Oligonucleotide-directed self-assembly of proteins: semisynthetic DNA – streptavidin hybrid molecules as connectors for the generation of macroscopic arrays and the construction of supramolecular bioconjugates

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ABSTRACT

Modified biomolecules were used for the non-covalent assembly of novel bioconjugates. Hybrid molecules were synthesized from short single-stranded DNA and streptavidin by chemical methods using a heterobispecific crosslinker. The covalent attachment of an oligonucleotide moiety to streptavidin provides a specific recognition domain for a complementary nucleic acid sequence, in addition to the four native biotin-binding sites. These bispecific binding capabilities allow the hybrid molecules to serve as versatile connectors in a variety of applications. Bifunctional constructs have been prepared from two complementary hybrid molecules, each previously conjugated to biotinylated immunoglobulin G or alkaline phosphatase. The use of nucleic acid sequences as a template for the formation of an array of proteins is further demonstrated on two size scales. A macroscopic DNA array on a microtiter plate has been transformed into a comparable protein chip. A nano-scale array was made by hybridizing DNA-tagged proteins to specific positions along a RNA or DNA sequence. The generation of supramolecular bioconjugates was shown by quantitative measurements and gel-retardation assays.

INTRODUCTION

Supramolecular chemistry is the chemistry beyond the molecule (1–4). Arrangements of molecules, supramolecules, bound via non-covalent interactions are under active investigation for potential applications in chemical synthesis, as detection systems and for new material properties (5–6). The development of supramolecular systems that use the combined functionalities of individual molecules is stimulated by natural models. Cells, as the ultimate molecular machines, use ensembles of proteins, nucleic acids, and other macromolecules to perform complicated

tasks. Natural recognition systems, ligand binding by receptor proteins, hybridization of nucleic acids and particularly combinations of these interactions, enable biological systems to perform highly complex functions; one impressive example is the photosynthetic machinery, consisting of the supramolecular structure of the thylakoid membrane of chloroplasts (7–10).

The highly specific functionality of single biological compounds like enzymes and immunoglobulins is very well recognized and utilized for an endless number of applications in biological and medical fields. However, very little has been reported about synthetic combinations of naturally occurring molecules or their modified analogs to make supramolecular constructs with new, and possibly enhanced, properties (11). Simple heterodimeric assemblies can be prepared by chemical crosslinking (12), but to take advantage of an enormous variety of functional macromolecules available, a conventional chemical crosslinking is not always possible for the purposeful combination of multiple biomolecules. There are several obstacles to making supramolecular constructs with a defined spatial arrangement. For example the limited chemical stability of most biomolecules discourages successive conjugation strategies. Here, a non-covalent assembly of biomolecules, previously connected to single-stranded DNA was used for the construction of novel bioconjugates. The DNA moiety of the biological macromolecule provides a specific recognition site for complementary nucleic acids, and can subsequently be used as a molecular handle for selective immobilization and construction purposes.

The attachment of DNA to a biomolecule can be achieved either directly or indirectly. Direct chemical methods using a heterobispecific crosslinker may not always be convenient. A universal connector between a protein and an oligonucleotide moiety might be superior for many applications. Therefore, hybrid molecules were synthesized from DNA and streptavidin (STV) as a novel coupling agent.

The tetrameric protein STV binds the small water-soluble molecule biotin (vitamin H) rapidly and with extraordinary

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specificity. Since biotinylated materials are often commercially available or can be prepared with a variety of mild biotinylation procedures, biotin-STV conjugates form the basis of many diagnostic and analytical tests (13–14). An additional great advantage of STV is the extreme chemical and thermal stability of the protein (15). The covalent attachment of an oligonucleotide moiety should provide STV with a specific binding domain for complementary nucleic acids in addition to its four native binding sites for biotin. This bispecificity of the hybrid molecules should allow them to serve as selective, efficient connectors in the oligonucleotide-directed self-assembly of proteins.

DNA-STV hybrid molecules can be used for a selective linking with additional molecules, bearing complementary oligonucleotide sequences, via simple annealing reactions. This concept allows the rapid and clean production of heterodimeric, bifunctional molecules under mild conditions without homodimer contaminants. Selective, non-covalent crosslinking was demonstrated with bifunctional constructs prepared from two complementary hybrid molecules, each previously conjugated to biotinylated immunoglobulin G (IgG) or alkaline phosphatase (AP). Furthermore, oligonucleotide-directed assembly can be used for the construction of macromolecular assemblies, supramolecules or nano-structures, with, in principle, no limitations on the nature and number of their constituents. A translation of DNA arrays into protein arrays on two different size scales was demonstrated using model systems. First, on a macro to microscopic scale, spatially separated DNA information on a microtiter plate was transformed into a functional protein array. Second, sequence information along a nucleic acid molecule was used to construct a supramolecular bioconjugate with a defined spatial arrangement of the single components.

MATERIALS AND METHODS

Oligonucleotides and antibodies

The oligonucleotides used in this study (Table 1) were purchased from Operon Technologies (Alameda, CA). Unless otherwise mentioned, the products were used without further purification. The thiolated oligonucleotides were delivered with a disulfide protection group (5'-PGS), which was removed by dithiothreitol (DTT) reduction immediately before use. An oligonucleotide solution (6 μ M) was treated with 15 mM DTT dissolved in PBE (phosphate-buffered EDTA; 100 mM phosphate buffer, pH 6.8, 5 mM EDTA) at 37°C for 1 h and purified by gel filtration chromatography using a disposable Sephadex G-25 column (NAP-10; Pharmacia).

