2D-MoS\textsubscript{2} Supported Copper Peroxide Nanodots with Enhanced Nanozyme Activity: Application in Antibacterial Activity

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Experimental section

Material and instrument:
Copper chloride (CuCl\textsubscript{2}.2H\textsubscript{2}O), Polyvinyl pyrrolidine (PVP), Molybdenum disulfide (MoS\textsubscript{2}), n-butyllithium, Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}, 30%), were purchased from Sigma-Aldrich. Chemicals involved in performing various assays such as DiSC\textsubscript{3}(5) (3,3’-Dipropylthiadicarbocyanine Iodide), DTNB (5,5’-dithiobis (2-nitrobenzoic acid)), 3,3’,5,5’-Tetramethylbenzidine (TMB), Glutathione, Luria broth media (LB, HiMedia), NaOH were purchased from commercial sources (Sigma Aldrich, SRL India Pvt. Ltd., and SDFCL chemicals) and used without any further purification. Milli-Q water was used to prepare all the buffer solutions. Agar(I) powdered form was purchased from SRL Chemicals to prepare agar plate.

For recording and processing AFM images, the JPK instrument was used. The height of nanosheets was measured using JPK software. The zeta potential of all materials and CP-nanodot size were determined using the Malvern Zetasizer Nano instrument. UV-vis spectra of ce-MoS\textsubscript{2}, CP-nanodots, and MoS\textsubscript{2}@CP were recorded using a UV-3600 Shimadzu UV-Vis-NIR Spectrophotometer (Japan Analytical Instruments). The peroxidase-like activity (absorbance) was measured using a Thermo Fisher Scientific plate reader. Fluorescence was measured by Thermo Scientific Varioskan Flash Multimode Reader. TEM images were recorded using JOEL JEM-2100F with an accelerating voltage of 200 kV instrument. The sample was drop cast on a Leccy-carbon grid for acquiring TEM elemental mapping images. Elemental TEM Images were acquired using a Transmission Electron Microscope (Titan Themis 300kV from FEI, now Thermo) with an accelerating voltage of 300 kV. The concentration of Mo and Cu in MoS\textsubscript{2}@CP, ce-MoS\textsubscript{2}, and CP-nanodots was determined using a quadrupole inductively coupled plasma mass spectrometer (ICP-MS) technique. The optical density of the bacterial solution was measured by Thermo Fisher Scientific Microplate.
Preparation of chemically exfoliated MoS\textsubscript{2} (ce-MoS\textsubscript{2}):  

The ce-MoS\textsubscript{2} was prepared by the well-standardized procedure from our group.\textsuperscript{1} 300 mg of bulk MoS\textsubscript{2} was carried in a two-neck round bottom flask under a nitrogen atmosphere inside the glove box. n-Butyllithium solution (5mL of 1.6M) in cyclohexane was added into the round bottom flask, which contains MoS\textsubscript{2}, and further kept for stirring for 48 hr. The reaction mixture was removed from the glove box and washed with hexane to remove excess n-butyl lithium (3 times). The lithium intercalated MoS\textsubscript{2} powder was resuspended into the water and sonicated for 60 min. The obtained solution was centrifuged at 10,000 rpm (three times), and collected the precipitate. Furthermore, resuspend the precipitate into the water and centrifuged at 2000 rpm to obtain monolayer nanosheets. The obtained solution was further characterized to confirm the formation of 2D-MoS\textsubscript{2} nanosheets called chemically exfoliated MoS\textsubscript{2}(ce-MoS\textsubscript{2}). Then, the solution was directly used for further preparation of the composite.

Preparation of Copper peroxide nanodots (CP-nanodots):  

Copper peroxide nanodots were synthesized by following the previous report\textsuperscript{2}. First, PVP (polyvinylpyrrolidone, 0.5g) was dissolved in milli-Q water containing CuCl\textsubscript{2}·2H\textsubscript{2}O (5 ml, 0.01M), and then, NaOH solution (5ml, 0.02M) was added slowly under stirring (observed bluish green color). Then, after 5 min, 100 \(\mu\)L of H\textsubscript{2}O\textsubscript{2} (30\% H\textsubscript{2}O\textsubscript{2}) was added, and the color of the solution turned from blue to green to yellow and then brown can be clearly seen in Figure S1. Finally, it was kept stirring for 45 min. PVP-coated CP nanodots were collected by using a dialysis membrane of 3.5KDa. The dialysis was continued for 18hr, and the water was changed after every 3hr interval.
Figure S1: Illustration of color change of solution during the CP nanodot synthesis.

