Supplementary Information

Gold nanozymes for efficient degradation of organic dye pollutants: outperforming natural enzymes

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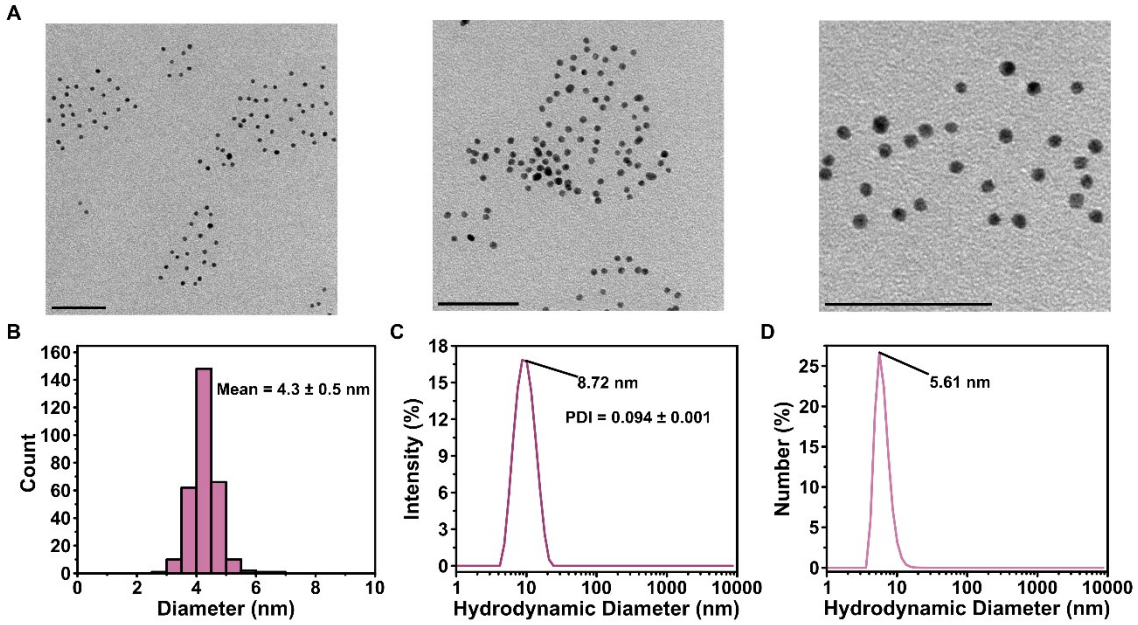

Figure S1: Characterization of AuNZ
(A) Sample TEM images of AuNZ (Scale Bar: 50 nm) and **(B)** TEM size distribution obtained counting more than 300 NPs from different spots on the grid. DLS analysis **(C)** by intensity and **(D)** by number, displayed as average of 3 measurements obtained from more than 10 runs.

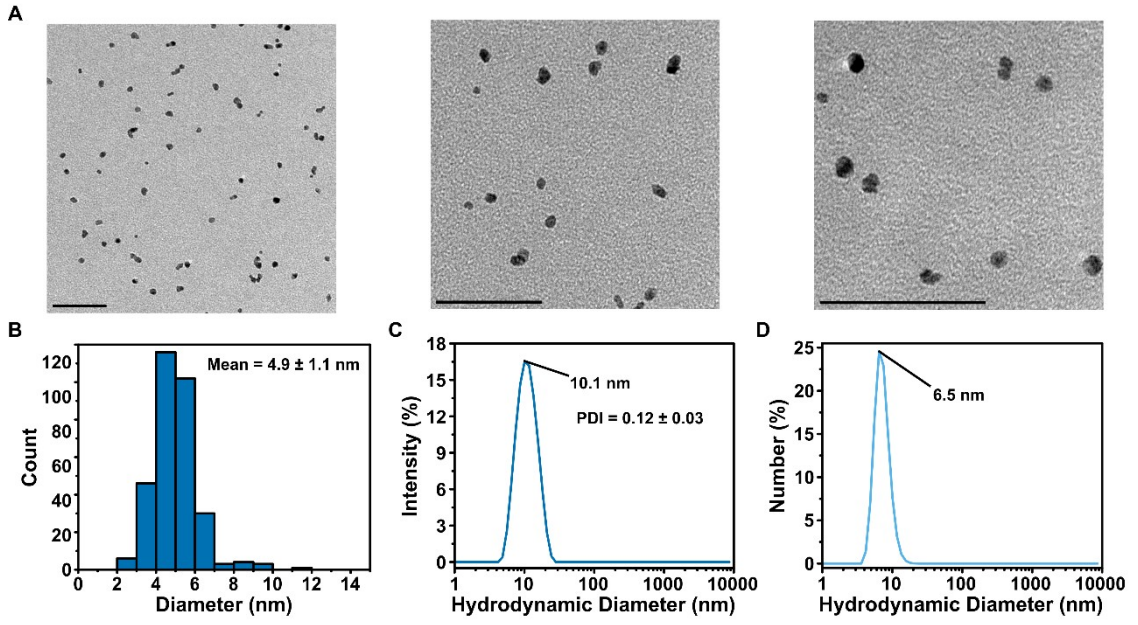

Figure S2: Characterization of PdNZ
(A) Sample TEM images of PdNZ (Scale Bar: 50 nm) and **(B)** TEM size distribution obtained counting more than 300 NPs from different spots on the grid. DLS analysis **(C)** by intensity and **(D)** by number, displayed as average of 3 measurements obtained from more than 10 runs.

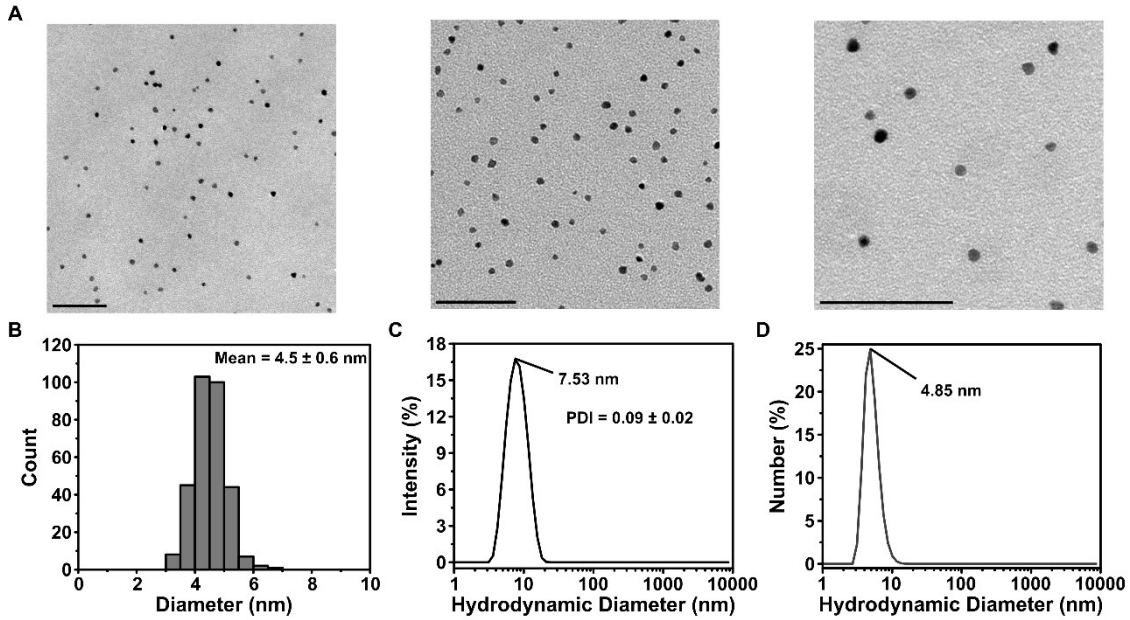

Figure S3: Characterization of PtNZ
(A) Sample TEM images of PtNZ (Scale Bar: 50 nm) and **(B)** TEM size distribution obtained counting more than 300 NPs from different spots on the grid. DLS analysis **(C)** by intensity and **(D)** by number, displayed as average of 3 measurements obtained from more than 10 runs.