The five thiolated oligonucleotides, UA, UB, UC, UD and UE, are complementary to a 108 base region of the plus strand of M13mp18 DNA between a reverse sequencing primer (# 1233; New England Biolabs) and a universal 17mer sequencing primer (# 1211; New England Biolabs).

All biotinylated antibodies used were polyclonal rabbit IgG, purchased from Sigma; biotinylated rabbit anti-bovine IgG (# B 7140); biotinylated rabbit anti-dog IgG (# B 7640); biotinylated rabbit anti-mouse IgG (# B 8520). Antibody-AP conjugates were also from Sigma; rabbit anti-bovine IgG-AP conjugate (# A-7914); rabbit anti-dog IgG-AP conjugate (# A-6042); rabbit anti-mouse IgG-AP conjugate (# A-2418). IgG antigens used were mouse IgG whole molecules (Pierce), bovine IgG whole molecules and dog IgG whole molecules (reagent grade, both from Sigma).

Synthesis of oligonucleotide carrier molecules

Single-stranded DNA carrier was prepared by denaturing a polymerase chain reaction (PCR) product. PCR was done using a biotinylated plus strand primer (BRSP-48), a non-biotinylated minus strand primer (M13-1211) and M13mp18 DNA template (New England Biolabs). The resulting 123 bp (base pair) fragment was desalted on a NAP-10 column and purified by ultrafiltration (30,000 MW cut-off membrane; Amicon, Beverly, MA). In some cases remaining primer contaminants were removed by ion exchange chromatography on a Mono Q HR 5/5 column using an FPLC system (Pharmacia). The carrier was immobilized on streptavidin-coated magnetic microbeads (M-280; Dynal) prepared as described in the product manual. Usually, non-specific binding sites of the beads were blocked with Tris-buffered saline (TBS; 20 mM Tris-HCl buffer, pH 7.3, 150 mM NaCl) supplemented with 0.5% non-fat dried milk powder, 5 mM EDTA and 1 mg/ml denatured herring sperm DNA (MESTBS). The immobilization step was carried out with a bead suspension (20 mg/ml) and biotinylated DNA (2–5 μ M) in 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA (TE) supplemented with 1 M NaCl (TEMN) for 1 h at room temperature. After washing the beads with TEMN, the DNA duplex was denatured by resuspending the beads in 0.1 M NaOH (10 μ l) for 10 min at room temperature. Removal of the supernatant was followed by washing with 0.1 M NaOH and then with biotin-saturated TBS supplemented with 0.05% Tween 20 and 5 mM EDTA (TETBS). The immobilized carrier was stored in TEMN at 4°C. The recovered single-stranded 123 base minus strand DNA was analyzed by 12% polyacrylamide gel electrophoresis (PAGE) under denaturing conditions.

RNA carrier was prepared by *in vitro* transcription using a 144 bp DNA as the template. The DNA template was prepared by PCR using M13-T7 and M13-1211 primers and was subsequently purified by ion exchange chromatography. *In vitro* transcription was carried out using T7 RNA polymerase as described (16). Unincorporated ribonucleoside triphosphates were removed using a spin column (Chroma Spin+TE-10; CLONTECH Laboratories). Transcription efficiency was determined by non-denaturing PAGE in the presence of an excess complementary, ³²P-labeled hybridization probe (CHP or M13-1211) with known specific activity (16). Quantitation of the bands, using a PhosphorImager (Molecular Dynamics), gave typical efficiencies of 8–10 mol RNA/mol DNA template.

Chemical crosslinking of thiolated DNA and streptavidin

STV (Boehringer Mannheim) was derivatized with excess sulfo-succinimidyl 4-[*p*-maleimidophenyl]butyrate (sulfo-SMPB; Pierce) in a 600 μ l reaction mixture, consisting of 3.8 mg/ml protein and 0.25 mg/ml sulfo-SMPB in PBS (100 mM K phosphate buffer, pH 7.3, 150 mM NaCl). After incubation for 1 h at room temperature, the products were desalted by ultrafiltration (30,000 MW cut-off membrane; Millipore) and the buffer changed to PBE. The derivatized STV (200 μ l, 3.5 mg/ml in PBE) was mixed with 200 μ l of a deprotected thiolated oligonucleotide (0.7 μ M in PBE) at room temperature. After 15 min and 30 min another 200 μ l aliquot of the DNA solution was added. The mixture was incubated for an additional 30 min and excess DNA was removed by ultrafiltration (Centricon 30). Purification and fractionation was carried out by ion exchange chromatography on a Mono Q HR 5/5 column using an FPLC system (Pharmacia). Best results were achieved with the following

