Assembling enzymatic cascade pathways inside virus-based nanocompartments using dual-tasking nucleic acid tags

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S1. DNA functionalization of GOx, GCK and GOx-GCK formation by DNA hybridization

The functionalization of GOx and GCK with single-stranded DNA was performed using adapted methods described in the literature.[1-3] In the first step, the outer lysine groups of GOx or GCK were functionalized with a bifunctional sulfo-EMCS linker containing a succinamide moiety and a maleimide-moiety. Thiol-modified DNA was added to the functionalized enzyme to obtain DNA coupled enzymes. GOx and GCK contain 15 and 13 lysine residues, respectively (per monomer). The number of DNA strands per enzyme was determined using the ratio between $\lambda = 260$ nm and $\lambda = 280$ nm absorbance of the free DNA and EMCS-linked enzyme using their respective extinction coefficients. In the case of GOx this resulted in an average of 15 – 20 ssDNA strands on each enzyme (in total there are 30 solvent-exposed lysines for GOx). The extinction coefficient of the enzyme-ssDNA was determined for each batch. The molecular masses were estimated to be 189 kDa (GOx–ssDNA), 160 kDa (non-functionalized GOx), 7.3 kDa (ssDNA), 71.8 kDa (GCK–csDNA), 43.4 kDa (non-functionalized GCK), 7.1 kDa csDNA. For both GOx and GCK, the excess DNA was effectively removed by spin-filtration. For GOx-ssDNA, a guanine-rich DNA sequence was chosen (Fig. S1A), which in the presence of hemin self-assembles into a supramolecular structure with horseradish peroxidase (HRP) like catalytic activity. The catalytically active DNAzyme has been used extensively in the past as a HRP mimic.[4] After DNA coupling, the excess DNA was removed by repeated washing using centrifugal filtration. For csDNA-GCK, a complementary DNA sequence (csDNA) to the DNAzyme was chosen (Fig. S1B). Upon mixing and hybridization of GOx-ssDNA and csDNA-GCK, the GOx-GCK can be formed (Fig. S1C).[5]

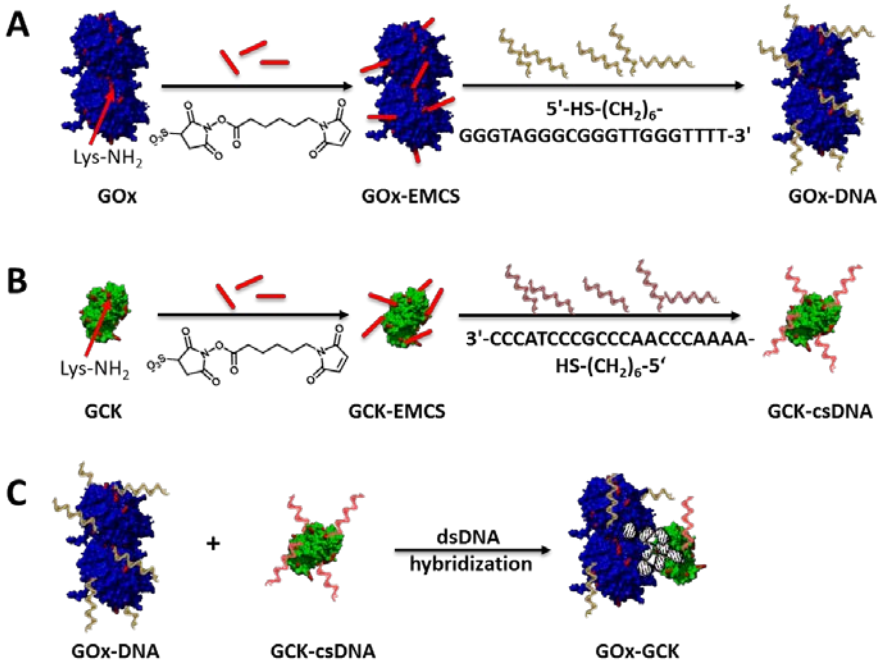
Preparation of enzyme-DNA hybrids

Preparation of GOx-ssDNA. 100 µM ssDNA (the catalytically inactive form of the DNAzyme) was dissolved in 10 mM PBS buffer containing 2 mM DTT (pH 7.4). The solution was incubated for 3 hr at RT to reduce any disulfide groups before the excess DTT was removed by repeated wash and buffer exchange steps using Amicon Ultra centrifugal filters (10 kDa MWCO). The eluent was tested for the presence of DTT using 100 µM Ni(II)-sulfate. For the preparation of the modified GOx, a solution containing 2 µM GOx and 100 µM sulfo EMCS in 10 mM PBS (pH 7.4) was

incubated for 1 hr at RT. The excess of sulfo EMCS was removed by buffer exchange against PBS buffer using Amicon Ultra centrifugal filters (30kDa MWCO). Modified GOx (2 µM) was mixed with 100 µM thiolated-DNA in a 2:1 (v/v) ratio and incubated for 1 hr at RT. The excess of DNA was removed by continuous washing and buffer exchange steps against PBS buffer using Amicon Ultra centrifugal filters (30 kDa MWCO). The eluent was tested for the presence of DNA by monitoring the absorbance at $\lambda = 260$ nm. Before the GOx-DNA was used for further experiments, the PBS buffer was exchanged with 50 mM Tris-HCl buffer, 100 mM NaCl, 10 mM MgCl2, 0.2 mM DTT, pH 7.5.

