Light-Trigged Release of Bioactive Molecules from DNA Nanostructures

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Supporting Information

ABSTRACT: Recent innovations in DNA nanofabrication allow the creation of intricately shaped nanostructures ideally suited for many biological applications. To advance the use of DNA nanotechnology for the controlled release of bioactive molecules, we report a general strategy that uses light to liberate encapsulated cargoes from DNA nanostructures with high spatiotemporal precision. Through the incorporation of a custom, photolabile cross-linker, we encapsulated cargoes ranging in size from small molecules to full-sized proteins within DNA nanocages and then released such cargoes upon brief exposure to light. This novel molecular uncaging technique offers a general approach for precisely releasing a large variety of bioactive molecules, allowing investigation into their mechanism of action, or finely tuned delivery with high temporal precision for broad biomedical and materials applications.

KEYWORDS: nanotechnology, DNA origami, controlled release, uncaging, photolabile cross-linker, bioactive cargo

Rapid advances in structural DNA nanotechnology allow the creation of intricately shaped nanostructures that can be functionalized with a high degree of control at precise locations.1−4 For example, DNA origami can be reliably and efficiently self-assembled by folding large, single stranded DNA with a set of specifically designed short oligonucleotide strands.5 This technique affords a tremendous amount of control over the size and shape of the nanostructure whose designs can now be assisted by well-developed software tools.6−8 Molecularily programmed, static9−11 or dynamic12−14 DNA architectures hold promise for applications in areas such as cell biology,15 NMR spectroscopy,16 super resolution microscopy,17 and nanotherapeutics,18 many of which would be advanced if DNA nanostructures were capable of releasing bound cargos at precise times.

Attempts to obtain controlled release from DNA origami nanostructures thus far have utilized two approaches through either noncovalent or covalent attachment of the cargo to origami. For example, the chemotherapy drug doxorubicin has been found to be able to noncovalently bind to DNA nanostructures through interactions with the DNA helices.19,20 By controlling the DNA origami structure configuration, it was shown that doxorubicin release from the nanostructures could elicit a cytotoxic response in regular and drug-resistant cancer cells. Noncovalent attachment strategies however critically depend on a chemical’s ability to intercalate into DNA helices. This binding mechanism cannot be generalized to most chemicals, and the binding sites within an origami cannot be easily controlled spatially. Direct covalent attachment of cargo to DNA origami nanostructures can overcome most of these limitations.

To covalently attach a cargo to DNA helices, short DNA strands can be designed to protrude at specific locations on the surface of the nanostructures, which can then bind to a variety of different chemical moieties including inorganic nanoparticles,21,22 proteins,23 antibodies,18 and fluorophores.24 Placement of cleavable linkages within these DNA strands permits the release of the bound cargo in a highly controllable fashion. However, such strategies often leave a chemical remnant, the chemical group being released, which may compromise their native biological function, limiting this approach to applications where the bioactivity of the cargo is important. Here, we demonstrate a novel and general method that releases chemically unaltered cargoes using brief pulses of light that can be broadly applied to a large variety of molecules.

We designed a novel, photolabile linker to append cargo molecules into the cavities of DNA nanostructures, so that light irradiation-induced breakage of the linker would allow the molecules to diffuse away from the protective cavity (Figure 1). This photolabile cross-linker possesses an o-nitrobenzyl (o-NB) motif for photocleavage, an azido group for attachment to alkyne functionalized oligonucleotides, and an activated carbonate group for attachment to cargo molecules possessing a free amino functional group (Figure 1A). The linker is designed to release cargo upon photo cleavage in its original state with no chemical remnants remaining attached. Given the fact that most

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peptides, proteins, and bioactive compounds contain exposed amino residues, the cross-linker design is broadly applicable to attach many molecules to DNA nanostructures, beyond the examples described here.

We first synthesized this photolabile cross-linker using conventional organic synthesis techniques. Gram scale product was easily produced from inexpensive, commercially available starting materials (Scheme S1). This photolabile cross-linker was then reacted with cargo molecules including glutamate, bovine serum albumin (BSA), and biotin amine, and subsequently conjugated to oligonucleotides allowing the cargo to be incorporated into preassembled DNA origami through DNA base pairing (Scheme S2).

In parallel, we computationally designed a multilayered, brick-like nanocage structure with a well-defined cavity in its center, similar to those previously reported.6,20,26 The nanocage contains 14 addressable, single-stranded DNA extensions in its cavity, which are complementary to those presented on the activated cargo (Figure 1B-D). Nanostructures were then self-assembled in a single step by slowly cooling a heated mixture of the DNA components. Analysis of the assembly by agarose gel electrophoresis showed a single, dominant product band that migrated faster relative to the single stranded DNA starting material (m13 DNA), consistent with that generally observed for multilayered DNA origami structures (Figure 1C).9,21 Further examination with transmission electron microscopy (TEM) revealed properly assembled structures with the desired shape and a clearly visible central cavity (Figure 1D). The short single-stranded DNA extensions, however, were too small to be resolved using TEM. Purification of fully formed nanostructures from excess oligonucleotides or subsequent cargo

Figure 1. Design and creation of light-triggered, cargo-releasing nanocages. (A) Scheme of the chemical activation of a cargo molecule with the photolabile cross-linker and an oligonucleotide. (B) Depiction of the DNA nanostructure formation. The solid cylinders represent DNA helices as shown by the inset. (C) Agarose gel electrophoresis showing the high folding yield of the crude DNA nanocage sample. Lane L contains the 1-kb ladder, lane m13 contains the single stranded DNA starting material, and lane cage contains the crude reaction mixture. (D) TEM images of DNA nanocages. Scale bars are 200 and 25 nm, respectively. (E) Schematic depiction of the encapsulation of cargo, the photochemical reaction, and subsequent cargo release.

