Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supporting Information

Magneto-Controlled Biocatalytic Cascade with a Fluorescent Output

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Experimental

Materials

Luciferase (Lucif; E.C. 1.14.14.3, from *Vibrio fischeri* (Photobacterium f)), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), phosphoenolpyruvate (PEP, ≥ 97%), adenosine triphosphate (ATP), and adenosine diphosphate (ADP), reduced nicotinamide adenine dinucleotide (NADH), L-lactic dehydrogenase (LDH; E.C. 1.1.1.27, from bovine muscle), were purchased from MilliporeSigma (former Sigma–Aldrich; https://www.sigmaaldrich.com/). D-Luciferin was purchased from Selleckchem (https://www.selleckchem.com/). Pyruvate kinase (PyrKin; E.C. 2.7.1.40, from rabbit muscle, lyophilized) was purchased from Lee Biosolutions, Inc. (https://www.leebio.com/). All other reagents were purchased from MilliporeSigma with 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 [1, 2].

Instrumentation

UV–Vis measurements were carried out using a Shimadzu P2041 spectrophotometer. Fluorescence (FL) measurements were performed with an excitation and emission wavelength of 460 and 535 nm, respectively, at room temperature by using a Cary 50 Fluorescence Spectrophotometer. The formation of the aggregation of the MNPs in the absence and presence of a magnetic field and the production of fluorescence around the aggregated MNPs were investigated with a confocal microscope (Leica Confocal Microscope LM6, Leica Microsystems, Buffalo Grove, IL, USA).

Synthesis of silica-coated MNPs

The MNPs were synthesized using a co-precipitation method as described elsewhere [1]. Iron chloride salts, 4.43 g $FeCl_3 \bullet 6H_2O$ and 1.625 g $FeCl_2 \bullet 4H_2O$ were dissolved in 190 mL of water with a mole ratio of 2 Fe^{3+} : Fe^{2+} at room temperature (22 $\pm$ 2 °C) under magnetic stirring. Then, 10 mL of 30% ammonium hydroxide was added immediately, and then the solution was stirred for additional 10 minutes. Formation of black MNPs was observed. MNPs were collected using a magnet, followed by washing 3 times with water. The surface of MNPs was stabilized with citrate ions by rapid washing with 2 M nitric acid and the addition of 5 mL of 0.5 M trisodium citrate in water, while maintaining pH 2.5. After stirring for 1.5 h, MNPs were magnetically isolated, washed with water 2 times, and diluted to 100 mL. The final concentration of MNPs was 2 % wt. The silica-coated MNPs were prepared for further surface modification following Stöber method [1]. 3 mL of the MNP stock solution was diluted in a mixture of 160 mL ethanol and 27 mL of water in a round bottom flask. Then, 5 mL of ammonium hydroxide was added. After the colloids were sonicated for 10 min, the colloid-containing flask was placed in an ice bath. While colloids were kept stirring, 500 µL of tetraethyl orthosilicate (TEOS) was added slowly. The reaction proceeded at 0 °C for 3 h under magnetic stirring. The reaction was stopped by adding 250 µL of 30 % hydrochloric acid. Precipitation of silica-coated MNPs was observed. Precipitation was magnetically collected and washed with water and ethanol 3 times. The final product was stable and dispersed in 80 mL of ethanol for further modification.

Grafting of PAA-b-PEGMA block-copolymer from the nanoparticles surface

The PAA-*b*-PEGMA block copolymer was grafted from the surface of the nanoparticles using an activator generated by electron transfer for atom transfer radical polymerization (AGET-ATRP mechanism) [1] First, ATRP initiator, α-bromoisobutyryl bromide (BIBB), was immobilized on the surface of the silica-coated MNPs. Prepared silica-coated MNPs were dispersed in 80 mL of ethanol and sonicated for 10 min, followed by the addition of 1 mL of (3-aminopropyl)-triethoxysilane (APTES) to introduce amino functional groups at the surface. The reaction was

carried out at room temperature for 20 h under magnetic stirring. The APTES immobilized nanoparticles were washed 2 times with ethanol and dichloromethane. The nanoparticles were dispersed in 80 mL of dry dichloromethane and sonicated for 20 min to achieve stable dispersion. Afterward, 1.6 mL of triethylamine (TEA) and 800 µL of BIBB were added into the dispersion while magnetically stirring. After 1.5 h. nanoparticles were collected using the centrifuge at 12000 rpm for 15 min, and the collected product was washed three times with ethanol. The nanoparticles bearing ATRP initiators were prepared. Polymerization was conducted in two consecutive AGET-ATRP steps. First, poly(*tert*-butyl acrylate) (PtBA) was grafted with tBA monomer. Then, the poly(ethylene glycol) methyl ether acrylate (PEGMA) block was continuously grafted using the same AGET-ATRP method. Finally, PtBA block was hydrolyzed to convert it into polyacrylic acid (PAA) block, bearing carboxylic functional groups for further conjugation of enzymes.