Table 1. Oligonucleotides used in this study

Name	Description	Sequence
UA	Thiolated, 24mer	5'-PGS-TCC TGT GTG AAA TTG TTA TCC GCT-3'
TC-UA	Thiolated, complementary to UA, 24mer	5'-PGS-AGC GGA TAA CAA TTT CAC ACA GGA-3'
UB	Thiolated, 21mer	5'-PGS-GTA ATC ATG GTC ATA GCT GTT-3'
BC-UB	Biotinylated, complementary to UB, 21 mer	5'-biotin-AAC AGC TAT GAC CAT GAT TAC-3'
TC-UB	Thiolated, complementary to UB, 21mer	5'-PGS-AAC AGC TAT GAC CAT GAT TAC-3'
UC	Thiolated, 21mer	5'-PGS-CCG GGT ACC GAG CTC GAA TTC-3'
BC-UC	Biotinylated, complementary to UC, 21mer	5'-biotin-GAA TTC GAG CTC GGT ACC CGG-3'
TC-UC	Thiolated, complementary to UC, 21mer	5'-PGS-GAA TTC GAG CTC GGT ACC CGG-3'
UD	Thiolated, 21mer	5'-PGS-CAG GTC GAC TCT AGA GGA TCC-3'
UDX	Thiolated, 33mer	5'-PGS-CAG GTC GAC TCT AGA GGA TCC AGT GCC AAG CT-3'
UE	Thiolated, 21mer	5'-PGS-AGT GCC AAG CTT GCA TGC CTG-3'
BRSP-48	Biotinylated primer for DNA carrier, 24-mer	5'-biotin-AGC GGA TAA CAA TTT CAC ACA GGA-3'
M13-1211	Non-biotinylated primer for DNA carrier, 17-mer	5'-GTA AAA CGA CGG CCA GT-3'
M13-T7	Primer for RNA carrier template with T7 promoter (underlined), 42mer	5'-TTC <u>TAA TAC GAC TCA CTA TAG</u> CGG ATA ACA ATT TCA CAC AG-3'
HP	Hybridization probe, 18mer	5'- ³² P-GCG GAT AAC AAT TTC ACA-3'
CHP	Hybridization probe, 18mer	5'- ³² P-TGT GAA ATT GTT ATC CGC-3'

run profile: 1 ml buffer A (20 mM Tris-HCl, pH 6.3, 0.3 M NaCl); sample injection; 4 ml buffer A, followed by a linear salt gradient of 22.6 mM/ml using buffer B (20 mM Tris-HCl, pH 6.3, 1 M NaCl); flow rate, 0.2 ml/min. The peak fractions were collected, concentrated by ultrafiltration and stored in TBS at 4°C for 3 months without noticeable degradation.

Biotinylated DNA-STV conjugates

Control samples were prepared from STV and the biotinylated oligonucleotide BRSP-48. STV (5 mg/ml, 900 pmol in 10 μ l) was diluted with 20 μ l of TE, and then 20 μ l of a BRSP-48 solution (48 pmol/ml, 1.06 equivalents) were added. The mixture was incubated at room temperature on a shaker for 30 min. Similar mixtures were prepared containing the same final STV concentration (1 mg/ml) but various biotinylated DNA concentrations, ranging from 4.7–144 pmol/ μ l (0.25–8 molar equivalents, respectively). Typically 5–8 μ l of these solutions were mixed with 2 μ l of a non-denaturing PAGE sample buffer and analyzed by gel electrophoresis.

Characterization of DNA-STV hybrid molecules

Non-denaturing PAGE studies were done as described (17) using 8.5% polyacrylamide gels. Samples were applied as 5–15 μ l portions containing 0.1–1 mg/ml protein (e.g. STV 1.8–18 pmol/ μ l) in a sample buffer (final concentrations 20 mM Tris-HCl, pH 8.9, 7.5% glycerol, 0.002% bromophenol blue). Staining was carried out either with Coomassie Brilliant Blue (CBB), a silver-staining kit (Biorad) or ethidium bromide.

DNA binding was studied using a 5'-[³²P]-labeled 18 base oligonucleotide (HP) (58 pmol/ μ l, 260 c.p.m./pmol) as a probe, which is complementary to the UA sequence of the STV-UA hybrid molecule. Various amounts of hybrid molecules (1–5 μ l, 3.2–16 pmol) were mixed with 5 μ l of the radiolabeled probe in an electrophoresis sample buffer. The samples were diluted with TE to a final volume of 15 μ l and subjected to non-denaturing PAGE. Analysis of the gel was carried out by autoradiography with a PhosphorImager. Additional gel-retardation experiments were carried out with UA-STV and UB-DNA hybrids, using

conventional STV conjugates with biotinylated DNA (BRSP-48 and BC-UB) as a complementary probe.

Assembly of heterodimeric constructs

General. Wells of a microtiter plate (Immulon-4; Dynatech) were coated with mouse, bovine or dog IgG (50 μ l protein solution at a concentration of 20 ng/ μ l in 50 mM borate buffer, pH 9.5) for ~14 h at room temperature. STV (50 ng/ml) was immobilized under the same conditions. The plates were washed with TBS (3 $\times$ 200 μ l) and the non-specific binding sites were blocked with 100 μ l MESTBS containing 0.02% NaN₃, followed by a TBS wash (3 $\times$ 200 μ l). Unless otherwise stated, the following incubation steps were carried out at room temperature. In a typical experiment, 2–5 pmol of a hybrid (1 μ M in TBS) were mixed with 2–5 pmol biotinylated material (IgG or AP, 1 μ M in TBS) and incubated for 15–30 min at room temperature. Undesired cross-coupling between multiple-biotinylated molecules and free biotin-binding sites of the hybrids was avoided by quenching the reaction with TETBS (10 μ l) saturated with free biotin. The preconjugated samples were diluted with a reagent dilution buffer (RDB; 9 parts TETBS and 1 part MESTBS) and applied to antigen-coated microtiter plates. After incubation, the plate was washed with TETBS (6 $\times$ 200 μ l) and TBS (2 $\times$ 200 μ l), and AP-dependent color reactions were carried out with 200 μ l of 1 M diethanolamine, pH 9.8, and 0.5 mM MgCl₂ containing 10 mM *p*-nitrophenyl phosphate (pNPP). After incubation for 60 min at 37°C the reaction was quenched by adding EDTA (125 mM final concentration). The absorbance at 405 nm of a 50 μ l aliquot was determined with an EIA-Reader Model EL-307 (BIO-TEK Instruments, Inc.).

