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# Quantitative Synthesis of Protein-DNA Conjugates with 1:1 Stoichiometry

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## 1. Materials

4-(2-Aminoethyl)benzenesulfonamide (SA), N-Succinimidyl 6-maleimidocaproate (EMCS), disuccinimidyl suberate (DSS), dithiobis(succinimidyl propionate) (DSP), Carbonic Anhydrase II (CAII) from bovine erythrocytes, 6-Carboxyfluorescein diacetate (CFDA), Dulbecco's phosphate buffered saline (PBS), tris(2-carboxyethyl) phosphine (TCEP), sodium acetate (NaAc), and 10x TBE buffer (1 M Tris, 0.9 M boric acid, and 0.01 M EDTA) were purchased from Sigma-Aldrich (St. Louis, MO). Human alpha-thrombin was purchased from Haematologic Technologies Inc. (Essex Junction, VT). Glycogen and SYBR® Gold Nucleic Acid Gel Stain were purchased from Invitrogen (Carlsbad, CA). DNA oligonucleotides were purchased from Integrated DNA Technologies (IDT) (Coralville, IA). Bio-Safe Coomassie Stain and Micro Bio-Spin Columns with Bio-Gel P-6 (a size exclusion limit of 6 kDa) were purchased from Bio-Rad Laboratories (Hercules, CA). Amicon Ultra-0.5 mL Centrifugal Filters were purchased from Fisher Scientific. HPLC grade methanol and ethanol were obtained from Merck KGaA (Darmstadt, Germany). Ultrapure water (18.2 MΩ) was obtained from a Milli-Q system (Millipore Filter Co., Bedford, MA) and used throughout this study. All chemicals and reagents were of analytical or higher grade.

## 2. HPLC separation and mass spectrometry detection

HPLC separation for small molecules was carried out on an Agilent 1100 series chromatographic system (Agilent Technologies, Palo Alto, CA). A C18 column (4.6 mm I.D. × 250 mm in length; particle size, 5 μm. Phenomenex, CA) was used with the following gradient elution program. Mobile phase B (0.05% tetrafluoroacetic acid, TFA in 100% methanol, MeOH) was increased linearly from 0 to 90% over 20 min with a flow rate of 1 mL/min, then 100% mobile phase A (0.05% TFA in 5% MeOH) was maintained for 5 min to re-equilibrate the column for the next analysis.

Proteins were analyzed using an Agilent 1290 Infinity Binary LC System coupled to a TripleTOF$^{TM}$ 5600 mass spectrometer (AB Sciex, Canada). An Agilent Zorbax 300SB C18 column (4.6 mm I.D. × 150 mm in length; particle size, 3.5 μm) was used for separation. The gradient program included MeOH in 0.05% TFA

from 0% to 90% over 20 min, remaining at 90% for 5 min, and finally returning to 0% for 5 min. The flow rate was 1 mL/min.

The parameter settings for the TripleTOF 5600 mass spectrometer were as follows: source type: DuoSpray ion source, ion spray voltage floating (ISVF) 5500 V, curtain gas (CUR) 15, interface heater temperature (IHT) 500 $^{o}$C, ion source gas 1 (GS1) 50, declustering potential (DP) 80 V. All data were acquired using information-dependent acquisition (IDA) mode with Analyst TF 1.5.1 software (AB SCIEX).

**3. Denaturing polyacrylamide gel electrophoresis (PAGE) analysis**

To 48.0 g urea was added 30 mL acrylamide/bis-acrylamide (40%, 19:1), 10mL 10X TBE buffer (1 M Tris, 0.9 M boric acid, and 0.01 M EDTA) and 10 mL $H_2O$. Then the solution was heated in a 50 $^{o}$C water bath to dissolve the urea. $H_2O$ was finally added to make 100 mL of denaturing 12% gel stock solution. Polyacrylamide gel was prepared by mixing 80 μL of 10% (W/V) APS and 12 μL of TEMED with 12 mL of the denaturing 12% gel stock solution. The gel was then poured onto a 1.5 mm thick mini PROTEAN plate (7.3 cm x 10.2 cm). The comb was then carefully placed into the plate.

Samples were heated at 95 °C for 5 min in 50% formamide prior to loading. Gel electrophoresis separation was operated under 80 V and with 1X TBE buffer. The separation was conducted in a 50 °C water bath for 80 min. PAGE gels were stained with Coomassie blue and SYBR Gold to visualize protein and DNA, respectively. The stained PAGE gels were finally imaged using a GE Healthcare ImageQuant LAS 4010 imaging system.

**4. Sodium dodecyl sulfate (SDS) PAGE analysis**

Separation gel stock solution (100 mL, 12%) was prepared by mixing 40 mL acrylamide/bis-acrylamide (30%, 29:1) with 26.0 mL Tris-HCl (1.5 M, pH=8.8), 1 mL 10% SDS and 32 mL $H_2O$. Stacking gel stock solution (100 mL, 4%) was prepared by mixing 13.3 mL acrylamide/bis-acrylamide (30%, 29:1) with 25 mL Tris-HCl (0.5 M, pH=6.8), 1.0 mL 10% SDS and 59.6 mL $H_2O$. The SDS polyacrylamide gel was prepared by mixing 45 μL 10% (W/V) APS and 4.5 μL TEMED with 4.5 mL separation gel stock

solution. The gel was then poured onto a 1.0 mm thick mini PROTEAN plate (7.3 cm x 10.2 cm). Once the separation gel was polymerized, 1.5 mL stacking gel stock solution was mixed with 15 µL 10% (W/V) APS and 1.5 µL TEMED, and added to the top of the separation gel, to obtain the stacking gel. The comb was then carefully placed into the plate.

Samples were heated at 95 °C for 5 min in a Laemmli Sample Buffer (10% 2-mercaptoethanol for reducing SDS-PAGE) before loading. Gel electrophoresis was run in 1X Tris-Glycine-SDS Buffer (2.5 mM Tris, 19.2 mM glycine, 0.01% SDS, pH 8.5) at 120 V for 80 min. Next, the gels were stained with Coomassie blue and SYBR Gold to visualize protein and DNA, respectively. The stained PAGE gels were imaged using a GE Healthcare ImageQuant LAS 4010 imaging system. Because protein-DNA conjugates have different signal responses to Coomassie blue and SYBR Gold stain compared with protein and DNA molecules, we calculated the conjugation yields using the signal intensity of the unconjugated protein in SDS-PAGE.

For conjugation reactions proceeding at 100 and 10 nM levels, the conjugation mixtures were concentrated using 3kDa Amicon Ultra-0.5 mL Centrifugal Filters before PAGE analysis.

DNA-protein conjugates have different signal responses to Coomassie and SYBR Gold stain as compared with protein and DNA molecules, we therefore calculated the conjugation yields using the signal intensity of the unconjugated protein in SDS-PAGE.

**5. Preparation of reactive DNA (NHS ester activated DNA, NHS-DNA)**

Amine-modified DNA (7 µL, 100 µM) was mixed with 7 µL ACN, 7 µL DSS or DSP (50 mM in DMF) and 1 µL TEA (10% in DMF) at room temperature for 30 min. The resulting NHS-DNA was then purified by ethanol precipitation. $H_2O$ (32 µL), NaOAc (5 µL, 3 M, pH 5.2), glycogen (2 µL, 20 mg/mL) and cold EtOH (140 µL, 96%) were then added to the reaction mixture, and then placed in a -80 $^oC$ freezer for 1h followed by centrifugation for 20 min (4 $^oC$, 16000 g). The supernatant was discarded and the pellet was

dissolved in $H_2O$. The NHS-DNA was used for conjugation immediately after preparation to minimize the hydrolysis of the NHS ester in aqueous solution.

**6. Procedures for the synthesis of ligand-DNA by conjugation of maleimide-labeled SA (SA-maleimide) to thiolated DNA**

**6.1. Synthesis of maleimide-labeled ligands:** EMCS (15uL, 100 mM in DMSO) and DIPEA (7.5 μL, 1 M in DMF) were added to 50 μL of 4-(2-Aminoethyl)benzenesulfonamide (SA, 50 mM in DMSO). After a 30 min reaction in room temperature, the products were analyzed by HPLC-ESIMS to confirm the synthesis of SA-maleimide.

