Electronic Supplementary Information

Kreb Cycle Metabolon Formation: Metabolite Concentration Gradient Enhanced Compartmentation of Sequential Enzymes

Fei Wu, Lindsey N. Pelster, and Shelley D. Minteer*

Department of Chemistry, University of Utah. 315 S 1400 E, Salt Lake City, UT 84112

Corresponding E-mail: minteer@chem.utah.edu

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Materials:

Pig heart mitochondrial malate dehydrogenase (mMDH), citrate synthase (CS), and all chemicals were purchased from Sigma-Aldrich unless otherwise noted. AlexaFluor® 488 and AlexaFluor® 555 for enzyme labeling was from Invitrogen. PDMS was purchased as the Silicone Elastomer Kit from Corning.

Enzyme labeling:

mMDH and CS were separately labeled by AlexaFluor® 488 and AlexaFluor® 555 as previously described with slight modifications.[1] About 1 mg of mMDH or CS were cleaned up by a pre-packed Sephadex™ G-25M column (GE Healthcare) to remove ammonium sulfate and other salts. After concentrating to about 2 mg/mL, enzymes were mixed with one vial of dyes (as purchased) dissolved in 50 μL of DMSO (prepared fresh just before use) in 50 mM sodium phosphate buffer (pH 8.3). Mixtures of enzymes and fluorophores was incubated in dark at room temperature for at least 1 h under shaking. After labeling, excessive fluorophores were removed by an overnight dialysis of mixture in a dialysis cassette with the mass cutoff at 10 kDa (Thermo Scientific) against 2 L of Tris-HCl buffer (50 mM, pH 7.4) at 4 C, followed by cleaning through the pre-packed Sephadex™ G-25M column again and multiple centrifugations in Amicon® centrifugal filters (10 kDa, Merck Millipore) at 5000 x g for 15 min until polarization of labeled enzymes was stabilized at about 0.3. Final fluorophore:enzyme molar ratio was determined to be approximately 1:1.

Sequential enzyme activity assay:

Reaction rate of citrate production from L-malate was measured in 100 mM potassium phosphate buffer (pH 7.4) containing 100 nM mMDH, 100 nM CS, 5 mM NAD, 0.2 mM acetyl-CoA, 0.4 mM DTNB and L-malate at 0, 0.5, 1.0, 2.5, 5.0, 10, 25, and 100 mM. Absorbance increase of DTNB was monitored every 1 sec over 2 min in a UV-Vis spectrophotometer (Revolution 260 Bio, Thermo Scientific). K_m values were determined by fitting the plot of reaction rate versus L-malate concentration to

a Michaelis-Menten curve. The citrate inhibition effect was tested in the same assay cocktail by adding in 10 mM citrate. ATP inhibition of mMDH was done in the same assay cocktail by adding in 25 mM ATP, and absorbance increase of NADH was monitored every 1 sec over 2 min.

Microfluidic channel fabrication:

The silicon master pattern for the microfluidic channel was fabricated with SU-8 Negative Photoresist (Microchem) to a height of 100 μm and a width of 240 μm according to the soft lithography protocol described by Xia and Whitesides.[2] An additional pattern was made close to the channel pattern at 400 μm from the start of channel to mark the position where the fluorescence would be measured. PDMS (pre-mixed with curing agent and degased) was poured onto the master and cured at 75 C for 2 h. PDMS was then cut and sealed by a microscopic glass slide after cleaning in a plasma cleaner (PDC-32G, Harrick Plasma). All chips were equilibrated overnight after sealing.