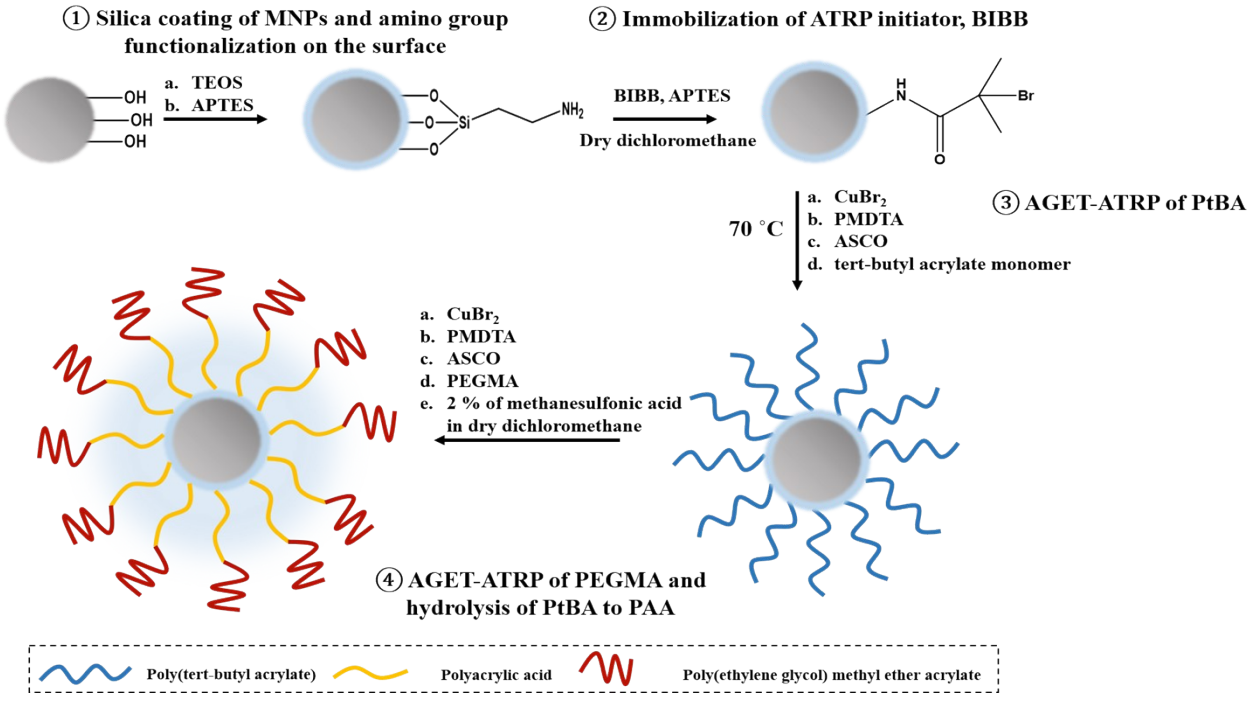


Figure S1. Schematic of modification of MNPs.

Polymerization: BIBB immobilized silica-coated MNPs, 210 µL of 0.1 M $CuBr_2$, and 320 µL of 0.5 M *N*,*N*,*N*′,*N*′′,*N*′′-pentamethyldiethylenetriamine (PMDTA) were added to 30 % of inhibitor-

removed tBA monomer in 45 mL of ethanol solution. Dissolved oxygen in the solution was removed by purging the solution with argon gas for 20 min. Then, 500 µL of ascorbic acid (ASCO) (0.1762 g/mL) was dropwise added into the polymerization reactor while degassing with argon. The polymerization proceeded for 30 min at 70 °C. The reaction was stopped by opening the cap and cooling the reactor. The particles were washed with ethanol three times and collected using a centrifuge. Grafting a second PEGMA block was prepared by a similar procedure. 25 % of inhibitor-removed PEGMA in 45 mL of ethanol solution was polymerized for 1 h at room temperature. The resulted MNPs were washed with ethanol three times, followed by the conversion of PtBA to PAA in 2 % methanesulfonic acid in 50 mL of dry dichloromethane for 5 min. After hydrolysis, particles were washed three times with ethanol and dried at 60 °C in the oven overnight. Powder form of PAA-b-PEGMA grafted silica MNPs were easily dispersed in water and formed stable colloidal status with an average size of 300 nm and zeta potential (ζ) = -30 mV at pH 7.4, characterized by dynamic light scattering (DLS).

