Deciphering the mechanism of hafnium oxide nanoparticles perturbation in the bio-physiological microenvironment of catalase

Farooq Ahmad, Tahir Muhmood and Asif Mahmood

1 College of Engineering and Applied Sciences, Nanjing National Laboratory of Microstructures, Jiangsu Key Laboratory of Artificial Functional Materials, School of Material Science and Engineering, Nanjing University, Nanjing, Jiangsu, 210093, People’s Republic of China
2 State Key Laboratory of Metal Matrix Composites, School of Materials Science and Engineering, Shanghai Jiao Tong University, Shanghai, 200240, People’s Republic of China
3 Beijing Key Laboratory of Photoelectric/Electro photonic Conversion Materials, School of Chemistry and Chemical Engineering, Beijing Institute of Technology, Beijing, 100081, People’s Republic of China

E-mail: ahmadchemist@hotmail.com

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Abstract

Nanoparticles (NPs) are extensively being used in state-of-the-art nano-based therapies, modern electronics, and consumer products, so can be released into the environment with enhancement interaction with humans. Hence, the exposures to these multifunctional NPs lead to changes in protein structure and functionality, raising serious health issues. This study thoroughly investigated the interaction and adsorption of catalase (CAT) with HfO₂-NPs by circular dichroism (CD), Fourier transform infrared (FTIR), absorption, and fluorescence spectroscopic techniques. The results indicate that HfO₂ NPs cause fluorescence quenching in CAT by a static quenching mechanism. The negative values of Vant Hoff thermodynamic expressions (ΔH°, ΔS°, and ΔG°) corroborate the spontaneity and exothermic nature of static quenching driven by van der Waals forces and hydrogen bonding. Also, FTIR, UV-CD, and UV−visible spectroscopy techniques confirmed that HfO₂ NPs binding could induce microenvironment perturbations leading to secondary and tertiary conformation changes in CAT. Furthermore, synchronous fluorescence spectroscopy confirmed the significant changes in the microenvironment around tryptophan (Trp) residue caused by HfO₂ NPs. The time depending denaturing of CAT biochemistry through HfO₂-NPs was investigated by assaying catalase activity elucidates the potential toxic action of HfO₂-NPs at the macromolecular level. Briefly, this provides an empathetic knowledge of the nanotoxicity and likely health effects of HfO₂ NPs exposure.

1. Introduction

Hafnium oxide (HfO₂) NPs due to extraordinary physicochemical behavior possess the characteristics such as high melting point (2758 °C), high dielectric constant (∼30), chemical stability, insulation potential, wide bandgap (>5.0 eV), and high neutron absorption cross-section, make them an excellent candidate to be used in a wide range of electronics and x-ray detection tools [1]. Recently, HfO₂-NPs composites have found their way into medicine due to the demand for safety evaluation [2, 3]. Europium doped hafnium dioxide NPs have developed for cellular optical imaging and as radiosensitive materials for clinical treatment [4]. In another study, inherently therapeutic polymeric silane conjugated hafnium oxide NPs (Hf-PS NPs) were proved very effective in eliminating the biofilms of Streptococcus mutans (most predominant and carious oral bacteria) by effectively killing the bacteria through ‘latch and kill mechanism’in rodent models. Furthermore, these Hf-PS NPs have also enhanced targeted x-rays imaging [3]. Therefore, the protracted use of these hafnia nanocomposites would make their way into the environment, which in turn might be associated with some health issues. While the studies show biocompatibility and lower toxicity of HfO₂-NPs toward human cells [6] and microorganisms [7],...
but there is no knowledge about the possible mechanism of HfO₂ NPs interaction with the biological molecules and affect their functionality. A lot of literature is available explaining the nano–material toxicity on human health [8–10]. There are also studies demonstrated that these NPs interact with biomolecules (proteins, lipids, and carbohydrates) and leads to alteration of native configuration and hence, the functionality of biomolecules [11, 12] and accelerate the initiation and progression of diseases [13]. As soon as these biomolecules come into physical contact with NPs, biomolecules competitively adhere to the surface of nano-particles, constructing the protein corona, resulting in the mutated fortune and mechanism of nanomedicine and increased the nanotoxicology [14]. The composition of the external coating is determined by the size and surface properties of nanoparticles and equilibrium binding constants of each protein corona [15, 16]. The final corona (hard corona) is permanent and able to mediate their interaction with other parts of the cell [17]. Hence, exploring the possible interactions of NPs with expected biomolecules in fluids of interest are of high significance. There is evidence that the NPs interaction induce the conformational alterations in the serum protein by distressing the secondary and tertiary architecture of albumins, and hence deviate from normal working [18, 19]. Nano–carbon relentlessly distresses the transportability of serum proteins by fluctuating their architecture and anatomy [12, 20]. Cobalt–doped iron oxide NPs also affects the 3D architecture and symmetry, so enhance the activity of acid phosphatases and bovine serum albumin [21, 22]. Furthermore, these studies unveil the conceivable transportations of NPs into the body, demands the safety by design synthesis of NMs for biomedical applications.

Catalase is a ubiquitous oxidative stress-reducing enzyme by a disproportionation of two molecules of H₂O₂ to one molecule of O₂ and two molecules of H₂O [23]. As an important antioxidant enzyme, CAT plays a key role in protecting the cell from xenobiotics-induced stress tolerance [24]. Some large number of evidences provide data that the catalase is a significant player in multiple uncontrolled states such as abnormal blood sugar, aging, oxidative stress (disturbed biochemical reactions), and cancer (unwanted propagation of cells). Though, denaturing and repetition mechanism of catalase in living body lacks comprehensive understanding [25, 26]. The inactivation of catalase would lead to H₂O₂ accumulation in the cells and amplified oxidative stress leading to protein and DNA oxidation [27, 28]. Furthermore, NPs exposure and inactivation of catalase links oxidative stress-mediated pathogenic pathways in tissue demolition [29]. Moreover, multiple studies ratified that the NMs attacking could significantly disturb the CAT free movement [30, 31]. Though, the specific information about the interface of CAT and NPs is urgently needed.