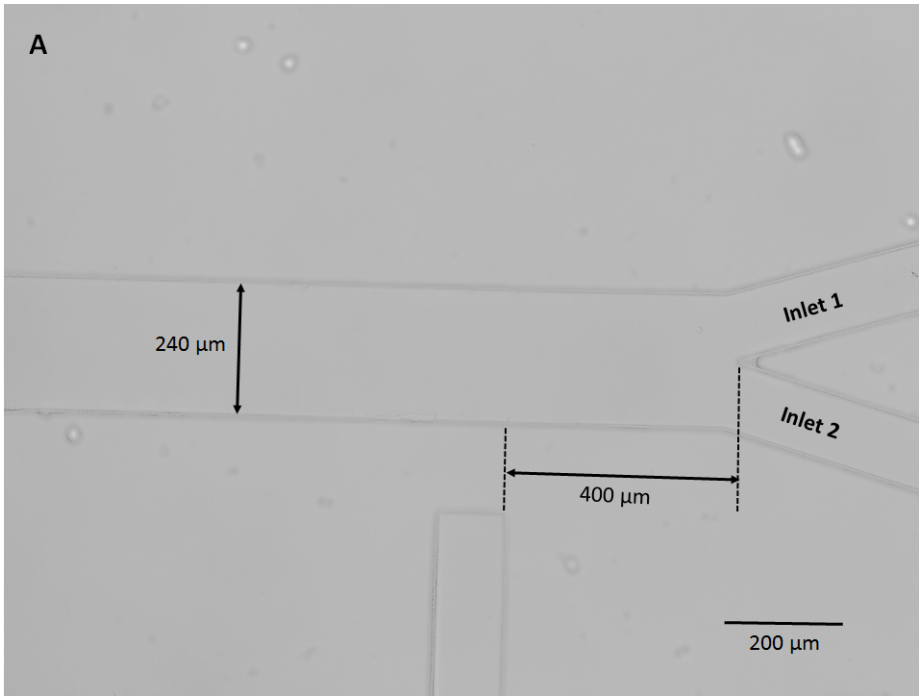
Fluorescent imaging setup:

Microdialysis tubing (Intech Laboratories) were used to connect the inlets of PDMS chip to a dual syringe pump (Harvard Apparatus) and the outlet to a beaker for solution waste. All solutions were filtered through syringe filters (0.2 μm, Corning) to remove particles and degassed for at least 15 min right before the experiment. The PDMS channel was filled with 30% isopropanol and then test buffer to avoid bubble generation. Flow (500 nL/min) in the chip was monitored by an inverted microscope (Olympus IX71). Using a FITC filter cube (excitation: 467-498 nm; emission: 513-556 nm) and a TRITC filter cube (excitation: 532-554 nm; emission: 570-613 nm), fluorescence of AlexaFluor® 488-labeled mMDH and AlexaFluor® 555-labeled CS was recorded simultaneously. Buffered solutions containing substrates and cofactors were injected through Inlet 1. Buffered solutions containing 100 nM AlexaFluor® 488-labeled mMDH or CS were injected through Inlet 2. The observed plane was adjusted to the middle of channel between the glass and PDMS plates to avoid any possible wall effects.

Calculation of t:

Since the dual syringe pump infusing rate was set at 0.5 μL/min, the total volumetric flow rate in the channel was 1 μL/min. Knowing that the channel is 240 μm in width and 100 μm in height, we calculated the cross-section area to be 2.4×10^4 μm^2. By dividing the total volumetric flow rate by cross-section area, the flow velocity was determined to be 694.4 μm/s. With $t_0 = 0$ s when the two flows met, total diffusion time (t) for enzyme molecules at 400 μm from the start of channel was calculated by 400 μm ÷ 694.4 μm/s = 0.576 s.

Supplemental figures:



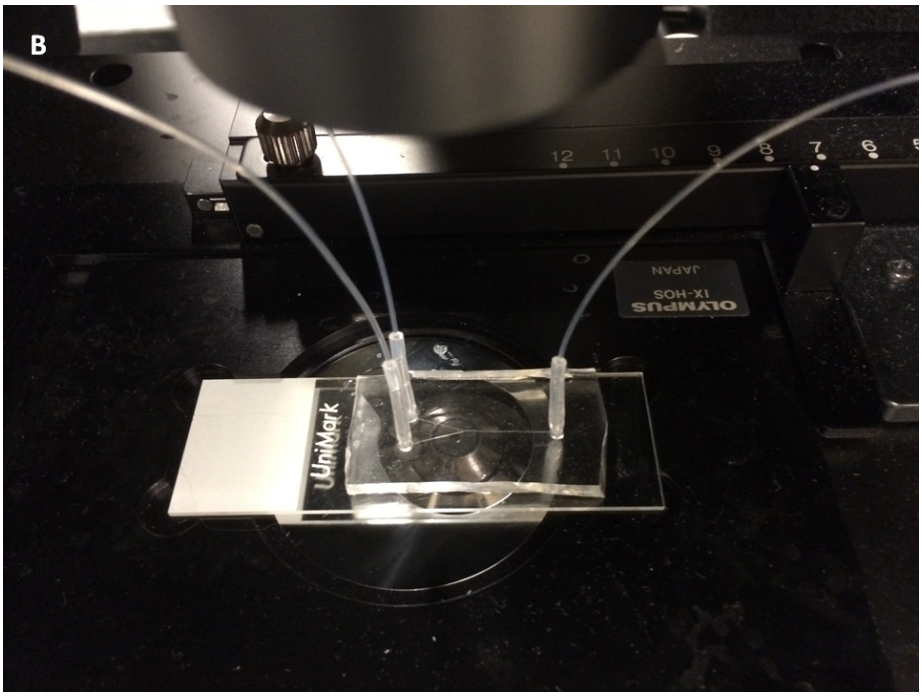


Fig. S1 Images of the microfluidic channel under microscope (**A**) and the actual chip with micro-tubing (**B**).

