**Enzyme-operated DNA-based nanodevices**
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**Supporting information**

**Reagents:**
Reagent-grade chemicals, including disodium phosphate, monosodium phosphate, citric acid, boric acid, tris-(2-carboxyethyl) phosphine hydrochloride, sodium chloride, magnesium chloride (all from Sigma-Aldrich, St Louis, Missouri) were used without further purifications. HPLC purified oligonucleotides modified with a pH-insensitive fluorophore (Quasar 670) and a quencher (Black Hole Quencher 2, BHQ2) were purchased from Biosearch Technologies (Risskov, Denmark) and employed without further purification. HPLC purified oligonucleotides modified with Cy3 or Cy5 were purchased from IBA (Gottingen, Germany) and employed without further purification. HPLC purified oligonucleotides modified with Quasar 570 and Quasar 670 were purchased from Biosearch Technologies (Risskov, Denmark) and employed without further purification.

In this work three different systems were employed. The following oligos modified and non-modified were used for each system:

1) **pH-dependent nanoswitch** (see Figure 2 and S1):
Optically-labeled pH-dependent nanoswitch:
5’-GGAGGGGAG-GTT(BHQ-2)A-CCTCCCCTCC-CTTG-CCTCCCCCTCC (Quasar670)-3’

2) **Enzyme-triggered toehold-mediated strand displacement** (see Figure 3 and S6):
Clamp-like triplex forming strand:
5’-TTCCTTTCTCCT-TCTTTT-AACTA-TTTCT-TCCTTTTCTT-GTTACATTGCACACT -3’
Output:
5’-AAGGAAAGAGGA-AGAAAA -3’
Invader:
5’-AGTGTGCAATGTAC-AAGGAAAGAG -3’
Reporters:
F: 5’- (Cy3) AAGGAAAGAGGA -3’
Q: 5’- TTTTCT-TCCCTTTCCCTT (Cy5) -3’

3) Enzyme-driven ligand load/release (see Figure 4 and S8):

Molecular Beacon:
5’- TTCCTT-TTTTT-TTCCTT-T(Quasar570)TTGGCTAGAG-AAGGAA -3’

Target used for load/release experiment:
5’- CTCTAGCCAAA(Quasar670)-3’

For all the sequences above the underlined bases represent the duplex portion, bold bases represent the triplex-forming domain.

Buffer conditions. All oligonucleotides were suspended to a final concentration of 100 µM and stored in phosphate buffer 50 mM at a concentration of 100 µM, pH 7 at -20°C (for DNA triplex switch) or in 0.01 M Tris + 0.01 M MgCl$_2$, pH 7, at -20 °C (for both the strand displacement and ligand-releasing systems).

Substrate preparation. All the substrate of the enzymes (GSH, Urea and Acetylthiocholine) were suspended in water, except for the CDNB, that was suspended in ethanol. The pH of the solutions was adjusted with the addition of HCl or NaOH to have GSH at pH 8.0, Urea at pH 5.0 and Acetylthiocholine at pH 8.0.

Fluorescence measurements. All fluorescence measurements were obtained using a Cary Eclipse Fluorometer (Varian) at 25°C. For each system the following procedures were used:

1) Triplex nanoswitch. All measurements were obtained with an excitation at 647 nm (± 5 nm) and emission at 665 nm (± 5 nm). We used a universal buffer prepared following the method reported elsewhere (Ostling and Virtama, 1946). Kinetics experiments shown in Figure 2A were performed using a concentration of switch of 10 nM in a universal citrate/phosphate/borate buffer (0.33 mM, 0.50 mM, 0.50 mM, respectively) + 2 mM MgCl$_2$ + 0.05 M NaCl, pH 7.8 in the presence of GST (2 µg/ml) and CDNB (1.00 mM). After a stable baseline was obtained (10-20 minutes), the enzymatic substrate GSH was added at the selected concentration (from 0.05 mM to 1.00 mM) and the fluorescence signal was followed continuously until stabilization.
Kinetics experiments shown in Figure 2B were performed using a concentration of switch of 10 nM in a universal citrate/phosphate/borate buffer (0.33 mM, 0.50 mM, 0.50 mM, respectively) + 2 mM MgCl₂ + 0.05 M NaCl, pH 5.0 in the presence of urease (0.15 mg/ml). After a stable baseline was obtained (10-20 minutes), the enzymatic substrate urea was added at the selected concentrations (from 0.2 mM to 1.0 mM) and the fluorescence signal was followed continuously until stabilization.

