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Magneto-Controlled Biocatalytic Cascade with a Fluorescent Output

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A biocatalytic cascade based on concerted operation of pyruvate kinase and luciferase with a bioluminescent output was switched reversibly between the low and high activity by applying external magnetic field at different positions or removing it. The enzymes participating in the reaction cascade were bound to magnetic nanoparticles to allow their translocation or aggregation/dispersion controlled by the magnetic field. The reaction intensity, measured as the bioluminescence output, was dependent on the effective distances between the enzymes transported on the magnetic nanoparticles controlled by the magnets.

Keywords: immobilized enzymes; biocatalytic cascade; magnetic nanoparticles; switchable reaction; luciferase; bioluminescence

Adaptivity of biological systems to changes of environment conditions and their responses to external signals are highly important for life.[1,2] These features are based on sophisticated regulatory mechanisms operating at different levels and with different kinetics, ranging from gene expression to enzyme catalyzed reactions. Replication of these processes in artificial systems might be attractive for various applications, however, because of their high complexity they cannot be copied from life systems directly. Therefore, even fragmental modeling of signal-responsive adaptive features, typical for biological systems, in artificial chemical models is challenging and very interesting. At the present state-of-the-art, signal-responding (bio)chemical systems are mostly researched for fundamental science advances, however, these studies keep important promises for future (bio)medical and (bio)technological applications. Some of these studies have been performed on systems of high complexity in the framework of Synthetic Biology.[3] Other approaches include much simpler systems, still demonstrating signal-responsiveness. The external signals applied to the artificial biochemical systems included changes in the solution composition (e.g., pH variation, addition of specific substrates or inhibitors, etc.), temperature changes, light illumination, electric or magnetic signals, etc. In order to make biochemical systems responsive to the signals, the biomolecules (e.g., enzymes) were modified with signal-recognition units changing their states upon receiving signals, then transducing the changes to the biocatalytic or biorecognition centers of the biomolecules affecting their activity. The signal-recognition units attached to enzymes have been in the form of photo-isomerizable species (e.g., spiropyran $\leftrightarrow$ merocyanine) responding to light[4,5] or biomolecules isomerized upon complexation with molecule signals (e.g., calmodulin).[6,7] These units were integrated with enzymes simply by their covalent binding or through complex genetic engineering resulting in chimeric enzymes. The conformational changes in the bound signal-recognition units are transduced to the enzyme molecules changing their activity.

Another commonly used approach is based on entrapping enzymes into polymeric matrices which can change their properties (hydrophilicity/hydrophobicity, density/permeability, etc.) in response to external signals (temperature, pH, light, etc.).[8,9] In this case, the effect of the

signals is mostly in changing access of the enzyme-substrates to the enzyme active centers, thus, changing the biocatalytic reaction rates. Notably, the enzymes themselves are not affected or little affected by the environment changes.

In order to respond to electric signals, the enzymes should be immobilized at electrode surfaces. In the majority of these systems, the enzymes are not directly affected by the electric potential applied and their activity is changing through indirect effects, for example, due to local pH changes generated by an electrochemical process.[10]

Enzymes have been bound to magnetic nanoparticles (MNPs), then responding to the external magnetic field.[11] In some systems, the enzyme-MNPs have been translocated (e.g., to/from an electrode surface) resulting in their reversible activation/inhibition.[12] In other systems, the enzyme-MNPs have been reversibly aggregated/disaggregated upon application/removal of the magnetic field.[13] The distances between the enzymes bound to the MNPs were changing, resulting in the change of the substrate/product transfer between the enzyme molecules. While in the dispersed state of the MNPs, the substrates/products were transported by bulk solution diffusion, in the aggregated state their transport changed to the “channeling” mechanism, which is more efficient for the substrate/product exchange between enzyme molecules.[13] This change particularly affects biocatalytic cascades with the substrate/product transport from one kind of enzyme to another one.

The present paper reports on the enzymatic cascade ended with the light emission controlled by the external magnetic field changing the distance between the enzymes included in the cascade process. Light-emitting enzyme reactions have numerous important applications.[14] While the present study is only aiming at the concept demonstration in a model system, future applications are highly feasible.

