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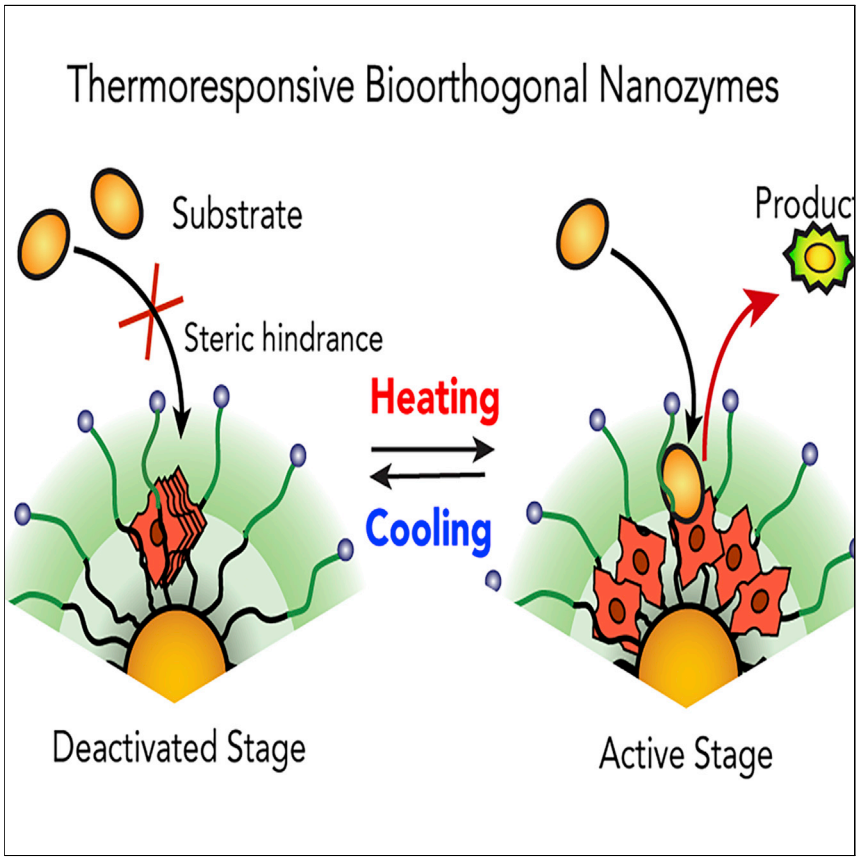
Thermally Gated Bio-orthogonal Nanozymes with Supramolecularly Confined Porphyrin Catalysts for Antimicrobial Uses



This paper describes the fabrication of thermoresponsive bio-orthogonal catalytic systems though the integration of transition metal catalysts into gold nanoparticles. The confined assemblies of the catalysts provide a temperature-regulated system able to controllably activate antibiotics within biofilms. This work presents a blueprint for synthesizing a family of reversible thermoresponsive nanozymes with tailored activation temperatures and preserved bio-orthogonal activity in complex biological environments.

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HIGHLIGHTS

Development of thermoresponsive bio-orthogonal nanocatalysts, i.e., nanozymes

Confined dynamics of iron catalyst assemblies in gold nanoparticle monolayers

Catalytic and thermoresponsive activity in complex biological systems

Eradication of biofilms through controlled activation of antibiotics using nanozymes

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Thermally Gated Bio-orthogonal Nanozymes with Supramolecularly Confined Porphyrin Catalysts for Antimicrobial Uses

Roberto Cao-Milán,[1] Sanjana Gopalakrishnan,[1] Luke D. He,[1] Rui Huang,[1] Li-Sheng Wang,[1] Laura Castellanos,[1] David C. Luther,[1] Ryan F. Landis,[1] Jessa Marie V. Makabenta,[1] Cheng-Hsuan Li,[1] Xianzhi Zhang,[1] Federica Scaletti,[1] Richard W. Vachet,[1] and Vincent M. Rotello[1,2,*]

SUMMARY

Bio-orthogonal catalysis has the capability of localized generation of imaging and therapeutic molecules *in vitro* and *in vivo*. The integration of these catalysts into thermoresponsive nanoparticle platforms would generate bio-orthogonal "nanozymes" that could be controlled through endogenous or exogenous thermal control. We have fabricated thermoresponsive nanozymes by confining supramolecular assemblies of porphyrins into the monolayer of gold nanoparticles. The resulting nanodevices feature an on-off gated thermal response occurring over a 3°C range with commensurate tunability of activation temperature from 25°C to 37°C. Reversible activation of catalysis was demonstrated in complex biological environments, and the efficacy of bi-stable thermoresponsive nanozymes demonstrated through thermal activation of antibiotic-based prodrugs to effectively treat bacterial biofilms.

INTRODUCTION

Bio-orthogonal chemistry provides a tool for biological and biomedical science that uses abiotic chemical reactions that do not interfere with endogenous enzymatic processes.[1,2] Bio-orthogonal catalysis extends this capability to generate imaging and therapeutic agents on demand through enzyme-like activation of prodrugs and profluorophores.[3–5]

The integration of bio-orthogonal catalytic systems such as transition-metal catalysts (TMCs) into nanoparticle hosts provides catalysts that replicate key features of enzymes.[6–8] The nanoparticle scaffolds of these nanozymes solubilize and stabilize catalysts in complex biological milieus.[1] Additionally, these scaffolds can be used to regulate the activity of the catalytic center, by using supramolecular interactions to provide exogenous activation of catalysis in cells, as well as providing complex kinetic behavior replicating enzymatic catalysis.[9–11]These bioinspired nanodevices with programable catalytic properties are especially appealing for biomedical applications where repetitive localized therapy is required such as biofilm infections.[12,13] Spatiotemporally controlled generation of antibiotics could facilitate generation of therapeutic strategies with reduced side effects.[12]

Thermally responsive systems provide a means of controlling catalysis that would allow the regulation of bioorthogonal processes by using endogenous or exogenous temperature gradients.[14] Most existing methods for creating thermoresponsive catalysts involve the insertion of either heterogeneous[15–18] or

The Bigger Picture

Bio-orthogonal chemistry is a medical tool that uses abiotic chemical reactions to generate diagnostic and therapeutic agents in the body without interfering with endogenous processes. The integration of catalytic systems, such as transition metal catalysts (TMCs) into nanoparticle scaffolds provides nanocatalysts that bio-orthogonally generate imaging and therapeutic agents through the enzyme-like activation of profluorophores and prodrugs. This study describes the fabrication of thermoresponsive nanozymes through the supramolecular confinement of TMC assemblies into biocompatible gold nanoparticles. The confined assemblies provide a means of regulating catalytic behavior through temperature. The potential application of this platform for the temperature-regulated generation of therapeutics was demonstrated by utilizing these thermoresponsive nanozymes for controlled activation of proantibiotics for the eradication of biofilms.

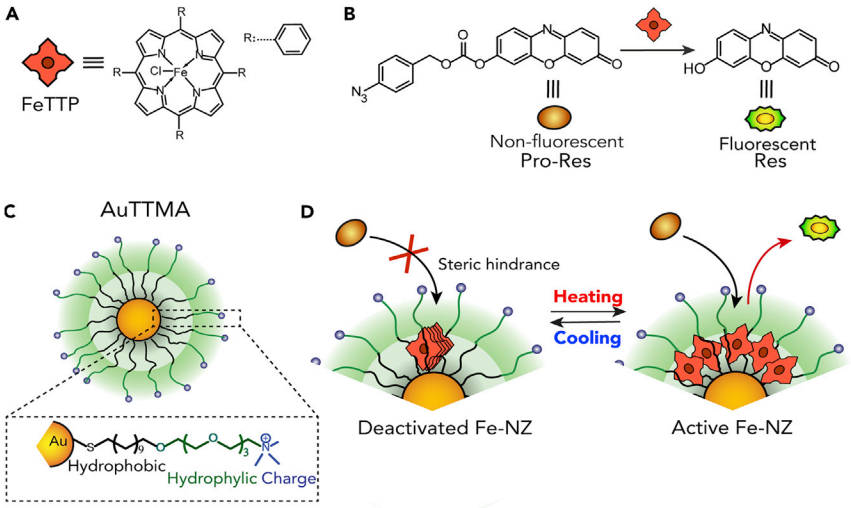

Figure 1. Thermoresponsive Nanozyme Design

(A) Structure of FeTPP.

(B) Conversion of profluorophore pro-Res through the catalytic reduction of aryl azides.

(C) Structure of AuTTMA.

(D) Confined dynamics of FeTPP assemblies within AuTTMA monolayer; FeTPP forms a packed structure with ligands preventing substrates from reaching active sites. Increasing temperature triggers the dissociation of FeTPP assemblies and redistribution of FeTPP molecules within the monolayer, forming structures that allow substrates to access active centers, enabling catalysis. This process is reversed by cooling down the system.

molecular[19,20] catalytic species into temperature-sensitive scaffolds such as polymers[6–10,21] or gels.[22–27] These systems modulate their catalytic activity through temperature-driven changes in volume and/or hydrophobicity of the scaffold,[28] thereby controlling the access of substrates to active sites.[7,16] As a result, these systems generally exhibit attenuation in their catalytic properties with increasing temperature,[7,15] providing turn-off-based regulation that is challenging to translate to many biomedical applications.

In our previous studies, we incorporated hydrophobic TMCs into the monolayer of 2-nm gold nanoparticles (NPs),[6,29–31] to generate nanozymes that were functional in complex biological environments.[19,20,22,23] We hypothesized that beyond solubilizing and stabilizing the TMCs, we could use the confinement provided by the monolayer[32] to regulate intercatalyst supramolecular assemblies, providing a new approach to the thermoregulation of catalysis (Figure 1; see also Data S1–S3). For these studies we employed iron (III) tetraphenyl porphyrin (FeTPP), a system readily capable of self-assembly through stacking (Figure 1A).[33,34] At low temperatures, the confined FeTPP assemblies display cooperative interactions with the ligands of the NP scaffold (Figure 1C), forming a compact structure that blocks substrate access to the active centers, thus deactivating the catalytic process.

An increase in temperature triggers the disassembly and redistribution of TMCs within the monolayer of NPs, enabling substrates to access the active centers and reactivating catalysis (Figure 1D). Activation occurred over a very narrow (3°C) range, providing bistable off-on control. Changing the FeTPP:NP stoichiometry enabled fine control of the gating temperature over the physiologically relevant range of 25°C–37°C, with a resolution of 3°C. The inherent biostability of the AuNP platform yielded nanozymes that retain their thermal gating properties in

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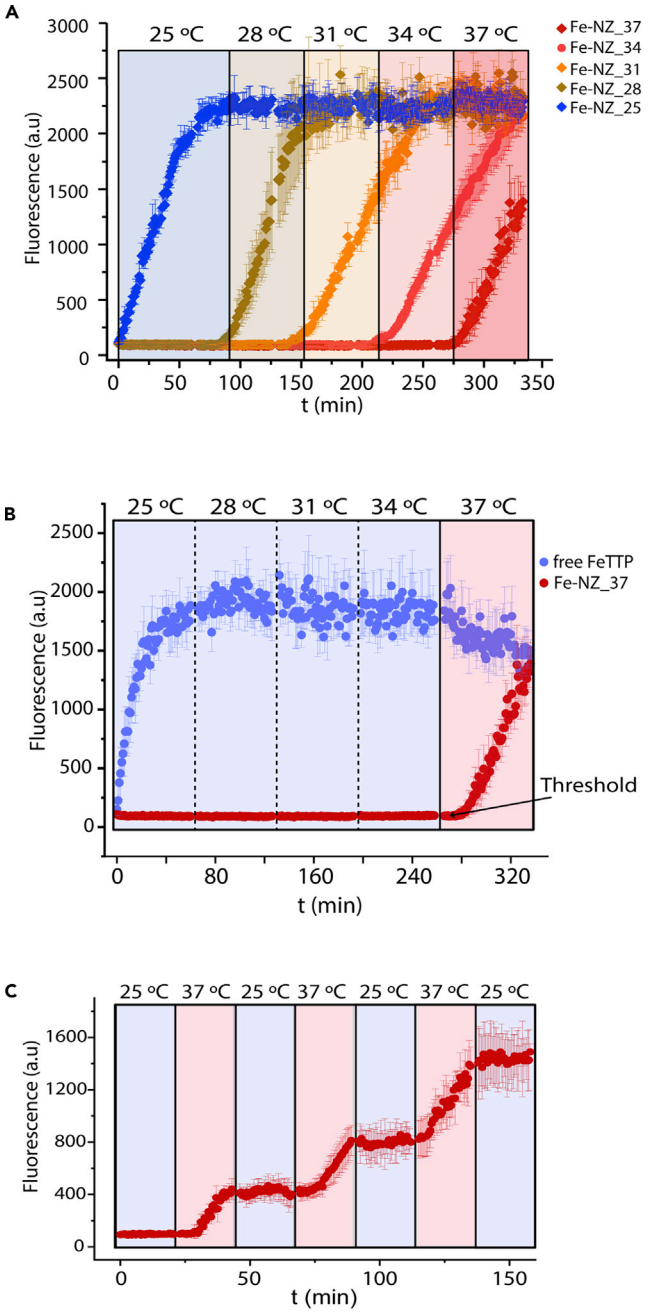

Figure 2. Demonstrating the Tunability and Reversibility of the Catalytic Activity of Thermoresponsive Nanozymes

(A) The thermoresponsive behavior of **Fe-NZ_25**, **Fe-NZ_28**, **Fe-NZ_31**, **Fe-NZ_34**, and **Fe-NZ_37** was monitored by measuring the catalytic activity of each nanozyme (at equal concentrations) in the temperature range of 25°C–37°C. Nanozymes were exposed to 25°C, 28°C, 31°C, 34°C, and 37°C for a fixed time interval (60 min each except at 25°C), represented by the appropriately colored columns.

(B) Thermal activation of the catalytic properties of **Fe-NZ_37** (red) at 37°C compared with free FeTPP (blue). Each column represents a 60-min period (except for the red column) of exposure to a given temperature. **Fe-NZ _37** is only active at 37°C (red column) while free FeTPP (blue plot) displays catalytic activity even at 25°C.

(C) Reversibility of catalytic properties of **Fe-NZ_37** was monitored through changes in catalytic activity because of alternate heating (to 37°C) and cooling (to 25°C) of **Fe-NZ_37**. Increase in

Figure 2. *Continued*
fluorescence intensity is observed upon heating. Exposure to both temperatures was limited to a duration of 20 min each as denoted by the alternately colored columns. Experiments were conducted in PBS (pH 7.4) solutions containing glutathione 5 mM as a cofactor for the catalytic process. Each experiment was replicated thrice, and error bars represent standard deviations of these measurements.

complex biological environments. The potential of this platform for bio-orthogonal therapy was demonstrated through a thermally regulated treatment of bacterial biofilms via the catalytic uncaging of antibiotic-based prodrugs. On the whole, this platform presents a new tool for the gated thermal regulation of biosystems through bioorthogonal catalysis.

RESULTS AND DISCUSSION

Design and Synthesis of a Thermoresponsive Nanozyme

Nanozymes (NZs) were prepared by incorporating FeTPP into AuTTMA scaffolds. The FeTPP molecule catalyzes the reduction of aryl azides to the corresponding amines[35] providing a robust and efficient bio-orthogonal catalyst that self-assembles into stacked aggregates (see Data S9 for substrate design strategies). The host NPs use the biocompatible scaffold used in our prior nanozyme systems that provides a hydrophobic environment for catalyst encapsulation.[6]

Nanozymes with different activation temperatures (Figure 2) were fabricated by mixing AuTTMA aqueous solutions (10 μM) with FeTPP solutions (THF) of concentrations ranging from 0.28 μM to 2.84 mM (see Data S5). Dynamic light-scattering measurements showed no change in size of the AuNPs after encapsulation of the FeTPP assemblies (see Data S1), indicating retention of nanopartricle architecture and absence of aggregation. The incorporation of FeTPP assemblies of different sizes into NPs produced a family of thermoresponsive nanozymes, **Fe-NZ_25**, **Fe-NZ_28**, **Fe-NZ_31**, **Fe-NZ_34,** and **Fe-NZ_37**, which had different activation temperatures ranging from 25°C to 37°C (see Data S2 and Data S5).

The catalytic properties of the nanozymes were studied in PBS buffer through the activation of a nonfluorescent resorufin-based profluorophore (pro-Res, Figure 1B), where reduction of the azide results in fragmentation and release of the fluorescent resorufin molecule (Figure 1B).

Demonstrating the Reversibility and Tunability of Thermoresponsive Behavior

Catalytic activity for each nanozyme was measured by monitoring changes in the fluorescence intensity of pro-Res solutions at different temperatures (Ex. 568 nm and Em. 588 nm). The FeTPP:AuTTMA stoichiometry was quantified using inductively coupled plasma mass spectrometry (ICP-MS). Table 1 summarizes the ratio of FeTPP:AuTTMA in each nanozyme and their corresponding rates for activation of substrates at their respective temperatures of activation. Figure 2A shows turn-on of catalysis for each nanozyme at their respective temperatures of activation. Similar catalytic activity across different nanozymes was observed at their activation temperature (Figure 2A and Table 1) consistent with the nanozymes having a similar number of accessible FeTPP when they activate. In contrast, at 45°C, the catalytic efficiency of nanozymes is proportional to the amount of encapsulated FeTPP, indicating that at such temperatures most of encapsulated FeTPP are dissociated and catalytically active (see Data S10).

The mixture of FeTPP solutions in THF with water instantly produced assemblies of different sizes on the basis of the concentration of FeTPP solution, as inferred from

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Table 1. Number of FeTPP Molecules Encapsulated in Each Fe-NZ and the Rate of Substrate Activation of Each Nanozyme at Its Activation TemperatureCatalytic activity was tracked by monitoring changes in fluorescence of pro-Res solutions (Ex. 568 nm, em. 588 nm).

Fe-NZ	FeTPP:NP (Molecules per Particle)	V_{TA} (Mol [NZ(Mol)] $^{-1}$ min^{-1})
Fe-NZ_25	4 ± 1	98.17 ± 8.448
Fe-NZ_28	8 ± 1	100.2 ± 30.11
Fe-NZ_31	9 ± 1	86.74 ± 4.7008
Fe-NZ_34	14 ± 2	123.2 ± 20.90
Fe-NZ_37	28 ± 3	113.1 ± 14.19

UV-vis analyses. These FeTPP assemblies are confined in the AuTTMA scaffold to obtain nanozymes. It is expected that FeTPP assemblies of smaller sizes would have lower contact with the ligands of the AuTTMA scaffold, thereby reducing the strength of hydrophobic interactions. As a result, the assemblies are expected to disaggregate into their open conformations at lower temperatures, thereby providing access of active centers to the substrates. Conversely, FeTPP assemblies of larger sizes would require higher temperatures to generate their open conformations because of stronger hydrophobic interactions with the ligands of the AuTTMA scaffold. Therefore, it was concluded that the transformation of substrates takes place only after the dissociation of the FeTPP assemblies within the AuTTMA scaffold.

Further studies were performed using **Fe-NZ_37** as 37°C is the ideal temperature for most bacterial and cell-culture studies. This would allow us to elucidate the thermoresponsive behavior in biological environments. Temperature-controlled activation of the nanozymes was verified by comparing the catalytic activity of **Fe-NZ_37** with that of free FeTPP assemblies. As shown in Figure 2B, **Fe-NZ_37** was activated at 37°C, whereas free FeTPP solution was activated at 25°C. Panels correspond to 60-min exposure periods for each temperature. We also investigated the reversibility of thermoresponsive behavior by using **Fe-NZ_37**. Figure 2C demonstrates the reversible nature of thermal activation of **Fe-NZ_37**, wherein the nanozyme can be returned to its dormant state by simply cooling the system.

We therefore conclude that confining FeTPP assemblies in the monolayer of our NPs produces nanozymes that respond to subtle changes in temperature by reversibly gating the access of substrates to active centers.

Elucidation of the Thermal Gating Mechanism of Thermoresponsive Nanozymes

We studied the UV-vis spectra of the nanozyme at different temperatures to validate that the mechanism of **Fe-NZ_37** activation is based on the dynamics of the confined FeTPP assemblies. Iron porphyrin aggregates present a characteristic absorption band (Soret band) located at ~410 nm, which tends to increase in intensity and blue shift when such assemblies dissociate into smaller structures.[36,37] The spectra of **Fe-NZ_37** show the electronic transitions of both chromophores, the gold core, and the confined FeTPP assemblies (Data S4). The absorption bands of the NP scaffolds show minimal changes within the studied range of temperatures (Data S3), which allows their subtraction from the **Fe-NZ_37** spectra to provide more insight into the behavior of the confined FeTPP assemblies at different temperatures. The subtracted spectra of **Fe-NZ_37** are presented in Figure 3B. At higher temperatures, the increased intensity of the Soret band is accompanied by a small blue shift. This

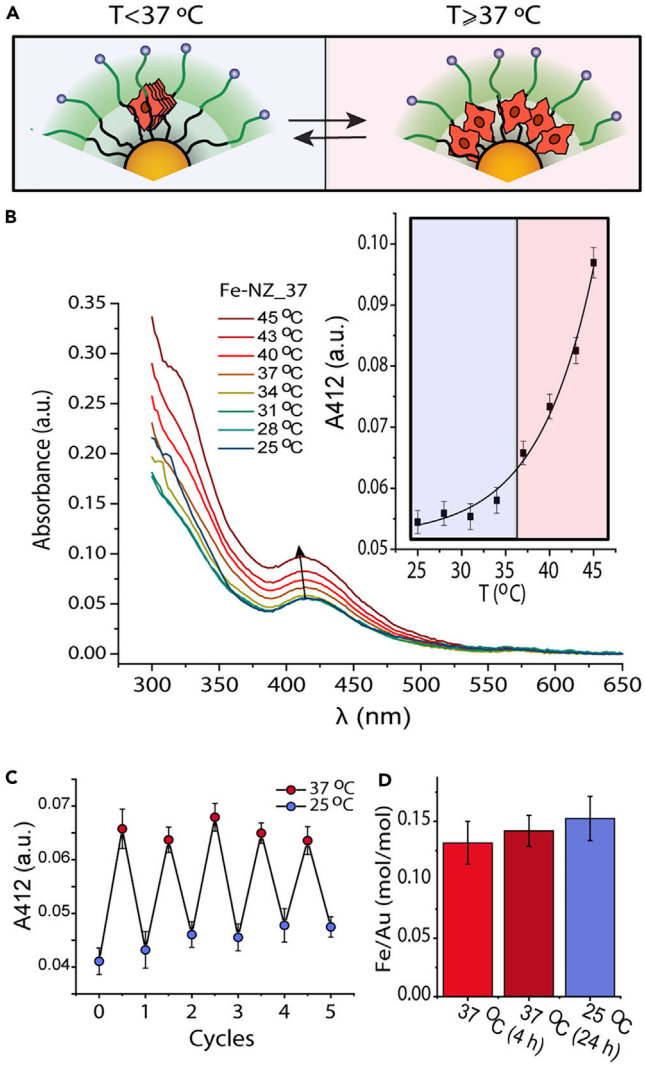

Figure 3. Thermally Triggered Reversible Dissociation of FeTPP Assemblies in the Monolayer of NPs

(A) Scheme of **Fe-NZ_37** at temperatures below (blue panel) and above (above) 37°C.

(B) Subtracted UV-vis spectra of **Fe-NZ_37** at different temperatures. The inset shows intensity of 412-nm band with increments of temperature. Significant variations in A412 are observed for **Fe-NZ_37** at temperatures above 37°C (red column).

(C) Intensity of the band at 412 nm during cycles of heating at 37°C (red circles) and cooling at 25°C (blue circles). Fe:Au ratio of **Fe_NZ-37** solutions subjected to 37°C (for different lapse of time) and to 25°C. Measurements were conducted by ICP-MS. Each experiment was made in triplicate. Error bars represent standard deviations of these measurements.

behavior indicates that confined FeTPP assemblies dissociate at increased temperatures. Analysis of the variations of intensity of the Soret band (412 nm) with temperature shows pronounced changes only when the temperature reaches the 37°C turn-on temperature, consistent with porphyrin catalyst dissociation at the activation temperature (Figure 3D). Next, we analyzed variations in the Soret band of confined FeTPP assemblies in **Fe-NZ_37** when exposed to different cycles of heating (at 37°C) and cooling (at 25°C) (see Data S4). Figure 3C shows that the intensity of the Soret band reversibly increases at 37°C and decreases at room temperature. These results are consistent with the observed reversible

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thermoresponsive behavior of the catalytic properties of **Fe-NZ_37** (Figure 2D). Resorufin showed no change in fluorescence due to changes in temperature (Data S6).

We next conducted ICP-MS experiments to detect whether the dissociation processes of the confined FeTPP assemblies occurred solely within the monolayer of the **Fe-NZ_37**, or if the catalysts could escape to solution. For this, **Fe-NZ_37** samples were incubated at 37°C for 4 and 24 h, then immediately filtered (Amicon ultra 4, 10K). Samples remaining in the filters were then collected, digested, and analyzed by ICP. Figure 3D shows that the heated **Fe-NZ_37** samples display the same Fe:Au ratio (mol:mol) as the samples kept at RT. These results confirm that all dynamic processes detected by UV-vis only occur within the confining environment of the monolayer of **Fe-NZ_37** as represented in Figure 1D. Furthermore, we demonstrated that only dissociated FeTPP contribute to catalysis. This can be seen in Data S10 where upon heating all **Fe-NZs** to 45°C, the rate of catalysis is directly proportional to the amount of catalyst encapsulated in each of the **Fe-NZs**.

Thermoresponsive Behavior of Fe-NZ_37 in Biological Systems

Having characterized our thermoresponsive nanozymes in solution, we subsequently studied their potential for the eradication of bacterial biofilms. The temperature-dependent properties of **Fe-NZ_37** in the biofilm were evaluated through the catalytic deprotection of pro-Res as determined through confocal microscopy. Biofilms comprising of green fluorescent protein-expressing *E. coli* (GFP-*E. coli*) were incubated with **Fe-NZ_37** in M9 media for 1 h to allow internalization of nanozymes within the biofilms.[20] Bacterial biofilms were then washed three times with lead sulfide (PBS) to remove excess nanozyme. The thermoresponsive behavior of the internalized nanozymes was then tested by incubating the biofilms in M9 media containing pro-Res (10 μM) at 25°C and 37°C for ~1 h before obtaining the images shown in Figure 4. Biofilms incubated only with pro-Res served as the negative control.

Figure 4 shows confocal studies that examine the penetration of nanozymes into bacterial biofilms. By analyzing the green channel, the GFP-*E. coli* biofilms can be visualized (Figures 4B-i, 4C-i, and 4D-i). Notably, the biofilms treated with **Fe-NZ_37** at 37°C (Figure 4D-i) resulted in higher bacterial density, presumably due to a combination of growth-stimulating effects (hormesis) of the cationic nanoparticles at low concentrations.[23] Analysis of the red channel illustrates negligible fluorescence in biofilms treated with pro-Res and **Fe-NZ_37** at 25°C (Figure 4C-i) and also those treated with only pro-Res at 37°C (Figure 4B-i). These results indicate that pro-Res deprotection occurs only in the presence of **Fe-NZ_37** at 37°C. The merged channel shows that the generated red fluorophore co-localizes with GFP-*E. coli*, implying that it is being activated within the biofilm. This suggests that the nanozymes successfully penetrated the bacterial biofilms and were able to retain thermoresponsive catalytic activity within the biofilms.

From an antimicrobial perspective, a strategy that relies on proantibiotic activation through bio-orthogonal thermoresponsive nanozymes can provide spatiotemporally controlled generation of antibiotics. As a result, these systems could facilitate the development of antimicrobial therapies with reduced side effects for mammalian tissues, organs, and beneficial microorganisms. We demonstrated this by using **Fe-NZ_37** for the activation of pro-Mox (a protected moxifloxacin derivative) (Figure 5 and Data S8).

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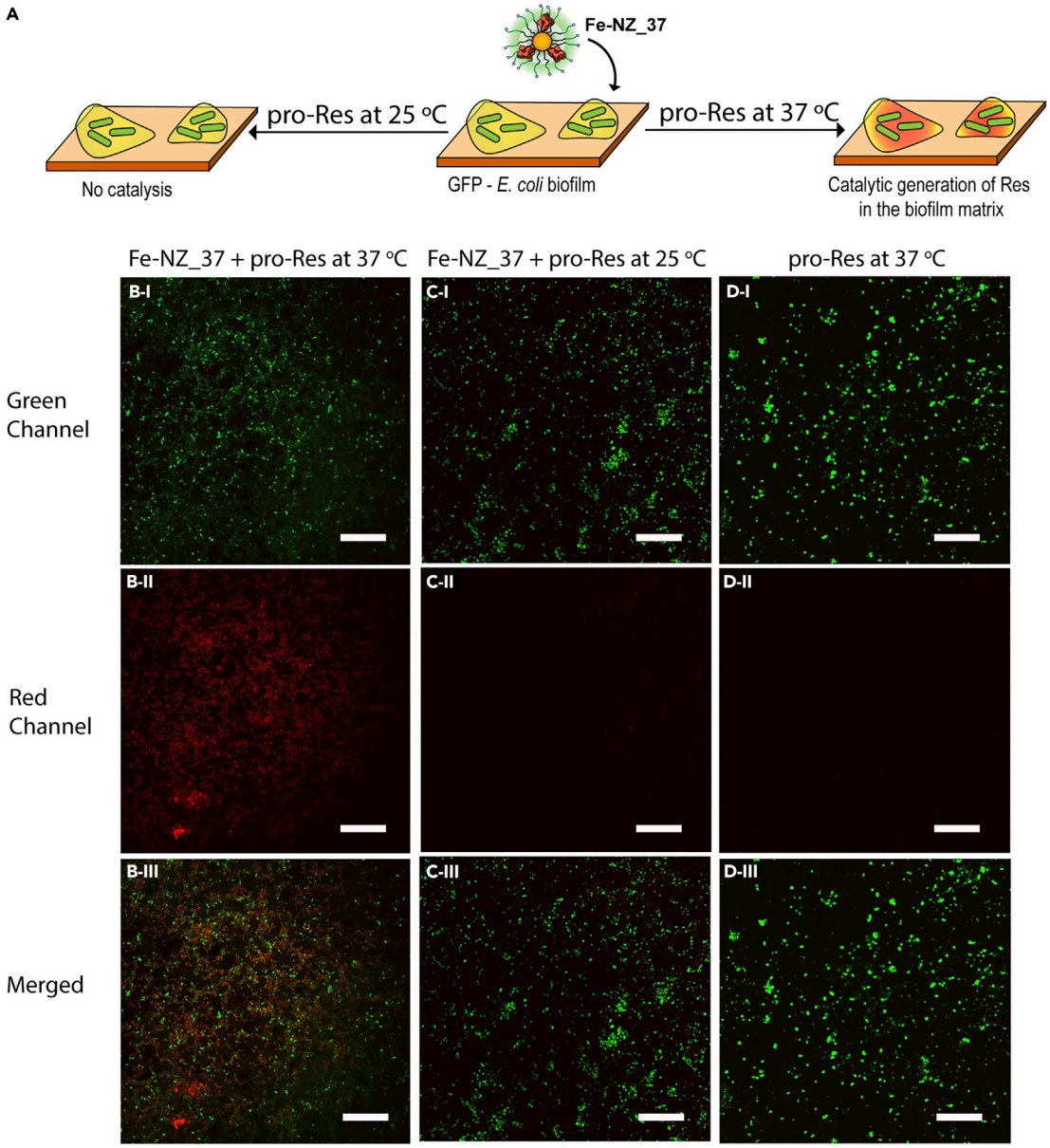

Figure 4. Thermoresponsive Behavior of Nanozymes in Biofilms

(A–D) Shown in (A) is the internalization of **Fe-NZ_37** in biofilms and temperature-controlled activation of pro-Res. Confocal microscopy images of biofilms treated (B) with pro-Res at 37°C, (C) with a combination of **Fe-NZ_37**, and (D) with pro-Res at 25°C, and with a combination of **Fe-NZ_37** and pro-Res at 37°C. Each channel is represented with sub index -i (green), -ii (red), and -iii (merged). Scale bars, 50 μM.

E. coli biofilms, treated with **Fe-NZ_37** and different concentrations of pro-Mox, were incubated at 25°C and 37°C for 6 h. The samples were then washed with PBS, and their viability was analyzed through an alamarBlue assay. Biofilms treated with varying concentrations of pro-Mox only and **Fe-NZ_37** only were tested as negative controls and varying concentrations of Mox + **Fe-NZ_37** were used as positive controls.

Figures 5B and 5C show the results obtained from bacterial biofilms exposed to each treatment described above at 25°C and 37°C, respectively. As expected, samples treated with different concentrations of pro-Mox displayed negligible variations in

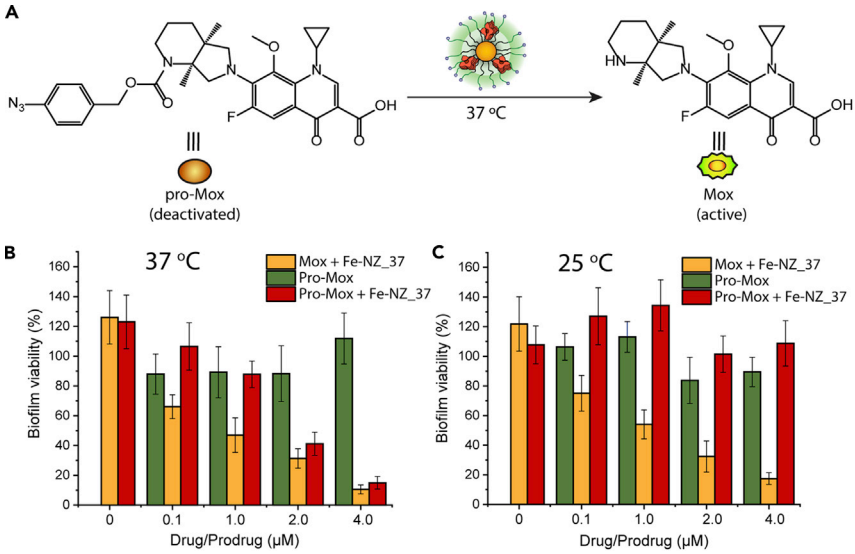

Figure 5. Deprotection of Antimicrobials in Biofilms Using Thermoresponsive Nanozymes

Fe-NZ _37 was used for the controlled activation of antimicrobial prodrugs that modulate biofilms viability.

(A) Scheme of activation of pro-Mox by p-p aggregates At 37°C.

(B and C) *E. coli* biofilms treated with pro-Mox and **Fe-NZ_37** (red bars) at (B) 25°C and at (C) 37°C. Biofilms treated only with pro-Mox (blue bars) or with a combination of moxifloxacin (Mox) and **Fe-NZ_37** (pink bars) were used in all experiments as negative and positive controls, respectively. Each experiment was replicated five times. Error bars represent standard deviations of these measurements. The thermoresponsive properties of **Fe-NZ_37** combined with its ability to penetrate biofilms is ideal for controlled deprotection of pro-Mox within biofilms. Mox was chosen as the antibacterial agent (Figure 5A, right) because of its broad-spectrum activity.[38] The secondary amino group is crucial for binding two target enzymes of the drug.[24] Masking this group with the bulky aryl-azide carbamate (Figure 5A, left) reduces the antibiotic activity of this drug by more than two orders of magnitude, as can be seen in **SI-7**. This large reduction in the bactericidal activity of pro-Mox (with respect to Mox) makes it ideal in combination with **Fe-NZ_37** to thermally control the viability of bacterial biofilms.

bacterial viability at both temperatures. On the contrary, biofilms responded in a dose-dependent manner to treatments containing **Fe-NZ_37** and different concentrations of active Mox, regardless of the temperature used. Biofilms treated with pro-Mox and **Fe-NZ_37** exhibited biofilm inhibitory effects at 37°C in a dose-dependent manner, whereas biofilms exposed to pro-Mox and **Fe-NZ_37** at 25°C (Figure 5B, red bars) displayed no significant variations in viability. Notable bactericidal effect was registered with pro-Mox concentrations of above 2 μM. These results show that **Fe-NZ_37** can thermally regulate bioprocesses via the uncaging of modulatory drugs even in the presence of complex biological environments.

Conclusions

In this report, we highlight a strategy for fabricating thermoresponsive nanocatalysts that reversibly switch between dormancy and high rates of substrate processing over slight variations in temperature. This strategy is based on the encapsulation of transition metal catalysts, in the form of supramolecular assemblies, into the monolayer of gold nanoparticles. We demonstrated that by confining supramolecular structures (p-p aggregates, in our case) into semi-rigid biocompatible scaffolds, the stimuli-responsive behavior of these supramolecular complexes is preserved in biological environments and can be employed in applications that include localized therapy and imaging. This enables the TMC assemblies to

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reversibly dissociate within the hosting scaffold upon heating, consequently resuming catalytic activity. This gating mechanism is based on the dynamics of confined TMC assemblies and allows the nanozymes to display thermoresponsive behavior in complex biological systems. Together with high rates of substrate processing, these properties make our nanozymes well-suited for biomedical applications that include not only antibacterial therapies, but also the self-modulation of inflammatory processes, which typically produce local heating. This strategy can be adapted to integrate other self-assembled chromophores and/or fluorescent molecules that can be used in the fabrication of nanometric sensors,[37,38] which detect subtle changes in cellular microenvironments, and programable nanobots that exert preventive regulatory functions in biosystems. This work presents a blueprint for synthesizing a family of reversible thermoresponsive nanozymes with tailored activation temperatures and preserved biorthogonal activity in complex biological environments.

EXPERIMENTAL PROCEDURES

Synthesis and characterization of ligands, NPs (Figure 1D), nanozymes, and substrates are described in the Supplemental Information. Detailed sample preparation for ICP-MS, UV-vis characterization of nanozymes and FeTPP assemblies, and additional bacteria culture studies can also be found in the Supplemental Information.

Fabrication of Thermoresponsive Nanozymes

0.5 mL of the FeTPP solution in THF was mixed with an equal volume of aqueous solution of NPs (10 μM) (see Data S11 and Data S12 for AuTTMA synthesis), and stirred for 10 min. THF was then slowly removed by evaporation to induce the encapsulation of FeTPP assemblies into the NP monolayer, with excess catalyst precipitating. **Fe-NZ** solutions were then purified by centrifugation at 14,000 rpm to eliminate precipitated FeTPP and filtered (Amicon ultra 4, 10K) six times to remove the remaining free catalyst. For synthesizing **Fe-NZ_37**, **Fe-NZ_34**, **Fe-NZ_31**, **Fe-NZ_28**, and **Fe-NZ_25**, 1 mL of FeTPP solutions with concentrations of 2.84 mM, 284 μM, 28.4 μM, 2.84 μM, and 0.28 μM, respectively, were mixed with equal volumes of NP solutions at 10 μM (Data S13). The resulting solutions were then concentrated by roto-evaporation and purified through multiple sessions of centrifugation and filtration (Amicon ultra 4, 10K). For long term preservation of **Fe-NZ** solutions (1–2 months), solid ascorbic acid was added to stock solutions at concentrations ranging from 1 to 6 mg/mL.

Determination of Temperature of Activation of Thermoresponsive Nanozymes

Each kinetic experiment was performed in a 96-well black plate with 100 μL of PBS solution containing the corresponding **Fe-NZ** (50 nm), pro-Res (20 μM), and 5 mM of glutathione (See Data S14 for pro-Res synthesis). Samples were then immediately inserted into the plate reader at 25°C to analyze the evolution of fluorescence (Ex. 568 nm, Em. 588 nm). At the end of each 60-min interval, the incubation temperature was increased by 3°C, and fluorescence was analyzed for another 60 min. When nanozymes were observed to become thermally active, fluorescence was monitored until reaching a maximum value (typically around 2,000 a.u.) representing pro-Res total transformation.

Thermoresponsive Activation of Prodrug in Bacterial Biofilms

E. coli strains CD 2 and GFP-*E. coli* were inoculated in 3 mL LB broth and grown to the stationary phase at 37°C. The cultures were then washed thrice in 0.85% sodium

chloride solution through centrifugation. The concentration of the resuspended bacteria solutions was estimated by measuring the optical density at 600 nm. Seeding solutions for both strains were prepared by diluting to 0.1 OD_{600} ($\sim 10^8$ colony forming units/ml) in M9 minimal media.

For the confocal studies, GFP-*E. coli* seeding solutions were spiked with IPTG (Isopropyl β-D-1-thiogalactopyranoside) so that the final solution contained 1mM of IPTG. The seeding solution (1 ml) was placed in confocal dishes and incubated at room temperature for ~3 days to grow biofilms. M9 minimal media was replaced each day. Biofilms were washed with PBS three times on day 3 and incubated with **Fe-NZ_37** in M9 media for 1 h. Bacterial biofilms were next washed three times with PBS to remove excess nanozyme and incubated pro-Res (100 μM) at 25°C and 37°C for ~1 h before obtaining images.

For the biofilm viability studies, 100 μL of the *E. coli* (CD 2) seeding solution was added into each well of a 96 well-plate and incubated overnight at room temperature to grow the biofilms. The biofilms were then washed thrice with PBS and treated with nanozymes (100 nM) and/or pro-Mox/Mox (see Data S15 for pro-Mox synthesis) at varied concentrations prepared in M9 media. One set of plates was incubated at 37°C for 5 h while the other was kept at 25°C. After this treatment, the biofilms were washed with PBS three times and their viability was determined using the alamarBlue assay according to the protocol established by the manufacturer

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.chempr.2020.01.015.

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AUTHOR CONTRIBUTIONS

R.C.-M. and V.M.R. conceived the idea. R.C.-M., L.D.H., R.H., R.F.L., and X.Z. synthesized nanozymes and substrates. R.C.-M., L.D.H., and L.S.W. designed and performed the experiments in solutions. R.C.-M., S.G., R.H., J.M.M., and C.H.L. conceived and designed bacteria experiments. R.C.-M., L.C., D.C.L., and R.W.V. conceived and performed ICP-MS analyses. All authors analyzed and discussed the data. R.C.-M., L.D.H., S.G., and V.M.R. co-wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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