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**Radiofrequency treatment enhances the catalytic function of an
immobilized nanobiohybrid catalyst**

Boi Hoa San^{1,2}, Eun-Ju Ha³, Hyun-jong Paik^{3*}, and Kyeong Kyu Kim^{1,2*}

¹Sungkyunkwan Advanced Institute of Nanotechnology (SAINT), Sungkyunkwan
University, Suwon 440-746, Korea

²Department of Molecular Cell Biology, Samsung Biomedical Research Institute,
Sungkyunkwan University School of Medicine, Suwon 440-746, Korea

³Department of Polymer Science and Engineering, Pusan National University, Busan
609-735, Korea

*Correspondence should be addressed to

Kyeong Kyu Kim (kyeongkyu@skku.edu); Tel.: +82-31-299-6136, Fax: +82-31-299-
6159;

Hyun-jong Paik (hpaik@pusan.ac.kr); Tel.: +82-51-510-2402, Fax: +82-51-513-7720;

Abstract

Biocatalysis, the use of enzymes in chemical transformation, has undergone intensive development for a wide range of applications. As such, maximizing the functionality of enzymes for biocatalysis is a major priority to enable industrial use. To date, many innovative technologies have been developed to address the future demand of enzymes for these purposes, but maximizing the catalytic activity of enzymes remains a challenge. In this study, we demonstrated that the functionality of a nanobiocatalyst could be enhanced by combining immobilization and radiofrequency (RF) treatment. Aminopeptidase PepA-encapsulating 2-nm platinum nanoparticles (PepA-PtNPs) with the catalytic activities of hydrolysis and hydrogenation were employed as multifunctional nanobiocatalysts. Immobilizing the nanobiocatalysts in a hydrogel using metal chelation significantly enhanced their functionalities, including catalytic power, thermal-stability, pH tolerance, organic solvent tolerance, and reusability. Most importantly, RF treatment of the hydrogel-immobilized PepA-PtNPs increased their catalytic power by 2.5 fold greater than the immobilized PepA. Our findings indicate that the catalytic activities and functionalities of PepA-PtNPs are greatly enhanced by the combination of hydrogel-immobilization and RF treatment. Based on our findings, we propose that RF treatment of nanobiohybrid catalysts immobilized on the bulk hydrogel represents a new strategy for achieving efficient biocatalysis.

1. Introduction

Enzymes are widely used as biocatalysts for many applications, such as synthetic chemistry, pharmaceuticals, environmental treatment, and food technology¹ because their ability to catalyze chemical transformations is highly selective under mild reaction conditions^{1,2}. Although many innovative techniques have been developed to meet the future demand for enzymes as biocatalysts³, their industrial applications are often hindered by several reasons including a lack of long-term stability, low catalytic power, difficulties in recovery and recycling, and protein contamination in the final product⁴. Thus there is great interest in developing new technologies necessary to improve the functionality of biocatalysts.

Recent efforts toward creating nanobiocatalysts or nanobiohybrid catalysts, which can be achieved by combining biological enzymes with inorganic nanomaterials, improved the functionality of biocatalysts and expanded their application repertoires. The versatility and reaction specificity of nanobiohybrid catalysts have been demonstrated with the generation of multifunctional hybrid materials with novel properties⁵⁻¹¹. For example, PepA, a dodecameric bacterial aminopeptidase from *Streptococcus pneumoniae*, has been used as a protein shell (PS) inside which platinum nanoparticles (PtNPs) with diameters ranging from 0.9 to 3.2 nm can be synthesized¹². PepA-PtNPs worked as a multifunctional bio-inorganic catalyst showing both hydrolysis and hydrogenation activities in addition to enhanced stability¹². Furthermore, this combination improves other functionalities of the final catalyst, including biocompatibility, stability in organic solvents, and sustainability¹². Using similar approaches it was also demonstrated that integration of various proteins with PtNPs enables the construction of biocompatible quenchers of the reactive oxygen species (ROS)¹³.

Immobilization of enzymes has been employed for process optimization, especially for industrial applications of biocatalyzed reactions¹⁴ because it can enhance enzyme stability, facilitate easy separation from the reaction media, and reduce enzyme consumption. Immobilized enzymes are also used in other applications including medical therapy, pharmaceuticals, biosensors, and water remediation¹⁵. Accordingly, many immobilization strategies have been developed such as adsorption, entrapment, and cross-linking or covalent binding to a support^{16, 17}. Hydrogels, which are water-insoluble hydrophilic polymeric networks made of biopolymers, synthetic resins, or inorganic polymers, are widely used as supports for enzyme immobilization¹⁸⁻²⁰. They have been proven to enhance the stability of enzymes against heat, pH, and organic solvents²¹⁻²⁶. Recently, a Ni²⁺-poly (2-acetamidoacrylic acid) (Ni²⁺-PAAA) hydrogel system was introduced for purification and immobilization of histidine-tagged proteins²⁷⁻²⁹. In addition, PAAA hydrogels have been utilized in diverse biotechnological applications on account of their water-absorbing capacity^{21, 22, 24}.

Radio frequency (RF) waves or currents have been intensively studied as a way to deliver heat or to activate the molecules for various applications including food technology^{30, 31} and inducing hyperthermia in medical treatment^{32, 33}. Although it was reported that acetylcholinesterase (AChE) in neuroblastoma cells showed enhanced activity when exposed to RF radiation of 147 MHz from 7.0 to 7.5 hours³⁴, the effect of RF on the enzyme was not directly investigated. In the current study, we introduced RF current to PepA-PtNPs in order to enhance the catalytic power of this nanobiocatalyst.

Engineering biocatalysts for industrial processes requires low cost, high stability, productivity, multi-functionality, and a combination thereof³. In this study,

we developed a new strategy to enhance the functionality of biocatalysts by combining three factors: integration with inorganic nanocatalysts, immobilization, and RF current treatment. We found that the histidine-tagged PepA-PtNP, a nanobiohybrid catalyst, immobilized on a Ni²⁺-PAAA hydrogel showed significantly enhanced functionality after RF treatment, and thus we propose that this technique may be useful for industrial applications of biocatalysts.

2. Materials and methods

2.1 Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received without further purification.