Figure 2. Light-triggered release of small molecules from nanocages. (A) Photolysis data showing increased irradiation duration results in an increase in the cleavage of Oregon Green/oligonucleotides conjugate. (B) Schematic depiction of the dye uncaging experiment. DNA nanostructures remain in the microdialysis chamber, whereas small dyes are able to diffuse out. (C) Absorption spectra of a dual dye tagged nanocage before (yellow curve) and after light irradiation (blue curve).
molecules was accomplished using poly(ethylene glycol) precipitation.27

Fully assembled and purified DNA nanocages were then incubated with the activated cargo to attach them to the interior of the nanocage cavity. When positioned inside of the nanostucture, the cargo is protected from the exterior environment and unable to bind to its native sites of action. Release from the cage was then achieved with light irradiation, which cleaved the photolabile bonds within the cross-linker (Figure 1E).

To first validate the photocleavage of our cross-linkers, we used it to conjugate an oligonucleotide to the small fluorescent molecule Oregon Green cadaverine (OG). We irradiated the compound with a low-power light source over time and quantified the degree of separation of OG from the oligonucleotide using HPLC (Figure 2A). We found that an increasing duration of light exposure led to a larger fraction of free OG dye. After 11 s of low-powered light irradiation, 50% of OG was released. Nearly complete cleavage was achieved after 40 s of exposure, consistent with the time course for the cleavage of the o-NB motif within the cross-linker.28

We then loaded the activated OG into the cavities of the nanostructures by incubating the OG/DNA conjugate with preassembled nanocages. To quantify loading efficiency, we incorporated a nonlabile dye (Alexa Fluor 647N, AF647) for comparison by attaching it to a region on the nanostructure distal to the cavity (Figure 2B). UV absorbance spectra analysis of the product showed two distinct absorption peaks centered around 500 and 647 nm, corresponding to the two dyes used (Figure 2C, yellow trace). The ratio of the dye concentrations for OG versus AF647 was 7.4 to 1, suggesting that about half of the 14 DNA extensions on each cage designed to bind OG were bound, which is likely a representative loading capacity for small molecules of similar size.

To measure the efficiency of the light-induced release of OG from the nanocages, we irradiated the structures with a low powered lamp for 60 s, and then analyzed the absorbance spectra of the reaction solution after extensive sample dialysis of released free OG (Figure 2B). We observed that the peak absorption at 500 nm corresponding to the photolabile OG dye was completely absent after irradiation, whereas the 647 nm absorption peak corresponding to the nonlabile AF647 remained (Figure 2C, blue trace). Together, these results demonstrate that our uncaging strategy can successfully release small molecular cargo from the DNA nanostructure upon brief low energy light irradiation.

We then explored the possibility of releasing large proteins from the nanocages, using bovine serum albumin (BSA) and streptavidin as examples that can be easily observed and analyzed using TEM. BSA was directly caged through the reaction of our cross-linker with the surface amino groups on the protein. Streptavidin was indirectly caged by attaching biotin-amine to the nanocage cavity and then subsequently mixing with the protein. TEM analysis of nanostructures at different orientations revealed clearly visible BSA and streptavidin proteins within the cavity of the DNA cage (Figure 3). None were seen tethered to the cage exterior. The number of DNA nanostructures with and without

![Figure 3. Light-triggered release of proteins, (A) streptavidin and (B) bovine serum albumin from nanocages. (i) Schematic depictions of the DNA nanocages with and without proteins. (ii) TEM images of nanocaged proteins before (left) and after (middle) irradiation with light. Scale bars are 25 nm. (iii) Graphs showing percentage of nanocages containing protein as determined by TEM image counting before and after light are shown on the right. Numbers in parentheses indicate the number of particles counted per condition.](image-url)
proteins was determined via particle counting of TEM images, and a loading efficiency of 93% for BSA and 71% for streptavidin was observed. After low power light irradiation for 60 s, we found only 19% of nanocages contained BSA, and 9% cages contained streptavidin, which corresponds to uncaging efficiencies of 79% for BSA and 87% for streptavidin. Together, these results demonstrate that full sized proteins can be effectively encapsulated and uncaged with high efficiency.

To demonstrate that molecules released from the DNA nanocages retain their bioactivity, we tested uncaging of the small molecule glutamic acid, an excitatory neurotransmitter that has been shown to be successfully uncaged in numerous instances (Figure 4A). The bioactivity of the released glutamate from the nanocages was measured by glutamate mediated calcium changes in cultured neurons using real-time fluorescence imaging. Primary hippocampal neuron cultures were incubated with the intracellular calcium dye Fluo-4 and the glutamate-containing DNA nanocages. Before light illumination, little basal calcium activity was observed in the nine-day-old cultures, consistent with the general activity patterns observed in neuron cultures of this age (Figure 4B and C). Immediately following a 1 ms light pulse illumination (240–400 nm), we observed an increase in intracellular calcium levels in 16.22% (N = 185 neurons, analyzed in two tests) (Figure 4C, Video S1). Activated cells exhibited heterogeneity in response amplitude with activation onsets ranged from 509 ms to 18.19 s after the light pulse, which could be due to difference in diffusion time from the releasing site to the cell surface, the concentration of released glutamic acid on a given cell, and intrinsic variability of cellular calcium responses (Figure 4D). The fact that light irradiation was delivered for 1 ms suggests that uncaging can be performed with millisecond temporal resolution. In the absence of the DNA nanocages, no cells exhibited a change in calcium levels upon light illumination (N = 124 neurons, analyzed in two tests) (Figure 4B, Video S2). Together, these results demonstrate that DNA nanocages can be used to release functional bioactive molecules with millisecond temporal precision.

