

The interaction of silica nanoparticles with catalase and human mesenchymal stem cells: biophysical, theoretical and cellular studies

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Aim: Nanoparticles (NPs) have been receiving potential interests in protein delivery and cell therapy. As a matter of fact, NPs may be used as great candidates in promoting cell therapy by catalase (CAT) delivery into high oxidative stress tissues. However, for using NPs like SiO₂ as carriers, the interaction of NPs with proteins and mesenchymal stem cells (MSCs) should be explored in advance.

Methods: In the present study, the interaction of SiO₂ NPs with CAT and human MSCs (hMSCs) was explored by various spectroscopic methods (fluorescence, circular dichroism (CD), UV-visible), molecular docking and dynamics studies, and cellular (MTT, cellular morphology, cellular uptake, lactate dehydrogenase, ROS, caspase-3, flow cytometry) assays.

Results: Fluorescence study displayed that both dynamic and static quenching mechanisms and hydrophobic interactions are involved in the spontaneous interaction of SiO₂ NPs with CAT. CD spectra indicated that native structure of CAT remains stable after interaction with SiO₂ NPs. UV-visible study also revealed that the kinetic parameters of CAT such as *K_m*, *V_{max}*, *K_{cat}*, and enzyme efficiency were not changed after the addition of SiO₂ NPs. Molecular docking and dynamics studies showed that Si and SiO₂ clusters interact with hydrophobic residues of CAT and SiO₂ cluster causes minor changes in the CAT structure at a total simulation time of 200 ps. Cellular assays depicted that SiO₂ NPs induce significant cell mortality, change in cellular morphology, cellular internalization, ROS elevation, and apoptosis in hMSCs at higher concentration than 100 µg/mL (170 µM).

Conclusion: The current results suggest that low concentrations of SiO₂ NPs induce no substantial change or mortality against CAT and hMSCs, and potentially useful carriers in CAT delivery to hMSC.

Keywords: silica nanoparticles, catalase, mesenchymal stem cells, interaction, spectroscopy, docking, molecular dynamics, cellular assays

Introduction

As nanotechnology is taking an increasing attention in fabrication of nanomaterial-based compounds for protein¹ and cell delivery agents,² revealing the impact of the physico-chemical properties of nanoparticles (NPs) such as their chemical composition on the protein structures and cells viability has become critically necessary.³ Based on the unique properties of silica (SiO₂) NPs, they offer potential stem cell delivery^{4,5} and protein delivery^{6,7} applications. Hence, synthesis and development of novel SiO₂-based multipotent carriers might be productive in the treatment of a wide range of diseases in vivo. However, before clinical applications of SiO₂ NPs, their adverse effects should

be examined against biological systems, such as proteins and cells *in vitro*. Actually, it may be indicated that NPs may induce some irreversible effects on the protein structure and cell viability.⁸ For example, it has been well documented that the interaction of iron NPs with BSA is spontaneous, causing some changes in the secondary structure of the protein.⁹ Data obtained from interaction of human hemoglobin with nanodiamond indicated that the hydrophobic interactions between nanodiamond and hemoglobin result in the significant secondary structural alteration of hemoglobin.¹⁰ Moreover, it was specified that bare or functionalized titanium dioxide (TiO₂) NPs may induce different effects on the protein structure.¹¹ It was also reported that composite NP like TiO₂/tungsten trioxide/graphene oxide can induce activity changes of catalase (CAT) and superoxide dismutase.¹² Nonetheless, it was revealed that the electrostatic interaction between zinc oxide NPs and BSA results in the slight quaternary structural changes of the protein and the secondary structure of BSA retained its identity.¹³ Therefore, based on the type of NPs or the physicochemical characteristics of NPs, their kind of interaction and corresponding induced structural changes of the protein may provide different outcomes. Regarding cellular effects of NPs, some conflicting results have been also depicted. For example, the cytotoxicity effects of silver NPs,¹⁴ zinc oxide doped TiO₂ nanocrystals¹⁵ and bismuth oxide NPs¹⁶ against human lung epithelial cells, human lung epithelial cells (A549) and human breast cancer (MCF-7) cells have been reported, respectively. But, it has been determined that some nanomaterials like iron NPs can serve as efficient and safe agents for internalization in the cells.¹⁷ In this regard, conflicting data have encouraged scientists to study the interaction of NPs with proteins and cells to explore the scenario behind the toxicity of NPs against biological systems. Meanwhile, to study the biological effects of SiO₂ NPs on protein structure, human erythrocyte CAT was chosen as the target macromolecule. CAT has a tetrameric structure with dominant α -helix conformation and it protects the cells against hydrogen peroxide.¹⁸ As a consequence, any conformational changes of CAT result in its deactivation and elevation of oxidative stress accompanied by cell death. In this study, CAT interaction with SiO₂ NPs was explored by spectroscopic and theoretical approaches.

Human mesenchymal stem cells (hMSCs) were also used to detect the cytotoxicity of SiO₂ NPs against normal cells. MSCs-based therapies have shown potential applications in preclinical and clinical cases for treating a wide range of disorders.^{19,20} NPs can be used to increase MSCs therapeutic efficacy for several disorders.^{21,22} However, before the

application of NPs as manipulating agents for MSCs differentiation,²¹ cytotoxicity of NPs like SiO₂ NPs as a function of viability and apoptosis should be explored to determine their biocompatibility. Also, it has been well documented that MSCs can be used as a potential cellular model for examining the cytotoxicity of engineered NPs.²³ Consequently, hMSCs were also used to detect the cytotoxicity of SiO₂ NPs.

Materials and methods

Material

Alpha-minimum essential medium (α -MEM), FBS, L-glutamine, streptomycin and penicillin were purchased from Sigma-Aldrich Co. All other materials were of analytical grade.

Methods

Fluorescence study

CAT sample with a concentration of 2 μ M was titrated with different concentrations of SiO₂ NPs (1, 5, 10, 15 and 20 μ M) at 298, 310 and 315 K. Samples were then excited at 280 nm and emission was read at 340 nm using a Cary Eclipse VARIAN fluorescence spectrophotometer. The excitation and emission slits were fixed at 5 and 10 nm, respectively. The phosphate buffer (pH 7.4, 20 mM) was used throughout the study. The fluorescence signal from the protein in the presence of NPs was subtracted from the fluorescence intensity of NPs and corrected for inner filter effect. In all experiments, CAT fluorescence intensity was measured in triplicate and the mean of the three data is reported.

Circular dichroism (CD) study

The CD signal of CAT with a concentration of 5 μ M in the absence and presence of SiO₂ NPs (5, 10, and 20 μ M) was recorded by employing an AVIV 215 spectropolarimeter (Aviv Associates, Lakewood, NJ, USA). Ellipticity change (θ) of the protein in the wavelength range 190–260 nm was reported as the NPs concentration was increased from 5 to 20 μ M. The experiment was performed at room temperature and the NPs and proteins were dissolved in the phosphate buffer solution (pH 7.4, 20 mM). The CD spectrum of CAT in the presence of NPs was subtracted from the spectra of NPs and buffer.

CAT assay

CAT activity was assayed based on the rate of H₂O₂ decomposition as determined by the reduction of absorbance at 240 nm. The reaction mixture contained 970 μ L phosphate

buffer (pH 7.4, 50 mM), 10 μ L of 1–20 mM H₂O₂ (3%), 10 μ L of 0.025 mg/mL CAT and 10 μ L of 10–50 μ M of SiO₂ NPs. The experiment was done at room temperature and the data was reported as the average of three independent experiments.

