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Modular Nucleic Acid Scaffolds for Synthesizing Monodisperse and Sequence-Encoded Antibody Oligomers

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SUMMARY

Synthesizing protein oligomers that contain exact numbers of multiple different proteins in defined architectures is challenging. DNA–DNA interactions can be used to program protein assembly into oligomers; however, existing methods require changes to DNA design to achieve different numbers and oligomeric sequences of proteins. Herein, we develop a modular DNA scaffold that uses only six synthetic oligonucleotides to organize proteins into defined oligomers. As a proof-of-concept, model proteins (antibodies) are oligomerized into dimers and trimers, where antibody function is retained. Illustrating the modularity of this technique, dimer and trimer building blocks are then assembled into pentamers containing three different antibodies in an exact stoichiometry and oligomeric sequence. In sum, this report describes a generalizable method for organizing proteins into monodisperse, sequence-encoded oligomers using DNA. This advance will enable studies into how oligomeric protein sequences affect material properties in areas

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AUTHOR CONTRIBUTIONS

P.H.W. and C.A.F. contributed equally.

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SUPPLEMENTAL INFORMATION

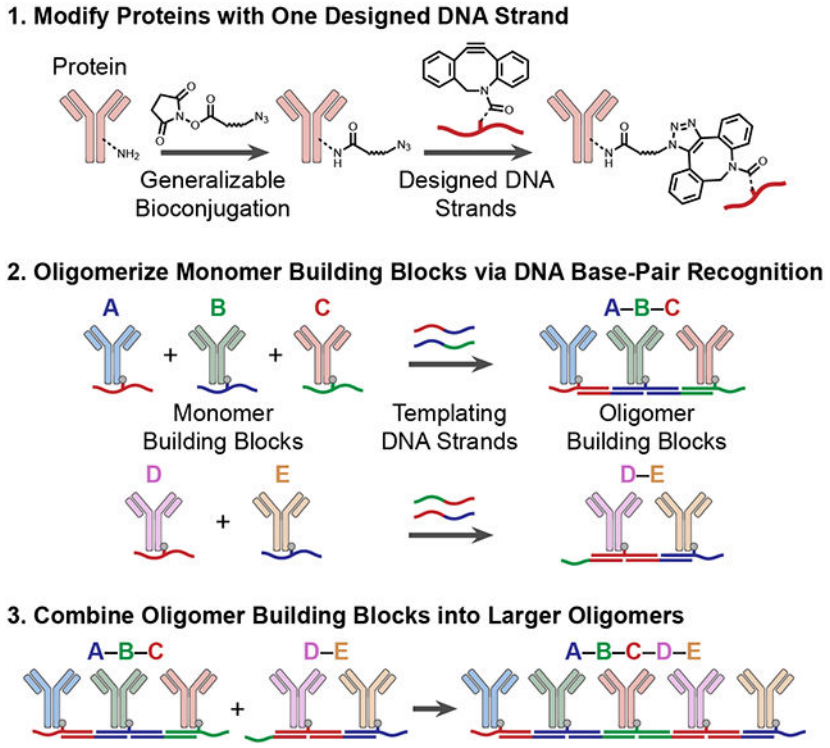
This section should include the titles and (optional) legends of all supplemental items. Document S1 is the main supplemental PDF: Document S1. Supplemental information regarding experimental procedures, results, and discussion, Tables S1-S3, Schemes S1-S5, and Figures S1-S13.

DECLARATION OF INTERESTS

"Synthetic Strategy to Polymerize Protein Into Molecularly Defined Polymers" Mirkin, C. A.; Winegar, P. H.; Figg, C. A., PCT/US2022/021359. March 22, 2022.

spanning pharmaceutical development, cascade catalysis, synthetic photosynthesis, and membrane transport.

Graphical Abstract



eTOC

In nature, many proteins organize into architectures where the exact number and spatial arrangement of each protein can dictate biological function, including catalysis and photosynthesis. To mimic and even surpass such function, the preparation of many different protein architectures is required. However, this is synthetically challenging. Herein, we developed a modular method that uses designed nucleic acid sequences to organize proteins into different monodisperse and sequence-encoded oligomers, including dimers, trimers, and pentamers.