Hence, this study aimed to analyze the conformational and purposeful transformations of CAT that began by interaction with HfO₂-NPs. This research work revealed the likely toxicity of HfO₂-NPs to the abundant universal antioxidant along with bonding parameters, sites, and conformational transformations in CAT with the help of bio-physical analytical techniques. Corresponding investigations would assist in understanding the toxic behavior of HfO₂ in-vitro and bring up to the minute scientific perceptions about a diverse range of nanomaterials.

2. Experimental details

2.1. Materials
Catalase (CAT) and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) were acquired from Aladdin industrial-Inc. Shanghai. HBS buffer was used to prepare CAT solutions with different gradations and stored at 4 °C. HfO₂ NPs was acquired from Sigma-Aldrich, China.

2.2. Characterization of HfO₂ NPs
Morphology of HfO₂ NPs was perceived by a transmission electron microscope. XRD was done for crystalline structure analysis (PNAlytical, Netherlands) equipped with CuKα radiation. FTIR (Thermo Fischer Nicolet 6700, FTIR) was used to obtain the surface coatings.

2.3. Circular dichroism measurement
Secondary structure of the CAT through Circular dichroism measurements and was performed on JASCO spectropolarimeter (J-810, scan range190–260 nm, cuvette path length = 100 mm) by fixing CAT at (2 μM) with graded HfO₂ NPs (50 to 250 μM). The mean residual ellipticity (MRE) was represented in deg cm² dem⁻¹ according to the equation given below:

\[
MRE = \frac{C_{\text{obs}}(m \text{ deg})}{C_p \text{ml}} \times 10
\]

Where \(C_p\) = Molarity of CAT, \(n\) = amino acid residues of CAT (240) and \(l\) = cuvette path-length (100 mm cm). The α-helical (% age) of free and conjugated CAT were mathematically determined at 208 nm
from MRE considering the equation (2)

\[
\alpha = \text{helix(\%age)} = \left[ \frac{(-\text{MRE}_{280} - 4000)}{(33000 - 4000)} \right] \times 100
\]

(2)

2.4. Fluorescence spectroscopy

Fluorescence spectra were documented with a spectra-Max M5 microplate reader with an emission maximum of 340 nm at excitation of 280 nm, at 298.15, 303.15, and 308.15 K by scanning from 300–420 nm. The fluorescence intensities of each spectrum were mathematically adjusted to avoid inner filter effect considering equation (3) [32].

\[
F_{\text{Cor}} = F_{\text{obsAntilog}} \left( \frac{A_{\text{ex}} + A_{\text{em}}}{2} \right)
\]

(3)

\(F_{\text{Cor}}\) and \(F_{\text{obs}}\) are adjusted and detected fluorescence strengths by detecting emission wavelength \(A_{\text{em}}\), respectively. \(A_{\text{ex}}\) and \(A_{\text{em}}\) are sums of the absorbencies of each suspension at specific excitation and emission wavelengths, respectively.

2.5. Fluorescence lifetime

Time Correlated Single Photon Counting (TCSPC) system was used to compute the Fluorescence lifetimes at 280 nm (IBH, nano led-07) to stimulate the CAT and fluorescence detected at 54.71 magic angles, to measure the Fluorescence lifetime. The FL decays were computed through Data Station v-2.5 decay analysis software. The appropriateness of the fits was assessed through \(\chi^2\) criterion and visual inspection of residuals of the fitted data and mean fluorescence lifetime \(\langle \tau \rangle\) was computed using equation (4)

\[
\langle \tau \rangle = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i}
\]

(4)

Here, \(\alpha_i\) is a pre-exponential factor matching to the \(i\)th decay time constant \(\tau_i\).

2.6. Synchronous fluorescence spectrometry (SFS)

The synchronous fluorescence spectra of each CAT suspension were measured on Spectrofluorimeter (1 cm quartz cell) (Shimadzu, Japan) at altered scanning intervals of \(\Delta \lambda (\Delta \lambda = \lambda_{\text{em}} - \lambda_{\text{ex}}) = 20\) and 60 nm, respectively at room temperature.

2.7. UV-spectrophotometry

Absorbance spectra of each suspension were graphed by scanning from 190 nm to 420 nm, through a double beam spectrophotometer (Shimadzu, Japan).

2.8. Fourier transforms infrared (FTIR) spectroscopy

The FTIR spectra of CAT were recorded with and without of HfO2 NPs in the range from 500 cm\(^{-1}\) to 1800 cm\(^{-1}\) by fixing CAT and HfO2-NPs concentration at 2.0 \(\mu\)M in HBS buffer.

2.9. Thermogravimetric analysis

Thermogravimetric analysis (TGA) of CAT@HfO2-NPs conjugates were completed using a Perkin Elmer Thermal-Analyzer (Waltham, PA). The weight drops of each sample (HfO2 NPs, CAT and CAT@HfO2 NPs) was noted from 25 \(^\circ\)C to 700 \(^\circ\)C at a rate of 10 \(^\circ\)C, with continuous N\(_2\) gas flow (50 ml min\(^{-1}\)).

2.10. CAT activity assay

CAT enzyme decays H\(_2\)O\(_2\) into water and oxygen. The H\(_2\)O\(_2\) and HBS buffer was homogeneously blended in a 3 ml quartz cell (reference solution). The catalyzing potential of CAT was graphed spectrophotometrically through recording the diminution of H\(_2\)O\(_2\) (36 mM) absorption at 240 nm [33, 34] at various incubation times (15, 30, 60 min) with graded concentrations of HfO2 NPs (after attaining equilibrium).

3. Results and discussion

3.1. Physicochemical characterization of HfO2 NPs

The Physico–chemical properties of employed HfO2 NPs provided in the supplementary information. XRD pattern (Supplementary figure S1 available online at stacks.iop.org/NANOX/1/030006/mmedia) of HfO2 NPs
elaborates on the cubic shape of HfO2-NPs with a mean crystallite size of 10 nm. TEM image conferred the 10 nm size with the round shape of HfO2 NPs. (Supplementary figure S2).