Molecular docking and dynamics

(SiO₂)₇₂ cluster was designed and used as a model of SiO₂ NPs. This model was constructed by Cartesian coordinates of atoms obtained from Munde et al²⁴ and Sabziparvar et al²⁵ studies. In order to compare the effect of silica and silicon clusters, a Si₂₀ cluster as a model of silicon NPs was designed using the Gaussian 98W suite of program.^{25,26} The B3LYP functional with the 6-31G(d) basis set was used to study the interaction of clusters with CAT. HEX 6.3 software as a molecular docking program was used to calculate and display attainable docking modes of CAT and NP clusters.²⁷ A spherical cluster of SiO₂ NPs with a diameter of 30 Å was constructed by repeating its unit cell for the simulation study. The molecular dynamic simulations were performed using Forcite code and universal force field.²⁸

Cell culture

hMSCs derived from bone marrow were purchased from Royan Institute of Tehran, Iran. The extraction and purification of these cells were performed under Ethical Procedures approved by Ethical Committee of Royan Institute. The characterization of extracted cells based on surface marker expression was previously reported.²⁹ Cells were cultured in α -MEM medium containing FBS (10%, v/v), L-glutamine (0.3 mg/mL), streptomycin (100 μ g/mL) and penicillin (100 U/mL) and kept at 37°C in a 5% CO₂. When hMSCs reached sub-confluence after five passages and after separation of non-adherent cells, varying concentrations of SiO₂ NPs (1, 10, 50, 100 and 200 μ g/mL) were added to the cell culture medium for 24 hrs.

Cell viability analysis

The viability percent of hMSCs in the presence of varying concentrations of SiO₂ NPs (1, 10, 50, 100 and 200 μ g/mL) was analyzed using the MTT assay. Briefly, after treatment of cells for 24 hrs, 10 μ L MTT with a concentration of 5 mg/mL was added to each well for 2 hrs. Then, the supernatant was gently removed and the formazone crystals were dissolved in 100 μ L DMSO. Finally, the absorbance intensity was measured at 570 nm using an ELISA reader (Expert 96, Asys Hitch, Ec

Austria). The cell viability percent was presented against the control samples.

Cellular uptake of SiO₂ NPs

The uptake of SiO₂ NPs was explored by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA) based on our previous paper.³⁰

Cell morphology assay

The cell morphology incubated with increasing concentrations of SiO₂ NPs (10, 50, 100 and 200 μ g/mL) for 24 hrs was observed employing invert microscope (Zeiss, Germany).

Lactate dehydrogenase (LDH) assay

The cytotoxicity of SiO₂ NPs against hMSCs was determined using a LDH assay kit (Colorimetric, ab102526) based on the manufacturer's protocol. Briefly, cells were cultured, treated with increasing concentrations of SiO₂ NPs (1, 10, 50, 100 and 200 μ g/mL) for 34 hrs, collected and incubated with reaction mixture. The output was read immediately at OD 450 nm to measure the LDH activity as a percentage of the control.

Intracellular determination of ROS

The generation of reactive oxygen radicals was analyzed using 2',7'-dichlorofluorescein diacetate [(DCFDA)/H₂DCFDA – cellular ROS assay kit (ab113851)]. The DCF fluorescence intensity is extensively correlated with the generation of intracellular reactive oxygen species. Briefly, the cells were treated with different concentrations of SiO₂ NPs (1, 10, 50, 100 and 200 μ g/mL), collected in tubes, stained with DCFDA and incubated at 37°C for 30 min. Afterward, the fluorescence intensity (λ_{ex} =495 nm, λ_{em} =529 nm) of solution was analyzed using FACScan cytometer (FACSCalibur; Becton-Dickinson, San Jose, CA, USA).

Caspase-3 activity assay

The assay of caspase-3 enzyme was carried out in cells treated with various concentrations of SiO₂ NPs (1, 10, 50, 100 and 200 μ g/mL) using a commercially available kit (Caspase-3 assay kit [Colorimetric, ab39401]). Briefly, the control and the SiO₂ NPs-treated cells were harvested after treatment for 24 hrs and re-suspended in 50 μ L of chilled lysis buffer for 10 mins followed by centrifugation at 10,000 g for 5 mins. Protein concentration was then determined by Bradford assay and BSA was used as a standard. Supernatants were then incubated with caspase-3 substrate (N-acetyl-DEVD-p-nitroaniline) at 37°C for 2 hrs and absorbance intensity was analyzed at 405 nm, using a microplate reader (Expert 96, Asys Hitch, Ec Austria).

Flow cytometry analysis

Apoptosis quantification was done based on the manufacturer protocol of Annexin V-FITC Apoptosis Staining/Detection Kit (ab14085). Briefly, after treatment with 200 $\mu\text{g}/\text{mL}$ of SiO_2 NPs for 24 hrs, hMSC (1×10^6) cells were collected by centrifugation, re-suspended, combined with 500 μL of 1X Annexin V binding buffer, mixed with 5 μL of Annexin V-FITC and 5 μL propidium iodide (PI), and incubated at room temperature for 5 mins in the dark. Finally, the cells were analyzed by FACScan cytometer (FACSCalibur; Becton-Dickinson, San Jose, CA, USA).

Statistical analysis

Statistical analyses were performed using the SPSS software. Data were expressed as mean \pm SD of three independent experiments. One-way ANOVA and Student's *t*-test were done to determine statistical significance. Differences between groups were considered to be significant at $P < 0.05$.

Results

Synthesis and characterization of SiO_2 NPs

SiO_2 NPs with a size of about 20 nm were fabricated in our previous study by sol-gel method and were characterized by different approaches.³¹

Quenching mechanism

CAT sample was titrated by different doses of SiO_2 NPs at 298, 310 and 315 K and plots of fluorescence quenching are displayed in Figure 1. It can be deduced that quenching efficacy is varied by temperature. To quantify the impact of temperature and identify the quenching mechanism, the Stern-Volmer Equation (1) was plotted.³²

$$F_o/F = K_{SV}[\text{SiO}_2 \text{ NP}] + 1 = k_q\tau_o[\text{SiO}_2 \text{ NP}] + 1 \quad (1)$$

where F_o , F , K_{SV} , k_q and τ_o represent the fluorescence in the absence of ligand, fluorescence in the presence of ligand, Stern-Volmer constant, CAT quenching rate and lifetime of CAT fluorescence without ligand, respectively.

It was seen that the K_{SV} values were $4.1 \pm 0.76 \times 10^4$, $4.3 \pm 0.064 \times 10^4$ and $5.0 \pm 0.61 \times 10^4 \text{ M}^{-1}$ at 298, 310 and 315 K, respectively (Figure 2, Table 1). Thus, as the K_{SV} value increases by the elevation of temperature, a dynamic quenching system may be involved in the quenching mechanism of CAT by SiO_2 NPs.³² Nevertheless, the k_q value was in the order of 10^{12} , which is significantly greater than the dynamic quenching limit (10^{10}), indicating a static quenching

mechanism of CAT by SiO_2 NPs (Table 1).³ Hence, it may be suggested that both dynamic and static quenching mechanisms are involved in the fluorescence quenching of CAT by SiO_2 NPs.^{32,33}

Binding parameters

Figure 3 presents the variation of $\log(F_o - F)/F$ against $\log([\text{SiO}_2 \text{ NPs}])$ for CAT at 298, 310 and 315 K, calculated by using Equation (2).³³

$$\text{Log}[(F_o - F)/F] = \log K_b + n \log[\text{SiO}_2 \text{ NP}] \quad (2)$$

It yields a linear plot with binding constant ($\log K_b$) of 2.28 ± 0.32 , 2.54 ± 0.27 and $2.98 \pm 0.41 \text{ M}^{-1}$ at 298, 310 and 315 K, respectively (Table 2). It was also revealed that there is almost a half binding site (n) for SiO_2 NPs in CAT. The half binding site suggests that two CAT molecules bind to one SiO_2 NP.