In conclusion, we describe a novel strategy to encapsulate bioactive molecules inside DNA nanostructures and release them using pulses of light. This strategy is realized through tagging DNA origami with a novel photolabile cross-linker that can be broadly used to encapsulate a large variety of molecules. With this cross-linker, a single, general chemical reaction scheme can be used to attach chemicals of interest to DNA origami through reacting with amino groups which are present on many biologically relevant compounds. This technique allows the release of cargo in its unaltered, bioactive state in contrast to existing labile conjugation chemistries, which often leave behind a chemical remnant that may interfere with the natural bioactivity of the cargo. This strategy was shown to be effective for a range of molecular sizes, from small molecules to full-sized proteins. Our nanocage design offers a high degree of addressability and customization, and future versions could be created that accommodate a larger variety of cargo molecules or cocktails of molecules in precise stoichiometries by controlling the shape and dimensions of the nanostructures as well as the sequences of the strands protruding from the cavity. Although light controlled uncaging techniques have been successful in releasing small molecules that rely on small, photochemical blocking chemical groups, our nanocaging platform could be easily designed to release many previously uncagable compounds and accelerate.

Figure 4. Light-triggered release of glutamate from DNA nanocages. (A) Schematic depiction of glutamate release from DNA nanocage using UV light at 240–400 nm and the subsequent activation of neurons by the freed glutamate. (B,C) Temporal derivative of the normalized fluorescence intensity indicating calcium concentration changes in the control group, neurons illuminated in the absence of nanocages (B, N = 124 neurons), and in the uncaging group, neurons illuminated in the presence of nanocages (C, N = 185 neurons). (D) Normalized fluorescence intensity indicating intracellular calcium activities of responsive cells in the uncaged group, aligned to light onset. Thick blue line indicates the mean, shaded gray indicates standard deviation, and red dots indicate the onset time (N = 30 neurons).
progress in understanding chemical receptor binding or controlled release of therapeutics.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.nanolett.6b00530.

Synthetic schemes, synthetic details, bioconjugation details, experimental details of glutamate uncaging, additional TEM images, and DNA sequences. (PDF)

Supplemental Video S1: In the presence of DNA nanocages containing glutamate, neurons exhibited an increase in intracellular calcium levels after 1 ms light pulse illumination. (AVI)

Supplement Video S2: In the absence of the DNA nanocages, no neurons exhibited a change in calcium levels after 1 ms light pulse illumination. (AVI)

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### Author Contributions

The research project concept was created by X.H. Molecular design, organic synthesis, nanostructure creation, TEM imaging, and cargo release characterization were performed by R.E.K. Glutamate uncaging experiments were performed by R.E.K. and S.S.C. S.S.C and X.H. analyzed the glutamate uncaging data. H.Y.M. provided neuron cultures and technical support for glutamate uncaging experiments. H.Y.M. supervised uncaging data. R.E.K. and S.S.C. S.S.C and X.H. wrote the manuscript.

### Notes

The authors declare no competing financial interest.

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Supplemental Information for

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Experimental Section

Cross-linker 1 synthesis (Scheme 1):

Reactions were monitored by TLC using glass-backed silica gel 60 F254 plates. Flash chromatography was performed in a quartz column with a fluorescent indicator (green 254 nm) added to the silica gel. TLC bands were visualized by UV. Solvent ratios used as elutants are reported in v/v. The purity of the final products was obtained through ¹H NMR and ¹³C NMR.

¹H NMR data were obtained on a 500 MHz Varian VMNRS spectrophotometer at the Chemical Instrumentation Center at Boston University. Chemical shifts are reported in parts per million (ppm) and coupling constants were reported in Hertz (Hz). ¹H NMR spectra obtained in CDCl₃ were referenced to 7.26 ppm and those obtained in DMSO-d6 were referenced to 2.50 ppm. ESIMS data were collected on an Agilent Single-Quad LC/MSD VL instrument at the Chemical Instrumentation Center at Boston University.

The following compounds were synthesized according to literature procedures: 5-hydroxy-2-nitroacetophenone (S1)¹ and ethylene glycol 2-azidoethyl ether tosylate (S2)².
1-(5-(2-(2-azidoethoxy)ethoxy)ethoxy)-2-nitrophenyl)ethan-1-one (S3). To a solution of 5-hydroxy-2-nitroacetophenone (2.46 g, 13.6 mmol) and ethylene glycol 2-azidoethyl ether tosylate (4.36 g, 13.2 mmol) in DMF (15 mL) was added potassium carbonate (3.77 g, 27.3 mmol), and the suspension was heated to 75°C. After 18 hours, the solution was concentrated in vacuo and partitioned between CH$_2$Cl$_2$ (40 mL) and NaHCO$_3$ (20 mL). The organic layer was washed with NaHCO$_3$ (3 x 10 mL), dried over Na$_2$SO$_4$, filtered, and concentrated in vacuo to produce 4.30 g (96% crude) of S3 as a dark brown oil that was taken on without further purification: $^1$H NMR (500 MHz,
DMSO-d6) δ 8.14 (d, $J = 9.1$ Hz, 1H), 7.21 (dd, $J = 2.8$ Hz, 9.1 Hz, 1H), 7.19 (d, $J = 2.8$ Hz, 1H), 4.29 (m, 2H), 3.79 (m, 2H), 3.59 (m, 6H), 3.38 (m, 2H), 2.53 (s, 3H).

1-(5-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)-2-nitrophenyl)ethan-1-ol (S4). To a solution of S3 (4.12 g, 12.2 mmol) in MeOH (30 mL) stirring in an ice bath was added sodium borohydride (723 mg, 18.7 mmol) in portions. After 2 hours, the solution was concentrated in vacuo and partitioned between CH$_2$Cl$_2$ (30 mL) and brine (20 mL). The organic layer was washed with NaHCO$_3$ (3 x 10 mL), dried over Na$_2$SO$_4$, filtered, concentrated in vacuo, and purified via flash chromatography (2:1 ethyl acetate : petroleum ether) to afford 3.63 g (88 %) of S4 as a yellow oil: $^1$H NMR (500 MHz, CDCl$_3$) δ 8.03 (d, $J = 9.1$ Hz, 1H), 7.37 (d, $J = 2.8$ Hz, 1H), 6.88 (dd, $J = 2.8$ Hz, 9.1 Hz, 1H), 5.55 (dq, $J = 4.0$ Hz, 6.3 Hz, 1H), 4.24 (m, 2H), 3.90 (m, 2H), 3.74 (m, 2H), 3.68 (m, 4H), 3.38 (m, 2H), 2.40 (d, $J = 4.0$ Hz, 1H), 1.54 (d, $J = 6.3$ Hz, 3H).