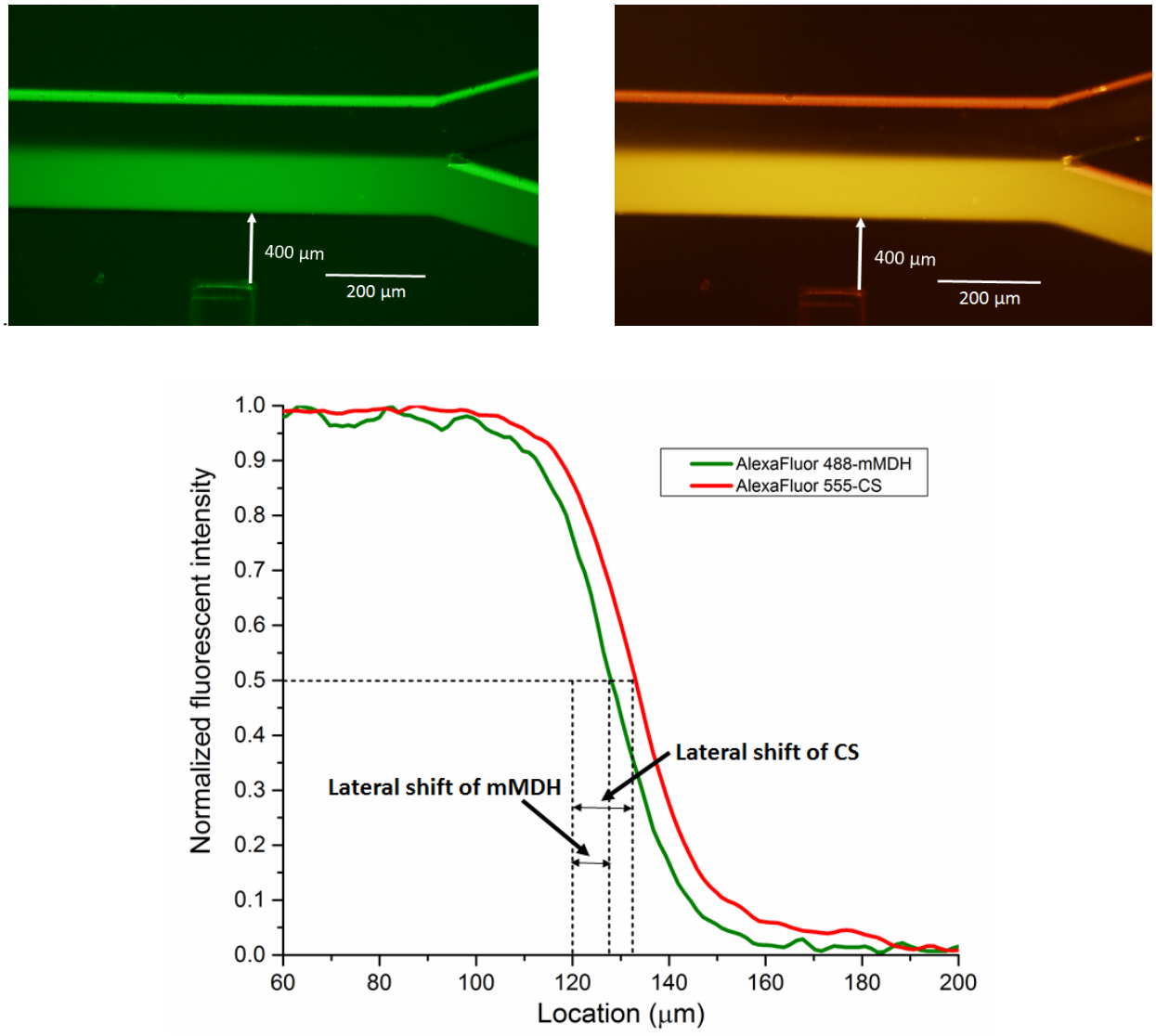


Fig. S2 Fluorescent images of AlexaFluor® 488-labeled mMDH (top left) and AlexaFluor® 555-labeled CS (top right). Normalized intensity profiles are shown at bottom.

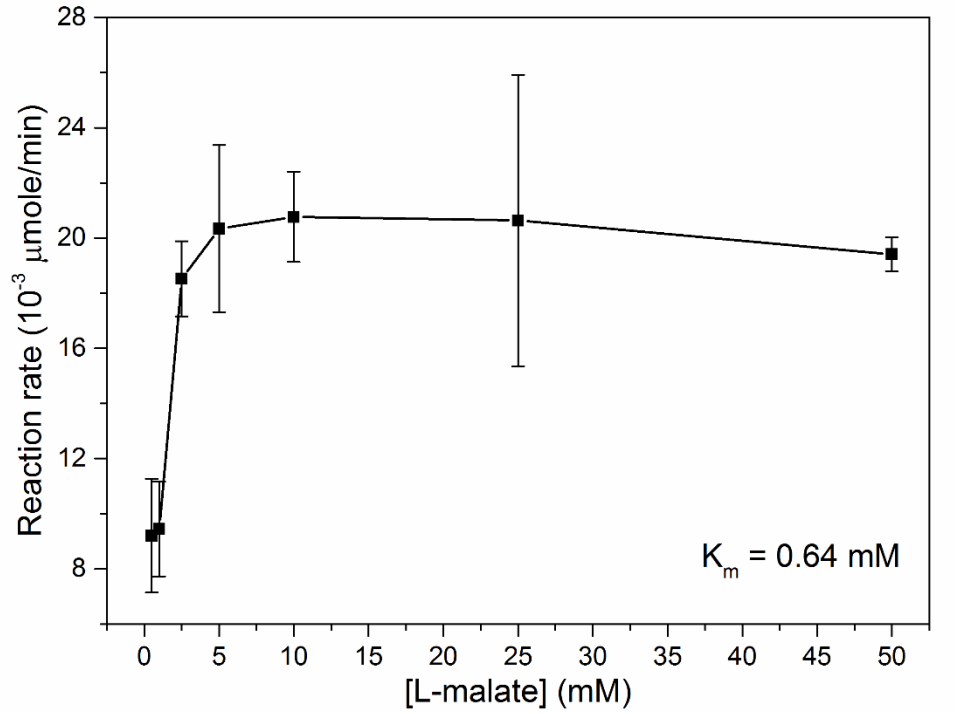


Fig. S3 Sequential activity assay of mMDH-CS.