Thermodynamic parameters

Figure 4 exhibits the variation of $\text{Ln} K_b$ against $1/T$ at 298, 310 and 315 K, estimated by using van't Hoff Equation (3).³⁴

$$\text{Ln} K_b = - (\Delta H/RT) + (\Delta S/R) \quad (3)$$

It gives a slope which demonstrates the enthalpy change (ΔH) with a value of 69.67 kJ/mol (Table 3). The entropy change ($T\Delta S$) was estimated from the intercept to be 81.62 ± 9.11 , 84.90 ± 10.28 and $86.27 \pm 13.35 \text{ kJ/mol}$ at 298, 310 and 315 K, respectively. The positive signs of ΔH and ΔS demonstrate that hydrophobic forces are the main contributing interactions in the formation of the SiO_2 NP/CAT complex.³⁵

Gibbs-Helmholtz Equation (4) was used to calculate the Gibbs free energy (ΔG).³⁵

$$\Delta G = \Delta H - T\Delta S \quad (4)$$

The ΔG values were shown to be -11.95 ± 1.81 , -15.23 ± 2.29 and $-16.60 \pm 2.41 \text{ kJ/mol}$ (Table 3) at 298, 310 and 315 K, respectively. The negative sign of ΔG points to a spontaneous interaction between SiO_2 NPs and CAT.

CD study

CD is a potential technique usually employed for the detection of changes in secondary and tertiary conformation of protein.^{35,36} Backbone configuration of the protein is explored by the far-UV CD ellipticity changes within the range of 190–260 nm. In this range of wavelength, the secondary structure of the protein represents a characteristic magnitude which can be denatured or

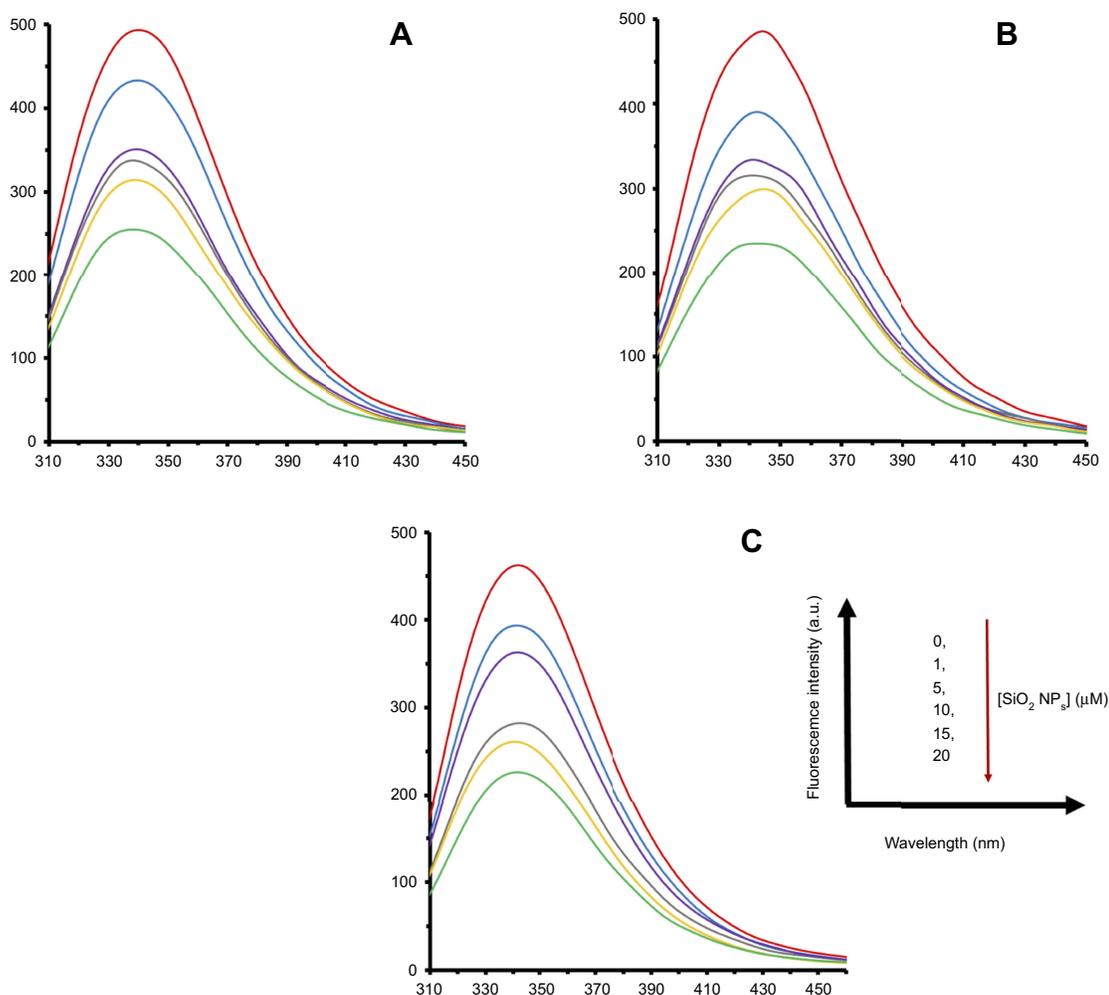


Figure 1 Fluorescence quenching of CAT in the presence of different concentrations of SiO₂ NPs: 0 (red), 1 (blue), 5 (purple), 10 (grey), 15 (yellow) and 20 μM (green) at 298 (A), 310 (B) and 315 K (C).

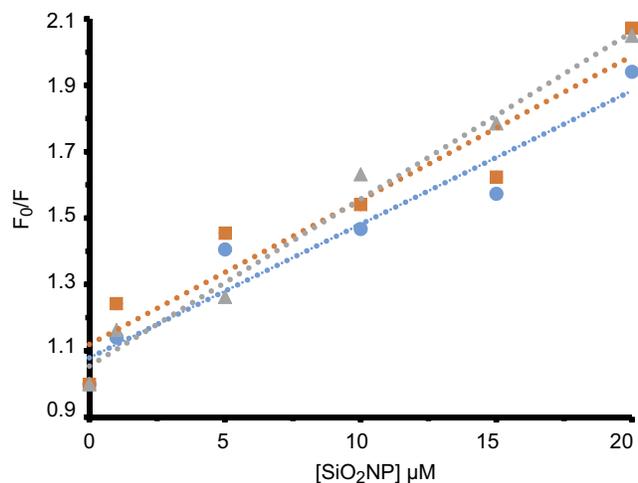


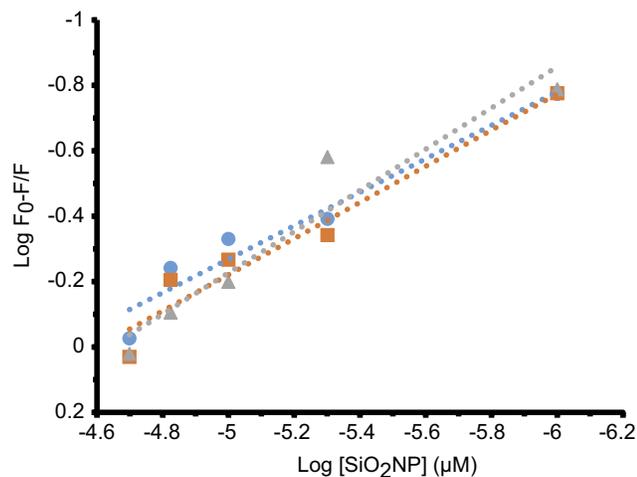
Figure 2 Stern–Volmer plots of CAT in the presence of different concentrations of SiO₂ NPs at 298 (blue), 310 (orange) and 315 K (grey).