3.2. Thermodynamics interaction

3.2.1. Steady-state fluorescence outcome by HfO2 NPs incubation

Chromophores inherit the characteristic fluorescence in proteins, amidst tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) accounted for strong fluorescence quantum yield. The fluorescence and synchronous fluorescence spectra indicate CAT conformation transformation by incubating HfO2 NPs as shown in figure 1. It is clear that CAT fluorescence intensity reduced progressively and extreme fluorescence emission wavelength shifted from 340 nm to 342 nm with increasing concentration of HfO2-NPs. Given the redshift behavior of fluorescence emission of CAT, corroborates the potential of HfO2 NPs to transform the CAT conformation results in shifting the microenvironment of CAT to hydrophilic. Steady stated FL data were computed through Stern–Volmer equation (5), (table 1):

$$\frac{F_0}{F} = K_{sv}[\text{HfO}_2] + 1$$

$F_0$ and $F$ indicate FL strengths of CAT, with and without HfO2 NPs, respectively. $K_{sv}$ is the Stern–Volmer constant and $[\text{HfO}_2]$ indicates absorption of HfO2 NPs. The mechanism of intrinsic FL quenching (static or dynamic) of Trp residue in CAT provide us very vital information about the dynamics between enzyme and quencher and examining $K_{sv}$ values at different temperatures could provide us with this information [35, 36]. Dynamic and static quenching can be distinguished through simultaneous variation in FL and viscosity by varying temperature. High temperature endorses dynamic quenching via boosting diffusion and restricts the static quenching subsequently formation of less stable complexes [37]. The reduction in $K_{sv}$ (table 1) with increasing temperature, validates the supremacy of static quenching, through ground-state complex construction. Also, the mechanism of ground–state complex computed through quenching rate constant ($k_q$) equation (6) and using FL lifetime ($\tau_o$) of biopolymer of $10^{-8}$ s:[38]

![Figure 1. Fluorescence spectra and Stern–Volmer plots.](image)

**Table 1.** The Stern–Volmer quenching constant ($K_{sv}$), quenching rate constant ($k_q$), binding constant ($K_b$), number of binding sites ($n$) and the thermodynamic parameters ($\Delta H^o$, $\Delta S^o$, and $\Delta G^o$) for CAT–HfO2 NPs interaction at different temperatures.

| T(K)  | $K_{sv} \times 10^3$ (l mol$^{-1}$) | $k_q \times 10^{12}$ (l mol$^{-1}$ s$^{-1}$) | $K_b \times 10^3$ (l mol$^{-1}$) | $\Delta G^o$ (kJ mol$^{-1}$ K$^{-1}$) | $\Delta H^o$ (kJ mol$^{-1}$) | $\Delta S^o$ (J mol$^{-1}$ K$^{-1}$) |
|-------|----------------------------------|--------------------------------------|---------------------------------|---------------------------------|-----------------|-----------------|
| 298.15| 5.04                             | 1.46                                 | 0.394                           | 0.05                            | -2.30           | -255.461        |
| 303.15| 3.77                             | 1.09                                 | 0.269                           | 0.08                            | -4.72           | -192.695        |
| 308.15| 2.27                             | 0.66                                 | 0.125                           | 0.11                            | -5.24           | -149.826        |
\[ k_q = \frac{K_b}{\tau_0} \]

The observed \( k_q \) value is 1.46 \times 10^{12} \text{ mol}^{-1}\text{s}^{-1} at 298.15 K, it's a higher quenching rate constant of the biopolymer (i.e., 2.0 \times 10^{10} \text{ mol}^{-1}\text{s}^{-1}). The information in Table 1 confirms a progressive reduction in quenching constant (\( k_q \)) with increasing temperature, indicating static quenching [11]. To extend the validation of static quenching, the number of binding sites and value of binding must decline with a temperature rise, after incubating with \( \text{HfO}_2 \) NPs, computed by the Lineweaver-Burk equation (7) [11, 39].

\[
\log \left( \frac{F_0 - F}{F} \right) = \log K_b + n \log[\text{HfO}_2] 
\]

Where \( K_b \) is the binding constant (i.e., evidence of the existence of binding sites in a molecule whose occupation depends on the affinity of the ligand to the receptor-such a binding behavior is very specific and can be interpreted as a particular characteristic between the ligand and the target molecules) and \( n \) is a number of binding sites [40, 41]. Thus, the plot of \( \log(\frac{F_0 - F}{F}) \) versus \( \log[\text{HfO}_2] \) can be used to determine \( K_b \) as well as \( n \), as summarized in Table 1. Reduction in \( n \) and \( K_b \) values with increasing temperature (Table 1), corroborates higher temperature efficiently compromise the ability of binding, results in unstable complex formation. Also, the above results authenticate the predominance of static quenching in the currently explored CAT-HfO_2 system.

3.2.2. Calculation of thermodynamic parameters \( \Delta H^0, \Delta S^0 \) and \( \Delta G^0 \)

Over-all, connections between proteins and NPs could be of numerous types and magnitude, such as electrosstatic, hydrogen-bonding, van-der-Waals, steric, hydrophobic/ hydrophilic inside the necessary active-sites, etc. The magnitude and symbols of thermodynamic parameters provide us with evidence in numbers for the approximate estimate of the participation of decisive binding forces in play [18, 42]. Considering a thermodynamic point of view, \( \Delta H^0 < 0 \) and \( \Delta S^0 > 0 \) designate electrostatic forces, \( \Delta H^0 > 0 \) and \( \Delta S^0 > 0 \) designate a hydrophobic force, and \( \Delta H^0 < 0 \) and \( \Delta S^0 < 0 \) designate van-der-Waals/hydrogen forces establishment. The enthalpy of \( \text{HfO}_2 \) NPs-CAT complex formation considered constant if there is no absorption/emission of heat. We computed the enthalpy transition (\( \Delta H^0 \)) free enthalpy transition (\( \Delta G^0 \)), and entropy transition (\( \Delta S^0 \)) through thermodynamic equations (8)–(10)

\[
\ln [\Delta K_b] = \frac{\Delta H^0}{R} \left( \frac{1}{T_1} - \frac{1}{T_2} \right) \quad (8)
\]
\[
\Delta G^0 = -RT \ln K_b \quad (9)
\]
\[
\Delta G^0 = \Delta H^0 - T \Delta S^0 \quad (10)
\]

Where ‘\( R \)’ is the gas constant (8.314 J mol\(^{-1}\)K\(^{-1}\)), and ‘\( T \)’ is the temperature in Kelvin (K) and \( K_b \) is the equilibrium binding constant at the particular temperature. The calculated indices of \( K_b, \Delta H^0, \Delta S^0 \), and \( \Delta G^0 \) are offered in Table 1. The negative values of \( \Delta G^0 \) demonstrated a spontaneous process. The positive values of \( \Delta S^0 \) and negative values of \( \Delta H^0 \) indicating the interaction for \( \text{HfO}_2 \) and CAT complex is primarily enthalpy driven. Furthermore, in our research, the hydrogen bonding and van-der-Waals interactions are the main bonding factors in \( \text{HfO}_2 \)-CAT complex emergence. [18, 42].