Anti-mouse IgG-alkaline phosphatase bifunctional construct. The TC-UA-STV hybrid (4 pmol) was preconjugated with 4 pmol of biotinylated anti-mouse IgG. Control A was the TC-UB-STV hybrid; control B contained IgG only. The three solutions diluted with biotin-saturated RDB to a final concentration of 0.2 μ M were mixed with an equimolar amount of a 50 nM solution prepared from UA-STV hybrid and

biotinylated AP (4 pmol each), as described above. The mixture was diluted with RDB to a final putative supramolecular construct concentration of 5 nM (which corresponds to an IgG concentration of $\sim 0.6 \mu\text{g/ml}$). Five-fold serial dilutions of the reagent were applied to mouse IgG-coated microtiter plates which were then incubated for 2 h. After washing, AP-dependent color reactions were carried out as described above and the absorbance at 405 nm of an 100 μl aliquot was determined.

Anti-bovine IgG-anti-dog IgG bifunctional and anti-bovine IgG-anti-mouse IgG bifunctional constructs. In separate tubes, 4 pmol of TC-UA-STV were conjugated to 4 pmol of biotinylated anti-mouse IgG or biotinylated anti-dog IgG (samples A and C, respectively). Controls were prepared in parallel, lacking the DNA-STV hybrid (samples B and D, respectively). In another tube, 16 pmol of the complementary hybrid (UA-STV) was pre-conjugated to biotinylated anti-bovine IgG (16 pmol). All samples were diluted to final hybrid concentrations of 0.2 μM . Aliquots (10 μl) of the anti-bovine conjugate were mixed with the same amount of samples A-D, and the mixtures were subsequently diluted with RDB to a final hybrid concentration of 8 nM. Five-fold serial dilutions were prepared and 50 μl of each of these solutions was applied to a microtiter plate, previously coated with mouse IgG and dog IgG antigen. The plate was incubated for 2 h and washed with TBS ($3 \times 200 \mu\text{l}$). Incubation with bovine IgG (50 μl of a 0.8 $\mu\text{g/ml}$ solution in RDB) was done to allow the immobilized construct to capture antigen. After washing with TBS ($2 \times 200 \mu\text{l}$) and TETBS ($3 \times 200 \mu\text{l}$), successful assembly and capture was tested by a 1 h incubation with an anti-bovine IgG-AP conjugate (50 μl of a 0.65 U/ml solution in RDB) and a subsequent AP reaction.

Macroscopic translation of a DNA array into a protein array

Biotinylated DNA was immobilized in wells of three rows on a microtiter plate, previously coated with STV (50 μl of 1 nM DNA solutions in TBS: row A, BRSP-48; row B, BC-UB; row C, BC-UC; total of nine wells per row). The plate was subsequently washed with biotin-saturated TETBS ($2 \times 100 \mu\text{l}$) and TBS (100 μl). Nine different pre-conjugates were prepared, each from 6 pmol of a DNA-STV hybrid and 6 pmol of biotinylated anti-bovine, anti-dog or anti-mouse IgG, respectively:

1. (AM) UA-STV hybrid and biotinylated anti-mouse IgG
2. (AB) UA-STV hybrid and biotinylated anti-bovine IgG
3. (AD) UA-STV hybrid and biotinylated anti-dog IgG
4. (BM) UB-STV hybrid and biotinylated anti-mouse IgG
5. (BB) UB-STV hybrid and biotinylated anti-bovine IgG
6. (BD) UB-STV hybrid and biotinylated anti-dog IgG
7. (CM) UC-STV hybrid and biotinylated anti-mouse IgG
8. (CB) UC-STV hybrid and biotinylated anti-bovine IgG
9. (CD) UC-STV hybrid and biotinylated anti-dog IgG

All samples were subsequently diluted with RDB to a final hybrid concentration of 0.2 μM (30 μl). Three of the pre-conjugate solutions were combined in order to yield mixtures containing UA, UB and UC DNA and anti-mouse, anti-bovine or anti-dog IgGs. In order to test nine possible permutations, three mixtures were prepared as follows:

mixture A, AM + BB + CD
mixture B, AB + BD + CM
mixture C, AD + BM + CB

Each of these mixtures was diluted with RDB to a final hybrid concentration of 40 nM, and 50 μl were applied to a total of nine wells per mixture (three columns each of the three different rows, previously coated with DNA complementary to either UA, UB or UC). If the annealing of the oligonucleotides is sufficiently selective, this should result in three blocks, each 3×3 wells, containing a different capture-antibody either against mouse, bovine or dog IgG. In parallel, a control experiment was carried out on the same STV-coated plate. Appropriate wells were incubated with biotinylated anti-bovine, anti-dog and anti-mouse IgG directly (50 μl of a 6.6 $\mu\text{g/ml}$ solution in RDB), mimicking the expected pattern of the DNA-directed sorting. The plate was incubated for 2 h and washed with TETBS ($3 \times 200 \mu\text{l}$). During capture, 50 μl of a mixture of three possible targets (bovine, dog and mouse IgGs, each at a concentration of 0.8 $\mu\text{g/ml}$ in RDB) was allowed to bind to the immobilized antibody. After incubation and washing with TETBS ($5 \times 200 \mu\text{l}$), successful binding was determined with three different secondary antibody-AP conjugates. For this purpose, each of the three conjugates was applied to one column (50 μl of a 1.3 U/ml solution in RDB) of a 3×3 block. After incubation and washing ($5 \times 200 \mu\text{l}$ TETBS, $2 \times 200 \mu\text{l}$ TBS), the AP reaction was carried out and the absorbance at 405 nm was determined.

