Bioelectronic System for Insulin Release Triggered by Ketone Body Mimicking Diabetic Ketoacidosis in Vitro

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Electronic Supplementary Information

Chemicals and reagents.

Polyclonal antibody to 3-hydroxybutyric acid (3-HBA) from rabbit was purchased from Cloud-Clone Corp. PQQ-dependent glucose dehydrogenase (PQQ-GDH; E.C. 1.1.5.2, from microorganism – not specified by the company) was purchased from Toyobo Co., Japan. 1-Pyrenebutanoic acid succinimidyl ester (PBSE) was purchased from AnaSpec Inc. Glucose oxidase (GOx; E.C. 1.1.3.4) from *Aspergirus niger sp.*, horseradish peroxidase type VI (HRP; E.C. 1.11.1.7), casein, bovine serum albumin (BSA), insulin (human), glutaric dialdehyde, β-D-(+)-glucose, sodium alginate from brown algae (medium viscosity, ≥2000 cP), 2,2'-azino-*bis*(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), phenazine methosulphate (PMS), 2,6-dichlorophenolindophenol (DCIP), 3-(*N*-morpholino)propanesulfonic acid (MOPS-buffer), 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris-buffer), (1-ethyl-3[3-(dimethylamino)propyl] carbodiimide (EDC), *N*-hydroxysuccimide (NHS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES-buffer), (3-aminopropyl)triethoxysilane (APTES), 4-amino-2-hydroxybutyric acid (2-HBA), 3-hydroxybutyric acid (3-HBA), Glu-1-Fibrinopeptide B and other standard organic and inorganic materials and reactants were obtained from Sigma-Aldrich or J.T. Baker. All commercial chemicals were used as supplied without further purification. Ultrapure water (18.2 MΩ·cm) from a NANOpure Diamond (Barnstead) source was used in all of the experiments.

Instrumentation

Cyclic voltammetry measurements were performed in a 3-electrode cell: Metrohm Ag|AgCl|KCl, 3M, reference electrode, graphite counter electrode (a rod 3 mm diameter, 1.9 cm^2 geometrical area), and buckypaper working electrode (0.7 cm^2 geometrical area counting for both sides). A single compartment cell was used with an electrochemical workstation (ECO Chemie Autolab PASTAT 10) and GPES 4.9 (General Purpose Electrochemical System) software. Cyclic voltammograms were recorded at a scan rate of 2 $mV \cdot s^{-1}$ in the absence and presence of glucose (20 mM). The potential measurements were performed under steady-state conditions in the open circuit vs. the reference electrode using a multimeter (Meterman 37XR). Anaerobic conditions (when needed) were achieved by purging the solution with argon. Shimadzu UV-2450 UV–Vis spectrophotometer with 1 mL cuvettes were used for optical spectrum measurements of released insulin, when Bradford method was used. High resolution field emission electron microscope

(JEOL-7400) was applied to characterize buckypaper used as a conducting support for the modified electrodes. Optical images of the alginate thin-film deposited on a graphite electrode were obtained with a confocal microscope (Nikon Eclipse C1 with 10×objective). The presence of insulin in the solution after the alginate film dissolution was also investigated using a QTOF Micro mass spectrometer (Waters Corp, Milford, MA), equipped with an electrospray ionization source and operated in positive mode, as previously described.[1] Instrument calibration was performed using a solution of 1 picomol Glu-1-Fibrinopeptide B, with a m/z of 785.84, doubly charged (2+). The sample was injected by direct infusion using a 100 µL Hamilton syringe. Prior to the MS analysis, the insulin sample was buffer-exchanged in water and then exchanged to a solution composed of 50% (v/v) water, 50% (v/v) acetonitrile and 0.1% (v/v) formic acid. Insulin sample obtained from Sigma-Aldrich, without including it in the alginate matrix, was also analyzed as a positive control (reference). Based on the mass-spectrum of the reference insulin, the peaks found in the released insulin were identified as the ionized species with various charge-states of insulin (3+, 4+, 5+ and 6+), which contained disulfide-linked alpha and beta subunits with a theoretical mass of 5,808 Da/atomic mass units or amu).