2.2 Sample preparation

The preparation of PepA and the synthesis of platinum nanoparticles (PtNP) using PepA were described previously^{12, 35, 36}. In brief, the histidine-tagged PepA was prepared by cloning the gene encoding PepA from *Streptococcus pneumoniae* R6 (GeneID: 15903724) into a pET-based vector with a tobacco etch virus (TEV) protease-cleavable N-terminal hexa-histidine tag³⁵. In situ synthesis of PtNPs was performed in solution by precipitating Pt^{II} inside PepA at room temperature (RT). The purified recombinant PepA mixed with K₂PtCl₄ in 10 mL buffer (50 mM HEPES, pH 8.0) at a molar ratio of 1:1000, followed by incubation for one hour with constant stirring. The final concentration of PepA and K₂PtCl₄ in the reaction mixture was 1 μM and 1 mM, respectively. After addition of 0.5 mL of 100 mM ice-cold NaBH₄ solution, the reaction mixture was incubated at RT for two hours with stirring. The reaction mixture containing the PepA-PtNPs was concentrated using Centricon centrifuge (30 kD cut-off, Sartorius, Gottingen, Germany) at 16,100 rcf for 30 minutes at 4 °C to remove the precipitate. Finally, PepA-PtNPs, which were further purified using a Superdex 200 gel filtration column (GE Healthcare, Princeton, NJ, USA) in 50 mM Tris-HCl at pH 7.5 (Buffer A), were prepared for enzyme immobilization. The concentration of PepA was determined by the Bradford assay³⁷ using bovine serum albumin (BSA) to generate the standard curve.

The poly (2-acetamidoacrylic acid) hydrogel was synthesized using a method reported previously²⁷. Briefly, 2-acetamidoacrylic acid (AAA) (500 mg; 4.00 mM),

2,2'-[(1,4-dioxo-1,4-butanediyl) diamino] bis(2-propenoic acid) (25.0 mg; 0.10 mM), and potassium persulfate (5.00 mg; 0.02 mM) were dissolved in dimethyl sulfoxide (DMSO) (1.50 mL). The poly (2-acetamidoacrylic acid) (PAAA) hydrogel was formed after heating the reaction mixture to 70 °C for two hours, and the product was washed thoroughly with distilled water (Fig. 1). The PAAA hydrogel was immersed in nickel chloride (NiCl_2 , 0.1 M) solution and stirred continuously at room temperature for 24 hours. The Ni-complexed hydrogel was washed with distilled water to remove any unbound Ni^{2+} . The amount of nickel in the Ni^{2+} -complexed PAAA hydrogel was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES).

Two-nanometer PtNPs were synthesized according to reported procedures with some modifications³⁸. Briefly, PtNPs were obtained by reducing 1 mM H_2PtCl_6 with 10 mM NaBH_4 in the presence of 3 mM sodium citrate in a 50-mL reaction mixture. The amounts of each component listed above were their final concentrations in the reaction mixture. The NaBH_4 solution was added drop-wise to the reaction mixture with constant stirring for 1 h. The final product was kept at 4 °C until further use. The concentration of citrate capped PtNPs was determined by ICP-AES.

2.3 Immobilization of PepA-PtNPs on the PAAA hydrogel

The His-tagged PepA-PtNP solution was incubated with the Ni^{2+} -PAAA hydrogel at 4 °C for two hours with continuous stirring in buffer A containing 20 mM imidazole, which was used to avoid non-specific binding of enzyme to the hydrogel. Typically, there was 60 μg of His-tagged PepA-PtNPs in 1.0 g of the fully swelled Ni^{2+} -PAAA hydrogel in the final product.

2.4 Hydrolysis activity of hydrogel-immobilized PepA-PtNPs

The aminopeptidase activities of the hydrogel-immobilized PepA-PtNPs were determined using glutamic acid-*p*-nitroanilide (Glu-*p*NA) (Bachem, Torrance, CA, USA) as a substrate³⁵. In a typical experiment, 400 μ L of reaction mixture containing 500 μ M substrate and 0.05 μ M of the soluble PepA-PtNPs or hydrogel-immobilized PepA-PtNPs in buffer A were incubated at 37 °C with shaking at 300 rpm in a thermomixer (Eppendorf, Hauppauge, NY, USA). The release of *p*-nitroanilide was then monitored using a GeneQuant™ 1300 spectrophotometer (GE Healthcare, Princeton, NJ, USA) at a wavelength of 405 nm for 30 minutes. *The concentration of PepA-PtNPs immobilized in the hydrogel was measured by eluting the PepA-PtNPs out of the hydrogel using buffer A containing 1 M imidazole. The immobilized PepA-PtNPs gel was then weighed to evaluate the final concentration of PepA-PtNPs remaining inside it.*

2.5 Hydrogenation activity of hydrogel-immobilized PepA-PtNPs

The catalytic activities of the hydrogel-immobilized PepA-PtNPs were measured using *p*-nitrophenol as a substrate in the liquid phase³⁹. Briefly, a 9 mL reaction mixture containing the hydrogel-immobilized PepA-PtNPs (~2 ppm PtNP) and 0.15 M NaBH₄ was stirred for 15 minutes at RT. One milliliter of *p*-nitrophenol was added to the mixture to give a final concentration of 0.1 mM, and this was stirred until the reaction was completed. The reaction progress was spectrophotometrically monitored at 405 nm using a UV-vis spectrometer (UV550, JASCO, Tokyo, Japan) for six minutes. Each 50- μ L sample was diluted in 450 μ L of distilled water. The hydrogenation activity was determined as a first order reaction. The hydrogenation activity of the sodium citrate capped PtNPs at 2 ppm was assessed in a similar way.

2.6 Hydrolysis and hydrogenation activities of hydrogel-immobilized PepA-PtNPs in organic solvents

The organic solvents used in this study were 25% tert-butyl alcohol, 25% DMSO (dimethyl sulfoxide), and 25% DMF (dimethyl formamide). The activities of the hydrogel-immobilized PepA-PtNPs in the organic solvents were determined relative to the activities of soluble PepA-PtNPs in the same organic solvents.

2.7 Thermal and pH effects on the hydrolysis activity of soluble and hydrogel-immobilized PepA-PtNPs

Different samples were kept in buffer A at 37, 40, 50, 60, 70 and 80 °C for 30 minutes with shaking at 300 rpm in a thermomixer (Eppendorf, Hauppauge, NY, USA). The activities of the hydrogel-immobilized PepA-PtNPs at various temperatures were determined relative to those at 37 °C. The hydrolysis activities were measured in various buffers with a pH of 3.0 (citrate buffer), 4.0 (citrate buffer), 6.0 [2,2-Bis(hydroxymethyl)-2,2',2''-nitrilotriethanol] (Bis-Tris), 7.0 [tris(hydroxymethyl)aminomethane-HCl] (Tris-HCl), 8.0 (Tris-HCl), 9.0 [(2-(cyclohexylamino)ethanesulfonic acid] (CHES), and 10.0 (N-cyclohexyl-3-aminopropanesulfonic acid) (CAPS). The buffer concentration was 50 mM in the final reaction mixture. The activities of the hydrogel-immobilized PepA-PtNPs in buffers with different pH values were determined relative to the activity of soluble PepA-PtNPs.

2.8 Sustained activity of hydrogel-immobilized PepA-PtNPs

The hydrogel-immobilized PepA-PtNPs were washed and recycled, and their hydrolysis and hydrogenation activities were determined after each cycle relative to

their activity in the first reaction. The first reaction activity was set to 100% and the relative activities of the subsequent reactions were plotted.