Preventing aggregation of the magnetic nanoparticles in the absence of a magnetic field is an extremely important issue in the present project, as well as in some other projects performed by us and reported recently [1]. The polymer brush bound to the solid core is ended with polyacrylic acid, which provides negative charges at the polymer shell. The negatively charged nanoparticles are electrostatically repulsed from each other, thus preventing their aggregation in the absence of the external magnetic field. Importantly, when the particles are magnetized in the presence of the magnetic field, the magnetic attraction overcomes the electrostatic repulsion, thus resulting in the nanoparticles aggregation. The repulsion (electrostatic) and attraction (magnetic) forces should be optimized to prevent the nanoparticle aggregation in the absence of the magnetic field and allow their aggregation in the presence of the magnetic field. This optimization was performed experimentally.

Magnetization of the magnetic nanoparticles

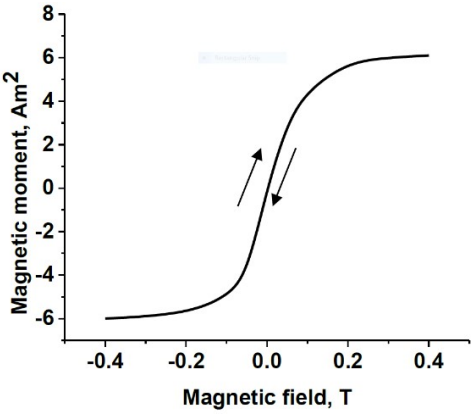


Figure S2. Magnetization function of the nanoparticles.

In order to aggregate in the presence of the external magnetic field the nanoparticles must be magnetized (note that they are paramagnetic). We characterized the magnetization features of the nanoparticles [1]. Magnetization of the nanoparticles functionalized with the polymer shell and immobilized enzymes, expressed as the dependence of the magnetic moment vs. magnetic field intensity corresponds to the function expected for the paramagnetic nanospecies. The magnetization reaches saturation at ca. 0.2 T and the experiments were performed upon applying magnetic field with the intensity of 0.4 T to ensure the complete (saturated) magnetization of the nanoparticles. The intensity of the applied magnetic field was controlled by the distance between the reaction system and the permanent magnet.

Functionalization of magnetic nanoparticles with Lucif and PynKin

Both enzymes (Lucif and PyrKin) were immobilized on the polymer-coated magnetic nanoparticles (MNPs) using the standard carbodiimide chemistry. Carboxylic acid groups of the poly(acrylic acid) (PAA) polymeric layer were reacted with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide – *N*-hydroxysuccinimide (EDC–NHS) reagents to form an intermediate active ester, which was then reacted with primary amino groups (lysine residues)

of Lucif or PyrKin to form covalent amide bonds. First, the MNPs (1 mg/ml) were dispersed in a 0.5 mL 2-(*N*-morpholino)ethanesulfonic acid (MES buffer) solution (10 mM, pH 8.0). Then, 19 mg of EDC and 11 mg of NHS were added to the nanoparticle dispersion and the mixture was shaken at a low speed at room temperature for 1 hr. The MNPs were then concentrated with a magnet and rinsed twice with (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid) solution (HEPES buffer). Thereafter, the MNPs were mixed with 0.5 mL (1 mg/mL) of the Lucif prepared in a MES buffer (20 mM, pH 5.0). The flask with the solution was covered with aluminium foil and was shaken at a low speed at room temperature for 1 hr. The Lucif-functionalized MNPs dispersion was magnetically collected and was washed twice with a MES buffer to remove unbound Lucif enzyme. Finally, the Lucif-functionalized MNPs were dispersed into 200 µL of 2-amino-2-hydroxymethyl-propane-1,3-diol solution (Tris-HCl buffer) (50 mM, pH 7.8) and were stored in dark in a refrigerator until used. A similar procedure was conducted for PyrKin, but using 0.5 mL PyrKin prepared in a MES buffer (20 mM, pH 6.3) due to the difference of the isoelectric point of the PyrKin enzyme compared with Lucif.