Hybridization of multiple proteins to nucleic acid carrier molecules

Quantification of hybrid binding. In separate tubes, the biotin-binding sites of purified UB-, UC-, UD- and UE-STV hybrid molecules (100 pmol each, in TBS) were saturated by the addition of 680 pmol of ^{14}C -biotin (120 c.p.m./pmol; Amersham). The final concentration of the hybrids was 1.2 μM . In order to avoid direct binding of excess biotin to the STV-coated beads, 5 μl (450 pmol) STV was added subsequently. Single kinds of either UB-, UC-, UD- or UE-STV hybrids (12 pmol) or equimolar mixtures of at least two of those (12 pmol total) were added to different tubes, each containing 0.1 mg magnetic microbeads with the immobilized 123 base single-stranded DNA carrier (~ 1 pmol). The suspensions were diluted with RDB to a final hybrid concentration of 0.5 μM . In parallel, control samples were prepared with identical contents of hybrids but with a different, non-complementary 261 base carrier. This carrier was prepared by PCR from pUC19 template with a biotinylated primer as previously described (18) and subsequently immobilized and denatured. Incubation of the samples was carried out for 14 h at room temperature. Subsequently, the beads were washed with TETBS ($7 \times 100 \mu\text{l}$ at 37°C), finally resuspended in 50 μl TETBS and transferred to scintillation vials. The radioactivity was determined by liquid scintillation counting. Experiments with ^3H -labeled biotin (38 c.p.m./pmol; Amersham) were carried out by a similar procedure.

Hybridization to RNA carrier molecules. The radiolabeled oligonucleotide (either M13-1211 or CHP) was diluted with unlabeled oligonucleotide in TE to a final concentration of 15 μM and a specific activity of ~ 500 c.p.m./pmol. RNA (0.25

μM , 40 μl , 10 pmol) was mixed with 7 μl of the probe solution (98 pmol) and 7 μl RNase inhibitor (140 U; Boehringer Mannheim). Mixtures of the DNA-STV conjugates (8 pmol, 1 μM) were prepared in a total volume of 8 μl . The RNA-probe mixture (5 μl) was added, and the solution was heated to 50°C for 1 min and allowed to cool to room temperature. A sample buffer (2 μl) was added immediately before application to the gel. Non-denaturing PAGE was performed at 100–150 V. The gel was exposed on a PhosphorImager screen (Molecular Dynamics) overnight (14 h), and analyzed with MD Image Quant software.

RESULTS AND DISCUSSION

Chemical synthesis of oligonucleotide-streptavidin hybrids

The electrophilic addition of thiols to maleimides yielding stable thioether conjugates was chosen as the crosslinking reaction, because 5'-thiol-modified oligonucleotides can be made using a standard DNA synthesizer and, if necessary, thiol groups can be incorporated into basically any protein (19). Amino groups of STV were first derivatized with a heterobispecific crosslinker,

sulfosuccinimidyl 4-[*p*-maleimidophenyl]butyrate, to provide a maleimide functionality, which was subsequently reacted with a thiolated oligonucleotide. The crosslinked products were prepurified by ultrafiltration, and then fractionated by ion exchange chromatography on a Mono Q HR 5/5 column using an FPLC system, which allowed quantitative separation of DNA-STV hybrid molecules with different numbers of DNA strands attached (Figure 1).

The electrophoretic behavior of conventional conjugates, prepared from biotinylated 24 or 30 base oligonucleotides (b-DNA) and STV, had been measured in control studies (data not shown). Titrations of STV with increasing amounts of b-DNA were analyzed by non-denaturing PAGE. A typical pattern observed showed free STV as the band with the lowest electrophoretic mobility. With addition of the biotinylated oligonucleotide, bands with higher mobility appeared, corresponding to b-DNA-STV conjugate molecules with different numbers of oligonucleotides attached. In the presence of 8 equivalents b-DNA/STV, two major bands were visible, which correspond to conjugates with 3 and 4 DNA strands attached. Free b-DNA migrated fastest and was visible at the very bottom of the gel. The higher electrophoretic mobility of the conjugates is caused by the additional negative charges of

Figure 1. Chromatogram of products from crosslinking reactions of streptavidin (STV) and thiolated DNA. DNA-STV adducts with different numbers of oligonucleotides attached were separated by ion exchange chromatography on a Mono Q HR 5/5 column. The peaks 1 and 5 represent unreacted STV and DNA, respectively. Note that the two curves, the absorbance at 280 nm and that at 260 nm, cross at around peak 1, where A_{280} is higher than A_{260} . Peaks 2–4 are hybrid molecules crosslinked to one, two or more nucleic acid moieties.

Figure 2. Analysis of ion exchange chromatography peak fractions 2–4 by 8.5% polyacrylamide gel electrophoresis. Lanes A and B, control samples of non-covalent b-DNA-STV conjugates; lanes C–E, ion exchange chromatography fractions 2–4 of Figure 1. The single band in lane C represents a hybrid species with a single DNA strand attached.

Table 2. Comparison of calculated A_{260}/A_{280} ratios of hybrid molecules with different numbers of oligonucleotides attached with observed ratios of ion exchange chromatography peak fractions

DNA molecules/ STV molecule	A_{260}/A_{280} calculated ^a	Peak number	A_{260}/A_{280} observed ^b	DNA molecules/ STV molecule ^c
1	1.05	1	0.58 (0.03)	0
2	1.27	2	1.03 (0.02)	1.0
3	1.40	3	1.23 (0.07)	1.9
4	1.49	4	1.42 (0.10)	3.2
		5	1.72 (0.10)	11.7

^aEach A_{260}/A_{280} is calculated from that of STV alone (0.58) or DNA alone (1.95) determined experimentally. The calculation is based on the following molecular masses: 60 kDa (STV), 6.93 kDa (21 base DNA).

^bStatistical average obtained from various preparations (standard deviation).