2.9 Radiofrequency (RF) effect on hydrolysis activity of hydrogel-immobilized PepA-PtNPs

RF was applied using a capacitive electric transfer (CET) system as reported previously (Figure 7A)⁴⁰. Insulated metal plates with a diameter of 2 cm, which were connected to the RF generator, were used as active and return electrodes. Two electrodes block each end of an incubation chamber, a glass cylinder 8 cm in length and 2 cm in diameter, harboring the immobilized PepA-PtNPs (Fig. 7A). Frequency and output power were measured using an oscilloscope TDS 210 (Tektronix, Inc., Beaverton, OR, USA). A RF current of 0.35 ± 0.05 MHz with a power of 40 W was applied for the treatment. The temperature of the reaction solution containing the hydrogel-immobilized or soluble PepA-PtNPs with substrate in the reaction chamber was monitored for 30 minutes using a fiber optic temperature sensor system with an accuracy of ± 0.1 °C. The hydrolysis activity of the hydrogel-immobilized PepA-PtNPs was monitored by measuring the product over 30 minutes with and without RF treatment in the reaction chamber (Fig. 7A).

3. Results and discussion

3.1 Immobilization of PepA-PtNPs on the PAAA hydrogel

A PAAA hydrogel was synthesized using 2-acetamido acrylic acid (AAA) and 2,2'-[(1,4-dioxo-1,4-butanediyl) diamino] bis(2-propenoic acid)²⁷ as shown in Fig. 1A. The inner structure of the PAAA hydrogel consisted of hydrogen bonds between the

hydrogel matrix and water. The PAAA hydrogel was able to swell up more than 100-fold in weight compared to the dry form²⁷. Ni²⁺ was coordinated to the fully swollen hydrogel in distilled water (Fig. 1B). ICP-AES analysis showed that the number of Ni²⁺ ions per carboxylic acid group in the dry Ni²⁺-PAAA hydrogel was 0.18 (Fig. 1B).

PepA-PtNPs were prepared by synthesizing 2 nm PtNPs inside aminopeptidase PepA shells, as reported previously^{12, 13} (Fig. 1C). In order to facilitate the immobilization of PepA-PtNPs onto a Ni²⁺-PAAA hydrogel, a six-histidine tag (His-tag) was genetically added to the N-terminus of PepA³⁵ (Fig. 1C). *This design allows His-tagged PepA-PtNPs to bind the Ni²⁺-PAAA hydrogel matrix, since the coordination of six consecutive histidine residues to Ni²⁺ is a fast and reversible reaction with high binding affinity. A histidine competitor like imidazole can be used to discharge the His-tagged PepA-PtNPs from the hydrogel matrix.* PepA-PtNPs were immobilized on the PAAA hydrogel by incubating the His-tagged PepA-PtNPs and the fully swelled Ni²⁺-PAAA hydrogel together. Typically, 60 µg PepA-PtNPs were mixed with 1 g hydrogel (Fig. 1D). The final product of the immobilization reaction, referred to as immobilized PepA-PtNPs, was used for further physiochemical characterization and functional analyses.

3.2 Catalytic activities of immobilized PepA-PtNPs

It has been reported that the catalytic activities of both PepA and PtNPs can be combined in the form of PepA-PtNPs to catalyze multistep reactions¹². To further improve the catalytic activity and functionalities of PepA-PtNPs, we immobilized PepA-PtNPs on a hydrogel. Their catalytic activities under various reaction conditions including different temperatures, pH values, organic solvents, and reusability were

then compared with those of soluble PepA-PtNPs (Figs. 3-7). Blanks of PepA-only and PtNP-only were also used for comparison. The hydrolysis activities of PepA-only, soluble and immobilized PepA-PtNPs were measured by monitoring the release of *p*-nitroanilide, a product of the PepA-catalyzed hydrolysis of Glu-*p*-nitroanilide, for 90 min (Figs. 2A-B). No significant difference was seen in hydrolysis activities of the soluble and immobilized PepA-PtNPs, implying that immobilization using the PAAA hydrogel did not affect the enzymatic activity of PepA. However, PepA-only had higher activity than PepA-PtNPs, soluble or immobilized, in the initial reaction period. It has been reported that the lower activity of encapsulated PepA is due to the steric hindrance¹². The hydrogenation activities of soluble and immobilized PepA-PtNPs which were measured by monitoring the hydrogenation of *p*-nitrophenol to *p*-aminophenol for 6 min were comparable with reaction rate constants of $9.86 \times 10^{-3} \text{ s}^{-1}$ and $9.96 \times 10^{-3} \text{ s}^{-1}$, respectively (Figs. 2C-D). The PtNP-only blank was synthesized using sodium citrate as a stabilizer (Fig. S1), and shown to have about two-fold lower activity than soluble PepA-PtNPs with a reaction rate constant of $5.34 \times 10^{-3} \text{ s}^{-1}$. It has been already proven that the strong stabilizing effect of protein shells enhance the reactivity of PtNPs¹². Taken together, these results show that immobilized PepA-PtNPs possess both hydrolysis and hydrogenation activities, like soluble PepA-PtNPs¹², but has lower hydrolysis activity than PepA-only, and higher hydrogenation activity than PtNP-only.

3.3 Effect of temperature on the hydrolysis activity of immobilized PepA-PtNPs

The thermal effect on the enzymatic activity of immobilized PepA-PtNPs was measured by monitoring the amount of products released for 30 min at various temperatures ranging from 40 to 80 °C in comparison to that observed for PepA-only

and soluble PepA-PtNPs. The results revealed that the hydrolysis activity of the immobilized PepA-PtNPs increased as a function of temperature up to 60 °C, while the catalytic activity of the soluble catalysts gradually decreased with increasing temperature (Fig. 3). At 60 °C, the catalytic activity of the immobilized catalysts was 50% higher than those of the PepA-only and soluble PepA-PtNPs. When the temperature was raised up to 80 °C, the immobilized PepA-PtNPs were still 65% active, but the PepA-only and the soluble PepA-PtNPs were only 57% and 30% active, respectively. These results suggest that the enzymatic activity of PepA-PtNPs at higher temperature can be significantly improved by immobilization. The enzymatic activity at elevated temperatures is positively affected by the increased kinetic rate constant and negatively affected by decreased protein stability. The enhanced enzymatic activity of the immobilized PepA-PtNPs at elevated temperatures seems to be the result of the increased thermostability of PepA. The hydrogel matrix helps to maintain enzyme conformation at high temperatures²⁵, and thus the thermostability of PepA seems to be improved by immobilization on the Ni²⁺-PAAA hydrogel.