Luciferase (Lucif; E.C. 1.14.14.3) enzyme produces bioluminescence in presence of two substrates, luciferin and adenosine triphosphate (ATP).[15] This reaction was organized as a two-

step biocatalytic cascade, where ATP is generated *in situ* through another reaction catalyzed, for example, by pyruvate kinase (PyrKin; E.C. 2.7.1.40) in the presence of adenosine diphosphate (ADP) and phosphoenolpyruvate (PEP). Both enzymes, Lucif and PyrKin, were immobilized on MNPs (ca. 45 nm). The superparamagnetic Fe_3O_4 core (ca. 15 nm) in the MNPs was coated with a SiO_2 shell (ca. 15 nm), which was then functionalized with a grafted polymeric brush of a block-copolymer PAA-*b*-PEG composed of poly(acrylic acid) (PAA) and poly(ethylene glycol) (PEG).[16] The carboxylic groups in the polymer brush were used to bind covalently enzymes using standard carbodiimide method.[17] Zeta (ξ)-potential of the functionalized MNPs was ca. -40 mV being dominated by the dissociated carboxyl groups in the PAA domains of the polymer brush. The negative charge associated with the MNPs prevented their aggregation, keeping them in the dispersed state in the absence of an external magnetic field. The ATP catalytically produced by PyrKin bound to the MNPs in their dispersed state was diluted in the bulk solution and its transport to the Lucif-MNPs was not efficient enough to produce bioluminescence (Figure 1, top). Application of an external magnetic field allowed manipulation with MNPs effectively changing distance between them. When the distance between two kinds of the MNPs (functionalized with Lucif and with PyrKin) was shortened, the transport of the produced ATP was facilitated and the bioluminescence was activated (Figure 1, bottom). We studied two example configurations of the system. In one example, the Lucif-MNPs and PyrKin-MNPs were collected separately and the distance between them was changed by manipulating them with two magnets (Figure 2). In another example, both enzyme-functionalized MNPs were mixed and we compared two states, when the MNPs are dispersed or aggregated (Figure 3). For both experimental setups, the substrates (PEP, ADP and D-luciferin) were present in the solution.

Figure 2A shows photos of the system in two different states, when the Lucif-MNPs and PyrKin-MNPs have a long distance (ca. 12 mm) and a shorter distance (ca. 6 mm) separating them from each other. The distance separating two kinds of the MNPs was easily changed with magnets moving them to different positions. The distance separating two kinds of the MNPs changed the fluorescence effectively between the high and low intensity (Figure 2B). Importantly, the

activation and inhibition of the light emission was reversible and easily controlled by re-positioning two magnets (Figure 2C). While we measured the light intensity for two magnet positions only, it is obvious that the separation between two enzymes bound to the MNPs can be tuned to different distances. Thus, the light emission can be not only switched between two distinct states, but also tuned precisely to any desired light emission intensity. The only limitation is the minimum distance still allowing to keep two kinds of the enzyme-functionalized MNPs separately. Once they are joint (in other words the magnetic particles are mixed), they cannot be separated again. However, then another switching option is possible.

Figure 3A shows photos of the system in two distinct states with the enzyme-MNPs dispersed and aggregated. In this case, the Lucif-MNPs and PyrKin-MNPs are mixed and controlled with one magnet. In the absence of the magnet and after stirring the solution, the enzyme-MNPs are dispersed. After the magnet is applied, all enzyme-MNPs are coming together to aggregate. These two states have different distances between the MNPs. The ATP is produced by PyrKin-MNPs in a local volume reaching the Lucif-MNPs effectively with a high local concentration. The aggregated state of this system produces higher bioluminescence comparing with the dispersed state (Figure 3B). Bringing the magnet closer to the solution and then removing it and stirring the solution result in reversible changes of the bioluminescence intensity (Figure 3C). The difference between two states was also visualized using a confocal microscope. The microscope fluorescent images show green light emission with very low (almost invisible) intensity in the dispersed state (Figure 3D, left) and high intensity in the aggregated state (Figure 3D, right).