destabilized by triggering the structural alterations during the binding of NPs to the protein.³⁵ In order to

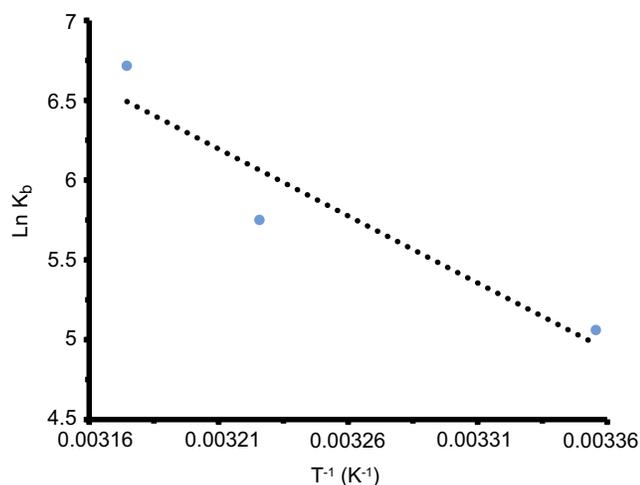
explore the change in the secondary structure of CAT in the presence of varying concentrations of SiO₂ NPs (5, 10 and 20 μM), far-UV CD spectra were measured (Figure 5). The CD spectrum of CAT demonstrates two characteristic negative peaks at 208 and 222 nm, indicating the alpha-helical configuration of CAT. Moreover, the CD spectra of CAT in the presence of varying concentrations of SiO₂ NPs illustrate similar patterns, revealing the predominance of the α-helical structure even in the presence of high dose of SiO₂ NPs. The α-helix content was not altered almost in the presence of SiO₂ NPs, which depicts that the interaction between CAT and SiO₂ NPs does not result in any significant change in the secondary structure of CAT. As deduced from Figure 5, the minimum positions and the ellipticity values of the CD spectra are not changed even after enhancing the molar ratio of NP, evincing that the native structure of CAT remains stable after the interaction.³⁵

Table 1 The K_{SV} and kq values for the SiO_2 NPs/CAT complex at three different temperatures

T	$K_{SV} (\text{M}^{-1})$	$kq (\text{M}^{-1} \text{s}^{-1})$	R^2
298	$(4.1 \pm 0.76) \times 10^4$	$(4.1 \pm 0.76) \times 10^{12}$	0.92
310	$(4.3 \pm 0.64) \times 10^4$	$(4.3 \pm 0.64) \times 10^{12}$	0.91
315	$(5.0 \pm 0.61) \times 10^4$	$(5.0 \pm 0.61) \times 10^{12}$	0.95

**Figure 3** Modified Hill plot of CAT in the presence of different concentrations of SiO_2 NPs at 298 (blue), 310 (orange) and 315 K (grey).**Table 2** The n and $\log K_b$ values for the SiO_2 NPs/CAT complex at different temperatures

T	n	$\log K_b (\text{M}^{-1})$	R^2
298	0.51 ± 0.07	2.28 ± 0.32	0.95
310	0.55 ± 0.08	2.54 ± 0.27	0.95
315	0.63 ± 0.08	2.92 ± 0.41	0.92

**Figure 4** van't Hoff plot of CAT in the presence of SiO_2 NPs at 298, 310 and 315 K.

Activity assay

The influence of varying the concentration of SiO_2 NPs (10–50 μM) on the CAT activity was explored. Table 4 summarizes the values of K_m , V_{max} , K_{cat} and the efficiency of CAT in the presence of different concentrations of SiO_2 NPs. It can be observed that SiO_2 NPs have not induced any significant effect on the CAT activity even at high concentrations. By increasing the concentration of SiO_2 NPs, the kinetic parameters and efficiency of CAT were almost consistent. In fact, the efficacy of the enzyme was 7.1×10^7 and $6.5 \times 10^7 \text{ min}^{-1} \text{ mM}^{-1}$ in the absence and presence of 50 μM SiO_2 NPs, respectively. This data manifests that the CAT efficiency dropped to only 8.5% relative to the native enzyme when the SiO_2 NPs concentration increased to 50 μM , indicating that SiO_2 NPs tend to keep the CAT protein in its native state with no significant denaturation.

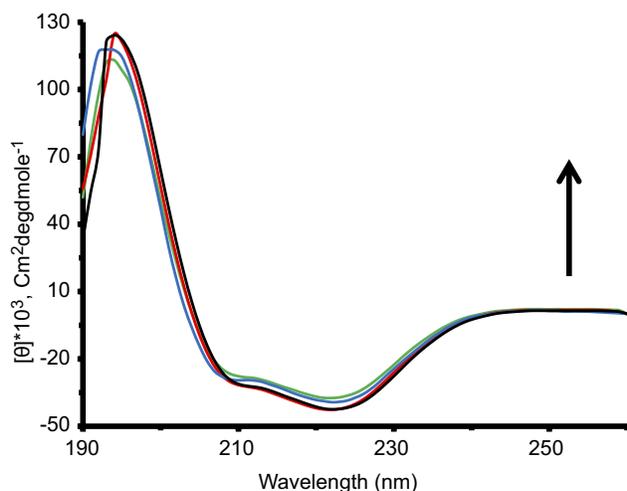
Molecular docking

At this stage, understanding the exact binding site of CAT is of crucial importance in order to understand the protein-NPs interaction. Administered or injected NPs may induce an affinity for the binding to the protein, formation of protein corona and results in the reduction of free fraction of the NPs. This binding affinity may play a pivotal role in the potential clinical consequence of NPs.

Molecular docking methods can predict the interaction between the protein and the NPs which have low or no similarity with real ligands. Accordingly, docking study can be used as a potential tool to define the binding affinity and the binding site of the protein that hosts the NPs. In the current study, the X-ray crystallographic structure of CAT was obtained from the protein data bank (IDGF) and molecular docking was carried out with NPs cluster as a ligand. The docked residues were visualized by using CHIMERA (www.cgl.ucsf.edu/chimera) and PyMOL (<http://pymol.sourceforge.net/>) graphical tools. The docked $(\text{SiO}_2)_{72}$ /CAT and Si_{20} /CAT systems are exhibited in Figure 6. The interacting residues of CAT with $(\text{SiO}_2)_{72}$ and Si_{20} clusters with a cutoff distance of 4 Å are shown in Figures 7 and 8, respectively. The nearest interacting residues for $(\text{SiO}_2)_{72}$ /CAT are Met-394.B, Met-394.A, Pro-374.A, Tyr-379.A, Gln-395.B, Asp-396.B, Gly-400.B, Gly-399.B, Gln-395.B and Val-323.A. For Si_{20} /CAT, the residues are Tyr-379.C, Pro-378.C, Cys-377.C, Ala-381.C, Arg-382.C, Val-383.C, Gln-395.C and Asp-396.C. This data suggests that hydrophobic residues are dominant amino acids in the binding pocket of CAT during the interaction with the NPs cluster. The calculated

Table 3 The thermodynamic parameters of SiO₂ NPs/CAT complex at three different temperatures

T	ΔH (kJ/mol)	$T\Delta S$ (kJ/mol)	ΔG (kJ/mol)
298	69.67±8.34	81.62±9.11	-11.95±1.81
310	69.67±8.34	84.90±10.28	-15.23±2.29
315	69.67±8.34	86.27±13.35	-16.60±2.41