3.3. Conformation transitions

3.3.1. Transitions in circular dichroism spectra

Circular dichroism spectroscopy (CDS) was practiced for conformation transformations of proteins solutions quantitatively due to its sensitive analytical measurements [43]. The CDS spectra (Figure 2) graphed two negative peaks at 210 and 225 nm, demonstrating the \( \pi \rightarrow \pi^* \) and \( n \rightarrow \pi^* \) electronic-transitions in CAT \( \alpha \)-helix segment, respectively. The contents of the \( \alpha \)-helix have shown in the inset of Figure 2. Furthermore, isodichroic or isosbestic point is the wavelength where the molar absorptivity is the same for two (or more) protein spectra [44, 45]. The existence of an isodichroic point for a given substance indicates a local two-state (\( \alpha \)-helix and random coil) population computed at 203–205 nm range [46, 47]. Therefore, analysis of the isodichroic point will distinguish a mutation that affects protein concentration calculation form that affecting native structure [48]. Given that, as shown in Figure 2, the isodichroic point for CAT is at 203 nm demonstrates hafnium oxide NPs and catalase complex formation significantly decreases the \( \alpha \)-helix complex formation with the increase in random coils in concentration-dependent manner. This complex formation also leads to compromising the intra protein H-bonding, hence decreasing the \( \alpha \)-helix content with increasing random coil. Compare to the pristine CAT, \( \alpha \)-helical number of CAT@\( \text{HfO}_2 \) NPs reduced from 81% to 53% (~34% reduction); suggesting strong interactions among \( \text{HfO}_2 \) NPs and CAT. The reduced amount of \( \alpha \)-helix and amplifie\( \beta \)-sheet amount in CAT designated that \( \text{HfO}_2 \) NPs have developed attractions with aromatic amino acid residues (Trp and Tyr) of the primary polypeptide tail of CAT, destructs the hydrogen bonding systems within protein [49, 50] leading to the exposing the active binding sites.
3.3.2. Transitions in synchronous fluorescence spectrometry (SFS)

Synchronous fluorescence spectrometry (SFS) used for fingerprinting the biological samples was introduced by Lloyd [11]. The salient features of SFS are sensitivity and simple spectra. In addition, SFS provides evidence about transformations in the molecular microenvironment of the fluorophore (e.g., residues of aromatic amino acids) functional group of CAT. In SFS, $\Delta \lambda (\Delta \lambda = \lambda_{em} - \lambda_{ex})$ values were fixed to 20 nm for tyrosine (Tyr) and to 60 nm for tryptophan (Trp), showed the essential information of microenvironment as well as amino acid residues [51] as shown in figure 3. According to figure 3, diminishing FL intensity of Tyr residues, and the position of maximum emission wavelength temporarily shifted to a longer wavelength (redshift), by fixing $\Delta \lambda$ to 60 nm. It corroborates transition in Trp microenvironment by the establishment of HfO$_2$-CAT NPs complex. The optimum emission wavelength ($\lambda_{max}$) located at 330 nm to 332 nm designates that Trp residues are positioned in the hydrophobic area of CAT; $\lambda_{max}$ positioned at 345–352 nm further designates, Trp residues are less positioned in the hydrophilic region [52]. The redshift recommended poor hydrophobic microenvironment of Trp residue and ground state HfO$_2$ NP-CAT complex formation. Our results corroborate that HfO$_2$-NPs probably bind to Trp residues of CAT, leading to subsequent conformation transition with amplified polarity around the Trp residues result in enhanced hydrophilicity [53, 54]. Hereafter reasonable communication position of HfO$_2$ with CAT is nearby Trp amino-acid. A trivial redshift of SFS spectra for $\Delta \lambda = 60$ nm stipulates conformation fluctuations in the micro-neighborhood of Trp residue with boosted hydrophilicity. These results are in good promise with the earlier studies [55]. Also, minor differences of SFS spectra at $\Delta \lambda = 15$ nm clarifies

![Figure 2](image_url). CD spectra of CAT in the absence and presence of HfO$_2$ NPs (0) 0, (1) 50, (3) 100, (4) 150, (5) 200 and (6) 250 $\mu$M ($T = 298$ K) the concentration of CAT was fixed at 20 $\mu$M in HBS buffer with pH = 7.4.

![Figure 3](image_url). Representative synchronous fluorescence spectra of CAT at different concentration of HfO$_2$ NPs ((0) 0, (1) 50, (2) 100, (3) 150, (4) 200 and (5) 250 $\mu$M), respectively.
non-significant transformations in the micro-environment of Tyr residue with an increasing concentration of HfO₂-NPs.

3.3.3 UV–vis Absorption spectroscopy

UV–vis absorption spectroscopy provides information related to structural transformations in a protein upon protein-NPs complex creation. The absorption band for active positioned around 192 nm is due to the \( \pi \rightarrow \pi^* \) transition in the polypeptide(C=O) structure [25]. The intensification of the absorbance at 192 and 278 nm indicates the HfO₂ NPs networked with CAT, shifting the CAT conformational conformation, results in a shifted micro-environment of the chromophores.