Overall, the two-step biocatalytic cascade catalyzed by two enzymes, PyrKin and Lucif, bound to the MNPs was effectively switched between the high and low intensity luminescent output simply by applying external magnets at different positions or removing them from the system. It was possible to change the distance between the enzymes without mixing them or to aggregate/disperse them in the mixed configuration. In all studied configurations the fluorescent output was related to the effective distance between cooperatively working enzymes. The number of the switchable cycles performed in the study included 3-4 cycles.

Obviously, more cycles should be possible, but the amplitude of the ON-OFF changes was decreasing. This is a quite typical problem for many different signal-switchable biomolecular systems. More sophisticated biocatalytic cascades with a larger number of participated enzymes bound to MNPs can be realized and controlled with a larger number of magnets.[18]

Experimental

Materials

Luciferase (Lucif; E.C. 1.14.14.3, from *Vibrio fischeri* (Photobacterium f) lyophilized powder), phosphoenolpyruvate (PEP) and adenosine diphosphate (ADP), were purchased from MilliporeSigma (formerly Sigma Aldrich). Pyruvate kinase (PyrKin; E.C. 2.7.1.40, from rabbit muscle, lyophilized powder) was purchased from Lee Biosolutions, Inc. D-luciferin was purchased from Selleckchem. All other standard reagents were purchased from MilliporeSigma with the highest grade and were used without further purification. All experiments were carried out using ultrapure water (18.2 MΩ·cm; Barnstead NANOpure Diamond). Preparation and full characterization of magnetic nanoparticles (MNPs) were reported elsewhere.[16,19] The covalent binding of the enzymes to the polymer-brush-functionalized MNPs was performed similarly to the procedure detailed elsewhere.[19] The activity of the enzyme-functionalized MNPs was determined according to the standard procedures suggested by the suppliers: Lucif-MNPs 4.3 U per mg MNPs; PyrKin 1.6 U per mg MNPs, which correlates with the immobilization efficiency of ca. 75% compared to the original amount of the enzymes in the solution.

Instrumentation

Bioluminescence spectra were measured using Varian, Cary Eclipse fluorescence spectrophotometer. Fluorescent images were obtained with Leica TCS SP5 II Tandem Scanning Confocal and Multiphoton Microscope.

Determination of activity of pyruvate kinase (PyrKin) before and after its binding to the MNPs

Pyruvate kinase (PyrKin) is involved in glycolysis in which it catalyzes transfer of a phosphate group from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP), producing one molecule of pyruvate and one molecule of adenosine triphosphate (ATP). To determine the activity of PyrKin, an indirect assay protocol was followed to measure pyruvate formed from PEP by PyrKin, which was measured by the formation of nicotinamide adenine dinucleotide (NAD^+) in presence of lactate dehydrogenase (LDH) [20]. In a typical procedure, the following assay was conducted in 1500 µL UV-Vis cuvette using a Tris-HCl buffer (50 mM, pH 7.8): $MgCl_2$ (4 mM); KCl (75 mM); ADP (0.5 mM); PEP (0.25 mM); NADH (0.25 mM); LDH (20 U/ml); and in the presence and absence of PyrKin (1 mg/mL). Thereafter, the UV-Vis spectra were measured before and after the addition of PyrKin. The maximum wavelength was found at λ_{max} = 340 nm and the optical absorbance at the λ_{max} was decreased with the reaction time. For the kinetics study, the reaction was performed using the λ_{max} = 340 nm versus time in the range of 0 – 30 min at room temperature. A similar procedure was conducted with the PyrKin-functionalized MNPs.

Determination of activity of luciferase (Lucif) before and after its binding to the MNPs

Luciferase (Lucif) enzyme utilizes D-luciferin substrate, ATP and Mg^{2+} as co-factors to emit a characteristic yellow-green light in the presence of oxygen. The activity of Lucif was determined according to the following assay protocol: Excess of ATP (2 mM) and $MgCl_2$ (2 mM) were added to Lucif (1 mg/mL) enzyme and the solution was prepared in a Tris-HCl buffer (50 mM, pH 7.8). Then, the D-luciferin substrate (200 µM) was added and fluorescence (FL) spectra were recorded using excitation and emission wavelengths of $\lambda_{ex.}$ = 488 nm, $\lambda_{em.}$ = 500-600 nm, respectively, using a fluorescent spectrophotometer. The difference in fluorescence between control (without the luciferin substrate) and sample (with the substrate) was recorded and calculated as ΔFL: FL($[D\text{-luciferin}]_{=X}$ - $[D\text{- luciferin}]_{=0}$. For the kinetics study, the reaction was performed versus time in the range of 0 – 130 min at room temperature. A similar procedure was used for the analysis of the Lucif-functionalized MNPs.