**6.2. Activation of the thiolated DNA:** The thiol group of the thiolated DNA ordered from IDT is a disulfide bond, which should be reduced before conjugation with the maleimide-labeled ligands. Thiolated DNA (8 μL, 100 μM) was mixed with HEPES buffer (3 μL, 500 mM, pH=8.0), TCEP (2 μL, 100mM) and $H_2O$ (7 μL). The reduction reaction was then allowed to proceed at room temperature for 30 min, and then the activated thiolated DNA was purified using P-6 Micro Bio-Spin Columns.

**6.3. Conjugation of SA-maleimide to thiolated DNA:** HEPES buffer (6 μL, 500 mM, pH=8.0) and SA-maleimide (6 μL) were added to the previously activated thiolated DNA. The mixture was left to react overnight at room temperature. The ligand-DNA was first purified using 3 kDa Amicon Ultra-0.5 mL Centrifugal Filters and then further purified using denaturing PAGE.

**6.4. Synthesis of protein-DNA conjugates**

The protein was first incubated with its corresponding ligand-DNA for 30 min in a conjugation buffer (1× PBS, 50 mM HEPES, pH = 7.5, 0.05% Tween-20). For thrombin, 10 mM $K^+$ and 1 mM $Mg^{2+}$ were added in the reaction mixture to facilitate the binding of thrombin aptamer (TBA) to thrombin in the G-quadruplex structure. NHS-DNA was subsequently added to initiate the binding-facilitated conjugation. After the completion of conjugation, free DNA was removed using 30kDa Amicon Ultra-0.5 mL Centrifugal Filters (16000g, 20 min).

**7. Optimization of reaction conditions to minimize the binding-independent conjugation**

Binding-independent reactions of NHS-DNA with lysine residues on the protein surface need to be avoided, as such reactions can produce heterogeneous conjugates. We controlled reaction concentrations of CAII and NHS-DNA to prevent the binding-independent DNA conjugation. To find out the optimum concentration, we incubated CAII with NHS-DNA at varying concentrations (1, 2, 5, 10, and 20 μM) overnight, and then characterized the reaction mixtures using denaturing PAGE and SDS-PAGE (Fig. S2). PAGE gels were stained with Coomassie blue and SYBR Gold to visualize the protein and DNA, respectively. No bands corresponding to DNA conjugation products were observed when protein and DNA concentrations were 1 and 2 μM. However, DNA conjugation products were observed from a concentration of 5 μM and onwards. Therefore, binding-independent DNA conjugation can be eliminated when the concentrations of CAII and NHS-DNA are lower than 2 μM. We then studied the conjugation rate by using a 1 μM reaction concentration of CAII and NHS-DNA. We first incubated CAII with SA-DNA for 30 min, and then added NHS-DNA to initiate the conjugation reaction. At each time point (5, 10, 20, 30, 60, 90, 120, and180 min), the reaction was terminated by adjusting the pH to 5.0. We found that only 30 min were needed to complete the reaction (Fig. S3). We also studied the impact of the spacer lengths on the conjugation yields, and found that the absence of any spacer residue resulted in the highest yield (Fig. S4). We further examined conjugation of DNA to CAII when the concentrations of CAII and the DNA hybrid were decreased to 100 nM and 10 nM, respectively. While a concentration of 100 nM for CAII led to a yield similar to that for 1 μM, negligible CAII-DNA conjugate was observed for a 10 nM concentration of the DNA hybrid (Fig. S5). Because 10 nM is much lower than the $K_d$ (3.2 μM) of binding of SA to CAII, about 3% of CAII molecules can be bound to SA under such reaction concentrations, which also confirms the importance of affinity binding to facilitate the conjugation of DNA to CAII.

**8. Iterative conjugation reactions after dissociation of hydrolyzed ligand and association of the intact NHS-DNA**

To confirm that the cycling of association and dissociation of affinity binding leads to the quantitative conjugation, we conducted a competitive binding experiment. We first incubated 0.1 μM CAII with 1.0

μM SA-DNA for 30 min to allow for sufficient binding of SA-DNA to CAII. We then added 1.0 μM NHS-DNA and varying concentrations of free SA (0, 5, 25, 125 μM). Because the excess amounts of the free SA were present in the reaction solutions, free SA could compete with SA/NHS-DNA hybrids in the process of association and dissociation of affinity binding. As expected, incomplete conjugation was observed when the free SA was applied (Fig. S7). Additionally, the conjugation yield decreased with increases of free SA concentration. Therefore, cycling of association and dissociation of affinity binding is the underlying reason of quantitative conjugation.

**9. Mass spectrometry analysis to confirm the 1:1 stoichiometry of the protein:DNA conjugate**

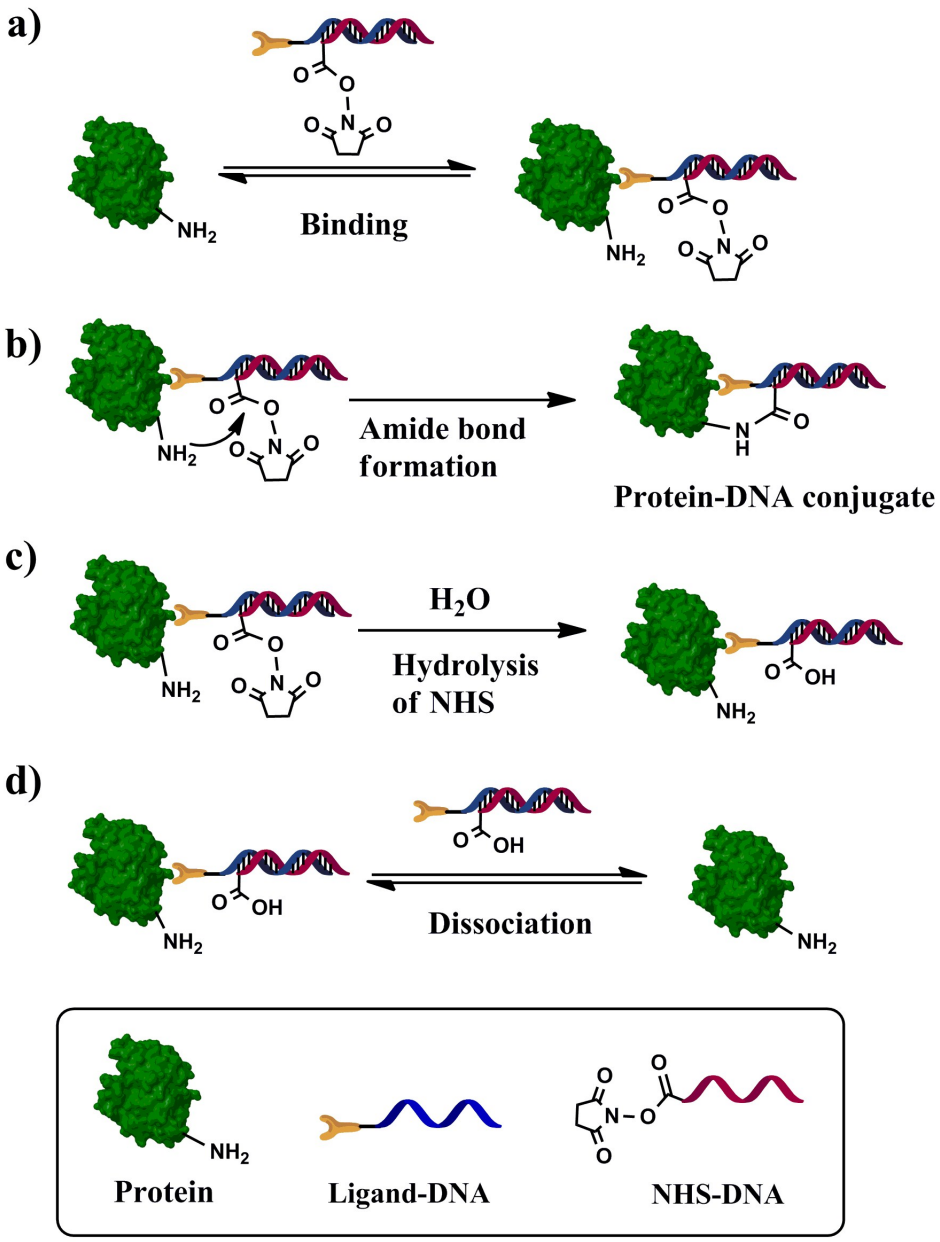
To further confirm the 1:1 stoichiometry of the protein:DNA conjugate, we used electrospray ionization high resolution mass spectrometry (ESIMS) to measure the molecular weights of CAII before and after conjugation. We used dithiobis (succinimidyl propionate) (DSP), a linker containing disulfide bonds, to prepare the NHS-DNA (Fig. S8a). A benefit of this approach is that simply reducing the disulfide bond could remove the DNA from the CAII-DNA conjugate, eliminating any interference of DNA on the ESIMS analysis. The remaining molecule with a molecular weight difference of 88 Da allows for the differentiation of the CAII conjugates from the free CAII. The single CAII conjugate appearing at 88 Da higher than that of the free CAII (Fig. S8b) indicates the 1:1 stoichiometry of the CAII-DNA conjugate.