Dispersibility of MoS$_2$@CP:

Figure S2. Demonstration of the Dispersibility of MoS$_2$@CP after 1 month of preparation.

Dynamic Light Scattering (DLS) analysis for the ce-MoS$_2$ and MoS$_2$@CP:

Figure S3. Analysis of the dispersibility through PDI index using DLS. DLS plot with PDI index of MoS$_2$@CP and ce-MoS$_2$ after 1 month of preparation.
IR spectrum analysis:

Figure S4. FT-IR spectra of MoS$_2$@CP, CP-nanodots and ce-MoS$_2$ depicting the presence of Polyvinyl pyrrolidone (PVP) peaks in the CP-nanodots, and MoS$_2$@CP.
AFM sample preparation:

The formation of monolayer MoS\(_2\) after exfoliation was characterised using AFM (JPK Instruments, USA) by drop-casting on atomically smooth mica foil. It shows the layer morphology and thickness of MoS\(_2\) layers. The raw data was processed by JPK software to obtain the height profile diagram.

Estimation of Copper and Molybdenum concentration using Inductively Coupled Plasma Mass Spectrometry (ICP-MS):

ICP-MS analysis was carried out using quadrupole inductively coupled plasma mass spectrometer. The unknown samples for analysis were prepared by adding HNO\(_3\) (2\%) in a 15 mL vial containing of functionalized material (20 \(\mu\)L) and incubated 3-4 hr at 100° C for digestion. The digested samples were made up to 5 mL by adding DI water. CuSO\(_4\)-5H\(_2\)O was used to prepare known concentrations of Cu as an external standard along with equimolar concentration of commercially available MoS\(_2\) standard was added. The external standards accuracy used for ICP-MS was confirmed by the linear regression fit to inductively
coupled plasma mass spectrometry (ICP-MS) values of external standards (R2>99.99). The copper (Cu) and molybdenum (Mo) concentration of the stock solution was determined by multiplying the dilution factor. In all cases, the concentration of composite reported in term of Mo and Cu.

**TEM sample preparation:**

TEM sample was prepared by drop casting the dilute solution of the material on formvar/carbon 200 mesh, copper grid. The sample was dried overnight and subjected for imaging. CP-nanodot images were acquired using a JOEL JEM-2100F with an accelerating voltage of 200 kV. For MoS$_2$@CP, Elemental mapping was done using Titan Themis from FEI, (MNCF facility of Cense Department, iisc) with accelerating voltage 300 kV.

**Assessment of photothermal capability of MoS$_2$@CP:**

The pointed laser with 808 nm was not suitable for measuring temperature in bulk material. So, MoS$_2$@CP was taken in a 1.5 mL Eppendorf tube and irradiated with the NIR LED light of wavelength 740 nm and images were captured using the photothermal camera.
Figure S6. Photothermal images of the Eppendorf containing different concentrations of the MoS$_2$@CP, irradiated with 740 nm light here 5x represent the dilution of the x.

Steady state kinetic analysis of peroxidase type catalytic activity:
To evaluate the kinetic parameters of POD-like nanozyme catalyst, similar assay was performed by independently changing the concentration of TMB substrate. Herein, we have done assay by keeping H$_2$O$_2$ concentration fixed and varying TMB concentration. All other condition were same as mentioned in above assay. The absorbance was recorded at 652 nm with lag time of 2 min for 1 hr. The value of kinetic constants was obtained by fitting data into origin software using Michaelis mentos non-linear fitting function. The initial velocity was obtained from the slope of absorbance versus time plot for each concentration of TMB plot. The absorbance value was converted in terms of the concentration by dividing slope by pathlength and molar extinction coefficient. Finally, $V_o$ versus concentration graph was plotted and $V_{max}$ and $K_m$ constant were obtained from Michaelis Menten non-linear fitting function using origin software.

![Graph showing absorbance versus time for different concentrations of H$_2$O$_2$.]

Figure S7. POD-like activity assay for the MoS$_2$@CP (Mo ~ 9 $\mu$g/mL : Cu ~ 2.4 $\mu$g/mL) using TMB as a substrate (0.5 mM) on varying the concentration of H$_2$O$_2$ from 0 to 5 mM in acetate buffer (100 mM, pH ~ 4.2) and absorbance was measured upto 40 min.