Cross-linker (1). To a solution of S4 (1.88 g, 5.51 mmol) and 4-nitrophenyl chloroformate (1.65 g, 7.86 mmol) in CH$_2$Cl$_2$ (21 mL) was added triethylamine (1.50 mL, 10.8 mmol). After stirring for 24 hours, CH$_2$Cl$_2$ (30 mL) was added and the solution was washed with NaHCO$_3$ (20 mL), dried over Na$_2$SO$_4$, filtered, concentrated in vacuo, and
purified via flash chromatography (gradient from 1:3 to 2:3 ethyl acetate : petroleum ether) to afford 2.16 g (78 %) of S4 as a tan oil: $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.26 (d, $J$ = 9.3 Hz, 2H), 8.12 (d, $J$ = 9.1 Hz, 1H), 7.37 (d, $J$ = 9.3 Hz, 2H), 7.24 (d, $J$ = 2.7 Hz, 1H), 6.95 (dd, $J$ = 2.7 Hz, 9.1 Hz, 1H), 6.53 (quart, $J$ = 6.3 Hz, 1H), 4.25 (m, 2H), 3.93 (m, 2H), 3.76 (m, 2H), 3.69 (m, 4H), 3.39 (m, 2H), 1.76 (d, $J$ = 6.2 Hz, 3H).

**General bioconjugate protocol (Scheme 2):**

Bioactive, amino-group containing compounds were first reacted in slight excess with Cross-linker 1 in organic solvents such as methylene chloride or dimethylformamide and trimethylamine. In cases where the starting material was insoluble, a dimethylsulfoxide/aqueous buffer mixture was used.

Carbonate intermediates were subsequently reacted with an alkyne functionalized oligonucleotide via the copper catalyzed azide alkyne cycloaddition (CuAAC) reaction using published procedures.$^3$ In brief, equal volumes of alkyne functionalized oligonucleotide (410 uM in PBS) and activated carbonate (1 mM in DMSO) were mixed. A solution of copper sulfate (10 equivalents, 20 mM in water) and tris(3-hydroxypropyltriazoylmethyl)amine (THPTA) (50 equivalents, 50 mM in water) were separately mixed together and added to the reaction mixture. Lastly, a solution of sodium ascorbate (120 equivalents, 100 mM) was added and the reaction was stirred overnight. The reaction was subsequently purified via HPLC (TSKgel OligoDNA RP column, Tosoh Bioscience) using a gradient from 1:19 to 3:2 acetonitrile : 100 mM ammonium acetate over 30 minutes.
Scheme S2
Glutamic Acid Conjugate. To a solution of cross-linker 1 (322 mg, 637 µmol) and L-glutamic acid di-tert-butyl ester hydrochloride (217 mg, 734 µmol) in CH$_2$Cl$_2$ (6 mL) was added triethylamine (570 µL, 4.09 mmol). After stirring for 48 hours, CH$_2$Cl$_2$ (25 mL) was added and the solution was washed with NaHCO$_3$ (2 x 15 mL). The combined aqueous layers were washed with CH$_2$Cl$_2$ (2 x 10 mL). The combined organic layers were dried over Na$_2$SO$_4$, filtered, concentrated in vacuo, and purified via flash chromatography (1:2 ethyl acetate : petroleum ether) to afford 383 mg of a yellow oil. To a solution of this intermediate in CH$_2$Cl$_2$ (5 mL) was added trifluoroacetic acid (700 µL, 9.15 mmol). The solution was concentrated in vacuo and purified via flash chromatography (5% MeOH in CH$_2$Cl$_2$) to afford 152 mg (47% over 2 steps) of product: $^1$H NMR (500 MHz, DMSO-d$_6$) δ 8.1 (dd, $J = 2.0$ Hz, $J = 9.0$ Hz, 1H), 7.8 (d, $J = 8$ Hz, 1H), 7.2 (dd, $J = 2.8$ Hz, $J = 17.3$ Hz, 1H), 7.1 (m, 1H), 6.1 (m, 1H), 4.3 (m, 2H), 3.9 (m, 1H), 3.8 (quart, $J = 3.2$ Hz, 2H), 3.6 (m, 4H), 3.4 (m, 2H), 2.3-2.2 (m, 2H), 2.0-1.7 (m, 2H), 1.5 (t, $J = 6.8$ Hz, 2H), 1.4 (d, $J = 8.5$ Hz, 3H). ESI-LRMS m/z 512.1 (M-) Product molecular weight = 513.46. Azido intermediate was reacted with the oligo-alkyne using the general bioconjugate protocol and purified via HPLC to afford a solution of the product.
Biotin bioconjugate. To a solution of cross-linker 1 (103 mg, 204 µmol) and biotin-amine (100 mg, 234 µmol) in CH₂Cl₂ (1 mL) and DMF (1 mL) was added triethylamine (150 µL, 1.08 mmol). After stirring for 2 hours the solvent was removed with a stream of air. CH₂Cl₂ (25 mL) was added and the solution was washed with NaHCO₃ (15 mL). The aqueous layer was washed with CH₂Cl₂ (10 mL). The combined organic layers were dried over Na₂SO₄, filtered, concentrated in vacuo, and purified via flash chromatography (gradient 2% to 10% MeOH in CH₂Cl₂) to afford 109 mg (80%) of a solid: ¹H NMR (500 MHz, DMSO-d6) δ 8.1 (d, J = 9.0 Hz, 1H), 7.7 (t, J = 5.8 Hz, 1H), 7.4 (t, J = 5.8 Hz, 1H), 7.1 (m, 2H), 6.4 (s, 1H), 6.3 (s, 1H), 6.1 (quart, J = 6.5 Hz, 1H), 4.2 (m, 3H), 4.21 (m, 1H), 3.8 (t, J = 4.5 Hz, 2H), 3.6 (m, 4H), 3.4 (t, J = 5.0 Hz, 2H), 3.1 (m, 2H), 3.0 (m, 2H), 3.0-2.8 (m, 2H), 2.6 (d, J = 12.5 Hz, 1H), 2.0 (t, J = 7.5 Hz, 2H), 1.5 (m, 5H), 1.6-1.2 (m, 8H). Azido intermediate (128 µL, 2 mM in DMSO, 256 nmol) was reacted with the oligo-alkyne (120 µL, 410 µM in 1x PBS, 49.2 nmol) using the general bioconjugate protocol. The product was isolated by ethanol precipitation and purified via HPLC to afford 11.3 nmol as a 100 µL, 113 µM solution of the product.
**Oregon Green Conjugate.** To a solution of cross-linker 1 (1.25 mg, 2.52 µmol) and Oregon Green cadaverine (1.21 mg, 2.39 µmol) in DMF (300 µL) and water (20 µL) was added triethylamine (20 µL, C). After stirring overnight the solution was purified via HPLC to afford 2.39 mg an orange solid: ESI-LRMS m/z 863.2 (M+) Product molecular weight = 862.80. Azido intermediate (150 µL, 1 mM in DMSO, 150 nmol) was reacted with the oligo-alkyne (150 µL, 410 µM in 1x PBS, 61.5 nmol) using the general bioconjugate protocol and purified via HPLC to afford 38 nmol as a 200 µL, 190 µM solution (62%) of the product.