Preparation of GCK-csDNA. GCK-csDNA was prepared using the same procedure as described above for the GOx ssDNA hybrid. A solution of GCK (1 mL) and 100 µM sulfo-EMCS in 10 mM PBS (pH 7.4) was incubated for 1 hr at RT before the excess sulfo-EMCS was removed by buffer exchange against PBS buffer using Amicon Ultra centrifugal filters (30 kDa MWCO). Modified GCK was mixed with (reduced) thiolated-DNA (100 µM) in a 2:1 (v/v) ratio and incubated for 1 hr at RT. The excess of DNA was removed by repeated wash and buffer exchange steps against PBS buffer using Amicon Ultra centrifugal filters (30 kDa MWCO). The eluent was tested for the presence of DNA by monitoring the absorbance at $\lambda = 260$ nm. Before the csDNA-GCK was used for encapsulation or kinetic experiments, the PBS buffer was exchange with 50 mM Tris-HCl buffer, 100 mM NaCl, 10 mM $MgCl_2$, 0.2 mM DTT, pH 7.5.

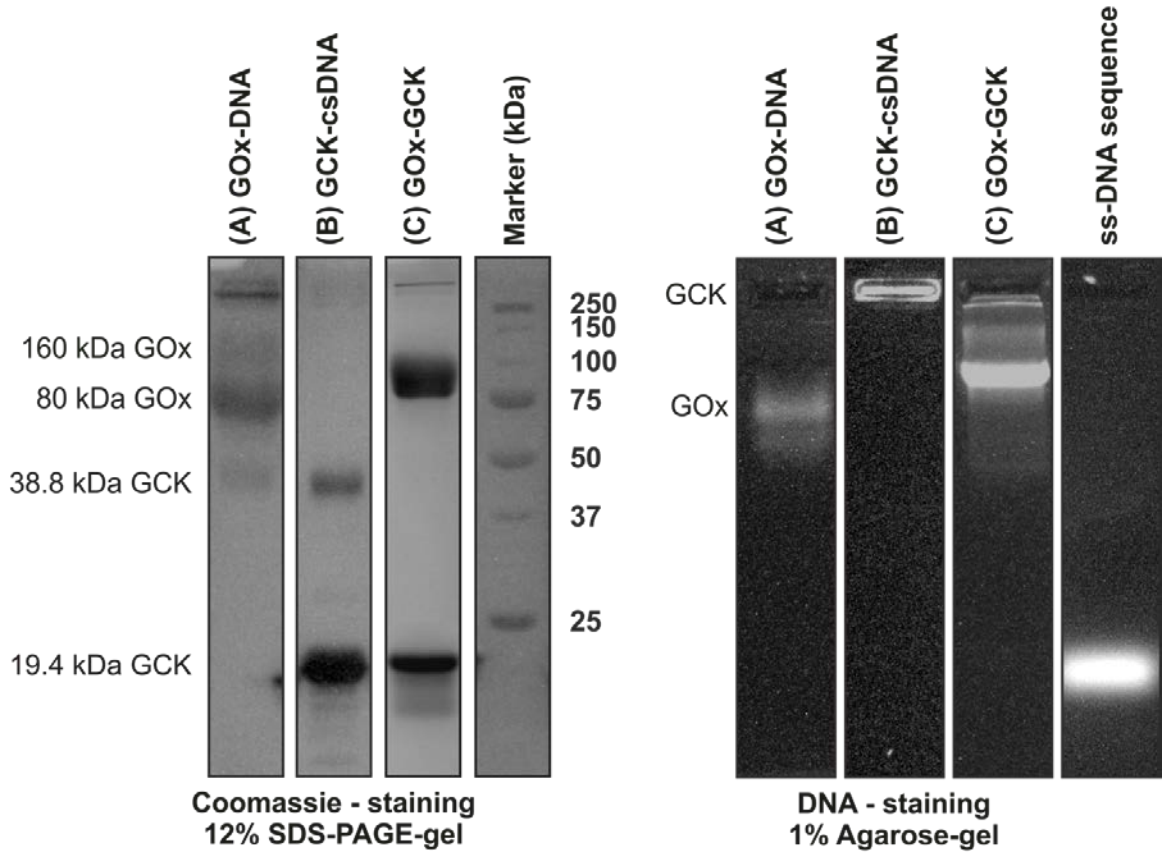
Preparation of GOx-GCK. Purified GOx-ssDNA and csDNA-GCK, both in PBS buffer, were mixed in a 1:1 (v/v) ratio and incubated at 27°C for 1 hr and subsequently cooled down to 20°C to induce hybridization between the two complementary DNA strands. The excess csDNA-GCK was removed by repeated wash steps using Amicon Ultra centrifugal filters (50 kDa MWCO). The eluent was tested for the presence of csDNA-GCK or non-functionalized GCK by monitoring the absorbance at $\lambda = 280$ nm. The excess ssDNA-GOx was removed by size-exclusion chromatography. Before the GOx-GCK complex was used for encapsulation or kinetic experiments, the PBS buffer was exchange against Tris-HCl buffer (50 mM, NaCl (100 mM), $MgCl_2$ (10 mM), DTT (0.2 mM), pH 7.5).