Experimental procedures

Silanization of silica nanoparticles (SiO_2-NPs). SiO_2-NPs (1.1 g) (Fiber Optics Center Inc., ca. 200 nm diameter) were dissolved in distilled water (pH 9.0 adjusted with NaOH) and sonicated for 30 min. Then water was replaced with ethanol by centrifuging three times and removing supernatant. The surface of the SiO_2-NPs was modified by reacting with APTES (1% (v/v) in ethanol) overnight at room temperature (23±2 ^oC). The silanized surface was washed with ethanol very carefully to remove unbound amino-silane.

Preparation of BSA-2-HBA conjugates: BSA (10 mg) was dissolved in 1 mL of carbonate buffer (0.1 M, pH 9.5) and mixed with 100 µL of 2-HBA solution (0.2 M). Then glutaric dialdehyde was added to the solution to obtain a 1% (v/v) final concentration and the mixture was let to react for 4 hours at 4°C, mixing the solution periodically. The 2-HBA-BSA conjugate was then separated from the supernatant and rebuffered in phosphate buffer (10 mM, pH 7.5).

Functionalization of SiO_2-NPs with 2-HBA-BSA conjugates: Silanized SiO_2-NPs (0.5 mg) were washed three times with phosphate buffer (10 mM, pH 7.5). The NPs were then incubated in a solution containing 10% (v/v) of glutaric dialdehyde in phosphate buffer (10 mM, pH 7.5) for one hour while shaking at room temperature. Then the modified NPs were washed three times with phosphate buffer (10 mM, pH 7.5) and incubated in the 2-HBA-BSA conjugate solution for 3 hours while shaking at room temperature. The 2-HBA-BSA-SiO_2-NPs conjugate was then separated from the supernatant and rebuffered in HEPES-buffer (10 mM, pH 7.5).

Functionalization of SiO_2-NPs with GOx and Ab. Silanized SiO_2-NPs (0.5 mg) were washed three times with HEPES-buffer (10 mM, pH 7.5). Then the NPs were incubated in a solution containing EDC (100 mM), NHS (100 mM), and GOx (50 $U \cdot mL^{-1}$) in HEPES-buffer (10 mM, pH 7.5) for three hours while shaking. Anti-3-hydroxybutyric IgG-antibody from rabbit (0.2 $mg \cdot mL^{-1}$) was then added to the solution along with fresh EDC (100 mM) and NHS (100 mM) and allowed to incubate for 3 hours while shaking. The antibody-GOx-SiO_2-NPs conjugate was then separated from the supernatant and rebuffered in HEPES-buffer (10 mM, pH 7.5).

Functionalization of buckypaper electrode. Buckypaper composed of compressed multi-walled carbon nanotubes (CNTs) (Buckeye Composites; NanoTechLabs, Yadkinville, NC) was used as the electrode material. Electrodes were washed with isopropyl alcohol with moderate shaking for 15 min at room temperature prior to their modification. The electrodes were incubated with PBSE, 10 mM, in ethanol with moderate shaking for 1 h at room temperature, subsequently rinsed with ethanol. The electrodes were prepared by immobilization of PQQ-GDH. The PBSE-functionalized electrodes were incubated for 1 hour

in the solution of PQQ-GDH (2.4 mg·mL^{-1}) in MOPS buffer (50 mM, pH 7.0) containing Na_2SO_4 (100 mM) and $CaCl_2$ (1 mM). Following, the electrodes were washed with MOPS-buffer while shaking and incubated in a solution containing EDC (50 mM), NHS (50 mM) for 1 hour. The electrodes were then washed with HEPES-buffer (10 mM, pH 7.5) and were incubated in a solution containing 2-HBA-BSA-SiO_2-NPs conjugate for 1 hour while shaking. Then the electrodes were rinsed with HEPES-buffer (10 mM, pH 7.5) on a shaker for 10 min, followed by 20 minutes in the presence of casein solution (2 % w/v) in HEPES-buffer solution while shaking. Next, the electrodes were rinsed in 10 mM HEPES-buffer and incubated in antibody-GOx-SiO_2-NPs for 1 hour while shaking. Before using, the electrodes were washed once more in HEPES-buffer (10 mM, pH 7.5) and stored in MOPS buffer.