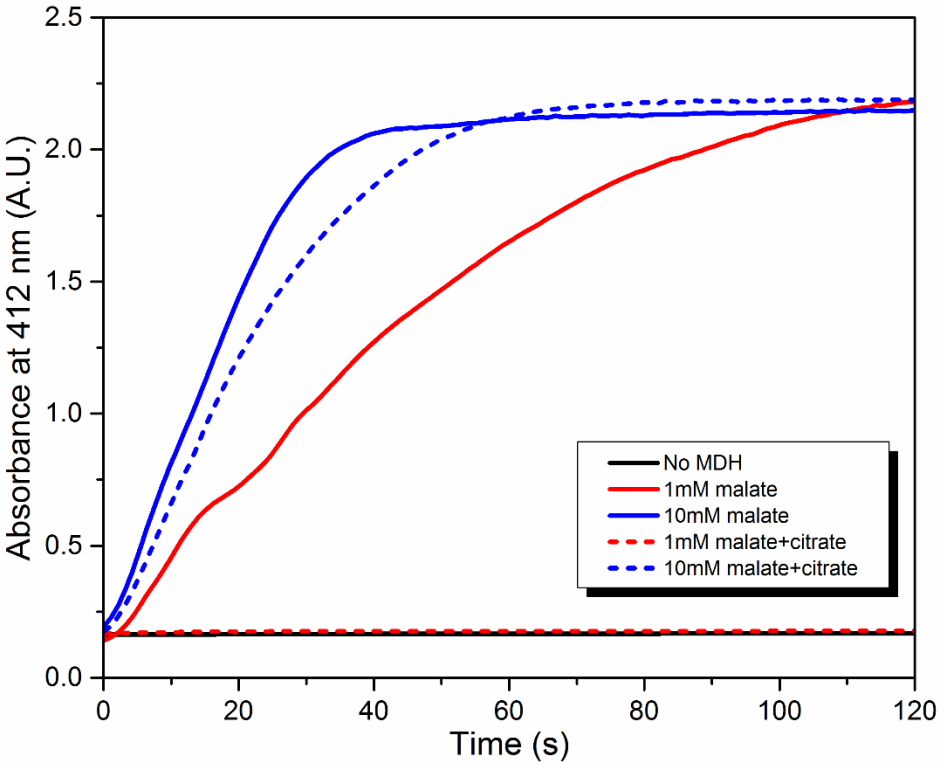


Fig. S4 Sequential activity of mMDH-CS in the presence of 10 mM citrate.