3.4 Effect of pH on the hydrolysis activity of immobilized PepA-PtNPs

The enzymatic activity of immobilized PepA-PtNPs at different pH values was investigated. A wide range of reaction buffers (pH 3.0 to 10.0) was used to evaluate the hydrolysis activity of the PepA-only, and the soluble and immobilized PepA-PtNPs. The PepA-only, the soluble and the immobilized PepA-PtNPs showed maximum activity at pH 10.0, 10.0 and 9.0, respectively. These results indicate that the optimal pH for PepA shifted from 10.0 to 9.0 (Fig. 4). Intriguingly, the hydrolysis activity of the immobilized PepA-PtNPs was higher than those of the PepA-only and

the soluble PepA-PtNPs at all pH levels examined, and was 20% higher at the respective optimal pH of each. These results also suggest that immobilization of PepA-PtNPs on the hydrogel promotes protein stability at various pH levels, which is consistent with findings for other immobilized enzymes^{23, 25}.

3.5 Catalytic activities of immobilized PepA-PtNPs in organic solvents

There are many advantages of using enzymatic catalysis in organic solvents, including broader range of substrate choices due to the increased solubility of the substrates in organic solvents as well as prevention of microbial contamination⁴¹⁻⁴³. However, enzymes often show relatively lower activity in organic solvents than in water, mostly due to the decreased enzyme stability in organic solvents⁴². Enzyme immobilization is often used for overcoming such problems⁴⁴. We further investigated the catalytic activity of immobilized PepA-PtNPs in various organic solvents (Fig. 5). The immobilized PepA-PtNPs exhibited significantly enhanced hydrolysis and hydrogenation activities in organic solvents (Fig. 5), implying that immobilization can contribute to the increased stability of PepA-PtNPs in organic solvents by preventing PepA deformation, which may adversely affect both the enzymatic and encapsulated PtNPs' activity.

3.6 Reusability of immobilized PepA-PtNPs

Reusability is one of the most important properties of biocatalysts in that it enhances the robustness of processes, allows greater duration of activity over multiple cycles, and increases the commercial value of biocatalysts. To test the effect of immobilization on the reusability of the PepA-PtNPs, we measured the hydrolysis and hydrogenation activities of the immobilized catalysts over several cycles (Fig. 6).

After four reaction cycles, the hydrolysis activity of the immobilized PepA-PtNPs remained at nearly 100%, but the hydrogenation activity during the fourth cycle was about 70% compared with the activity during the first cycle (Fig. 6). The decrease in hydrogenation activity was expected because the surface of PtNPs is known to be damaged during catalysis^{12, 45, 46}. These results also support the hypothesis that immobilization of PepA-PtNPs enhances protein stability and consequently the reusability of PepA-PtNPs.

3.7 The effect of RF currents on the hydrolysis activity of immobilized PepA-PtNPs

These days, electromagnetic energy has been successfully applied to the enzyme-catalyzed chemical reactions for the purpose of enhancing the enzyme efficiency. For example, irradiation of microwaves can increase reaction rates of proteases through the thermal effect⁴⁷. Since PtNPs have been proven to be an efficient thermal irradiator in response to the RF current⁴⁰, we hypothesized that the enzyme activity of PepA encapsulating PtNPs can be increased upon RF treatment. In order to investigate the use of RF in enhancing enzymatic activity, we applied an RF current of 0.35 ± 0.05 MHz with a power of 40 W to immobilized PepA-PtNPs in a reaction chamber (Fig. 7A). The temperature of the reaction chamber and the enzymatic activity of the immobilized PepA-PtNPs were monitored accordingly. Interestingly, we found that the enzymatic activity of the immobilized PepA-PtNPs accelerated considerably with RF treatment compared to that of untreated samples (Fig. 7B). Over 30 minutes of RF exposure, the temperature of the reaction chamber containing PepA-PtNPs gradually increased to about 65 °C, while the chamber temperature was maintained close to 37 °C in the absence of RF

treatment (Fig. 7B). During this period, the amount of product released from the immobilized PepA-PtNPs was 2.5-fold greater than that from the control (immobilized PepA-PtNPs without RF treatment, open circle in Fig. 7B, top). The RF-treated immobilized PepA-PtNPs consistently showed 1.6- and 4.5-fold higher activities than the soluble PepA-PtNPs with and without RF treatment, respectively (Fig. 7C). Its activity was also 2.7- and 4.5-fold higher than RF-treated and untreated PepA-only, respectively (Fig. 7C). Notably, RF treatment for 30 min was shown to enhance the activity of immobilized catalysts 3.0-fold, when compared with the incubation at 60 °C which is the optimum temperature for immobilized PepA-PtNPs, and also the final temperature achieved though 30 minutes of RF treatment (Fig. 7B). This result implies that RF treatment is more powerful than simple heat treatment. *In addition, since we have previously shown that the heat released from the PtNPs or the temperature increment in the incubation chamber was proportional to the exposure time, the concentration of PtNPs encapsulating inside PepA shell, and the power of the RF field⁴⁰, the catalytic activities of enzyme is expected to be highly maintained by properly optimizing those RF parameters.* To investigate the enhancing effect of RF on the hydrolysis activity, the temperature of the reaction chamber was placed in a 37 °C water bath during RF exposure (Fig. 7C). The reaction temperature was maintained in this way to study the net effect of RF. Under these conditions, RF-treated samples showed similar activity to the samples incubated at 37 °C (Fig. 7C). These results suggest the enhanced enzyme activity by RF-treatment is mostly contributed by the elevation of temperature due to the heating properties of platinum nanoparticles under RF current.

RF heating is promising for industrial applications because RF produces heat rapidly and uniformly with a large penetration depth and low energy consumption³⁰,

³¹. It is apparent that RF heating is advantageous over conventional methods for economic reasons, and proper selection of reactions may increase the reaction efficiency and/or reduce side products. PepA-PtNPs immobilized on a hydrogel matrix are considered as a candidate for new-generation biocatalysts that meets the needs of industrial applications³. Therefore, further design and optimization studies are needed to obtain the highest reaction activity of immobilized PepA-PtNPs with RF treatment.

4. Conclusions

We have developed a simple method for immobilizing a bioinorganic nanocatalyst, PepA-PtNPs, in a Ni²⁺-PAAA hydrogel matrix, which increases the hydrolysis activities and functionalities under various conditions compared to PepA-only or soluble PepA-PtNPs. Immobilized PepA-PtNPs showed enhanced catalytic power, thermal-stability, pH tolerance, activity in organic solvents, and reusability. We also showed that exposure of PepA-PtNPs to RF dramatically improved the catalytic power of PepA. Taken together, the results of our study indicate that combining immobilization and RF treatment is an effective strategy for the future design of nanobiocatalysts to enable their use in industrial applications.

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Figure legends

Figure 1. Schematics of hydrogel synthesis and PepA-PtNPs immobilization. A, Synthesis of the poly(2-acetamidoacrylic acid) hydrogel. B, Schematic of the Ni²⁺-PAAA hydrogel. C, Ribbon models of PepA-PtNPs. Each PepA subunit is shown in different colors, and a PtNP with a size of 2 nm is located inside a PepA shell to display the overall structure of the PepA-PtNP complex. A His-tag in the N-terminus of each PepA subunit is shown in red and indicated by a black arrow. D, Schematic of the His-tagged PepA-PtNPs bound to the Ni²⁺-PAAA hydrogel matrix.