^cAn average number of DNA molecules/STV molecule of each peak fraction is estimated based on the calculated A_{260}/A_{280} values shown on the left and those of pure compounds.

the nucleic acid backbone. However, with increasing numbers of attached oligonucleotides, the increased steric bulk of the conjugates induces loss in mobility. Therefore, in the case of a 30 base oligonucleotide, the order of mobility observed was as follows: free STV tetra-b-DNA–STV tri-b-DNA–STV di-b-DNA–STV mono-b-DNA–STV conjugate free b-DNA.

The peak fractions from ion exchange chromatography were characterized by non-denaturing PAGE (Figure 2). Peaks 1 and 5 contained unreacted STV and DNA, respectively (data not shown). Generally, peak 2 gave a single band (lane C, position 6) co-migrating with a control sample containing mainly a mono-b-DNA–STV conjugate (lane A). In some batches, however, small amounts of contaminants were observable as a slower migrating band. The nature and composition of these impurities is currently unknown. The main band resulting from peak 3 (lane D, position 5) co-migrated with a conventional di-b-DNA–STV conjugate, whereas peak 4 gave several bands (lane E, positions 1–5) probably representing tri-, tetra- and higher DNA-containing species as well as free DNA (position 7).

The A_{260}/A_{280} ratio of each peak fraction was determined to estimate the DNA/protein ratio, and was compared with theoretical values, calculated for species with different numbers of oligonucleotides attached (Table 2). The values confirmed the results obtained by gel electrophoresis. Peaks 1 and 5 contained mostly unreacted STV and DNA, the latter slightly contaminated with protein-containing compounds. The observed fractional DNA/protein ratios of peaks 3 and 4 indicated mixtures of different species present. The good conformity of the value for the mono-DNA adduct and the value for peak 2, the major product of the crosslinking reaction, provided additional evidence that only a single species, namely the desired mono-DNA–STV hybrid, was present in this fraction.

The biotin binding of the purified hybrids was quantified by a chromatographic shift assay as previously described (20). A

ratio of 4.1 mol bound biotin/mol DNA–STV hybrid was observed. The result was in good agreement with the expected value of 4.0. DNA binding was investigated by gel-retardation experiments with a 5'-labeled complementary oligonucleotide as a probe. In the presence of the hybridization probe, materials recovered from peak 2 showed a single band with a decreased mobility, whereas materials from peaks 3 and 4 gave complex band patterns, indicating the presence of multiple species. In control experiments, addition of non-complementary hybridization probes caused no band-shift.

A series of DNA–STV hybrid molecules with oligonucleotide moieties, of various sequences and lengths, was prepared. All results obtained so far indicate a 1:1 molar ratio of DNA to STV for the hybrid molecules present in ion exchange chromatography peak 2 (Figure 2). Although this fraction showed high homogeneity, judged from chromatography and gel electrophoresis, multiple species with different sites of chemical crosslinking may be present, unresolved by the separation methods used. Therefore, due to steric reasons, some individual molecule species might lack the full, proposed bispecific binding capabilities expected for four biotins and one complementary DNA.

Non-covalent assembly of bifunctional constructs

Anti-mouse IgG–AP bifunctional construct. As a first test of this new class of molecules, a bifunctional construct was built by self-assembly of two complementary DNA–STV hybrids, one pre-conjugated to biotinylated AP and the other to biotinylated anti-mouse IgG (Figure 3A). The conjugation of the mono-DNA–STV hybrid molecules with the biotinylated molecules was carried out by mixing the two components in equimolar amounts. As previously investigated by gel electrophoresis, one main product resulted from this stoichiometric ratio of the two compounds, likely the 1:1 conjugate. To test their binding capability and enzymatic activity, microtiter plate wells were coated with mouse IgG, and a mixture of the two complementary

Figure 3. Schematic representation of bifunctional constructs. (A) An anti-mouse IgG–AP bifunctional construct. Two complementary DNA–STV hybrids were used for the non-covalent crosslinking of an antibody (anti-mouse IgG) and a color generating enzyme (AP). (B) Supramolecular bispecific antibody constructs.

Figure 4. Demonstration of the bifunctionality of the antibody–AP construct (see Fig. 3A). The heights of the bars, representing the absorbance at 405 nm, correlate with the enzymatic activity of AP, bound via an IgG moiety to immobilized antigen. Two control experiments, each lacking one of the necessary assembly compounds, gave no significant signal.

conjugates was added to allow binding and assembly of the construct. Subsequently, the enzymatic activity of AP attached was determined. Two control experiments, one with a DNA-STV-IgG conjugate with non-complementary sequence (control A) and the other in which the DNA-STV-AP conjugate was incubated with the biotinylated IgG only (control B), were also carried out. As shown in Figure 4, a signal was obtained only when the two complementary DNA-tagged components were present. From absorbance measurements at 405 nm, the signal-to-noise ratio was greater than 60 at a reagent concentration of 6 $\mu\text{g/ml}$.

Bispecific antibody constructs. Two bifunctional constructs bearing an anti-bovine IgG molecule and either anti-mouse IgG or anti-dog IgG (Figure 3B) were tested for their ability to bind to dog or mouse IgG, respectively, immobilized on microtiter plate wells. In analogy to a sandwich ELISA, after binding and immobilization, the construct was used to capture bovine IgG. Two control experiments, each lacking one of the two DNA-STV hybrids, were carried out in parallel. Successful

assembly was tested with a commercial AP-labeled secondary antibody against bovine IgG. The absorbance readings obtained from the pNPP color reaction indicated functionality of both tethered ends. Average signal-to-noise ratios obtained were 1.42 and 3.46 with bispecific constructs containing anti-mouse IgG or anti-dog IgG, respectively. Results obtained in control experiments with no functional bispecific construct present suggested that the relatively high backgrounds, especially in the case of immobilized mouse IgG, are primarily due to non-specific binding of the polyclonal antibodies.