In order to cleave the 2-HBA/antibody immune-complex, the modified electrodes were incubated in a solution containing 10 mM of 3-HBA (HEPES-buffer 10 mM, pH 7.5) for 1.5 hours while shaking and washed in MOPS-buffer as described previously.

Activity assays of the PQQ-GDH-modified electrode. The activity of PQQ-GDH immobilized on the modified buckypaper electrode was determined according to the protocol published elsewhere[2] by measuring decrease in absorbance of 2,6-dichlorophenolindophenol (DCIP) upon its biocatalytic reduction with glucose. The PQQ-GDH-modified electrode was immersed in a MOPS-buffer solution (10 mM, pH 7.0) containing 10 mM glucose, 120 μM PMS, 200 μM DCIP, 1 mM $CaCl_2$ and the change of the absorbance (λ = 600 nm; ε_{DCIP} = 21 mM^{-1}·cm^{-1}) in time was measured. The PQQ-GDH activity was measured as 700 mU per electrode.

Potential measurements on the modified electrodes. The potential measurements of buckypaper modified electrodes were performed in MOPS-buffer (50 mM, pH 7.5, containing 100 mM Na_2SO_4, 1 mM $CaCl_2$ and 20 mM glucose). The potential measurements were performed under steady-state conditions in the open circuit vs. an Ag|AgCl|KCl, 3M, (Metrohm) reference electrode

Amount of GOx immobilized on the buckypaper modified electrodes. The amount of GOx immobilized on the electrode surface was measured by performing an enzymatic assay of GOx. The modified electrodes described above (obtained after the last modification step resulting in GOx immobilization) were incubated in a glucose solution (2 mM) in citrate buffer (100 mM, pH 5.0) and allowed to react for 30 minutes. After that, ABTS (4 mM) and HRP (1.5 U) were added and the absorbance was measured at 415 nm. The amount of GOx was calculated using the amount of converted H_2O_2 and the enzyme's activity to give the approximate loading of 15 mU of GOx per electrode.

Deposition of alginate on a graphite support. Sodium alginate (1.5% w/w) was dissolved in 100 mM Na_2SO_4 (pH 4.5) and stirred for 30 minutes at 45 °C. The solution was cooled to room temperature (23±2 ºC) and $FeSO_4$ (35 mM) and insulin (1 mg·mL^{-1}) were added and mixed well. Insulin was entrapped into the Fe^{3+}-cross-linked alginate film upon its electrochemical deposition on the electrode surface. The mixture was deposited on an electrode (a well-polished graphite rod, pencil lead, 0.9 mm diameter, geometrical area of 0.3 cm^2 exposed to the solution) upon oxidation with a potentiostat (+0.8 V, 60 sec) of Fe^{2+} cations, yielding Fe^{3+} and resulting in an alginate cross-linked film on a graphite surface containing entrapped insulin. The resulting electrode was washed thoroughly with water. The detailed thickness analysis and electrochemical characterization were reported elsewhere.[3] The amount of released insulin was determined by using the Bradford method.[4]

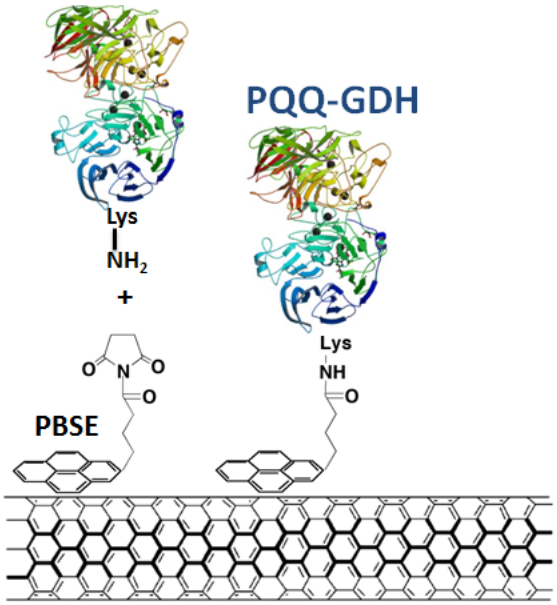

Figure S1. Immobilization of the PQQ-GDH on CNTs with the help of the heterobifunctional linker 1-pyrenebutanoic acid succinimidyl ester (PBSE), which provides covalent binding with amino groups of protein lysine residues through the formation of amide bonds and also interacts with CNTs via π-π stacking of the polyaromatic pyrenyl moiety.