In the open/close cycles experiment (Figure 2C) a universal citrate/phosphate/borate buffer (0.33 mM, 0.50 mM, 0.50 mM, respectively) + 2 mM MgCl₂ + 0.05 M NaCl, pH 7.8 containing GST (2 µg/ml), CDNB (2 mM) and urease (0.15 mg/ml) was used. After a stable baseline was reached the enzymatic substrate GSH was added at a concentration of 2 mM and after 45 minutes the second substrate (urea) was added at a concentration of 1 mM.

2) Enzyme-triggered toehold-mediated strand displacement. All measurements were obtained with an excitation at 548 nm (± 5 nm) and emission at 563 nm (± 5 nm). Strand displacement experiments were performed using a concentration of reporter (R) of 30 nM and a concentration of Target complex of 10 nM in 0.01 M Tris buffer + 0.01 M MgCl₂, pH 5.0, in the presence of urease (0.15 mg/ml). After a stable baseline was achieved the Invader was added (Figure 3B). Again, after a new stabilization of the signal baseline the enzymatic substrate urea was added at the selected concentrations (from 0.2 mM to 5.0 mM) (Figura S7). The signal increase of Cy3 was followed for 30 minutes after substrate addition.

3) Enzyme-driven target load/release. All measurements were obtained with an excitation at 548 nm (± 5 nm) and emission at 565 nm (± 5 nm). Ligand-releasing kinetics experiments (Figure S9) were performed using a concentration of 10 nM for the molecular beacon and of 100 nM of ligand, in a phosphate buffer solution (phosphate 0.01 mM + 10 mM MgCl₂ pH 8.0) containing Acetylcholinesterase (0.03 µg/ml). The enzymatic substrate Acetylthiocholine was added, after a stable baseline, at the selected concentrations (from 0.1 mM to 0.8 mM).

Ligand-loading kinetics experiments (Figure S10) were performed using a concentration of 10 nM for the molecular beacon and of 100 nM of ligand, in a phosphate buffer solution (phosphate 0.01 mM + 10 mM MgCl₂ pH 5) containing urease (0.15 mg/ml). The enzymatic substrate urea was added, after a stable baseline, at the selected concentrations (from 0.01 mM to 0.20 mM).

In both cases the signal change of Quasar 570 was followed for 10 minutes after substrate addition.

In the load/release cycles experiment (Figure S11) a phosphate buffer solution (phosphate 0.01 mM + 10 mM MgCl₂ pH 5) containing both urease (0.15 mg/ml) and Acetylcholinesterase (0.03 µg/ml) was used. After a stable baseline was reached the enzymatic substrate, urea, was added at a concentration of
0.3 mM and after 10 minutes the second substrate, Acetylthiocholine, was added at a concentration of 5 mM. Finally urea was added again, after 10 minutes, at a concentration of 1.0 mM.