Translation of a macroscopic DNA array into a protein array

A number of efforts are currently under way to develop methods for the immobilization of biomolecules on surfaces in a spatially defined way. Such techniques would facilitate a variety of applications, ranging from atomic force microscopy to the generation of spatially defined receptor arrays for biosensors and parallel ligand-binding assays. Recently, immobilization of biomolecules using photolithographic techniques was demonstrated (21–22). Although this method allows the

Figure 5. Translation of a DNA array into a protein chip. Immobilized DNA was used for selective binding of a complementary DNA-STV hybrid molecule from a mixture of hybrid molecules. The hybrids were previously conjugated with biotinylated antibodies with specificities against mouse, bovine or dog IgG. Subsequently, the immobilized IgGs were used as capture antibodies for binding specific antigen targets. Successful binding was determined with an AP-labeled secondary antibody and pNPP color reaction.

Table 3. Absorbance readings obtained for the self-sorting biochip ELISA

	Column no. (secondary antibody)								
	1 (aM-AP)	2 (aD-AP)	3 (aB-AP)	4 (aM-AP)	5 (aD-AP)	6 (aB-AP)	7 (aM-AP)	8 (aD-AP)	9 (aB-AP)
A-DNA	533	8	45	146	20	729	73	673	84
B-DNA	153	9	591	31	481	57	346	4	30
C-DNA	26	102	23	49	5	8	24	9	90
Control A	177	17	15	50	7	279	20	334	28
Control B	64	10	319	24	413	26	189	6	31
Control C	35	941	75	186	5	19	61	0	265

Each 3×3 block of wells was incubated with the same mixture of three different DNA-STV-IgG conjugates. Each row contained a different immobilized DNA (A, B or C) and each column was analyzed for the captured target with a different secondary antibody (anti-mouse, anti-dog or anti-bovine IgG-AP conjugate). The bold face positions indicate the expected positions for positive reactions. Each value is an A_{405} value $\times 1000$.

preparation of checkered patterns of a single kind of protein (e.g. immunoglobulins), it seems unsuitable for spatially addressing many types of compounds. One major difficulty in the preparation of protein arrays is the instability of most biomolecules, which prevents a stepwise, successive immobilization of multiple delicate proteins. A single regioselective process under chemically mild conditions for the attachment of multiple compounds is desired to circumvent this problem. DNA molecules and the concept of self-organization would provide an optimal solution to the process of highly parallel immobilization. Because of the exceptionally high chemical and physical stability of DNA, arrays containing separated spots of different oligonucleotides can be

prepared by successive attachment strategies (23). Furthermore, spatially separated DNA synthesis directly on small silicon-based surfaces can be carried out by photolithographic procedures in automated processes (24). In the present work, DNA-STV hybrids were used to translate a DNA array into a macroscopic protein array (Figure 5).

A DNA chip was simulated using three different oligonucleotides immobilized in different wells of a STV-coated microtiter plate via biotin-STV interaction. This array was used for the selective immobilization of three different DNA-STV-IgG conjugates. Specific hybridization to their counterparts allowed sorting of a mixture containing three different DNA-STV hybrids, each previously conjugated to a different biotinylated rabbit antibody, with specificity against mouse, bovine or dog IgG, respectively. The translated DNA array was then used to bind specifically IgG antigens from a mixture of possible IgG targets. Successful capture was determined using a commercially available AP-labeled secondary antibody.

The result of this sandwich ELISA is illustrated in Table 3. Each of the six blocks contains three different capturing oligonucleotides (rows) and three different specific labels (secondary antibody-AP conjugates, columns). Each row, and each column, is expected to have only one positive if the experiment is successful. In fact, in each block, one signal was dominant in each row and column. All control experiments (lower row) show the expected results, indicating the successful capture and detection of the antigen. In some self-sorting experiments (upper panel), false or weak positives were observed in columns 4 and 9, respectively. Repetition of the experiment gave false positives again in the same positions, indicating that the hybridization step was not optimized for the particular DNA-IgG combinations. Nevertheless, in these first experiments only two translation tests out of nine failed. Note that these results were obtained in a multiple sandwich assay with polyclonal antibodies. Furthermore, the melting temperatures, as well as the GC content of the oligonucleotides used, were not yet optimized for this specific application. The use of monoclonal antibodies, as well as a more careful selection of nucleic acid sequences, could undoubtedly improve the signal-to-background ratio.

Assembly of a single linear protein array

The nucleic acid moieties of DNA-STV hybrids can also be utilized for the construction of biological supramolecules. A single-stranded nucleic acid carrier molecule, bearing the complementary sequences, was used to provide the necessary spatial information for a self-assembly reaction (Figure 6A). Both single-stranded DNA and RNA were used to construct supramolecules, consisting of multiple DNA-STV hybrids as components.