Keywords

biomaterial; protein; antibody; DNA; DNA nanotechnology; polymer; oligomer; sequence-encoded; self-assembly; protein assembly

INTRODUCTION

In Nature, many proteins assemble into defined oligomeric architectures that contain exact numbers and oligomeric sequences of multiple different proteins.[1, 2, 3] Herein, oligomeric sequence of proteins and oligomeric protein sequence are defined as the order of proteins within an oligomeric architecture. This assembly can dictate the biological (*e.g.*, human

IgM antibodies contain five protein subunits),[4] catalytic (*e.g.*, eukaryotic RNA polymerase II contains twelve protein subunits),[5] photophysical (*e.g.*, cyanobacteria photosystem I contains twelve protein subunits),[6] and membrane transport (*e.g.*, *Streptomyces lividans* potassium channel contains four protein subunits)[7] properties of proteins. To mimic and potentially surpass these properties, the modular synthesis of different protein oligomers is needed. A versatile synthetic protein oligomerization method would: (1) provide access to a large number of proteins per oligomer, (2) provide access to any oligomeric sequence of the same or different proteins, (3) be generalizable with regard to proteins, and (4) not require mutations of the amino acid sequence of proteins and recombinant protein expression. A method that meets all four criteria would enable the study of how the identity, number, stoichiometry, oligomeric sequence, and architecture of proteins affects the emergent properties of oligomers. While strategies have been developed to prepare synthetic protein oligomers[8, 9, 10, 11] and study how oligomerization affects protein properties,[12, 13, 14, 15] there is no current method that meets all four listed criteria (Figures 1A and 1B). In this work, we explored the design and synthesis of a single modular nucleic acid scaffold that can be used to organize proteins into a near limitless array of monodisperse and sequence-encoded protein oligomers (Figure 1C).

Protein oligomers are frequently prepared using techniques from molecular biology, including genetic engineering and recombinant expression of mutated proteins (Figure 1A). Generally, fusion proteins are designed via genetic engineering and recombinantly expressed to achieve the desired oligomerization structure. Three common methods for oligomerization using fusion proteins include: direct expression of protein oligomers as a single polypeptide[16, 17, 18]; fusion of proteins to a subunit of a multimeric protein (*e.g.*, streptavidin) that assembles into supramolecular constructs (Figure 1A, i)[19, 20, 21, 22]; or fusion of proteins to a subunit that can selectively form covalent bonds with a complementary group (*e.g.*, SpyTag/SpyCatcher,[23] SnoopTag/Snoopcatcher,[24] SNAPtag,[25] HALOtag,[26] or cutinase,[27] Figure 1A, ii).[24, 28, 29, 30, 31] Furthermore, recent advances in the design of protein–protein interfaces enable sophisticated control over synthetic protein architectures (Figure 1A, iii).[32, 33, 34, 35, 36, 37] Each of these methods requires mutations of the amino acid sequence of proteins and recombinant protein expression. However, many proteins are challenging to prepare via recombinant expression (*e.g.*, proteins with post-translational modifications,[38] proteins with disulfide bonds,[39] toxic proteins,[40] or proteins that aggregate[41]), potentially limiting the scope of proteins that can be oligomerized through these methods.

Chemical approaches to assemble proteins are another powerful method to control oligomerization (Figure 1B). The amino acid sequence of proteins can be mutated to incorporate (un)natural amino acids at defined positions for interactions such as electrostatic,[42] supramolecular host–guest binding,[43, 44, 45, 46] metal coordination,[47, 48, 49] or covalent linking.[50, 51] However, without extensive chemical design, modification of protein amino acid sequences and/or recombinant protein expression, it is challenging to access monodisperse and sequence-encoded oligomers that are larger than dimers or trimers. Chemical approaches to directly oligomerize proteins post-expression without modifying the amino acid sequence of proteins or using an assembly template (*vide infra*) were expanded with the introduction of bioorthogonal “click” reactions (Figure 1B, i).[52, 53, 54] While these

reactions have been used to oligomerize therapeutically relevant proteins (*e.g.*, antibodies), it is challenging to achieve oligomers larger than dimers or trimers.[55]

Proteins can be oligomerized using attachment to chemical scaffolds (*e.g.*, polymers[56, 57, 58, 59, 60] or DNA[61, 62, 63, 64, 65], Figure 1B, ii). Templated assembly of proteins using DNA is one of the most promising and versatile approaches to organize proteins into oligomers larger than dimers or trimers. The utility of this approach is a result of the programmability of nucleic acids where specific, defined assemblies can be accessed solely based on DNA sequence design.[61, 62, 63, 64, 65] For example, proteins have been covalently[66, 67, 68] or noncovalently[67, 69] modified with oligonucleotides and the resulting constructs have been organized via DNA–DNA interactions into a multitude of protein oligomers with one-,[70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81] two-,[82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104] and three-dimensional[105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119] architectures. However, in each of these systems, DNA design must be changed to synthesize defined protein oligomers that contain different numbers or oligomeric sequences of proteins. For example, a protein tetramer can be readily synthesized using a DNA tetrahedron scaffold[82] or a four-arm Holliday DNA junction scaffold.[83, 84, 85] However, to synthesize a protein pentamer, every DNA sequence must be redesigned to account for the one additional protein. Modular multi-protein constructs can be realized on large DNA origami structures,[86, 87, 105] but most of the composition (*e.g.*, >80%) of these constructs is DNA instead of protein. Molecular constructs that minimize the required amount of DNA such that most of the chemical properties are dictated by the identity and organization of proteins are inaccessible using these techniques.[62] Together, these limitations significantly hamper any studies where access to libraries of different protein oligomers with discrete stoichiometries and oligomeric sequences of proteins could provide insight into how protein–protein interactions and cooperativity can be exploited for enhanced properties of oligomers.

