

Supporting Information

Cellular Metabolic Activity and Electrochemical Stability Assay of Embedded Oxidoreductase Enzyme Confined in Nanospace of Framework Exoskeleton

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S1. Materials and Instrumentation.

Chemicals:

All the chemicals were obtained commercially and used without additional purification. Zinc nitrate hexahydrate (99.0 %), hydrogen peroxide (H₂O₂, 30.0 %), hydrochloric acid, sodium chloride, and ammonium fluoride (NH₄F), 97.0 %) were obtained SHOWA. Imidazole-2-carboxaldehyde (ICA, 97.0 %), 2-methylimidazole (2-mIm, 99.0 %), 3-amino-1,2,4-triazole (3-AT, 96.0 %) and xylenol orange tetra sodium salt (ACS reagent) was purchased from Alfa Aesar, while catalase (CAT), Glucose Oxidase (GOx), and polyvinylpyrrolidone (PVP, MW:40,000) were purchased from Sigma-Aldrich. The cell lines A549 and NIH3T3 are purchased from National Centre for Cell Science (NCCS), Pune.

S2. Experimental

ZIF-8 Synthesis:

ZIF-8 nanocrystals were prepared following the procedures previously reported.¹⁹ Typically, 0.587 g of Zn(NO₃)₂·6H₂O and 1.298 g of 2-methylimidazole (Hmim) were dissolved in 40 mL of methanol (MeOH) separately, and then the Hmim solution was poured into the Zn(NO₃)₂·6H₂O solution under magnetic stirring. The mixture was stirred at room temperature for 2 h. The solid product was separated from the milky colloidal dispersion by centrifugation. After being washed with anhydrous ethanol and centrifugation three times, the white ZIF-8 product was dried at 60°C.

Synthesis of the enzymes@ZIF-8.

Catalase@ZIF-8

The enzymes@ZIF-8 were synthesized *via* a water-based mild *de novo* approach. In a typical synthetic process, CAT (1.0 mg, ca.14 nM) was firstly dispersed into 5 mL deionized water, followed by adding 38 mg zinc acetate dehydrate and 970 mg HmIM. Then, the mixed solution was stirred for 10 min at room temperature. Finally, the precipitate was collected by

centrifugation (8000 rpm, 10 min), and then washed, sonicated, and centrifuged three times to remove loosely adsorbed enzymes.

ZIF-90-Enzyme encapsulation:

Zinc nitrate (371.3 mg) was added into DI water (3.0 mL). Then this solution was added to DI water (25 mL) in which ICA (2-Imidazolecarboxaldehyde) (480.0 mg), PVP (50 mg) and catalase from bovine liver (25.0 mg) was dissolved at 42 °C. After mixing the solutions above, the resulting powder was stirred for a few minutes. All reactions were carried out at room temperature. Finally, the as-obtained products were collected by centrifugation (14,000 rpm), washed with excess DI water, and vacuum dried at room temperature.

Linking Fluorescein Isothiocyanate (FITC) with Catalase:

Catalase (10 mg) was suspended in 10 mL of PBS (phosphate buffer saline) (pH 7.4) under 4 °C. 5 mg of EDC and 2.5 mg of NHS (N-Hydroxysuccinimide) were added into the catalase-containing solution, and the whole solution was stirred for 1h. After that, FITC (63 µg) was dissolved in PBS solution and then was added into the catalase-containing solution. The whole solution was stirred for another 4h at 4 C in the dark. The unreacted EDC, NHS, and FITC in the FITC-catalase containing solution were removed by dialysis (MWCO: 12k-14k) for one day.

Hemocompatibility Assay

A coagulation assay was performed on the blood samples collected through retro-orbital puncture of a healthy mice in an EDTA coated vial to avoid the coagulation of hematopoietic cells. Blood fractionation at 3000 rpm for 10 min was done to separate out the Erythrocytes from plasma cells. This freshly collected plasma was washed thrice with saline solution and further transferred to PBS buffer (PH 7.4). The final sample was incubated with 2mg/mL concentration of catalase loaded ZIF-8 framework at 37°C for 2 h in a temperature control

incubator. Here, blood with 1% surfactant triton-X-100 and Normal saline treated blood was considered as positive and negative controls, respectively. The absorbance was recorded at 540 nm to check the total hemoglobin content in blood plasma of the incubated samples. Both saline and MOF treated suspensions were smeared on a glass slide and observation was done under an inverted microscope at 40X magnification.