Figure 2. Reactivity of PepA-PtNPs immobilized on a hydrogel. A, The hydrolysis activity of PepA-only (top), soluble PepA-PtNPs (middle) and immobilized PepA-PtNPs (bottom) was estimated by measuring the released product for 90 min at 37 °C. The activity was expressed as the percentage relative to the saturated point. B, Reaction scheme of the hydrolysis of Glu-*p*-nitroanilide by PepA to yield *p*-nitroanilide and glutamic acid. C, Hydrogenation activity of PtNP-only (top), soluble PepA-PtNPs (middle) and immobilized PepA-PtNPs (bottom) were measured monitoring UV-Vis spectral changes over 6 min at 1-min intervals. D, Reaction scheme of the PtNP-catalyzed hydrogenation of *p*-nitrophenol to *p*-aminophenol.

Figure 3. Thermal effects on the hydrolysis activities of PepA-only (gray bar), and soluble (light gray bar) and immobilized PepA-PtNPs (black bar). The hydrolysis activity was monitored by calculating the amount of hydrolyzed substrate over 30 min at various temperatures. Activities were expressed relative to hydrolysis at 37 °C.

Figure 4. pH effects on the hydrolysis activities of PepA-only (gray bar), and soluble (light gray bar) and immobilized PepA-PtNPs (black bar). The hydrolysis activity was monitored as described in Figure 3.

Figure 5. Hydrolysis activities (top panel) of PepA-only (gray bar), and soluble (light gray bar) and immobilized PepA-PtNPs (black bar), and hydrogenation activities (bottom panel) of PtNP-only (white bar), and soluble (light gray bar) and immobilized PepA-PtNPs (black bar) in the following organic solvents: 25% DMF (dimethyl formamide), 25% DMSO (dimethyl sulfoxide), and 25% tert-butyl alcohol. The catalytic activities of PepA-only, PtNP-only and immobilized PepA-PtNPs are shown relative to the activity of soluble PepA-PtNPs in the same organic solvents and were monitored in the same way described in Figure 3.

Figure 6. Reusability of immobilized PepA-PtNPs. The hydrolysis and hydrogenation activities of immobilized PepA-PtNPs were measured repeatedly to examine their reusability. After each measurement, the immobilized PepA-PtNPs were washed with excess buffer to remove all remaining reactants and products for the next cycle of catalysis. The activity was expressed relative to the first measurement.

Figure 7. Radiofrequency (RF) effects on the hydrolysis activity of immobilized PepA-PtNPs. A, Schematic of the CET system and incubation chamber. A RF current generated from the RF generator was applied from the active electrode (red) to the return electrode (black) through the incubation chamber. Each electrode was coated with an insulation material. An oscilloscope and a thermometer measured the electric parameters applied to the active electrode and temperatures in the incubation chamber,

respectively. Immobilized PepA-PtNPs were placed in the incubation chamber (black dot). RF of 0.35 MHz with a power of 40 W was used for the treatment. The hydrolysis activity of the immobilized PepA-PtNPs in the RF reaction chamber was monitored by measuring the concentration of products over 30 min with (closed circle) and without (open circle) RF treatment (B, top panel). The temperature change as a function of time with RF treatment was also recorded (closed circle) (B, bottom panel). C. The hydrolysis activities of PepA-only, soluble PepA-PtNPs and immobilized PepA-PtNPs are expressed relative to the activity of soluble PepA-PtNPs incubated at 37 °C without RF treatment (second lane). The activities of PepA-only, soluble and immobilized PepA-PtNPs measured at 37 °C (first, second and third lanes) and 60 °C (4, 5, and 6th lanes) are also shown for comparison. To eliminate the effect of temperature during RF treatment, the reaction chamber was placed in a water bath and the temperature was maintained at 37 °C during the reaction with RF treatment (7, 8, and 9th lanes). The relative activities of PepA-only, soluble PepA-PtNPs and immobilized PepA-PtNPs in the presence of RF treatment are indicated in the 10, 11 and 12nd lanes, respectively. The name of each sample is indicated on the x-axis along with the maintained temperatures during reaction (@37 °C or @60 °C) and RF treatment status [(-) RF and (+) RF].

Radiofrequency treatment enhances the catalytic function of an immobilized nanobiohybrid catalyst

Boi Hoa San^{1,2}, Eun-Ju Ha³, Hyun-Jong Paik^{3*}, and Kyeong Kyu Kim^{1,2*}

¹*Sungkyunkwan Advanced Institute of Nanotechnology (SAINT), Sungkyunkwan University, Suwon 440-746, Korea*

²*Department of Molecular Cell Biology, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon 440-746, Korea*

³*Department of Polymer Science and Engineering, Pusan National University, Busan 609-735, Korea*

*Correspondence should be addressed to

Kyeong Kyu Kim (kyeongkyu@skku.edu): Tel.: +82-31-299-6136, Fax: +82-31-299-6159;

Hyun-jong Paik (hpaik@pusan.ac.kr): Tel.: +82-51-510-2402, Fax: +82-51-513-7720.

Figure 1

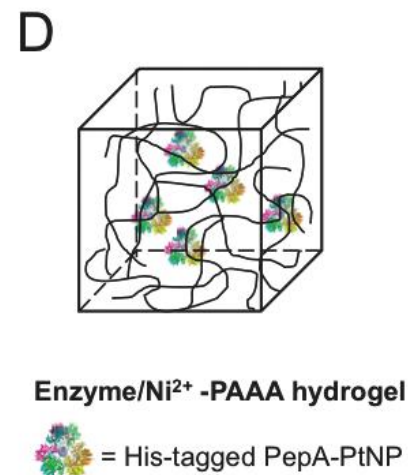
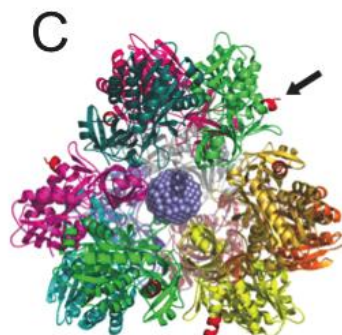
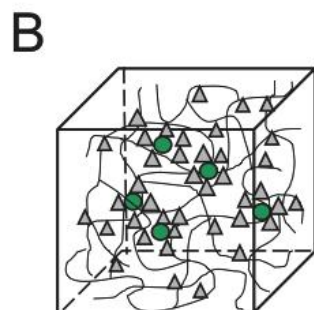
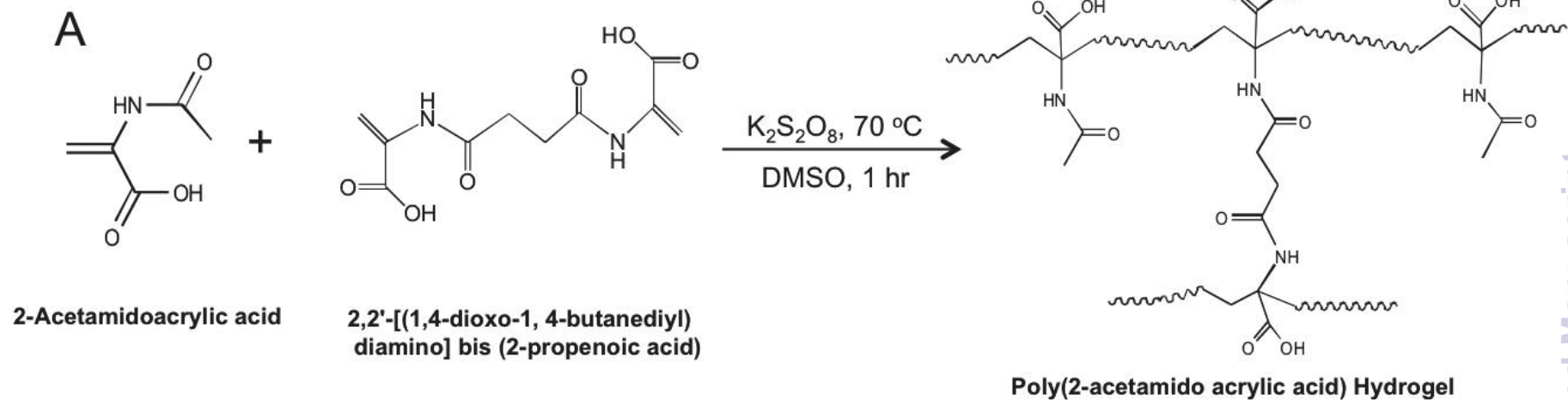


