

Electronic Supplementary Sheet for

ATP foster tuning of Nanostructured CeO₂ Peroxidase-like activity for promising Anti-bacterial performance

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Outline: Supporting information includes tabulated scheme discussing the present limitations, issues addressed in the manuscript and following sections, figures, and Tables:

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ESS 1.1 Synthetic protocol for preparing catalysts (CeO₂ NCs).

ESS 1.2 XPS analysis to identify % Ce³⁺ and Ce⁴⁺ concentration in CeO₂ NCs.

ESS 1.3 Optimization of Peroxidase (POD) assay parameters.

ESS 1.4 Influence of pH on catalyst POD reaction; role of ATP.

ESS 1.5 Fluorometric Resazurin Microplate assay (f-REMA).

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S8 (a) Influence of pH on formation of oxidised TMB and **(b)** heat map for (%) Relative activity at pH 4.5 & 7.4.

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(AMP, ADP, ATP) and histidine in stabilizing cation radical (TMB^+) at neutral pH (7.4); inset shows the related color reactions after 60 minutes.

S10 Reusability Experiment: POD-like-activity in CeO_2 NCs. after every cycle at **(a)** pH 4.5; **(b)** pH 7.4.

S11 XRD pattern of CeO_2 NCs. post synthesis and recovered after 10 catalytic cycles performed at pH 4.5 and 7.4.

S12 Fluorescence spectra of 2- hydroxy terephthalic acid for detection of hydroxyl radical ($\cdot\text{OH}$) using terephthalic acid (TA) as probe.

S13 Calibration curve for **(a)** *S. aureus* **(b)** *E. coli*.

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ESS Table

Table S1 Binding energies and Peak areas for Ce^{3+} and Ce^{4+} ion concentration

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Table S3 Comparative Assessment of CeO_2 NCs. Anti-bacterial potential with earlier reports

Limitations of the present POD mimic addressed

Limitations/Challenges	Issues address in this manuscript
Catalytic activity within narrow pH range.	POD-mimic activity in ATP- CeO_2 NCs shows broad pH scope (3-8) with maximum activity at neutral pH (7.4).
Clinically use concentration of H_2O_2 (traditional bactericide) inhibits wound healing and damages normal tissues.	Catalyst exhibits low K_m for H_2O_2 ; reduces the effective concentration to ~ 285 times promoting anti-bacterial performance of H_2O_2 and cause no harm to normal tissues at low doses.
Antibiotic-resistant bacterial strains	POD-mimic nanozyme (CeO_2 NCs.) catalyse low dose H_2O_2 transformation into $\cdot\text{OH}$ radicals which helps to combat bacterial growth.

1.1 Material synthesis

CeO₂ NCs. were synthesized using co-precipitation method. In a typical reaction, 24 mg CeCl₃·7H₂O was dissolved in 20 mL DI water and magnetically stirred for 1 hr to obtain homogenous solution of 7 mM. 25% (W/V) ammonia (NH₃) solution was added drop wise under continuous stirring to achieve pH 10.5 followed by heating at 60°C in water bath for 1 hr to promote deprotonation of product before precipitation. The solution was stirred for 22 hrs at room temperature to obtain the uniform growth distribution. White precipitate was washed several times with DI water to remove excess ions and unreacted salts; confirmed from solution pH 7.2; followed by drying in an oven at 70°C gives ceria NCs. The characterization and other assays were performed at room temperature and pressure unless and otherwise mentioned.

1.2 XPS Analysis

Gaussian fitting applied to Ce (3d) spectrum and analysed for the deconvoluted peaks. These multiple peaks arise from mixed valence state (+3 and +4) in cerium as well as due to multiple d-splitting. Ten deconvoluted peaks show in Fig. S1 was used to calculate % of Ce³⁺ and Ce⁴⁺ ion concentration using integrated area under curve in the following equation (i & ii): [1]

$$[\%Ce^{3+}] = \frac{Av_o + Av' + Au_o + Au'}{\Sigma A_i} \quad \text{Equation (i)}$$

$$[\%Ce^{4+}] = \frac{Av + Av'' + Av''' + Au + Au'' + Au'''}{\Sigma A_i} \quad \text{Equation (ii)}$$

Where, $Av_o, Av', Au_o, Au', Av, Av'', Av''', Au, Au''$ and Au''' are areas of the respective band given in Table S1; A_i = Integrated peak areas

Table S1 Binding energies and Peak areas for Ce³⁺ and Ce⁴⁺ ion concentration

Sample	Peak Assignment	Binding energy (eV)	Ce contribution (+3/+4)	Peak Area
CeO ₂	u	900.4	+4	22179.10
	u _o	899.01	+3	17732.95
	u'	902.3	+3	5958.83
	u''	907.4	+4	15515.97
	u'''	916.3	+4	37981.32
	v	881.9	+4	40056.95
	v _o	879.9	+3	3969.42
	v'	883.9	+3	31986.63

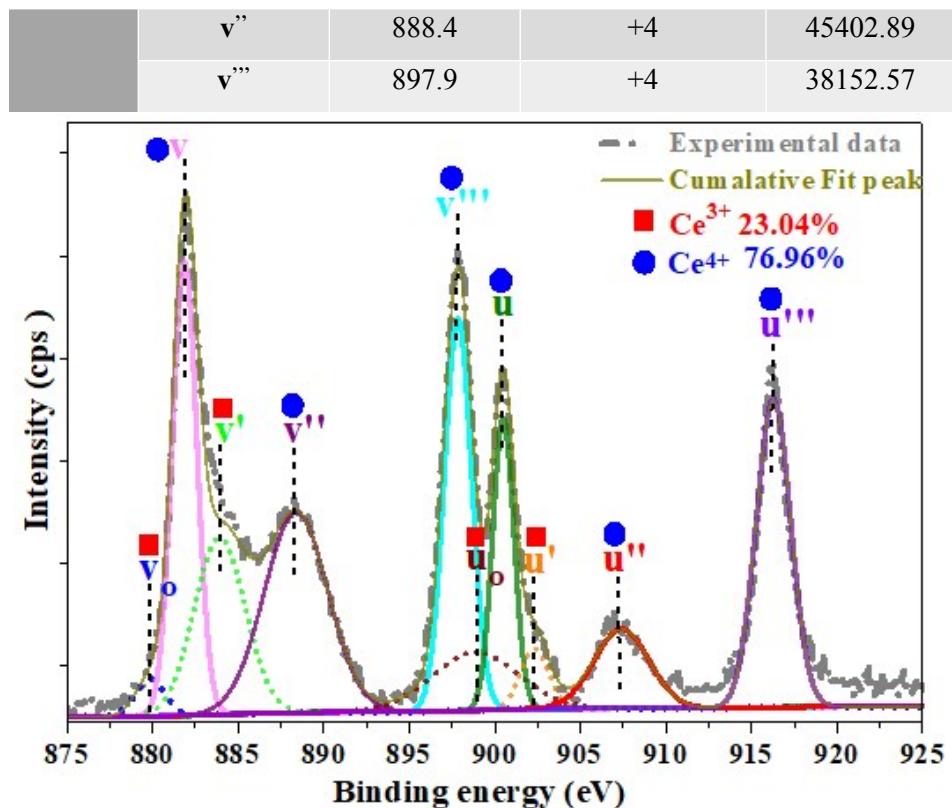


Fig. S1 Deconvoluted XPS spectra of Ce (3d) region

1.3 Optimization of Assay Conditions:

To ensure highest catalytic activity of CeO₂ NCs, influence of several parameters on experimental performance were investigated (Fig. S2, S3, S4, S7, S8 and S9).

It can be seen in Fig. S2 the catalytic reaction rate at pH 4.5 was found to be maximum for 0.3 mM TMB and 3.5 mM H₂O₂; same concentration of TMB and H₂O₂ was used at neutral pH to compare the catalytic potential. To examine the optimal incubation time under acidic (pH 4.5) and neutral (pH 7.4) condition, after every 180 seconds (sec.) we examine the catalytic capability at 652 nm. Fig. S7 shows for first 900 sec. the absorbance increases; later after 900 sec. shows no significant increase. Absorbance at 652 nm suggests the degree of reaction from TMB to oxTMB, and its intensity shows the catalytic ability. CeO₂ NCs shows the best catalytic activity after 900 sec. of reaction time. So, we selected an incubation period of 900 sec. for all the experiments at both the pH.