We hypothesized that a single set of designed DNA strands could be used as a modular scaffold to organize proteins into oligomers with exact stoichiometries and oligomeric sequences (Figure 1C). This DNA design would enable different proteins to be precisely organized into an expansive array of monodisperse, sequence-encoded oligomers (Figure 1C, i). Herein, we tested our hypothesis by designing a modular six-strand DNA scaffold and using it to oligomerize commercially available and therapeutically relevant proteins (*i.e.*, antibodies). The scaffold consists of three distinct DNA strands that can be conjugated to proteins and three distinct DNA strands that template the assembly of DNA-modified proteins into oligomers via DNA–DNA interactions (Scheme S1). Importantly, each of the six DNA strands contains two distinct binding domains and the sites for attachment to proteins can be located anywhere on the DNA strands (Scheme S3). Using the designed DNA scaffold, monodisperse, sequence-encoded monomer, dimer, and trimer building blocks are synthesized. Next, these building blocks are used to access a larger oligomer (*i.e.*, pentamer) that contains a defined number and oligomeric sequence of proteins. Importantly, the foundational examples shown herein are a fraction of the possible oligomeric sequences that are accessible using the modular six-strand DNA scaffold (Figure 1C, ii, Table S3). For example, if five different proteins are used, there are, in principle, 3,125 different accessible pentameric sequences. Overall, this generalizable synthetic route

will enable future investigations into how the identity, stoichiometry, oligomeric sequence, and architecture of proteins in oligomers affect the properties of these constructs.

RESULTS

Three commercially available IgG antibodies commonly used as checkpoint inhibitors (*i.e.*, **anti-mouse-PD-1** (**A**), **anti-mouse-TIGIT** (**B**), and **anti-mouse-CTLA-4** (**C**)) were chosen for the sequence-encoded oligomerization of proteins using a modular DNA scaffold. To install a single DNA strand onto either **A**, **B**, or **C**, each antibody was reacted with 2 equiv. of an oligo(ethylene glycol) molecule containing an *N*-hydroxysuccinimide activated ester and an azide (NHS–PEG_{12}–N_3) for 45 min (Figure 2A). This chemistry targets the primary amines (*e.g.*, ε-amines on lysines and α-amines on N-termini)[120] on both the Fc and Fab regions of the antibody and was chosen because it is generalizable with regard to proteins. Although the exact location of conjugation cannot be controlled, we expected that the number of azide modifications per antibody would be controlled by low numbers of equivalents of NHS–PEG_{12}–N_3 added. We hypothesized that the low number of primary amines modified would not inhibit the target binding characteristics of antibodies. After purification by size-exclusion chromatography (SEC), the azide on the surface of each antibody underwent a strain-promoted azide-alkyne cycloaddition reaction with 5 equiv. of DNA strands containing dibenzocyclooctyne (DBCO) and a fluorophore (*i.e.*, Cyanine 3 (Cy3), Cyanine 5 (Cy5), or fluorescein (FITC)) and two distinct 20 base nucleic acid sequences (Table S1, Schemes S1A and S2, *i.e.*, S2–DBCO–Cy3–S3, S4–DBCO–Cy5–S5, or S6–DBCO–FITC–S1). After 16 h, roughly 25–30% of antibodies were modified with one DNA strand (Figure S1). Next, unreacted DNA was removed from the reaction mixture using SEC. Anion exchange chromatography was used to isolate antibodies that were functionalized with a single DNA strand from unreacted antibodies and antibodies that were functionalized with multiple DNA strands (Figure 2A). Three different protein–DNA conjugates (*i.e.*, S2–**A**–Cy3–S3, S4–**B**–Cy5–S5, and S6–**C**–FITC–S1) were prepared and confirmed to contain a single DNA functionalization via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Figure 2B) and SEC (Figure S2).