**10. Relevance of binding affinity of the ligands to the conjugation reaction**

Although the quantitative conjugation can be achieved by using various affinity ligands, the binding affinity is relevant to the required concentration of protein and DNA. With the use of aptamer HD22 ($K_d$ 0.5 nM), only 10 nM DNA was necessary to form the thrombin-DNA conjugates (Fig. S11). In the case of weaker binding using the small molecule inhibitor SA to CAII ($K_d$ 3.2 μM), negligible CAII-DNA conjugate was observed when the concentration of DNA was 10 nM. Therefore, higher binding affinity enables conjugation under lower reaction concentrations.

**Table S1** DNA sequences used in this study

| DNA | Sequences (5' → 3' ) |
|---|---|
| Thiolated ligand DNA | HS-TCA ACA TCA GTC TGA TAA GCT A |
| **Aptamer TBA**-DNA (The nucleotides in **bold** represent the aptamer sequence) | **GGT TGG TGT GGT TGG** TTT TTC AAC ATC AGT CTG ATA AGC TA |
| **Aptamer HD22**-DNA (The nucleotides in **bold** represent the aptamer sequence) | **AGT CCG TGG TAG GGC AGG TTG GGG TGA CT** T TTT TCA ACA TCA GTC TGA TAA GCT A |
| Amino-modified reactive DNA (0T spacer) | TTA TGT AGC CGT ATG ATT CAG ACT GAT GTT GA-$NH_2$ |
| Amino-modified reactive DNA (6T spacer) | TTA TGT AGC CGT ATG ATT CAG ACT GAT GTT GA $(T)_6$-$NH_2$ |
| Amino-modified reactive DNA (12T spacer) | TTA TGT AGC CGT ATG ATT CAG ACT GAT GTT GA $(T)_{12}$-$NH_2$ |
| Amino-modified reactive DNA (18T spacer) | TTA TGT AGC CGT ATG ATT CAG ACT GAT GTT GA $(T)_{18}$-$NH_2$ |
| Initiator DNA | TAG CTT ATC AGA CTG ATG TTG A |




**Scheme S1** Binding-facilitated synthesis of protein-DNA conjugates using an affinity ligand and the NHS-labeled DNA strand. (a) The 1:1 protein-ligand binding brings a single NHS-DNA molecule to close proximity with the protein molecule. (b) The NHS-DNA strand reacts with the protein by formation of a covalent amide bond between the NHS and a lysine residue. As a result, a 1:1 protein-DNA conjugate is formed. (c) NHS can also hydrolyze in aqueous solution. The DNA strand with hydrolyzed NHS cannot react with the protein. (d) Because the affinity binding between the ligand and protein is reversible, the DNA strand with hydrolyzed NHS can dissociate from the protein, leaving the protein available to bind with another active NHS-DNA probe (a). These iterative affinity interaction and covalent binding processes continue until all protein molecules are conjugated with the DNA, in a 1:1 stoichiometry.

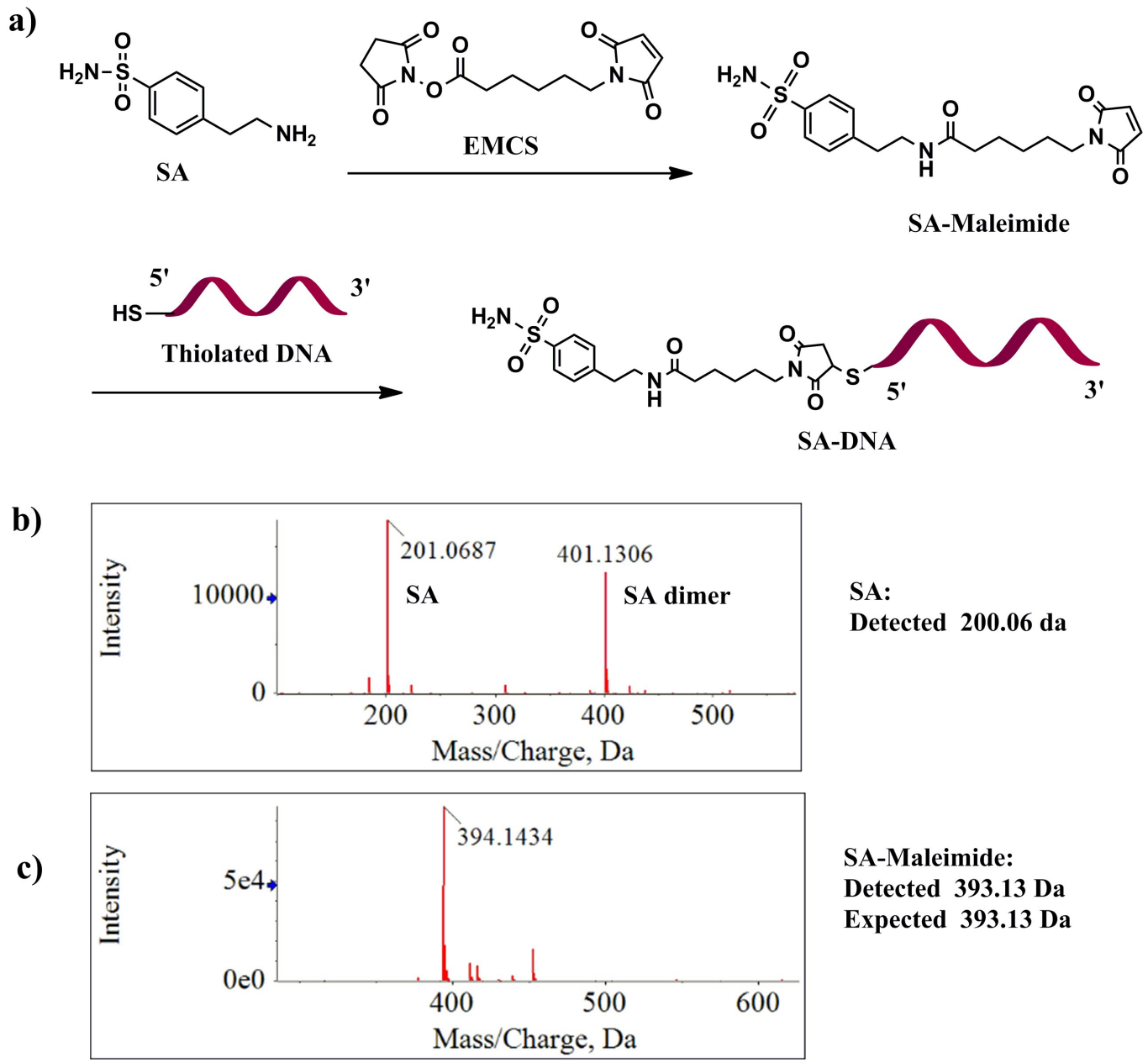



**Fig. S1. (**a) Chemical reactions for synthesis of SA-DNA. The amine group of 4-(2-Aminoethyl) benzenesulfonamide (SA) first reacts with the NHS group of EMCS, resulting in SA-maleimide. Thiolated DNA is then added to react with SA-maleimide. The reaction between thiol and maleimide forms SA-DNA. (b) ESIMS spectrum of SA. (c) ESIMS spectrum of SA-Maleimide. The detected molecular weight of SA-maleimide (393.13 Da) matches exactly with the expected molecular weight (393.13 Da), representing the addition of an EMCS (308.10 Da) to SA (200.06 Da) with the loss of a NHS group (115.03 Da). The detected peaks in the spectra represent the $[M+H]^+$ ions.