**BSA Conjugate.** A solution of BSA (200 µL, 500 µM in 1x PBS, 100 nmol) and cross-linker 1 (20 µL, 5 mM in DMSO, 1000 nmol) in 80 µL DMSO was mixed overnight. The reaction was centrifuged at 17000 rcf for 5 minutes to pellet insoluble materials. The supernatant was dialyzed in 1x PBS against a 25 kDa cutoff to afford 300 µL (333 µM) of product. Azido intermediate (50 µL, 333 µM in PBS, 16.7 nmol) was reacted with the oligo-alkyne (200 µL, 410 µM in 1x PBS, 82 nmol) using the general bioconjugate protocol and purified using Amicon spin filters (3 spins with 30 kDa cutoff tube and 3 spins with 50 kDa cutoff tube) against buffer (5 mM Tris, 1 mM EDTA, and 16 mM MgCl₂) to produce 50 uL of product solution.
Design and assembly of DNA nanostructures. Nanostructures were designed using caDNAno. Single stranded M13mp18 bacteriophage DNA was prepared as described previously. All oligonucleotides were purchased from Integrated DNA Technologies (IDT) and used with no additional purification. Creation of nanostructures was performed by first heating a solution containing a final concentration of 40 nM m13 scaffold DNA and 200 nM of each staple in a folding buffer containing 5 mM Tris, 1 mM EDTA, and 20 mM MgCl$_2$ to 80°C, followed by cooling from 80°C to 60°C over 80 minutes, and then from 60°C to 24°C over 48 hours. Removal of excess staple strands was accomplished by three rounds of precipitation with polyethylene glycol solutions. Pellets were re-dissolved in 5 mM Tris, 1 mM EDTA, and 16 mM MgCl$_2$.

Cavity functionalization. Nanostructures were mixed with 70 equivalents of oligo bioconjugates (5 equivalents per handle, with 14 handles in the cavity interior) and incubated overnight at 40°C and subsequently purified by at least two rounds of PEG precipitation.

Gel electrophoresis. Reaction solutions were electrophoresed on 1.5% agarose gels containing 0.5x TBE, supplemented with 10 mM MgCl$_2$. DNA dye SybrSafe was mixed with gel solutions before loading onto the gel. The gel box was submerged in an ice water bath to prevent excessive heating.

TEM sample preparation and imaging. TEM samples were prepared by placing 3 µL of sample solution onto a carbon coated grid (FCF400-Cu, Electron Microscopy Sciences)
which was previously charged using a plasma etcher (30 seconds of irradiation). After 2 minutes, the solution was wicked away from the grid with filter paper (Whatman 50 hardened). The grid was immediately treated with stain for 30 seconds and excess solution was wicked away. The remaining solution on the grid was evaporated at room temperate prior to imaging. TEM images were acquired with an FEI Tecnai Spirit Transmission Electron Microscope operated at 80 kV. Saturated uranyl formate (in ddH$_2$O prepared freshly before usage) was used for protein caging experiments and 2% uranyl acetate (diluted with ddH$_2$O from 4%, Electron Microscopy Sciences) was used for all other samples.

**Kinetics of o-NB cleavage.** Samples of the Oregon Green cadaverine oligonucleotide bioconjugate were irradiated with a handheld UV lamp (UVM-57, 6W, 302 nm) for varying lengths of time and analyzed using HPLC (TSKgel OligoDNA RP column, Tosoh Bioscience) using a gradient from 1:19 to 3:2 acetonitrile : 100 mM ammonium acetate over 30 minutes. Irradiation durations used were 5, 10, 15, 20, 25, 30, 35, 40, and 60 seconds. A UV detector monitoring at 490 nm was used to collect traces containing Oregon Green. The degree of o-NB cleavage was obtained by comparing the areas under the peaks corresponding to the Oregon Green-oligo conjugate starting material with the released Oregon Green cadaverine (Figure S1).
2 dye labeling experiment. The general cavity functionalization protocol was followed but with two different oligos. 5 oligos per binding site were used. The cavity contained 14 binding sites for the activated Oregon green oligo, whereas 1 binding site on the unfolded loop was available for the Alexa Fluor 647 oligo. Reactions were incubated overnight at 40°C and subsequently purified by at least two rounds of PEG precipitation. The final solution was analyzed using the UV setting of a Nanodrop 2000. The ratio of the dyes was obtained by comparing the concentrations of each dye in solution as calculated with Beer’s law.