Supplementary Figure S1. Schematic representation of the reaction scheme for the covalent coupling of (A) ssDNA to GOx leading to the formation of GOx-ssDNA, (B) the complementary csDNA to GCK leading to the formation of csDNA-GCK. (C) DNA hybridization of the two functionalized enzymes, GOx-ssDNA and csDNA-GCK leads to the formation of GOx-GCK.

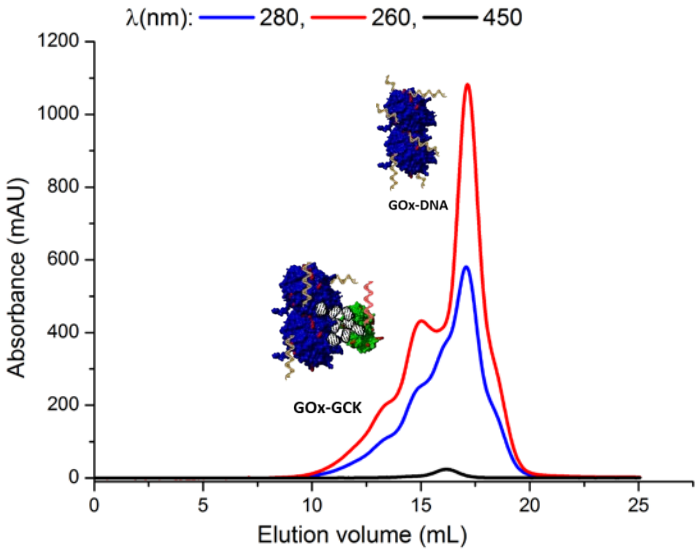
S2. Purification and characterization of GOx-ssDNA, csDNA-GCK and GOx-GCK

The coupling of the single-stranded DNA to the appropriate enzyme was confirmed by denaturing SDS-PAGE analysis (Fig. S2). From the SDS-PAGE (Fig. S2 (left), lanes A, B, C), the first lane (A) shows three bands for GOx-ssDNA, which corresponds to the GOx dimer (160 kDa), the GOx monomer (80 kDa) and a third, unknown band at 40 kDa. The second lane (B) confirms the presence of the GCK dimer (38.8 kDa) and GCK monomer (19.4 kDa). Finally, the third lane (C) shows two bands, corresponding to GOx and GCK. However, since Coomassie staining does not allow for the visualization of DNA, a 1% agarose gel was prepared to confirm that the enzymes are functionalized with DNA. Fig. S2 (right, lanes A, B, C) shows bands corresponding to both monomeric and dimeric functionalized GOx-ssDNA, csDNA-GCK and the hybridized GOx-GCK (as indicated by the presence of both GOx-ssDNA and csDNA-GCK), respectively. The presence of two bands suggests an unequal hybridization between GOx and GCK.



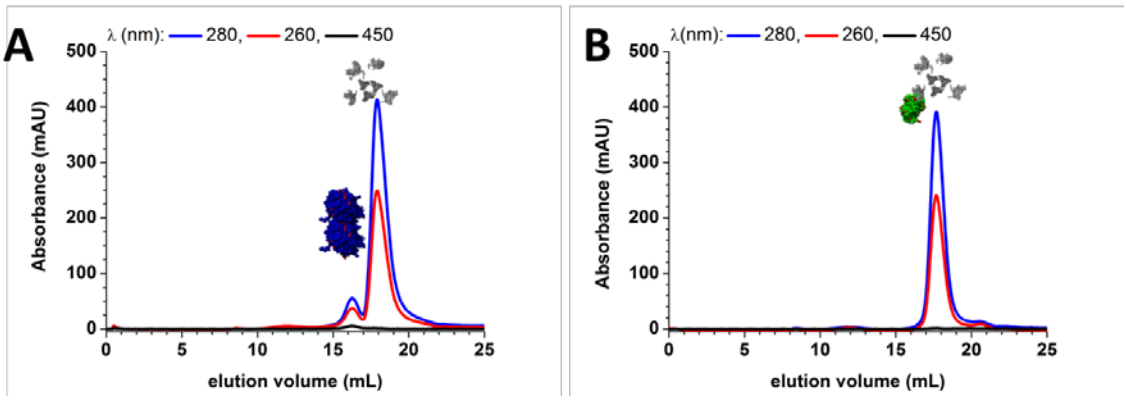
Supplementary Figure S2. Gel analysis of the DNA-functionalized enzymes used in this study. (left) SDS-PAGE analysis showing (A) GOx-ssDNA, (B) GCK-csDNA, (C) GOx-GCK and (right) the corresponding agarose gel for the detection of ssDNA and csDNA.