Figure 2

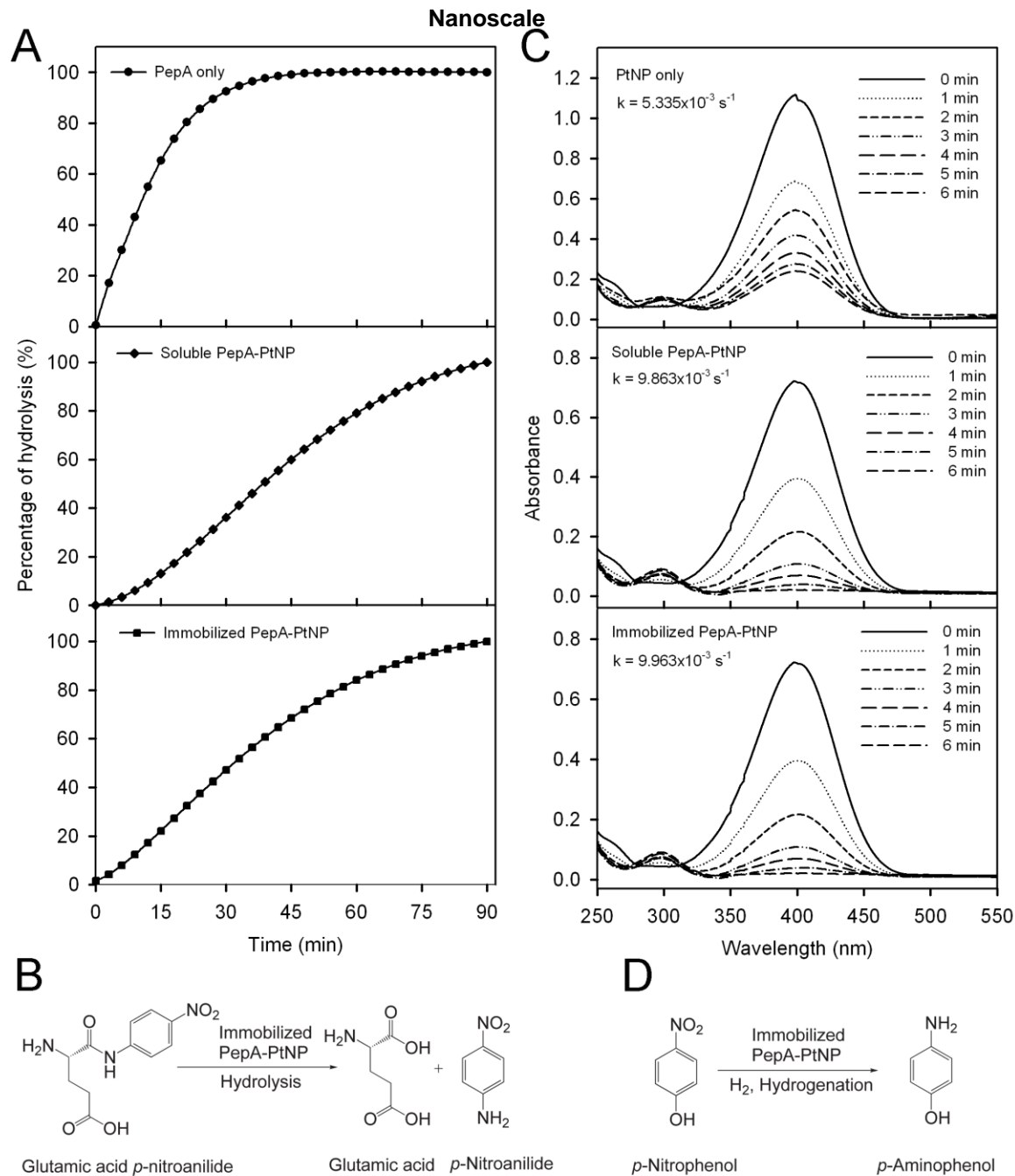


Figure 3

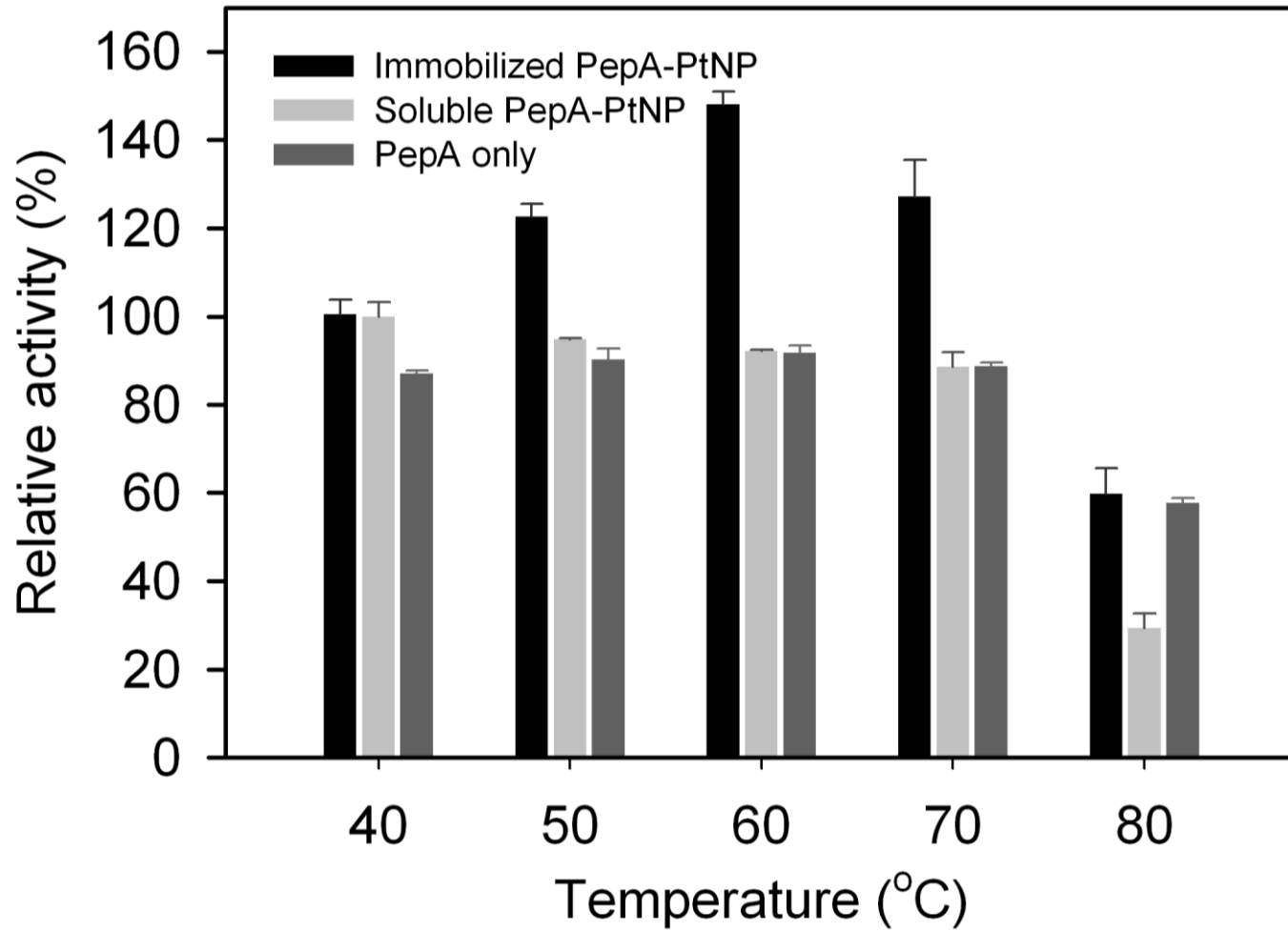