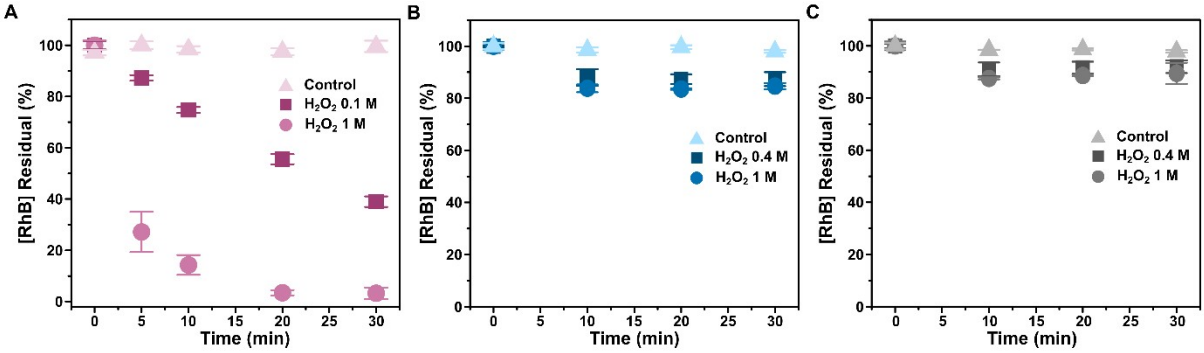

Figure S4: Optimizing conditions for RhB degradation
Kinetics of RhB degradation with different concentrations of H_2O_2 using **(A)** Au, **(B)** Pd, or **(C)** PtNZs as catalysts. Data are plotted as average ± st. deviation of three replicates.

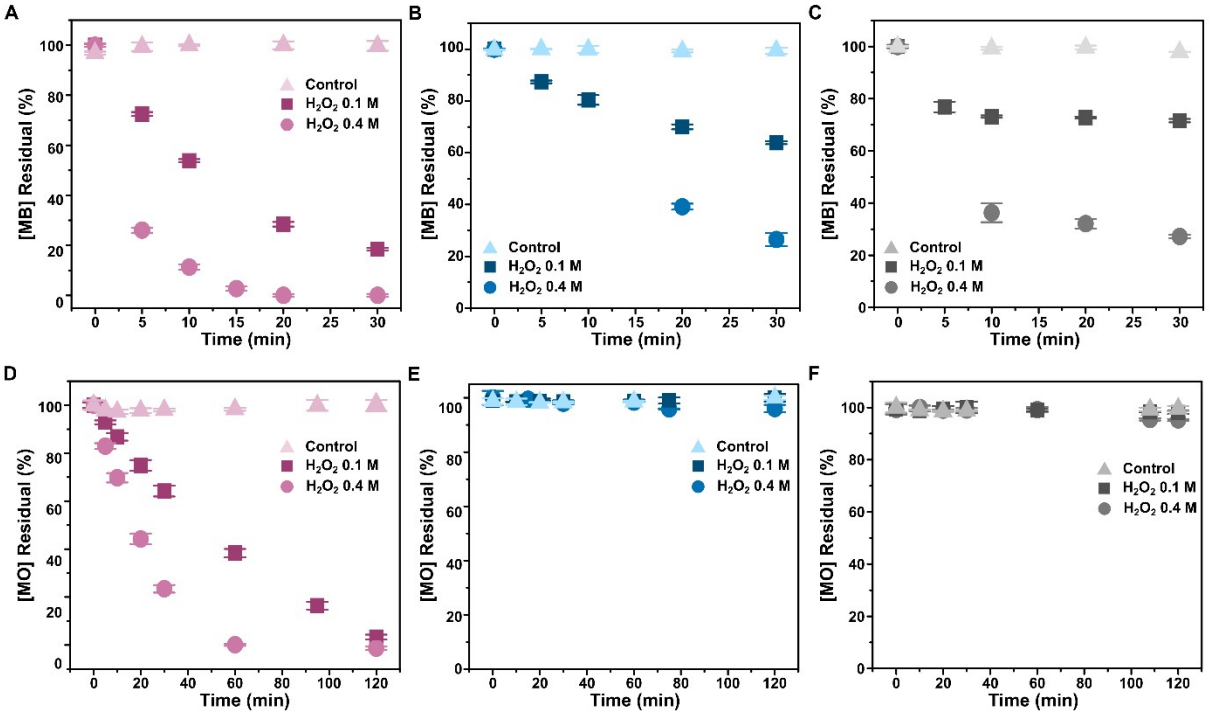

Figure S5: Optimizing conditions for MB and MO degradation
Kinetics of MB degradation with different concentrations of H_2O_2 using **(A)** Au, **(B)** Pd, or **(C)** PtNZs as catalysts. Kinetics of MO degradation with different concentrations of H_2O_2 using **(D)** Au, **(E)** Pd, or **(F)** PtNZs as catalysts. Data are plotted as average ± st. deviation of three replicates.

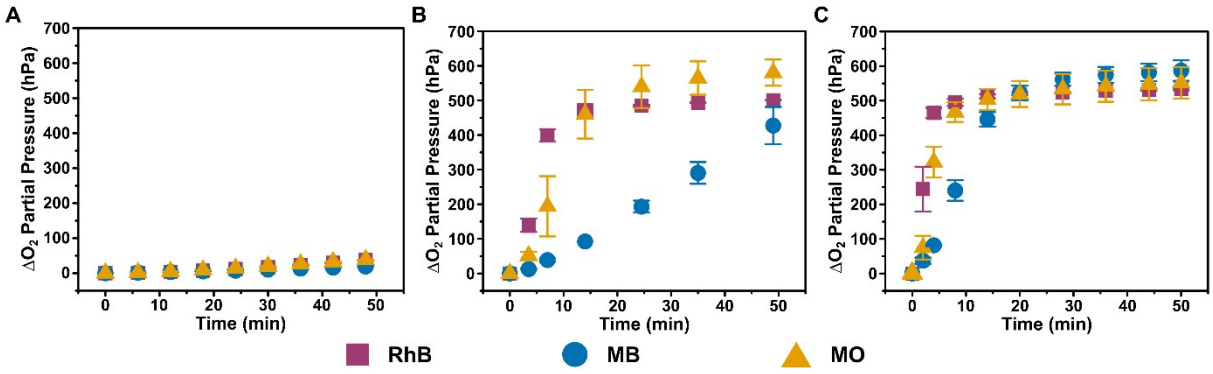

Figure S6: NZs oxygen production in the presence of different dyes
Kinetic of CAT-like activity in the presence of the different dyes (40 μM) with the same concentration of H_2O_2 (500 mM) for **(A)** Au, **(B)** Pd, and **(C)** PtNZs. Data are plotted as average ± st. deviation of two replicates.