The absorption peak at about 404 nm corresponds to the Soret absorption band belonging to \( \pi \rightarrow \pi^* \) transitions of hematoporphyrin in catalase (inset of figure 4) [56]. The hyperchromic effect in the Soret region may be attributed to a conformational change in the vicinity of the heme moiety that leads to an alteration in the intensity of the \( \pi \rightarrow \pi^* \) transitions [57]. Hence, both the hyperchromic and slight red around 404 nm suggests that HfO₂ NPs are bound close to the active center of CAT and have developed relatively stronger interaction with the heme group [38, 58]. Since the changes of the spectra in the Soret region correlate with the changes of CAT activity, we considered that HfO₂ NPs may result in an alteration of CAT activity. UV–vis absorption spectra have been generally commissioned to learn the mechanism of ground state protein-ligand complex formation ultimately leads to the quenching of the protein-ligand system [38]. Static quenching stimulates changes in the absorption spectra of the protein after complex formation at ground-state, on the contrary, dynamic quenching only disturbs the excited state of the fluorophores, and therefore absorption spectra indicate the slight transformations. Concisely, ground-state complex formation of HfO₂ NPs with CAT was a spontaneous process and static quenching was dominant quenching mechanism of CAT. This verified the results comprehended the time-resolved fluorescence assay.

3.3.4 Transitions in fourier transform infrared spectroscopy

FTIR spectroscopy is an admirable device for the scanning of protein-NPs interactions [43]. Figure 5 illustrates the comparative FTIR spectra of pristine CAT and CAT–HfO₂ NPs. The 1655.5, 1538.5, and 1393.8 cm⁻¹ bands were linked to the \( \alpha \)-helix primary amide, secondary amide, and tertiary amide, respectively [59]. Two bands, 1655.5 and 1538.5 cm⁻¹ were attributed to elevated \( \alpha \)-helix content indicated by the primary amide and secondary amide of CAT. The \( \alpha \)-helix configuration injury was illustrated by intensity zone out at 1655.5, 1538.5, and 1393.8 cm⁻¹ bands. The profound intensity destruction was assigned to enduring attractive forces among CAT and HfO₂ NPs [60] and initiating the transformations in secondary and tertiary structure conformation.

3.3.5 Energy transfer between HfO₂ NPs and CAT

Förster’s non-radiative energy transfer will occur when the donor emission and absorption spectrum of the acceptor overlaps, provided the donor and acceptor distance is less than \( \sim 8.0 \) nm [32, 61]. The efficacy of energy transfer, \( E \), was computed through equation (11).

*Figure 4. UV–vis absorption spectra of CAT in various concentrations of HfO₂ NPs, (i) 0, (ii) 50, (iii) 100, (iv) 150, (v) 200 and (vi) 250 (μM), respectively. The concentration of CAT was fixed at 20 μM in HBS buffer with pH = 7.4.*
Here, ‘r’ is the mean distance linking the donor and acceptor and ‘R0’ is critical distance with 50% transfer efficiency. The value of ‘R0’ is computed through equation (12) [61]:

$$R_0^6 = 8.8 \times 10^{-23} K^2 N^{-4} \Phi J$$

Here $K^2$ is the spatial direction linked with donor and acceptor dipoles, $K^2 = 2/3$ for arbitrary orientation, for liquids; $N$ is the refractive index of the medium.; and $\Phi$ is the donor quantum yield. $J$ is the extent of spectral overlap amid donor fluorescence and acceptor absorption spectrum, and computed through equation (13) [61, 62]:

$$J = \frac{\int_0^\infty F(\lambda) \varepsilon(\lambda) \lambda^4 d\lambda}{\int_0^\infty F(\lambda) d\lambda}$$

Here, $F(\lambda)$ is the donor fluorescence intensity at wavelength $\lambda$, and $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength $\lambda$. Figure 6 illustrated the intersection of the absorption spectrum of HfO2 NPs and the CAT emission spectrum. Considering equations (11)–(13), by means of $K^2 = 2/3$, $n = 1.336$ and $\Phi = 0.15$ for CAT, we calculated; $J = 92.5 \times 10^{-17}$ cm$^2$/L/mol, $R_0 = 2.55$ nm, $r = 2$ nm and $E = 68.2\%$. In the case of HfO2 NPs–CAT system, observed donor-acceptor distance ($r$) <8 nm further confirming the static quenching was predominant. The mean tiny distance amid CAT–HfO2 NPs advocate the stronger attractive electrostatic forces that exist between HfO2 NPs and CAT conjugate.

3.3.6. Transitions in fluorescence lifetime analysis

Fluorescence lifetime is the state of the art analytical tool practiced for identification of transitions around the microenvironment of a fluorophore, and for differentiating the static and dynamic fluorescence quenching [63]. Therefore, FL lifetime analysis further confirms the dominance of static quenching in the HfO2–CAT system (figure 7). CAT (Without HfO2 NPs) illustrates the mean FL lifetime of 4.03 ns from equation (4), while a negligible decrease in FL 4.02 ns was observed with a graded concentration of HfO2 NPs, clearly represents the agitations in the microenvironment [64].

The data obtainable in table 2 demonstrate the insignificant transformations in the FL lifetime of CAT in the presence of HfO2 NPs. Also, the data corroborate the establishment of a non-fluorescent ground state complex between CAT and HfO2 NPs. It is also a deep-rooted reality that the establishment of static ground state complex does not reduce the decay-time of the non-complexed fluorophore since only the life-times of unquenched fluorophores are sensed in the time-resolved spectroscopy [65]. This further corroborates the outcomes of the steady-state FL quenching. Typically, Trp lifetimes are reduced in polar environments [64].
3.3.7. Red-edge excitation energy shifts (REES)

For polar fluorophore with incomplete solvent reduction emission spectra shows redshifts at excitations on a longer wavelength region of the absorption spectrum, this phenomenon is known as REES\[32\]. This phenomenon is a signal for an abruptly dense dynamic microenvironment of Trp residue with reduced access to the solvent and stimulated through electronic coupling amid Trp indole rings and neighboring dipoles\[61, 66\]. Consequently, REES is predominantly advantageous in tracking dynamic shifting around the Trp residues in proteins. Calculating the FL emission and red edge excitation shift (REES) of CAT after incubation with HfO$_2$.