Figure 4

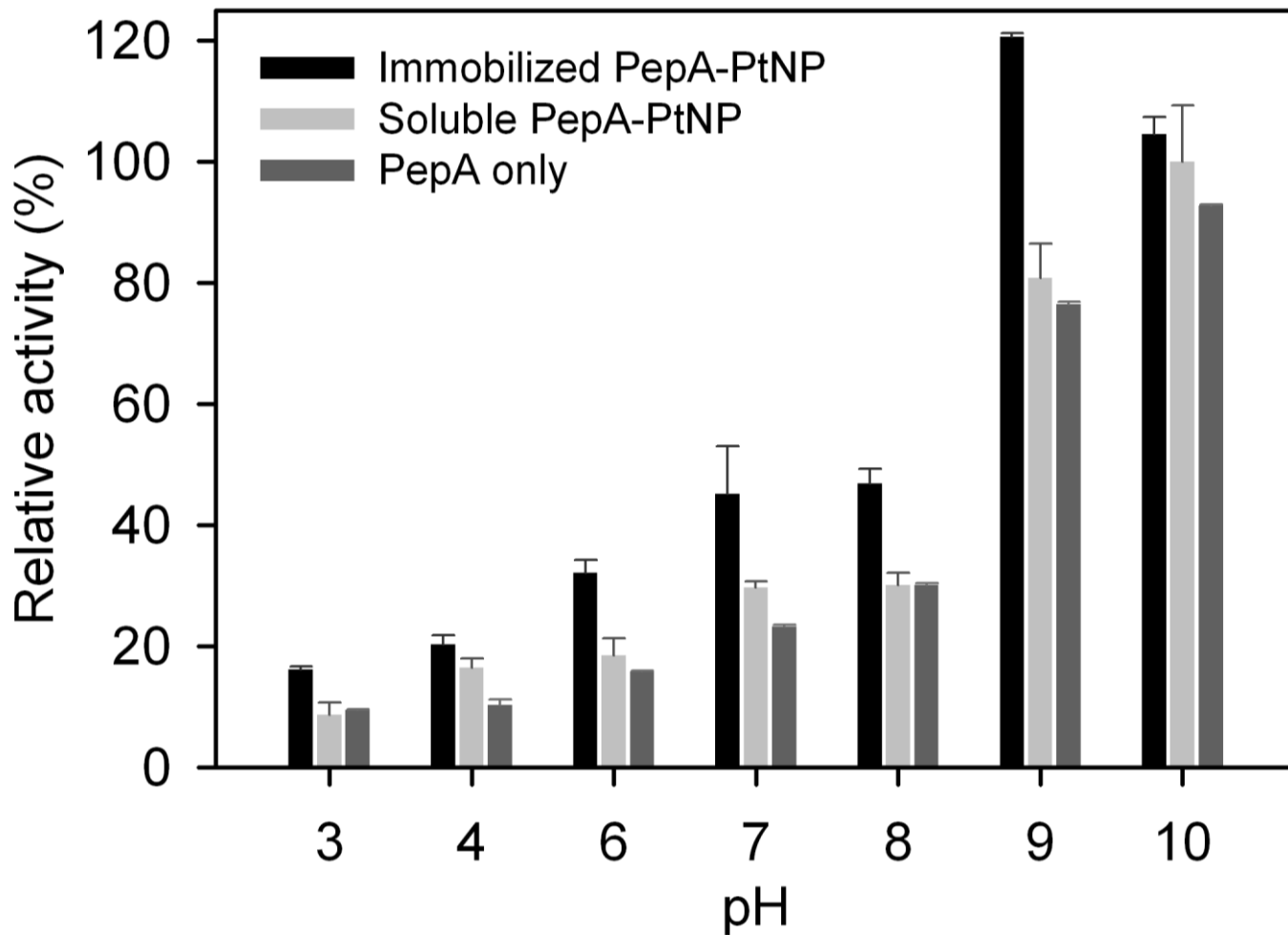


Figure 5