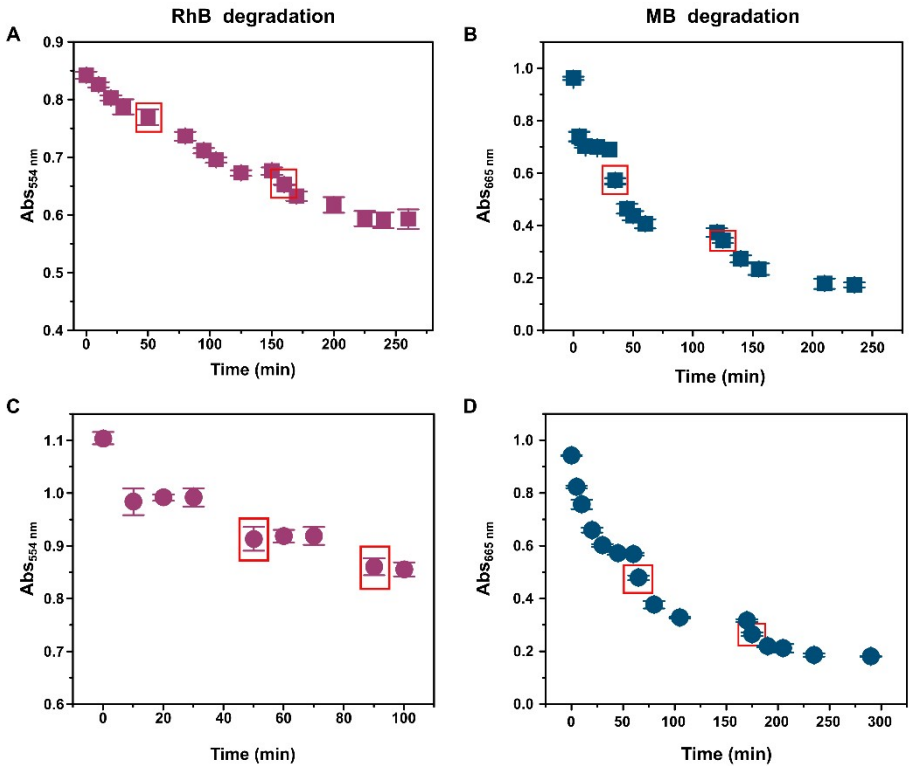

Figure S7: Addition of consecutive aliquots of H_2O_2.
Kinetic of **(A)** RhB and **(B)** MB degradation in the presence of PtNZ when consecutive aliquots of H_2O_2 are added in the reaction solution. Kinetic of **(C)** RhB and **(D)** MB degradation in the presence of PdNZ when consecutive aliquots of H_2O_2 are added in the reaction solution. The red squares highlight the first time point acquired after adding a fresh aliquot of H_2O_2.

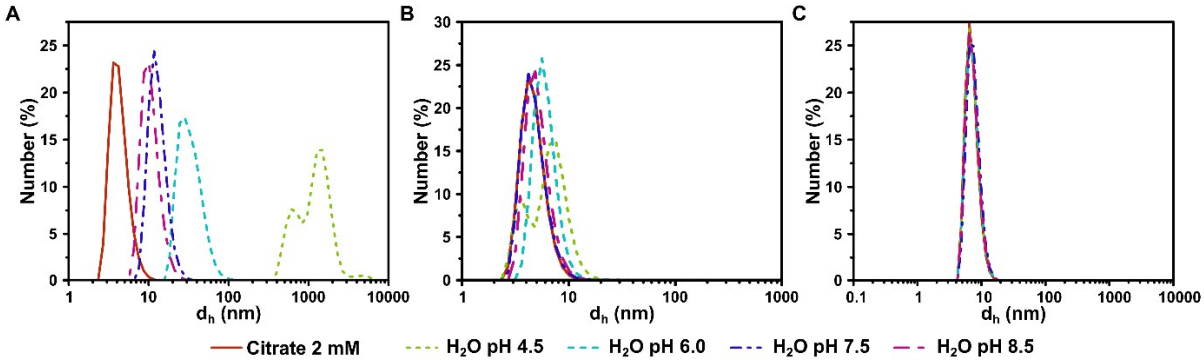

Figure S8: Stability of AuNZ in the presence of different dyes
DLS analysis of AuNZs in the presence of **(A)** RhB, **(B)** MB, and **(C)** MO, in water at different pH and in citrate 2 mM (stock solution). The data are plotted as average of 3 consecutive measurements with 12 run each.

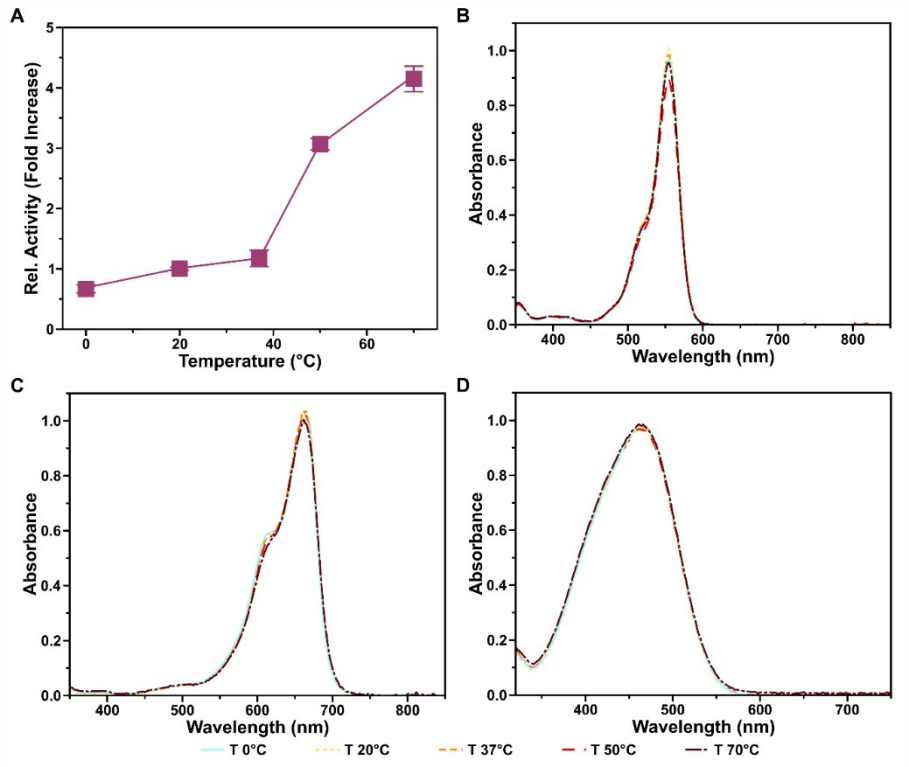

Figure S9: AuNZ POD-like activity temperature dependence and dye temperature stability

(A) Temperature dependence of AuNZ obtained using Amplex Red as chromogenic substrate. Temperature stability of **(B)** RhB, **(C)** MB, and **(D)** MO.

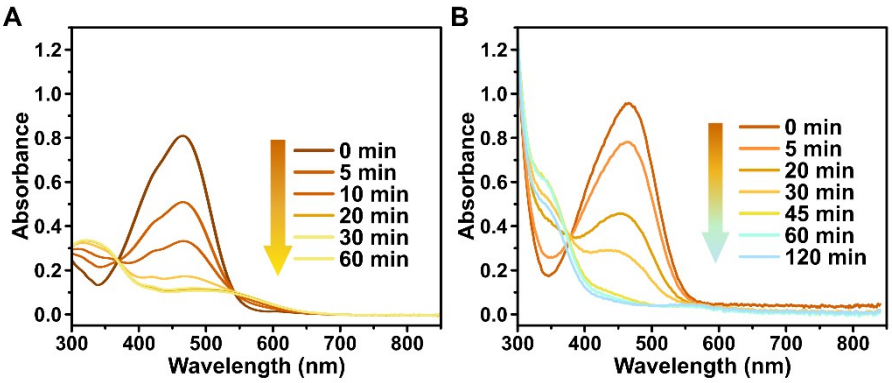

Figure S10: Absorption spectra of MO solution degraded by HRP and AuNZ.