References:
(1) Östling, S.; Virtama, P. Acta Phys. Scandinav. 1946, 11, 289.
Figure S1. **(Left)** pH-dependent nanoswitch. The DNA-based triplex pH-triggered nanoswitch is designed to form an intramolecular triplex structure through the formation of a Watson–Crick (dashed) pH-insensitive hairpin and a second Hoogsteen (dots) pH-sensitive hairpin. Because they require the protonation of the N3 of cytosine in the third strand, CGC triplets in this nanoswitch are only stable at acidic pHs (average pKa of protonated cytosines in triplex structure is ≈6.5). It is thus possible to open-close such nanoswitch at different pHs. To follow opening/closing of the triplex portion of the switch, we labeled it with a fluorophore/quencher pair. More specifically, a fluorophore is conjugated at one end of the DNA sequence, and a quencher is internally inserted in the loop of the hairpin duplex DNA so that the triplex-to-duplex transition (unfolding) brings the fluorophore away from the quencher and increases the fluorescence signal observed. We note that the fluorophore used in this work (Quasar 670) is insensitive to pH over a wide pH window. **(Right)** Shown is the pH-titration curve of the triplex nanoswitch (10 nM) in a universal citrate/phosphate/borate buffer (0.33 mM, 0.50 mM, 0.50 mM, respectively) + 2 mM MgCl₂ + 0.05 M NaCl.
Figure S2. Enzyme-driven closing of a DNA nanoswitch. We demonstrate the use of an enzyme (Glutathione transferase, GST) that in the presence of its specific substrate (GSH) can decrease the pH of the solution thus leading to the closing of the nanoswitch. Here we show the degree of nanoswitch’s closing as a function of GSH concentration. Here fluorescence measurements were obtained in a solution of nanoswitch (10 nM) in a universal citrate/phosphate/borate buffer (0.33 mM, 0.50 mM, 0.50 mM, respectively) + 2 mM MgCl₂ + 0.05 M NaCl at 25 °C, pH 7.8, in the presence of GST (2 μg/ml).
Figure S3. We demonstrate that by changing the enzyme’s concentration (Glutathione transferase, GST) and using a fixed concentration of the substrates (GSH and CDNB), we can control the rate of the nanoswitch’s closing. By doing so we can rationally control the half-time ($t_{1/2}$) of nanoswitch’s closing (from 14 to 27 min changing the concentration of the enzyme from 0.5 to 0.3 mg/ml). The nanoswitch’s closing is followed by fluorescence measurements obtained in a solution of switch (10 nM) in a universal citrate/phosphate/borate buffer (0.33 mM, 0.50 mM, 0.50 mM, respectively) + 2 mM MgCl₂ + 0.05 M NaCl at 25 °C, pH 7.8 containing the indicated concentration of GST. GSH and CDNB were added at a concentration of 1 mM after 5 minutes.
**Figure S4.** Enzyme-driven opening of a DNA nanoswitch. We demonstrate here that an enzyme (urease) in presence of its specific substrate (urea) can lead to the opening of a DNA-nanoswitch. Here we show the degree of nanoswitch’s opening as a function of urea concentration. Fluorescence measurements were obtained in a solution of pH-nanoswitch (10 nM) in a universal citrate/phosphate/borate buffer (0.33 mM, 0.50 mM, 0.50 mM, respectively) + 2 mM MgCl$_2$ + 0.05 M NaCl at 25 °C, pH 5, in the presence of urease (0.15 mg/ml).
Figure S5. We can control the rate of the nanoswitch’s opening by changing the enzyme’s concentration (urease) and using a fixed concentration of the substrate (urea, 1 mM). By changing the concentration of the enzyme (from 0.06 to 0.12 mg/ml) we can rationally control the half-time ($t_{1/2}$) of nanoswitch’s opening (from 13 to 43 min respectively). The nanoswitch’s opening is followed by fluorescence measurements obtained in a solution of switch (10 nM) in a universal citrate/phosphate/borate buffer (0.33 mM, 0.50 mM, 0.50 mM, respectively) + 2 mM MgCl$_2$ + 0.05 M NaCl at 25 °C, pH 5 containing the indicated concentration of urease. Urea was added at a concentration of 1 mM after a stable baseline was obtained (where indicated by the arrow).
**Figure S6.** The OH⁻-activated strand-displacement system used in this work. In this system we have used a clamp-like DNA strand that, under acidic pHs, forms a triplex inactive complex (Target complex \( \text{OFF} \)) with the strand to be released (output). The additional Hoogsteen interactions in this triplex structures provide an increased stabilization to the complex that prevents strand-displacement upon Invader addition. At basic pHs, the destabilization of the Hoogsteen interactions leads to an active complex (Target complex \( \text{ON} \)) characterized by a simple duplex conformation. Because this structure is not stabilized anymore by Hoogsteen interactions, it can undergo displacement through a classic toehold-exchange mechanism. In this study the progress of strand displacement is always followed using an optically labeled reporter complex (R) that stoichiometrically reacts with the released strand (Output) to produce an unquenched fluorophore-labeled single strand DNA molecule (F). We note that the reaction between the reporter complex (R) and the released strand (Output) is not sensitive to pH in the pH range we have investigated and does not directly take part to the strand displacement reaction. We also note that the fluorophore used in this system is insensitive to pH.