S3. Purification of GOx-GCK



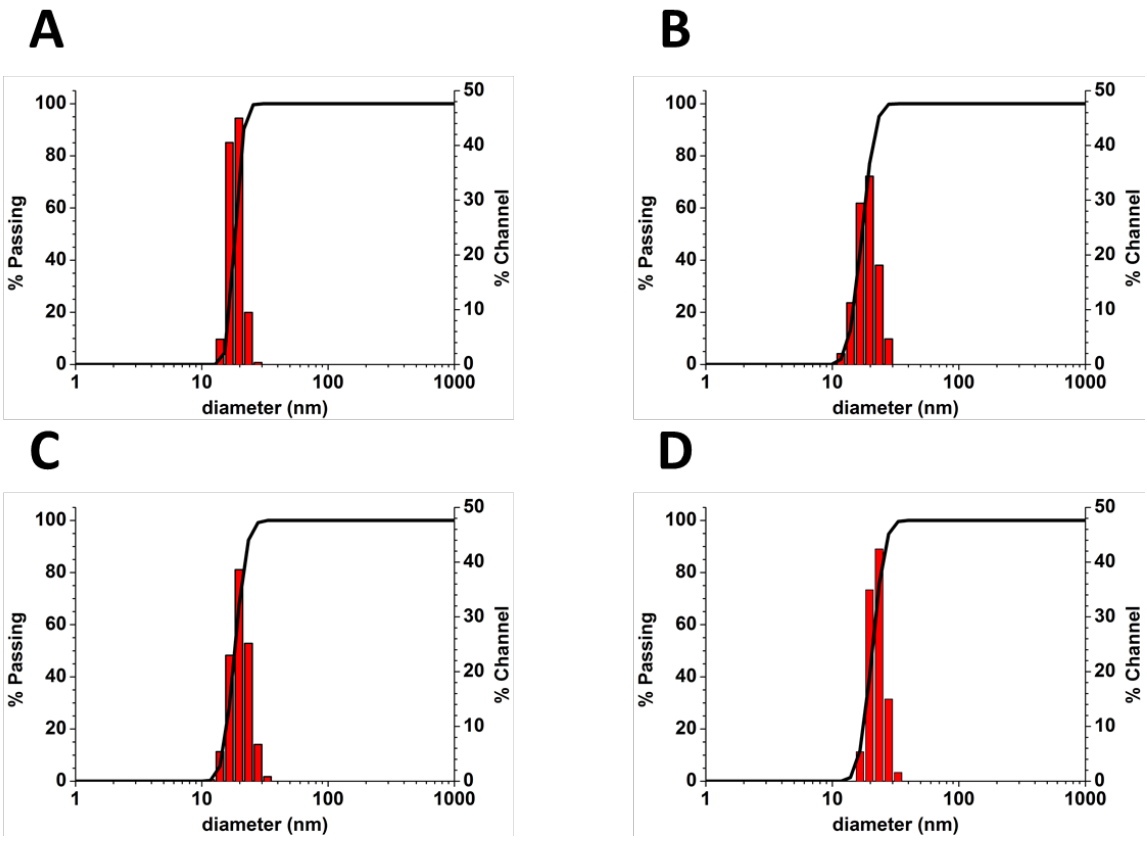
Supplementary Figure S3. FPLC-chromatogram of hybridized GOx-GCK via ssDNA-csDNA interaction, monitored at λ = 260 (red), 280 (blue) and 450 nm (black), for DNA, CCMV and flavin (GOx), respectively. The elution peaks at earlier fractions (10-15 mL) correspond to hybridized GOx-GCK forms as non-hybridized GOx eluted at around 16 mL. The earlier fractions were collected for encapsulation.

S4. Control experiments for non-functionalized enzymes



Supplementary Figure S4. FPLC-chromatogram of non-functionalized GOx or GCK incubated with CCMV-CP at pH 7.5, monitored at λ = 260 (red), 280 (blue) and 450 nm (black), for DNA, CCMV and flavin (GOx), respectively. (A) Non-encapsulated GOx elutes at 16 mL and non-encapsulated CCMV-CP at 18 mL. (B) Non-encapsulated GCK and CCMV-CP both elute at 18 mL.