![Figure 6. Fluorescence resonance energy transfer. Spectral overlap of absorbance of HfO$_2$ NPs and Catalase (CAT) fluorescence emission.](image)

![Figure 7. Fluorescence lifetime decays of CAT (\(\lambda_{ex} = 280\) nm, \(\lambda_{em} = 340\) nm) at minimum (20 \(\mu\)M) and (50 \(\mu\)M), (250 \(\mu\)M) concentrations of HfO$_2$ NPs in HBS buffer, pH 7.4. The concentration of CAT was fixed at 2 \(\mu\)M.](image)

| Sample                  | \(\tau_1\) (ns) | \(\tau_2\) (ns) | \(\tau_f\) (ns) | \(\chi^2\) |
|-------------------------|------------------|------------------|-----------------|------------|
| CAT                     | 2.45 (52\%)      | 5.7 (48\%)       | 4.03            | 0.915      |
| CAT + 50 \(\mu\)M HfO$_2$ NPs | 5.25 (36\%)      | 4.47 (44\%)      | 4.67            | 1.35       |
| CAT + 100 \(\mu\)M HfO$_2$ NPs | 2.57 (38\%)      | 6.05 (42\%)      | 4.62            | 1.01       |
| CAT + 150 \(\mu\)M HfO$_2$ NPs | 5.32 (40\%)      | 4.42 (60\%)      | 4.68            | 1.55       |
| CAT + 200 \(\mu\)M HfO$_2$ NPs | 5.4 (51\%)       | 4.44 (49\%)      | 4.65            | 1.50       |
| CAT + 250 \(\mu\)M HfO$_2$ NPs | 5.1 (81\%)       | 4.81 (91\%)      | 4.73            | 1.12       |

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For polar fluorophore with incomplete solvent reduction emission spectra shows redshifts at excitations on a longer wavelength region of the absorption spectrum, this phenomenon is known as REES\[32\]. This phenomenon is a signal for an abruptly dense dynamic microenvironment of Trp residue with reduced access to the solvent and stimulated through electronic coupling amid Trp indole rings and neighboring dipoles\[61, 66\]. Consequently, REES is predominantly advantageous in tracking dynamic shifting around the Trp residues in proteins. Calculating the FL emission and red edge excitation shift (REES) of CAT after incubation with HfO$_2$.
NPs (CAT–HfO2 NPs complex), provide us the information about the microenvironment and mobility of Trp residue.

The Aftermath of REES study for the CAT-HfO2 conjugate (table 3) demonstrates the REES value is 20. This is significantly elevated than the REES value of CAT (without HfO2 NPs), demonstrating HfO2 NPs enforce the unfolding of CAT revealing the fundamental fluorophore by early complex creation by establishing the attractive forces with the hydrophobic domains of CAT limiting free motion of Trp microenvironment [61, 67].

### 3.4. Adsorption of CAT on HfO2 NPs

Furthermore, quantitative analysis was performed to investigate the adsorption of CAT on HfO2 NPs through TGA. Thermograms at 700°C reveal that HfO2 NPs (Supplementary figure 3, blue thermogram) exhibit the lowest weight loss due to solid Hf core, whereas CAT (Supplementary figure 3, black thermogram) showed optimum weight loss due to extreme protein degradation at higher temperatures. The weight loss of all CAT@HfO2 NPs (Supplementary figure 3, red thermogram) was between those of the HfO2 NPs and the CAT sample.

### 3.5. Influence of HfO2 NPs on the functionality of CAT

According to the above experimental results, attractive forces were operational between HfO2-NPs and CAT leads to the configurational alteration in the conformation of CAT. We are familiar that enzyme activity correlates directly to its conformation [38]. Therefore, a sensitive assay is imperative to perform with and without incubating with HfO2 NPs to elaborate on the relationship between configurational changes and activity disparity of CAT. Every subunit possesses a single heme, an integral part of the CAT active center. The effect of HfO2 on CAT activity was presented in figure 8; the catalase activity revealed a decline in CAT activity is associated with the incubation with HfO2 NPs.

As the concentration of HfO2 increased from 0 to 250 mM, the catalytic activity CAT decreased to 44%, 63%, and 69.2% compared to control with incubation times of 15, 30, and 60 min, respectively. The conformational transformation of the CAT complex in combination with the above spectral computation

| System       | $\lambda_{em}$ (nm) | REES | $\Delta \lambda_{em}$ (nm) |
|--------------|---------------------|------|---------------------------|
| CAT          | 340                 | 354  | 14                        |
| CAT + 250 μM HfO2 NPs | 340     | 360  | 20                        |

![Figure 8](image_url) Effect of HfO2 on CAT activity in HBS buffer. CAT (20 μM) were exposed to graded concentrations of HfO2 for 15, 30, or 60 min. Data were pooled from at least three independent experiments and analyzed with one-way ANOVA. Error bars indicate standard deviation.
displayed that activity inhibition of CAT instigated by incubating with HfO2 NPs, and results in structural transformations. However, HfO2 NPs could also influence the activity of CAT directly through binding with the active center of CAT, (figure 8). Similar results could be found in the work of Wei et al which evidenced the graphene oxide inhibited CAT activity through attacking the active center. This hypothesis was validated by confirming the change of the microenvironment of Tyr, Phe which binds near to the intense center and has a close relationship with the activity of CAT [56].

4. Conclusion

The attractive forces amid CAT and HfO2 NPs were explored by a diverse range of spectroscopic tools. We also concluded that the HfO2 NPs attached with CAT through ground-state complex generation directing the dominance of static FL quenching and the bonding course is greatly impulsive with loss in energy. The computation of UV-visible, FTIR, and UV-CD, spectroscopy exemplify obvious transformations in secondary (2°) and tertiary (3°) configurations of CAT upon incubation with HfO2 NPs. Likewise, the SFS spectra at Δ60 nm authenticate the disorder simulated around the Trp residue microenvironment instigated through HfO2 NPs. Furthermore, TGA thermograms again established a profuse coating of CAT on HfO2 NPs surfaces. In brief, results advocated that HfO2 NPs markedly weaken the catalytic activity and ultimately the performance of proteins. Consequently, demanded a profound consideration of NPs’ impressions on biological systems before their extensive utilization in daily consumer goods.

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Competing interest

Authors declare no competing interest.