![Graph showing absorbance versus time for different concentrations of TMB.]

**Figure S8.** POD-like activity assay for the MoS$_2$@CP (Mo ~ 9 µg/mL : Cu ~ 2.4 µg/mL) using H$_2$O$_2$ (5 mM) on varying the concentration of TMB as substrate from 0 to 5 mM in acetate buffer (100 mM, pH ~ 4.2) and absorbance was measure upto 40 min.

![Absorbance vs Wavelength plot](image)

**Figure S9.** POD-like activity assay for the MoS$_2$@CP (7 µg/mL, Mo : 1.7 µg/mL, Cu ) using OPD as a substrate (0.5 mM) in presence of 5 mM (H$_2$O$_2$) in acetate buffer (100 mM, pH ~ 4.2) and absorbance was measure after 60 min of incubation.

![Kinetic plot](image)

**Figure S10.** a) Kinetic plot for POD-activity of ce-MoS$_2$ (16.6 µg/mL, Mo) on varying concentration of TMB substrate from 0-0.9 mM in presence of H$_2$O$_2$ (5.0 mM) in acetate buffer (100 mM, pH ~ 4.2). b) corresponding Michaelis Menten curve obtained by fitting using enzyme kinetic function in origin software.
**Figure S11.** a) Kinetic plot for Fenton’s like activity of CP-nanodots (30 µg/mL, Cu) on varying concentration of TMB substrate from 0-0.5 mM in presence of H₂O₂ (5.0 mM) in acetate buffer (100 mM, pH ~ 4.2). b) corresponding Michaelis Menten curve obtained by fitting using enzyme kinetic function in origin software.

**Table S1:** Summary of the MBC (minimum bactericidal concentration) value of bacteria treated with material.

<table>
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<th>MBC value (µg/mL)</th>
<th>MRSA</th>
<th>E.coli</th>
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<tr>
<td></td>
<td>With light</td>
<td>Without light</td>
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<tr>
<td>MoS₂@CP (Mo:Cu)</td>
<td>14.4 : 3.8</td>
<td>57.6 : 15.2</td>
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<td>CP-nanodots (Cu)</td>
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<td>MoS₂ (Mo)</td>
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**Table S2:** Comparison of the POD-like activity based antibacterial action with reported nanozymes.

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<th>POD-like nanozyme</th>
<th>Material dose (µg/mL)</th>
<th>H₂O₂ doses (mM)</th>
<th>Bacteria</th>
<th>References</th>
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### Table

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<th>Material</th>
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<th>CP@Tf NPs</th>
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<th>Graphene/CuO₂</th>
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### Estimation of oxidative stress:

Oxidative stress induced by MoS₂@CP nanocomposite was assessed by Ellman’s assay. In the procedure, MoS₂@CP (Mo ~ 5 mg/mL : Cu ~ 1.319 mg/mL) composite were taken in a vial. Then 0.4 mM GSH was added, which was then dissolved in 50 mM bicarbonate buffer with pH 8.6. In the negative control, no material was added whereas in the positive control, 10 mM H₂O₂ was added. The tubes were wrapped with aluminium foils to prevent any photochemical oxidation and solutions were incubated at 37°C. From the stock at different time intervals (0 min, 15 min, 30 min, 1 h, and 2 h), 100 µL of solution was added to 100 µL of 2 mM 5,5′- dithiobis(2-nitrobenzoic acid) (DTNB, SRL Chem) in 50 mM Tris-HCl buffer (pH 8.3, SRL Chem) taken in a 96-well plate. Then, the absorbance of the resulting solution was measured immediately after 5 min at 412 nm using a UV−visible spectrometer by plate reader. The percentage loss of glutathione was calculated using following formula and finally plotted against time.

\[
\text{%loss of Glutathione} = 1 - \frac{\text{absorbance at 412 nm of the sample at a particular time}}{\text{absorbance at 412 nm of negative control at 0 min}} \times 100
\]

### Estimation of type of ROS:
For the determining the type of ROS species involve in the oxidative stress, Ellman’s was performed in presence of different type of quencher molecules. The experimental procedure is similar to that of Ellman’s assay. Quencher molecules such as hole-quencher sodium oxalate (0.4 mM), electron quencher sodium chromate (0.4 mM), superoxide anion radical quencher TEMPO (0.4 mM), peroxide-quencher catalase (1 μM), singlet oxygen species L-Histidine and hydroxyl radical-quencher isopropanol (0.4 mM) were added to vial with contain Glutathione and MoS$_2$@CP(5μg/mL, Mo : 1.31 μg/mL, Cu) in presence of PBS buffer. The concentrations of all the quenchers mentioned are the final concentrations. After 30 min of incubation, the absorbance was measured of DTNB was measured using plate reader. Finally, calculated the loss of glutathione using above formula and plotted.