Oregon Green cadaverine uncaging. 25 µL 2 dye labeled nanostructures was irradiated with a handheld UV lamp (UVM-57, 6W, 302 nm) for 60 seconds. The solution was placed in half of a microdialysis chamber and dialyzed against 2 L of buffer (5 mM Tris, 1 mM EDTA, and 16 mM MgCl₂) overnight. The resulting solution was analyzed using the UV setting of a Nanodrop 2000.
**Protein uncaging.** Protein containing nanocages were created following the general cavity functionalization protocol. 5 equivalents per oligo handle were used. For BSA encapsulation, the BSA/oligo bioconjugate was used. For streptavidin, the nanocage was first modified with the biotin-amine/oligo bioconjugate and subsequently with streptavidin (5 equivalents per oligo handle). Each round of modification was purified using two rounds of PEG precipitation. Uncaging experiments were performed by irradiating a PCR tube containing 5 μL of a 0.5 nM solution of protein-containing nanocage for 60 seconds with a handheld UV lamp (UVM-57, 6W, 302 nm). Samples were heated at 40°C for 30 seconds and then imaged by TEM. The extent of uncaging was analyzed using particle counting of the TEM images. The entirety of each TEM image was analyzed to avoid bias.

**Glutamate uncaging.** Glutamate containing nanocages were created following the general cavity functionalization protocol. 5 equivalents of activated glutamate/ oligo handle were used. Reactions were incubated overnight at 40°C and subsequently purified by three rounds of PEG precipitation. For cell testing, the structures were PEG precipitated and dissolved in a modified Tyrode buffer (25 mM HEPES, 119 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 10 mM MgCl₂, pH 7.4), at a final concentration of 180 nM. 9 days old primary rat hippocampal neurons were prepared on 12 mm diameter glass coverslides. The calcium dye Fluo-4 AM (life technologies) was dissolved in DMSO to yield a stock concentration of 2.3 mM. Neurons were loaded for 30 min in Fluo-4 AM at 2.3 μM, diluted in the modified Tyrode buffer at room temperature. Neurons were then
rinsed three times with Tyrode buffer, and incubated at 37C for another 30 min to allow complete de-esterification of intracellular AM esters. Glass coverslides were fractured into smaller pieces (approximately 1 mm²) with a pointed tungsten-carbide glass cutter to limit the use of nanocage reagents. The buffer was wicked off the surface of the fractured glass and replaced with 2 uL glutamate containing nanostructures in Tyrode. Neurons were then placed under a custom microscope with a 10x objective, equipped with a 5W LED (LZ1-00B200, 460 nm; LedEngin, San Jose CA) for excitation, an excitation filter (HQ 470/50), a dichroic mirror (FF506-Di02), an emission filter (FF01-536/40), and imaged with a Hamamatsu camera (C11440-42U) at 20 Hz. After baseline activity was collected for 5 s, the flash lamp (JML-C2, Rapp OptoElectronic GmbH, Hamburg, Germany) was triggered to deliver a light pulse for 1 ms at 240 – 400 nm, and the calcium activities of neurons were measured for another 25 s.

**Calcium signal processing.** All analysis was conducted with MATLAB (MathWorks, Massachusetts, US). Individual neurons were manually identified, and the mean fluorescence intensity averaged for all pixels within each neuron was then further processed to represent individual neuron calcium changes. Due to the saturation effects of the high intensity uncaging flash light that lasted for 6 frames (300 ms) following the light illumination, the fluorescence intensities of these 6 frames were removed and replaced by a linear fit connecting the end values that were not affected by the flash. The fluorescence of each neuron was first baseline subtracted using its linear fit for the 5 second baseline period, and then normalized by the standard deviation of the baseline and smoothed using a built-in function, Smooth, with a moving average filter with the span of
25 frames. The temporal derivative of the signal was calculated and smoothed using the moving average filter with the span of 6 frames. To screen for activated cells, we first calculated the root mean squared error (RMSE) for each 5 second intervals throughout the 30 second recording sessions, and thus 6 RMSE values were calculated. We then used the maximum RMSE of these 6 values to represent the RMSE of each neuron, and obtained the 95% confidence interval of the RMSE for the control group. We then calculated the threshold value for the instantaneous temporal derivative that would correspond to the 95% confidence interval of the RMSE. Cells were deemed as activated when their temporal derivative exceeds the threshold. To determine the onset of calcium responses, we calculated the z score of the fluorescence trace of the activated cells. Onset threshold were set as the first time point of 10 consecutive points in which the temporal derivative values had a z score bigger than 3.
Figure S2. TEM images of unmodified DNA nanocage. Scale bars equal 100 nm (top three images) and 50 nm (remaining six images).
Figure S3. TEM images of streptavidin containing DNA nanocages before (top) and after (bottom) light irradiation. Scale bars equal 50 nm.
Figure S4. TEM images of bovine serum albumin containing DNA nanocages before (top) and after (bottom) light irradiation. Scale bars equal 50 nm.
**Video S1:** Neuronal responses evoked by released glutamatic acid from DNA nanocages. A representative video showing intracellular calcium changes in neurons loaded with calcium indicator Fluo 4, before and after flash lamp illumination in the presence of DNA nanocages containing glutamate. Video is speed up 5 times.

**Video S2:** Control experiments showing lack of neuronal responses upon light illumination in the absence of DNA nanocages. A representative video showing intracellular calcium changes in neurons loaded with calcium indicator Fluo 4, before and after flash lamp illumination, without DNA nanocages. Video is speed up 5 times.

