Electronic supplementary information

Control of Allosteric Protein Electrochemical Switch with Magnetic Signals †

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Chemicals and Materials

L-Glutamate oxidase from *Streptomyces* sp. (GlutOx; E.C. 1.4.3.11), alanine aminotransferase from porcine heart (AAt; E. C. 2.6.1.2), horseradish peroxidase (HRP; E.C. 1.11.1.7), D-(+)-glucose, L-alanine, 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), α-ketoglutarate, pyrroloquinoline quinone (PQQ), 3-(aminopropyl)triethoxysilane (APTES), poly(ethyleneimine) (PEI, 50% w/v solution in H_2O, MW≈750,000), sodium alginate from brown algae (medium viscosity, ≥2000 cP), polyethylene glycol (PEG), fluorescein isothiocyanate isomer I (FITC), 4-mercaprobenzoic acid (4-MBA), *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), 2-amino-2-(hydroxymethyl)propane-1,3-diol (TRIS-buffer), (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES-buffer), 2-(*N*-morpholino)ethanesulfonic acid (MES-buffer), Sephadex G-50, and other standard organic and inorganic materials and reactants were obtained from MilliporeSigma (former Sigma-Aldrich) or Fisher Scientific (Hampton, NH, USA). Buckypaper composed of compressed multi-walled carbon nanotubes and Screen Printed Gold Electrodes (SPAuE, diameter = 4 mm) were purchased from Buckeye Composites-NanoTechLabs (Yadkinville, NC, USA) and Metrohm USA (Riverview, FL, USA), respectively. M13 skeletal muscle myosin light chain kinase peptide (H-Lys-Arg-Arg-Trp-Lys-Lys-Asn-Phe-Ile-Ala-Val-Ser-Ala-Ala-Asn-Arg-Phe-Lys-Lys-Ile-Ser-Ser-Ser-Gly-Ala-Leu-OH; KRRWKKNFIAVSAANRFKKISSSGAL) was purchased from AnaSpec (Fremont, CA, USA). All commercial chemicals were used as supplied without further purification. All experiments were carried out in ultrapure water (18.2 MΩ·cm; Barnstead NANOpure Diamond).

Supplementary Table 1. Summary of protein sequences used in this study. The sequences are colored according to the functional elements.

PQQ-GDH-CaM	MDVPLIPSQFAKAKSENFDKKVILSNLNKPHALLWGPDNQI WLTERATGKILRVNPESGSVKTVFQVPEIVNDADGQNGLL GFAFHPDFKNNPYIYISGTFKNPKSTDKELPNQTIIRRYTYN KSTDTLEKPVDLLAGLPSSKDHQSGRLVIGPDQKIYYTIGD QGRNQLAYLFLPNQAQHTPTQQELNGKDYHTYMGKVLRL NLDGSIPKDNPSFNGVVSHIYTLGHRNPQGLAFTPNGKLLQ SEQGPNSDDEINLIVKGGNYGWPNVAGYKDDSGYAYANY SAAANKTIKDLAQNGVKVAAGVPVTKESEWTGKNFVPPL KTLYTVQDTYNYNDPTCGEMTYICWPTVAPSSAYVYKGG KKAITGWENTLLVPSLKRGVIFRIKLDPTYSTTYDDAVPMF KSGCGGTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSL GQNPTEAELQDMINEVDADGNGTIDFPEFLTMMARKMKD TDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDE EVDEMIREADIDGDGQVNYEEFVQMMTAGGSCGNRYRDV IASPDGNVLYVLTDTAGNVQKDDGSVTNTLENPGSLIKFTY KAKKLAAALEHHHHHH
Caged-peptide (Low-Affinity-CaM-28aaLinker-TVMVsite-M13peptide)	MGHHHHHHSSGTRVAILWHEMWHEGLEEASRLYFGERNV KGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLMEAQE WCRKYMKSGNVKDLTQAWDLYYHVFRRISGGSGGSGSGS GGSGGTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLG QNPTEAELQDMINEVDADGNGTIDFPEFLTMMARKMKDT DSESEIREAFRVADKDGNGYISAAEARHVMTNLGEKLTDE EVDEMIREADIDGDGQVNYEEAVQMMTAGGSGGS**ETVRF QS**GGSGGSGGSGSGSGGKRRWKKNFIAVSAANRFKKISSS GAL
TVMV-protease	SHHHHHHSKALLKGVRDFNPISACVCLLENSSDGHSERLFG IGFGPYIIANQHLFRRNNGELTIKTMHGEFKVKNSTQLQMK PVEGRDIIVIKMAKDFPPFPQKLKFRQPTIKDRVCMVSTNFQ QKSVSSLVSESSHIVHKEDTSFWQHWITTKDGQCGSPLVSII DGNILGIHSLTHTTNGSNYFVEFPEKFVATYLDAADGWCK NWKFNADKISWGSFTLVEDAPEDDFMAKKTVAAIMD

Methods and Procedures

Chimeric Gene Construction and Protein Expression and Purification

The chimeric PQQ-GDH-CaM, CaM-BP and TVMV-protease genes were generated by Gibson Assembly method according the manufacturer's instruction (New England Biolabs) and cloned into pET28a vector.[1] The gene fragments for this assembly were either generated by a polymerase chain reaction (PCR) or ordered as synthetic gBlock gene from IDT (Integrated DNA Technologies). The protein expression and purification were described recently.[2] After Ni-NTA purification, the eluted fractions were pooled and dialyzed against buffer containing 20 mM TRIS-buffer, pH 7.2, with 0.1 M NaCl for 10 hours. Purified PQQ-GDH-CaM was reconstituted with its cofactor by adding PQQ in a 1.0:1.5 molar ratio.

In order to reduce the binding affinity of calmodulin with M13 peptide, we generated the low binding affinity version with combined mutations including E83S, F92A, L105A, F141A. The M13 peptide was genetically fused with low affinity CaM through 28 amino acid linker that includes TVMV protease recognition site (ETVRFQS). The CaM-BP chimera (caged peptide) was expressed and purified as described above.

Preparation of the PQQ-GDH-CaM-Modified Electrode

Screen Printed Gold Electrodes (SPAuE, diameter = 4 mm, Metrohm USA, Riverview, FL, USA) were electrochemically cleaned in 0.1 M H_2SO_4 by running cyclic voltammograms (CVs) between -0.3 and +1.7 vs. Ag|AgCl (3 M KCl; all electrochemical measurements were performed using this reference electrode) at a scan rate of 0.3 V s^{-1} until a well-defined CV was obtained. Afterwards, SPAuE were modified by electrodeposition of highly porous gold (hPG) by initially sweeping the potential for 25 scans between +0.8 and 0 V at a scan rate of 50 mV s^{-1} and then applying a fixed potential of −3 V in a 10 mM $AuCl_3$ solution containing 2.5 M NH_4Cl.[3]
Then, the modified electrodes were activated in 0.1 M H_2SO_4 by running CVs between -0.3 and +1.7 V at a scan rate of 0.1 V s^{-1} until a well-defined CV was obtained. Then, the modified electrode was incubated overnight in a 10 mM ethanol solution containing 4-mercaprobenzoic acid

(4-MBA). Next, the electrode was thoroughly rinsed with ethanol and distilled water. Afterwards, the electrode was incubated with a mixture of 25 mM NHS and 100 mM EDC prepared in phosphate buffered saline (PBS-buffer) (50 mM, pH 7.2) for 30 mins at room temperature and subsequently rinsed in MES-buffer (50 mM, pH 6, to avoid hydrolysis of the NHS-ester). The functionalized electrodes were immediately incubated with enzyme solutions 1.5 μM of PQQ-GDH-CaM (25 mM HEPES-buffer, pH 6.0, containing 1 mM $CaCl_2$) for 1 h at room temperature under moderate shaking. The modified electrodes were stored (4 ºC) in HEPES-buffer (25 mM, pH 7.2, containing 100 mM Na_2SO_4) until further use.

Electrochemical Experiments

Cyclic voltammetry measurements were carried out with an ECO Chemie Autolab PASTAT 10 electrochemical analyzer, using the GPES 4.9 (General Purpose Electrochemical System) software package. A BASi Ag|AgCl|KCl, 3M, electrode served as a reference electrode and a graphite slab was used as a counter electrode. Cyclic voltammograms were recorded at a scan rate of 2 $mV \cdot s^{-1}$ in an electrolyte solution composed of 50 mM buffer, pH 7.2 containing 100 mM Na_2SO_4. After that CVs were performed adding 125 μM $Ca(CH_3COO)_2$, then 20 mM glucose and finally a mixture 1:1 of TVMV-protease (1 μM) and caged peptide (1 μM) (pre-incubated separately for 20 min before the addition into the electrochemical cell). Anaerobic conditions were achieved by purging the solution with argon.

Amperometric experiments were performed to check the real activation of PQQ-GDH-CaM after releasing one of the machinery components from Fe^{3+}-cross-linked alginate. These experiments were performed by applying +400 mV vs. Ag|AgCl (3M KCl) while the releasing electrode was triggered by *in situ* production of H_2O_2 through a biocatalytic cascade including L-alanine and α-ketoglutarate that were converted from alanine aminotransferase (AAt) immobilized onto magnetic nanoparticles (MNPs) to L-glutamate that was further oxidized by GlutOx immobilized at the alginate electrode. The activation of PQQ-GDH-CaM was checked by monitoring current vs. time accounting for glucose oxidation operated by PQQ-GDH-CaM upon proper activation. Secondly, a magnet was applied to attract the MNPs to the alginate-modified electrode, thus allowing product (substrate for second enzyme) channeling and not diffusion, resulting in a faster alginate dissolution process. Two magnets (NdFeB, grade N42, plating/coating: Ni-Cu-Ni,

dimensions: 1/2" × 1/4" × 1/8", B842 from K&J Magnetics, Inc.) were placed outside the electrochemical cell at the distance of about 5 mm from the electrode (back side) to create a nearly uniform (homogeneous) magnetic field in the liquid system with the intensity of 0.4 T measured at the electrode surface. The magnetic field was measured with Bell-5180 Gauss/Tesla meter (OECO LLC, Milwaukie, OR, US) with a detachable axial probe that was placed in the immediate proximity of the electrode surface.

FITC-labeling of Caged Peptide

First, the caged peptide was dissolved in 100 mM carbonate solution (pH 9.0) at a concentration of 1 mg mL^{-1}. Then, a 50 µL fluorescein isothiocyanate solution (FITC) (10 mg mL^{-1}) in dimethyl sulfoxide (DMSO) was added and the protein solution was stirred at 4 °C in a dark room for 4 h. Aqueous NH_4Cl solution (200 mM, 1 mL) was added and the mixture was stirred for another 2 h to stop the reaction. After the reaction was completed, the mixture was applied on a Sephadex G-50 column. Elution was performed with 20 mM HEPES-buffer, pH 7.0. The eluted yellow solution was collected in different fractions and the presence of labeled protein was confirmed by using 1D-gel electrophoresis. The final product was lyophilized, further dissolved in 10 mM HEPES-buffer pH 7.2 (with a final concentration 25 µM) and stored at -20 °C.[4]

Preparation of Fe^{3+}-cross-linked Alginate-modified Electrode

Sodium alginate (1.5% w/w) was dissolved in 100 mM Na_2SO_4, pH 6.0, and stirred for 4 h at 45 °C. The uniform alginate solution was cooled to room temperature (23 ± 2 °C) and $FeSO_4$ (35 mM) and FITC-caged peptide (25 µM) were added and well mixed. The FITC-caged peptide-loaded alginate thin-films were deposited on a buckypaper electrode (geometrical area, 0.5 cm^2) upon potentiostatic (+0.9 V) oxidation of Fe^{2+} cations resulting in the formation of Fe^{3+} and yielding Fe^{3+}-cross-linked alginate film on the electrode surface. The electrode modification was performed for 180 s and then the modified electrodes were rinsed 3 times with 1 mM TRIS-buffer pH 8.0. After that, the alginate-modified electrode was rinsed with water, immersed in 1 % w/v PEI solution for 180 s, rinsed with water again, and put in HEPES-buffer (25 mM, pH 7.4) for 2 hours resulting in deposition of PEI on the alginate film.[5]

Immobilization of GlutOx onto Fe^{3+}-cross-linked alginate based electrode

The PEI-alginate-modified electrode was immersed in a HEPES-buffer (25 mM, pH 7.4) containing 1 % (w/w) PEG, 50 mM of EDC, 50 mM of NHS and 10 U of GlutOx for 2 hours. After that, the electrode was rinsed with a HEPES-buffer (25 mM, pH 7.4). After immobilization, enzyme-functionalized alginate electrode (EAE) was rinsed with a HEPES-buffer (25 mM, pH 7.2 containing 100 mM Na_2SO_4), and left for 2 hours in a HEPES-buffer until uncontrolled leakage of FITC-caged peptide mostly stopped.[5]

Synthesis and Modification of Magnetic Nanoparticles (MNPs)

A co-precipitation method was used to obtain magnetic nanoparticles. Briefly, 3.75 mmol of $FeCl_2 \cdot 4H_2O$ and 5 mmol of $FeCl_3 \cdot 6H_2O$ were mixed in a beaker and dissolved in 87.5 mL of distilled water. The solution was mechanically stirred and after complete dissolution of the salts the pH was 2.4. It is known that ferrous ions can easily be oxidized to ferric ions, therefore an excess of Fe^{2+} was used. Concentrated ammonium hydroxide (NH_4OH) was added dropwise until pH 10 was obtained. On adding the first drop of ammonia a black precipitate of nanoparticles appeared. The suspension turned completely black at pH 10. The resulting suspension was then sonicated for 20 mins, followed by magnetically induced sedimentation, which was performed at least five times. The precipitate was washed with a solution of ammonia (pH 10), water and acetone. Thereafter, MNPs suspension was lyophilized.[6]

MNPs (100 mg) were dispersed in 1 mL H_2O that had been adjusted to pH 9.0 using NaOH. The dispersion was sonicated for 40 minutes until homogenous suspension was obtained, then it was centrifuged for 10 minutes at 10,000 rpm. The H_2O was replaced with ethanol three times, centrifuging the particles each time. The MNPs in the ethanolic dispersion (1 mL) were reacted with 3-(aminopropyl)triethoxysilane (APTES, 3 % v/v) for 3 hours under mild shaking. The silanized MNPs were carefully rinsed 3 times with ethanol by centrifuging and replacing ethanol each time. Then, the ethanol was replaced with 25 mM HEPES-buffer, pH 7.5, 3 times, centrifuging each time.

The silanized MNPs were reacted with alanine aminotransferase (ALT, 10 U/mL), 100 mM EDC and 100 mM NHS in 25 mM HEPES buffer, pH 7.5, under mild shaking for 4 hours, followed by

washing steps with the 25 mM HEPES buffer pH 6. The modified MNPs were precipitated by magnetically induced sedimentation and mechanically dispersed in 25 mM HEPES buffer, pH 6, for 5 times in order to wash out any enzyme molecule loosely bound.[6]

The activity was further assayed by using optical absorbance measured at λ = 415 nm that corresponds to the maximum absorbance of the oxidized ABTS. A Shimadzu UV-2450 UV–Vis spectrophotometer with 1 mL (10 mm optical path) poly(methyl methacrylate) (PMMA) cuvettes was used for optical absorbance measurements. The following concentrations were used: 5 mM L-alanine, 5 mM α-ketoglutarate, GlutOx (1 U/mL), HRP (1 U/mL) and 0.25 mM ABTS. A calibration curve was performed by injecting different amounts of ALT solution (10 U/mL). In a separate experiment, 5 μL of ALT modified MNPs were used to evaluate their activity.

Releasing Experiment

The uncontrolled leakage and signal-triggered release of the FITC-caged peptide were followed by measuring fluorescence at λ=517 nm in a HEPES-buffer solution (25 mM, pH 7.2 containing 100 mM Na_2SO_4). For these measurements, EAE was placed in a cuvette (1 cm optical path) containing 1.5 mL HEPES-buffer solution (25 mM, pH 7.2 containing 100 mM Na_2SO_4). The uncontrolled leakage of the FITC-caged peptide was measured without any additional component in the solution. Next, the releasing electrode was triggered by *in situ* production of H_2O_2 through a biocatalytic cascade including L-alanine and α-ketoglutarate that were converted from L-alanine aminotransferase (AAt) immobilized onto magnetic nanoparticles (MNPs) to L-glutamate that was further oxidized by GlutOx immobilized onto the alginate electrode. The activation of PQQ-GDH-CaM could be easily checked by monitoring current vs. time accounting for glucose oxidation operated by PQQ-GDH-CaM upon proper activation. Secondly, a magnet was applied to attract the MNPs to the alginate-modified electrode, thus, allowing product (substrate for second enzyme) channeling and not diffusion, resulting in a faster alginate dissolution process.

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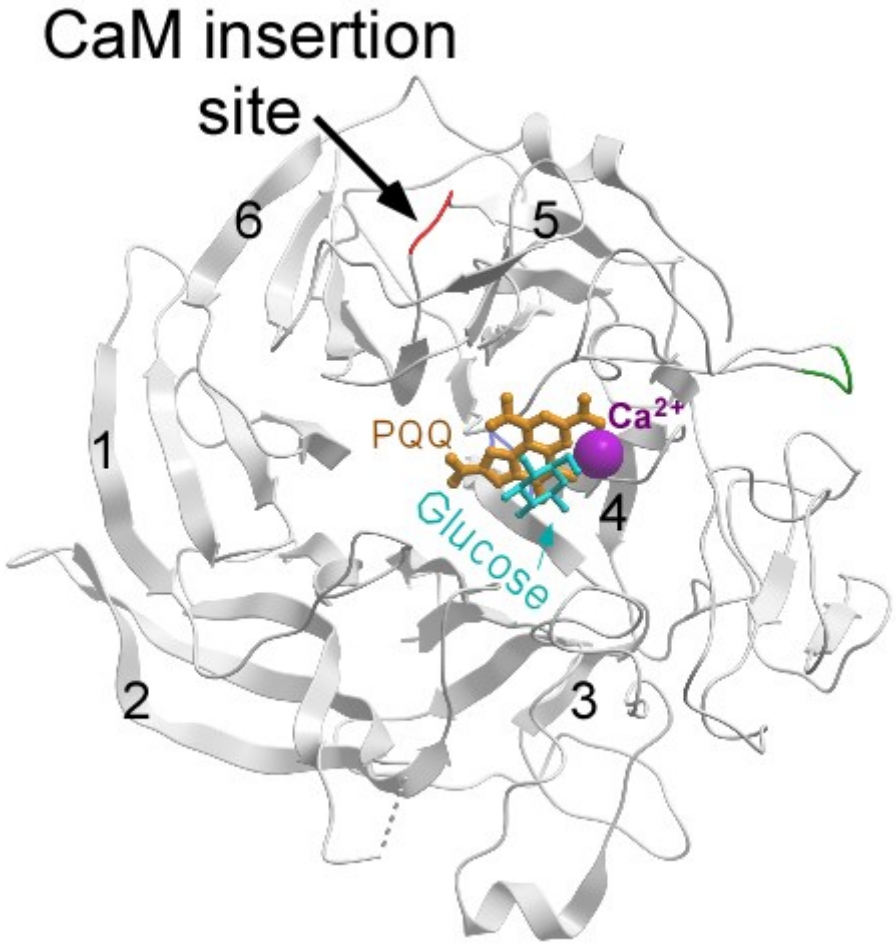

Figure S1. The PQQ-GDH structure and the position of the CaM insertion. Structure of PQQ-GDH (PDB: 1C9U) in ribbon representation with labeled calmodulin insertion sites. PQQ cofactor is displayed in ball and stick representation colored in golden while glucose colored in green. The coordinated Ca^{2+} in the active site is shown as a magenta ball. The β-sheets of the molecule are numbered 1 to S206.

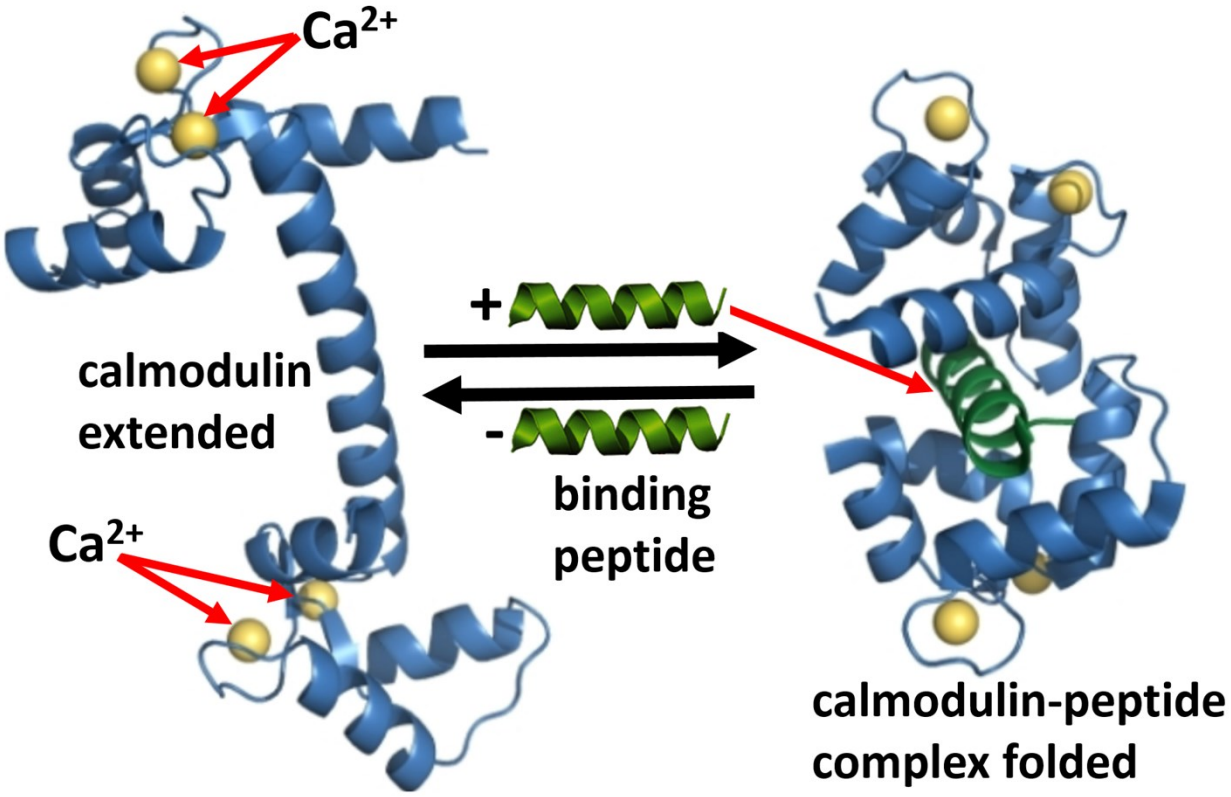

Figure S2. Reversible conformational changes in the CaM receptor upon binding/removing specific peptide molecule.

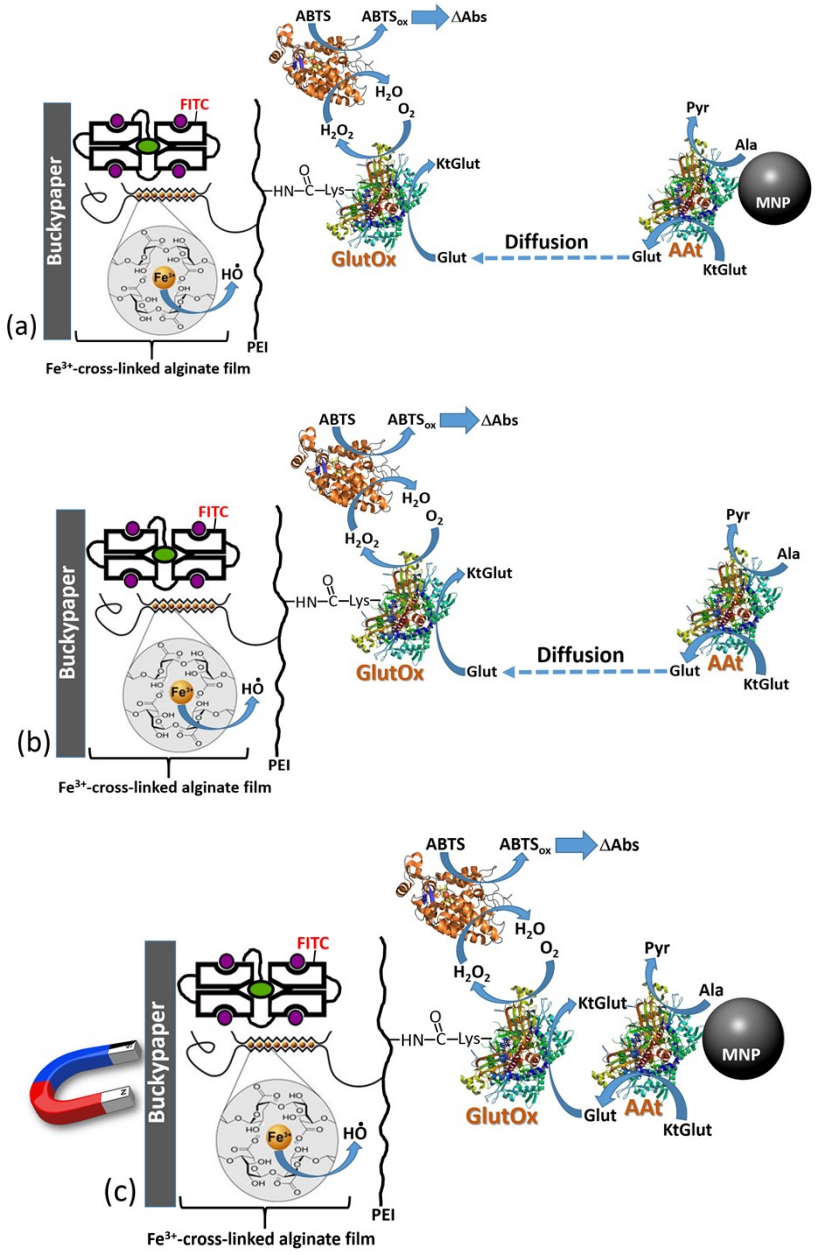

Figure S3. Schematic representations of the release-triggering mechanisms: (a) AAt immobilized onto MNPs converts L-alanine and α-ketoglutarate to produce L-glutamate that diffuses from bulk solution to the electrode surface where GlutOx catalyzes its oxidation with the *in situ* production of H_2O_2, (b) AAt (freely diffusing) converts L-alanine and α-ketoglutarate to produce L-glutamate that diffuses from bulk solution to the electrode surface where GlutOx catalyzes its oxidation with the *in situ* production of H_2O_2 and (c) AAt immobilized onto MNPs converts L-alanine and α-ketoglutarate to produce L-glutamate that can easily undergo channeling upon application of magnetic field in the proximity of the electrode surface where GlutOx catalyzes L-glutamate oxidation with a faster *in situ* production of H_2O_2. The H_2O_2 production, corresponding to the AAt activity, can be easily assayed by measuring optical density of ABTS oxidation in the presence of HRP.

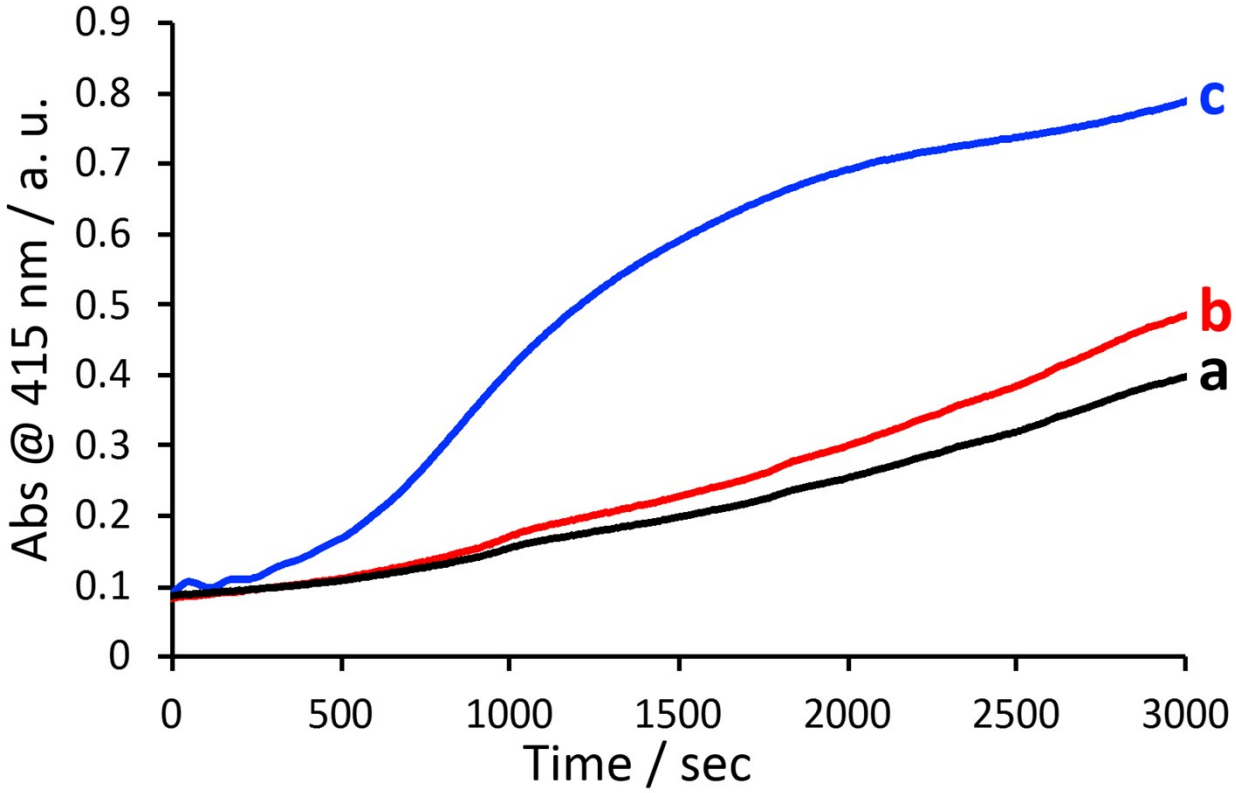

Figure S4. Optical absorbance measurements performed in 25 mM HEPES-buffer, pH 7.2, with 100 mM Na_2SO_4. The initial assay solution contains 5 mM L-alanine, 5 mM α-ketoglutarate, HRP (1 U/mL) and 0.25 mM ABTS. GlutOx was immobilized onto Fe^{3+}-cross-linked alginate modified buckypaper electrode. Next, 5 μL of AAt-MNPs stock solution (corresponding to 0.1 U/mL in the final assay volume) were added in the assay solution (curve a), 5 μL of AAt (not immobilized) stock solution (corresponding to 0.1 U/mL in the final assay volume) were added in the assay solution (curve b) and 5 μL of AAt-MNPs stock solution (corresponding to 0.1 U/mL in the final assay volume) were added in the assay solution upon application of magnetic field (curve b).