Hemocompatibility Assessment

The hemocompatibility test was done to determine the hemolysis percentage and also to check the Agglutination of the blood cells which revealed that the hemolysis rate was less than 5% at the High concentration of 200 µg. Moreover, results revealed that there were no significant differences regarding the values of white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB) between the treated sample and those in the normal saline blood solution.

Cell culture materials and reagents:

A549 cells were grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% heat-inactivated fetal bovine serum, [+] L glutamine, [-] Sodium bicarbonate, 100 µg/mL penicillin and 100 U/mL streptomycin. A549 cells were obtained from National Center for Cell Science, Pune, India) and cultured in a humidified incubator at 37 °C temperature and 5% CO₂.

In vitro cell viability using MTT assay

200 µL of A549 containing media (25000 cells/mL) was added to each well in 96 well plate and incubated overnight to allow cells to adhere on to the surface of the plate. The cells were treated with different dosages (0 µg/mL, 25 µg/mL, 50 µg/mL, 250 µg/mL, 500 µg/mL, 1250 µg/mL) of ZIFB, ZIF90, Catalase, ZIFB-cat and ZIF90-cat for 48 h. After 48 h of incubation, the media was removed and the cells were washed with PBS. A 10 µL of an MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) aqueous solution (10 mg/mL) was added

to each well of the 96-well plate and incubated for 4 h. At the end of 4 h, the solution was removed and 100 μ L of DMSO was added to each well. The absorbance was recorded at 570 nm using a plate reader (Polar Star Omega Plate Reader Germany).

Calcein AM-PI cell viability assay

For Calcein AM-PI cell viability assay experiment, trypsinize a subconfluent monolayer culture, and the cells were collected in the growth medium containing serum. The suspension was centrifuged at 1700 rpm for 5 min to pellet the cells. Cells were resuspended into the growth medium and counted them. The cells were seeded in a 96-well flat bottom tissue culture plate at a concentration of 10^4 cells/well. The cells were allowed to grow and stabilize for overnight. The as prepared cells were treated with different concentrated nanomaterials prepared (in duplicates 250 μ g/mL). Then the plate was incubated for 4 hours. After incubation with nanomaterials, media was removed, and cells were treated with Calcein AM (final conc 2 μ M) and propidium iodide (final conc 3 μ M) in PBS. Then the cells were again incubated for 30 min at 37 $^{\circ}$ C. The cell images were taken with fluorescence microscope (Olympus IX73).

Methods and materials: Calcein AM and Propidium Iodide (PI) were purchased from Merck and SRL respectively.

LIVE/DEAD assay: The LIVE/DEAD assay was performed using NIH3T3 and A549 cell lines. In a typical experiment, 200 μ L of media containing 5×10^4 cells/mL was added to each well in 96 well plate. The cells were incubated overnight to adhere to the surface of the plate. The cells were then treated with 250 μ g/ml of catalase, ZIF8, ZIF90, Cat@ZIF8 and Cat@ZIF90 respectively for 4 hours at 37 $^{\circ}$ C. After the incubation, the cells were treated with Calcein AM and PI in PBS with the final concentrations of 2 μ M and 3 μ M respectively and incubated at 37 $^{\circ}$ C for 30 min. The images were taken with the Olympus IX73 fluorescence microscope using FITC and TRITC filters.