Protein oligomers were synthesized by mixing the purified protein–DNA conjugates (Figure 3A, 3E: lanes 1–3) with template DNA strands (Table S1, Scheme S1B, *i.e.*, S1'–S2', S3'–S4', or S5'–S6'). The template strands were designed as complements to two 20 base nucleic acid sequences on different antibody–DNA conjugates (Table S2, Scheme S1C). For example, the S5' DNA sequence on the template strand S5'–S6' is complementary to the S5 DNA sequence on S4–**B**–Cy5–S5 and the S6' DNA sequence is complementary to the S6 DNA sequence on S6–**C**–FITC–S1. Equal amounts of the **B**–DNA conjugate, **C**–DNA conjugate, S5'–S6' template strand, and S1'–S2' template strand were mixed to synthesize a protein dimer with the oligomeric sequence S4–**B**–**C**–S2' (Figure 3B, Scheme S5A) at an assembly yield of 68% (Figure S5A, D). Oligomers that contain greater than two antibodies were not observed in the assembly mixture because there are no DNA sequences that are complementary to either the S4 or S2' DNA sequences. Protein dimers were isolated from unreacted monomers and template strands in the assembly mixture using SEC purification and characterized with agarose gel electrophoresis (Figure 3E: lane 4). Importantly, the agarose gel showed a single band for dimers with only the expected

Cy5 and FITC fluorescence and lower electrophoretic mobility than either antibody–DNA conjugate alone. Therefore, monodisperse and sequence-encoded protein dimers with the oligomeric sequence S4–**B**–**C**–S2' were successfully synthesized.

Next, the synthesis of a sequence-encoded protein trimer was targeted. Equal amounts of the **A**–DNA conjugate, **B**–DNA conjugate, **C**–DNA conjugate, S3'–S4' template strand, and S5'–S6' template strand were mixed to synthesize a protein trimer with the oligomeric sequence S2–**A**–**B**–**C**–S1 (Figure 3C, Scheme S5B) at an assembly yield of 27% (Figure S5B, D). Oligomers that contain greater than three antibodies or trimers containing alternative oligomeric antibody sequences were not observed in the assembly mixture because there are no DNA sequences that are complementary to either the S2 or S1 DNA sequences. Protein trimers were isolated from the assembly mixture using SEC purification and characterized with agarose gel electrophoresis (Figure 3E: lane 5). The agarose gel showed a single band for the trimers with the expected Cy3, Cy5, and FITC dye fluorescence as well as lower electrophoretic mobility on an agarose gel than the dimers. Therefore, these results indicate that monodisperse and sequence-encoded protein trimers with the oligomeric sequence S2–**A**–**B**–**C**–S1 were successfully synthesized. Importantly, no disassembly of S4–**B**–**C**–S2' or S2–**A**–**B**–**C**–S1 oligomers were observed over 10 days of storage at 4 °C.

To ensure that this synthetic technique is generalizable, different protein oligomers were synthesized, including a protein dimer with the oligomeric sequence **A**–**B** (Figure S13) and a protein trimer with the oligomeric sequence, **A**–**B**–**B** (Figure S6). Furthermore, another antibody, **anti-human-PD-1** (**D**), was functionalized with a single DNA strand of S2–DBCO–Cy3–S3, S4–DBCO–Cy5–S5, or S6–DBCO–FITC–S1 (Figure S3 and S4), and the resulting constructs were organized into protein dimers with the oligomeric sequence **D**–**D** (Figures S7, S8, and S10) and trimers with the oligomeric sequence **D**–**D**–**D** (Figure S7). Analytical SEC analysis of antibody–DNA conjugates, dimers, and trimers show a single peak for each sample with decreases in retention time as degree of oligomerization increases (Figures S6C and S7C).

The target binding characteristics of human antibodies after functionalization with DNA and oligomerization with the modular DNA scaffold were investigated using antigen binding and checkpoint inhibitor activity cellular assays. We studied **D**, **D**–DNA conjugates, **D**–**D** dimers, and **D**–**D**–**D** trimers using these assays and confirmed that antigen binding and checkpoint inhibitor activity were retained in each sample (Figures S11 and S12, see the supplemental information (SI) for additional details). Importantly, an antibody dimer, **A**–**B**, exhibited minimal degradation in the cellular media used in these experiments (Figure S13, see the SI for additional details).