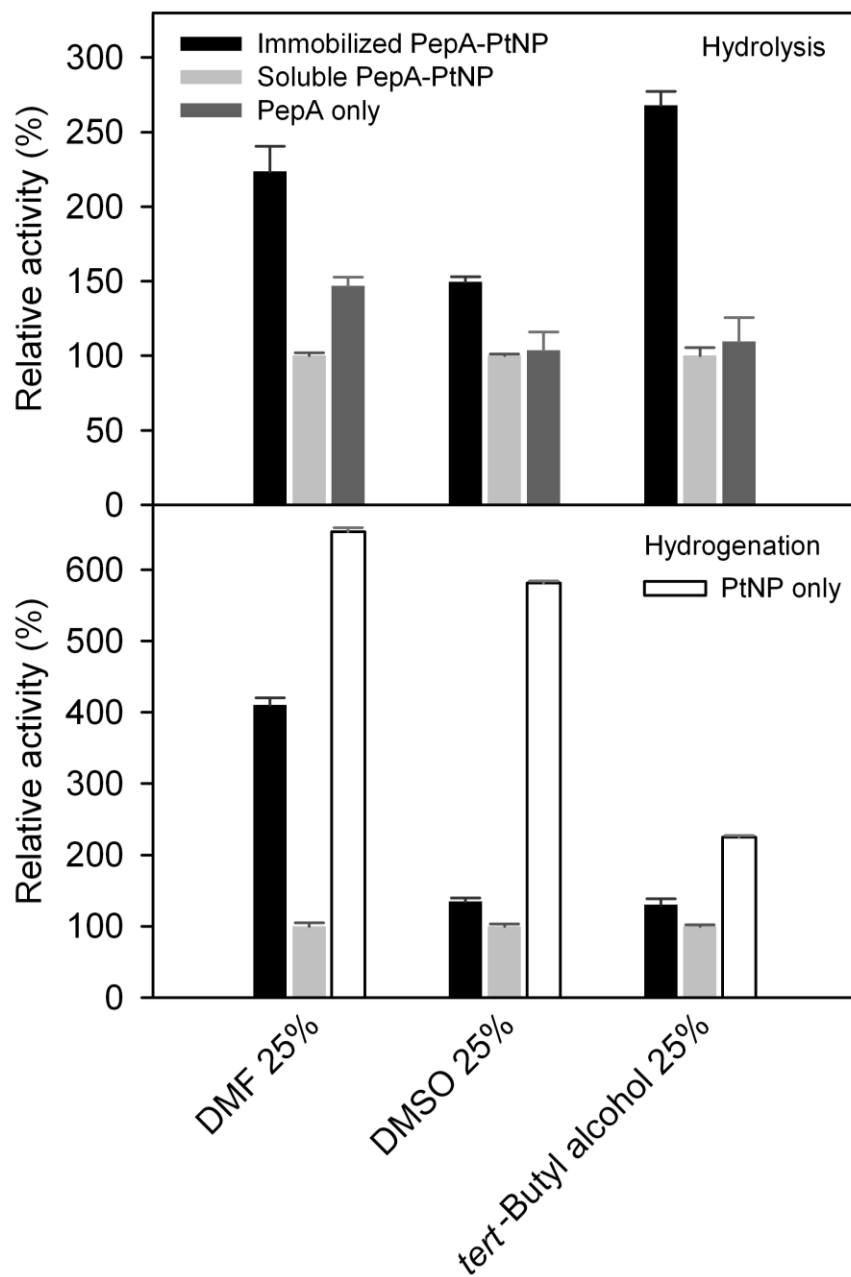


Figure 6

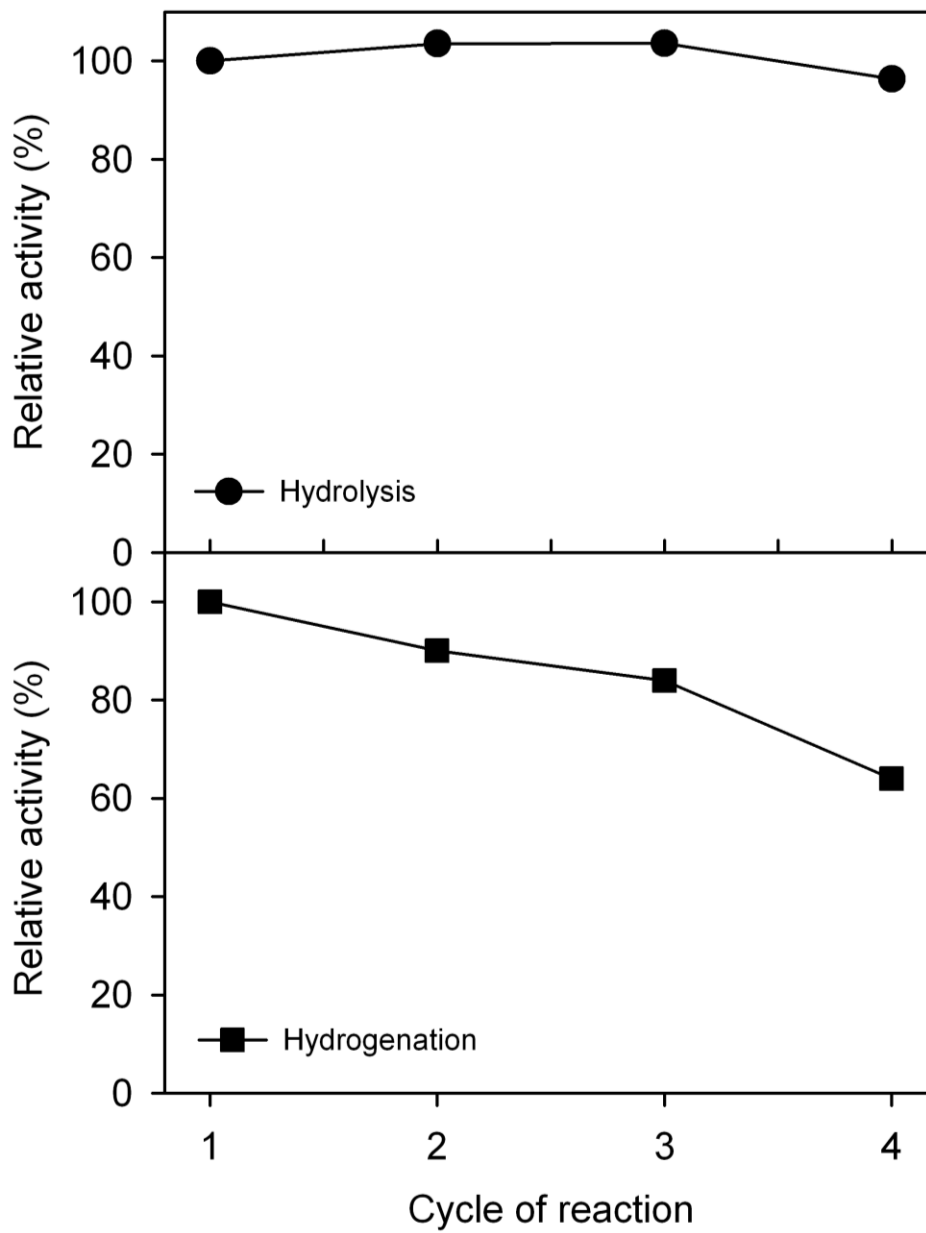


Figure 7