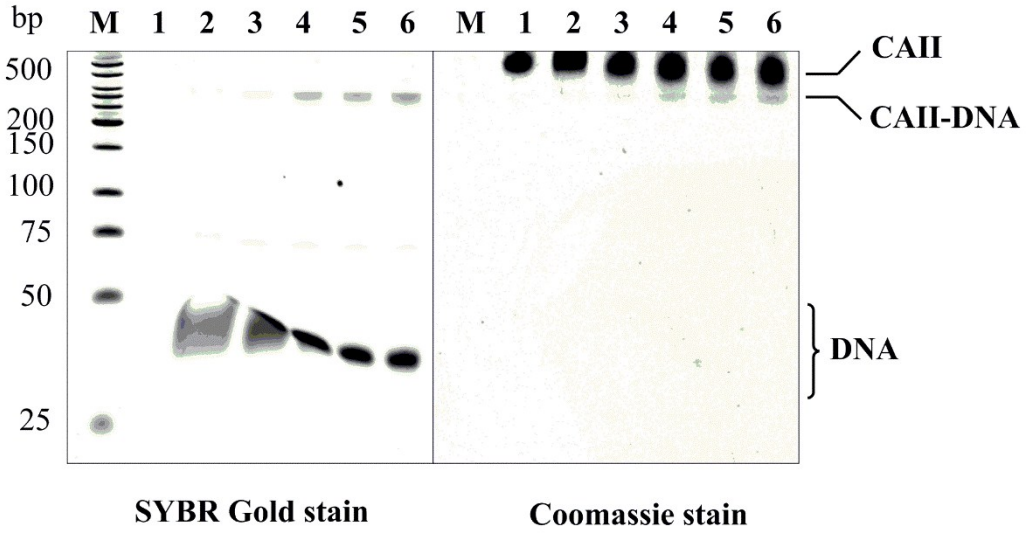



**Fig. S2.** Random conjugation of NHS-0T-DNA to CAII at varying concentrations of CAII and NHS-0T-DNA. The conjugation products were characterized by denaturing PAGE. Lane M, low molecular weight DNA ladder; Lane 1, 1 μM CAII; Lane 2, 1 μM CAII and NHS-0T-DNA; Lane 3, 2 μM CAII and NHS-0T-DNA; Lane 4, 5 μM CAII and NHS-0T-DNA; Lane 5, 10 μM CAII and NHS-0T-DNA; Lane 6, 20 μM CAII and NHS-0T-DNA. CAII and NHS-0T-DNA were finally diluted to 1 μM for denaturing PAGE analysis. PAGE gels were stained with Coomassie blue and SYBR Gold to visualize protein and DNA, respectively. DNA conjugation products were observed from a concentration of 5 μM and onwards. No band corresponding to DNA-protein conjugation products were observed when protein and DNA concentrations were 1 μM and 2 μM. Therefore, binding-independent DNA conjugation can be obviated when the concentrations of CAII and NHS-DNA are lower than 2 μM.

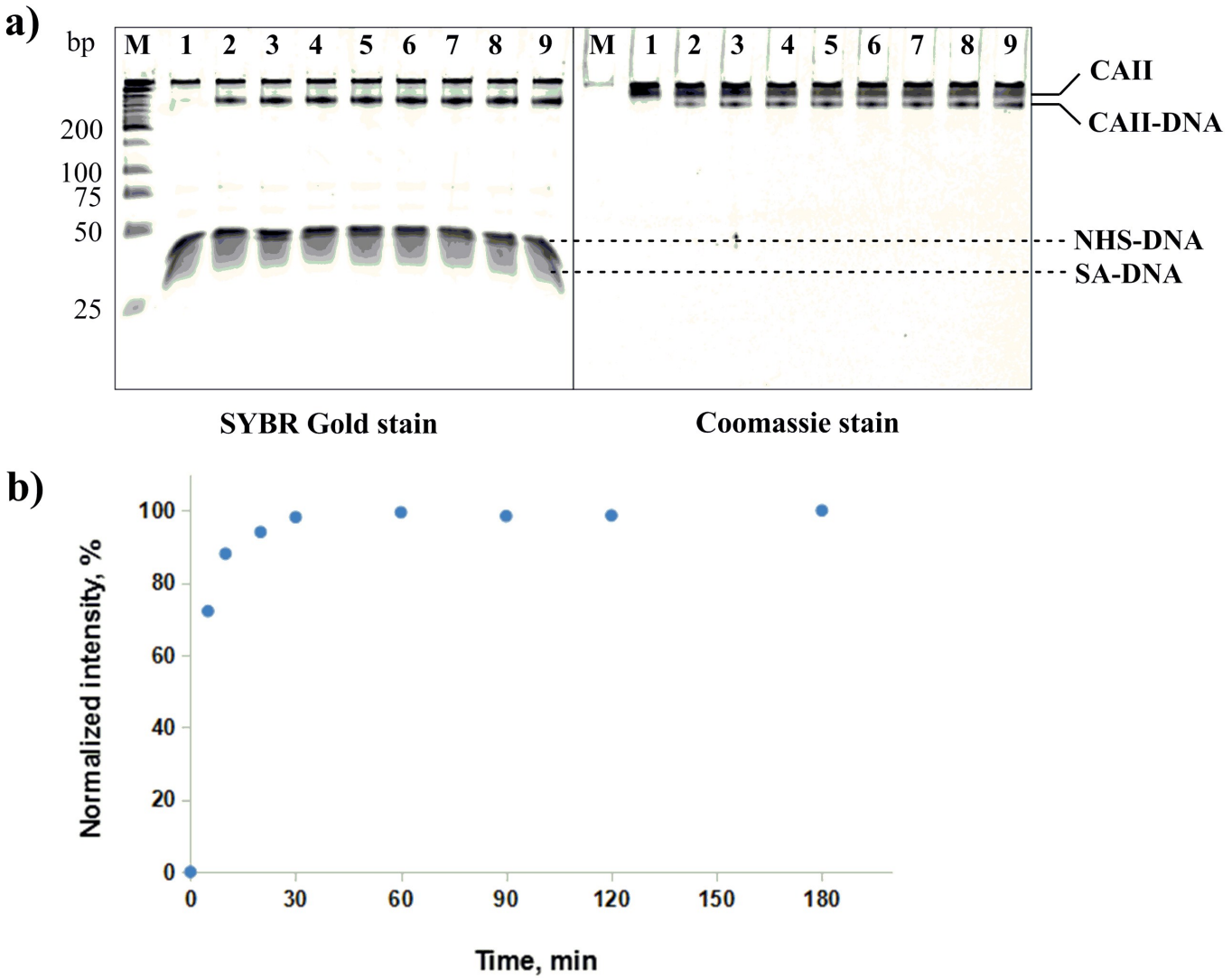



**Fig. S3.** Optimizing the reaction time that is needed to complete the binding-facilitated conjugation. CAII is first incubated with SA-DNA for 30min, and then NHS-DNA is added to initiate the conjugation reaction. The reaction is terminated by adjusting pH from 7.5 to 5.0 at 0 min (Lane 1), 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 60 min (Lane 6), 90 min (Lane 7), 120 min (Lane 8), 180 min (Lane 9). (a) Conjugation products characterized by denaturing (12%) PAGE. (b) Plot of normalized band intensities of CAII-DNA conjugates from SYBR Gold stain of denaturing PAGE over 180 min. At 30 min, the signal for the protein-DNA conjugate reach plateau, indicating the completion of the conjugation reaction.