Author contributions

Ali Othman – Conceptualization, Investigation, Methodology, Writing – original draft

Oleh Smutok - Investigation, Methodology, Writing – original draft

Yongwook Kim - Investigation, Methodology

Sergiy Minko - Investigation, Methodology

Artem Melman – Methodology, Supervision

Evgeny Katz – Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition

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Supporting Information provides technical details on the system preparation, optimization and operation. Additional experimental results (figures) are available.

Conflict of Interest

The authors declare no conflict of interest.

[1] Systems Biology for Signaling Networks, S. Choi (Ed.), Springer-Verlag, New York, **2010**.

[2] J. Hancock, Cell Signalling, Oxford University Press, 3rd ed., **2010**.

[3] Systems and Synthetic Biology, V. Singh, P. K. Dhar (Eds.), Springer, New York, **2015**.

[4] M. Aizawa, K. Namba, S. Suzuki, *Arch. Biochem. Biophys.* **1977**, *182*, 305–310.

[5] E. Katz, A. N. Shipway, I. Willner, In: Photoreactive Organic Thin Films, S. Sekkat, W. Knoll (Eds.), Elsevier, San Diego, USA, **2002**, Chapter II-7, pp. 219–268.

[6] Z. Guo, W. A. Johnston, J. Whitfield, P. Walden, Z. Cui, E. Wijker, S. Edwardraja, I. Retamal Lantadilla, F. Ely, C. Vickers, J. P. J. Ungerer, K. Alexandrov, *J. Am. Chem. Soc.* 2019, *141*, 8128–8135.

[7] M. Gamella, Z. Guo, K. Alexandrov, E. Katz, *ChemElectroChem* **2019**, *6*, 638–645.

[8] T. Shimoboji, E. Larenas, T. Fowler, S. Kulkarni, A. S. Hoffman, P. S. Stayton, *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 16592–16596.

[9] T. Shimoboji, E. Larenas, T. Fowler, A. S Hoffman, P. S. Stayton, *Bioconjug. Chem.* **2003**, *14*, 517–525.

[10] P. K. Wells, O. Smutok, A. Melman, E. Katz, *ACS Appl. Mater. Interfaces* **2021**, *13*, 33830–33839.

[11] M. Bilal, Y. Zhao, T. Rasheed, H. M. N. Iqbal, *Int. J. Biol. Macromol. B* **2018**, *120*, 2530–2544.

[12] R. Hirsch, E. Katz, I. Willner, *J. Am. Chem. Soc.* **2000**, *122*, 12053–12054.

[13] Y. Filipov, A. Zakharchenko, S. Minko, E. Katz, *ChemPhysChem* **2018**, *19*, 3035–3043.

[14] A. J. Syed, J. C. Anderson, *Chem. Soc. Rev.* **2021**, *50*, 5668–5705.

[15] S. M. Marques, J. C. G. Esteves da Silva, *Life* **2009**, *61*, 6–17.

[16] A. Zacharchenko, N. Guz, A. M. Laradji, E. Katz, S. Minko, *Nature Catalysis* **2017**, *1*, 73–81.

[17] I. Willner, E. Katz, *Angew. Chem. Int. Ed.* **2000**, *39*, 1180–1218.

[18] S. Vasilyev, M. Pita, E. Katz, *Electroanalysis* **2008**, *20*, 22–29.

[19] K. Szekeres, P. Bollella, Y. Kim, S. Minko, A. Melman, E. Katz, *J. Phys. Chem. Lett.* **2021**, *12*, 2523-2527.

[20] M. Malcovati, G. Valentini, *Methods in Enzymology* **1982**, *90*, 170–179.