**Figure 5** CD spectra of CAT in the presence of different concentrations of SiO₂ NPs: 0 (black), 5 (red), 10 (blue) and 20 μM (green), at room temperature.**Table 4** The kinetic parameters of CAT in the presence of varying concentrations of SiO₂ NPs

[SiO ₂ NP] (μM)	K _m (mM)	V _{max} (mM/min)	K _{cat} (min ⁻¹)	Efficiency (min ⁻¹ mM ⁻¹)
0	3.9±0.21	2.8±0.11×10 ⁻¹	2.8×10 ⁸	7.1×10 ⁷
10	3.9±0.19	2.8±0.25×10 ⁻¹	2.8×10 ⁸	7.1×10 ⁷
20	3.9±0.33	2.7±0.17×10 ⁻¹	2.7×10 ⁸	6.9×10 ⁷
50	4.0±0.39	2.6±0.28×10 ⁻¹	2.6×10 ⁸	6.5×10 ⁷

binding energies were found to be -519.95 and -248.30 E-value for (SiO₂)₇₂ and Si₂₀ clusters, respectively. As a result, it can be suggested that CAT displays a higher affinity toward (SiO₂)₇₂ cluster than Si₂₀ cluster.

Molecular dynamics simulation

Molecular dynamics study can potentially complement the experimental data for the protein/NPs systems. The models of NPs and protein were covered by 500 water molecules and the annealing process was used to set the equilibrium temperatures at 298 K. The constant-energy, constant-volume ensemble with a time step of 1 fs, and a total simulation time of 200 ps were fixed in the simulations. **Figure 9**

delineates the complex in the beginning of the simulation and after 200 ps. As can be seen, the SiO₂ cluster causes partial unfolding of the CAT structure in the binding site over time. In fact, the α -helical structure of the protein remains almost intact after the interaction with SiO₂ NPs. These results are in good agreement with CD spectroscopic results.

MTT assay

hMSCs cells were exposed to increasing concentrations of SiO₂ NPs (1, 10, 50, 100 and 200 μg/mL) as detected by MTT assays (**Figure 10**). MTT data indicated a negligible cytotoxicity of SiO₂ NPs against hMSCs up to 100 μg/mL. However, increasing the concentration of SiO₂ NPs to 200 μg/mL causes a significant reduction (63.94±4.72) in the percent of viable cells after 24 hrs compared to the control sample (**P*<0.05) (**Figure 10**). The MTT assay demonstrates a dose-dependent cytotoxicity from which reliable data about the percentage of viable cells could be determined.

Cellular internalization of SiO₂ NPs

The cytoplasmic internalization of SiO₂ NPs by hMSCs was evaluated by flow cytometry analysis. A significant reduction in the fluorescent intensity of forward scatter (FSC) versus side scatter (SSC) subset (**Figure 11A and B**) was detected after hMSCs cells were incubated with 200 μg/mL of SiO₂ NPs for 24 hrs. This is well established to be due to light reflection that derived from cellular NP internalization.³⁶ Indeed, the fold enhancement in SSC of hMSCs and fold reduction in FSC of hMSCs exposed to SiO₂ NPs for 24 hrs reveal the cellular internalization of NPs.

Morphology of cells

We also studied the morphological changes of hMSCs induced by different concentrations of SiO₂ (0 [**Figure 12A**], 10 [**Figure 12B**], 50 [**Figure 12C**], 100 [**Figure 12D**] and 200 μg/mL [**Figure 12E**]). It was shown that morphological changes activated by increasing concentrations of SiO₂ NP (1–100 μg/ml) were not obvious in hMSCs (**Figure 12A–D**). However, hMSCs have experienced a shrinkage in cytoplasm and roundness of the cells following exposure to 200 μg/mL of SiO₂ NP (**Figure 12E**). This data suggests the occurrence of hMSCs morphological changes after exposure to high concentration of SiO₂ NPs.

LDH assay

After 24 hrs incubation of hMSCs with SiO₂ NPs, LDH assay demonstrated a very low level of toxicity for all

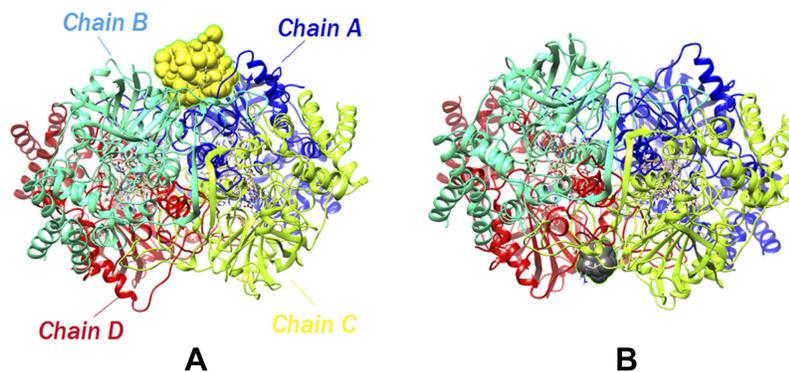


Figure 6 The docked $(\text{SiO}_2)_{72}/\text{CAT}$ (A) and $\text{Si}_{20}/\text{CAT}$ (B) systems.

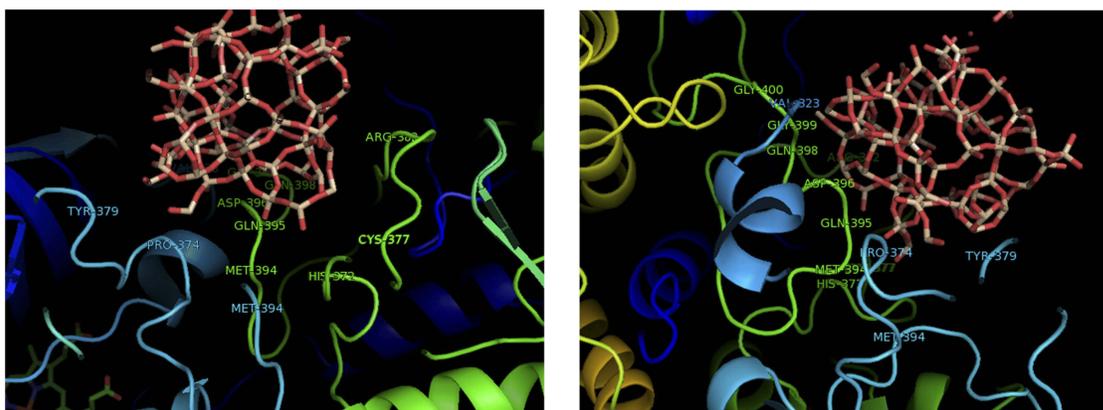


Figure 7 The interacting residues of CAT with $(\text{SiO}_2)_{72}$ cluster from two different view angles.