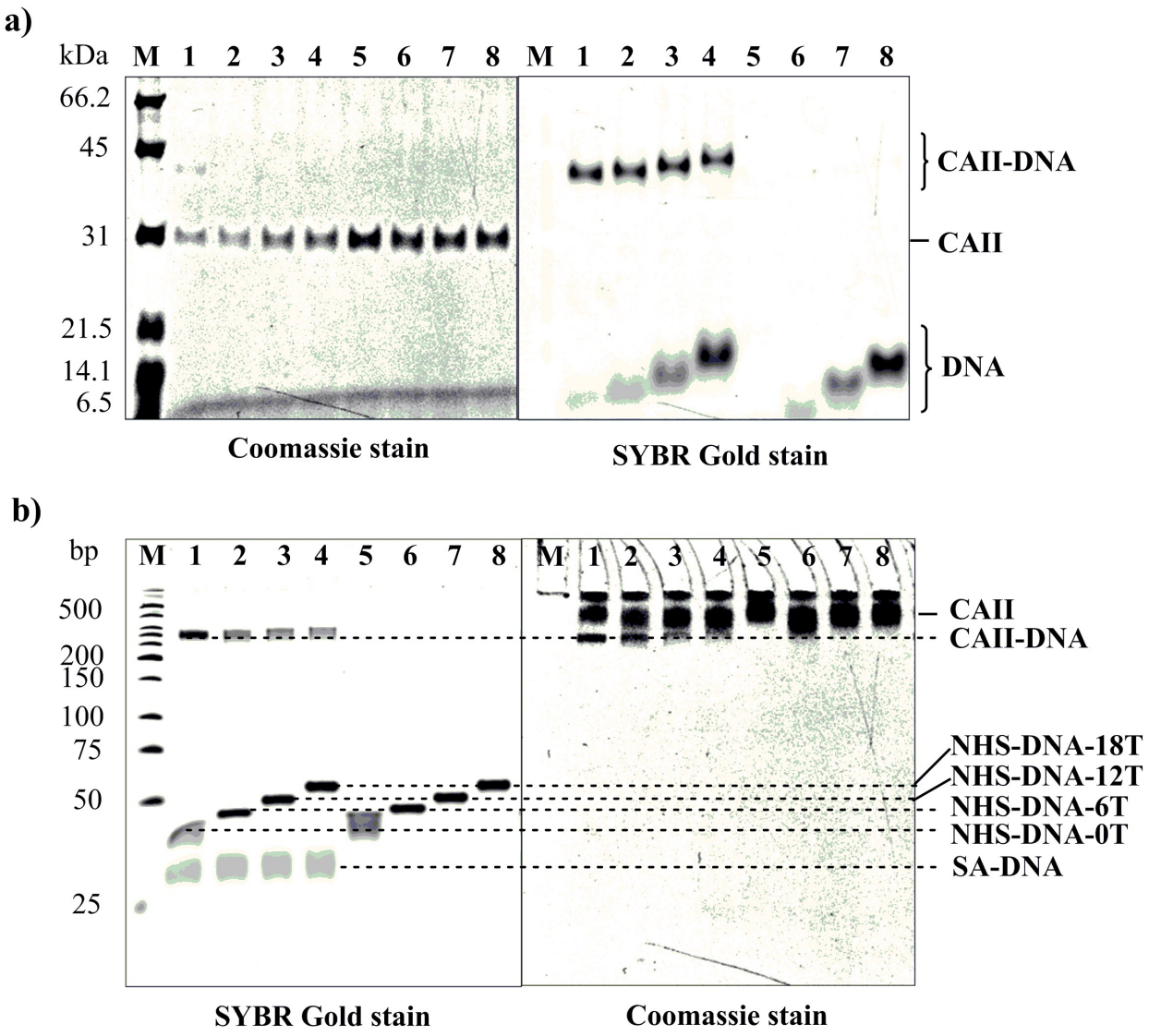



**Fig. S4.** (a) SDS-PAGE and (b) Denaturing PAGE characterizing reaction mixtures of CAII-DNA using NHS-DNA with different spacer lengths. Lane M, protein ladder for SDS-PAGE and low molecular weight DNA ladder for denaturing PAGE; Lane 1, CAII, SA-DNA and NHS-0T-DNA; Lane 2, CAII, SA-DNA and NHS-6T-DNA; Lane 3, CAII, SA-DNA and NHS-12T-DNA; Lane 4, CAII, SA-DNA and NHS-18T-DNA; Lane 5, CAII, NHS-0T-DNA; Lane 6, CAII, NHS-6T-DNA; Lane 7, CAII, NHS-12T-DNA; Lane 8, CAII, NHS-18T-DNA. Because protein-DNA conjugates have different signal responses to Coomassie blue and SYBR Gold stains compared with proteins and DNA molecules, we calculated the conjugation yields through the signal intensity of unconjugated protein in SDS-PAGE. The yields for spacer lengths with 0, 6, 12 and 18T were calculated to be 61.8, 60.5, 42.1 and 36.3%, respectively. These results demonstrate that the conjugation yields increase with the decrease of the spacer length. The absence of any spacer residue (0T) resulted in the highest yield.

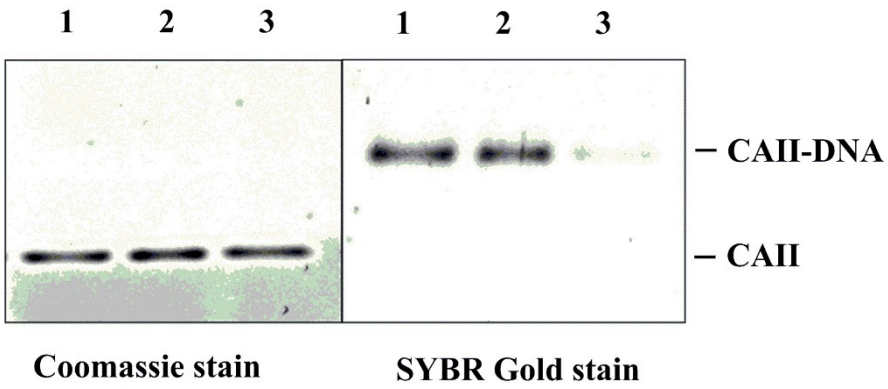



**Fig. S5.** SDS-PAGE characterizing the concentration dependent conjugation of DNA to CAII. The molar ratio of CAII:SA-DNA:NHS-0T-DNA is kept at 1:1:1. Lane 1, 1 μM of CAII, SA-DNA and NHS-0T-DNA; Lane 2, 100 nM of CAII, SA-DNA and NHS-6T-DNA; Lane 3, 10 nM of CAII, SA-DNA and NHS-12T-DNA. PAGE gels were stained with Coomassie blue and SYBR Gold to visualize protein and DNA, respectively. The concentration of 100 nM led to a yield similar to that for 1 μM, and negligible CAII-DNA conjugate was observed for the 10 nM concentration. Because 10 nM is much lower than the Kd (3.2 μM) of binding of SA to CAII, about 3% of CAII molecules bind to SA under such reaction concentrations. This result demonstrates the importance of affinity binding to facilitate the conjugation of DNA to CAII.