Finally, a protein dimer and trimer were used as building blocks to synthesize a monodisperse and sequence-encoded protein pentamer where three different antibodies are organized into a precise oligomeric sequence. The S4–**B**–**C**–S2' protein dimer and S2–**A**–**B**–**C**–S1 protein trimer were mixed together at a 1:1 ratio and the specific binding between the S2' DNA sequence on the dimer and the S2 DNA sequence on the trimer leads to the synthesis of a sequence-encoded protein pentamer with the oligomeric sequence S4–**B**–**C**–**A**–**B**–**C**–S1 (Figure 3D, Scheme S5C) at an assembly yield of 58% (Figure

S5C, D). Oligomers that contain greater than five antibodies were not observed in the assembly mixture. Protein pentamers were isolated from other unreacted dimers, trimers, and template strands in the assembly mixture using SEC purification and characterized with agarose gel electrophoresis (Figure 3E: lane 6). The pentamers showed a single band with the expected Cy3, Cy5, and FITC dye fluorescence as well as lower electrophoretic mobility on an agarose gel than the trimers. Therefore, monodisperse and sequence-encoded protein pentamers with the oligomeric sequence S4–**B**–**C**–**A**–**B**–**C**–S1 were successfully synthesized. This is the first reported monodisperse antibody pentamer that contains different antibodies in a predefined oligomeric sequence.

DISCUSSION

The designed set of six DNA strands was used as a modular scaffold to organize proteins into oligomers with programmed identity, stoichiometry, and oligomeric sequence. This scaffold provided access to monomer, dimer, and trimer building blocks that could be modularly combined independent from the identity of proteins. Therefore, all of the criteria for a versatile synthetic protein oligomerization method were met: (1) providing access to a large number of proteins per oligomer, (2) providing access to any oligomeric sequence of the same or different proteins, (3) being generalizable with regard to proteins, and (4) not requiring mutations of the amino acid sequence of proteins and recombinant protein expression.

Established chemistry was used to functionalize a primary amine (*e.g.*, ε-amine on a lysine or α-amine on a N-terminus) on proteins with a single DNA strand.[120] Nearly all proteins contain primary amines, so this approach is generalizable with regard to proteins, including proteins that are commercially available, isolated from natural sources, or recombinantly expressed. Many other covalent[66, 67] and noncovalent[67, 69] bioconjugation methods could also be used to modify proteins with one of the DNA strands reported here. By taking advantage of these approaches, nearly any protein can be modified with a single DNA strand and organized into monodisperse, sequence-encoded oligomers using the designed DNA scaffold.

While modular multi-protein constructs can be prepared using large DNA origami scaffolds, proteins comprise less than 20% of the mass of these constructs.[86, 87, 105] This large amount of DNA compared to protein means that most of the solution properties and interactions of these constructs are dictated by the DNA scaffold instead of by the proteins. In cases where protein binding interactions are integral to function (*e.g.*, antibody–antigen binding), this large amount of DNA may affect target recognition and accessibility. In contrast, using the modular DNA scaffold reported in this work, proteins make up most of the mass of oligomeric constructs. For example, proteins comprise 84%, 86%, and 85% of the mass of the sequence-encoded **B**–**C** dimer, **A**–**B**–**C** trimer, and **B**–**C**–**A**–**B**–**C** pentamer, respectively. While other protein assembly techniques using nucleic acids also provide access to oligomers mostly comprised of proteins (*e.g.*, oligomerization using a DNA tetrahedron scaffold[82] or a four-arm Holliday DNA junction scaffold[83, 84, 85]), they lack modularity to access different numbers of proteins per construct.

In principle, the modular DNA scaffold described herein provides access to vast numbers of different oligomeric sequences and sizes. For example, 3 different proteins could be oligomerized into trimeric constructs with 27 different oligomeric sequences, including homooligomers of one protein, and hetero-oligomers of 2 or 3 proteins (Figure 1c, ii). Oligonucleotides in the DNA scaffold interact through Watson–Crick–Franklin base pairing to form a right-handed double helix. Therefore, two oligomers with reversed oligomeric sequences of proteins (*e.g.*, A–A–B and B–A–A) form different structures and are considered as different sequences. Likewise, 2 different proteins could be oligomerized into dimeric constructs with 4 different oligomeric sequences, 4 different proteins could be oligomerized into tetrameric constructs with 256 different oligomeric sequences, and 5 different proteins could be oligomerized into pentameric constructs with 3,125 different oligomeric sequences (Table S3). Furthermore, each oligomer building block synthesized using this method inherently contains living chain ends where more units could be added to access larger oligomers (*e.g.*, hexamers, heptamers, and octamers, Scheme S4). Considering the growing number of discovered proteins, the foundational oligomers synthesized in this work illustrate the unlimited number of protein oligomers that could be accessed via a single modular DNA scaffold.