ORCID iDs

Farooq Ahmad  https://orcid.org/0000-0001-6019-436X
Tahir Muhmood  https://orcid.org/0000-0002-7441-1617

References

[1] Wan Y and Zhou X 2017 Formation mechanism of hafnium oxide nanoparticles by a hydrothermal route RSC Adv. 7 7763–73
[2] Guangxu L et al 2017 Nanoscale metal–organic layers for deeply penetrating x-ray-induced photodynamic therapy Angew. Chem. Int. Ed. 56 12102–6
[3] Wang C et al 2014 Synergistic assembly of heavy metal clusters and luminescent organic bridging ligands in metal–organic frameworks for highly efficient x-ray scintillation JACS 136 6171–4
[4] Villa I et al 2018 Demonstration of cellular imaging by using luminescent and anti-cytotoxic europium-doped hafnia nanocrystals Nanoscale 10 7933–40
[5] Ostadhossein F et al 2018 Dual purpose hafnium oxide nanoparticles offer imaging Streptococcus mutans dental biofilm and fight it in vivo via a drug free approach Biomaterials 181 252–67
[6] Field J A et al 2011 Cytotoxicity and physicochemical properties of hafnium oxide nanoparticles Chemosphere 84 1401–7
[7] García-Saucedo C et al 2011 Low toxicity of HfO2, SiO2, Al2O3, and CeO2 nanoparticles to the yeast, Saccharomyces cerevisiae J. Hazard. Mater. 192 1572–9
[8] Stern S T and McNeil S E 2007 Nanotechnology safety concerns revisited Toxicol. Sci. 101 4–21
[9] Balbus John M et al 2007 Meeting report: Hazard assessment for nanoparticles—report from an interdisciplinary workshop Environ. Health Perspect. 115 1654–9
[10] Lu S et al 2015 Comparison of cellular toxicity caused by ambient ultrafine particles and engineered metal oxide nanoparticles Part. Fibre Toxicol. 12 5
[11] Paul B K et al 2012 A spectroscopic investigation on the interaction of a magnetic ferrofluid with a model plasma protein: effect on the conformation and activity of the protein Phys. Chem. Chem. Phys. 14 15482–93
[12] Xu Z Q et al 2016 Interactions between carbon nanodots with human serum albumin and gamma-globulins: the effects on the transportation function J. Hazard. Mater. 301 242–9
[13] Ahmad F, Wang X and Li W 2019 Toxicoco-metabolomics of engineered nanomaterials: progress and challenges Adv. Funct. Mater. 29 1904268
[14] Selva Sharma A and Ilanchelian M 2015 Comprehensive multispectroscopic analysis on the interaction and corona formation of human serum albumin with gold/silver alloy nanoparticles The Journal of Physical Chemistry B 119 9461–76
[15] Ahmad F et al 2016 Systematic elucidation of interactive unfolding and corona formation of bovine serum albumin with cobalt ferrite nanoparticles RSC Adv. 6 35719–30
[16] Madathiparambil Visalakshan R et al 2020 The influence of nanoparticle shape on protein corona formation Small 16 2000285
[17] Blume J E et al 2020 Rapid, deep and precise profiling of the plasma proteome with multi-nanoparticle protein corona Nat. Commun. 11 3662
[18] Peng X et al 2015 Elucidating the influence of gold nanoparticles on the binding of Salvinianolic acid B and Rosmarinic acid to bovine serum albumin PLoS One 10 e0118274
[19] Sekar G, Mahlerjese A and Chandrasekaran N 2015 Comprehensive spectroscopic studies on the interaction of biomolecules with surfactant detached multi-walled carbon nanotubes Colloids Surf., B 128 315–21
[20] Cano P et al 2015 A quantitative binding study of fibrinogen and human serum albumin to metal oxide nanoparticles by surface plasmon resonance Biosens. Bioelectron. 74 376–83
[21] Ahmad F et al 2016 Probing the interaction induced conformation transitions in acid phosphatase with cobalt ferrite nanoparticles: relation to inhibition and bio-activity of Chlorrella vulgaris acid phosphatase Colloids Surf., B 145 338–46
[22] Ahmad F, Abubshait S A and Abubshait H A 2021 Untargeted metabolomics for Achilles heel of engineered nanomaterials’ risk assessment Chemosphere 262 128058
[23] Glorieux C and Calderon P B 2017 Catalase, a remarkable enzyme: targeting the oldest antioxidant enzyme to molecular docking approach Biol. Chem. 398 1095–108
[24] Mandil R et al 2020 In vitro and in vivo effects of flubendiamide and copper on cyto-genotoxicity, oxidative stress and spleen histology of rats and its modulation by resveratrol, catechin, curcumin and α-tocopherol BMC Pharmacology and Toxicology 21 1–7
[25] Guan J et al 2014 Spectroscopic investigations on the interaction between carbon nanotubes and catalase on molecular level J. Biochem. Mol. Toxicol. 28 211–6
[26] Teng Y, Zhang H and Liu R 2011 Molecular interaction between 4-aminoantipyrine and catalase reveals a potentially toxic mechanism of the drug Mol. Biosyst. 7 3157–63
[27] Masduz Z et al 2017 Elucidating the interaction of clofazimine with bovine liver catalase; a comprehensive spectroscopic and molecular docking approach J. Mol. Recognit. 30 e2619
[28] Yang B et al 2013 Binding of chrysoideine to catalase: spectroscopy, isothermal titration calorimetry and molecular docking studies J. Photochem. Photobiol., B 128 35–42
[29] Li N, Xia T and Nel A E 2008 The role of oxidative stress in ambient particle matter–induced lung diseases and its implications in the toxicity of engineered nanomaterials Free Radical Biol. Med. 44 1689–99
[30] Klaper R et al 2009 Toxicity biomarker expression in daphnids exposed to manufactured nanoparticles: changes in toxicity with functionalization Environ. Pollut. 157 1152–6
[31] Prabhaskar PV et al 2011 Oxidative stress induced by aluminum oxide nanomaterials after acute oral treatment in Wistar rats J. Appl. Toxicol. 32 436–45
[32] Lakowicz JR 2010 Principles of Fluorescence Spectroscopy. (Berlin: Springer) 3rd Edition
[33] Naik GH et al 2003 Comparative antioxidant activity of individual herbal components used in Ayurvedic medicine Phytochemistry 63 97–104
[34] Jiang Y et al 2009 Stimulatory effect of components of rose flowers on catalytic activity and mRNA expression of superoxide dismutase and catalase in erythrocytes Environ. Toxicol. Pharmacol. 27 396–401
[35] Li C et al 2017 The antiproliferative activity of di-2-pyridylketone dithiocarbamate is partly attributed to catalase inhibition: detailing the interaction by spectroscopic methods Mol. Biosyst. 13 1817–26
[36] Santalucia J 1998 A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics Proc. Natl Acad. Sci. 95 1460–5
[37] Zhang Y-Z et al 2008 Studies of the interaction between Sudan I and bovine serum albumin by spectroscopic methods J. Mol. Struct. 888 152–9
[38] Zhang K, Wu Q and Liu R 2017 Characterizing the binding interaction between ultrafine carbon black (UFCB) and catalase: electron microscopy and spectroscopic analysis RSC Adv. 7 42549–58
[39] Vivian J T and Callas P R 2001 Mechanisms of Tryptophan Fluorescence shifts in proteins Biophys. J. 80 2093–109
[40] Delghani Sani F et al 2018 Changes in binding affinity between ofloxacin and calf thymus DNA in the presence of histone H1: spectroscopic and molecular modeling investigations J. Lumin. 203 599–608
[41] Chamani J 2010 Energetic domains analysis of bovine α-lactalbumin upon interaction with copper and dodecyl trimethylammonium bromide J. Mol. Struct. 979 227–34
[42] Ross P D and Subramanian S 1981 Thermodynamics of protein association reactions: forces contributing to stability Biochemistry 20 3096–102
[43] Shen X-C et al 2007 Spectroscopic studies on the interaction between human hemoglobin and CdS quantum dots J. Colloid Interface Sci. 311 400–6
[44] Purdie N 1996 Circular Dichroism and the Conformational Analysis of Biomolecules ed G D Fasman (New York: (Brandeis University), Plenum Press) x + 738 pp, $125.00. ISBN 0-306-45142-5 Purdie N 1996 Journal of the American Chemical Society 118 12871
[45] Johnson W C 1988 J. Secondary structure of proteins through circular dichroism spectroscopy Annual Review of Biophysics and Biophysical Chemistry 17 145–66
[46] Corrêa D H and Ramos C H 2009 The use of circular dichroism spectroscopy to study protein folding, form and function Afr. J. Biochem. Res. 3 164–73
[47] Holtzer M E and Holtzer A 1992 α-helix to random coil transitions: determination of peptide concentration from the CD at the isodichroic point Biopolymers: Original Research on Biomolecules 32 1675–73
[48] Ribeiro E A Jr and Ramos C H 2004 Origin of the anomalous circular dichroism spectra of many apomyoglobin mutants Anal. Biochem. 329 300–6
[49] Li D et al 2011 Characterization of the baicalein–bovine serum albumin complex without or with Cu2+ or Fe3+ by spectroscopic approaches Eur. J. Med. Chem. 46 588–99
[50] Johnston MJ, Nemr K and Hefford MA 2010 Influence of bovine serum albumin on the secondary structure of interferon alpha 2b as determined by far UV circular dichroism spectropolarimetry Biologicals 38 514–20
[51] Yue H-L et al 2014 Development of morin-conjugated Au nanoparticles: exploring the interaction efficiency with BSA using spectroscopic methods Spectrochim. Acta, Part A 130 402–10
[52] Tang J, Luan F and Chen X 2006 Binding analysis of glycyrrhetinic acid to human serum albumin: fluorescence spectroscopy, FTIR, and molecular modeling *Bioorganic & Medicinal Chemistry* **14** 3210–7