Calculation of enzyme activity

One unit (U) of enzyme (either PyrKin or Lucif) activity is defined as the amount of the enzyme required to produce 1.0 µmole of a product from substrate in one minute, and was calculated using the following equation [3,4]:

$$\text{Enzyme Activity} = \frac{\Delta OD \times V_{total}}{[\text{Enzyme}] \times \varepsilon \times V_{enzyme}}$$

Where, enzyme activity: (nmol $min^{-1}mg^{-1}$)

ΔOD: Difference in optical signal from the absorbance or fluorescence spectra (min^{-1})

V_{total}: Total volume in a cuvette (mL)

[Enzyme]: enzyme concentration (mg/mL)

ε: Molar extinction coefficient; (for NADH (at 340 nm) and luciferin (at 530 nm) are 6.3 and 2.0 L/mmol, respectively, for an optical path length of 1.0 cm)

V_{enzyme}: enzyme solution volume in cuvette (mL)

Characterization of cascade OFF-ON assay by fluorescent confocal microscopy

The formation of the green fluorescence gradient upon applying external magnetic field was investigated with a confocal microscope (Leica Confocal Microscope LM6, Leica Microsystems, Buffalo Grove, IL, USA). Green fluorescence originates from oxidation of D-luciferin substrate in the presence of ATP (2 mM) in a Tris-HCl buffer (pH 7.8). Excitation and emission wavelengths are $\lambda_{ex.}$ = 488 nm, $\lambda_{em.}$ = 500-600 nm, respectively.

Magnetic Particles Characterization

The average size and surface charge of the MNPs were analyzed by Dynamic Light Scattering (DLS) and zeta (ζ)-potential, resulting in ca. 278 nm and -30 mV, respectively [5]. High resolution transmission electron microscopy (HRTEM) image and an average size are presented in **Figure S3**, confirming spherical shape and well dispersed particles. The number and volume of the MNPs, determined from the DLS results, are 4.65×10^{10} MNPs/mL and 50 mm^3/mL, respectively, for each enzyme bound to the MNPs.

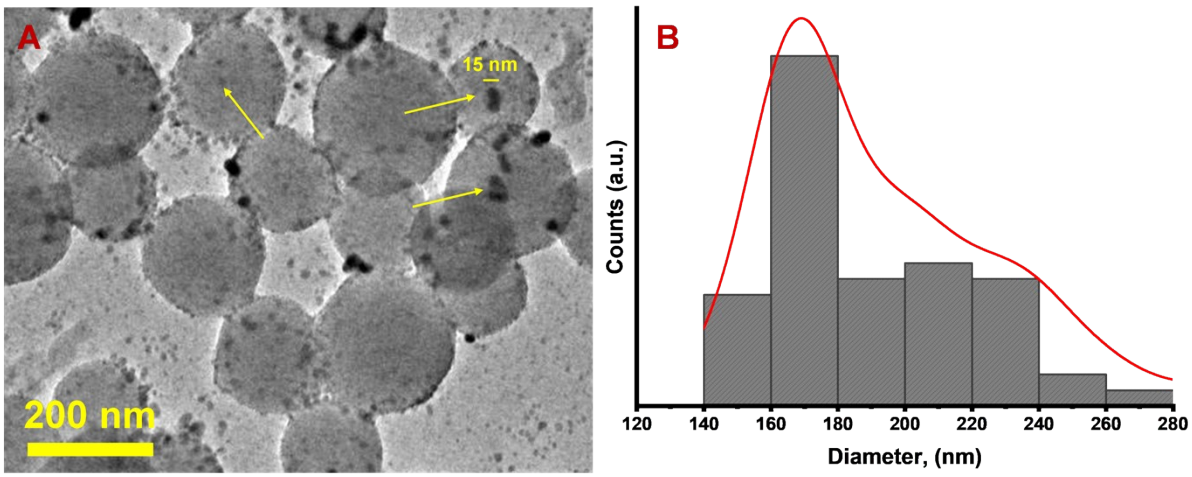


Figure S3. (A) An HRTEM image of the MNPs with **(B)** their corresponding average size. The DLS size was estimated as 276 nm, as reported elsewhere [6].

Optimization of the Lucif biocatalytic activity

The activity of the Lucif enzyme was first optimized. Different concentrations of the Lucif enzyme in the range of 0 – 1.5 mg/mL (i.e., 0, 0.5, 1, 1.25, and 1.5 mg/mL) were tested and the FL responses upon addition of the D-luciferin substrate (50 µM) are shown in **Figure S4AB**. Based on the obtained results, we selected 1 mg/mL Lucif concentration to move forward to the next step. Thereafter, different concentrations of D-luciferin in the range of 0 – 200 µM were added to the Lucif solution (1 mg/mL) and the ΔFL: FL($[D\text{-Luc.}]_{=X}$ - $[D\text{-Luc.}]_{=0}$ was recorded (**Figure S4C**). The result shows that the FL response of the solution increases with the increasing concentration of the substrate D-luciferin with saturation in the range of 150-200 µM.