CONCLUSION

In conclusion, this work shows how monodisperse, sequence-encoded protein oligomers can be synthesized using generalizable bioconjugation chemistry with a judiciously designed DNA scaffold. This versatile protein oligomerization approach is powerful and useful because oligomers with different stoichiometries and oligomeric sequences can be synthesized without the need to redesign the proteins or the DNA scaffold. Importantly, this synthetic advance will enable subsequent studies to understand the fundamental relationships between protein oligomer structures and properties, which have significant implications for many fields (*e.g.*, therapeutics, catalysis, photosynthesis, and membrane transport).

EXPERIMENTAL PROCEDURES

Resource availability

Lead contact—Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Chad A. Mirkin (chadnano@northwestern.edu).

Materials availability—All materials generated in this study can be obtained upon reasonable request from the corresponding author with a valid materials transfer agreement and an assumption of costs.

Data and code availability—This study did not generate any datasets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Bigger Picture Statement

In nature, many proteins combine to form architectures that contain exact numbers and oligomeric sequences of different subunits, and such hierarchical structural control influences their biological, catalytic, photophysical, and membrane transport properties. To mimic such structures and potentially surpass their properties, the synthesis of different sequence-encoded protein oligomers is needed.

Herein, a method to organize proteins in such a way using designed nucleic acid sequences is reported. Generalizable bioconjugation reactions are used to install one DNA strand on each protein, and assembly occurs via base-pair recognition of different DNA sequences. Therefore, nearly any protein can be used without requiring mutations or recombinant expression. Using this method, monomers, dimers, and trimers are synthesized that can be further assembled into larger structures, laying the foundation for the study of sequence-encoded protein materials across many areas.

Highlights

- A modular DNA scaffold was designed to organize proteins into defined oligomers
- Oligomers were synthesized with exact numbers and sequences of up to five proteins
- This oligomerization strategy is generalizable to nearly any protein