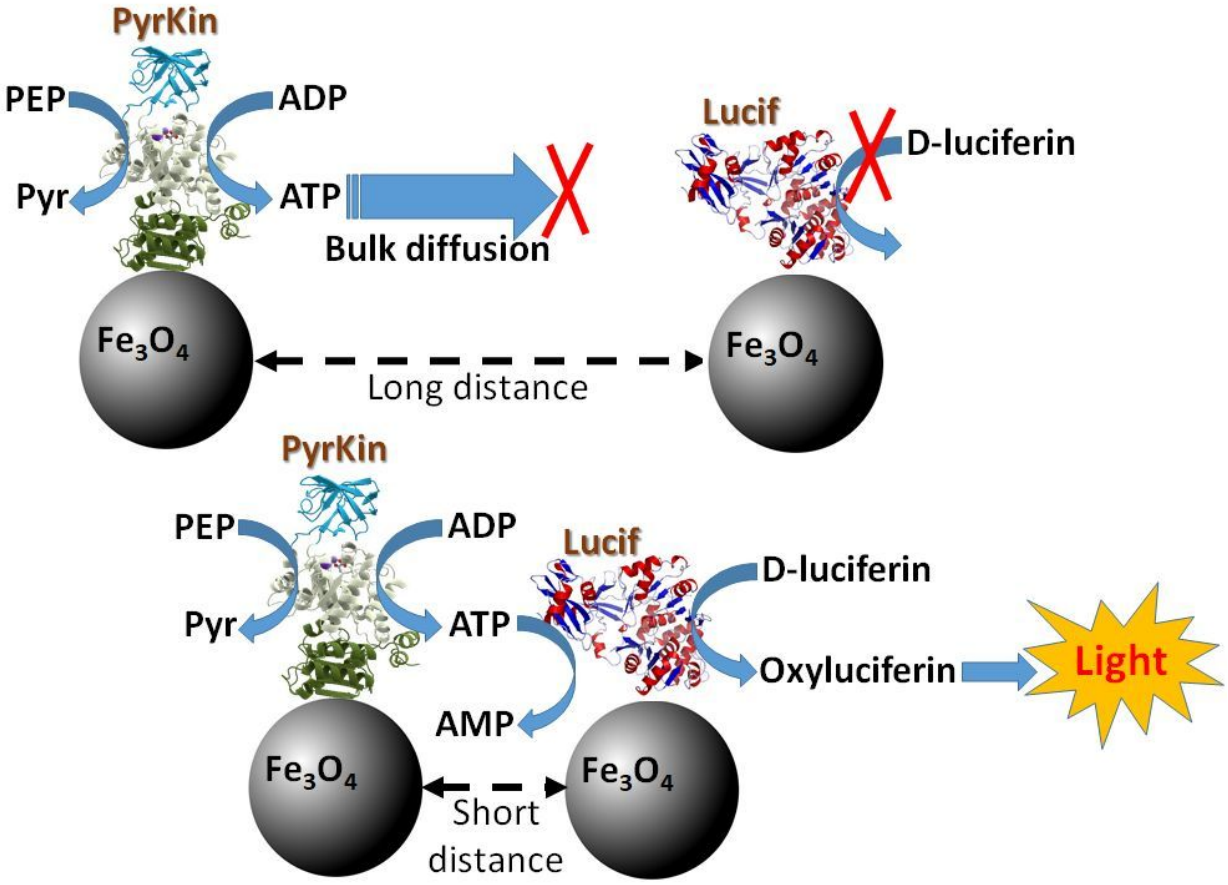

Figure 1. Schematic presentation of the reaction cascade catalyzed by pyruvate kinase (PyrKin) and luciferase (Lucif) bound to Fe_3O_4 magnetic nanoparticles. The reactions proceed differently for a long or short distance separating the enzymes. The distance separating them is controlled by the external magnetic field. The used abbreviations: PEP – phosphoenolpyruvate; Pyr – pyruvate; ATP, ADP and AMP – adenosine triphosphate, adenosine diphosphate, and adenosine monophosphate, respectively.