**Figure S12.** Estimation of the type of ROS species by Ellman’s assay in the presence of different ROS scavengers, 0.4 mM of isopropanol, oxalate, sodium chromate, (2,2,6,6-tetramethylpiperidin-1-yl) oxyl (TEMPO), L-histidine and 10 μm of catalase.

**Membrane depolarization assay:**
The MRSA bacteria were cultured, followed by harvesting at mid-log phase culture ($A_{600nm} \sim 0.3$) and centrifuged at 5000 rpm for 5 min. The pellet was washed with 5 mM glucose and 5 mM HEPES buffer (pH 7.2) mixed in 1:1 ratio. The washed pellet was resuspended in 5 mM HEPES buffer, 5 mM glucose, and 100 mM KCl solution mixed in a 1:1:1 ratio. Then, 100
μL of bacterial suspension was taken in a 96-well plate, 2 μL of 5 mM DISC3 dye (3,3’-dipropylthiadicarbocyanine iodide) was added, and the plate was incubated for 30 min. Then MoS2@CP (5μg/mL, Mo : 1.31 μg/mL, Cu) nanocomposite was added to the 96-well plate containing bacterial suspension and DISC3 dye. Then the fluorescence was monitored with an excitation wavelength of 622 nm and an emission wavelength of 670 nm, after a period of 1.5 hr. The increase in the fluorescence indicates membrane depolarization of the bacteria membrane as compared to the control.

**Inner membrane permeabilization assessment by ONPG assay:**

The damage in the bacterial membrane leads to release of the b-galactosidase enzyme which is known to decompose o-nitrophenyl-β-d-galactopyranoside (ONPG) into β-galactose and ortho-nitrophenol. The obtained ortho-nitrophenol (ONP) is yellow in colour and has maximum absorbance at 405 nm. Therefore, this quality is used for the quantification of any kind of membrane permeability using UV-visible spectrophotometer. In this assay, first, The MRSA bacteria were cultured, followed by harvesting at mid-log phase culture (A_{600nm} ~ 0.3) and centrifuged at 5000 rpm for 5 min. Then, bacterial suspension was treated with MoS2@CP (11.450g/mL, Mo : 2.8 mg/mL, Cu), CP-nanodots(3 mg/mL, Cu) in acetic buffer at pH ~ 5 and incubated for 2h. After this, ONPG was added into each vial. In the positive control, only b-galactosidase (100 nM) was taken instead of MRSA bacteria (b-galactosidase + acetate pH~5 +ONPG) and in negative control, Bacteria suspension was not treated with MoS2@CP(MRSA + acetate (pH ~ 5) + ONPG). The final concentration of ONPG in all cases were 0.25 mM). The formed ortho-nitrophenol (specific absorbance at 405 nm) was measured to quantify membrane damage.

**Sample preparation for the live/dead imaging:**

MRSA bacteria sample was harvested for live/dead imaging of bacteria cells using confocal microscope. Bacteria suspension centrifuged at 5000rpm for 5 min and then washed with PBS. Then adjusted OD_{600} ~ 0.3 in acetate buffer (pH ~ 5), subsequently this 100 μL of bacteria solution was treated with the material MoS2@CP(Mo ~ 30 μg/mL : Cu ~ 8μg/mL), ce-MOS2(Mo ~ 30 μg/mL) and CP-nanodots(Cu ~ 8μg/mL), along with NIR@808nm irradiation of light for 10 min, followed by incubation for 3hr. Treated bacteria was centrifuged at 10000 rpm to remove the supernatant. Further washed bacteria pellet using PBS (two times). Finally, added dye solution to the bacteria pellet, syto-9 (4 μM) and PI (4.5
μM) incubate for 45 min. At last, 20 mL of the bacteria solution drop cast on the glass slit and covered by cover slip and used for taking images under confocal microscope.