Further to establish a correlation between the increased POD-like activity of CeO₂ NCs (at neutral pH) and concentration of ATP, we examine the catalytic activity as a function of ATP concentration (ranging from 0.1 mM to 1.5 mM). As expected, increase in concentration of ATP observed a progressive increase in absorbance at 652 nm (Fig. S9(a)) and the highest catalytic activity observed at 1.5 mM concentration of ATP, demonstrating that the activity of

CeO_2 NCs can be tuned by controlling the ATP concentration. Similar study was performed with other adenosine analogues (ADP, AMP) and histidine; evidently ATP shows an augmented POD-like activity in CeO_2 NCs at neutral pH and catalytic order agrees to $\text{ATP} > \text{Histidine} \geq \text{ADP} > \text{AMP}$, Fig. S9(a).

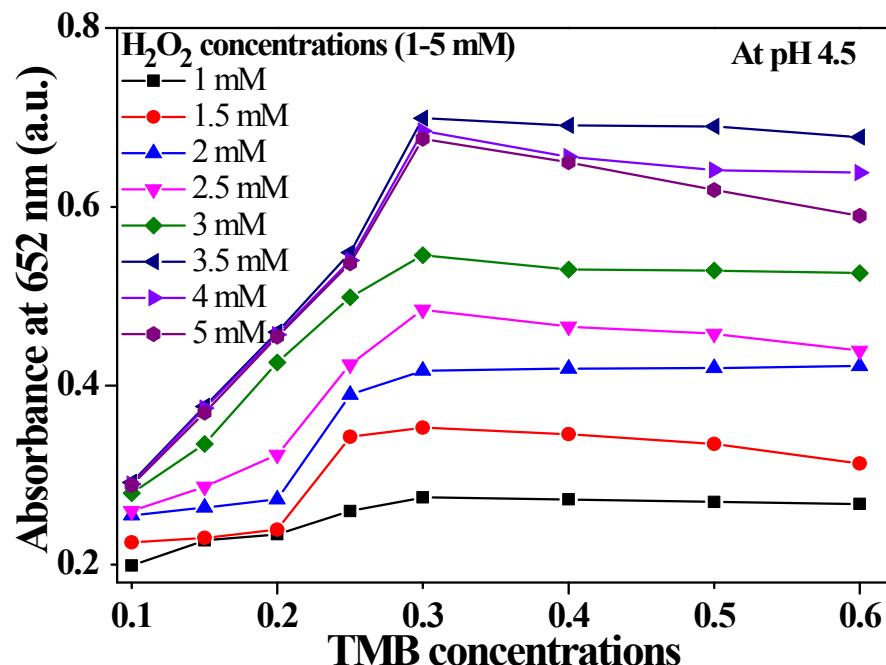


Fig. S2 Optimization of substrate (TMB & H_2O_2) concentration for POD-like-activity in CeO_2 NCs. at pH 4.5.

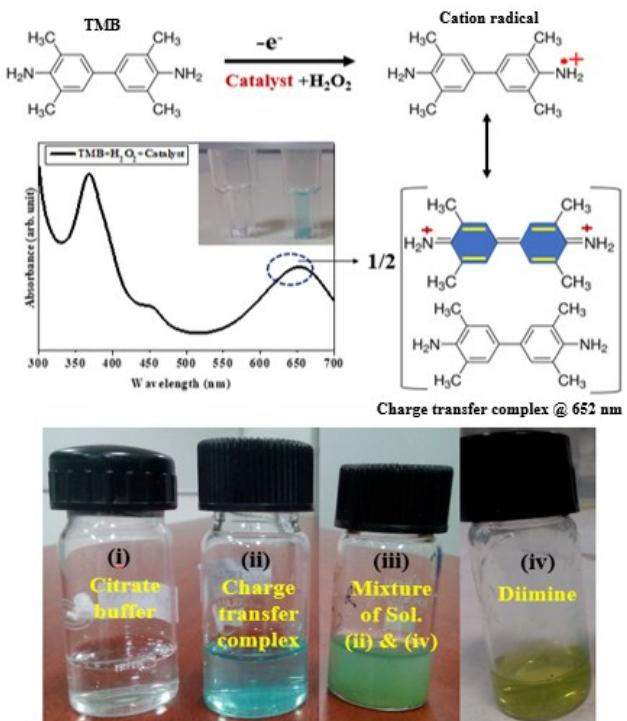


Fig. S3 Schematic illustration of catalyst (CeO_2 NCs.)-catalyzed oxidation of TMB and typical photography of TMB- H_2O_2 reaction system (i) with buffer only; (ii) Blue charge-transfer complex; (iii) Mixture of solution (ii)&(iv), (iv) Yellow Diimine.

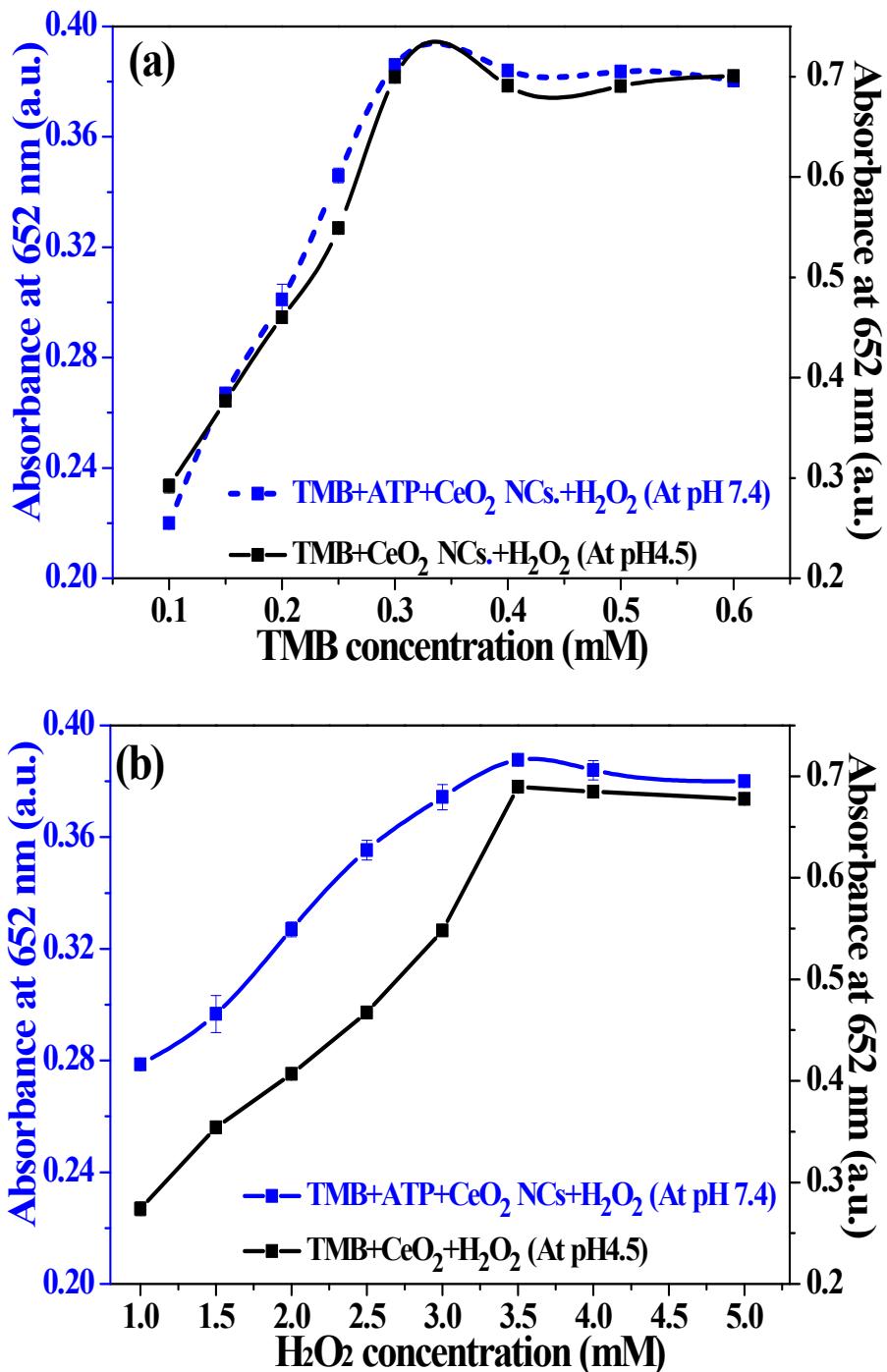


Fig. S4 Substrate concentration dependent POD-like activity of CeO₂ NCs. at pH 4.5 & 7.4 for (a) TMB 0.1-0.6 mM; (b) H₂O₂ 1-5 mM.

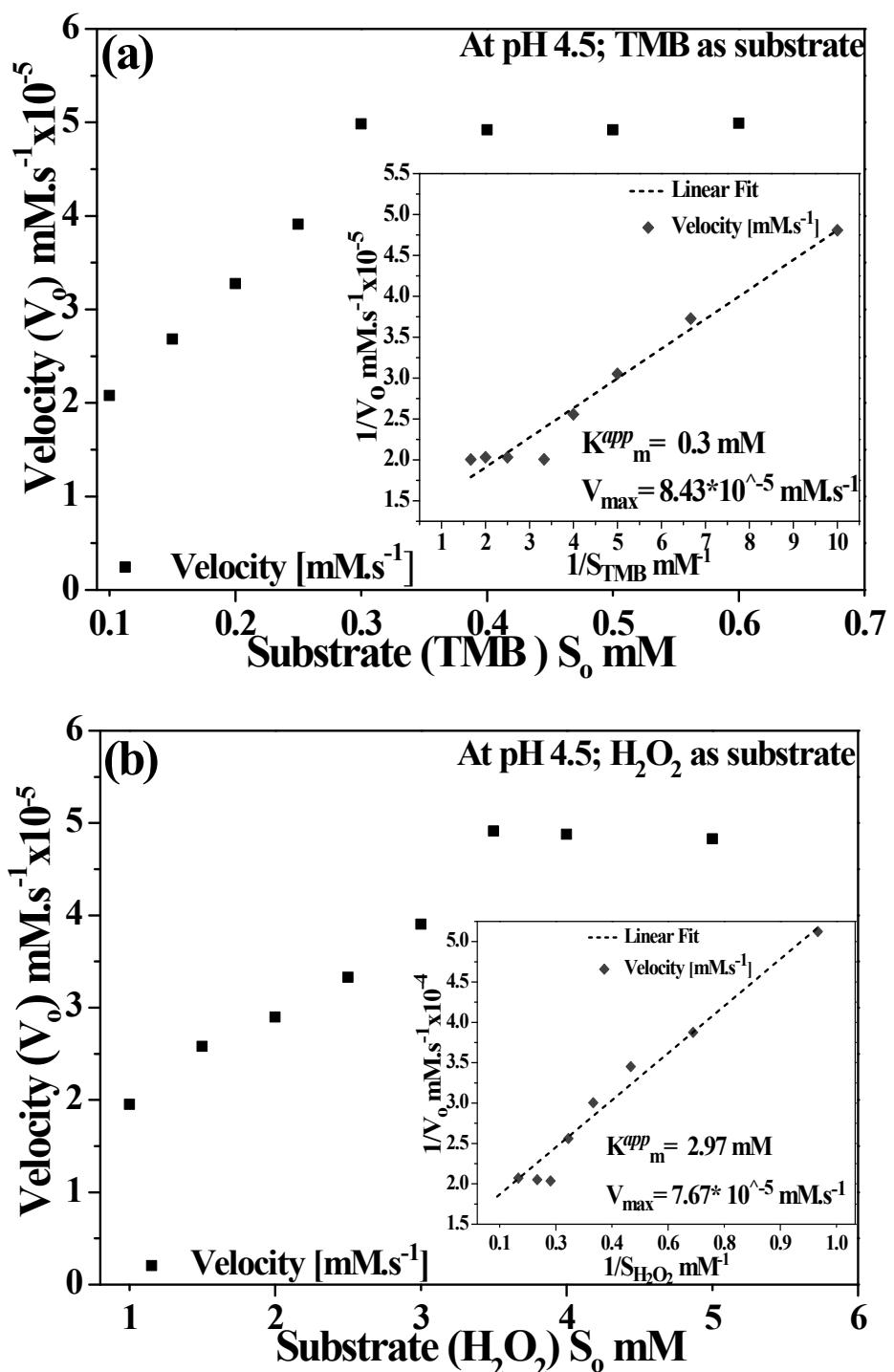


Fig. S5 Steady-state kinetic assay of CeO_2 NCs. at pH 4.5 (a) 3.5 mM of H_2O_2 with different concentrations of TMB (0.1-0.6 mM); (b) 0.3 mM of TMB mM with different concentrations of H_2O_2 (1-5 mM). Double reciprocal given as inset.

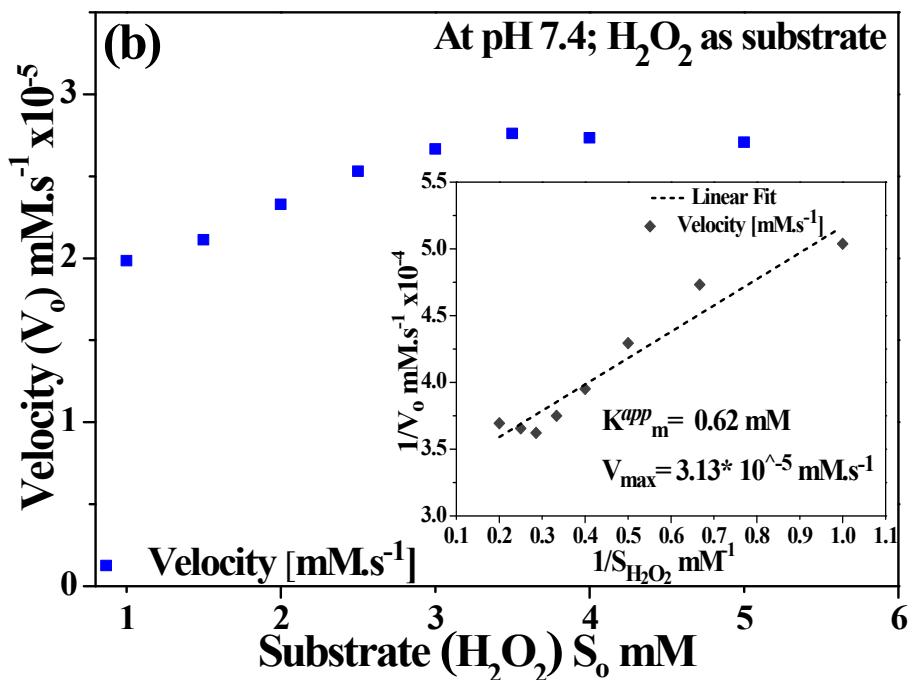
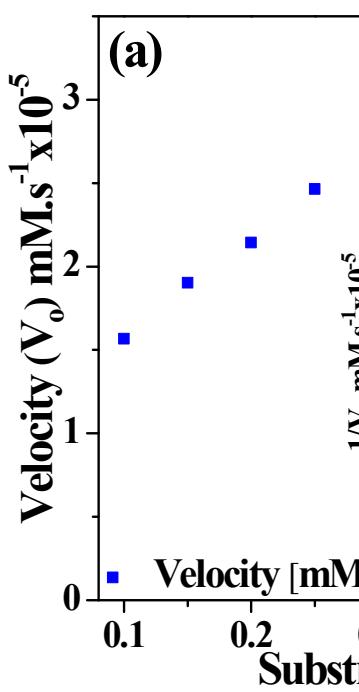
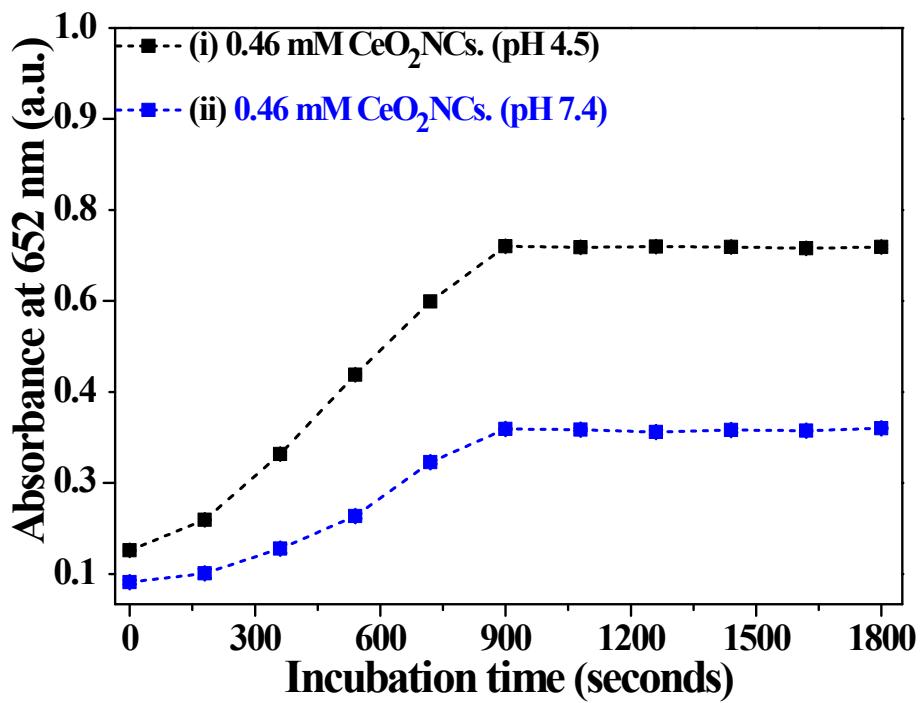


Fig. S6 Steady-state kinetic assay of CeO₂ NCs. at pH 7.4 **(a)** 3.5 mM of H₂O₂ with different concentrations of TMB (0.1-0.6 mM); **(b)** 0.3 mM of TMB mM with different concentrations of H₂O₂ (1-5 mM). Double reciprocal given as inset.

Fig. S7 Effect of incubation time on the absorbance of TMB-H₂O₂ reaction catalysed by CeO₂ NCs. at (i) pH 4.5 in citrate buffer; (ii) pH 7.4 in PBS. The concentrations of CeO₂ NCs., ATP, H₂O₂ and TMB are 0.46 mM, 1.5 mM, 3.5 mM and 0.3 mM respectively.

1.4. ATP ambit Catalyst POD activity to a broad pH range

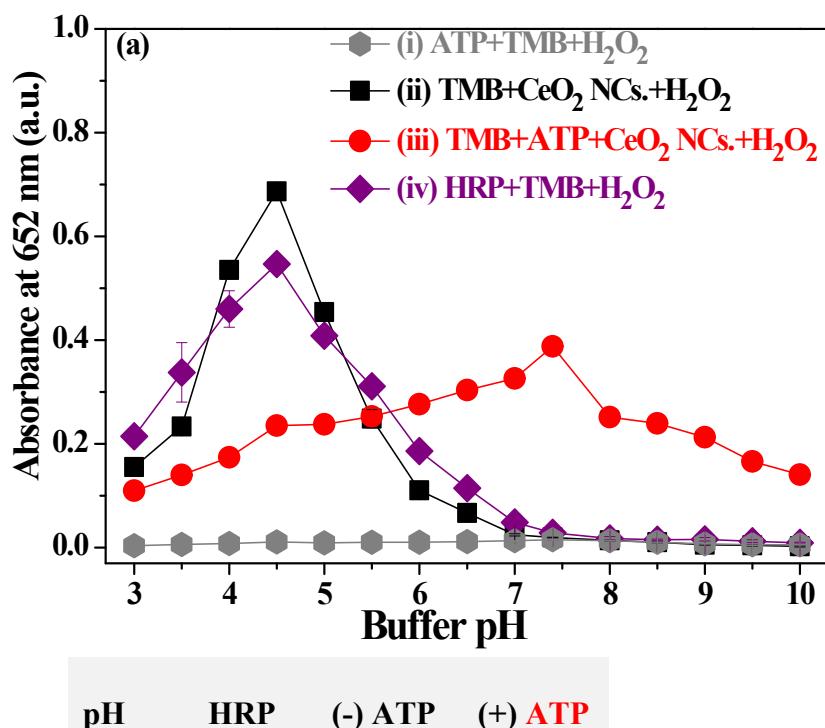
Investigated catalyst (CeO₂ NCs.) POD activity by the oxidation of TMB in buffer solutions maintained at different pH values (3-10). Catalytic activity characterized by deep blue color solution, recorded absorbance at 652 nm after 15 min. of reaction at room temperature for four groups: (i) ATP+TMB+H₂O₂; (ii) TMB+CeO₂ NCs.+H₂O₂; (iii) TMB+ATP+CeO₂ NCs.+H₂O₂ and (iv) HRP+TMB+H₂O₂. Reactants optimum concentration acquired through a pre-optimization experiment, ESS 1.3.

We found group (i) shows a very weak absorption signal (Fig. S8a (i)) indicate no contribution towards POD-like-activity of the catalyst at all pH. This rules-out self-promoted activity of ATP while group (ii) clearly suggest catalyst POD-like activity is critical to pH. It was found catalysts at excessive low pH (below 4) shows a steep decline in the reaction, possibly indicates protonation of both the amino group of TMB leads to insusceptibility towards catalytic oxidation. Studies reported on aromatic amines under acidic medium (pH 2.5-3.5) have shown compromised sensitivity and product stability [2]. Catalyst POD reaction rises gradually (from pH 3.5) with optimal reaction at pH 4.5 evocative of an acid dependent reactivity on account of protonation in one of amino group in TMB and further the reaction declines with increase in pH; precisely after pH 6.5 insignificant POD reaction noted. The apparent decrease in the efficiency of colored product at alkaline pH implies an impeded electron transfer process due to H₂O₂ instability and its rapid decomposition into H₂O and O₂ [3]. Compared with group (iv),

optimal pH for POD reaction found to be identical for CeO_2 nanzyme and HRP. As known, activity of HRP strongly influenced by pH (Fig. S8a (iv)) this features similarity of CeO_2 NCs as POD mimic with natural equivalent for ‘pH limitation’ as prime factor that controls catalytic activity. Catalyst POD reaction at pH 4.5 operates \sim 1.25-fold high over natural enzyme i.e. HRP. Subsequent experiments with catalyst in acidic condition performed at pH 4.5.

In pursuit to emulate POD reaction at physiological condition (at pH 7.4). We introduced catalytic modulator ‘ATP’ to CeO_2 +TMB+ H_2O_2 system. It can be seen, catalyst POD reaction in presence of ATP shows a steady increase over a broad range of pH, Fig. S8a (iii). ATP found to improve catalyst POD activity \sim 20 times at pH 7.4. Here we presumed ATP imitates distal histidine residue of HRP which triggers H_2O_2 molecules available on $\text{Ce}^{+3}/\text{Ce}^{+4}$ sites for $\cdot\text{OH}$ radical production, thus realizes catalyst POD feasibility at neutral pH.

Relative activity (%) summarized as heat map (Fig. S8 (b)) classified into four groups shown in different color schemes: maximum (green: $\geq 75\%$), moderate (blue: 50-74%), moderately low (light gold: 25-49%) and minimum (pink: 0-24%). The highest point specified as 100% relative activity. It reveals relative activity in group (iii) i.e. TMB+ATP+ CeO_2 NCs.+ H_2O_2 shows no activity below 24% and observes the maximum activity at pH 7.4, identifies ATP synergistic role for catalyst POD reaction at neutral pH; while relative activity in both group (ii) TMB+ CeO_2 NCs.+ H_2O_2 and group (iv) HRP+TMB+ H_2O_2 demonstrates POD reaction in a narrow pH range. This underscore ATP stimulated catalyst POD execution over broad pH scope.



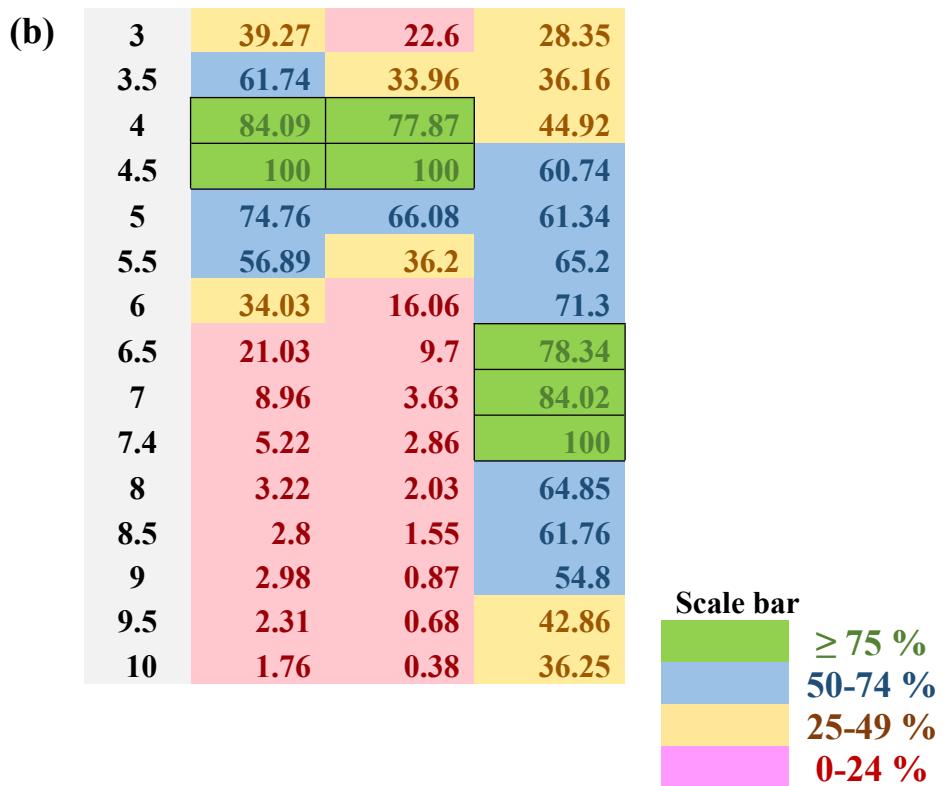


Fig. S8 (a) Influence of pH on formation of oxidised TMB. Comparative performance of CeO_2 NCs. (0.46 mM) with HRP (2.2×10^{-11} M); (b) Heat map for pH dependent relative activity (%). Reaction conditions at different pH (3-10) includes 1.5 mM, 3.5 mM and 0.3 mM concentrations of ATP, H_2O_2 and TMB respectively.

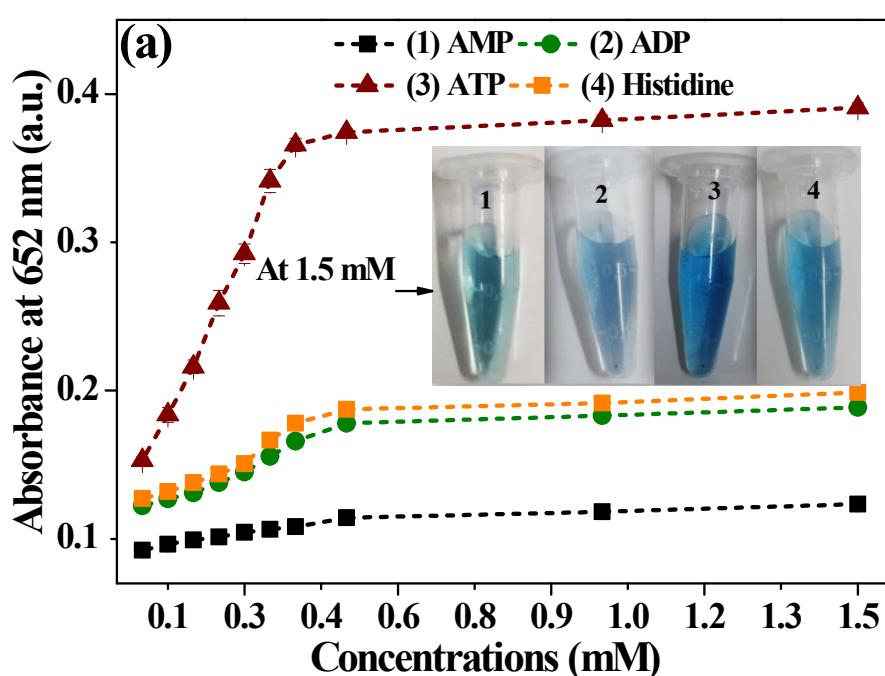


Fig. S9(a) Effect of adenosine analogues (AMP, ADP and ATP) and histidine on the TMB- H_2O_2 reaction catalysed by CeO_2 NCs. in PBS (1X) at pH 7.4.

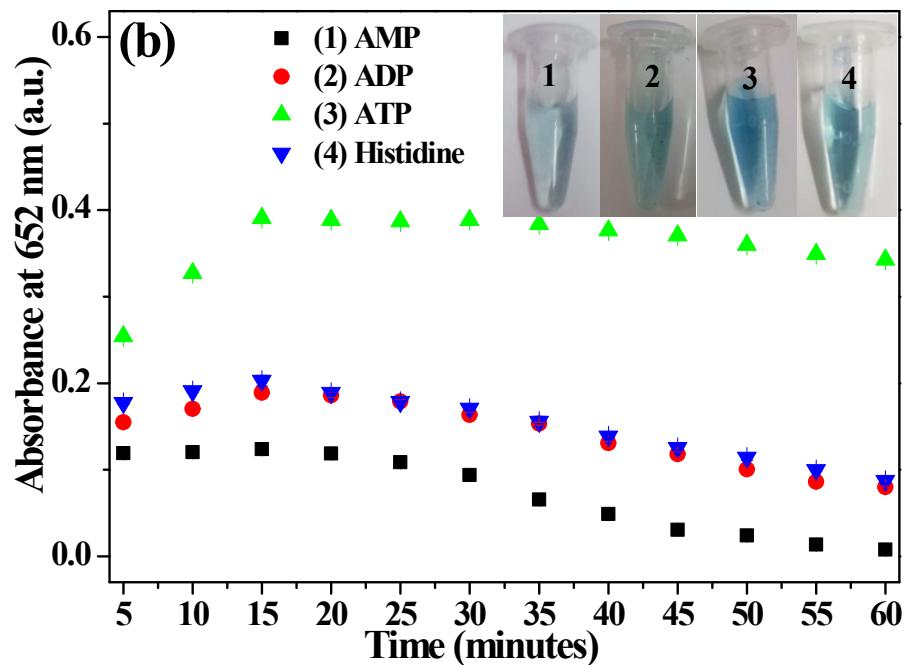
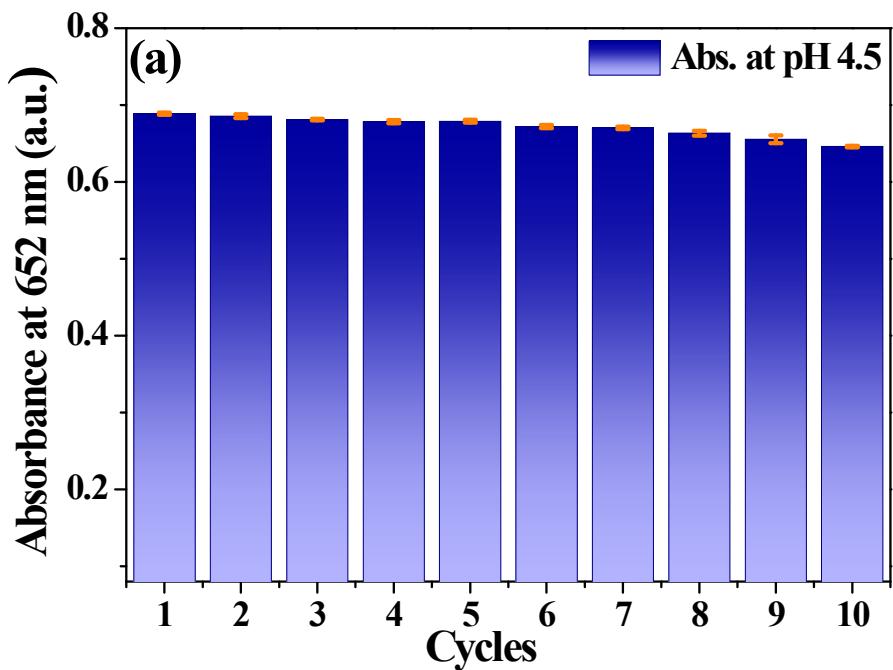


Fig. S9(b) Effect of adenosine analogues (AMP, ADP, ATP) and histidine in stabilizing cation radical (TMB^+) at neutral pH (7.4); inset shows the related color reactions after 60 minutes.



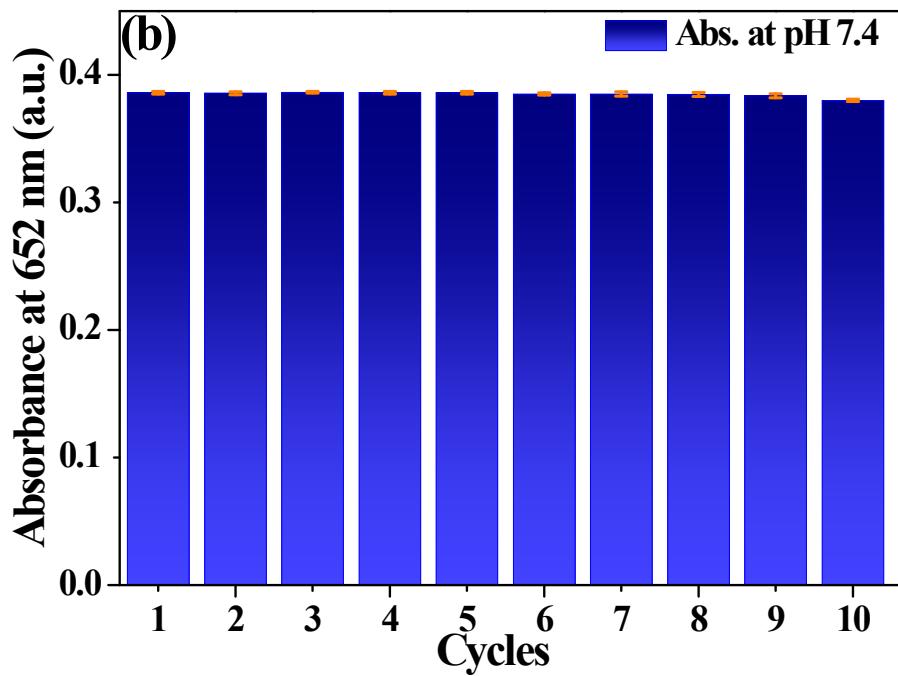


Fig. S10 Reusability Experiment: POD-like-activity in CeO_2 NCs. after every cycle at (a) pH 4.5; (b) pH 7.4.

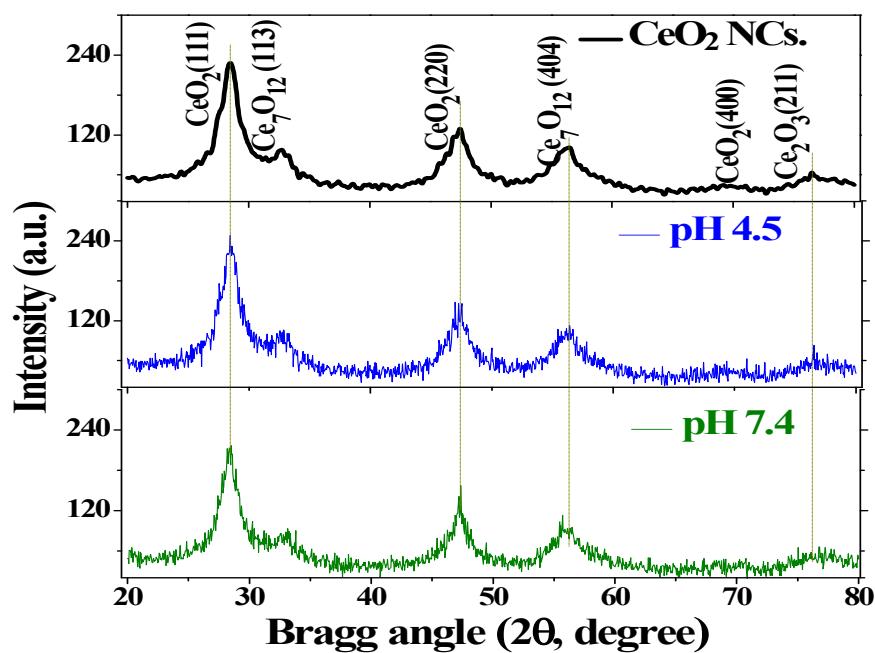


Fig. S11 XRD pattern of CeO_2 NCs. post synthesis and recovered after 10 catalytic cycles performed at pH 4.5 and 7.4.

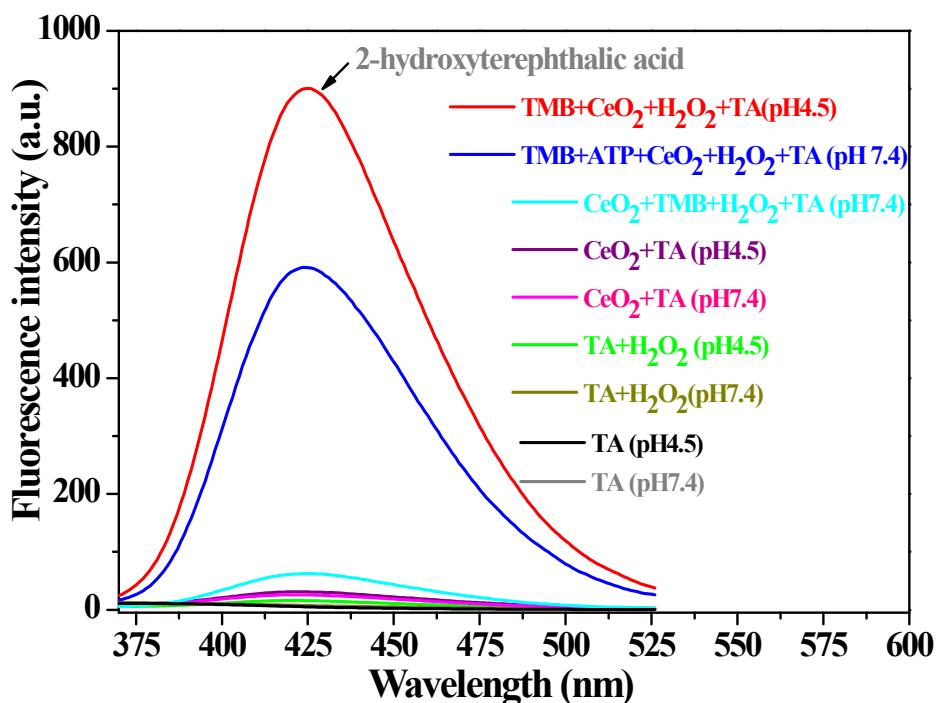


Fig. S12 Fluorescence spectra of 2- hydroxy terephthalic acid for detection of hydroxyl radical ($\cdot\text{OH}$) using terephthalic acid (TA) as probe

1.5 Fluorometric Resazurin Microplate assay (f-REMA)

Anti-bacterial activity of catalyst alone and in combination was studied using resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) reduction assay, recorded as mean fluorescence values for two bacterial species, Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) at pH 4.5 and 7.4. This method overcome solubility issues of test materials and evaluated both fluorometrically and visually [4]. Chemical reduction based on cell metabolism monitored for irreversible reduction of resazurin into highly fluorescent pink color substance ‘resorufin’, indicates presence of metabolic activity/growth while non-fluorescent blue color interpreted as absence of metabolic activity/bacterial cell death.

Preliminary to f-REMA, calibration curves constructed for both the bacterial species to correlate bacterial cell concentration (CFU mL^{-1}) with resorufin fluorescence intensity (RFI), Fig. S13 (a & b). Using two and tenfold dilution bacterial suspension prepared to 0.001, 0.005, 0.01, 0.05, 0.1, 0.5 and $1*10^5$ CFU mL^{-1} . An optimization experiment on resazurin metabolism finds 0.002 % (20 μL) reduction in 35 min. used in the present study to record stable resorufin fluorescence in both the test isolates. f-REMA carried out to monitor reduction

in resorufin RFI for test samples (NCs/H₂O₂/ATP) alone or in combination with bacterial culture + redox dye, growth medium with redox dye as positive control and bacterial culture in growth medium with redox dye (without NCs/H₂O₂/ATP) as negative control. Fluorometric measurement carried out at excitation/ emission wavelength of 530 nm/ 580 nm for 180 minutes.

Curve fitting analysis on bacterial cell density verses resorufin fluorescence were made in Origin Pro 8.5. Linear regression equation (i) and correlation coefficient quotient (R²) estimated for both the bacterial isolates. Calibration curve derived average bacterial number, represented as log (CFU mL⁻¹± SD) where n=3 verses were used to calculate % bacterial inhibition using equation (ii), [5].

$$FI(\text{resorufin}) = \text{slope} * (\text{CFU mL} - 1) + \text{intercept} \quad (\text{i})$$

$$\% \text{ Bacteria Inhibition} = 100 - (\text{Log} \frac{(S_{180 \text{ min.}})}{(C_{-ve \text{ 180 min.}})} * 100) \quad (\text{ii})$$

Where, S_{180 min.}= Logarithm of average bacterial cells (CFU mL⁻¹) in treated group after 180 min.; C_{-ve 180 min.}= Logarithm of average bacterial cells (CFU mL⁻¹) in untreated group (negative control) after 180 min.

1.6 Minimum Inhibitory Concentration (MIC):

Broth microdilution was performed following Clinical and Laboratory Standards Institute (CLSI) guidelines with some modification, coupling redox indicator ‘resazurin’ to distinguish metabolically viable cells from non-viable cells [6]. Prior to MIC experiment, to eliminate false positive results it was ensured that the catalyst (CeO₂), ATP and H₂O₂ do not interfere with redox dye at both the pH (4.5 and 7.4). This was considered as positive control and negative control includes medium + bacterial culture + redox dye.

MIC₉₉ was determined against both the bacterial cells (*S. aureus* and *E. coli*) with a final cell density corresponds to 1*10⁵ CFU mL⁻¹ for (a) CeO₂ NCs, (b) H₂O₂, (a)+(b) and (a)+(b) +ATP at pH 4.5 and 7.4. Followed an incubation of 18 hrs. added redox indicator (20 µL; 0.002%) to all wells and observed for color change after 2.5 hrs. Number of viable cells is directly proportion to the intensity of color produced. Lowest concentration prior to color change was considered as MIC value where cells losses its metabolic potential to reduce resazurin (blue) into resorufin (pink fluorescent dye).

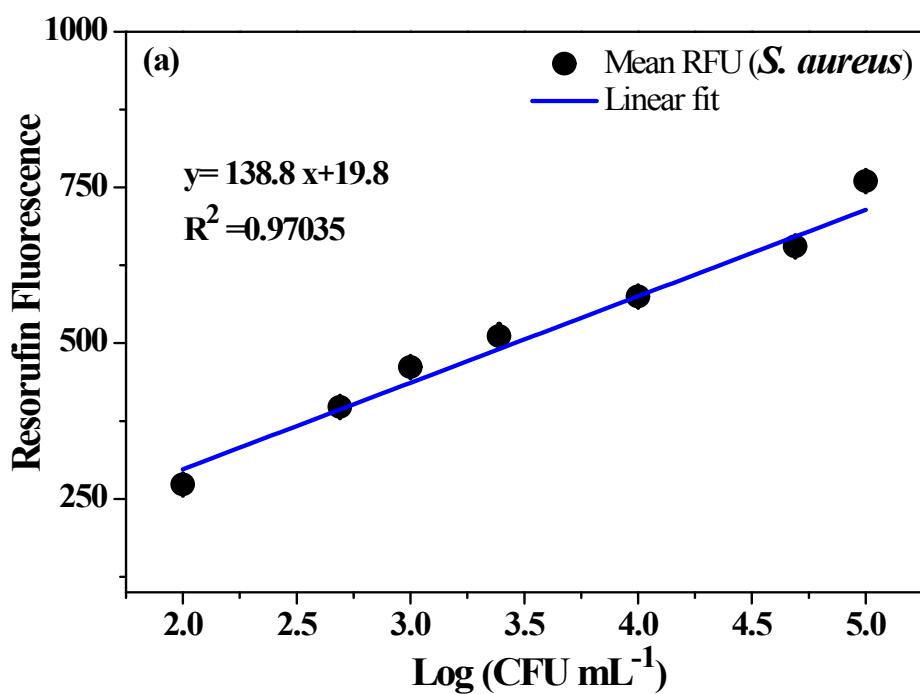


Fig. S13 (a) Calibration curve for *S. aureus*

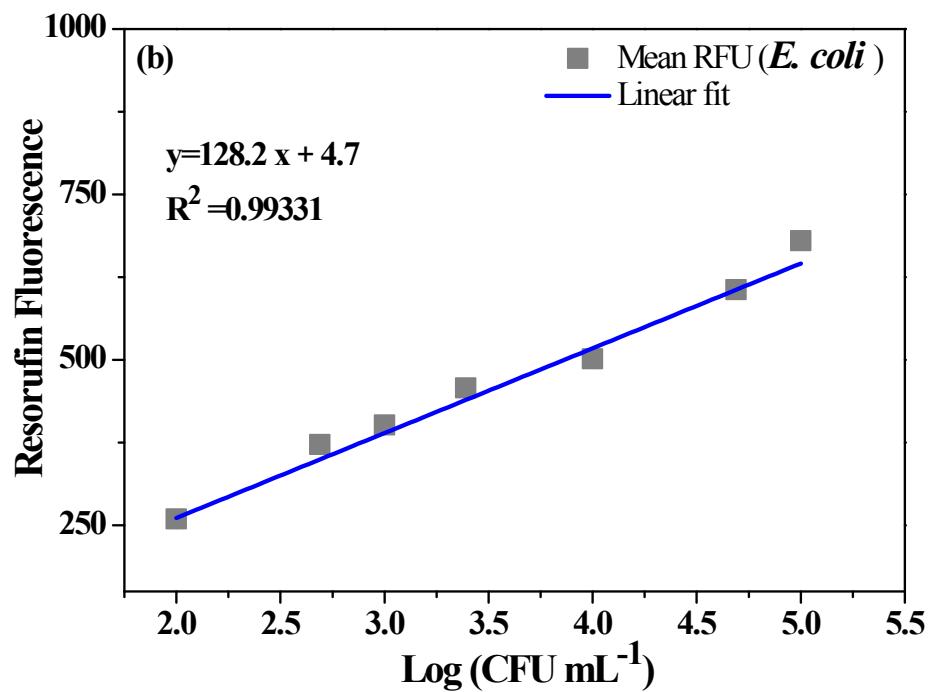


Fig. S13 (b) Calibration curve for *E. coli*

Table S2 Catalyst MIC_{99%} ($\mu\text{g mL}^{-1}$) alone and in combination:

Test compound	pH 4.5		pH 7.4	
	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>
CeO₂ NCs	250	186.7	225	200
H₂O₂^a	5	4	7.5	5.5
CeO₂ NCs+ H₂O₂	108	50	216	170
CeO₂ NCs+H₂O₂+ATP	100	47	115	78

Note: MIC values corresponds to minimum 3 concordant values collected from 3-6 replicates

a: concentration in mM

1.7 Influence of H₂O₂ on bacterial growth (at pH 4.5 & 7.4):

To exhibit CeO₂ nanzyme POD approach in suppressing bacterial growth *in vitro*, we optimize the concentration of H₂O₂ that shows bactericidal effect. Investigated the effect of oxidant (H₂O₂) at different concentrations (1, 2.5, 3.5 and 5 mM) against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacterial cells at pH 4.5 and 7.4.

Fig. S14 (a) shows the (%) bacterial survival as a function of oxidant concentrations. At both the pH a concentration dependent effect observed, *E. coli* being more susceptible over *S. aureus*. This well accords to MIC₉₉ values given in Table S2. Further the oxidant exposed cells were quantified for malondialdehyde-thiobarbituric acid (MDA-TBA) adduct using the standard calibration curve (Y= 0.06848X+ 0.07535), given in inset of Fig. 14 (b). As a measure of H₂O₂ induced oxidative damage incurred in bacterial membrane, Lipid peroxidation was estimated [7]. It was found exposure of 3.5 mM and 5 mM of H₂O₂ reveals maximum MDA equivalents in *E. coli* over *S. aureus* at both the pH, Fig. S14 (b). It is seen clearly, H₂O₂ exposure at 5mM reveals an effective bacterial growth inhibition at both the pH..

Through a substrate (H₂O₂) optimization experiment at pH 4.5 and 7.4, it was found catalyst gave optimal POD reaction at 3.5 mM H₂O₂ concentration (Fig. 14 (c)), further increase in H₂O₂ concentration causes decline in POD activity. This might possibly be due to decrease in

Ce^{3+} species and excess H_2O_2 concentration disrupts $\text{Ce}^{4+} \rightarrow \text{Ce}^{3+}$ transitions and obstruct $\cdot\text{OH}$ generation potential at both the pH, inset Fig. 14 (c).

Consequently, all anti-bacterial study performed at 3.5 mM concentration of oxidant (H_2O_2). This does not impede catalyst regeneration ability, catalyse conversion of H_2O_2 into $\cdot\text{OH}$ radical mediated bacterial killing.

Our finding reinforces CeO_2 nanorods anti-bacterial (in acidic and neutral pH) effect without any external irradiation source. Catalyst excellent POD activity at pH 4.5 foresees application to eradicate pathogenic microflora in dental regime. Achieving POD feasibility at physiological pH open doors to a variety of biological applications.

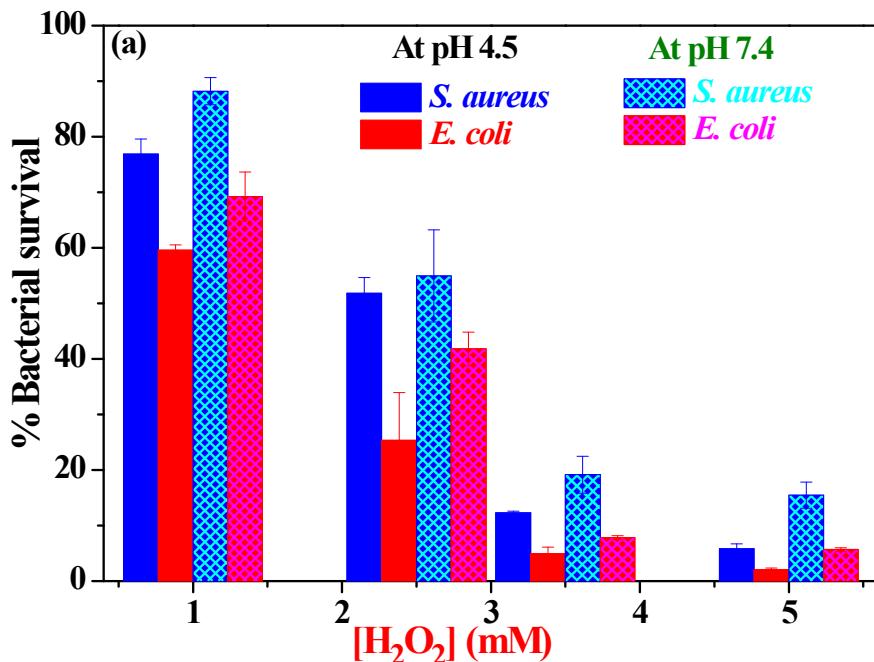


Fig. 14 (a) Bacterial Survival (%) verses oxidant (H_2O_2) concentrations at pH 4.5 and 7.5.

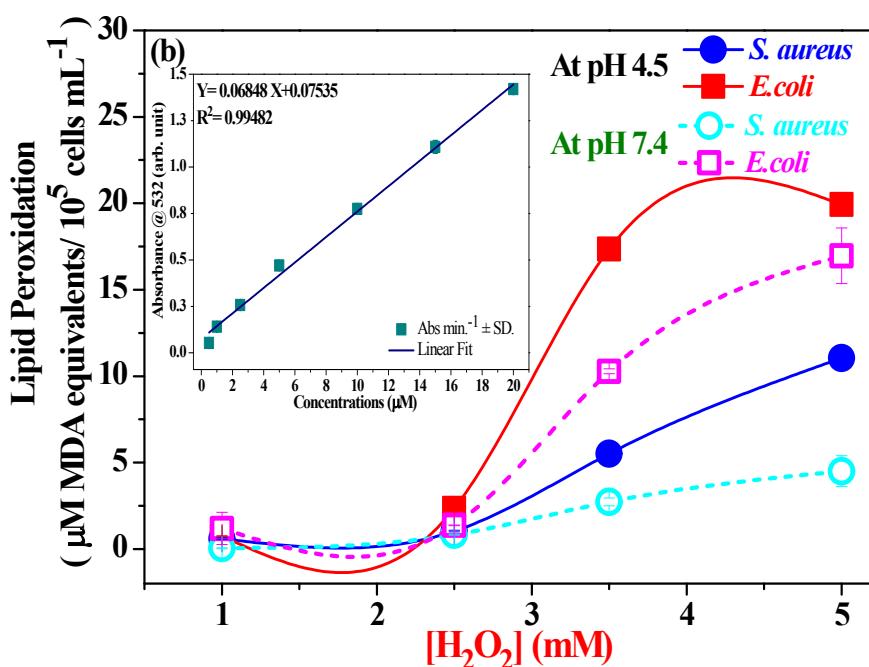


Fig. 14 (b) Lipid Peroxidation in oxidant (H_2O_2) treated bacterial cells at pH 4.5 and 7.5.

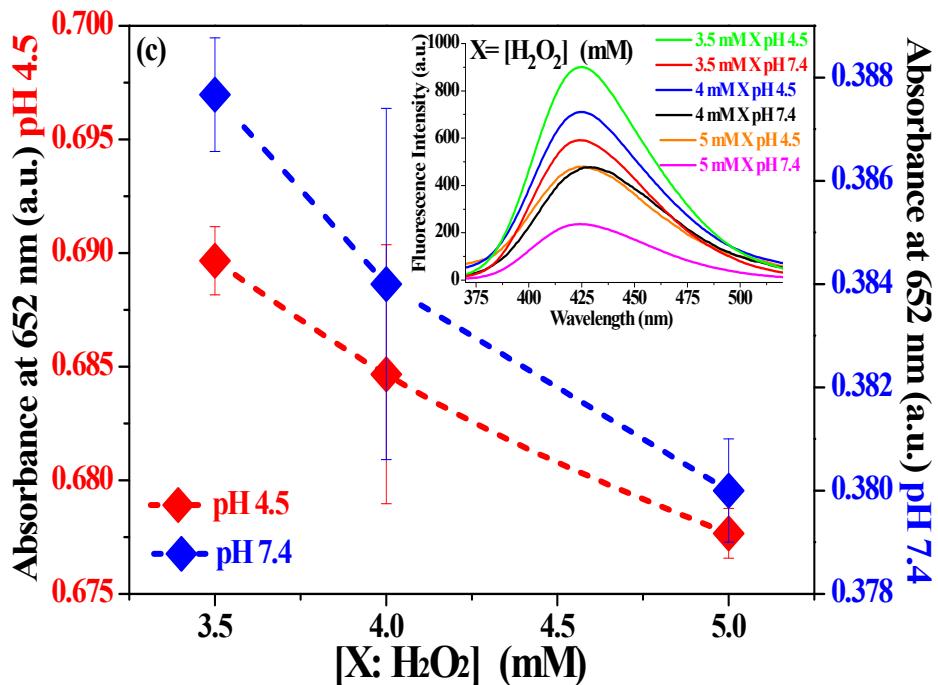


Fig. 14 (c) Catalyst substrate (H_2O_2) dependent POD activity, inset shows $\cdot\text{OH}$ radical generation using TA probe at pH 4.5 and 7.5.

Table S3 Comparative Assessment of CeO₂ NCs. Anti-bacterial potential with earlier reports:

Salt precursor, Synthesis method & Nanoproduct	Shape & Average Size (nm)	Microbiological technique, pH & Concentration	Test organism		Mode of action	Ref. & Year
			<i>S. aureus</i>	<i>E. coli</i>		
(NH ₄) ₂ Ce(NO ₃) ₆ ; microwave assisted ; CeO ₂	Spherical ^a (~45 nm)	Agar diffusion	5 mm	7 mm	NPs absorption on bacterial cell wall	[8] 2016
CeCl ₃ .7H ₂ O; hydrothermal; CeO ₂	honeycomb	Agar diffusion (at 100 mg)	24 mm	26 mm	Photogeneration of ROS, electrostatic bond between NPs. and bacterial cell membrane.	[9] 2019
Ce(NO ₃) ₃ . 6H ₂ O; hydrothermal; CeO ₂	Cube and octahedral	Agar diffusion	25 mm	22 mm	CeO ₂ NPs. oxygen vacancies	[10] 2019
Ce(NO ₃) ₃ . 6H ₂ O; Green synthesis; CeO ₂	Cube	Agar diffusion	9 mm	No inhibition	-	[11] 2019
Ce(NO ₃) ₃ . 6H ₂ O; Green synthesis; chitosan-CeO ₂	Spherical ^b (23.12- 89.91 nm)	Agar diffusion	-	11 mm	Direct interaction of NPs-bacterial cell wall causes membrane disruption	[12] 2017

Ce(NO ₃) ₃ . 6H ₂ O; hydrothermal; CeO ₂	-	Agar diffusion	-	0 mm	-	[13] 2013
		MIC (mg mL ⁻¹) pH 7.4		3		
Ce(NO ₃) ₃ . 6H ₂ O; Sol-gel; CeO ₂	^a (~4.2 nm)	Log (CFU mL ⁻¹) at 1 mM -24 hrs.	1.53±0.07	1.11±0.02	-	[14] 2012
CeCl ₃ .7H ₂ O; Coprecipitation; CeO ₂	Nanorods Mean length (nm) ^a (197±13.4) ^b (214±6.4)	At pH 4.5 -3hrs Log (CFUmL ⁻¹); (MIC µg mL ⁻¹) CeO₂	4.25±0.47 (250)	2.90±0.19 (186.7)	CeO ₂ NCs POD mimic based •OH radical enabled bacterial growth reduction at pH 4.5	Present work
		CeO₂+H₂O₂	1.47±0.18 (108)	0.82±0.28 (50)		
		CeO₂+H₂O₂ +ATP	1.27±0.14 (100)	0.63±0.31 (47)		
		At pH 7.4 -3hrs Log (CFUmL ⁻¹); (MIC µg mL ⁻¹) CeO₂	3.43±0.07 (225)	3.18±0.25 (200)		
		CeO₂+H₂O₂	3.03±0.01 (216)	2.77±0.03 (170)		
		CeO₂+H₂O₂ +ATP	1.41±0.02 (115)	1 ±0.11 (78)		

Note: MIC: Minimum Inhibitory Concentration; POD: Peroxidase; a: TEM; b: FE-SEM; (-) Not reported

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