Single-stranded DNA as construction template. A DNA carrier was prepared from a 123 bp DNA segment, generated by PCR as described in Materials and Methods. The plus strand contained five blocks of sequences, complementary to five synthetic 21 base oligonucleotides (UA, UB, UC, UD and UE) which were used in the preparation of hybrid molecules. This strand was also 5'-biotinylated, which allowed immobilization on STV-coated magnetic microbeads. The double-stranded DNA was denatured under alkaline conditions, and the minus strand in the supernatant was removed. The immobilized carrier was then used for the binding of radiolabeled DNA-STV hybrid molecules:

Figure 6. Assembly of linear protein arrays. (A) Schematic representation of a supramolecular complex consisting of four DNA-STV hybrid molecules positioned along a single-stranded nucleic acid. (B) Immobilized single-stranded DNA was used as a carrier. The chart represents relative molar amounts of DNA-STV adducts bound to the carrier as determined by quantification with radioactivity. The error bars indicate the maximum deviation observed. (C) Autoradiograph of a polyacrylamide gel-retardation experiment using RNA as a carrier. A radiolabeled RNA carrier (position 6) is retarded upon addition of complementary DNA-STV hybrids (position 5). The presence of additional hybrids with other complementary oligonucleotide moieties causes a progressive reduction in the mobility of the products.

Four different hybrids, each with a different DNA sequence (UB, UC, UD or UE), were saturated with radiolabeled biotin. In separate tubes, each of these hybrids was allowed to bind to immobilized DNA carrier. Other experiments were carried out with mixtures of two or more hybrids, but at the identical hybrid to carrier molar ratio of $\sim 20:1$. After annealing and removal of excess protein, the amount of bound STV was determined by the biotin-derived radioactivity. Control experiments were run in parallel with a non-complementary 261 base carrier. The radioactivity obtained from several independent experiments using either ^{14}C - or ^3H -labeled biotin was analyzed statistically, and relative molar amounts of bound hybrid molecules were calculated (Figure 6B).

In the presence of a single adduct, the differences in the amount of each individual immobilized hybrid were sub-stoichiometric. The variations may reflect differences in the stability of the corresponding double helices, which could lead to loss of material due to extensive washing. When a mixture of different species was used, a considerable increase in immobilized material was observed. However, the data also indicated that sub-stoichiometric amounts of different hybrids bound to the carrier. This may be due to steric reasons, since the length of a 21 bp double helix is only slightly longer than the radius of a STV molecule. Therefore, a mixture of species with a different number of hybrids may have been generated. A quantification with hybrids bearing longer DNAs should allow more detailed studies of the influence of secondary structures and possible cooperative binding effects.

Single-stranded RNA as construction template. RNA carriers were also investigated. Despite the greater chemical stability of single-stranded DNA, an RNA carrier has several attractive advantages. For instance, RNA–DNA duplexes are more stable than DNA–DNA duplexes. This means the use of an RNA carrier could provide greater stability to the constructs. Furthermore, RNA can be readily produced in large amounts by *in vitro* transcription and can, if desired, be selectively degraded by various enzymes.

A transcription template containing the T7 promoter region was used to generate an RNA carrier. In gel-retardation format experiments, the RNA carrier was tested for binding of various DNA–STV hybrids which were, in some cases, pre-conjugated with biotinylated molecules such as oligonucleotides or IgGs. For detection purposes, a ^{32}P -labeled deoxyoligonucleotide probe was hybridized to either the 3'- or 5'-terminal region of the carrier. The molar ratio of the components was $\sim 50:10:1$ for the radioactive probe, the proteinaceous components and the carrier, respectively. After annealing, the products were analyzed by non-denaturing PAGE, followed by autoradiography (Figure 6C). The radiolabeled RNA (lane 1, position 6) was retarded upon addition of the complementary oligonucleotide–STV adduct (lanes 2 and 6, position 5). Only a single band was observed in the presence of a solitary kind of hybrid, despite its 10-fold excess concentration. This implies that non-specific hybridization between the carrier and the adduct was not significant. However, addition of DNA–STV hybrids with different nucleotide sequences caused a progressive reduction in the mobility of the product (lanes 3–6, positions 4, 3 and 2, respectively), indicating the generation of higher molecular weight species. In lane 7, a pre-formed conjugate of a DNA–STV hybrid and biotinylated anti-mouse IgG was hybridized to the carrier molecule. The band of the hybridization product (lane 7, position 1) appeared in the same high molecular weight range as the putative, four STV-

containing supramolecule in lane 5. The comparable molecular masses of the two aggregates, indicated by their comparable electrophoretic behavior, supports the assumption of the presence of supramolecular bioconjugates. Furthermore, addition of mouse IgG antigen caused complete retardation of the band (data not shown), indicating the intact antigen-binding ability of the IgG-containing construct.

CONCLUSIONS

DNA–STV hybrid molecules were synthesized by chemical methods using an amino-thiol bispecific crosslinker. Purification and fractionation of the products yielded the mono-adduct as the major product. Some physical and biochemical properties of these molecules have been investigated by gel electrophoresis, UV absorbance and binding studies with complementary nucleic acids and biotin. These novel coupling agents were used for the non-covalent crosslinking of a biotinylated antibody molecule with a biotinylated color-generating enzyme, as well as with a second IgG. Furthermore, the semisynthetic hybrids allow a translation of DNA arrays into protein and, in principle, other macromolecular arrays. As demonstrated in this work, the conversion of DNA information to a protein arrangement on a macroscopic scale can be utilized for the selective self-assembly of functional protein chips. This concept is particularly powerful, since the new hybrid molecules are readily available in sufficiently large amounts to be used as constituents of a kit to convert a given DNA array into any desired protein array. On a molecular scale, the positioning along a DNA sequence allows the efficient, selective construction of spatially defined supramolecular assemblies. The concept of oligonucleotide-directed protein assembly was demonstrated by the binding of multiple DNA–STV hybrid molecules to one target nucleic acid, however, comparison of the results obtained using either RNA or DNA as a carrier is not possible at this stage. Although some refinement is needed for particular applications, this new class of hybrid molecules will serve as rapid, selective connector molecules for generating a wide variety of nano-structures useful in supramolecular chemistry and biotechnology.

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