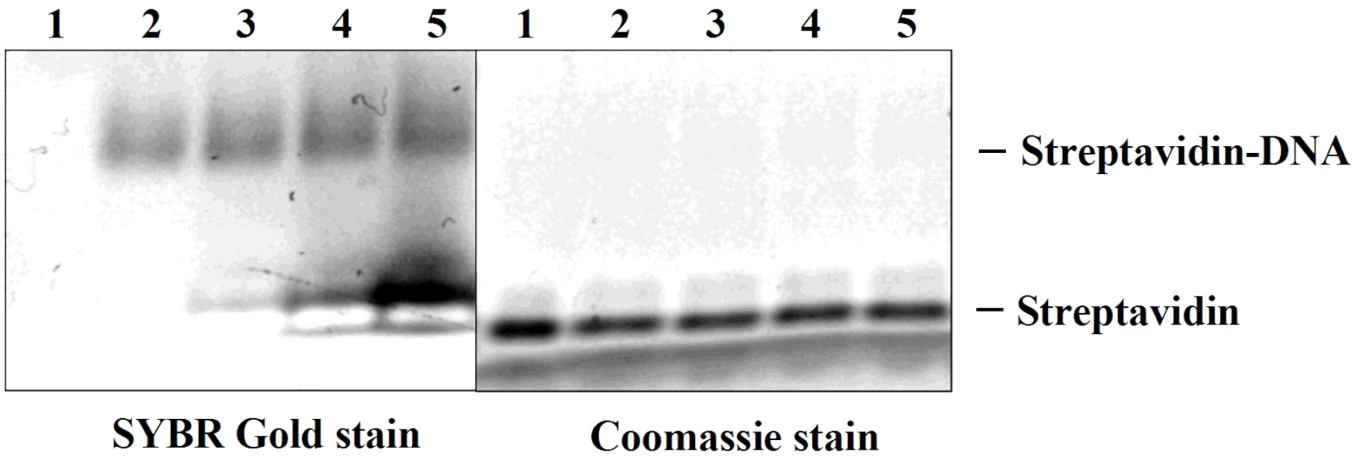



**Fig. S6.** Reducing (2-mercaptoethanol) SDS-PAGE (12%) was used to characterize the conjugation products of streptavidin-DNA with varying DNA:protein ratios. Concentration of streptavidin was kept at 0.1 μM, and biotin/NHS-DNA concentrations were 0 μM (Lane 1), 0.1 μM (Lane 2), 0.2 μM (Lane 3), 0.5 μM (Lane 4), and 1 μM (Lane 5). PAGE gels were stained with SYBR Gold and Coomassie blue to visualize the proteins and DNA, respectively. The yield of streptavidin-DNA conjugate was calculated to be ~28% for the different DNA:streptavidin ratios. Because of the extraordinarily high binding affinity ($Kd=10^{-15}$) of biotin to streptavidin, the biotin/NHS-DNA can bind to streptavidin with 100% efficiency. This result demonstrates that the diminishing conjugation efficiency is attributed to the hydrolysis of NHS group, rather than incomplete binding.

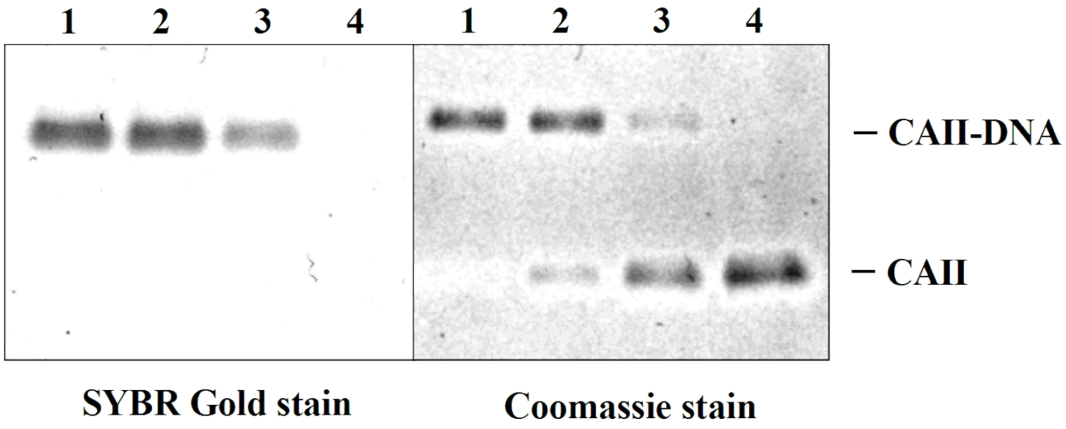



**Fig. S7.** Complete conjugation of CAII-DNA (lane 1) and the tests of competing SA (lanes 2, 3, and 4). Reducing (2-mercaptoethanol) SDS-PAGE (12%) was used to characterize the conjugation products of CAII-DNA under competition with varying concentrations of free SA. CAII (0.1 μM) was first incubated with 1.0 μM SA-DNA for 30 min to allow for sufficient binding of SA-DNA to CAII. NHS-DNA (1.0 μM) and 0 (lane 1), 5 μM (lane 2), 25 μM (lane 3), 125 μM (lane 4) of free SA were then added to the mixture.

In the absence of the competing free SA, complete conjugation was achieved, yielding quantitative CAII-DNA product (lane 1), as expected. Because the affinity binding between the ligand and the protein is reversible, the DNA strand with hydrolyzed NHS can dissociate from the protein, leaving the protein available to bind with another active NHS-DNA probe. These iterative affinity interaction and covalent binding processes continue until all the protein molecules are conjugated with the DNA, in a 1:1 stoichiometry.

When the free SA was added as the competitor to interfere with the association and dissociation of affinity binding, the conjugation reaction was incomplete, leaving unconjugated CAII (lanes 2, 3, 4). The conjugation yield decreased with increasing concentration of the free SA. The iterative affinity interaction and covalent binding processes was affected by the competing free SA.