(A) Full absorption spectra of the degradation process of a solution containing 40 μM MO with 20 ppm of HRP. The colour gradient follows the incubation time. While the main absorption peak of MO at 465 nm decrease, other two peaks appear, one centred at around 550 nm and the other in the UV region, below 350 nm. **(B)** Full absorption spectra of the degradation process of a solution containing 40 μM MO with 20 ppm of AuNZ. The colour gradient follows the incubation time. While the main absorption peak of MO at 465 nm decrease, one peak at around 350 nm progressively appears, but at late time points also this second peak decreases in intensity.

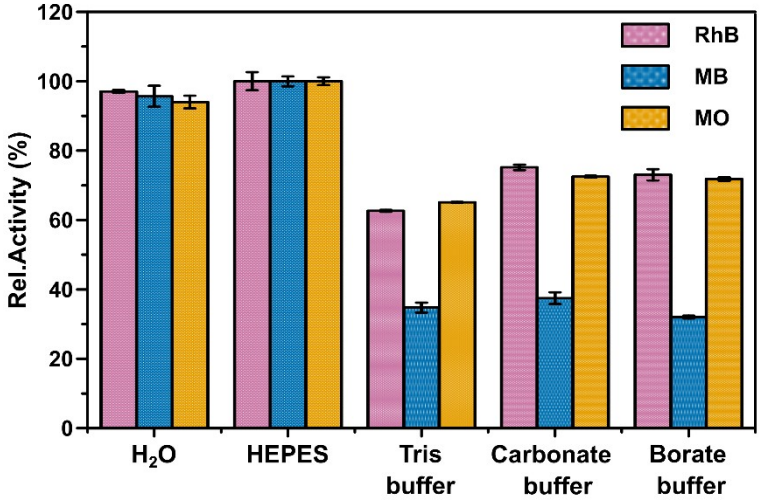

Figure S11: Buffer dependence of AuNZ catalytic activity.
Activity of AuNZ in HEPES, Tris, Carbonate, and Borate buffers (pH 8) for the degradation of the three different dyes, compared to their activity in pure H_2O at the same pH. All data are presented as mean ± st. deviation of three replicates.

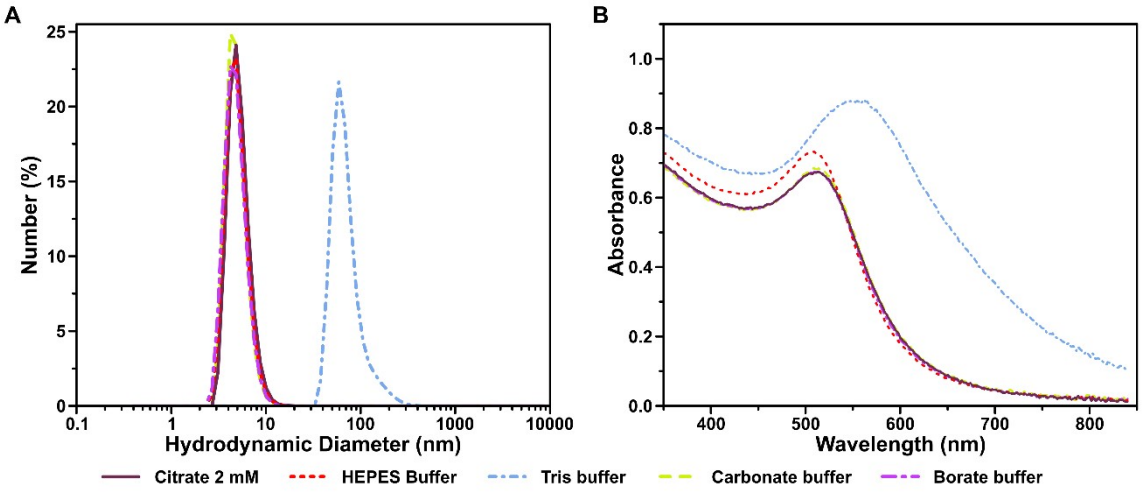

Figure S12: AuNZ stability in different buffers.
(A) AuNZ colloidal stability in different buffers compared to their stability in storage buffer (citrate 2 mM). The data are plotted as average of 3 consecutive measurements with 12 run each. **(B)** UV/vis spectra of AuNZ incubated in different buffers showing the plasmon resonance peak. Samples were incubated for 30 minutes in the buffer solution before analysis.