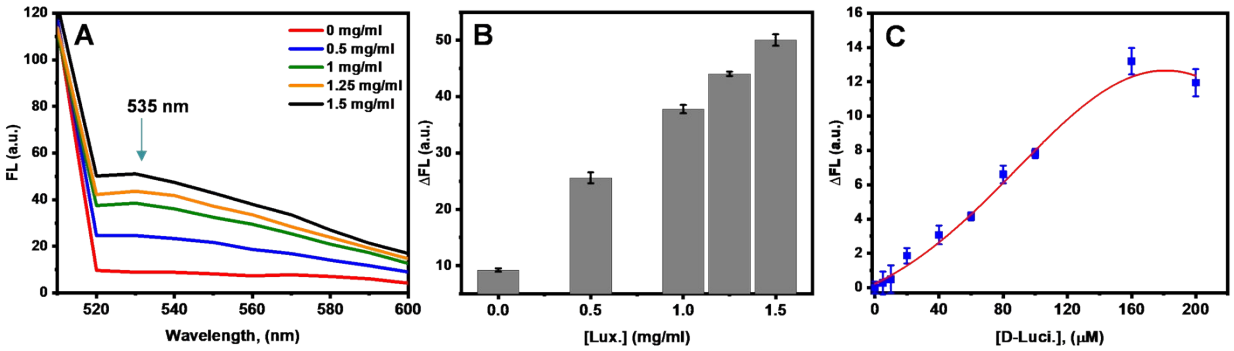


Figure S4. (A) The FL spectra of D-luciferin (50 µM) in response to the addition of different concentrations of luciferase. **(B)** Histograms of the ΔFL of D-luciferin (50 µM) at the maximum ($\lambda_{em.}$ = 535 nm) vs different concentrations of luciferase. **(C)** The ΔFL of different concentrations of D-luciferin ($\lambda_{em.}$ = 535 nm) using luciferase concentration of 1 mg/mL.

The Lucif enzyme was used to modify the surface of the MNPs at the optimum concentration of 1 mg/mL (**Figure S5A**). The enzyme was immobilized by covalent conjugation (using EDC-NHS chemistry). The enzyme activity assay, using the FL as an output, performed before and after the enzyme immobilization exhibited slower kinetics of the enzyme activity after immobilization (**Figure S5BC**) with saturation obtained after 120 min. The activity of Lucif enzyme before and after binding to the MNPs was calculated and the values were 6.75×10^3 and 3.50×10^3 nmol min^{-}

^{1} mg^{-1}, respectively. This result reveals that the enzyme retains about 50% of its original activity after immobilization at the surface of the MNPs.