**SEM sample preparation of bacteria:**
MRSA bacteria was cultured in LB media for the preparation of the SEM sample, bacteria were collected by centrifugation at 5000 rpm for 5 min. The bacterial pellet was washed in PBS and measured the OD$_{600}$ value and prepared bacteria solution of OD$_{600}$ ~ 0.3 in the acetate buffer (pH ~ 5). Then treated the bacteria with MoS$_2$@CP composite (3.8 μg/mL ,Cu) and H$_2$O$_2$ (0.5 mM) with 808 nm NIR light for 10 min and then kept for 3 hr incubation. After the incubation period of 3 hr, the bacterial solution was centrifuged again at 5000 rpm to recollect the bacteria pellets. Afterward, fixed the bacteria with 3% glutaraldehyde solution. The bacterial solution was centrifuged again at 5000 rpm to recollect the bacteria pellets. Then after glutaraldehyde fixation again centrifugation done for 5 min at 5000 rpm and repeatedly washed to dehydrate using 30, 50, 70, 90 and 100% ethanol gradient solutions. And finally, small amount of the solution was drop casted on the clean silicon wafer, desiccated under high vacuum overnight and sputtered with gold prior for SEM imaging.

**Hemolysis test:**
In this process, hemolysis assay was performed using rabbit erythrocytes. The blood samples were collected from central animal facility of Indian Institute of Science, Bangalore following the guidelines of Institutional Animal Ethics Committee (IAEC). The 2 mL of blood sample was collected from rabbit, immediately RBCs washed with PBS (three times) and centrifuged at 5000 rpm. After washing, 1 mL RBCs re-dissolved in 25 mL of PBS. Then, 200 μL of RBC (final RBCs Concentration, 2%) added to a vial containing 200 μL of MoS$_2$ with PBS. For positive control, 0.1% of Triton X-100 (200 μL) and PBS (200 μL) as a negative control, incubated at 37°C. Triton X-100 is a surfactant causes the complete lysis of the blood cell which ultimately resulted in the release of the hemoglobin pigment from the blood cell. PBS can mimic the biological medium for the cell therefore, we expect negligible hemolysis of blood cells. After 1 hr, samples were centrifuged at 5000 rpm and taken 100 μL of supernatant and measured absorbance of hemoglobin at 570 nm. The percentage of hemolysis calculated as follows:
%Hemolysis = \frac{\text{sample absorbance} - \text{absorbance of negative control (average)}}{\text{absorbance of positive control (avg)} - \text{absorbance of negative control (avg)}} \times 100

Figure S13. Quantitative analysis of the amount of hemolysis after treatment with different concentrations of MoS$_2$@CP. Here, Triton X 100 was used as the positive control, whereas PBS was used as the negative control.

Cytotoxicity:
**Figure S14.** Cytotoxicity study of the MoS$_2$@CP nanocomposite with HEK 293 cells.(x represent the bactericidal concentration Mo ~ 14.4 mg/mL : Cu ~ 3.8 mg/mL ). Data are mean ± SD (n = 4).

In the procedure, first we have revived the freeze stock of the HEK 293 using the growing media which comprises of DMEM(Dulbecco’s Modified Eagle’s Medium),20% FBS (Fetal Bovine Serum),1% antibiotic–antimycotic solution, and 2 mM of L-glutamine. Subsequently, the cells were cultured in a CO$_2$ incubator at 37°C.(5% CO$_2$ and 95% humidity). Once the growing cells covers 90 % of cell culture flask, they were detached using 0.05% trypsin–EDT followed by centrifugation at 1800 rpm for 5 min at 4°C. Further, cells were culture using above mention cell culture media solution and used for the MTT assay. For the MTT assay, the 15000 cells/mL were seeded in each well of 96 well plate and kept in the incubator for 24 h. After 24 h, the media was changed to remove the dead cells followed by PBS wash. The adhere cells were further incubated with different concentrations MoS$_2$@CP for 24 h. After treatment, the media is carefully removed from the wells and washed with PBS. The treated cells further incubate with MTT solution (5mg/mL) for 3 hr. After incubation, the MTT media solution was removed carefully without disturbing the formazan crystal. The formazan crystals present in each well were solubilize in the 200 μL of DMSO. Finally, The OD of the solubilized solution was recorded using the plate reader at 570 nm.

**References:**