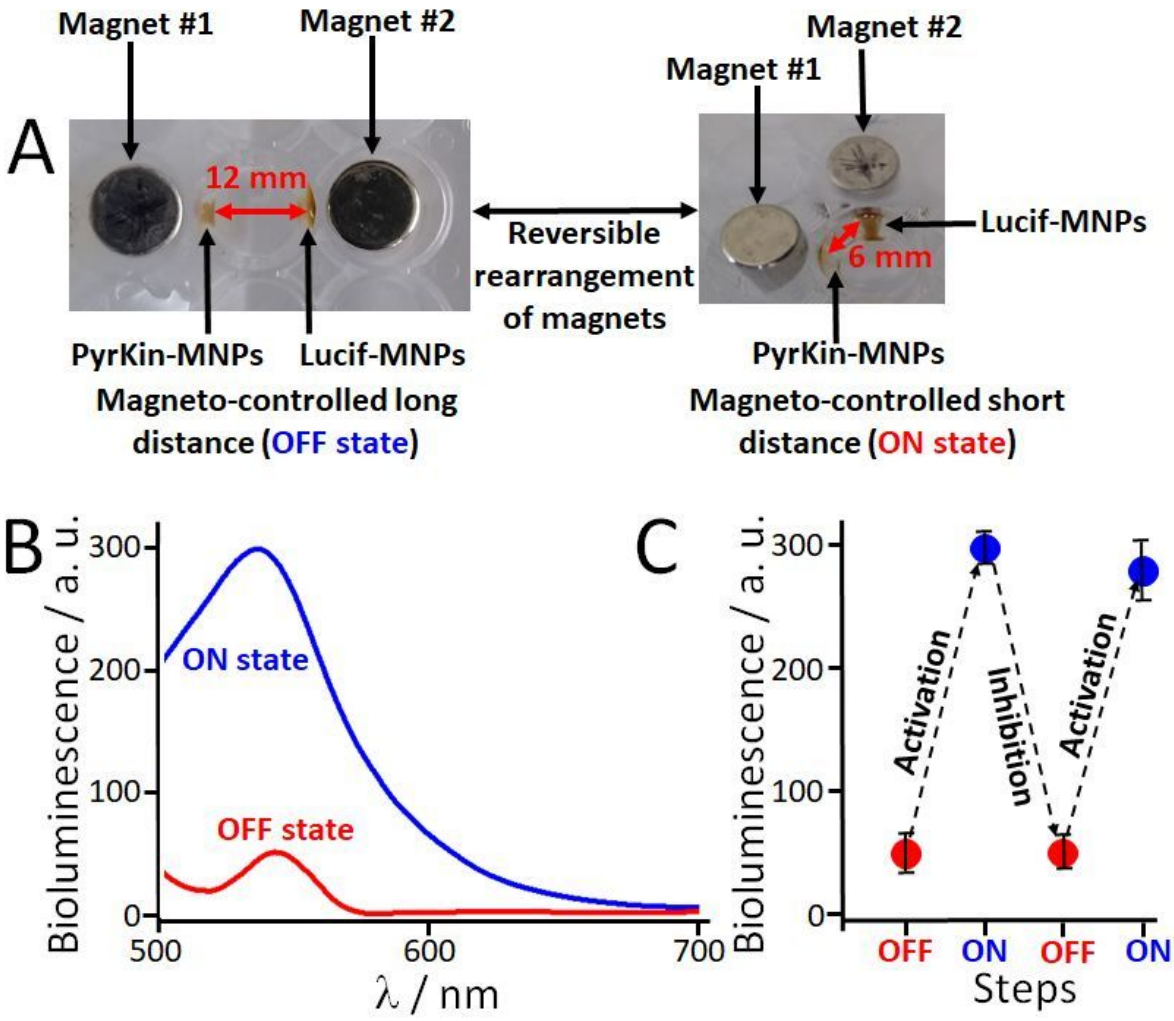

Figure 2. (A) Photos of the system setup with two kinds of the enzyme-MNPs (PyrKin-MNPs and Lucif-MNPs) separated with different (long and short) distances controlled by two magnets. (B) Bioluminescence spectra ($\lambda_{ex.}$ = 460 nm; $\lambda_{em.}$ = 535 nm, reaction time 60 min) obtained with the short (ON state) and long (OFF state) distances separating two kinds of the enzyme-MNPs. (C) Reversible activation/inhibition of the bioluminescence produced by moving the magnets to different positions: OFF states – long distance; ON state – short distance. The solution composition: 5 µM D-luciferin, 1 mM ADP, 500 µM PEP, 1 mg/mL PyrKin-MNPs, 1 mg/mL Lucif-MNPs, 50 mM TRIS buffer, pH 7.8.