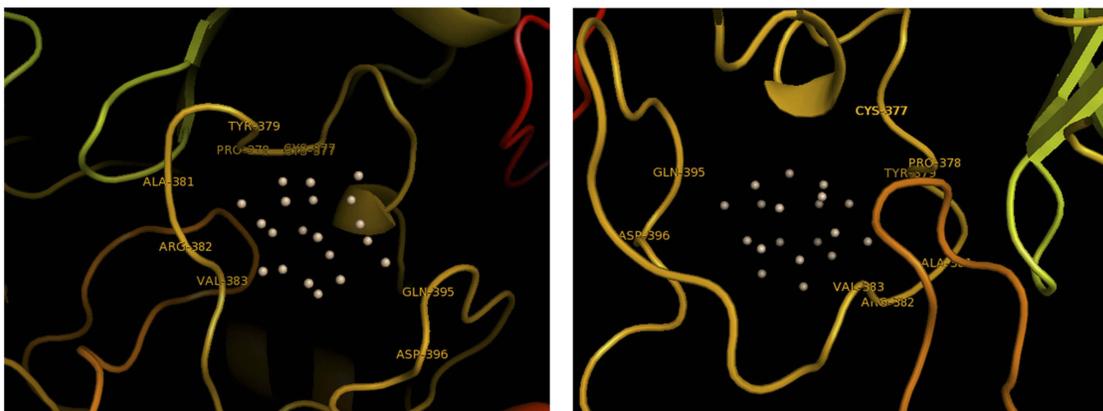


Figure 8 The interacting residues of CAT with Si_{20} cluster from two different view angles.

SiO_2 NPs concentrations with membrane damage similar to the control samples (Figure 13). Indeed, LDH assay did not indicate a dose-dependent manner. The discrepancy between the MTT and the LDH assay data raised the probability that SiO_2 NPs-induced cytotoxicity may occur through apoptosis rather than necrosis.

Production of ROS

hMSCs cells exposed to different concentrations of SiO_2 NPs (1, 10, 50, 100 and 200 $\mu\text{g}/\text{mL}$) for 24 hrs also revealed a significant increase in the production of ROS only at high concentrations (100 and 200 $\mu\text{g}/\text{mL}$), compared to the controls

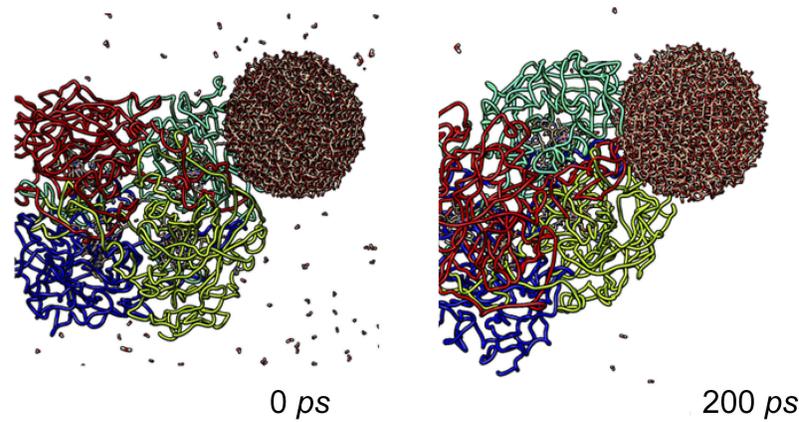


Figure 9 SiO₂ cluster/CAT complex in the beginning and after 200 ps simulation.

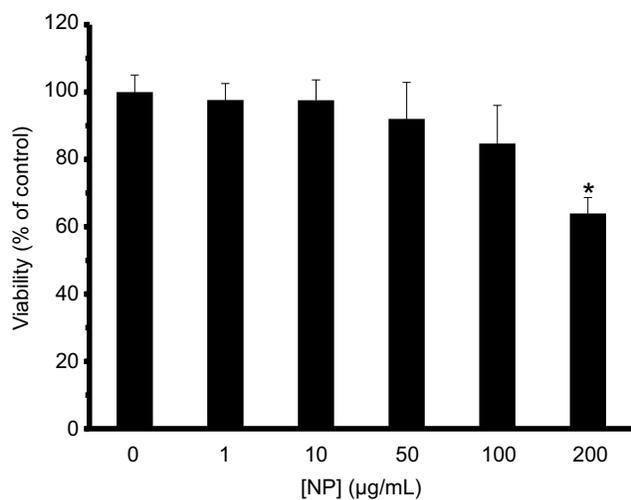


Figure 10 SiO₂ NPs (up to 200 µg/mL) decrease the viability of hMSCs. The cells were incubated with varying concentrations of SiO₂ NPs for 24 hrs before MTT assay was carried out. **P*<0.05 compared to the control group.

(**P*<0.05) (Figure 14). We observed that different concentrations of SiO₂ NPs (1, 10, 50, 100 and 200 µg/mL) produced 116.33±17.07, 125.01±8.42, 136.66±24.05, 161.33±18.66 (**P*<0.05), 185.11±9.90 (**P*<0.05), respectively. While for the negative control group, the level of ROS was determined to be 114.66±6.54. On that account, the largest effect was observed for 100 and 200 µg/mL of SiO₂ NPs as shown in Figure 14.

Caspase-3 assay

Apoptotic stimuli including NPs result in apoptosis induction which is executed by the activation of caspases. Caspase-3 activation was assessed by colorimetric assay after 24 hrs, by measuring the absorbance change at 405 nm. Figure 15 displays the results, where SiO₂ NPs-induced activation of caspase-3 as an effector enzyme can only be

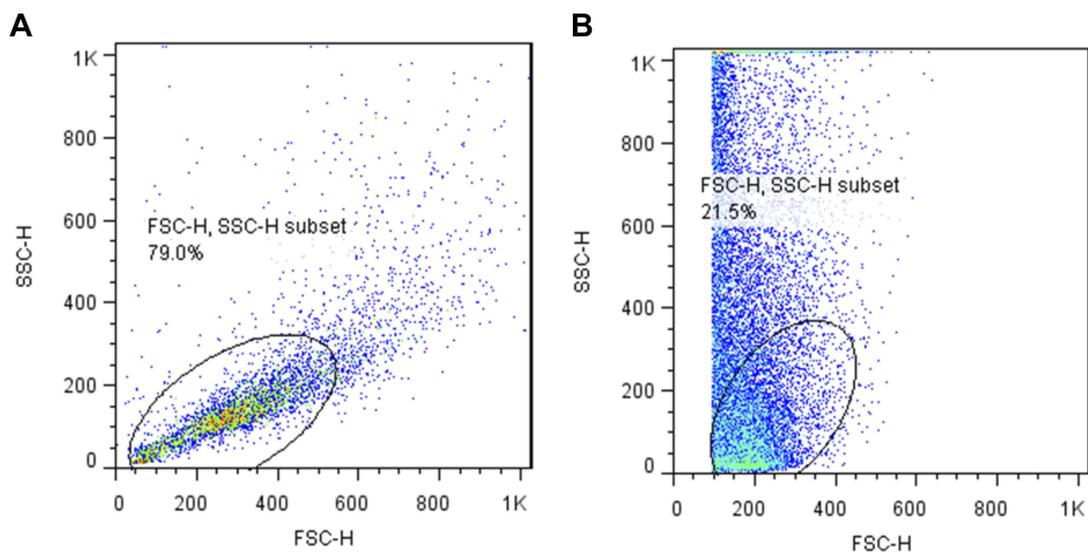


Figure 11 Cellular internalization of SiO₂ NPs into the hMSCs. (A) Control cells and (B) treated cells with SiO₂ NPs (200 µg/mL).