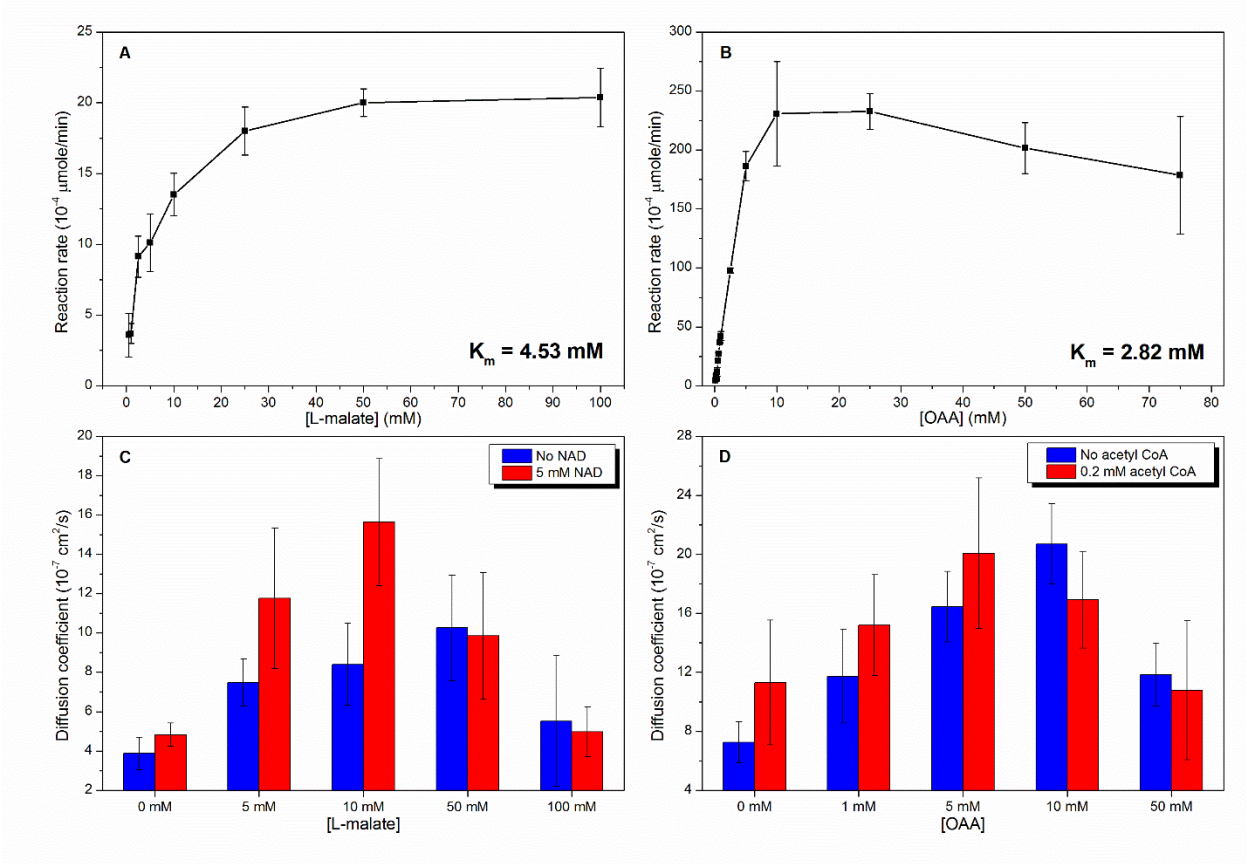


Fig. S5 Single enzyme activity and apparent diffusion coefficients. **A**: mMDH assay. **B**: CS assay. **C**: Diffusion of mMDH (100 nM) against L-malate with or without NAD^+. **D**: Diffusion of CS (100 nM) against OAA with or without acetyl CoA.

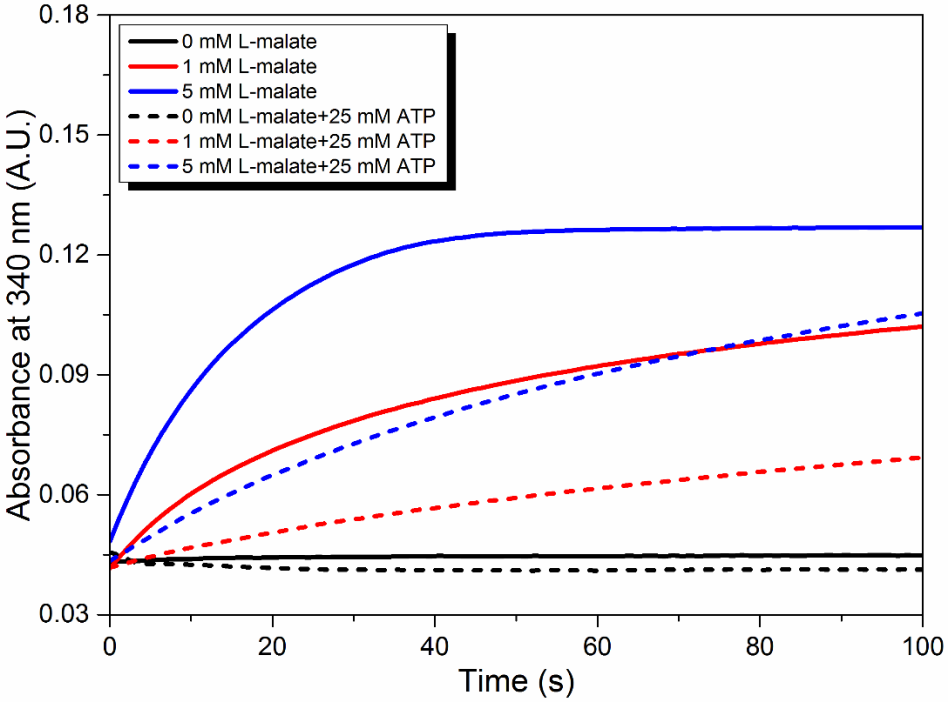


Fig. S6 ATP inhibition of mMDH in sequential assay of mMDH-CS.

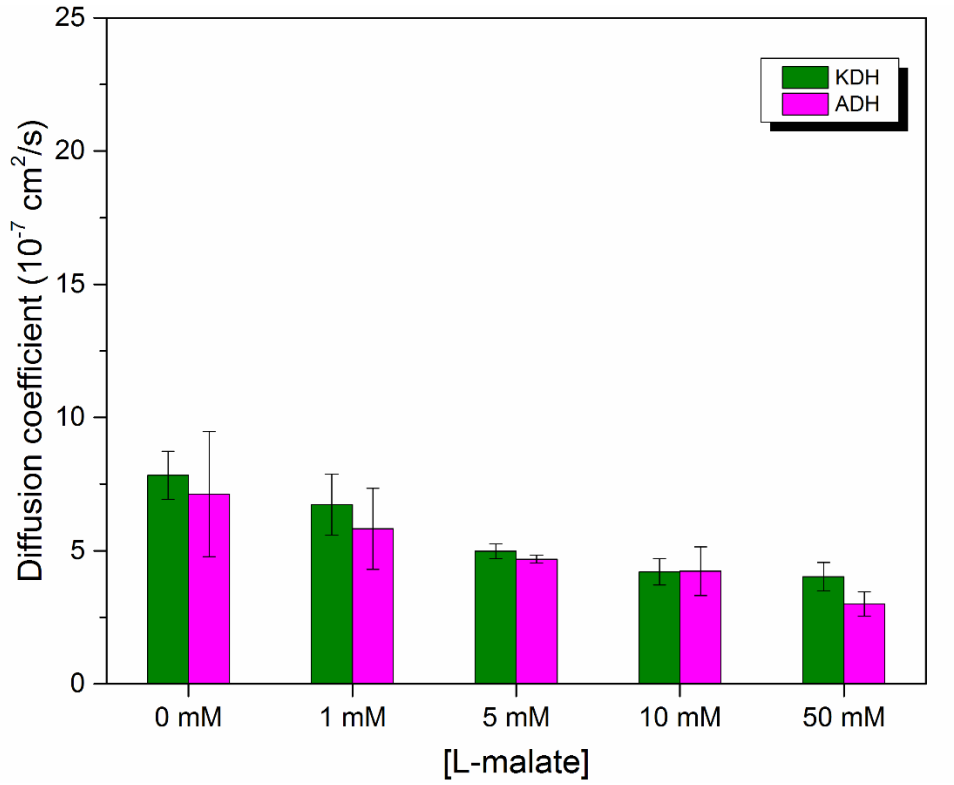


Fig. S7 Apparent diffusion coefficients of KDH (100 nM) and ADH (100 nM) measured with L-malate and 5 mM NAD^+.

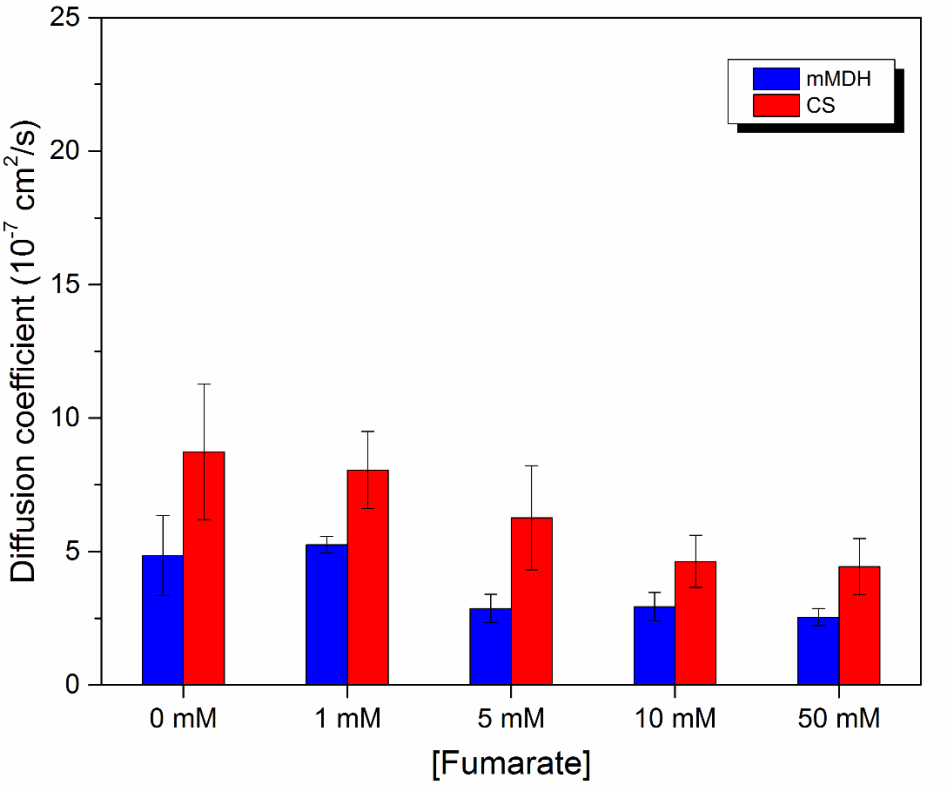


Fig. S8 Apparent diffusion coefficient of mMDH (100 nM) and CS (100 nM) measured with fumarate, 5 mM NAD^+ and 0.2 mM acetyl CoA.