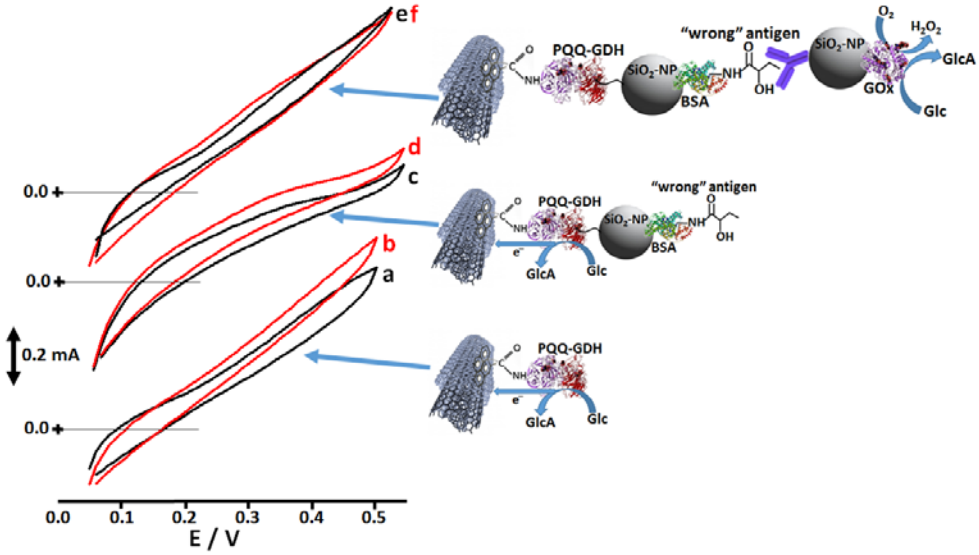

Figure S2. Cyclic voltammograms obtained on the buckypaper electrode after various modification steps in the absence and presence of glucose: (a-b) The electrode modified with PQQ-GDH. (c-d) The electrode modified with PQQ-GDH and covalently bound SiO_2-NPs functionalized with 2-HBA. (e-f) The electrode after the final modification step including SiO_2-NPs functionalized with Ab and GOx. Cyclic voltammograms a, c and e were obtained in the absence of glucose. Cyclic voltammograms b, d and f were obtained in the presence of glucose (20 mM). Potential scan rate, $2 \cdot mV\ s^{-1}$. Note that the modified electrode demonstrates the electrocatalytic anodic current corresponding to glucose oxidation after the first two modification steps. However, after attaching GOx-functionalized NPs the electrode becomes insensitive to the presence of glucose.