[53] Patra D, Barakat C and Tafseth R M 2012 Study on effect of lipophilic curcumin on sub-domain IIA site of human serum albumin during unfolded and refolded states: a synchronous fluorescence spectroscopic study *Colloids Surf., B* **94** 354–61

[54] Li G et al 2015 Investigation on the effect of fluorescence quenching of bovine serum albumin by cefoxitin sodium using fluorescence spectroscopy and synchronous fluorescence spectroscopy *Luminescence* **31** 1054–62

[55] Song M et al 2011 Interaction of human serum album and C60 aggregates in solution *Int. J. Mol. Sci.* **12** 4964

[56] Wei X-L and Ge Z-Q 2013 Effect of graphene oxide on conformation and activity of catalase *Carbon* **60** 401–9

[57] Wang Y and Zhang H 2014 Comprehensive studies on the nature of interaction between catalase and SiO2 nanoparticle *Mater. Res. Bull.* **60** 51–6

[58] Floris R et al 1995 Heme-protein interaction in myeloperoxidase: modification of spectroscopic properties and catalytic activity by single residue mutation *JACS* **117** 3907–12

[59] Tattini V et al 2005 Effect of lyophilization on the structure and phase changes of PEGylated-bovine serum albumin *Int. J. Pharm.* **304** 124–34

[60] Meng G et al 2005 Study of Protein—Lipid Interactions at the Bovine Serum Albumin/Oil Interface by Raman Microspectroscopy *J. Agric. Food Chem.* **53** 645–52

[61] Sarkar D 2013 Probing the interaction of a globular protein with a small fluorescent probe in the presence of silver nanoparticles: spectroscopic characterization of its domain specific association and dissociation *RSC Adv.* **3** 24389

[62] Shahabadi N, Maghsudi M and Rouhani S 2012 Study on the interaction of food colourant quinoline yellow with bovine serum albumin by spectroscopic techniques *Food Chem.* **135** 1836–41

[63] Engelborghs Y 2001 The analysis of time resolved protein fluorescence in multi-tryptophan proteins *Spectrochim. Acta, Part A* **57** 2255–70

[64] Chakraborti S, Sarwar S and Chakrabarti P 2013 The effect of the binding of ZnO nanoparticle on the structure and stability of α-lactalbumin: a comparative study *The Journal of Physical Chemistry B* **117** 13397–408

[65] Zheng C et al 2014 Study on the interaction between histidine-capped Au nanoclusters and bovine serum albumin with spectroscopic techniques *Spectrochim. Acta, Part A* **118** 897–902

[66] Sharma A and Ilanchelian M 2015 Comprehensive multispectroscopic analysis on the interaction and corona formation of human serum albumin with gold/silver alloy nanoparticles *The Journal of Physical Chemistry B* **119** 9461–76

[67] Lakowicz J R and Weber G 1973 Quenching of fluorescence by oxygen. A probe for structural fluctuations in macromolecules *Biochemistry* **12** 4161–4170