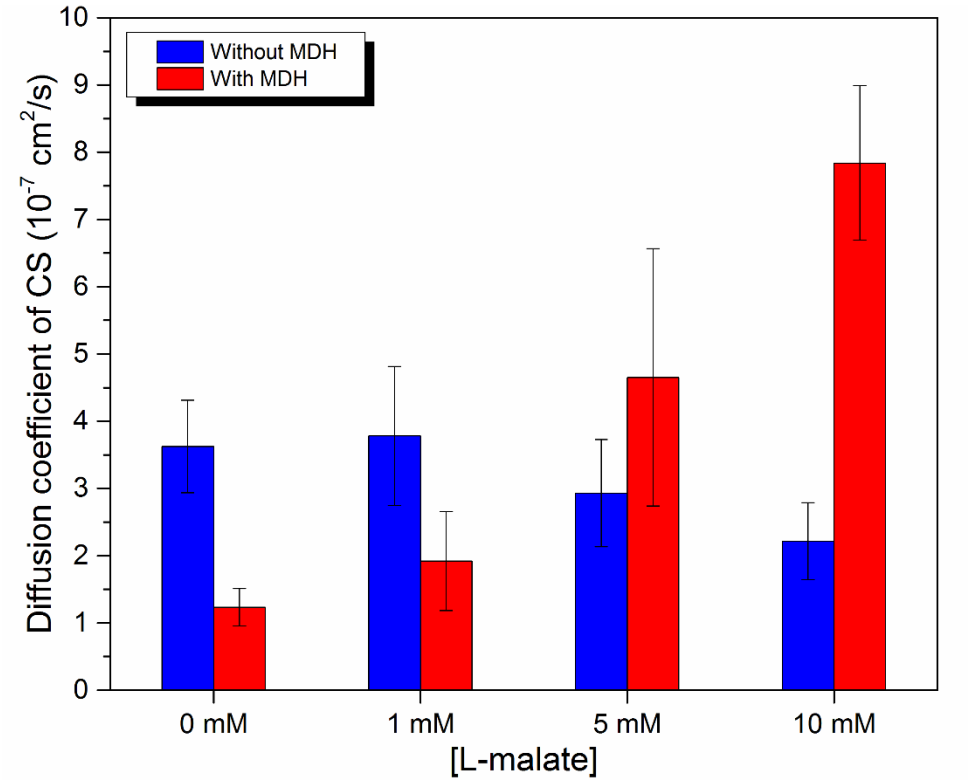


Fig. S9 Apparent diffusion coefficient of CS toward L-malate in the absence and presence of mMDH.

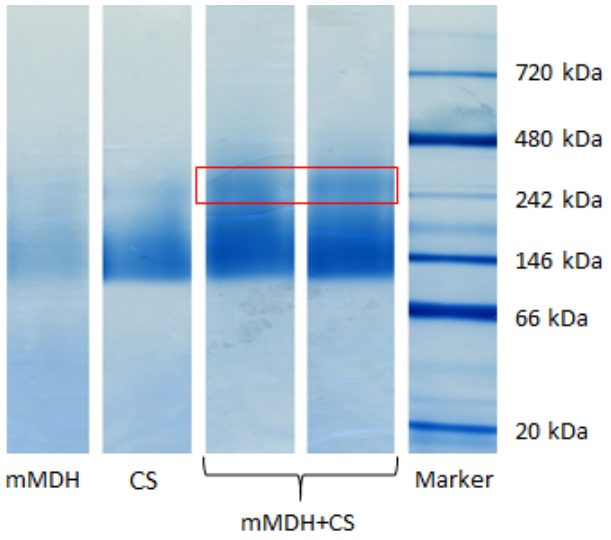


Fig. S10 Reducing SDS-PAGE of the mixture of 100 nM mMDH and 100 nM CS. A protein complex (2mMDH-1CS) band is denoted by red square.

References

1 Wu, F.; Minteer, S. D. *Biomacromolecules* **2013**, *14*, 2739.
2 Xia, Y.; Whitesides, G. M. *Annu. Rev. Mater. Sci.* **1998**, *28*, 153.