Figure S7. Enzyme-driven activation of the strand-displacement process. We demonstrate that we can trigger the displacement process with an enzymatic reaction and we can finely modulate this process by varying the concentration of enzymatic substrate (i.e. urea) from 0.2 to 5.0 mM in a solution containing a fixed concentration of the enzyme urease (0.15 mg/ml). By doing so we can rationally control the release of the output strand. The strand displacement system is followed by fluorescence measurements obtained in a solution of target complex of 10 nM in the presence of the reporter (R) (see cartoon in Figure S6) at a concentration of 30 nM in a 0.01 M Tris buffer + 0.01 M MgCl₂, at 25 °C, pH 5.0. The invader (30 nM) and urea were added at the times indicated in the figure.
Figure S8. The pH-controlled DNA receptor we have used in this work (Figure 4) can act as a DNA-based nanomachine that, through pH changes, can reversibly load and release its target in a controlled fashion. To follow load/release of the ligand (purple strand) we labeled the ligand strand with a quencher and the DNA-nanomachine with a fluorophore. The binding of the target (load) is thus associated with a decrease of fluorescence signal while its release results in an increase in fluorescence signal (see Figure 4B-E). While at acidic pHs the triplex stem of this DNA-nanomachine is highly stable, it completely unfolds to a simple duplex stem at higher pHs. Because the additional interactions of the triplex structure will make the stem (and thus the non-binding state) more stable and difficult to be opened by the target, we observe a poorer affinity for the target at acidic pHs. An enzymatically induced pH change of the solution will thus allow to achieve a reversible loading and release of the ligand strand from the DNA nanomachine (Figure 4, S11).
Figure S9. In the first strategy (enzyme-driven ligand release) we demonstrate that the enzymatic reaction catalyzed by AchE can be used to release a ligand strand previously hybridized to the molecular beacon (Figure 4B). Here we demonstrate that we can finely modulate the amount of ligand released from the molecular beacon by gradually varying the concentration of the enzyme’s substrate (i.e. acetylthiocholine) from 0.1 to 0.8 mM in the presence of a fixed concentration of acetylcholinesterase (0.03 μg/ml). Here the release of the ligand strand is followed by fluorescence measurements obtained in a solution containing the molecular beacon (10 nM) and its complementary ligand (100 nM) in a 0.01 mM Phosphate buffer + 0.01 M MgCl₂, at 25 °C, pH 7.8. The acetylthiocholine was added at the time indicated in the figure.
Figure S10. In the second strategy (enzyme-driven ligand loaded) we demonstrate that the enzymatic reaction catalyzed by Urease can be used to bind a ligand strand (Figure 4E). Here we demonstrate that we can finely modulate the amount of ligand bound to the molecular beacon by gradually varying the concentration of the enzyme’s substrate (i.e. urea) from 0.01 to 0.20 mM in the presence of urease (0.15 mg/ml). Here the loaded of the ligand strand is followed by fluorescence measurements obtained in a solution containing of molecular beacon (10 nM) and its complementary ligand (100 nM) in a 0.01 mM phosphate buffer + 0.01 M MgCl₂, at 25 °C, pH 5.0. The urea was added at the time indicated in the figure.
Figure S11. We also demonstrate a fast and reversible possibility to cyclically load and release the ligand to the DNA receptor by using both enzymes by sequentially adding urea (loading), acetylthiocholine (releasing) and urea again (loading). Here the reversible loading/releasing of the ligand strand is followed by fluorescence measurements obtained in a solution containing of molecular beacon (10 nM) and its complementary ligand (100 nM) in a 0.01 mM Phosphate buffer + 0.01 M MgCl₂, at 25 °C, pH 5.0. Urease and AchE were used at a concentration of 0.15 mg/ml and 0.03 µg/ml respectively. The two substrates (urea and acetylthiocholine) were added at the time indicated in the figure.