnight, allow for some attachment of the C_{MMAE} strand to the wireframe cube.

However, using only seven eq. of C_{MMAE} pr. cube strand shows no clear single band formation, on native gel electrophoresis. Only a smear is observed, and we therefore investigated the overnight incubation using different eqs. of C_{MMAE} strands. After incubation, excess unbound C_{MMAE} strand is removed using a 30 K molecular weight cut off (MWCO) filter. We find that using 30 eq. of the C_{MMAE} strand overnight lead to the formation of a single higher lying band, compared to the naked wireframe cube, which is presumably the fully loaded MMAE cube (Cube-C_{MMAE}) (Figure 2b). The use of a MWCO filter for purification of the cube constructs is used in all the following experiments. The Cube-MMAE is expected to migrate slower on native gel electrophoresis, as the diameter of the construct is larger than the naked cube.

With the fully loaded MMAE wireframe DNA cube at hand, we started preparing the antibody DNA conjugate. We were concerned that direct binding of an antibody DNA construct to the remaining single chain on the cube might be unfavorable because of steric repulsion. We therefore design the antibody DNA binding with a “connector” strand (CS), as shown in Figure 1. We chose the antibody Trastuzumab, as multiple ADCs are prepared using this antibody.[38–40] Furthermore, Trastuzumab targets the cancer marker human epidermal growth receptor 2 (HER2), and it is well-known that Trastuzumab upon binding to HER2 can be recycled into the endosome and later the lysosome,[41–43] as is required for the release of MMAE in linker **1a**. The DNA antibody construct is prepared using a lysine directed labeling reagent (LDLR) recently published from our lab.[44] The method allows for easy fabrication of antibodies functionalized with azides, with high control of number of labels pr. antibody. The method requires that the functionality of interest is dibenzocyclooctyne (DBCO) modified. A DNA-DBCO strand is therefore prepared using standard DNA chemistry. The DBCO strand is given to azide functionalized Trastuzumab, and the mono-functionalized DNA antibody construct is purified by anion exchange and analyzed by reducing SDS-PAGE (Supporting information Fig. S13).

The antibody DNA construct does not tolerate the temperature ramp used for folding the cube strands. Therefore, it and the connector strand are incubated at room temperature overnight with the C_{MMAE} strand to the already folded cube strands. The complete construct is then analyzed by native gel electrophoresis (Figure 3a). We were pleased to see only one band, after

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incubation of the cube with the antibody DNA construct overnight. The construct, which is presumably the fully MMAE loaded cube with the antibody attached (Cube-C_{MMAE}-Ab), migrates even slower than the Cube-C_{MMAE} as expected. The Cube-C_{MMAE}-Ab, along with the other prepared DNA wireframe cube constructs, are further analyzed by atomic force microscopy (AFM), (Figure 3. Zoom out AFM images of all structures can be found in the Supporting information Figs. S15-18). The AFM analysis of the different cube structures, both with and without antibody, shows clear formation of all analyzed constructs. The DNA wireframe cube, is to some extend distorted by the AFM tip, but observed to be close to the reported size of approximately 15 nm for the structure.[29] The Ab with its more thick and dense structure shows significantly higher contrast in the AFM images than the Cube. In most images containing the Ab, it is viewed just as a dot, however, in some of the images the characteristic Y-shape of the antibody is observed.