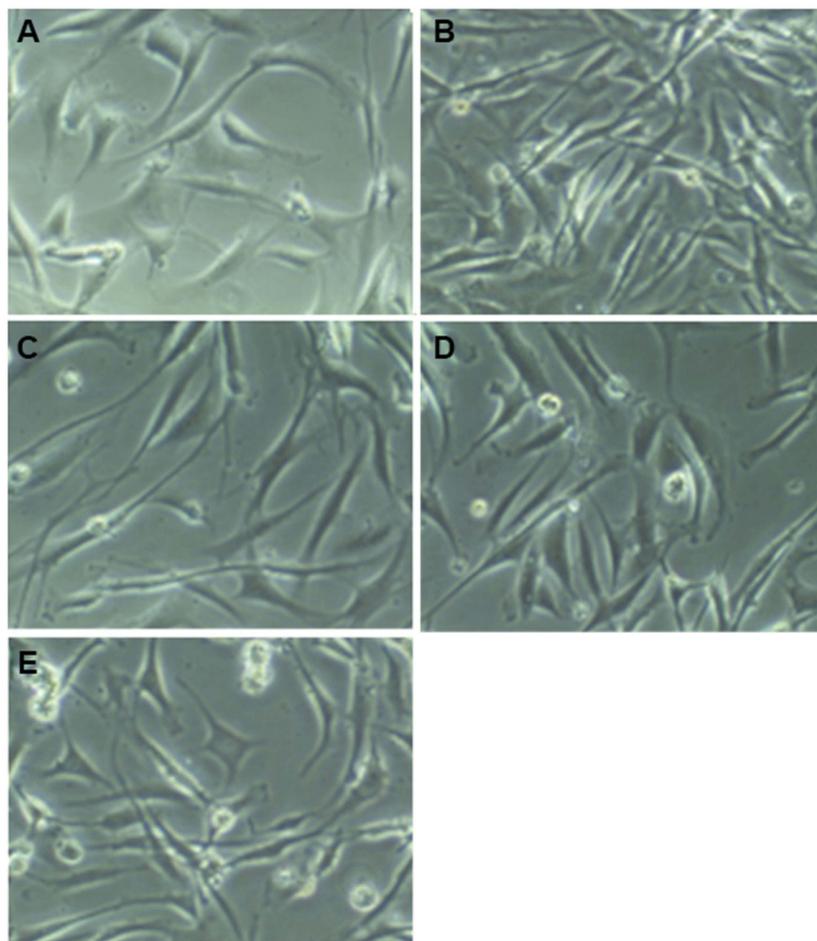


Figure 12 Morphological changes of hMSCs induced by different concentrations of SiO₂ [0 (A), 10 (B), 50 (C), 100 (D) and 200 µg/mL (E)].

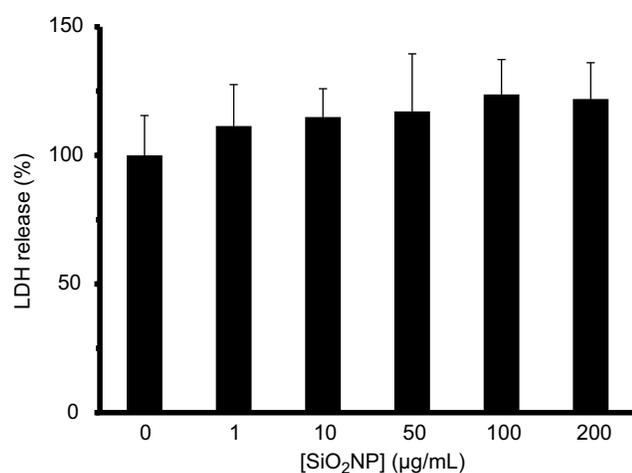


Figure 13 SiO₂ NPs cause no increase in the level of LDH in cell culture medium. hMSCs cells were incubated with varying concentrations of SiO₂ NPs for 24 hrs before examining the LDH activity of cell culture medium.

observed at high concentration (200 µg/mL, [**P*<0.05]). Consequently, it is evident that all concentrations of SiO₂

NPs did not cause activation of caspases, and the effect is most significant at high concentrations of SiO₂ NPs.

Flow cytometry analysis

To further quantify cell apoptosis and necrosis, hMSCs were exposed to SiO₂ NPs with a concentration of 200 µg/mL and then stained by employing Annexin V-FITC/PI double-staining method. The results are depicted in Figure 16, where A and B correspond to the representative plots of flow cytometry analysis in hMSCs in the absence and presence of SiO₂ NPs (200 µg/mL), respectively. It can be seen from Figure 16A that almost no apoptotic or necrotic cells were discovered in negative control cells. Nevertheless, in the SiO₂ NPs-treated sample, a significant increase in the population of apoptotic cells could be detected (Figure 16B). After 24 hrs of incubation with 200 µg/mL of SiO₂ NPs, the percentage of hMSCs cells going through early-stage apoptosis was 8.45% (***P*<0.01), indicating apoptosis induction in the hMSCs cells. As expected, lower concentrations of

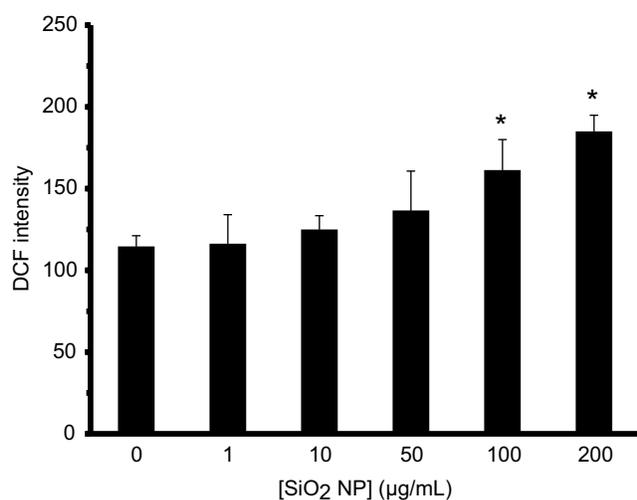


Figure 14 SiO₂ NPs at concentrations of 100 and 200 µg/mL increase the level of ROS in hMSCs. hMSCs cells were incubated with SiO₂ NPs for 24 hrs before the ROS level was examined. **P*<0.05 compared to control group.

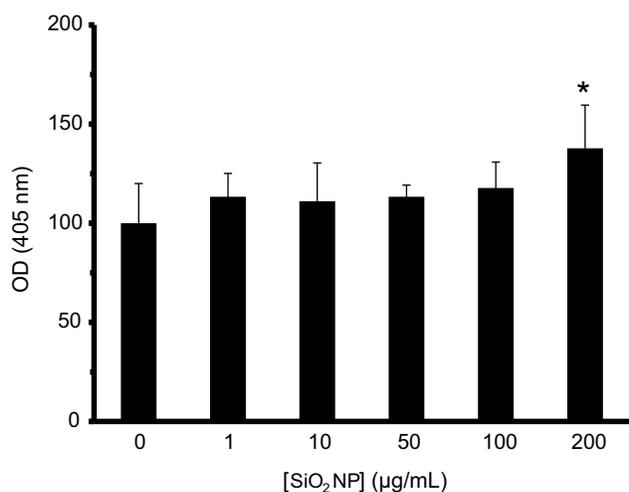


Figure 15 SiO₂ NPs at concentration of 200 µg/mL activate caspase-3 in hMSCs. hMSCs cells were incubated with varying concentrations of SiO₂ NPs for 24 hrs before the caspase-3 activity was assessed. **P*<0.05 compared to control group.

SiO₂ NPs did not induce any significant changes in the number of viable or apoptotic cells relative to the control cells (data not shown). These data confirm again that SiO₂ NPs induce apoptosis in hMSCs only at high concentration.