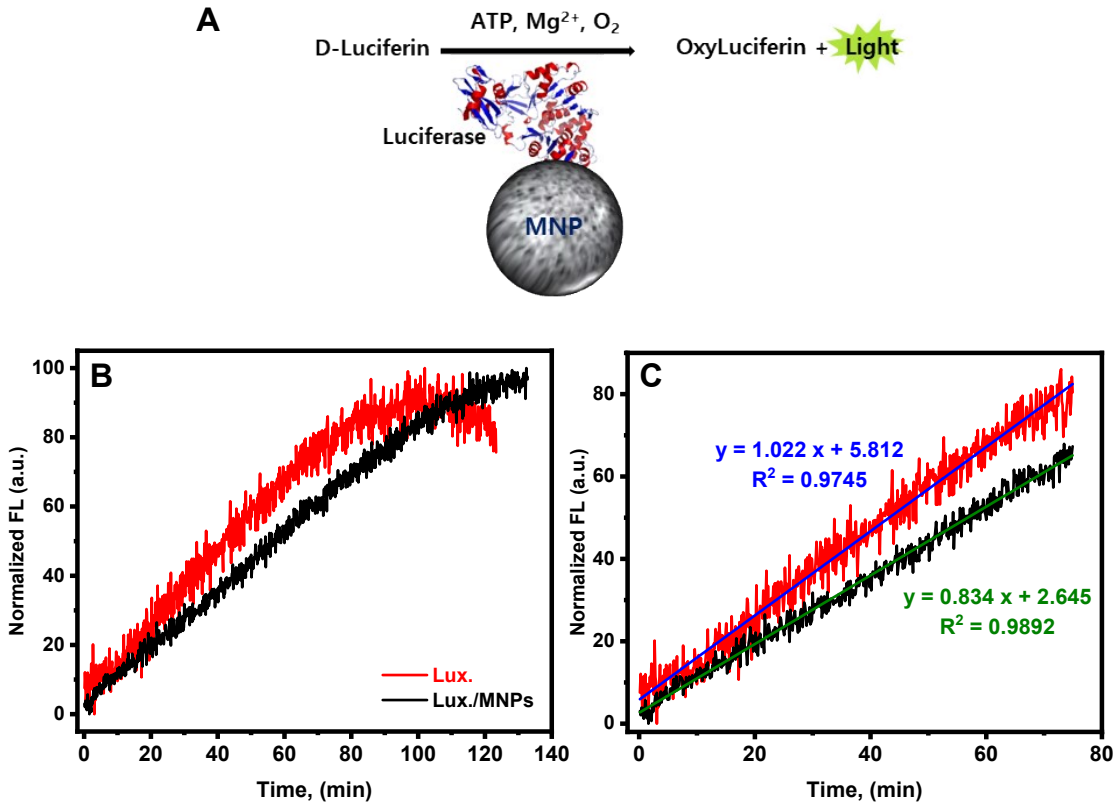


Figure S5. (A) A schematic representation of the Lucif/MNP biocatalytic operation, **(B)** Kinetics study of Lucif activity before and after immobilization onto the MNPs with **(C)** their corresponding calibration curves. [Lucif] = 1 mg/mL, [D-luciferin] = 200 µM, and ATP (2 mM) at pH 7.8, ($\lambda_{em.}$ = 535 nm).

The bioluminescence activity of the as-prepared functional Lucif/MNPs was also analyzed with a confocal fluorescent microscope as a function of the distance between the MNPs being in the aggregated and disaggregated states (**Figure S6**). The fluorescence was significantly increased upon concentrating (aggregating) the MNPs in the presence of magnetic field.

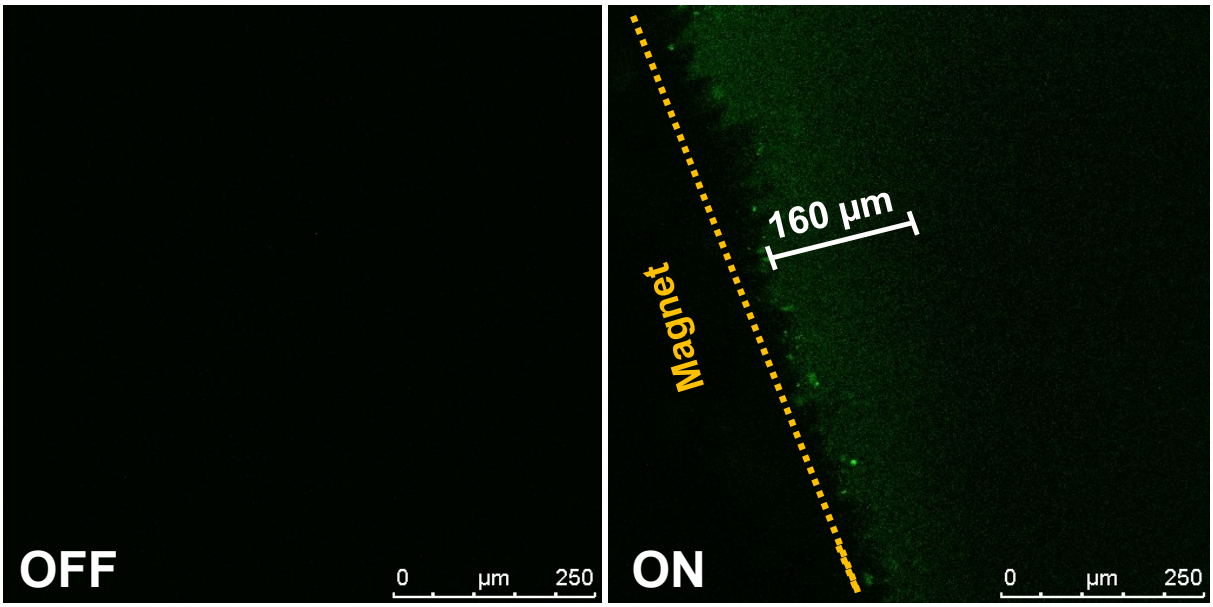
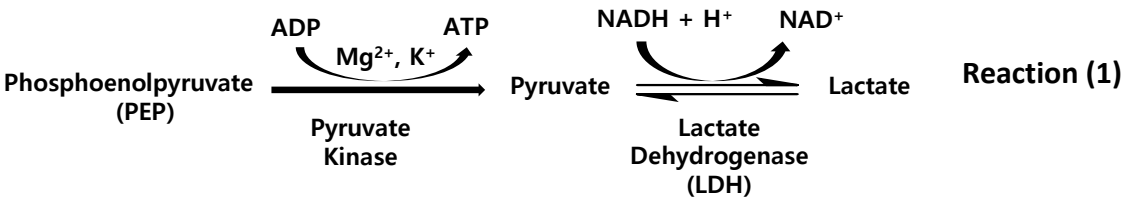