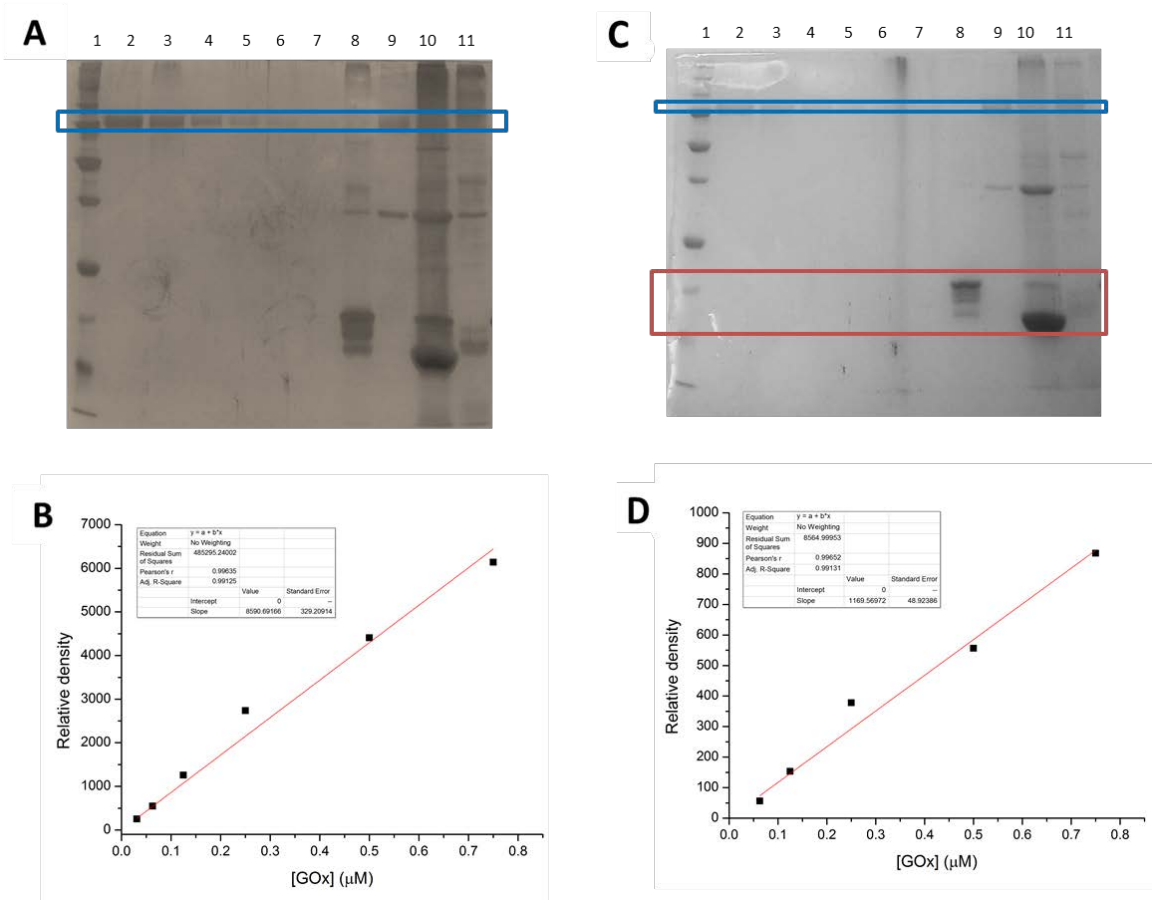
S5. Hydrodynamic size of enzyme(s)-encapsulating virus-like particles



Supplementary Figure S5. Dynamic light scattering (DLS) of encapsulated (A) ssDNA, (B) GOx-ssDNA, (C) csDNA-GCK and (D) GOx-GCK inside CCMV virus-like capsids. An average particle size of 19 ± 5 nm, 19 ± 3 nm, 20 ± 4 nm, 20 ± 4 nm was observed, respectively.

S6. Densitometry analysis

To determine the concentration of GOx-ssDNA (either encapsulated in CCMV or in bulk solution), SDS-PAGE densitometry analysis was performed (Fig. S6). The relative intensity of the protein bands was compared against a calibrated standard curve of known GOx concentrations run identically with all samples in the same gel. The Coomassie-stained gel was used to visualize the GOx and GCK bands in GOx-GCK filled VLPs and non-encapsulated GOx-GCK (Fig. S6C-D). Based on the non-encapsulated system, the relative ratio of GOx:GCK was determined to be 1:1.48, indicating a mixture of 1:1 and 1:2 GOx-GCK. To optimize the visualization of GOx band in GOx-ssDNA filled VLPs, the same gel was subsequently silver-stained (Fig. S6A-B). The average number of GOx per VLP was determined based on the relative density of GOx band and the capsid subunit band resulting in the ratio of 1:63.8, indicating that 1 VLP (T=1 particles, 60 subunits) can only accommodate 1 GOx.



Supplementary Figure S6. Estimation of overall GOx and GCK concentrations and the average number of enzymes within an VLP by gel densitometry. (A) Silver stained SDS-PAGE showing lane (1) Protein marker, (2-7) GOx standards, (8) GOx-ssDNA filled VLPs, (9) non-encapsulated GOx-ssDNA, (10) GOx-GCK filled VLPs, (11) non-encapsulated GOx-GCK; (B) Corresponding calibration densities for known concentrations of GOx as obtained and determined from the silver-stained SDS-PAGE. (C) Coomassie stained SDS-PAGE[6] showing lane (1) Protein marker, (2-7) GOx standards, (8) GOx-ssDNA filled VLPs, (9) non-encapsulated GOx-ssDNA, (10) GOx-GCK filled VLPs, (11) non-encapsulated GOx-GCK; (D) Corresponding calibration densities for known concentrations of GOx determined from the Coomassie-stained SDS-PAGE. The ratio of GOx:GCK was determined based on the relative densities of the GOx band and that of the GCK band in non-encapsulated GOx-GCK, whereas the ratio of GOx:CCMV capsid subunit was determined based on the relative densities of the GOx band and the CCMV capsid band in encapsulated GOx-ssDNA sample.[7] It should be noted that additional protein bands at molecular weights below 20 kDa are assigned to degradation of the CCMV N-terminus, which has been observed in the past.[8-9]

To confirm the number of enzymes encapsulated inside one T=1 capsid, the exterior of the native CCMV virus was first modified with a Pacific blue NHS ester (Thermo Fischer). The dye to virus

mass ratio was 0.075 and the reaction mixture was incubated for 1 hr in PBS pH 7.4. The RNA and non-bound dye were subsequently removed from the virus particles by dialysis. The absorbance at $\lambda = 280$ and $\lambda = 410$ nm was measured to determine the degree of labeling (DOL), using equation (1) to (3). A DOL of 0.2 (dyes/capsid protein) was used for subsequent encapsulation of the enzyme, which was found to have the optimal reassembly characteristics without any aggregation occurring. The correction factor (CF_{260}) of the CP+dye combination was experimentally determined for each batch.