**Staple List:**

Center staples – those which do not come in contact with the edges of the nanostructure

GGATTAGCAATATAAAAAAGCG
AATCGTCATAAATATTCAGAATTTG
CAATAGAAAGGCGACATTAACTGT
CGAAGAAGGCTTTTGAGGAGCACAG
GCCGAATTGCATTGGAAGTGC
TACCTTTTTACATTACAACATACC
ATTATCGGAATTATCATCGTGCCTTA
CAGACGAGCATTGAAGAACCATGAAAC
GAAAAAGAAATCCAATCGCAGCCAGGT
GCATGATCAAGAAAAATTAGATGAATAG
GCTATAATGCAGTACGGATTTGGCAAT
GGGTATACCTACCATATCGAGTT
TCGGCAGCATCGCCATTTAGGACG
TTTGTCAGGCAACAGCTAGAGCAACTG
ATACGTTTATCGAACCAGCTACTGCAT
CCACCACCACCGGAATCCAAAAAGGGTCTTTACCCTGATCCATAA
GAATACACTAACGCAATACATAACCTCTTTTGAATAAAATACCAA
TATTTGCAAGAAGATAAAAACAGCTCGAAGAACCACCTTGCATGC
TTCTGCCGCCTCCCTCAGCCACCACCCTCTCTTTGATAAAATACCAA
AAATCTCGCGTAACGATCTAAAGACAGCTGAGTTTCGTCACCCTAA
ACCAGAAAAATCAACCAAGCAATAAAGCAAACATTTAGCTATGCTG
CGCCACCCAGGAGGTTGCTCCTTTTGATAATTTGCTCATTCCAAATTC
CTATCTTAGCCGAACATGAGGCTGTGGAAAAACGCAACCCGATCA
TGTTTCCAACCTGTCACATAATATCACCAGCAGTGAATATACCC
AATAAATACAAAACCAACCAGTTGAGATTAAGGТГААГТААТААГТТАТ
ACAGTTГАGGAСТCСААСAAАССAGCCTTСССААСАСТТТГГТААТАА
AGAGАGССGСTTGССTTTAGCГАATAGCAGCAGCACCAGTTATTCГГА
CCGACAATATTCCGГТАТТААААССCGCAAAАААТАСССССАГCATCAGCAG
ГТААТАТАТГГТГГГТААТАТГГГТААГГГАААААТГГГАААААГТГГГГГ
ГГТГГГГГГГГГГГГГГГГГГГГГГГГГГГГГГГГГГГГГГГГГГГГГГГГГ
CAAGАAAAATCTACTACAGGTTTGСТТСТАААААГТТТГАСААААСТСГТССТ
AAA

Right edge staples – those which come in contact with the right edge (when visualized in cadnano) of the nanostructure; helix ends contain TT overhangs to prevent nanostructure aggregation

TTGACTACCTTTTTAACCTCC
TTATGAAAGGTCCCGGTGATT
TTTCAATTCCATAAGGCTTTT
TTAACCACCAGAGGCCGAGATT
AGCTTTTCGGGAGAGATAGCGATAGCTTAGATT
TTCCGCTTTTGCGGGATCTGCAGGGACCGATATGAC
TTAACGCGCTGTAGCATTCCACAGTATTCTGCTTT
TTGCCGGAAACCAGGCCACGGCACCGAAGATCGGGG
TTTTGTAACGTTAAATATTAAGCAAAAAAGCCCTATG
TTTACCGAGCTCGAATTCGTAGAATGATAGCCTT
TTTAATGCAATGCTGAGAACCCTTTCAACGATAC
TTCAAGGGCATAAGTGTGCAGGGGATGTGCTGTT
TTCAATCGGCTGCAGCACCAGGCCATTCGCTT
TTGGTTTCCAGTCACGCACTGGGTAAACGCCAGGTT
TACCCCTGTCAAAAGGATTACACCATCAATATGATATT
TTCGCCAGCTGGGCGAAGAAACCTCTTTGCTATTATT
TTGCGATCGGTGCCGCCGTCAACTGTGGGGAAGGTT
TGGCGAGTGCGGGCCGTCAACCTGTTGGGGAAGGTT
TTTACAAAGCTATCGCTATGCGCAAAGAGAGGTATTATC
TTGCAACGGCAAAGATTAGCAGAAAATTAGGCAATAGAAGAC
TTCAAGGATAGAGATACCTTTGAATTGACAGACCGGAAGCAATCGA
TTTACAACTTTATGAGGCTGTCACCCCGGCAAATCTGTGTTAGTT
TTTTTCAACACTAATGTAGCTAGAGCTTAAGAGGTCATTTTTCTT
CCTGATTCAAGGCGAAATGGGCAAGTCCACTATTAAAGAACGTT
TTCTATTAATTATTCTTTAACAATAACCAGAAGAGCTTTCG
TGAAACACATATCGAGAGAAATAAAGGTCATAAAGATTTCAAAGGGTGAAGTT
Left edge staples – those which come in contact with the left edge (when visualized in cadnano) of the nanostructure; helix ends contain TT overhangs to prevent nanostructure aggregation