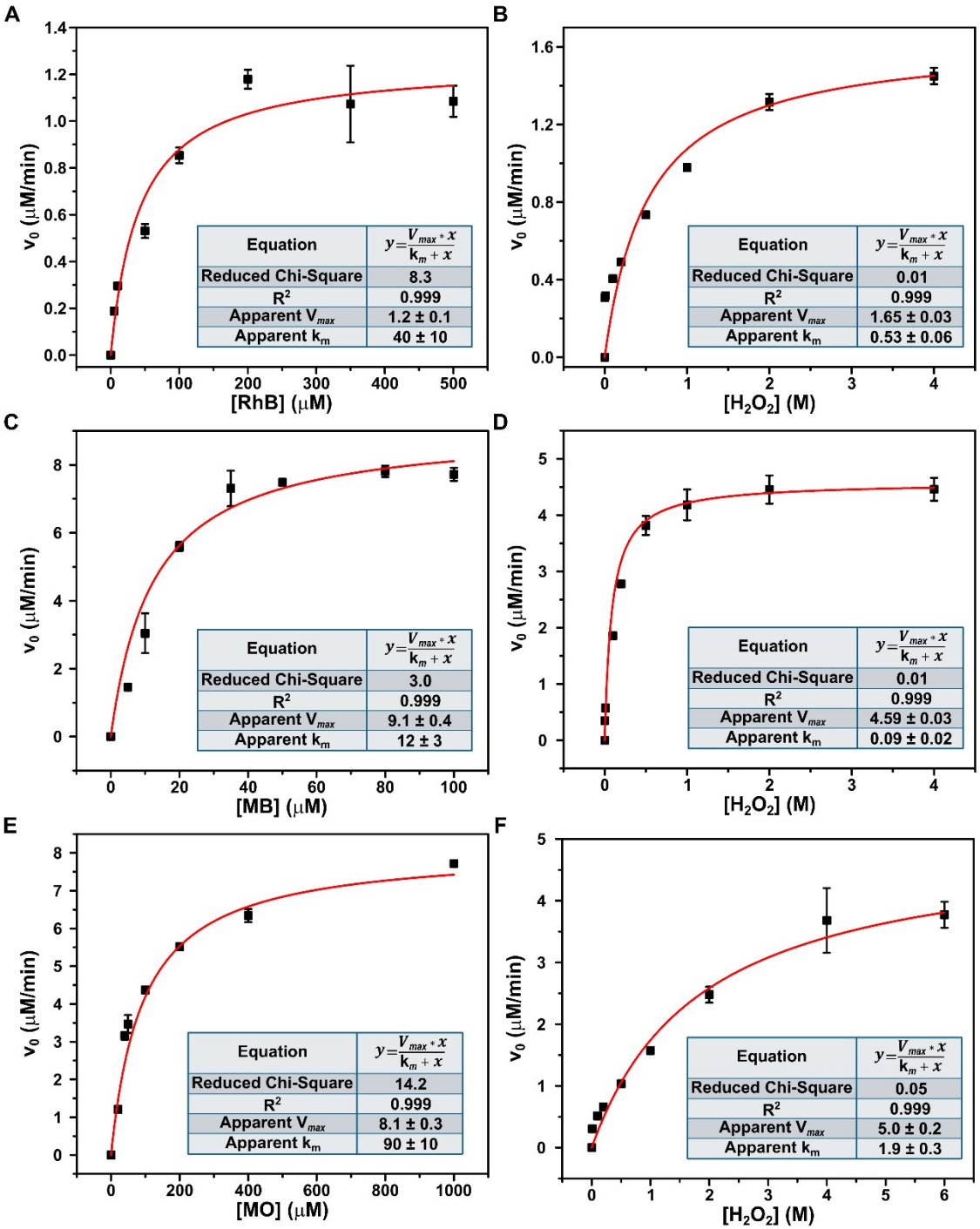

Figure S13: Michaelis-Menten fitting for degradation of synthetic dyes.
Speed of degradation reaction obtained at different concentration of **(A)** RhB **(C)** MB, and **(E)** MO at the same concentration of H_2O_2 (0.4 M), and different concentrations of H_2O_2 using the same amount of **(B)** RhB (10 μM), **(D)** MB (14 μM), and **(F)** MO (40 μM). Michaelis-Menten fittings were generated with Origin. Data are plotted as average ± st. deviation of three replicates.

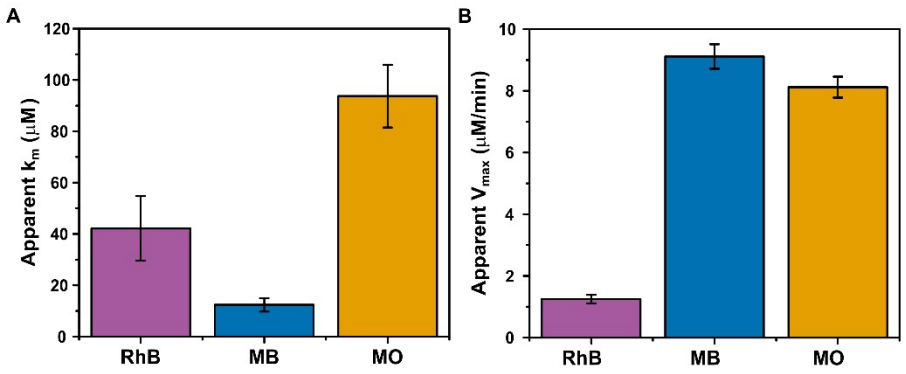

Figure S14: Michaelis-Menten parameters comparison. Comparison between **(A)** the k_m constants and **(B)** the V_{max} values obtained for the dyes.

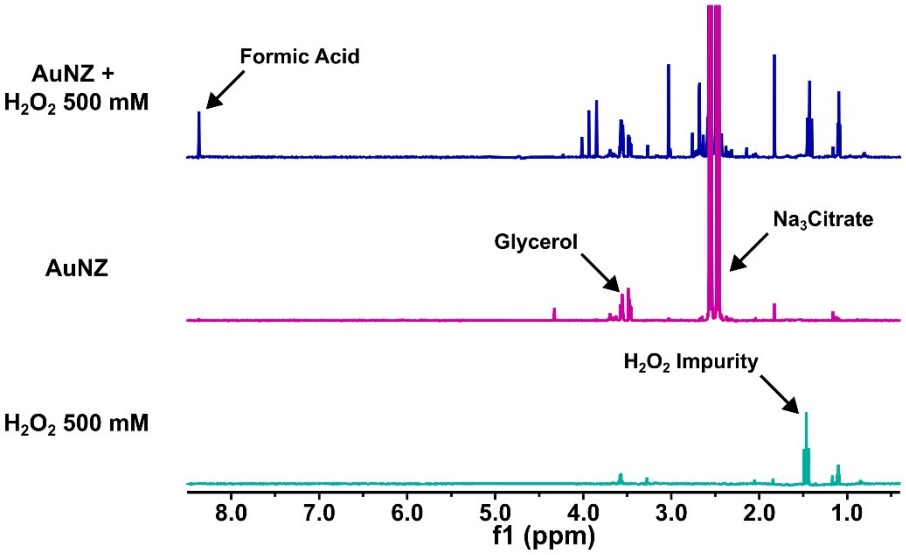

Figure S15: ^{1}H NMR of Controls.
^{1}H 1D NMR spectra of controls containing only H_2O_2 500 mM, AuNZ 15 ppm in NaCitrate 666 μM (working concentration for dye degradation experiments), and the combination of the two (from bottom to top). The spectra highlight the presence of some impurities in the starting reagents (H_2O_2 contains stabilizers, while AuNZ contain, besides the citrate, some glycerol, probably adsorbed during work-up with Amicon® centrifugal filters). The combination of AuNZ and H_2O_2 shows other signals, probably associated to the degradation product of those contaminants.

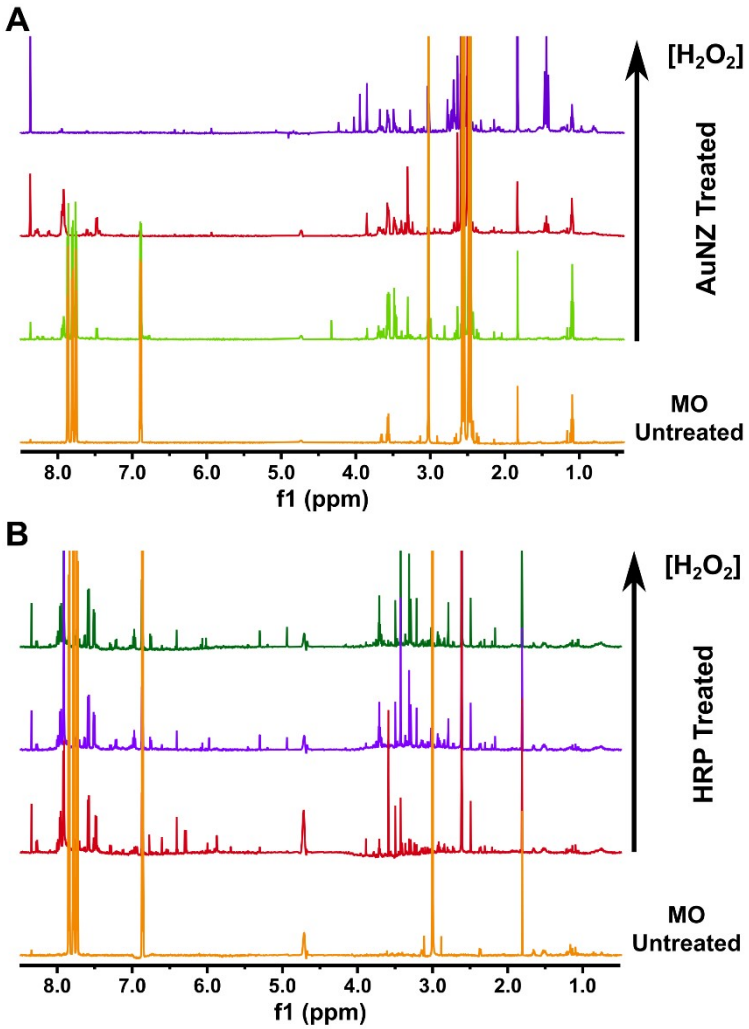

Figure S16: ^{1}H NMR of MO Degradation.
^{1}H 1D NMR spectra of MO degraded **(A)** with AuNZ (15 ppm) and increasing concentration of H_2O_2 (10, 100 and 1000 mM), and **(B)** with HRP (20 ppm) and increasing concentration of H_2O_2 (1, 3 and 5 mM). All the ^{1}H 1D NMR spectra and pictures were acquired after 24 h of treatment to guarantee all the H_2O_2 was consumed.

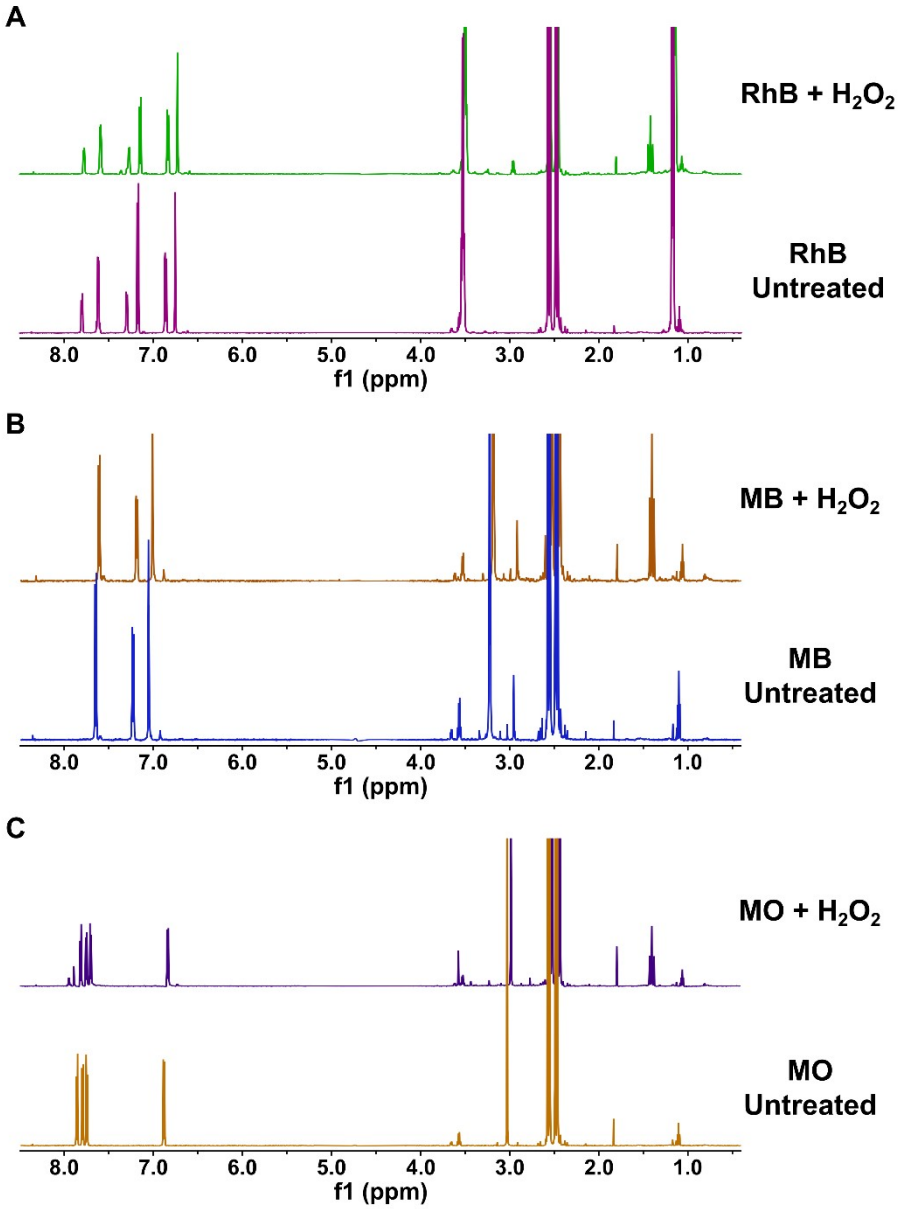

Figure S17: ^{1}H NMR of dyes.

^{1}H 1D NMR spectra of solutions containing 100 μM **(A)** RhB, **(B)** MB, or **(C)** MO with and without H_2O_2 (500 mM) after >7 days of incubation. No significant differences are visible from the spectra in presence of H_2O_2 compared with the spectra of the dyes alone, confirming that the effect of H2O2 on the dyes are negligible in the absence of AuNZ. All the ^{1}H 1D NMR spectra and pictures were acquired after 24 h of treatment to guarantee all the H_2O_2 was consumed.

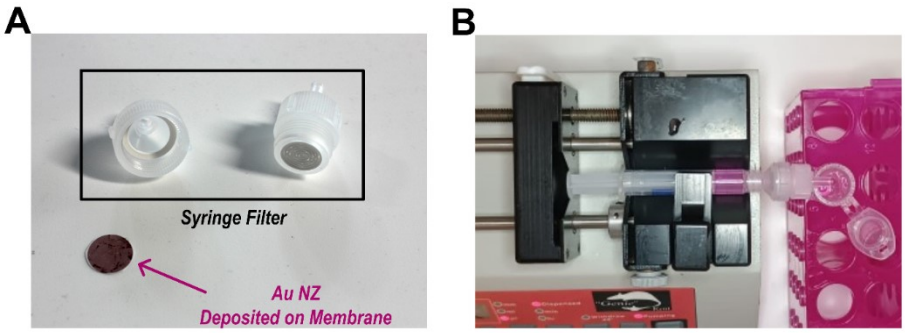

Figure S18: Pictures of filtration system.
Pictures depicting **(A)** the syringe filter, and the AuNZ deposited on the nylon membrane, and **(B)** the system in action during the degradation of a solution of RhB assisted by the syringe pump.

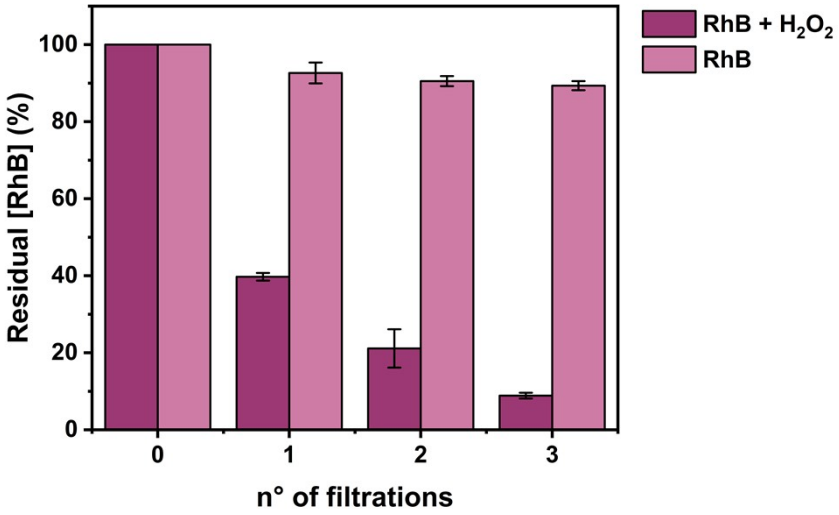

Figure S19: Optimization of filtration protocol on AuNZ membrane.
Residual concentration of RhB after 1, 2, and 3 consecutive filtrations through the AuNZ membrane. The control without H_2O_2 shows that RhB is not significantly retained by the filter (after the conditioning process described in the methods section). Data are plotted as average ± st. deviation of three replicates.

Materials and methods

Chemicals

Ammonium hydroxide, Sodium hydroxide, Sodium citrate tribasic dihydrate BioUltra, ≥99.0% (NaCit), Sodium borohydride granular, 99.99% , Glycerol ≥99.5%, Tannic acid, Hydrogen Peroxide 30%, Ethanol (EtOH) 96.0-97.0%, Hydrochloric acid 37%, Nitric acid 65-67 %, Citric acid, Chloroplatinic acid hexahydrate, Palladium (II) chloride, Rhodamine B (RhB), Methylene blue (MB), Methyl orange (MO), Horseradish peroxidase (HRP), Ultra-15 Centrifugal Filter Unite were purchased from Merck. In addition, 10-Acetyl-3,7-dihydroxyphenoxazine (Amplex Red, AR), Hydrogen tetrachloroaurate (III) hydrate, 99.9 % and the UltraPure Agarose were purchased by Thermo Fisher Scientific.

Synthesis of PtNZs

Platinum nanozymes (PtNZs) were synthetized following published method.[1] Briefly, in a round bottom flask, 162 µL of $H_2PtCl_6 \cdot 6H_2O$ 0.5 M previously dissolved in water and 192 µL of NaCit 0.5 M were added to 87 mL of Ultrapure water under vigorous magnetic stirring. 5.4 mL of $NaBH_4$ 0.06 M were added drop by drop (one drop per second) into the vessel at room temperature, observing a change in color from pale yellow to black. Then, the vessel was placed in a glycerol bath, pre-heated at 75 °C, and let react under magnetic stirring. After 30 minutes, the vessel was removed from the glycerol bath and cooled down to room temperature while stirring. The NZs were concentrated and washed multiple times with NaCit water solution (2 mM) using centrifugal filter (50 kDa cut off), spinning at 1000 G for periods of 3 minutes.

Synthesis of PdNZs

Palladium nanozymes (PdNZs) were synthetized adapting a published method.[2] Briefly, in a round bottom flask, 300 µL of $PdCl_2$ 0.05 M, previously diluted in HNO_3 0.1 M, and 8.8 mL of NaCit 0.03 M and citric acid 2 mM were added to 80 mL of Ultrapure water under magnetic stirring. Then, 3 mL of $NaBH_4$ 0.02 M were rapidly added into the vessel at room temperature, observing a change in color from pale yellow to black. The vessel was placed in a glycerol bath pre-heated at 100 °C under magnetic stirring. After 20 minutes, the vessel was removed from the glycerol bath and cooled down to room temperature while stirring. The NZs were concentrated and washed multiple times with NaCit water solution (2 mM) using centrifugal filter (30 kDa cut off) spinning at 1000 G for periods of 5 minutes.

Synthesis of AuNZs

Gold nanozymes (AuNZs) were synthetized following published method.[3] Briefly, in a round bottom flask, 150 mL of a solution containing NaCit 2.2 mM, tannic acid 0.0017 mM, and K_2CO_3 1 mM was warmed up to 70°C under stirring. Then, 304 µL of $HAuCl_4$ 82 mM were added into the vessel, observing a change in color from pale gray to brown/orange. After 5 minutes, the vessel was removed from the glycerol bath and fast cooled down using an ice bath. Then, 150-200 µL of NaOH 0.5 M were added to reach a pH of ~8.5, stopping the reaction and stabilizing the NZs. The NZs were concentrated and washed several times with NaCit water solution (2 mM) using centrifugal filter (50 kDa cut off), spinning at 1000 G for periods of 3 minutes.

Transmission Electron Microscopy (TEM): the samples were prepared by drop casting 3 µL of the stock solution on TEM grid Formvar/Carbon 200 Mesh, Copper (PE01800-F) by Nanovision and let dry. The images were obtained by JEOL JEM-1400 – Analytical 120 kV and analyzed with ImageJ to estimate the mean diameter.

Dynamic light scattering (DLS): DLS measurements were performed with a Malver Zetasizer Nano-S, using disposable cuvette. The temperature equilibrated for 60 seconds before measurements. Each sample was measured three times with at least 12 runs per measurement.

ICP-OES: The elemental concentrations of NZs were obtained with ICP-OES analysis: 50 µL of the stock solutions were dissolved in 1 mL of aqua regia (3 parts of HCl 37% and 1 part of concentrated HNO_3 65-67%) overnight, then brought to a volume of 10 mL with deionized ultrafiltered H_2O before analysis with a ThermoFisher iCAP 7600 DUO Thermo spectrometer.

Optimization of reaction conditions

Solutions of NZs and RhB (10 µM), MB (14 µM) or MO (40 µM) were incubated with solutions of H_2O_2 from 0.1 to 1 M in 10 mM HEPES buffer pH 8.2 for 30 and 120 minutes. The UV/vis spectra were acquired, and the relative concentration of the residual dye was calculated monitoring the absorbance of the characteristic peak of RhB, MB and MO at 554, 664 and 465 nm respectively. The NZ concentrations were normalized to work with the same total surface area (1.5 x 10^{15} nm^2/mL, for RhB, and 1.1 x 10^{15} nm^2/mL, for MB and MO) for all the NZs, thus their molar concentrations in the reaction solution were: 46.2 and 34.6 nM PtNZs, 67.1 and 51 nM PdNZs, 44.3 and 33.2 nM AuNZs.

Comparison among NZ for dyes degradation

UV/Vis measurements

Solutions of 40 µM RhB, MB, or MO were incubated in the presence of the different NZs and H_2O_2 400 mM, in 10 mM HEPES buffer pH 8.2. The UV/vis spectra were acquired, and the relative activity was calculated monitoring the decrease of the characteristic absorbance peak of RhB, MB and MO at 554, 664 and 465 nm respectively. The NZ concentrations were normalized to work with the same total surface area (1.5 x 10^{15} nm^2/mL, for RhB, and 1.1 x 10^{15} nm^2/mL, for MB and MO) for all the NZs, thus their molar concentrations in the reaction solution were: 46.2 and 34.6 nM PtNZs, 67.1 and 51 nM PdNZs, 44.3 and 33.2 nM AuNZs.

O_2 sensor measurements

Solutions of 40 µM RhB, MB, or MO were incubated in the presence of the different NZs and H_2O_2 400 mM, in 10 mM HEPES buffer pH 8.2. The needle-type oxygen probe of a 2 channel FireSting-O_2 was inserted through the septum to measure the O_2 concentration (%) in the headspace of the vial. A small needle was inserted to avoid an increase of the total pressure inside the vials during the reaction. O_2 production was monitored, and the relative activity was calculated based on the partial pressure of O_2 recorded. The NZ concentrations were normalized to work with the same total surface area (1.5 x 10^{15} nm^2/mL, for RhB, and 1.1 x 10^{15} nm^2/mL, for MB and MO) for all the NZs, thus their molar concentrations in the reaction solution were: 46.2 and 34.6 nM PtNZs, 67.1 and 51 nM PdNZs, 44.3 and 33.2 nM AuNZs.

pH dependence

Solutions of 10 µM RhB, 14 µM MB, or 40 µM MO were incubated for 5, 3 and 30 minutes, respectively in the presence of AuNZs 20 ppm (for RhB) and 15 ppm (for MB and MO) and H_2O_2 0.4 M (for RhB and MB) and 1 M (for MO), in water, adjusting the pH from 4.5 to 8.5 with diluted HCl or NaOH. The UV/vis spectra were acquired, and the relative activity was calculated monitoring the decrease of the characteristic absorbance peak of RhB, MB and MO at 554, 664 and 465 nm respectively.