Discussion

Protein delivery³⁷ or stem cell delivery² can be used to treat several human disorders. For example, Scaletti et al³⁸ revealed that NP-therapeutic protein supramolecular assemblies can be used to deliver therapeutic proteins into the cells. Cheng et al³⁹ reported that metal-organic framework NPs can be applied as promising agents for protection and delivery of proteins. Zhang et al⁴⁰ disclosed that NPs can be used as useful systems

in stem cell delivery for treating some neurological disorders. Singhal et al⁴¹ reported that NP-mediated CAT delivery can mitigate the oxidative stress in human neurons. Song et al⁴² divulged that CAT-loaded nanoshells can be used as bio-nanoreactors and enzyme delivery carrier for increasing radiotherapy. Song et al⁴³ showed that liposomes can be employed as excellent candidates for intracellular delivery of CAT. Li et al⁴⁴ also suggested that some kind of nanosomes can be utilized as potential delivery and for increasing the bioactivity of CAT. In fact, as CAT delivery can be used to treat several diseases such as cancer therapy,⁴⁵ ischemia/reperfusion,⁴⁶ and vascular oxidative stress,⁴⁷ the applications of NPs as efficient carriers can be used to deliver CAT to the target tissues. However, before application of NPs in delivery of therapeutic proteins or stem cells, the structure of proteins or cell viability should be evaluated in the presence of NPs. For these applications, the interaction of NPs with CAT should be investigated in order to explore the conformational changes and corresponding activity of CAT in the presence of NPs. In this paper, we demonstrated that SiO₂ NPs did not significantly change the structure and activity of CAT. However, different studies have shown that multi-walled carbon nanotubes,⁴⁸ TiO₂ NPs,⁴⁹ N-acetyl-L-cysteine-capped CdTe quantum dots,⁵⁰ superparamagnetic iron oxide NPs⁵¹ and TiO₂/WO₃/GO nanocomposites¹² caused substantial structural and functional alterations of CAT.

hMSCs are being widely used for cell therapy and as targeted therapeutic agents.⁵² NPs can be used to develop a system including hMSCs-based cell engineering for tissue delivery.⁴ It has been also demonstrated that NPs can be used as agents to track the stem cells^{53,54} and promote their differentiation.^{55,56} On that account, it also seems necessary to explore the cytotoxicity of NPs against hMSCs to introduce some promising NPs which can be implemented with hMSCs as promising agents for therapeutic purposes. In the current study, we unveiled that SiO₂ NPs up to 100 µg/mL do not induce a remarkable cytotoxicity against hMSCs. In spite of that, Mancuso et al⁵⁷ and Kim et al⁵⁸ depicted that ZnO NPs induce mortality, morphological changes, cell cycle arrest and apoptosis in hMSC. Yan et al⁵⁹ also revealed that the surface charge of carbon quantum dots can play an important role in the NP-induced cytotoxicity against hMSCs.

Therefore, it may be concluded that SiO₂ NPs do not induce remarkable changes on the CAT activity and cytotoxicity against hMSCs. Future studies may involve functionalization of SiO₂ NPs with different moieties and integrating with CAT in order to increase the activity of

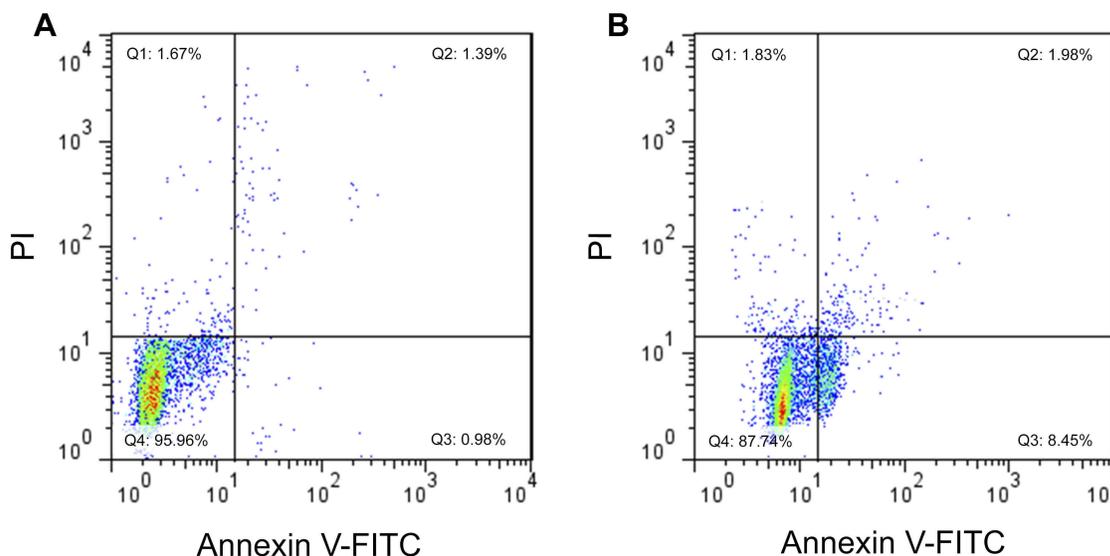


Figure 16 hMSCs cells were incubated with 200 $\mu\text{g}/\text{mL}$ of SiO_2 NPs for 24 hrs, then co-stained with Annexin V-FITC/PI to quantify the apoptosis induction. **(A)** Control cells and **(B)** SiO_2 NPs-treated cells.

CAT and promote the differentiation of hMSCs for stem cell therapy in high oxidative stress regions. Indeed, CAT delivery by NPs can mitigate the oxidative stress and promote the efficacy of hMSCs in cell therapy.

Perspectives

Both the toxicity assay and therapeutic potency of NPs are critically urgent for their utilization in biomedical applications. For both plans, it is vital to explore some basic queries regarding how the biomolecules or cells contact with NPs. One question is to know the factors influencing the cellular effects of NPs. In the present study, we have mainly developed our information about the biological impacts of SiO_2 NPs; however, comprehensive investigations about the mechanisms of toxicity of bare NPs and NPs/protein complex should be performed. The complexity in the characteristic of NPs, the kind of cells and proteins and even physicochemical features affect the biological system–NP interactions. It would be important to explore how these parameters influence the protein structures and cellular vitality. This brand new information will receive an advantage in the perceptive development of monohybrids in the time ahead. The second query is the methodological challenge as well as the type of biological systems as models in exploring NP-protein and NP-cell interactions. These challenges should be addressed by selecting the best models and appropriate techniques for understanding the NP-protein/cell interactions.

Well-established methods are necessary to disclose how NPs like SiO_2 NPs affect conformations and viability of proteins and cells, respectively. It is crucial to use the proper techniques to speculate prospective adverse effects to protein and cells. Some biophysical techniques as well as bioinformatical investigations are potential to unveil some information regarding the protein structure incubated with NPs. Cellular assays will also help scientists to clarify signal pathways to explore the outcome from cell-NP interactions. The third query is how to reveal the interfacial interaction details among NPs, proteins and cells. This research may help to provide comprehensive affirmation regarding the chemical mechanisms for protein and cellular impacts of SiO_2 NPs. By addressing these matters, we can acquire more exhaustive data regarding the interaction of NP with proteins or cells and this data will be useful for the reasoned development of potential and biocompatible nanovehicles in the future.

Conclusion

CAT delivery by NPs may enhance the therapeutic efficacy of hMSCs by reducing the oxidative stress. However, NPs may induce some unwanted effects on the CAT structure and function and hMSCs viability. In this study, we explored the interaction of SiO_2 NPs with CAT and hMSCs by different approaches. It was depicted that hydrophobic interactions are the main contributing forces in the spontaneous interaction of SiO_2 NPs with CAT. Also, native structure and activity of CAT remain

unchanged after interaction with SiO₂ NPs. It was also demonstrated that SiO₂ NPs triggered no remarkable cytotoxic effect against hMSCs till 100 µg/mL. Finally, SiO₂ NP in combination with CAT as a multipotent complex may enable efficient cell therapy in future.

Disclosure

The authors report no conflicts of interest in this work.

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