Before using the Cube-C_{MMAE}-Ab construct for cell cytotoxicity, we wanted to ensure that the antibody retains antigen binding. As the conjugation approach used is not 100% site specific,[44] and considering the size of the attachment, we were concerned that the antibody might be sterically to hindered to bind its antigen favorably. To investigate the antigen binding of the cube antibody construct we prepared a Sulfo-Cyanine 3 (Cy3) loaded wireframe DNA cube. This is done by first preparing a Cy3 modified strand using the same sequence as for C_{Alk} and C_{MMAE}. We hypothesized that the Cy3 DNA strand (C_{Cy3}) would be quite soluble, and therefore have similar hybridization towards the wireframe cube as C_{Alk}. For initial attempts of forming the fully loaded Cy3 cube (Cube-Cy3), we therefore used nine equivalents of C_{Cy3} during the temperature ramp with the cube strands. This and the preparation of Cube-Cy3 with Trastuzumab proved successful, as shown by the appearance of a single band on native electrophoresis gel (Figure 3b). The band is furthermore visualized both by DNA staining and by fluorescence scanning for Cy3. The cube Cy3 constructs are then investigated with flow cytometry on a HER2 positive cell line, to confirm that the antibody retained binding. The breast cancer cell line SKBR3 is chosen and treated with either Cube-Cy3 or Cube-C_{Cy3}-Ab (Figure 3c). As a control, a population of the cells is pretreated with unconjugated Trastuzumab to block the available HER2 epitopes, these cells are then given Cube-Cy3-Ab. This is done to ensure that binding of the cube antibody construct to the cells, remains through the original epitope. From these experiments we can conclude that the cube antibody construct retains binding to HER2 through the original epitope.

Finally, we investigate the application of the developed ADC for targeted drug delivery. The cell line SKBR3 is used again, along with the cell line A431, which displays very low HER2 expression, as a control.[45] To ensure that targeted drug delivery is occurring an experiment with multiple washing steps was set up. The study is illustrated in Figure 4a. Trastuzumab with only one C_{MMAE} attached was also prepared as a control for this study. The antibody was modified with DNA using the same method, as has already been described, and was prepared with a DNA strand complementary to C_{MMAE}. To this antibody DNA conjugate was then given C_{MMAE} and the final structure (Ab-C_{MMAE}) was verified on native SDS-PAGE (Supporting information Fig. S14). The toxicity study is performed in triplicates, and four different constructs are tested: The Cube-C_{MMAE}-Ab, the Cube-C_{MMAE}, native Trastuzumab, Ab-C_{MMAE} and C_{MMAE}. Both DNA cube constructs are gel purified before usage. Each sample and a control are added with 10% PBS in cell media to a population of cells and left at 37 °C with 5% CO_2 for 30 min. The cell media is then removed, and the cells are washed three times with PBS. Then new cell media is provided, and the cells are left for 3 days, whereafter the number of live cells is quantified (Figure 4a). Except a small reduction for the samples treated with Trastuzumab or Ab-C_{MMAE}, only the developed ADC (Cube-C_{MMAE}-Ab) shows a decline in cell viability and only on the HER2 positive cell line SKBR3. This confirms that the DNA wireframe cube ADC construct can be used for targeted drug delivery *in vitro*