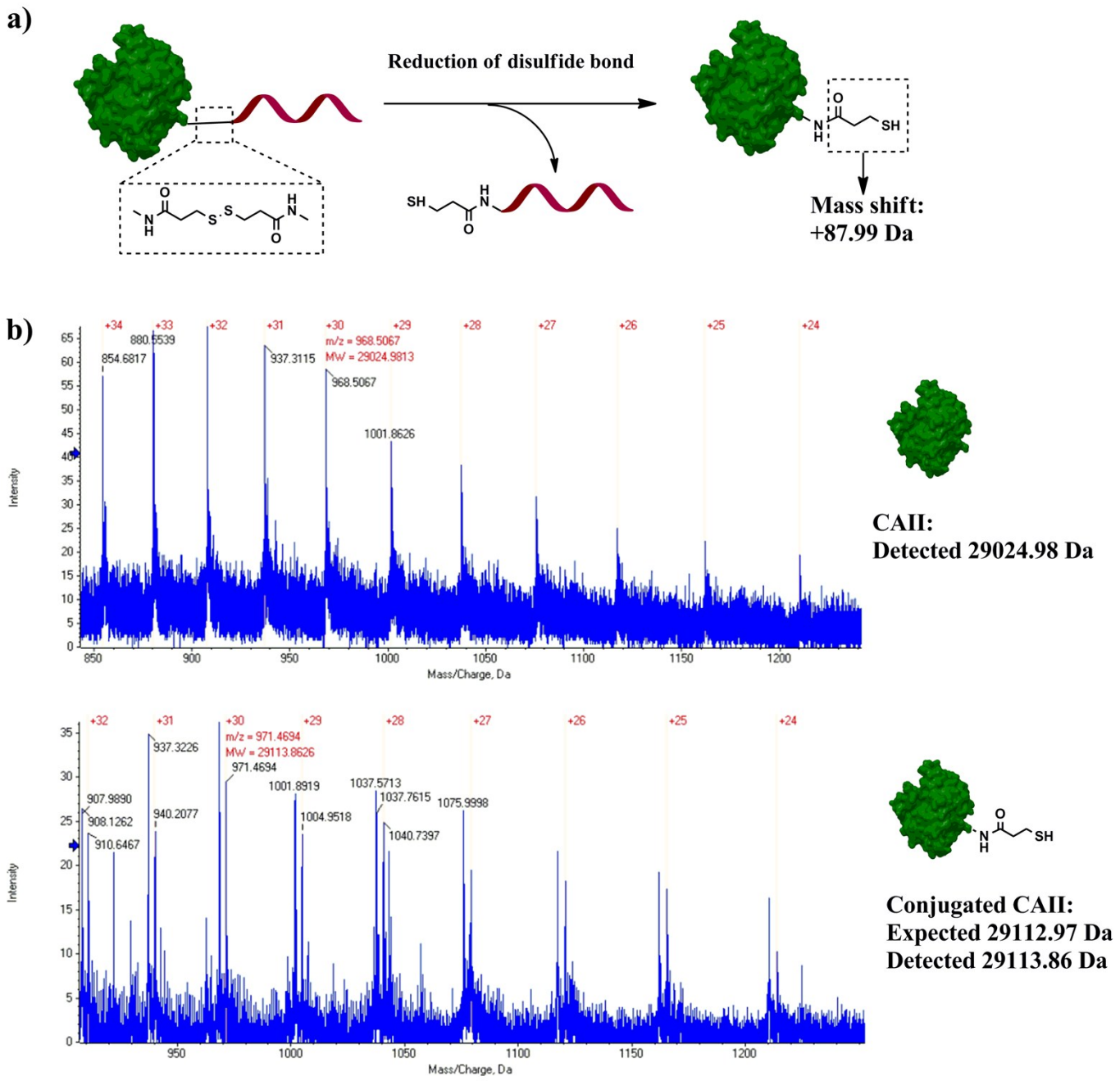



**Fig. S8.** (a) Prior to ESIMS analysis, DNA was removed from the CAII-DNA conjugates by reducing the disulfide bonds between the proteins and DNA. The remaining chemical residue with a molecular weight of 87.99 Da allows for the differentiation of CAII conjugates from free CAII. (b) ESIMS spectra of conjugated CAII spiked with free CAII. The CAII conjugates only presented as a single molecular weight, which is 88Da more than that of free CA, indicating a 1:1 ratio of CAII-DNA conjugates.

Note that reaction conditions were chosen to intentionally have both the conjugation product and the unconjugated protein present, allowing for their detection in the same MS spectrum. Under the optimum reaction conditions for conjugation, the protein-DNA conjugation was complete and the unconjugated protein was not detectable in the MS spectra.

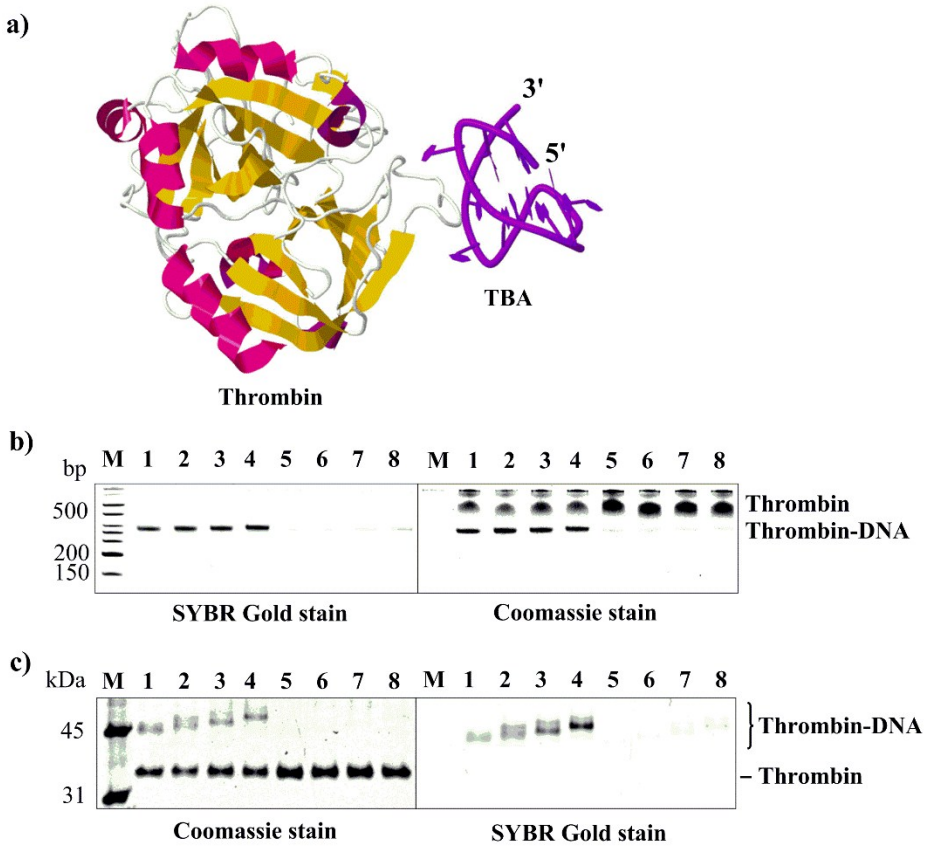



**Fig. S9.** (a) Structure of human α-thrombin in complex with TBA shown in purple (PDB entry 4DII). This figure was prepared using Jmol: an open-source Java viewer for chemical structures in 3D. http://www.jmol.org/. (b) Denaturing PAGE and (c) reducing (2-mercaptoethanol) SDS-PAGE (12%) characterizing the conjugation products of α-thrombin-DNA. 10 mM $K^+$ and 1 mM $Mg^{2+}$ were added in the reaction mixture to facilitate the binding of TBA to TB in G-quadruplex structures. Lane M, low molecular weight DNA ladder for (b), SDS-PAGE molecular weight standards, high range for (c); Lane 1, α-thrombin, TBA and NHS-0T-DNA; Lane 2, α-thrombin, TBA and NHS-6T-DNA; Lane 3, α-thrombin, TBA and NHS-12T-DNA; Lane 4, α-thrombin, TBA and NHS-18T-DNA; Lane 5, α-thrombin, NHS-0T-DNA; Lane 6, α-thrombin, NHS-6T-DNA; Lane 7, α-thrombin, NHS-12T-DNA; Lane 8, α-thrombin, NHS-18T-DNA. The conjugation yields for spacer lengths of 0T, 6T, 12T and 18T were 39.6%, 41.3%, 24.1% and 28.1%, respectively. The DNA-thrombin conjugation yields increased with the decrease of the spacer length, when using TBA as affinity ligand.