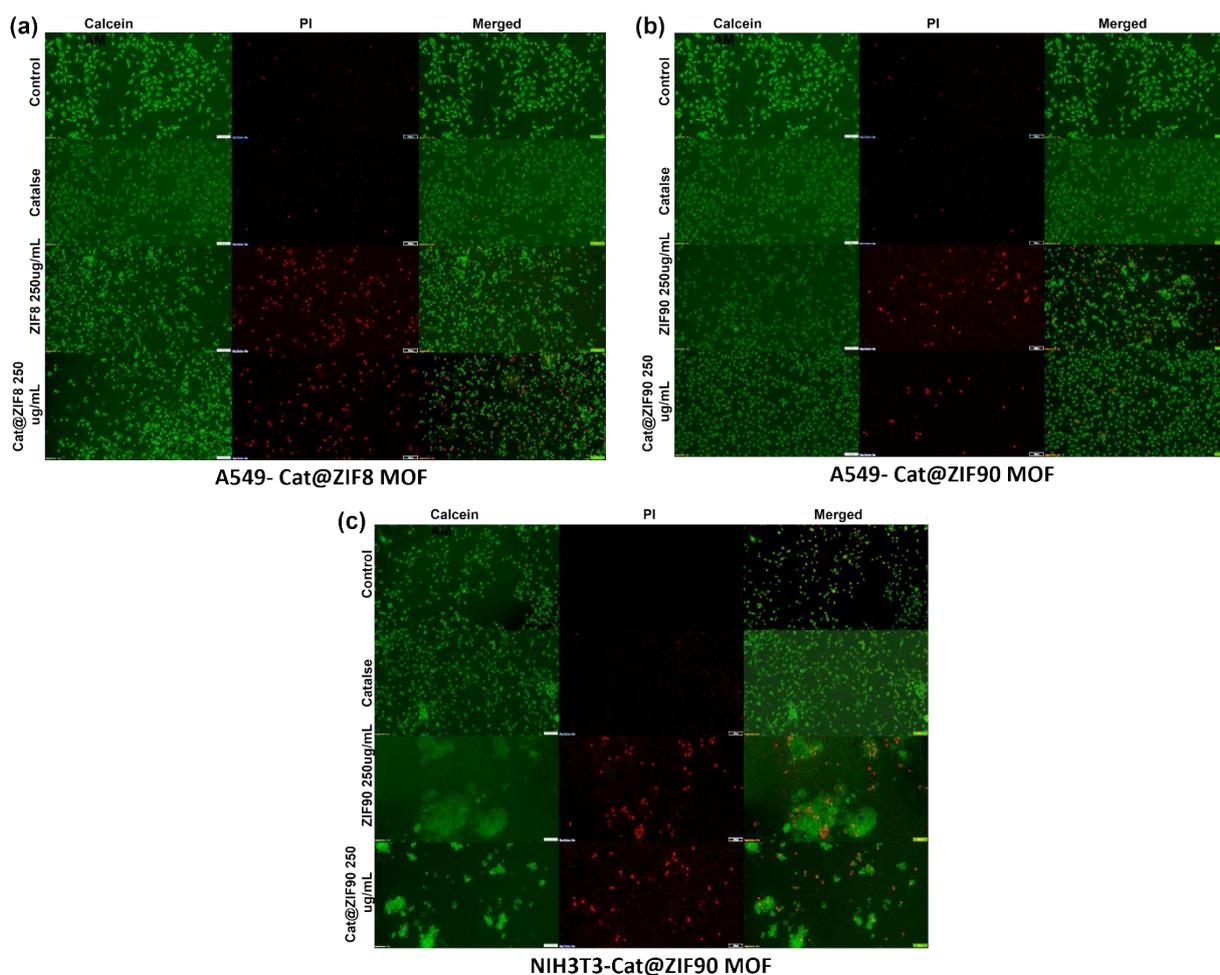


Figure S1. Fluorescence microscopy of (a) Control, Catalase, ZIF8 and Cat@ZIF8 on A549 cells (b) Control, Catalase, ZIF90 and Cat@ZIF90 on A549 cells (c) Control, Catalase, ZIF90 and Cat@ZIF90 on NIH3T3 cells using Calcein AM and PI dye.

Characterizations

Field-emission scanning electron microscopy (FE-SEM) was carried out with a FESEM- JEOL 7900F (at an acceleration voltage of 5 kV). Powder X-ray diffraction patterns (PXRD) were collected on a Japan Rigaku Mini flex 600 rotation anode X-ray diffractometer equipped with graphite monochromatized Cu K α radiation ($\lambda = 1.54 \text{ \AA}$). The transmission electron microscopy (TEM), high-resolution TEM (HRTEM) were acquired on JEOL-2010 instrument. Microscopic imaging of fluorescent tagged Catalase containing ZIF-8 and ZIF-90 was recorded by using a

Laser Confocal Microscope with Fluorescence Correlation Spectroscopy (FCS) - Olympus FluoView™ FV1000 with FLIP, FRAP, FRET, FLIM and FCS (PicoQuant).