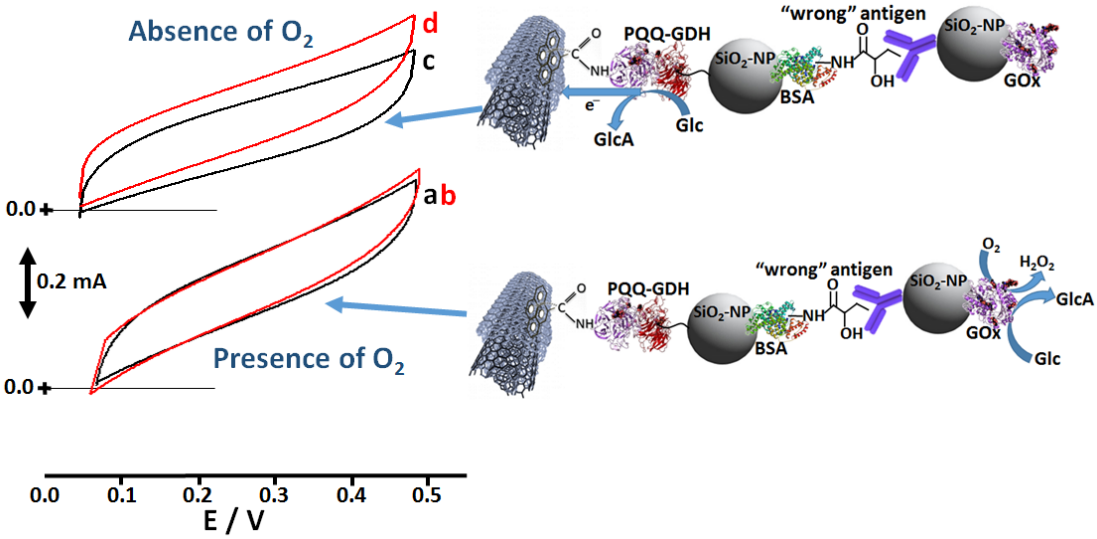

Figure S3. Cyclic voltammograms obtained on the buckypaper electrode after the final modification step including SiO_2-NPs functionalized with Ab and GOx. Cyclic voltammograms a, b were obtained in the presence of oxygen (oxygen was dissolved in the solution in equilibrium with air). Cyclic voltammograms c, d were obtained in the absence of oxygen (the solution was deaerated with Ar). Cyclic voltammograms a, c were obtained in the absence of glucose. Cyclic voltammograms b, d were obtained in the presence of glucose (20 mM). Potential scan rate, $2 \cdot mV\ s^{-1}$. Note that the modified electrode does not show electrocatalytic oxidation of glucose in the presence of oxygen, however, in the absence of oxygen the electrode clearly shows the electrocatalytic anodic current corresponding to glucose oxidation.

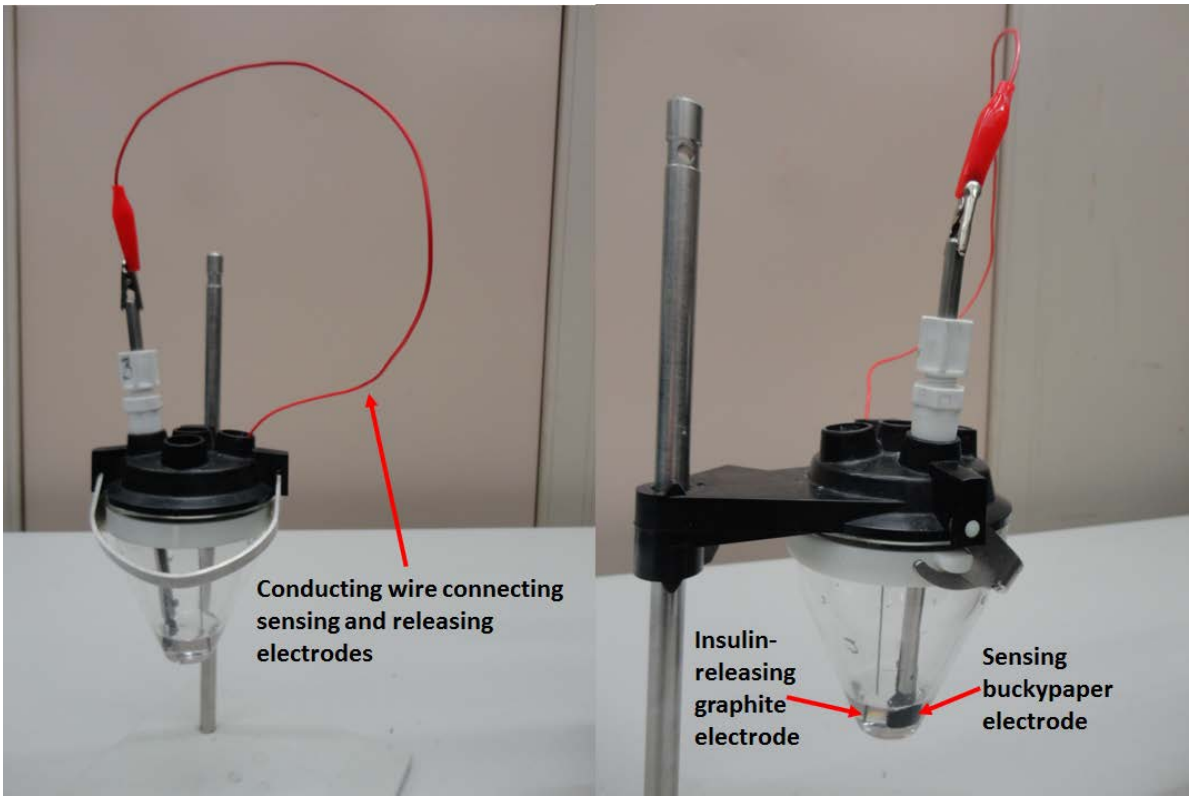

Figure S4. Experimental setup. Electrically connected sensing and releasing electrodes in the cell.