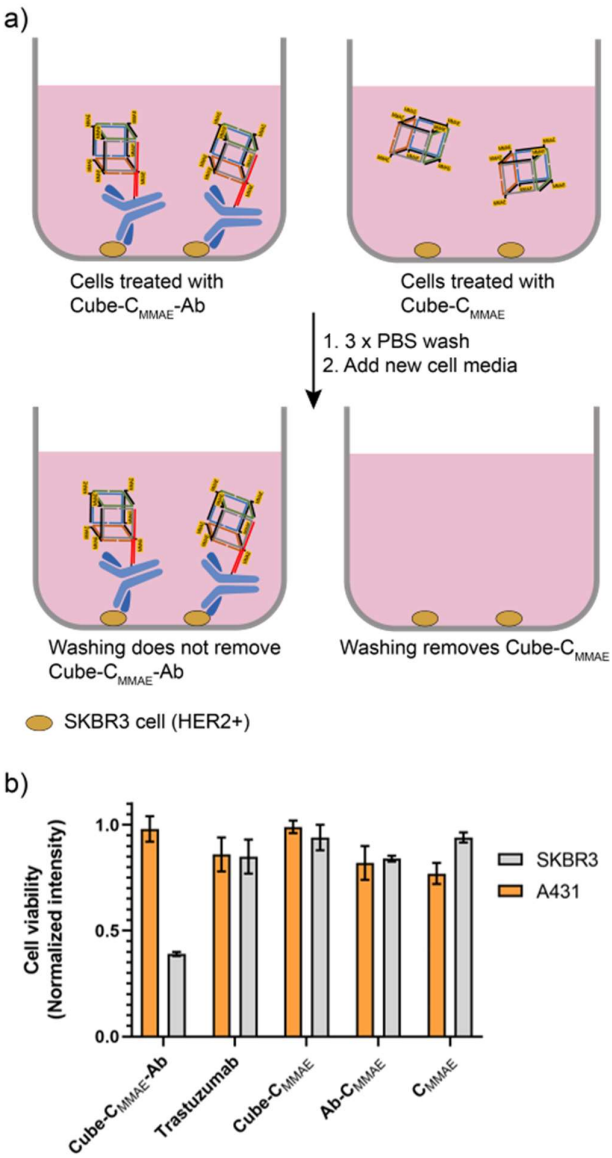

Figure 4. a) Illustration of the washing protocol used for showing targeted drug delivery on SKBR3 (HER2+) cells. Two wells with SKBR3 cells treated with Cube-C_{MMAE}-Ab or Cube-C_{MMAE} are shown before and after washing with PBS. b) Cell viability of SKBR3 (HER2+) or A431 cells treated with either Cube-C_{MMAE}-Ab, Cube-C_{MMAE} or Trastuzumab using the washing protocol. The experiment is done in triplicates.

(Figure 4b), and that both targeting and high drug loading is necessary in the tested setup to see lowered cell viability.

The construct developed herein would likely have a low *in vivo* half-life as a result of degradation of the DNA nanostructure used. To investigate this the serum stability of the final construct was investigated *in vitro*, and it was found that the structure is completely degraded over a 24 h period in 10% FBS, Fig. S11 in the supplementary information. This would likely mean that if the structure developed here was used *in vivo* untargeted release of MMAE would occur. Another issue for the construct used here is that its large scale production would likely be difficult. With this study we hope to inspire the development and use of simpler oligonucleotide nanostructures combined with antibodies, with life-time prolonging modifications such as PEG chains or non-natural oligonucleotides implemented.

To conclude, we have demonstrated a new approach to generate an ADC with an exact number of drug molecules, in this case seven copies of MMAE. Each of the drugs are covalently linked to ssDNA strands via a cleavable linker. Seven of the MMAE modified strands are assembled in a small DNA cube, which was used as the drug carrier and easily attached to a Trastuzumab-DNA conjugate by hybridization. DNA nanostructures are available in a plethora of different sizes and shapes and offers great control and opportunities to vary the number of drug molecules associated with the complex. Beside the high control of drug loading, the structures also offer advantages such as the charge of the oligonucleotides, which serves to keep multiple copies of a hydrophobic drug such as MMAE in solution. A dramatic decline in viability to approximately 40% is observed only for cells that express the HER2 receptor and are exposed to the full conjugate prepared here. While this approach provides proof-of-concept, some challenges of the system must be addressed before this system can be applied *in vivo*. Most importantly the DNA sequences must be modified or replaced with nucleotide analogues to resist enzymatic degradation and we will address this in future studies.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: antibody drug conjugate, controlled DAR, protein conjugation, DNA nanotechnology, atomic force microscopy

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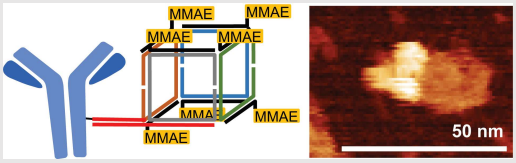

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A Wireframe DNA Cube - Antibody Conjugate for Targeted Delivery of Multiple Copies of Monomethyl Auristatin E

A method for preparation of an antibody - DNA wireframe cube - drug conjugate with exactly seven copies of the cytotoxic drug MMAE is presented. The drug is conjugated to a DNA strand via a cleavable linker and the conjugate is used for preparing a DNA wireframe cube. The drug loaded cube is furthermore attached to the antibody Trastuzumab. The construct is used for targeted drug delivery of MMAE to a HER2 positive cell line *in vitro* and a dramatic decline in viability is observed only for cells that overexpress the HER2 receptor.