Figure S6. Confocal microscope microphotographs of the fluorescence assay in response to an external magnetic field. The OFF state (*left*): 0 min reaction; and the ON state (*right*) after 30 min of reaction, both measured in the presence of an external magnetic field. The green fluorescence originates from the oxidation of D-luciferin (50 µM) substrate in the presence of ATP (2 mM) in a Tris-HCl buffer (pH 7.8). Excitation and emission wavelengths are $\lambda_{ex.}$ = 488 nm, $\lambda_{em.}$ = 500-600 nm, respectively.

Similarly, the biocatalytic operation of pyruvate kinase (PyrKin) was optimized in the soluble and immobilized states. To optimize the PyrKin concentration, the enzyme activity was first tested in a solution. PyrKin catalyzes the irreversible transfer of a phosphate ion from phosphoenolpyruvate (PEP) to ADP yielding pyruvate and ATP (**Reaction 1**). The assay is based on a reaction in which the regeneration of the hydrolyzed ATP is coupled to the oxidation of NADH. The LDH-catalyzed reaction speed can be easily measured using UV-Vis spectroscopy by monitoring decrease in absorbance peak at 340 nm attributed to the oxidation of NADH to NAD^+ [7].



The PyrKin enzyme activity assay, as expressed above in **Reaction 1** and **(Figure S7A)**, was performed before and after the enzyme immobilization on the MNPs. **Figure S7B** shows the oxidation of NADH to NAD^+ with the corresponding UV-Vis characteristic peak at 340 nm. The kinetic study of the enzyme activity before and after immobilization (**Figure S7CD**) exhibited almost a similar behavior with a saturation achieved within about 15 min. The activity of Lucif enzyme before and after functionalization its immobilization on the MNPs was calculated and the values were 2.16×10^3 and 1.58×10^3 nmol min^{-1} mg^{-1}, respectively. From this result, one can conclude that the PK enzyme maintains about 70% of its activity after immobilization at the surface of the MNPs.