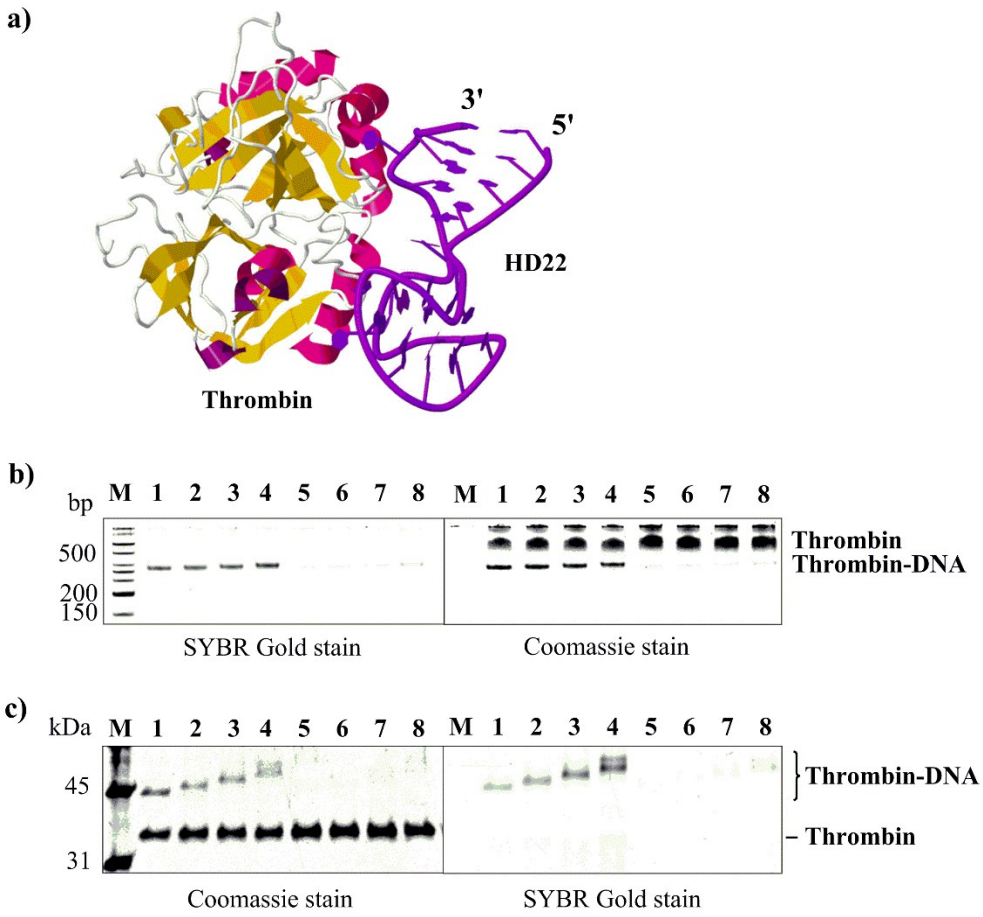



**Fig. S10.** (a) Structure of human α-thrombin in complex with HD22 shown in purple (PDB entry 4I7Y). This figure was prepared using Jmol: an open-source Java viewer for chemical structures in 3D. http://www.jmol.org/. (b) Denaturing PAGE and c) reducing (2-mercaptoethanol) SDS-PAGE (12%) characterizing the conjugation products of α-thrombin-DNA. 10 mM $K^+$ and 1 mM $Mg^{2+}$ were added in the reaction mixture to facilitate the binding of HD22 to TB in G-quadruplex structures. Lane M, low molecular weight DNA ladder for (b), SDS-PAGE molecular weight standards high range for (c); Lane 1, α-thrombin, HD22 and NHS-0T-DNA; Lane 2, α-thrombin, HD22 and NHS-6T-DNA; Lane 3, α-thrombin, HD22 and NHS-12T-DNA; Lane 4, α-thrombin, HD22 and NHS-18T-DNA; Lane 5, α-thrombin, NHS-0T-DNA; Lane 6, α-thrombin, NHS-6T-DNA; Lane 7, α-thrombin, NHS-12T-DNA; Lane 8, α-thrombin, NHS-18T-DNA. The conjugation yields for spacer lengths of 0T, 6T, 12T and 18T were 31.4%, 22.1%, 18.6% and 16.5%, respectively. The DNA-thrombin conjugation yields increased with the decrease of the spacer length, when using HD22 as affinity ligand.

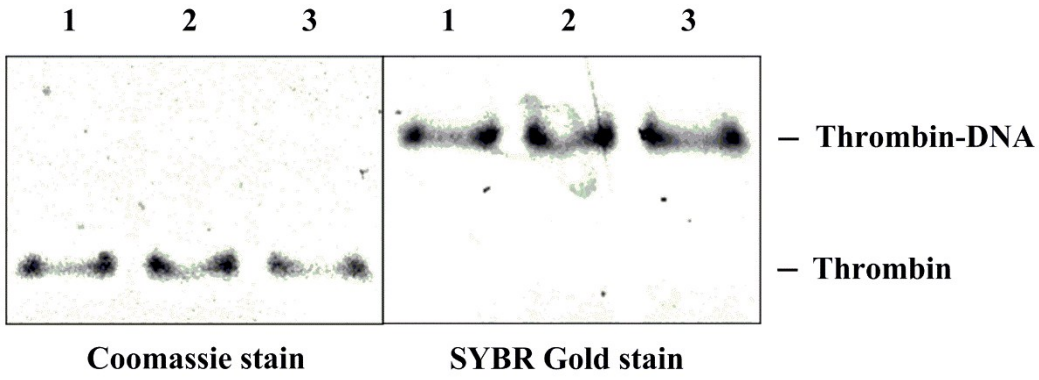



**Fig. S11.** SDS-PAGE characterizing the concentration dependent conjugation DNA to α-thrombin. The molar ratio of α-thrombin:HD22:NHS-0T-DNA is kept at 1:1:1. Lane 1, 1 μM of α-thrombin, HD22 and NHS-0T-DNA; Lane 2, 100 nM of α-thrombin, HD22 and NHS-0T-DNA; Lane 3, 10 nM of α-thrombin, HD22 and NHS-0T-DNA. PAGE gels were stained with Coomassie blue and SYBR Gold to visualize protein and DNA, respectively. Due to the high affinity of HD22 ($k_d$ = 0.5nM), thrombin-DNA conjugates were observed when the DNA concentration was 10 nM, with a similar yield to that of 0.1 and 1 μM. This result demonstrate that high binding affinity enables conjugation under low reaction concentrations.

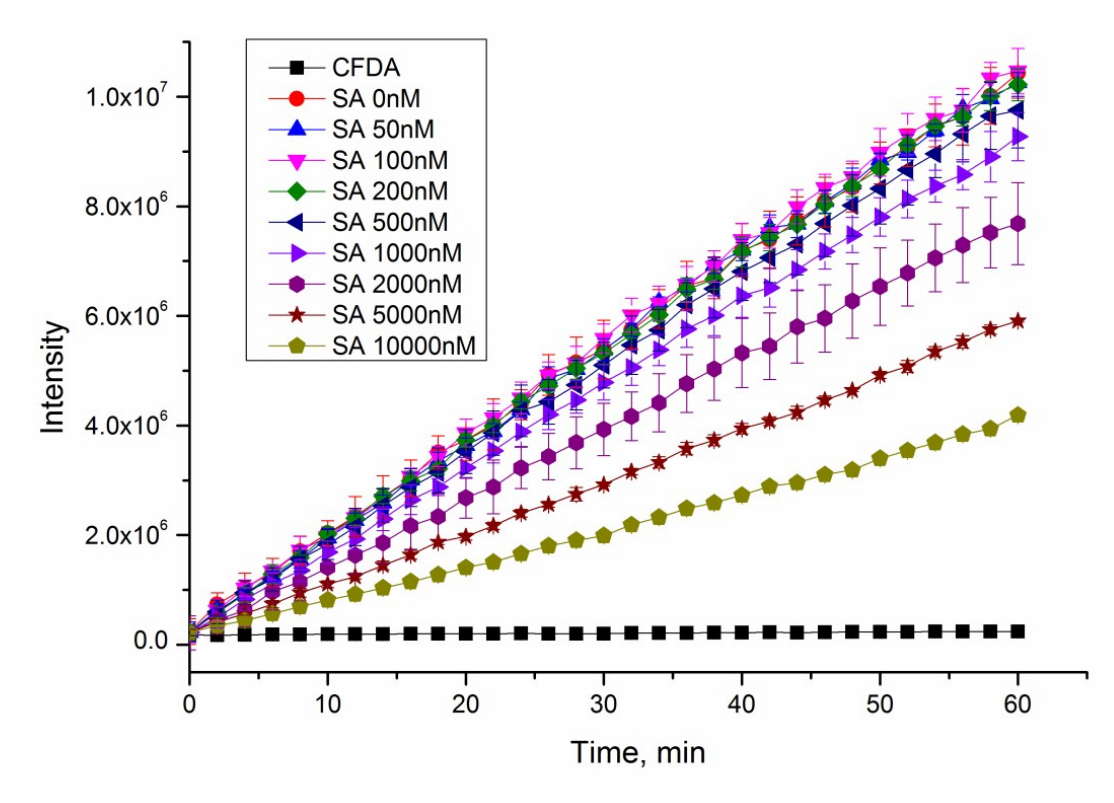



**Fig. S12.** Inhibition of 50 nM CAII by varying concentrations of SA. 6-Carboxyfluorescein diacetate (CFDA) (1 μM) was used as the substrate. Samples were transferred onto a 96-well plate (Fisher Scientific, Ottawa, Canada), and then analyzed using a fluorescence microplate reader (DTX 800, Beckman Coulter). Even though the free SA was in large excess, the activity of CAII was not effectively inhibited, confirming the weak inhibition ability of the free SA.