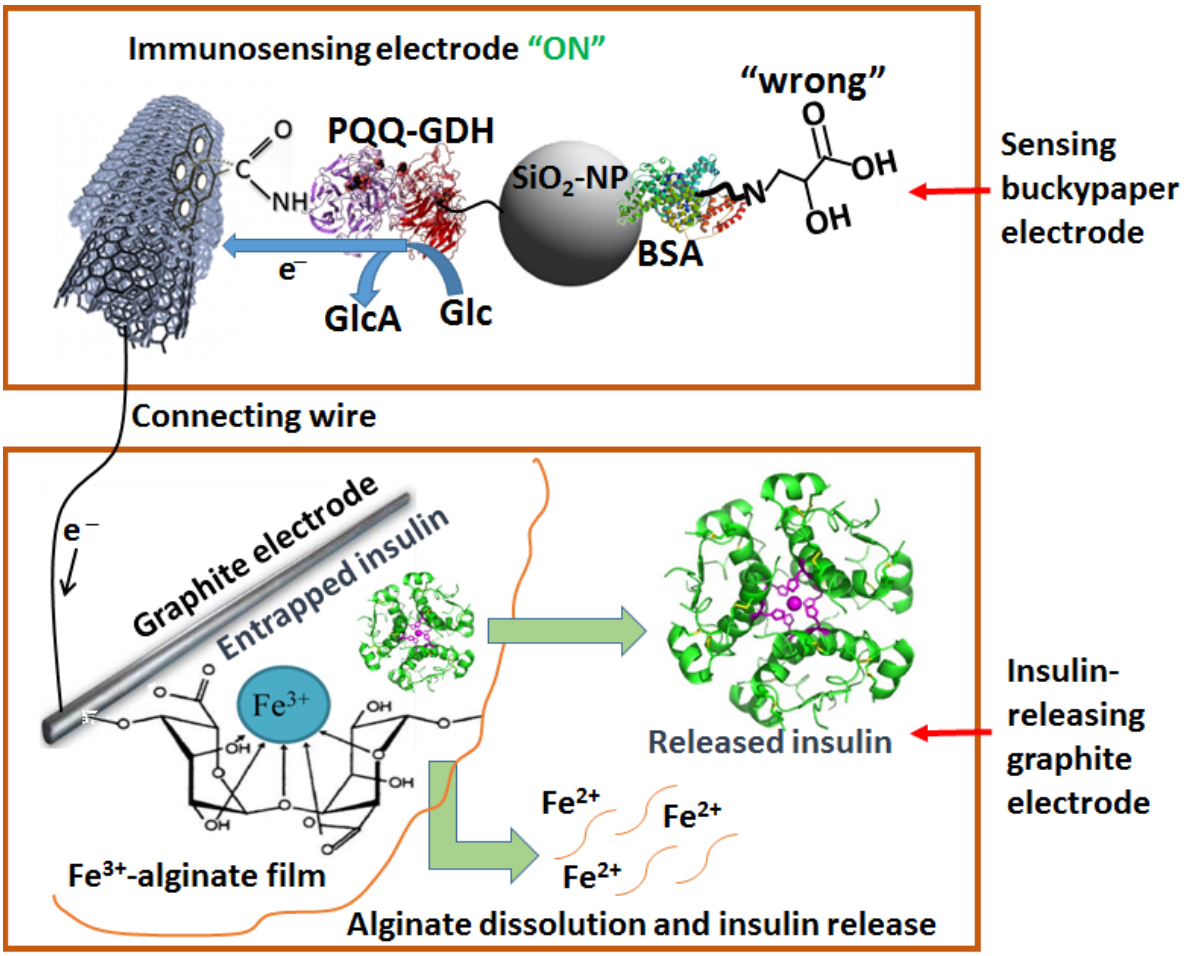

Figure S5. Schematics showing the layered composition and operation of the modified electrode. The scheme details the experimental photo (see Figure ESI-4 shown above). All abbreviations used in the scheme are explained in the paper.

References

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