Preparation of Cat@ZIF-8, GO_x@ZIF-8, Cat@ZIF-90 electrode

To make a carbon electrode for electrochemical characterizations in a three-electrode cell, C-ZIF and 5 wt% Nafion with C-ZIF:dry ionomer weight ratio of 8:2 were dispersed in methanol, followed by ice-bath ultrasonication for 1 h to generate a homogeneous ink. The ink was transferred by a micropipette to coat $1 \times 1 \text{ cm}^2$ area of carbon paper. Carbon papers were dried at room temperature, and successively in a vacuum oven at 80 °C overnight before use. Four pairs of commercial carbon electrodes (BP-2000, KJ-600, XC-72 and Super P) were also prepared through the same procedures for comparison.

Electrochemical Characterization in a three-electrode cell

All electrochemical measurements were conducted at room temperature in 0.5 M NaCl in a traditional three-electrode electrochemical cell. The cell was connected to a computer-controlled CHI 760D electrochemical workstation (CH Instruments, USA). A KCl-saturated Ag/AgCl and a Pt wire were used as the reference electrode and the counter electrode, respectively. Cyclic voltammetry was conducted over the potential range of -0.10 to 0.10 V vs. Ag/AgCl under various scanning speeds (2, 5, 10, 20, 50 and 100 mV s^{-1}). The galvanostatic charge-discharge measurements were measured by setting the upper potential limit at 0.50 V vs. Ag/AgCl at a current density of 0.4 mA cm^{-2} . Electrochemical impedance spectroscopy (EIS) for the samples was carried out at open-circuit potential by setting the amplitude to 5 mV and the frequency range from 1 M Hz to 0.1 Hz . EIS equivalent circuit simulation was performed in a Zview software.

Table S1. Advantages of encapsulating/embedding of oxidoreductase enzyme in the ZIF/s with respect to free enzyme systems.

Entry	Sample	Advantages	References
1	CAT@GZIF-67 vs Free CAT	1) Widely usable range of temperature 2) Higher residual activity at longer storage time (day)	¹
2	HRP-A@ZIF-8 vs peroxidase-minicking enzymes (HRP horseradish peroxidase)	1) ZIF-8 armor protection from denaturation. 2) higher catalytic activity than the free HRP	²
3	Cat@ZIF-8 (confined catalase) Cat@h-ZIF-8 (free-standing catalase)	Interfacial interactions between catalase and internal surface of ZIF-8. Tryptophan protein remain unfolded, no fluorescence red-shift. Catalase is shielded from inhibitor proteinase-K	³
4	FA-L@MD@CAT and free CAT	Protease K digestion inhibition at 37 °C and O ₂ production in H ₂ O ₂ before and after protease K digestion.	⁴
5	CAT@ZIF-8	pH 8 with proteinase K shows biological activity with rate constant 2.5 x 10 ⁻⁴ S ⁻¹ size-sheltering functionality	⁵
6	CAT@ZIF-90	Decomposition of H ₂ O ₂ in presence of Urea (denaturing agent) as compared to free Catalase Structural conformation of the embedded CAT changes less.	⁶
7	ICG/Cyt c@ZIF-8 NPs modified with zoledronate (ZOL)	Catalase like activity of Cyt c decompose H ₂ O ₂ to O ₂ enhance PDT efficiency. ZIF allows not only storing and protecting Cyt c also allows a surface modification by a bone targeting moiety ZOL	⁷
8	ZIF-8:Trp nanocarrier for GOx	Exhibit fluorescent red shift effect, used as nanoreactor for cascade reaction for O ₂ production, nanosizing and nanocarrier role of ZIF-8 provide cascade bioreactor and O ₂ induced therapy.	⁸
9	Cat@ZIF-8 and Cat@ZIF-90	A549-lung adenocarcinoma (lung cancer cell) and NIH3T3 (mouse fibroblasts) cells exhibit concentration dependent behavior of a fixed catalase wt% loading on ZIFs as compared to free Catalase	This work

Table S2. Comparison of enzyme activity of current systems with that of literature reported enzyme embedded system.

CAT-system	Cell line	Cell viability	Reference

1. ZCM	SW1990 cancer cells	97.5%	9
2. HA-CAT@aCe6 NPs	Normoxic cells	90%	10
3. MPA-CdSe/ZnS QDs-CAT	Hepatocytes C57BL/6J mice	98%	11
4. CAT-IONP	4T1 cells under hypoxic and normoxic conditions	65%	12
5. CAT@ZIF-8	A549-lung adenocarcinoma (lung cancer cell)	Concentration dependent cell viability	This work
6. CAT@ZIF-90	A549-lung adenocarcinoma (lung cancer cell)	Concentration dependent cell viability	This work

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