TTACCACATTCTACGAGGCATT
TTATTTAACGGACCTAAAAACTT
TTAGGAGGTTTAGTACCGCCA
TTGTAGCAACGTAGAAAGGATT
TTTTCTAAGAAATAACATAAAAAATT
TTGCATTTTCGGTCATAATCAAATT
TTACCAACGCTTTACAAATAAATT
TTCTAACAACTTGAGGATTTAGATT
TTCAGTGAGACCTTGAGAGAGTTTT
TTAATCAACAGTTGTTAGGAGCATT
TTCAAGCCATATTATCCCTTTTTATT
TTCTTAAATCAATTTTTTGTTTATT
TTCCGCTGCAAAAAATCTAAAGCTT
TTAGTACAATAATCTTTAGGAATTT
TTGCAAGCAAGCGTTGGAAGGAGGTT
TTACAGAATCAAGTCACCCTCAGTT
TTAGACTGGAATTCGGAACCTATTTT
TTTTGAGCAATAATCAGTAGCGTT
TTTCCGCGCCATTGGCCTTGATATT
TTTAGTAAGAGATAAGTGCCGTCTT
TTCAATAATAACGGGAGCCATTTTT
TTCAAAATGCTTGCTTGAGTAACCTT
TTGAAACGTCACCACCACCAGAGTT
TTAACCGCCACGAAACGCGAGCTCTT
TTTTACGCAGTATGAGGTAAATATT
TTTAGCGAGAGCTCAAGAGAAGGTT
TTGGGTAATTGAAACGCAAAGATT
TTACAACGCCAAGTAATAAGGAGATT
TTCAAGGGAAGCGCAAAAGTCAGATT
TTGAAAGAGGCATCACCCTACTCTT
TTCCAGCGCCAGCGTTTTCATCGT
TTAGTATTAGACTTTGAAAGTTTCTT
TTACCACGGAATATATGGTTTATT
TTTCCTAATTGTTCCTTTCTATT
TTGGCAGGGGAGACTTCTTTCCTTT
TTAGAAAGTAACCGAGGAAACGTT
TTCAAAATTATAATTAAGGAAACTT
TTAACGTCAGGGAAGACAATATT
TTATTTCAATTTCAGAAATATT
TTGGGAAATTAGTTAGCAAGGGCCTT
TTAGTCAAATAATGTAAAGCCCAATT
TTGCCGTGGGTGTTGCGCTCTTT
TTATCCCGGATTTTGATGATACCTT
TTACAACCTAAACTCAGAACCGCCTT
TTTCTGACCTAAATAAGGCGTTTT
TTTAATAAGAGTAAGACTCCTTTATT
TTACGGATTCGCCTCAGAGGCGAATTTT
TTGCCCGCTTTCCAGAATCGGCCAACTT
TTATCACCTTGCTGGGTCAGTTGGCATT
GCCTATTAGCGTCTCTAATAGTAAAATGTTTTT
GGTCAGTTAAACAGTTTACTGAATCCCCCTT
GAGACTCGCTTTTGACGATAAAAAACAAAAATT
TTTTATCCGCCTCACAATTCACACAATGTATAGC
TTATTCTGAACATGAAAGTATTAAGCAACCCCTT
TTTTCAAAAACAAATATTCCTCATACGGCAGGT
TTAGGAGTGATCTGTAATAAAGTTTTTTCAAATGTAT
TTATTTAGGATTACGCCTGTTTTGCTGCTGTAGGCT
TTATGTCGGATCAAAACAGTTAATGCAGATAACGG
TTTGTAACACCCTCATAGTTTACGGGATAGCAAGCCTT
TTCAATAGGAACCGATCTACCCACGGGACGAAATAACTAC
GATAATATCTAAAGGAACATTTAATGTGCGGATGTTGAAATTGTT
CAGCCCCATGAAAATAAGGAACAGATTAGCGGGAGGTTTTGGAAGCTT
AATCCAGGCCCTAAATTTGCCAGAAACGAGCAGCTTTTATCTGAATCTTTT
AATCGCGGATTGCTCAAAATGAACAGTGCGTCAGTATTAACATT
TTACCCTCAGAGCCACCCCTCATTTAAAGGAAACTCAAGGCAACGGAA
TTTCTGAATTTACCGTCTCCAGTAAAGCTAGAAAAGCCAGAATGCTCTC
TTGAGAGGGTTGATATAAGTATAGCCTTTTAGTACCAGGC
TGAAATATCTAACCCTCATAATTGCGCCTAATAAGCATAAAAGTGTAAATT
ATAGGAGAATATTTTACAGAGAGACGCAGGGCAAGGGCTTATCCGATT
CTACCTGAACCTAGCAGATCGCTACGAGCAGAAATAATTATCCCATATT
TTAAATAAGAATAAACACCGGTACATCGATGAATACGTAGATTTTCAGGGTTT
TTATATAAAGTACCGACAAAAGTGTGATAATTGTAATTTTATCATTCTTT
TTCATTTCCAAGAACGGGTATTTTCAGCCACATGTAAGAATCGCCATATTTATT

**Cavity staples** – those which protrude into the cavity of the nanostructure exposing AAAAAAAAAAAAAAAA handles

AACATTTTTAGTATGTGTTAGGATGAAPAABBBBBBBBBBBBBBBB
CGAATAGATAGTGAGTGTGTTTGAATGAAAAABBBBBBBBBBBBBBB
CTAATAGAGCCTGATGAAATAACATGAAAAABBBBBBBBBBBBBBBBB
ACATGGAACAGAGTCTTTTCATAGCCCGAAAAABBBBBBBBBBBBBBB
GACTTTTAGCTAATTTTCATCGAGATAGAAAAABBBBBBBBBBBBBBB
GGGACACACAGGCGATGAAATTCTAGTGGAAAAABBBBBBBBBBBBBBB
ACTCATCGCTAATGCAGATAATTCTCAAAAAABBBBBBBBBBBBBBB
GTCCAGAAACATTCTTACATATTACTACAAAAABBBBBBBBBBBBBBB
TTAAATTTTTTCATCGAGAATGGACAGTCGCCGAAAAABBBBBBBBBBBBB
ACGAGTAGCAGACAGAATGACTTCGTAACAGAAAAABBBBBBBBBBBBB
GGCGTATCTCAGCTGGAGAATCTCACTCGCCAGAAGATTCAAGAAAAABBBBBBBBBBBBB
AAAAA
ATCAAAAACCCAGGCAGCAGATGATGCTCATTCCAGAACAABBBBBBBBBBBBBBB
AAAAAA
CCTTTATGCTCAGACTGTAGCAAGACAAAAATTTCAAGTTTATGAAAAABBBBBBBBBBBBBBB
AAAAA
TTGCCGGAGACAGTCAAATCAGATTGTATTGTTAAAATTACGAAAAAAAAAAAAAA

Cavity binding oligo

/5Hexynyl/TTTTTTTTTTTTTTT

Loop binding oligo

/5Alex647N/TGAGTAGAAGAACTCAAACTATCGGCCTTGCTGGTAATAT

References:


