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ORIGINAL ARTICLE

Unveiling the role of ATP in amplification of intrinsic peroxidase-like activity of gold nanoparticles

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Abstract

Peroxidase enzyme-like activity of gold nanoparticles (AuNPs) is currently being investigated for the potential application in the several realms of biomedicines. However, little is explored about the peroxidase activity of AuNPs decorated with different surface charges. It is well-documented that the catalytic activity and the interaction with mammalian cells are significantly different among AuNPs carrying different surface charges. We have recently reported that ATP enhances the peroxidase-like activity of AuNPs and iron oxide nanoparticles. However, a comprehensive and systematic study to reveal the role of surface charge on nanoparticles peroxidase-like activity has not been studied. In this work, we have shown that AuNPs coated with PEG (PEG AuNPs), citrate (citrate AuNPs) or CTAB (CTAB AuNPs) exhibit varying peroxidase-like activity and the boosting effect imparted by ATP was also different. We found that the peroxidase-like activity of PEG AuNPs and citrate AuNPs is dependent on hydroxyl radical formation, whereas CTAB AuNPs did not show any significant activity under the same experimental conditions. We also studied the boosting effect of ATP on the peroxidase-like activity of PEG and citrate AuNPs. Although the use of ATP resulted in enhanced peroxidase-like activity; however, contrary to the expectation, it did not facilitate the enhanced production of hydroxyl radical. In further studies, we found that the likely mechanism of boosting effect by ATP is the stabilization of oxidized TMB after peroxidase reaction. ATP imparts stabilization to the oxidized TMB produced due to PEG AuNPs, citrate AuNPs as well as HRP.

Keywords Nanozymes · Biomimetic nanoparticles · Artificial enzymes · Metal nanoparticles · Hydroxyl radicals

Introduction

Currently, immense interest has been developed for constructing artificial enzymes due to the several disadvantages associated with natural enzymes such as extreme sensitivity of catalytic activity towards environmental conditions, less operational stability (prone to digestion and degradation) and high cost of synthesis and purification. These limitations restrict the broad spectrum applications of natural enzymes. Therefore, to circumvent the aforementioned limitations, nanomaterials have been developed as artificial enzymes (nanozymes), exhibiting the properties and catalytic activities demonstrated by natural enzymes (Lin et al. 2014b; Manea et al. 2004a; Yang et al. 2017). Nanomaterials-based artificial enzymes offer several advantages such as facile synthesis, tunability of catalytic efficiency, high stability under stringent reaction conditions, long-term storage and easy availability (Lin et al. 2014a). Several nanomaterials have been recently discovered which possess unique enzyme mimetic activity, such as AuNPs (Comotti et al. 2004; Luo et al. 2010; Manea et al. 2004b; Pengo et al. 2005; Zheng et al. 2011), cerium oxide (Asati et al. 2009a, 2011b; Heckert et al. 2008; Singh et al. 2011), graphene oxide (Song et al. 2010a), carbon nanotubes (Cui et al. 2011; Song et al. 2010b), V_2O_5 nanowires (Andre et al. 2011), Co_3O_4 (Mu et al. 2012), CuO (Hong et al. 2013), and iron oxide (Fu et al. 2017; Gao et al. 2007; Vallabani et al. 2017). These nanomaterials exhibit excellent peroxidase-like activity by catalyzing the oxidation of peroxidase substrate in the presence of H_2O_2. Due to the well-established surface chemistry, the enzyme-like activities of AuNPs are further explored

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in several applications such as biosensing, environmental chemistry and therapeutics (Ahmed et al. 2017; Deng et al. 2016; Ni et al. 2014; Sharma et al. 2014; Zhan et al. 2014; Stefan et al. 2012). Although due to high surface to volume ratio, AuNPs offer several advantages as nanozymes, however, suffer from several limitations as well such as lower binding affinity and specificity for the substrate, relatively low catalytic activity as compared to natural enzymes and lower stability in biologically relevant buffers of high salt concentration. Such events may compromise the catalytic efficiency and efficacy of AuNP-based enzyme mimetics in the real system and impede further development and application. To circumvent these concerns, it is imperative to develop strategies which could lead to improving the nanozymatic activity of AuNPs. In this context, Lin et al. have demonstrated Au/SiO_2 based hetero-nanocomposite as a robust and recyclable artificial peroxidase enzyme performing the catalytic activity at high temperature under the influence of ionic liquid. Although ionic liquid acts as a positive modulator of catalytic activity and enhanced the thermal stability of the product, it completely inhibited the catalytic activity of nanozymes due to high viscosity and ionic strength (Lin et al. 2013). Nonetheless, compounds exhibiting boosting effect to the catalytic activity of nanozymes as well as imparting enhanced thermal stability to the reaction product would be ideal to be explored.

Surface charge is another factor which influences the properties of nanomaterials such as fabrication process, stability, redox property, and catalytic properties. It is well-established that AuNPs are stabilized by the dynamic balance between electrostatic repulsion and van der Waals attraction forces operational between the charged particles, which are controlled by the ionic strength of the suspension (Singh et al. 2007). The addition of ions causes interruption to these forces leading to the aggregation of the colloidal particles, which may decrease the peroxidase-like activity of AuNPs. Wang et al. have investigated the role of charge present on AuNPs surface in peroxidase-like activity by considering the amino-modified (positively charged) or citrate-capped nanoparticles (negatively charged) and unmodified (no charge) AuNPs (Wang et al. 2012). It was found that unmodified AuNPs exhibited better peroxidase-like activity than charged nanoparticles. Furthermore, Jv et al. have compared the peroxidase-like activity of positively charged (cysteamine coated) and negatively charged (citrate coated) AuNPs and reported that the former exhibited excellent activity than the latter (Jv et al. 2010).

Additionally, a report suggests that certain ions such as Ag^{+}, Bi^{3+}, Pb^{2+}, Pt^{4+} and Hg^{2+} ions, induce boosting effect to the nanozyme activity of AuNPs and showed significant improvement in catalytic activity (Lien et al. 2013). It was argued that these ions may deposit on the surface of AuNPs and facilitate the catalytic activity through competitive and synergistic interaction between them and AuNPs. We have previously reported that ATP also imparts boosting effect to the peroxidase-like activity exhibited by citrate-capped (negatively charged) AuNPs as well as iron oxides (Shah et al. 2015). With our AuNPs study, we found that the boosting effect is size-dependent as ~ 30 nm size AuNPs exhibited strong peroxidase-like activity than 15, 50 and 70 nm. However, the effect of ATP on the peroxidase-like activity of positively and uncharged AuNPs is so far unexplored. Furthermore, the mechanism behind the boosting effect of ATP over artificial peroxidase activity of differently charged AuNPs remains to be studied.

In this study, we have used polyethylene glycol (PEG), citrate and CTAB-coated AuNPs to explore their peroxidase-like activity in presence of ATP. The reaction kinetics and enzyme kinetic parameters are also determined. We found that PEG AuNPs and citrate AuNPs show strong peroxidase-like activity than CTAB AuNPs. Furthermore, the peroxidase reaction performed in presence of ATP showed lower K_m and higher V_{max} than the reaction executed in absence of ATP. We also found that ATP stabilizes the oxidized TMB for a longer period of time, which is probably the reason behind the observed boosting effect.

Materials and experimental methods

Materials

Cetyl-*N*,*N*,*N*-trimethylammonium bromide (CTAB), adenosine triphosphate disodium salt (ATP), citric acid monohydrate, trisodium citrate dehydrate, horse radish peroxidase (HRP), terephthalic acid were purchased from Hi-Media (Mumbai, India). 3,3′,5,5′-Tetramethylbenzidine (TMB), polyethylene glycol (6000) was purchased from Acros Organics (Geel, Belgium). 30% hydrogen peroxide (H_2O_2), sodium borohydride LR, chloroauric acid ($HAuCl_4 \cdot 3H_2O$) were purchased from SD fine chemicals (Mumbai, India).

Synthesis of PEG AuNPs

For a preparation of PEG AuNPs, 100 µL of 100 mM $HAuCl_4$ was added to 5.9 mL of Milli-Q water and heated with stirring until it starts refluxing, then it was mixed with 4 mL of PEG solution (325 mg mL^{-1}-PEG 6000 MW.) and allowed to mix for 3 min. 300 µL of NaOH 1% was added dropwise to this solution under vigorous stirring with heating until the transparent solution turns into ruby red color (Stiufiuc et al. 2013). The particle size distribution histogram was obtained by measuring the size of 200 individual nanoparticles from 15 different images using the software (image-J).

Synthesis of citrate AuNPs

150 µL of 100 mM $HAuCl_4$ solution was added in 14.85 mL of Milli-Q water and heated until it started refluxing. 1.0 mL of 38.8 mM trisodium citrate was added dropwise with vigorous stirring, and solution was boiled for 5 min until solution turns dark red (Shah et al. 2015).

Synthesis CTAB AuNPs

5 mL of $HAuCl_4$ (2 mM) aqueous solution was mixed with 5.0 mL (0.2 M) CTAB solution and allowed to mix at 37 °C until solution develops a dark yellow color. Then, reduction was accomplished by adding 600 µL of ice-cold $NaBH_4$ (0.1 M) dropwise. The vials were stirred vigorously for ~ 24 h and kept occasionally opened to release any evolved hydrogen gas (Narayanan et al. 2008). The suspension was in red color and allowed to age for 5 days. This seed solution was used for experiment.

Characterization of AuNPs

UV–Vis absorption spectra of AuNPs were acquired using Biotek (Synergy HT spectrophotometer) at room temperature in a quartz cuvette of 1 cm path length. Particle size was measured using transmission electron microscope (TEM) equipped with 120 kV (Jeol, JEM1400 plus) on a carbon-coated copper TEM grid. Zeta potential studies of AuNPs were carried out using dynamic light scattering (Zetasizer Nano-Zs, ZEN3600 Malvern Instruments Ltd) using a laser with wavelength of 633 nm.

Preparation of buffer

Citric acid and trisodium citrate solutions were prepared in 100 mL of Milli-Q water. Into this, stock citric acid and trisodium citrate was added in a 59:41 ratio to obtain the citrate buffer solution 0.1 M which was further diluted to obtain the 0.01 M of concentration which was used in the peroxidase-like activity.

Peroxidase-like activity

Kinetic study was performed in a total reaction volume of 500 µL with different concentrations of AuNPs (5–100 µg) of PEG, citrate and CTAB AuNPs and fixed concentrations of H_2O_2 (1 M), TMB (1 mM) and 2.0 mM ATP in citrate buffer solution (pH = 4) at 37 °C for 20 min. To investigate the effect of ATP on the peroxidase-like activity of AuNPs, the study was performed in presence and absence of ATP. Absorbance was monitored at 652 nm.

Effect of temperature and pH on peroxidase-like activity

Experiments were carried out using 55 µg PEG and citrate AuNPs, 2 mM ATP, 1 mM TMB and 1 M H_2O_2 in 500 µL citrate buffer. The reactions were carried out at different pH (2–10) and a wide range of temperature (20–70 °C) in presence and absence of ATP. For pH reactions, temperature was set as 37 °C and temperatures studies were carried out at pH 4. Change in absorbance was measured at 652 nm after 20 min of time interval.

Kinetic parameter analysis

Kinetic assays were carried out at 37 °C using 35 µg of PEG AuNPs and citrate AuNPs, 0.5 mM TMB with varying concentrations of H_2O_2 0.05–2 M in presence and absence of 2 mM ATP and a fixed concentration of H_2O_2 (1 M), citrate and PEG AuNPs 55 µg with varying concentrations of TMB 0.05–2 mM in 500 µL of citrate buffer at pH = 4. All reactions were monitored at 652 nm using kinetics mode. The kinetic parameter was calculated based on Lineweaver–Burk plot.

$$\frac{1}{v} = \frac{K_m}{V_{max}}\left(\frac{1}{[S]} + \frac{1}{K_m}\right)$$

where v is the initial velocity, K_m is Michaelis constant, V_{max} represents the maximal reaction velocity and $[S]$ is the substrate concentration.

Terephthalic acid based test of hydroxyl radicals

Terephthalic acid readily reacts with ·OH radicals to generate 2-hydroxyl terephthalic acid, a highly fluorescent product. 2.1 mL of citrate buffer (pH = 4) containing 0.5 mM aqueous solution of terephthalic acid in 50 mM NaOH was subjected to react in presence of H_2O_2 (1 M) with different concentrations of PEG and citrate AuNPs (35–100 µg) for 20 min at an ambient temperature. Citrate AuNPs samples were centrifuged at 10,000 rpm for 10 min and PEG AuNPs samples were centrifuged four times at 10,000 rpm for 10 min and prior to the fourth centrifugation, add 0.4% NaCl to complete sedimentation of the AuNPs. Supernatants were used for measurements of PL intensity with an excitation wavelength of 315 nm using Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies).

Comparison of the stability of PEG, citrate and CTAB AuNPs

In 3.0 mL of citrate buffer (pH 4), 0.5 ng HRP and 15 µg PEG and citrate AuNPs were oxidized by 1 mM TMB using 10 mM H_2O_2 and incubated at 37 °C in presence and absence of ATP (2 mM) for different time intervals till 24 h. Peroxidase-like activity was measured by change in absorbance at 652 nm. Before recording absorbance, all samples were centrifuged at 13,000 rpm for 3 min and supernatants were used to monitor the absorbance.

Results and discussion

Characterization of AuNPs

Figure 1a shows the UV–Vis spectra of PEG AuNPs, citrate AuNPs, and CTAB AuNPs deciphering clear SPR band at ~ 521, ~ 524 and ~ 523 nm, respectively, which is contributed to the excitation of transverse surface plasmon resonance (Turkevich et al. 1951). Figure 1b–g represents the TEM image (Fig. 1b, d and f) and average particle size distribution (Fig. 1c, e, and g) of PEG AuNPs, citrate AuNPs, and CTAB AuNPs. As clearly evident from TEM image (Fig. 1b), PEG AuNPs are spherical in shape with high polydispersity as the particles size distribution is scattered from 4 to 16 nm with an average size distribution of ~ 8.1 nm (Fig. 1c). Citrate AuNPs are also spherical in shape (Fig. 1d) with relatively monodispersed distribution of particles with average particle size distribution of ~ 15.3 nm (Fig. 1e). Similarly, TEM image of CTAB AuNPs (Fig. 1f) reveals that particles are spherical in shape with an average size distribution of ~ 7.2 nm (Fig. 1g). The three types of AuNPs were synthesized to represent the positively charged (CTAB AuNPs), negatively charged (citrate AuNPs) and uncharged (PEG AuNPs) nanoparticles. Table 1 represents the average zeta potential values of these AuNPs suspension. CTAB AuNPs and citrate AuNPs carry a zeta potential value of + 50.87 ± 5.41 and − 23.45 ± 4.06 mV, respectively. However, PEG AuNPs suspension showed − 5.86 ± 0.97 mV zeta potential, suggesting that the particles have nearly zero charge. It is expected that PEG AuNPs are stabilized by the steric repulsion between the PEG chains, which is dependent on the PEG chain length (Stiufiuc et al. 2013).

Table 1 Zeta potential values of aqueous suspended PEG AuNPs, citrate AuNPs and CTAB AuNPs

	Zeta potential (mV)
PEG AuNPs	− 5.86 ± 0.97
Citrate AuNPs	− 23.45 ± 4.06
CTAB AuNPs	+ 50.87 ± 5.41

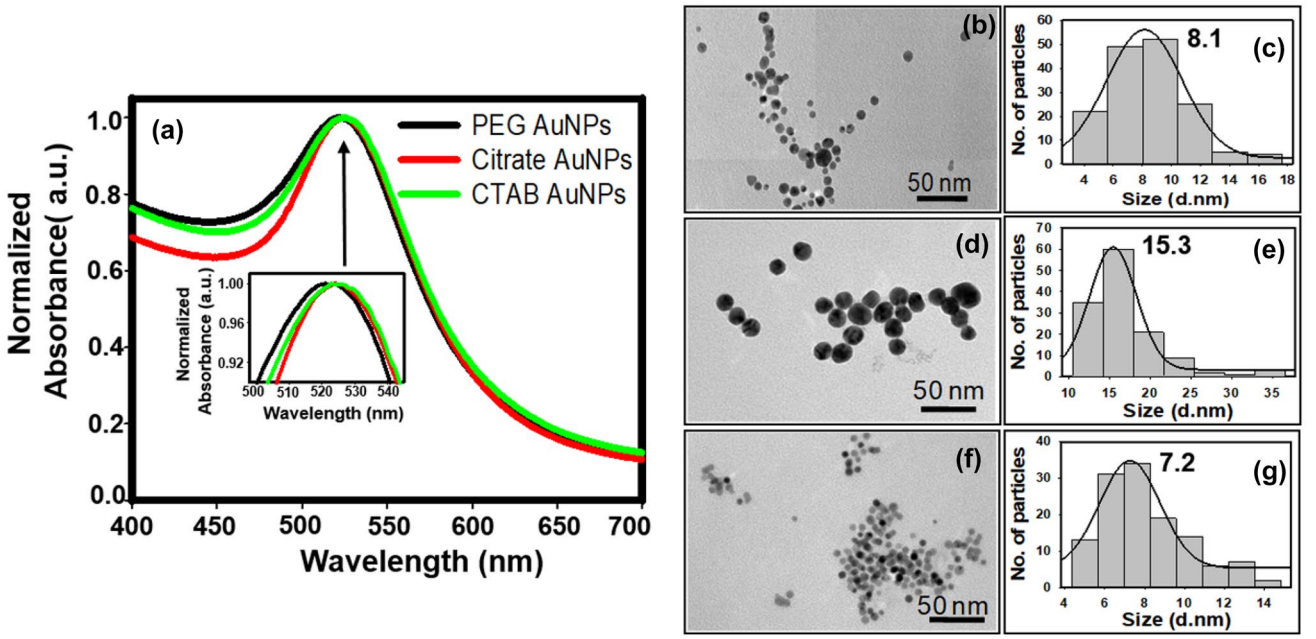

Fig. 1 Characterization of AuNPs: **a** Normalized UV–Visible absorption spectra of PEG AuNPs (black curve), citrate AuNPs (red curve) and CTAB AuNPs (green curve). Inset shows zoom image of absorbance spectrum. The representative transmission electron micrograph of PEG AuNPs (**b**), Citrate AuNPs (**d**) and CTAB AuNPs (**f**) are obtained from the respective suspension. Mean particle size distribution of PEG AuNPs (**c**), Citrate AuNPs (**e**) and CTAB AuNPs (**g**) were calculated from ~ 200 particles

Artificial peroxidase activity of AuNPs is surface charge dependent

It has been reported that the catalytic activity of nanomaterials is predominantly controlled by superficial charge present on the nanoparticle surface (Campbell et al. 2014; Pirmohamed et al. 2010). However, studies on the effect of superficial charge on the peroxidase-like activity are unexplored, which motivated us to study the effect of different charges on the peroxidase-like activity of PEG AuNPs, citrate AuNPs and CTAB AuNPs, representing neutral, negatively and positively charged AuNPs, respectively. To demonstrate the peroxidase-like activity of AuNPs, the catalytic oxidation of peroxidase substrate (TMB) in presence of H_2O_2 was tested. Figure 2 clearly reveals that the colorless TMB (reduced) solution is converted into blue colored TMB (oxidized) when incubated with H_2O_2 in presence of AuNPs. Different concentrations (5, 15, 25, 35, 45, 55, 65, 75 and 100 µg) of PEG AuNPs (Fig. 2a), and citrate AuNPs (Fig. 2b) showed a concentration-dependent increase in absorbance at 652 nm, which is the characteristic absorbance–wavelength of oxidized TMB. The concomitant intensity of the blue color solution of oxidized TMB can be seen in the respective photographs of reaction tubes (Fig. 2a, b, insets). Interestingly, CTAB AuNPs did not exhibit any absorbance at 652 nm suggesting that positively charged AuNPs are not peroxidase active. The color of the solution in reaction tubes (Fig. 2c, inset) remains colorless, which further confirms our observation. Additionally, we also followed the peroxidase reaction kinetics for 20 min (Fig. 2d–f). PEG AuNPs and citrate AuNPs showed time and concentration-dependent increase in the absorbance at 652 nm. As expected, CTAB AuNPs did not show any

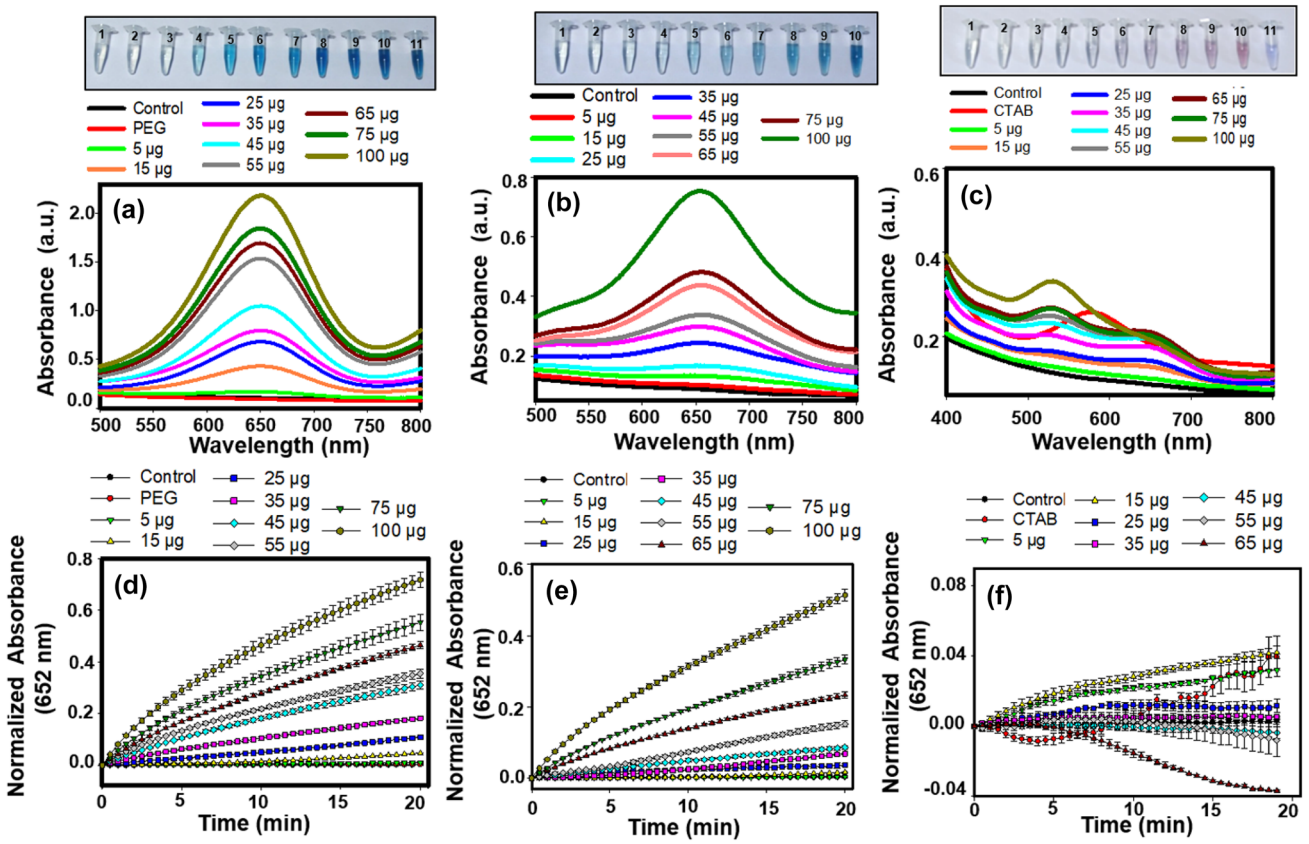

Fig. 2 Evolution of oxidized TMB followed by recording the absorbance at 652 nm in presence of different concentrations of PEG AuNPs (**a**), Citrate AuNPs (**b**) and CTAB AuNPs (**c**). Images of tubes represent the production of color intensity of TMB in presence of H_2O_2, ATP, and different concentrations of AuNPs. **a** (inset), tubes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 represent control (no AuNPs), PEG only, 5, 15, 25, 35, 45, 55, 65, 75 and 100 µg of PEG AuNPs, respectively. **b** (inset), tubes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 represent control (no AuNPs), 5, 15, 25, 35, 45, 55, 65, 75 and 100 µg of Citrate AuNPs, respectively. **c** (inset), tubes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 represent control (no AuNPs), CTAB only, 5, 15, 25, 35, 45, 55, 65, 75 and 100 µg of CTAB AuNPs, respectively. Time-dependent change in absorbance of oxidized TMB at 652 nm in presence of different concentrations of PEG AuNPs (**d**), Citrate AuNPs (**e**) and CTAB AuNPs (**f**). All reactions were carried out in 500 µl 10 mM citrate buffer in presence of 1 M H_2O_2 and 1 mM TMB. Error bars represents the standard deviation (SD) calculated from one of the best representing experiment performed in triplicate

significant increase in absorbance at 652 nm (Fig. 2f). The control experiments were also conducted where the solution containing only TMB and H_2O_2 neither showed any development of blue color nor increase in absorbance at 652 nm, suggesting that the oxidation of TMB requires AuNPs. The intensity of the blue color of the solution was much deeper for PEG AuNPs than the other two systems with the same concentration of citrate AuNPs and CTAB AuNPs, suggesting that PEG AuNPs shows the best peroxidase activity.

ATP enhances the artificial peroxidase activity of PEG AuNPs and citrate AuNPs

As described earlier by Singh and co-workers that ATP enhances the peroxidase-like activity of citrate AuNPs and Fe_3O_4 NPs (Shah et al. 2015; Vallabani et al. 2017), therefore, we asked the question that how ATP influences the catalytic activity of AuNPs with varying surface charges (Fig. 3). As expected, citrate AuNPs exhibited ~ 1.5 fold increase in peroxidase-like activity in presence of ATP when compared with the reaction performed without ATP (Fig. 3b and ESM 1b). Interestingly, PEG AuNPs exhibited ~ 3.0

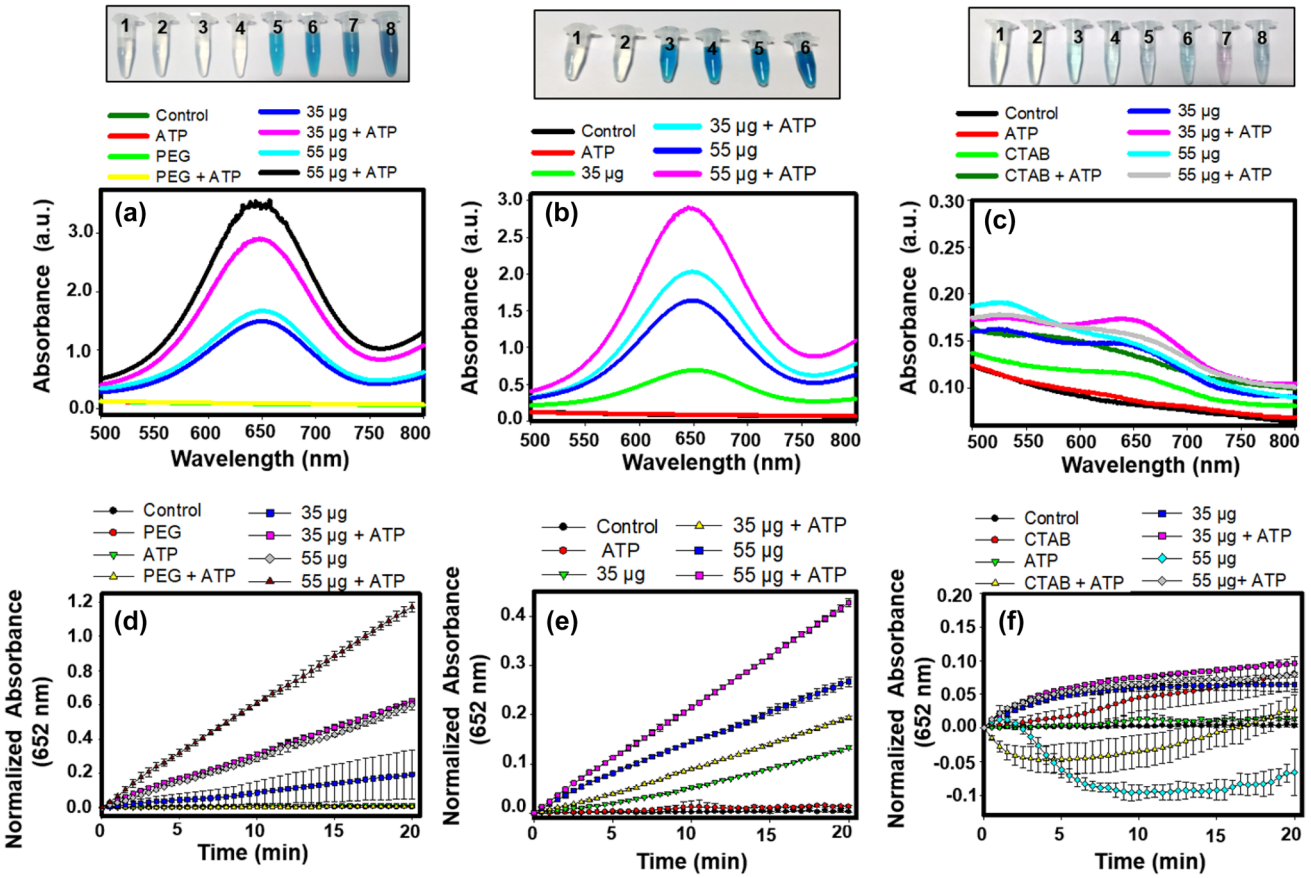

Fig. 3 Evolution of oxidized TMB followed by recording the absorbance at 652 nm in presence of ATP (2 mM) and different concentrations of PEG AuNPs (**a**), Citrate AuNPs (**b**) and CTAB AuNPs (**c**). Images of tubes represent the production of color intensity of TMB in presence of H_2O_2, ATP, and different concentration (35 and 55 µg) of AuNPs. **a** (inset), tubes 1, 2, 3, 4, 5, 6, 7 and 8 represents the color of reaction mixture containing TMB + H_2O_2, TMB + H_2O_2 + ATP, PEG + TMB + H_2O_2, PEG + TMB + H_2O_2 + ATP, PEG AuNPs (35 µg) + TMB + H_2O_2, PEG AuNPs (35 µg) + TMB + H_2O_2 + ATP (2 mM), PEG AuNPs (55 µg) + TMB + H_2O_2, and PEG AuNPs (55 µg) + TMB + H_2O_2 + ATP (2 mM), respectively. **b** (inset), tubes 1, 2, 3, 4, 5, and, represents the color of reaction mixture containing TMB + H_2O_2, TMB + H_2O_2 + ATP, Citrate AuNPs (35 µg) + TMB + H_2O_2, Citrate AuNPs (35 µg) + TMB + H_2O_2 + ATP, Citrate AuNPs (55 µg) + TMB + H_2O_2, Citrate AuNPs (55 µg) + TMB + H_2O_2 + ATP (2 mM), respectively. **c** (inset), tubes 1, 2, 3, 4, 5, 6, 7 and 8 represents the color of reaction mixture containing TMB + H_2O_2, TMB + H_2O_2 + ATP, CTAB + TMB + H_2O_2, CTAB + TMB + H_2O_2 + ATP, CTAB AuNPs (35 µg) + TMB + H_2O_2, CTAB AuNPs (35 µg) + TMB + H_2O_2 + ATP (2 mM), CTAB AuNPs (55 µg) + TMB + H_2O_2, and CTAB AuNPs (55 µg) + TMB + H_2O_2 + ATP (2 mM), respectively. Time-dependent change in absorbance of oxidized TMB at 652 nm in presence of different concentrations of PEG AuNPs (**d**), Citrate AuNPs (**e**) and CTAB AuNPs (**f**). All reactions were carried out in 500 µL 10 mM citrate buffer in presence of 1 M H_2O_2 and 1 mM TMB. Error bars represents the standard deviation (SD) calculated from one of the best representing experiment performed in triplicate

fold increase in peroxidase-like activity when performed in presence of ATP (Fig. 3a and ESM 1a) suggesting that ATP synergizes better with uncharged AuNPs than charged (positively and negatively) particles. We performed this study with two concentrations of PEG AuNPs (35 and 55 µg), however, the maximum enhancement in peroxidase-like activity was observed with 35 µg concentration, other higher or lower concentrations did not produce much higher activity. The color of resultant oxidized TMB solution is shown in respective insets from Fig. 3a, b, which clearly show that in presence of ATP, deep blue color solution was obtained. Additionally, the peroxidase reaction kinetics was also performed, which further supported that in presence of ATP, PEG AuNPs (Fig. 3d) and citrate AuNPs (Fig. 3e) catalyze the peroxidase reaction much faster than without ATP. CTAB AuNPs were also examined for any peroxidase-like activity in presence of ATP. However, we did not observe any enhancement in peroxidase-like activity (Fig. 3c). The color of TMB also remains colorless even after long time incubation with CTAB AuNPs with ATP (Fig. 3c, inset). The pH and temperature-dependent peroxidase activity of PEG AuNPs and citrate AuNPs (ESM 3 and ESM 4) further revealed that inclusion of ATP improves the enzyme-like activity of nanoparticles for a broad range of pH (2–10) and temperature (20–70 °C). The optimum pH and temperature remains similar to the peroxidase activity of AuNPs with or without ATP suggesting that inclusion of ATP does not alter the properties of AuNPs-based nanozyme but only enhances the catalytic activity at a broad range of physiological conditions.

Study of kinetic parameters (K_m and V_{max}) during artificial peroxidase activity of PEG AuNPs and citrate AuNPs

The steady state kinetic studies of TMB oxidation by PEG AuNPs and citrate AuNPs showed a hyperbolic relationship between the substrate concentration and rate of reaction, like a typical Michaelis–Menten reaction (Figs. 4, 5). Since only PEG AuNPs and citrate AuNPs showed peroxidase-like activity, we did not study the reaction kinetic parameters for CTAB AuNPs. The kinetic parameters K_m and V_{max} were determined for substrate (TMB) and H_2O_2, from Lineweaver–Burk plot. In the case of PEG AuNPs, the K_m and V_{max} values for TMB was found 0.155 mM and 8.32×10^{-8} Ms^{-1}, respectively (Fig. 4a, b). However, when oxidation of TMB performed in presence of ATP, the K_m and V_{max} values for TMB was found 0.197 mM and 14.7×10^{-8} Ms^{-1}, respectively (Fig. 4c, d). These results suggest that although the affinity of the substrate with catalyst does not improve, due to increase in K_m value, but the overall velocity of the reaction is enhanced when peroxidase-like activity was performed in presence of ATP. We also studied the K_m and V_{max} values for H_2O_2 was during the peroxidase reaction performed with PEG AuNPs. The K_m and V_{max} values for H_2O_2 were found to be 191 mM and 6.38×10^{-8} Ms^{-1}, respectively (Fig. 4e, f), whereas when reaction was performed in presence of ATP, the K_m and V_{max} values were 175 mM and 8.76×10^{-8} Ms^{-1} (Fig. 4g, h). These kinetic parameter values suggest that H_2O_2 also facilitates the enhancement of peroxidase-like activity of PEG AuNPs.

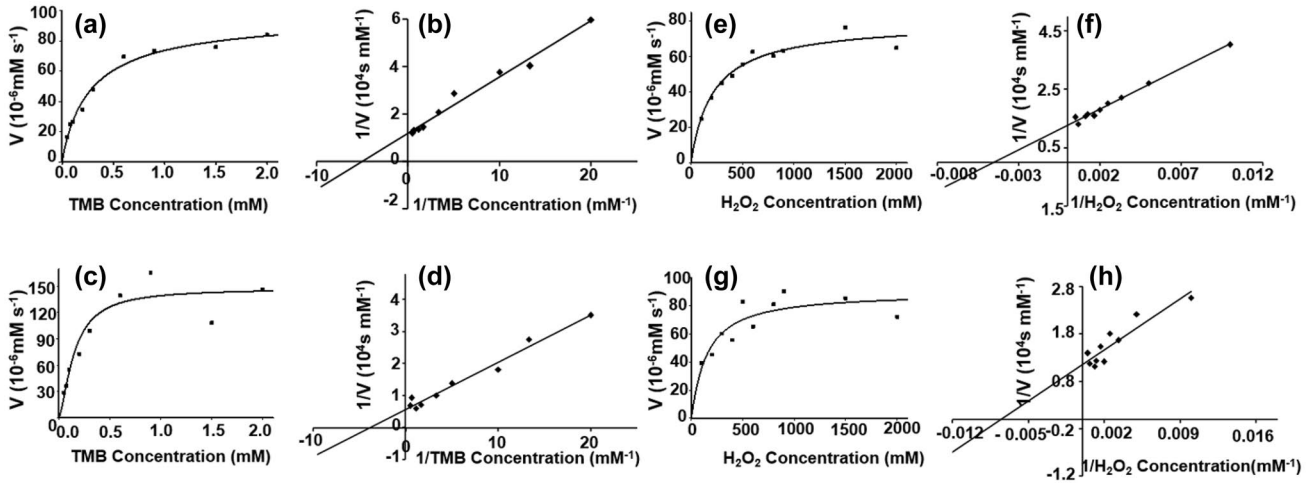

Fig. 4 Effect of ATP on the kinetic parameters (K_m and V_{max}) of PEG AuNPs: The Michaelis–Menten curves for the peroxidase-like activity of PEG AuNPs at fixed concentration of H_2O_2 (**a**, **c**) and TMB (**e**, **g**) in absence (**a**, **e**) and presence (**c**, **g**) of ATP were drawn. For varying concentrations of TMB, the concentrations of H_2O_2 was fixed 1 M and a concentration of TMB was varied from 0.05 to 2 mM (**a**–**d**) and for varying H_2O_2, the concentration of TMB was fixed 0.5 mM and a concentration of H_2O_2 was varied from 0.05 to 2 M (**e**–**h**). The double reciprocal plots of TMB (**b**, **d**) and H_2O_2 (**f**, **h**) was made from the respective Michaelis–Menten Curve in absence (**b**, **f**) and presence (**d**, **h**) of ATP

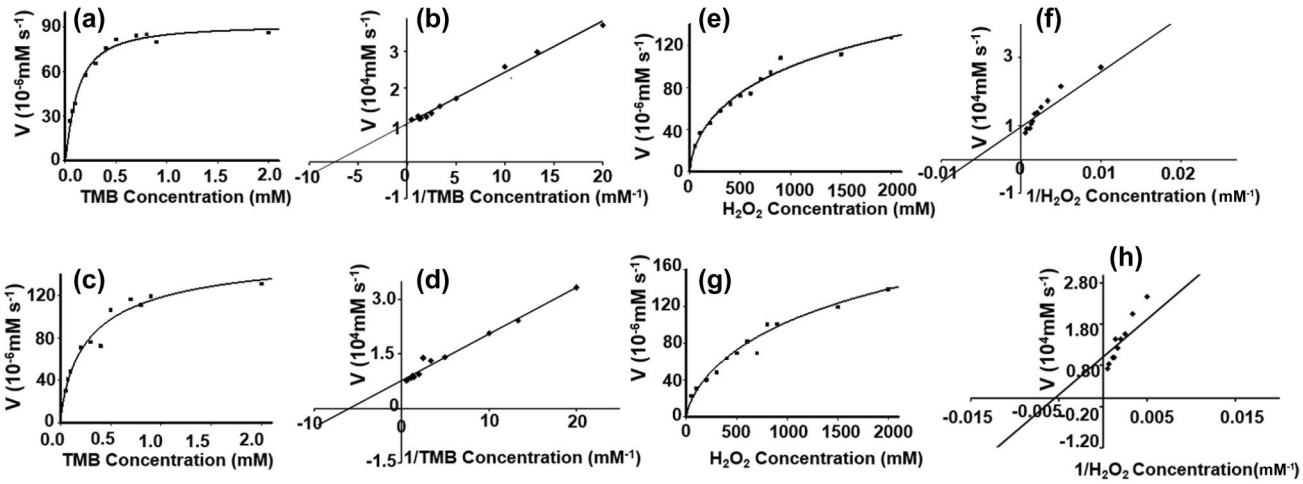

Fig. 5 Effect of ATP on the kinetic parameters (K_m and V_{max}) of citrate AuNPs: The Michaelis–Menten curves for the peroxidase-like activity of citrate AuNPs at fixed concentration of H_2O_2 (**a**, **c**) and TMB (**e**, **g**) in absence (**a**, **e**) and presence (**c**, **g**) of ATP were drawn. For varying concentrations of TMB, the concentrations of H_2O_2 was fixed 1 M and a concentration of TMB was varied from 0.05 to 2 mM (**a**–**d**) and for varying H_2O_2, the concentration of TMB was fixed 0.5 mM and a concentration of H_2O_2 was varied from 0.05 to 2 M (**e**–**h**). The double reciprocal plots of TMB (**b**, **d**) and H_2O_2 (**f**, **h**) was made from the respective Michaelis–Menten Curve in absence (**b**, **f**) and presence (**d**, **h**) of ATP

Furthermore, when studying the kinetic parameters for citrate AuNPs, the K_m and V_{max} values for TMB was found to be 0.134 mM and 9.65×10^{-8} Ms^{-1}, respectively (Fig. 5a, b), whereas, when oxidation of TMB was performed in presence of ATP, the K_m and V_{max} values for TMB was found to be 0.168 mM and 13.1×10^{-8} Ms^{-1}, respectively (Fig. 5c, d). These results clearly suggest that ATP enhances the velocity of peroxidase activity exhibited by citrate AuNPs. Furthermore, it must also be noted that the increase in reaction velocity (due to ATP) is more in PEG AuNPs than citrate AuNPs, which is in accordance with the data shown in Figs. 2, 3. The K_m and V_{max} values for H_2O_2 were found to be 213 mM and 10.6×10^{-8} Ms^{-1}, respectively (Fig. 5e, f), whereas when the reaction was performed in presence of ATP, the K_m and V_{max} values were 196 mM and 9.83×10^{-8} Ms^{-1} (Fig. 5g, h). Unlike PEG AuNPs, citrate AuNPs did not influence the kinetic parameters of H_2O_2 a lot suggesting that the peroxidase-like activity of citrate AuNPs is not significantly dependent on H_2O_2, therefore, the K_m and V_{max} values are not improved when the reaction was performed in presence of ATP.

The kinetic parameters (K_m and V_{max}) of TMB and H_2O_2 for PEG AuNPs and citrate AuNPs were compared with literature reported values for HRP (Table 2). The comparison data clearly shows that the K_m value of HRP for TMB is higher than PEG AuNPs and citrate AuNPs, suggest that AuNPs (with or without ATP) have better substrate affinity than HRP, which leads to the strong peroxidase-like activity. Furthermore, the velocity of the reaction was found more when PEG AuNPs and citrate AuNPs are used in peroxidase reaction with ATP than HRP (based on V_{max} values). Similarly, the V_{max} value of HRP for H_2O_2 is lesser than PEG AuNPs and citrate AuNPs (with ATP) suggesting that peroxidase reaction is faster when AuNPs are used.

Table 2 Comparison of kinetic parameters (K_m and V_{max}) of PEG AuNPs, citrate AuNPs and HRP in absence and presence of ATP, where K_m is the Michaelis–Menten constant and V_{max} is the maximum reaction velocity

Catalyst	Substrate	K_m (mM)	V_{max} (M s^{-1})	References
PEG AuNPs	TMB	0.155	8.32×10^{-8}	This work
	H_2O_2	191	6.38×10^{-8}	This work
PEG AuNPs + ATP	TMB	0.197	14.7×10^{-8}	This work
	H_2O_2	175	8.76×10^{-8}	This work
Citrate AuNPs	TMB	0.134	9.65×10^{-8}	This work
	H_2O_2	213	10.6×10^{-8}	This work
Citrate AuNPs + ATP	TMB	0.168	13.1×10^{-8}	This work
	H_2O_2	196	9.83×10^{-8}	This work
HRP	TMB	0.434	10×10^{-8}	Gao et al. (2007)
	H_2O_2	3.7	8.71×10^{-8}	Gao et al. (2007)

Role of hydroxyl radicals in artificial peroxidase activity of PEG AuNPs and citrate AuNPs

It has been reported that peroxidase-like activity exhibited by nanomaterials are dependent on the formation of hydroxyl radicals due to the breakdown of H_2O_2. Therefore, we explored that if there is any relation between the charge of AuNPs surface and hydroxyl radical (˙OH) production during the peroxidase reaction. The formation of ˙OH radical was determined by fluorescence method using terephthalic acid as probe molecule where ·OH radical reacts with terephthalic acid to generate 2-hydroxy terephthalic acid which is a highly fluorescent product (Dalui et al. 2015). Figure 6 clearly reveals that there is PEG AuNPs and citrate AuNPs concentration-dependent increase in the ·OH radical formation during the peroxidase-like activity exhibited by nanoparticles. It is expected that AuNPs would catalyze the degradation of H_2O_2 into ·OH radicals that can oxidize TMB and develop a blue color solution. Although there is ~ fourfold increase in ·OH radical formation when citrate AuNPs used instead of PEG AuNPs (~ threefold), which is in accordance with the V_{max} values of H_2O_2 for these particles (Table 2), where citrate AuNPs (10.6×10^{-8} Ms^{-1}) show high V_{max} value than PEG AuNPs (6.38×10^{-8} Ms^{-1}). However, when compared the peroxidase activity, PEG AuNPs show better activity than citrate AuNPs, which suggest that ·OH radicals are not the only factor that controls the peroxidase activity. We also compared the ·OH radical formation by PEG AuNPs and citrate AuNPs in presence of ATP (Fig. S2). To our surprise, a decrease in ·OH radical production was observed, which was proportional to the concentration of AuNPs used. We tested the ·OH radical production at two concentrations (55 and 100 µg) of PEG AuNPs and citrate AuNPs, which resulted in almost same trend of decrease in ·OH radical formation in both the nanoparticle types, suggesting that nanoparticle charge or capping molecule does not play any significant role in hydroxyl radical generation.

ATP imparts stability to oxidized TMB

Since our ·OH radical formation study revealed that there are other factors which control the peroxidase-like activity of AuNPs, we explored if ATP imparts any potential stability to the oxidized TMB. In this investigation, we performed the peroxidase-like activity of PEG AuNPs, citrate AuNPs and HRP enzyme in presence or absence of ATP for 20 min at 37 °C. Next, we followed the absorbance of oxidized TMB at 652 nm from 20 min to 24 h (Fig. 7). As evident from Fig. 7a, in absence of ATP, as time progress, the absorbance intensity of oxidized TMB was significantly reduced in all the three test conditions. Although it was found that TMB oxidized by PEG AuNPs (Fig. 7a) and citrate AuNPs (Fig. 7b) show lesser decrease in intensity than HRP (Fig. 7c). The inset image shows the representative color of oxidized TMB (due to peroxidase reaction of PEG AuNPs, citrate AuNPs, and HRP) in presence or absence of ATP after 24 h. This observation suggests that ATP facilitates the stability to the oxidized TMB, however, TMB oxidized in absence of ATP results in the quick reduction of TMB due to which the absorbance intensity decreases. It has been reported that ATP stabilizes the colored end products of TMB and ABTS via single electron transfer reaction (Kong et al. 2010; Lin et al. 2014a; Shah et al. 2015; Stefan et al. 2012).

Mechanistically, it has been reported that peroxidase-like activity of nanomaterials is due to the ability of nanoparticles to catalyze the reduction of H_2O_2 by transfer of electron by charge transfer to produce hydroxyl radicals (Shi et al. 2011). However, in the present study, this process does not seem to dominate when the peroxidase-like activity of PEG AuNPs and citrate AuNPs are performed in presence of ATP, which rather promotes the decrease in hydroxyl radical production. This clearly suggests that use of ATP in peroxidase reaction promotes the stability of oxidized TMB, than enhanced hydroxyl radical production (Stefan et al. 2012). Furthermore, reports show that ATP acts similar to the distal histidine residue of the biological peroxidase enzyme.

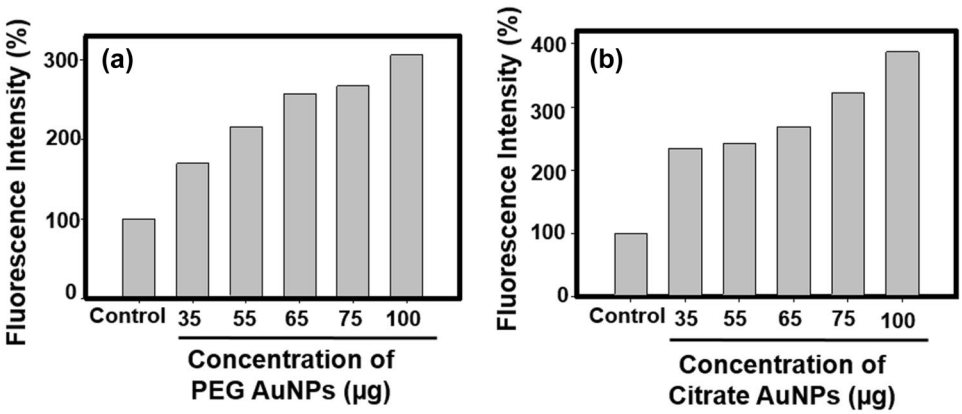

Fig. 6 Effect of AuNPs concentrations in the generation of hydroxyl radicals using terephthalic acid as a fluorescent probe: Evaluation for generation of ·OH radicals during peroxidase-like activity by photoluminescence using terephthalic acid (0.5 mM) in presence of different concentrations of PEG AuNPs (**a**) and Citrate AuNPs (**b**) in citrate buffer. The excitation and emission wavelengths were 315 and 425 nm, respectively

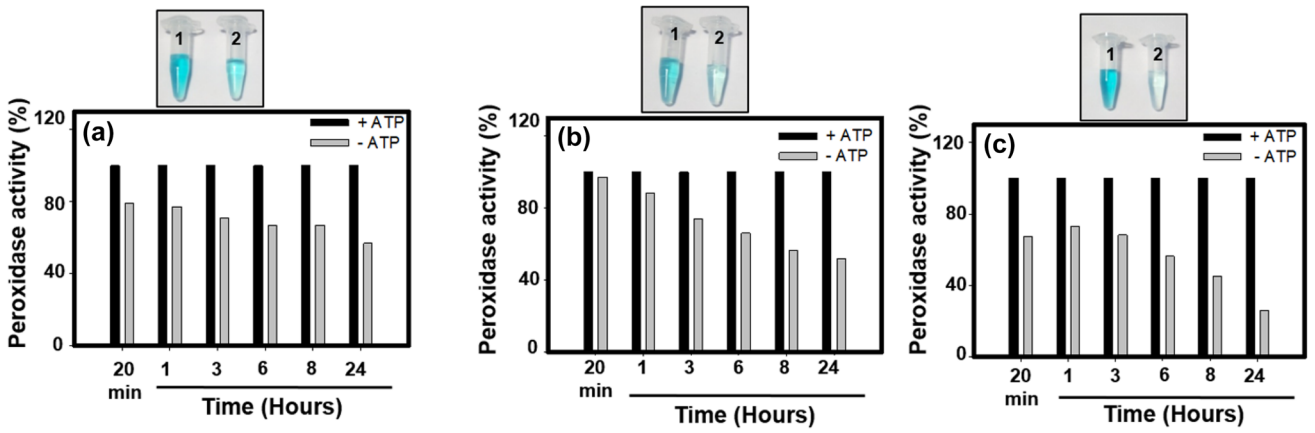

Fig. 7 ATP imparts stability to the oxidized TMB: 15 µg of PEG AuNPs (**a**), Citrate AuNPs (**b**) and 0.5 ng of HRP (**c**) was incubated for 24 h during the peroxidase reaction for at 37 °C in absence or presence of 2 mM ATP. The change in the absorbance of oxidized TMB (1 mM) by H_2O_2 (10 mM) was followed at 652 nm by recording the absorbance at different time intervals. Image (inset) shows the representative color of oxidized TMB over a period of 24 h with (1) and without ATP (2). Activity in presence of ATP at each time point was set as 100%

However, it must also be noticed that the surface capping molecules also play a major role in determining the catalytic activity of AuNPs, therefore, the CTAB AuNPs did not exhibit any significant peroxidase-like activity.

Conclusion

In summary, we report here that the peroxidase-like activity of AuNPs significantly depends on the surface charge and type of capping molecule on the surface of nanoparticles. Uncharged AuNPs (PEG AuNPs) exhibited the maximum peroxidase-like activity than citrate AuNPs or CTAB AuNPs. It has been reported that citrate AuNPs show better activity when positively charged peroxidase substrate such as TMB is used. Therefore, it is expected that the electrostatic attraction between negatively charged citrate AuNPs and positively charged TMB may temporarily exhibit peroxidase-like activity, however, the same extent of enhancement would not be possible with negatively charged peroxidase substrate such as ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid). In this context, PEG AuNPs will be an excellent choice to be used with both positively and negatively charged peroxidase substrate with similar activity. ATP acts as a boosting agent for PEG AuNPs and citrate AuNPs but works well with PEG AuNPs, which could be due to the imparting stability to the oxidized TMB. The reaction kinetic study (V_{max}) revealed that ATP enhances the velocity of peroxidase activity exhibited by PEG AuNPs and citrate AuNPs. The results from PEG AuNPs suggest that although the affinity of the substrate with catalyst (PEG AuNPs) does not improve, (high K_m value), the overall velocity of the reaction is enhanced when peroxidase-like activity was performed in the presence of ATP. However, the kinetic parameter values for H_2O_2 suggest that hydrogen peroxide also facilitates the enhancement of peroxidase-like activity of PEG AuNPs. Contrary to PEG AuNPs, the peroxidase-like activity of citrate AuNPs is not significantly dependent on H_2O_2, therefore, the K_m and V_{max} values are not improved when reaction was performed in presence of ATP. Hydroxyl radicals are found to play a significant role in the peroxidase-like activity of PEG AuNPs and citrate AuNPs, however, did not show any effect when ATP was used to boost the peroxidase activity. Interestingly, ATP was observed to impart stability to the oxidized form of TMB in the order of PEG AuNPs > citrate AuNPs > HRP, suggesting that ATP can also be used in conjunction with natural enzymes to boost their biocatalytic activity. Taken together, this study could be used to tune the peroxidase and other enzyme-like activities of nanomaterials and find a wide range of new applications in biosensing, theranostics, and nanomedicines.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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