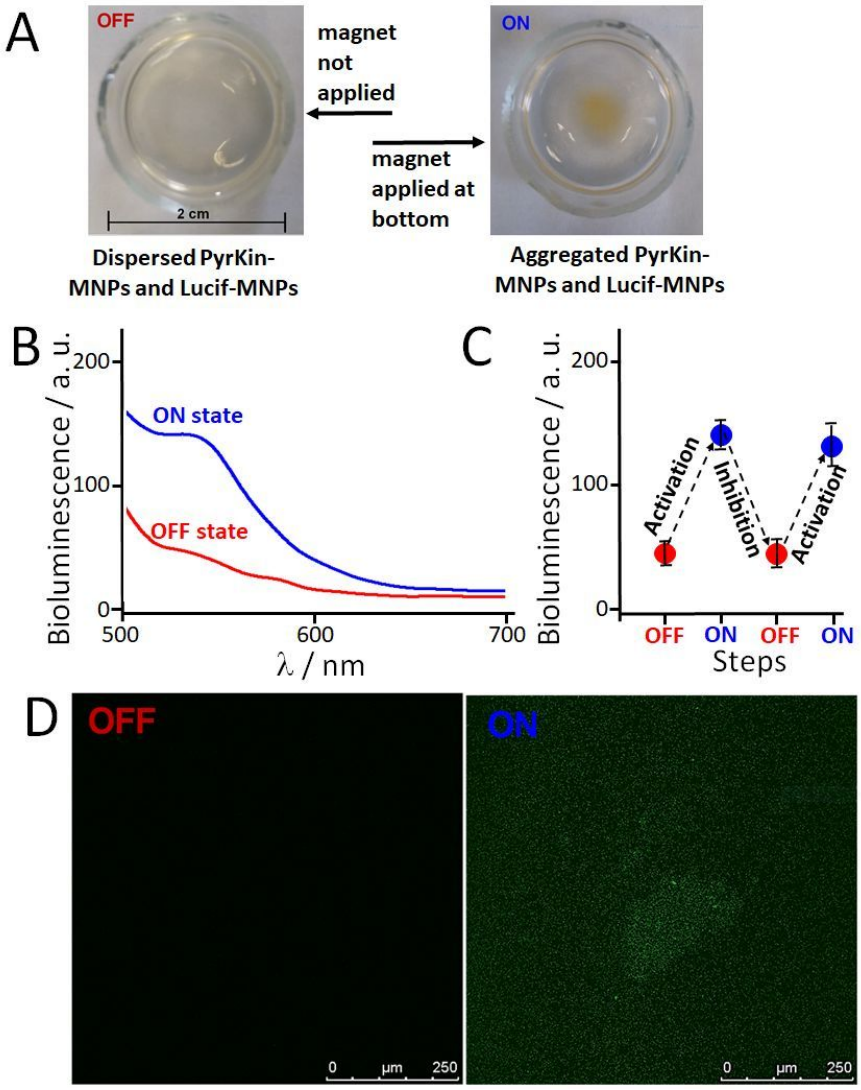

Figure 3. (A) Photos of the system setup with two kinds of the enzyme-MNPs (PyrKin-MNPs and Lucif-MNPs) in the dispersed and aggregated states (in the absence and presence of a magnet, respectively). (B) Bioluminescence spectra ($\lambda_{ex.}$ = 460 nm; $\lambda_{em.}$ = 535 nm, reaction time 60 min) obtained with the aggregated (ON state) and dispersed (OFF state) of the mixed enzyme-MNPs. (C) Reversible activation (ON state) and inhibition (OFF state) of the bioluminescence produced by adding and removing magnet. (D) Confocal microscope luminescent images of the system in the OFF and ON states controlled with a magnet. The solution composition: 5 μM D-luciferin, 1 mM ADP, 500 μM PEP, 1 mg/mL PyrKin-MNPs, 1 mg/mL Lucif-MNPs, 50 mM TRIS buffer, pH 7.8.