Comment: From these experiments we were able to prove that the application of magnetic field allows faster *in situ* production of H_2O_2, thus resulting in faster dissolution of the alginate layer with the release of the larger amount of the FITC-caged peptide. In the absence of magnetic field, the kinetics are very slow, both for AAt-MNPs and AAt freely diffusing, due to slow diffusion process of the reaction product of the first enzyme that is also operates as the substrate of the second enzyme.

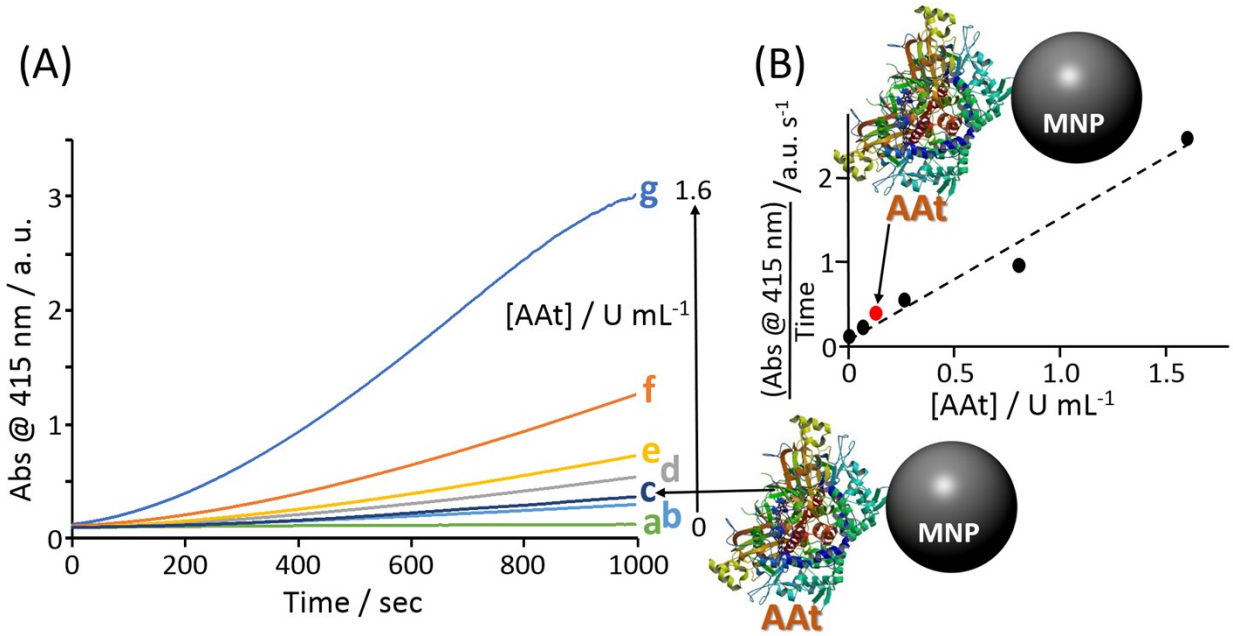

Figure S5. Optical absorbance measurements performed in 25 mM HEPES-buffer, pH 7.2, with 100 mM Na_2SO_4. The initial assay solution contains 5 mM L-alanine, 5 mM α-ketoglutarate, GlutOx (1 U/mL), HRP (1 U/mL) and 0.25 mM ABTS. Next, different volumes of AAt stock solution were added to the assay: 0 U/mL (a), 0.1 U/mL (b), 0.2 U/mL (d), 0.4 U/mL (e) 0.8 U/mL (f) and 1.6 U/mL (g), while in (c) 5 µL of AAt-MNPs stock solution were added instead of freely diffusing enzyme. Inset: Calibration plot of absorbance kinetics vs. [AAt] reported in U/mL.

Comment: From these experiments, we were able to estimate the activity of AAt-MNPs.