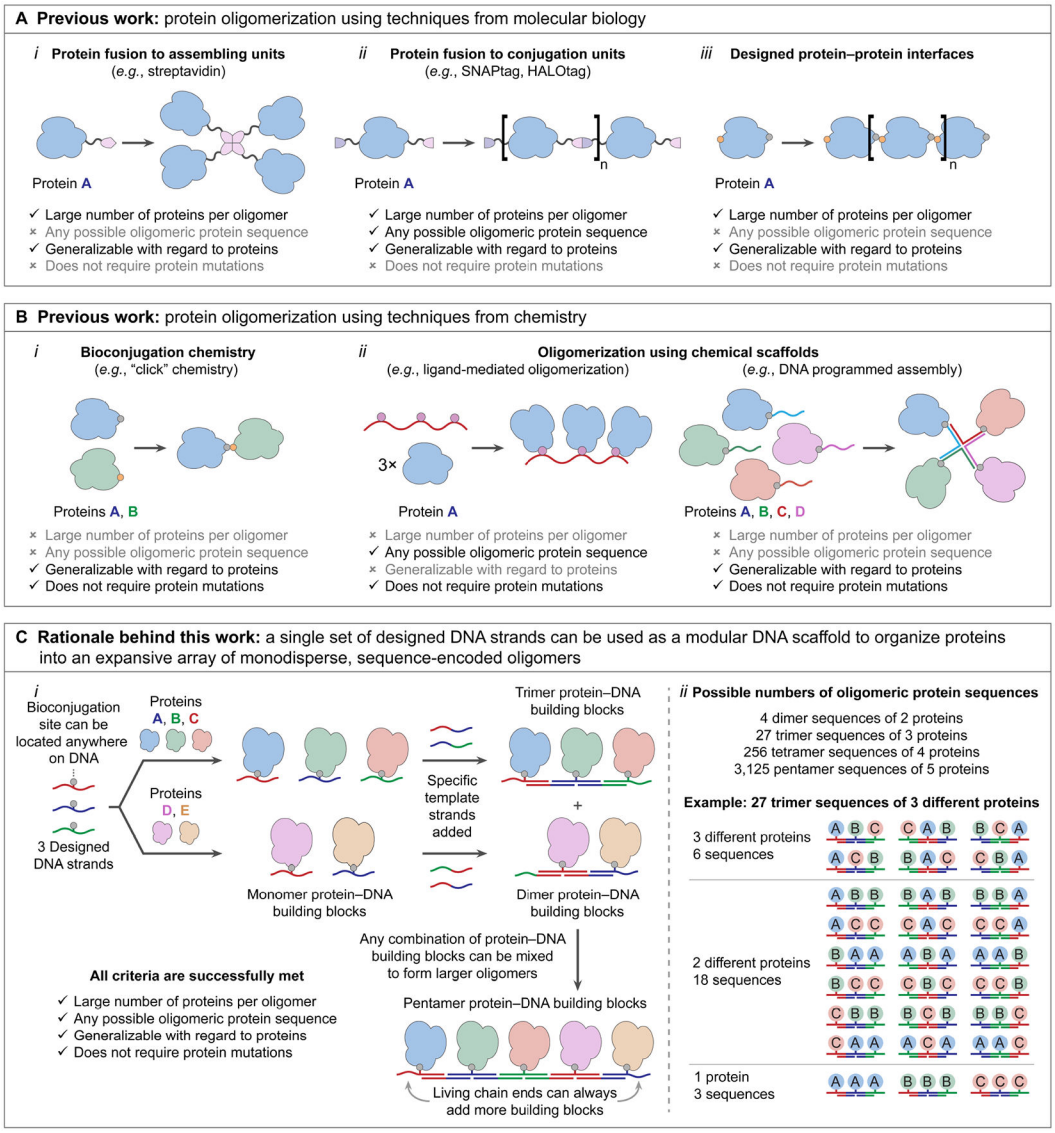

Figure 1. Protein oligomerization techniques and limitations.

(A) Protein oligomers can be synthesized using techniques from molecular biology including (*i*) recombinant expression of linear oligomers, (*ii*) protein fusion to assembling units, and (*iii*) protein fusion to conjugation units. (B) Oligomerization can also be achieved using techniques from chemistry, such as (*i*) bioconjugation chemistry and (*ii*) scaffold-directed oligomerization of proteins. (C) In this work, (*i*) we design a set of six DNA strands that can be used as a modular DNA scaffold to organize proteins into an expansive array of monodisperse and sequence-encoded oligomers. (*ii*) This generalizable method will enable the synthesis of different oligomeric sequences of proteins.

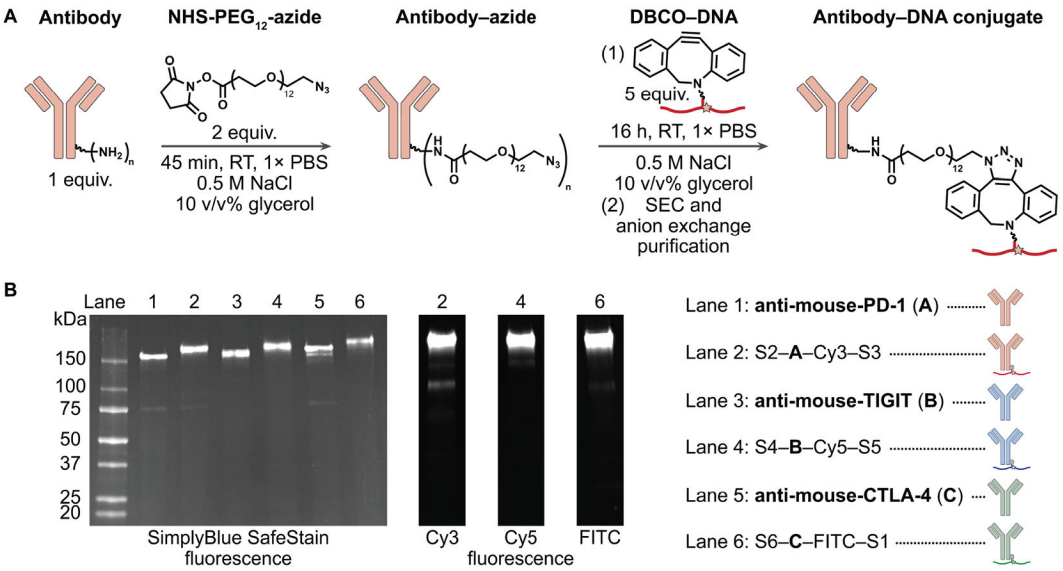

Figure 2. Antibody functionalization with a single DNA strand.

(A) Primary amines on the surface of antibodies were functionalized with azides using an NHS-PEG_{12}-N_3 linker. Next, dibenzocyclooctyne (DBCO)-modified DNA was conjugated to azide-modified antibodies via a strain-promoted azide-alkyne cycloaddition (SPAAC). (B) SDS-PAGE characterization of mouse antibodies (*i.e.*, lane 1: **anti-mouse-PD-1** (**A**), lane 3: **anti-mouse-TIGIT** (**B**), **and lane 5: anti-mouse-CTLA-4** (**C**)) and antibody–DNA conjugates (*i.e.*, lane 2: S2–**A**–Cy3–S3, lane 4: S4–B–Cy5–S5, and lane 6: S6–**C**–FITC–S1). A single gel was imaged for SimplyBlue SafeStain, Cy3, Cy5, and FITC fluorescence.