The dye-labeled capsid proteins were used to encapsulate the enzymes. Following the usual purification using size-exclusion chromatography, the resultant peak was further examined with UV-Visible spectrophotometry. The CP content was determined based on the absorbance at $\lambda =$ 410 nm (equation 4). The concentration of encapsulated GOx-ssDNAwas calculated based on equation (5). Finally, using equation (6), the number of enzymes/capsid can be determined. The calculations resulted in 1.2 of GOx per capsid, considering that the capsid particles are all T=1 (i.e. 60 subunit as confirmed by cryo-EM).

Dye-labeled capsid protein

$$[dye] = A_{410\ Measured} / \varepsilon_{410dye} \quad (1)$$

$$[CP] = (A_{280Measured} - CF_{280dye} * A_{410\ measured}) / \varepsilon_{280\ CP} \quad (2)$$

$$DOL = [dye]/[CP] \quad (3)$$

Enzyme/Capsid

$$[CP_{assembled}] = A_{410\ Measured} / (\varepsilon_{410dye} * DOL) \quad (4)$$

$$[Enzyme\text{-}ssDNA_{encapsulated}] = (A260 - CF_{260CP\text{-}dye} * A_{410\ measured}) / \varepsilon_{260\ enzyme\text{-}ssDNA} \quad (5)$$

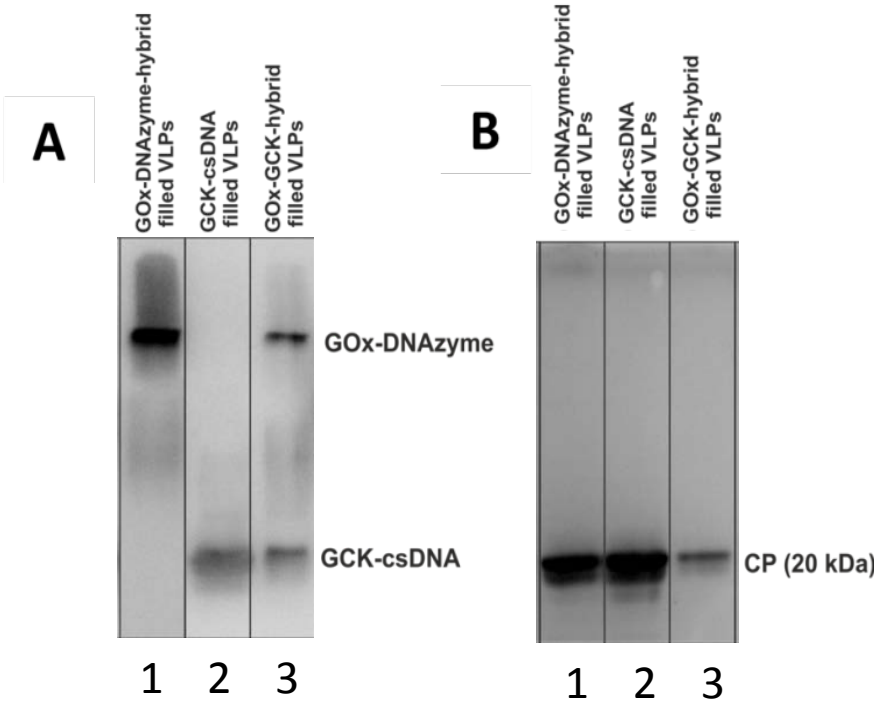
$$Enzyme/T=1\ Capsid = [Enzyme\text{-}ssDNA_{encapsulated}] * 60 / [CP_{assembled}] \quad (6)$$

CF_{280} = Correction factor at 280 from pacific blue dye = 0.20.
ε = extinction coefficient, $\varepsilon_{260\ DNA} = 201500\ M^{-1}cm^{-1}$, $\varepsilon_{280\ DNA} = 114915\ M^{-1}cm^{-1}$, $\varepsilon_{280\ CP} = 24075\ M^{-1}cm^{-1}$, $\varepsilon_{280\ GOx} = 267200\ M^{-1}cm^{-1}$, ε_{410} pacific blue dye = $48000\ M^{-1}cm^{-1}$.

S7. Western blot analysis

To further confirm the encapsulation, the GOx-ssDNA, GCK-ssDNA, and GOx-GCK in CCMV VLPs were analyzed by western blot analysis combined with immunostaining (Fig. S7A). For the immunostaining, the GOx and GCK primary antibodies were detected by chemiluminescence using a HRP-linked secondary antibody. Fig. S7A, lane (1) and lane (2) show single bands corresponding to GOx-ssDNA and GCK-csDNA, respectively, individually encapsulated in CCMV. Lane (3) confirmed the co-encapsulation of GOx and GCK indicated by the presence of two bands. After protein transfer, the gel was subsequently Coomassie stained (Fig. S7B). The result revealed only a single band at 20 kDa, corresponding to non-transferred monomeric CCMV capsid subunit (CP).