Temperature dependence

For the temperature dependence experiments, different solutions were prepared:

- 100 µM AR, 10 ppm AuNZs and 100 mM H_2O_2
- 10 µM RhB, 20 ppm AuNZs and 400 mM H_2O_2

- 14 μM MB, 20 ppm AuNZs and 100 mM H_2O_2
- 40 μM MO, 20 ppm AuNZs and 400 mM H_2O_2

The solutions were incubated for 3 minutes (5 minutes in case of MO) in 10 mM HEPES pH 8.2, at different temperatures (from 0 to 70 °C). The UV/vis spectra were acquired, and the relative activity was calculated monitoring the decrease of the characteristic absorbance peak of AR, RhB, MB and MO at 570, 554, 664 and 465 nm respectively. Controls without AuNZ or H_2O_2 were performed in the same conditions to confirm the stability of the dye at the different temperatures.

Buffer dependence

Solutions of 10 μM RhB, 14 μM MB, or 40 μM MO were incubated for 5, 3 and 30 minutes, respectively, in the presence of 20 ppm AuNZs and 400 mM H_2O_2, in water, adjusting the pH to 8 with diluted NaOH, or in the presence of a 10 mM buffer at the same pH. The UV/vis spectra were acquired, and the relative activity was calculated monitoring the decrease of the characteristic absorbance peak of RhB, MB and MO at 554, 664 and 465 nm respectively.

Estimation of Michaelis-Menten Constants

Estimation of K_m for dyes

For determining Michaelis Menten constant of AuNZ with the different dyes, the following reaction solutions were prepared varying the concentration of the dye from 10 to 500 μM.

- RhB: 20 ppm AuNZs and 2 M H_2O_2
- MB: 15 ppm AuNZs and 0.8 M H_2O_2
- MO: 15 ppm AuNZs and 4 M H_2O_2

The solutions were incubated in 10 mM HEPES buffer pH 8. After 10 (for RhB), 3 (for MB) or 8 minutes (for MO), the absorbance of the dye was recorded by UV/vis spectroscopy and the amount of dye consumed was estimated from the intensity of the characteristic peak of RhB, MB and MO at 554, 664 and 465 nm respectively. Data were elaborated with Origin, using the Michaelis-Menten fitting function.

Estimation of K_m for H_2O_2

AuNZ (20, for RhB, and 15 ppm) was incubated in the presence of 10 μM RhB, 14 μM MB, or 40 μM MO, varying the concentration of H_2O_2 from 0.01 to 6 M. After 5, 3 and 10 minutes the absorbance of the dye was recorded by UV/vis spectroscopy and the amount of dye consumed was estimated from the intensity of the characteristic peak of RhB, MB and MO at 554, 664 and 465 nm respectively. Data were elaborated with Origin, using the Michaelis-Menten fitting function.

NMR Analysis

Samples preparation

AuNZ (15 ppm) was incubated in the presence of the different dyes at a constant concentration (100 μM) and with different amounts of H_2O_2 (0, 10, 100, 500 and 1000 mM). Experiments were performed in D_2O. After 72 hours, 600 μL of each sample were transferred into 5 mm NMR for analysis.

Similarly, HRP (20 ppm) was incubated in a 3 mL solution containing MO 100 μM with or without 1 mM H_2O_2. After 3 h, 1 mL of the solution was withdrawn (sample 1 mM), and 2 μL of concentrated H_2O_2 (1 M) was added to the remaining reaction solution. After another 3 h, a second aliquot of 2 μL of concentrated H_2O_2 (1 M) was added to the reaction solution. After 3 h, 1 mL of the solution was withdrawn (sample 3 mM), and 1 μL of concentrated H_2O_2 (1 M) was added to the remaining reaction solution. One last aliquot of 1 μL of concentrated H_2O_2 (1 M) was added to the reaction solution after another 3 h (sample 5 mM). This process aimed to avoid enzyme inactivation due to high H_2O_2

concentration. Experiments were performed in PB 10 mM pH 6. After 24 hours from the last aliquot of H_2O_2, 540 mL of each sample were mixed with 60 mL of D_2O and the NMR spectra were recorded.

Samples analysis

Each NMR experiment was recorded at 298 K using a Bruker FT-NMR 600 MHz ADVANCE NEO equipped with a QCI $^{1}H/^{19}F–^{13}C/^{15}N–D$ cryoprobe and a Samplejet™ autosampler with temperature control. For all samples, a 1D ^{1}H NMR spectrum was acquired (Bruker sequence: noesygppr1d) with 256 scans, a relaxation delay (d1) of 4s, a spectral width of 30 ppm, an acquisition time of 1.83 s and a mixing time of 10 ms. The data were multiplied by an exponential function with line broadening of 0.3 Hz before Fourier transformation.

Membrane Filtration

Membrane preparation

The nylon membrane was cut in a circle to perfectly fit the filter. 1 mL of a dispersion of AuNZ (60 ppm) were deposited on the membrane and let dry under vacuum for 10 minutes. The filter was then immersed in an aqueous solution and the concentration of AuNZ in the solution was monitored by UV/vis spectroscopy for 24 hours, observing no release of the NZ from the membrane surface.

Reaction conditions optimization and recyclability test

RhB showed significant unspecific adsorption on the nylon membrane, so before filtering samples and controls, the AuNZ-membrane was equilibrated (up to saturation) passing through the filter 3 mL of a solution of RhB 10 μM.

Solutions (1 mL) containing 10 μM RhB and 1 M H_2O_2 were passed through the filter with a flow of 0.1 mL/min. The flow through was collected, its absorbance acquired by UV/vis spectroscopy and passed through the filter 2 more times to obtain a dye removal > 90%.

Control experiments were performed passing through the filter a solution 10 μM RhB, without H_2O_2.

For testing the recyclability of the filter, after each sample the filter was thoroughly washed with ultrapure water (3 times), before injecting the next one.

Simulated real samples

Solutions (1 mL) containing 10 μM RhB and 1 M H_2O_2 in water collected from different basins were passed through the filter with a flow of 0.1 mL/min. The flow through was collected, its absorbance acquired by UV/vis spectroscopy and passed through the filter 2 more times to obtain a dye removal > 90%.

Control experiments were performed passing through the filter a solution 10 μM RhB, without H_2O_2.

Unspecific adsorption of the dye on the filter equal to about 10 % the total concentration was observed after the first filtration, but no further adsorption was observed during the second and third filtration (a partial release of the adsorbed dye was observed instead).

Tap water was collected from a sink in the IIT building in Via Morego 30, Ge, Italy. Lake water was collected from “Lago della Busalletta”. River water was collected from Polcevera river, at the height of “Via Parodi 2, Santa Marta, Ge, Italy”. Sea water was collected from Boccadasse beach, Ge, Italy.

References

1. Moglianetti, M.; De Luca, E.; Pedone, D.; Marotta, R.; Catelani, T.; Sartori, B.; Amenitsch, H.; Retta, S. F.; Pompa, P. P., *Nanoscale* **2016,** *8* (6), 3739-3752. DOI https://doi.org/10.1039/C5NR08358C.
2. Mastronardi, V.; Kim, J.; Veronesi, M.; Pomili, T.; Berti, F.; Udayan, G.; Brescia, R.; Diercks, J. S.; Herranz, J.; Bandiera, T.; Fichthorn, K. A.; Pompa, P. P.; Moglianetti, M., *Nanoscale* **2022,** *14* (28), 10155-10168. DOI https://doi.org/10.1039/D2NR02278H.
3. Piella, J.; Bastús, N. G.; Puntes, V., *Chemistry of Materials* **2016,** *28* (4), 1066-1075. DOI https://doi.org/10.1021/acs.chemmater.5b04406.