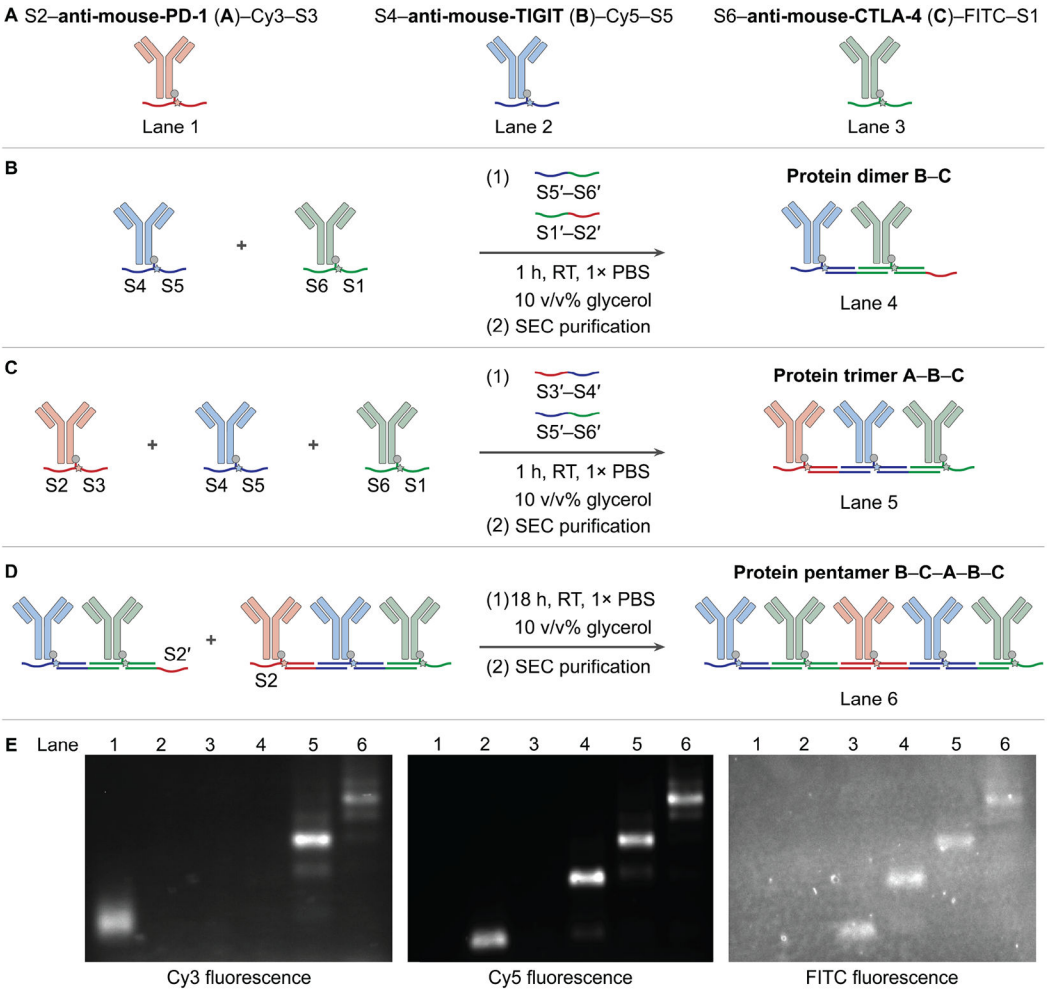

Figure 3. Antibody oligomerization into encoded sequences using DNA–DNA interactions.

(A) Antibody–DNA conjugates and template DNA strands were assembled using DNA–DNA interactions into sequence-encoded protein (B) dimers and (C) trimers. Dimers and trimers were subsequently assembled using DNA–DNA interactions into sequence-encoded protein (D) pentamers. (E) Agarose gel characterization of antibody–DNA conjugates (*i.e.*, lane 1: S2–**anti-mouse-PD-1** (**A**)–Cy3–S3, lane 2: S4–**anti-mouse-TIGIT** (**B**)–Cy5–S5, and lane 3: S6–**anti-mouse-CTLA-4** (**C**)–FITC–S1) along with sequence-encoded antibody dimers (*i.e.*, lane 4: S4–**B**–**C**–S2'), trimers (*i.e.*, lane 5: S2–**A**–**B**–**C**–S1), and pentamers (*i.e.*, lane 6: S4–**B**–**C**–**A**–**B**–**C**–S1). A single gel was imaged for Cy3, Cy5, and FITC fluorescence and these images are merged into one composite image in Figure S9.