The absence of GOx and GCK bands in the Coomassie-stained gel after the transfer indicated a successful transfer for both enzymes.

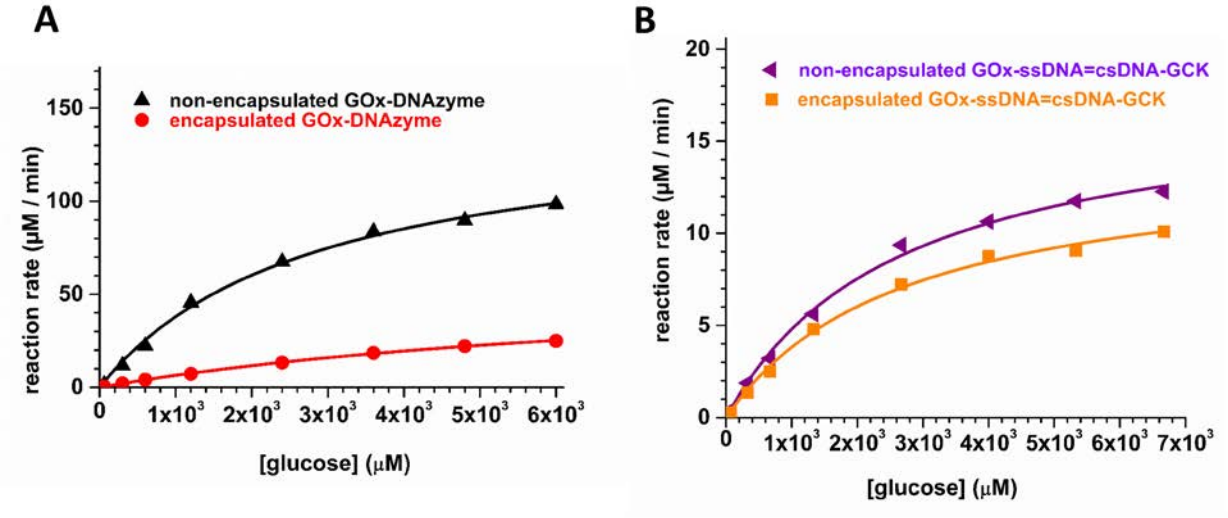


Supplementary Figure S7. Western blot analysis and immunostaining of GOx-GCK filled CCMV VLPs. (A) The GOx and GCK primary antibodies were detected by chemiluminescence using a HRP-linked secondary antibody. (B) Coomassie-stained gel after protein transfer for Western blot analysis, showing the bands of non-transferred capsid subunit of CCMV (CP). The absence of GOx and GCK bands in the gel after the transfer indicated a successful transfer for both enzymes.

S8. Kinetic profiles and kinetic parameters of the cascade pathways

Two cascade systems inside the virus-like particles with UV-visible spectroscopy were monitored. In the first confined cascade system, the catalytic activity of GOx–DNAzyme was monitored via the production of $ABTS^{+\bullet}$ at λ = 410 nm upon addition of glucose to the system. The obtained activity plots for both free non-encapsulated GOx–DNAzyme (in the bulk solution) and encapsulated GOx–DNAzyme show that both GOx and DNAzyme remain catalytically active after encapsulation (Fig. S8A). Notably, a direct comparison between the two systems cannot be made due to the difference in GOx concentrations (the free system contains a higher GOx concentration than the encapsulated system). The concentration of the GOx was 0.25 μM for non-encapsulated system and 0.04 μM for encapsulated system. For both systems, the concentration of the nucleic acid tags (and therefore the DNAzyme) was kept to 30 ng/mL. The encapsulated system is likely

to contain excess nucleic acid tags (not attached to the enzymes), which are also encapsulated by the CCMV capsids due to their negative charges. This explains why the ratio of the nucleic acid to GOx is higher for the encapsulated system than non-encapsulated system.



Supplementary Figure S8. Kinetic investigation of the cascade pathways. (A) Kinetic measurements for the production of ABTS radical (cascade system I) at different glucose concentration for non-encapsulated system (black) and encapsulated system (red). (B) Kinetic measurements for the production of NADPH (cascade system II) at different glucose concentration for non-encapsulated system (purple) and encapsulated system (orange).

In the second cascade system consisting of GOx, GCK and 6-PGDH, we monitored the formation of the end product, NADPH at λ = 340 nm upon addition of glucose to the system. As shown in Fig. S8B, we found that the reaction rate for encapsulated system is slightly lower than non-encapsulated system as well. We attribute this difference to the different concentrations of the enzymes in the two systems. The concentrations of the GOx and GCK were 0.021 μM and 0.024 μM, respectively for non-encapsulated system and 0.007 μM and 0.010 μM, respectively for encapsulated system. For both systems, the concentration of the nucleic acid tags was kept to 10 ng/mL. Based on these concentrations, the encapsulated system is likely to contain excess nucleic acid tags (not attached to the enzymes), which were also encapsulated by the CCMV capsids due to their negative charges. This species cannot be further removed because they were confined together with the enzymes inside the virus-like particles.