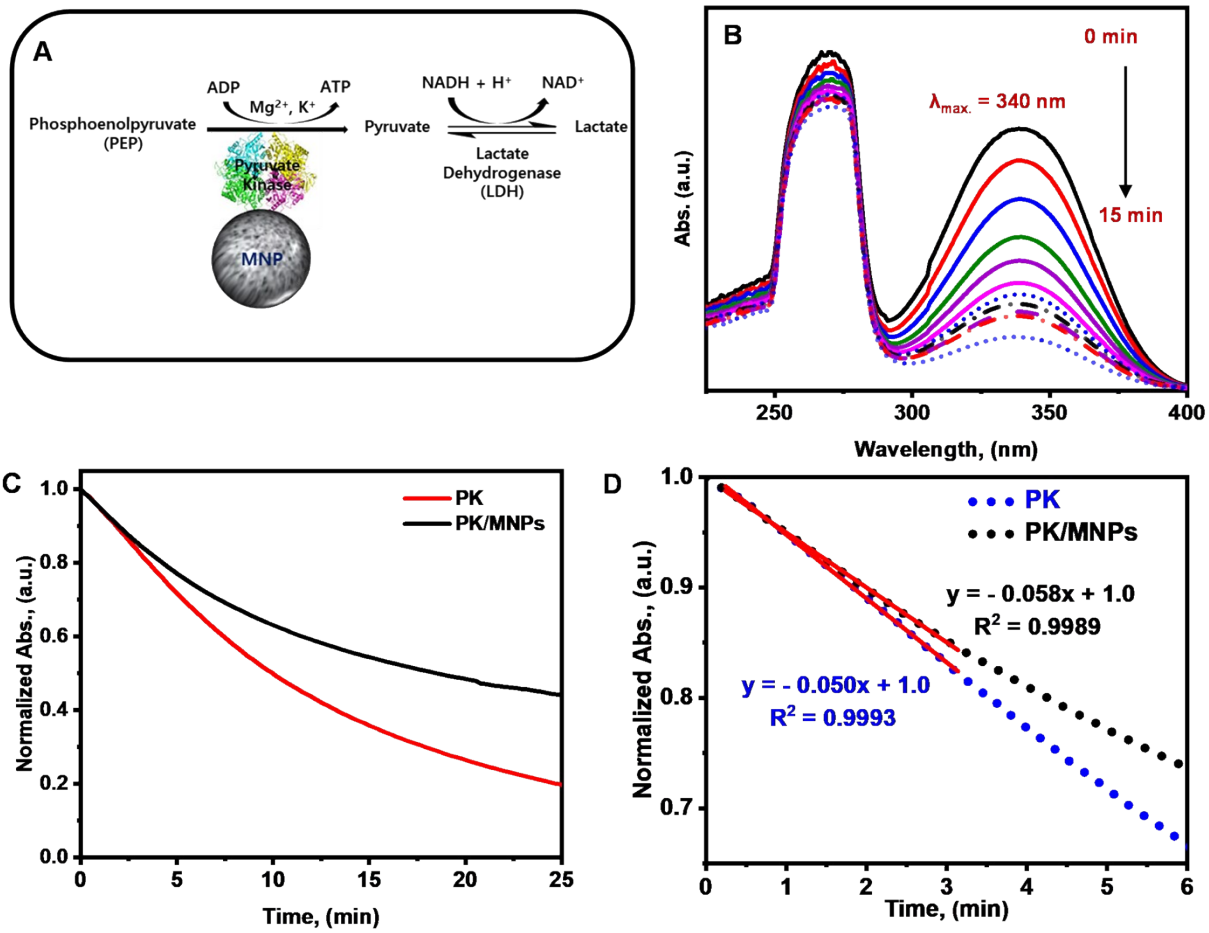


Figure S7. (A) A schematic representation of the functionalized PyrKin/MNP with **(B)** the corresponding UV-Vis spectra of the assay versus time (0 - 15 min). **(C)** Kinetic study of PyrKin activity before and after immobilization on the MNPs with **(D)** their corresponding calibration curves. [PK] = 1 mg/mL, at pH 7.8, ($\lambda_{max.}$ = 340 nm).

Further confirmation of the successful immobilization of both enzymes (Lucif and PyrKin) onto the surface of the MNPs was also provided by the UV-Vis spectra (**Figure S8**). As can be seen from the spectra of both systems, the enzyme-functionalized MNPs (blue line) shows the presence of the MNPs (black line) indicating that the enzymes were successfully functionalized onto the surface of the MNPs.

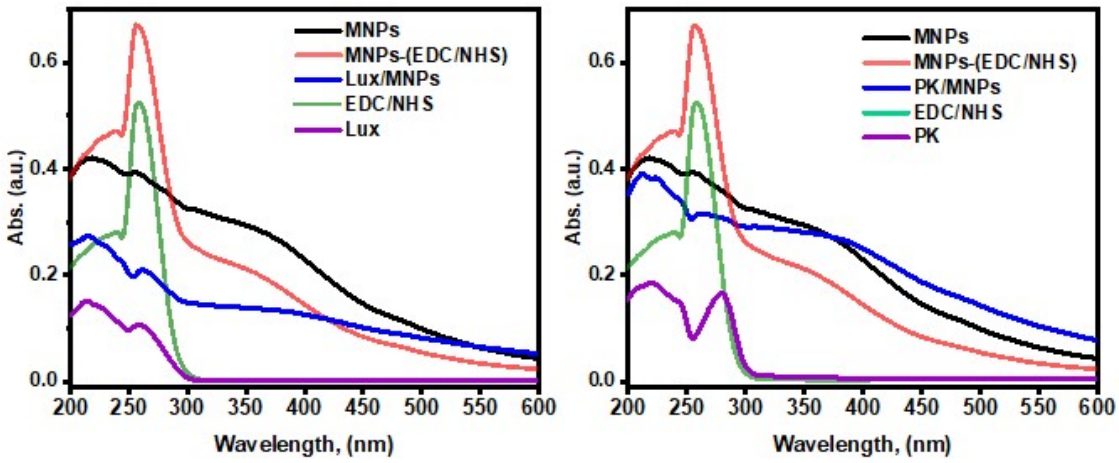


Figure S8. Absorbance spectra of MNPs before and after Lucif (*left*) and PyrKin (*right*) immobilization.

References

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