The kinetic parameters for both systems were calculated based on Michaelis-Menten equation (7).

$$\mathbf{v_0} = \frac{\mathbf{V_{max,app}} \cdot [\mathbf{S}]}{\mathbf{K_{m,app}} + [\mathbf{S}]} \qquad (7)$$

The turnover number (kcat, app) was obtained by dividing the Vmax, app by the concentration of the first enzyme in the cascade (i.e. the GOx) determined by densitometry. Although the characterization with densitometry inherently comes with approximately 20% error, as the concentration estimation was performed as a relative ratio in one gel for all samples and hence all samples were treated in the exact same fashion, the inherent error would have been the same for each relative values that we obtained.

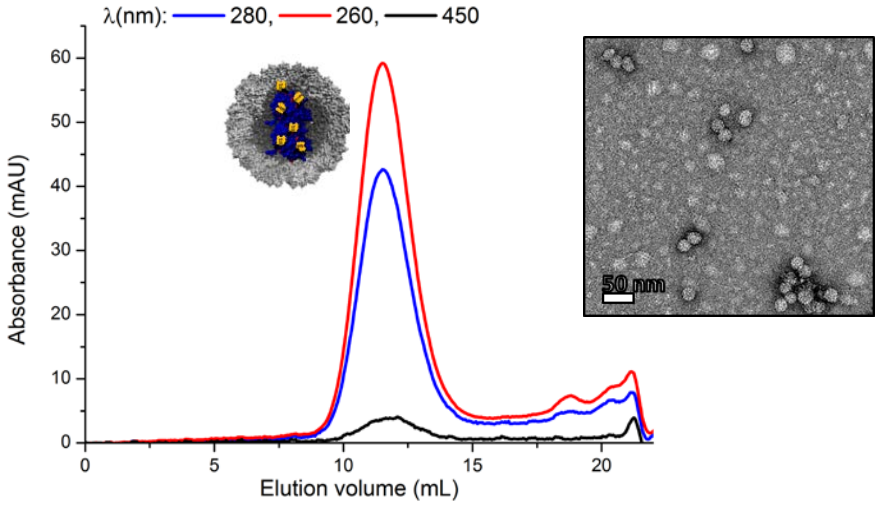
The obtained kinetic parameters are summarized in Table S1.

Supplementary Table S1. Kinetic parameters of the cascade pathways

Cascade system I	Km, app (mM)	kcat, app (min^{-1})	[GOx] (μM)	[DNAzyme] (ng/mL)
Non-encapsulated	0.6 ± 0.1	23 ± 1	0.25	30
Encapsulated	1.3 ± 0.2	40 ± 3	0.04	30
Cascade system II	Km, app (mM)	kcat, app (min^{-1})	[GOx] (μM)	[GCK] (μM)
Non-encapsulated	2.8 ± 0.3	4971 ± 286	0.021	0.024
Encapsulated	2.5 ± 0.4	10853 ± 735	0.007	0.010

S9. Competing catalase pathway and particles stability after kinetic assay

Stock solutions containing both substrates of glucose (ranging from 0–1 M) and 4 mM ABTS/10 μM catalase were prepared. Individual solutions containing either (1) free GOx-DNAzyme (30 μL) and hemin (30 μL, 5 μM), or (2) encapsulated GOx-DNAzyme (30 μL) and hemin (30 μL, 5 μM) were prepared and incubated at RT for 2 hr. The kinetic reactions were performed under the exact same conditions as described above, monitoring at $\lambda = 410$ nm in 100 sec time intervals over 2 hr. At this wavelength, the consumption of H_2O_2 is spectroscopically silent since catalase does not absorb at $\lambda = 410$ nm and the DNAzyme can no longer convert the ABTS to $ABTS^{+\bullet}$. In order to inactivate the catalase, the pH of the assay solution was decreased after 1 hr to pH 3–4, upon addition of gluconic acid (2 M, pH 2–3). After the inactivation of catalase the conversion of ABTS to $ABTS^{+\bullet}$ was observed and the reaction was continued for an additional 2 hrs. The stability of the particles after the assay is characterized with size-exclusion chromatography and transmission electron microscopy (Fig. S9).



Supplementary Figure S9. The GOx-DNAzyme filled VLPs remain stable upon lowering the buffer to pH 4 during catalase inactivation assay confirmed by FPLC-chromatogram of GOx-ssDNA filled VLPs after catalase inactivation assay, monitored at λ = 260 (red), 280 (blue) and 450 nm (black), for DNA, CCMV and flavin (GOx), respectively, and based on negatively stained transmission electron microscopy.

References

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5. It should be noted that DNAzyme is enzymatically inactive when hybridized.
6. It should be noted that the SDS-PAGE gel shown are the same gel, initially stained with Coomassie dye (Fig. S7C) and subsequently silver stained (Fig. S7A).
7. All band intensities and relative densities were determined using ImageJ analysis software. It should be noted that the GOx:GCK ratios were determined from the Coomassie stained gel since the silver-stained gel shown in (A) shows enhanced intensity in the relative bands which led to large errors.
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