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Surface defect rich ZnO quantum dots as antioxidant inhibiting α -amylase and α -glucosidase: A potential anti-diabetic nanomedicine†

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Preventing chronic hyperglycaemia and associated oxidative stress is utmost important for the treatment and management of Type 2 Diabetes Mellitus (T2DM). Here we report the role of different size surface defect rich ZnO quantum dots (D-QDs) for inhibiting metabolic enzymes and scavenging free radicals, which plays a key role in reducing hyperglycaemia and oxidative stress. Quantitative analysis of radical scavenging and metabolic enzyme inhibition activity of D-QDs demonstrates a size dependent behaviour, where D-QDs with smaller diameter shows superior activity compared to larger size D-QDs. Considering the size dependence in surface defect formation, the increased surface defect density in smaller size D-QDs can be considered as the reason behind this enhancement. Detailed studies establishing the underlying mechanism behind potent free radical scavenging and enzyme inhibition provides an intense scientific rationale for considering D-QDs to design safe and effective nanomedicine for T2DM.

1. Introduction

Type 2 Diabetes Mellitus (T2DM) is a persistent metabolic disorder characterised by chronic hyperglycaemia, which leads to an abnormally enhanced level of free radicals and concurrent decline of antioxidant defence mechanisms, finally resulting in oxidative stress (OS).¹ Increasing evidence in both clinical and preclinical studies indicates that OS plays a major role in the disturbances of physiological homeostasis, manifested by an increase in lipid peroxidation, insulin resistance, damage of cellular organelles and enzymes²⁻⁶. Hence, OS promotes the development of pathophysiological complications of T2DM like nephropathy, neuropathy, retinopathy and cardiomyopathy⁷. These chronic diseases are responsible for majority of morbidity and mortality.

Inhibition of α -glucosidase and α -amylase activity is reported as suitable targets for controlling postprandial hyperglycaemia in T2DM^{8,9}. Use of antioxidants is recommended for controlling OS and related complications^{6, 10, 11}. Therefore, designing a potential multifunctional therapeutic agent with enzyme inhibitory as well as antioxidant property is of utmost importance for the treatment of T2DM and prevention of associated problems.

“Jasada Bhasma” is a unique herbo-metallic formulation used in Indian traditional medicine (Ayurveda) for the treatment of diabetes and its progression¹². A comprehensive physico-chemical characterization of Jasada Bhasma by

Bhowmick *et al.*, reveals the presence of ZnO nanoparticles (NPs)¹³. These ZnO NPs present in Jasada Bhasma are predominantly of wurzite crystal structure and contains a considerable amount of oxygen deficiency¹³. Hence, the presence of defect rich ZnO NPs in medicinal formulation like Jasada Bhasma strongly rationalise the possibility that surface oxygen vacancies (V_O) can be the key attributing factor giving its medicinal value. Recently, *in vivo* evaluation of anti-diabetic activity and safety assessment of ZnO NPs were reported¹⁴. However, till date, there are no reports on the detailed mechanistic study of the therapeutic role for V_O present in ZnO NPs in T2DM.

Recent experimental and theoretical work shows size dependence in the formation of defects in nanocrystals^{15, 16}. These defects are found predominantly at the surface of nanocrystals and its density increases with decreasing nanocrystal size^{15, 17}. In previous works on defect rich ZnO quantum dots (D-QDs) synthesis, a similar trend for size dependence was observed for V_O formation even when ZnO QDs are grown under defect promoting conditions^{17, 18}. Thus, it is expected that the surface chemistry of D-QDs will vary with different sizes. Further, the presence of this surface defects can contribute a net surface charge for D-QDs and the density of this surface charge will increase with decreasing particle size. Hence, this increase in surface charge density for smaller D-QDs can enhance charge based interaction of D-QDs with biomolecules. Earlier reports indicate that the NPs surface

charge is a contributing factor for protein adsorption onto NPs via electrostatic interaction¹⁹⁻²⁵. In ZnO NPs, V_O is the most cited surface defects, and its presence results in a positively charged surface²⁶.

A recent study on cerium oxide NPs by Lee *et al.*, revealed that V_O clustered at the surface of NPs have a size dependent antioxidant activity and smaller diameter NPs (containing more surface V_O) were found to possess high antioxidant activity²⁷. This study suggests that size dependent V_O formation in NPs may be the main reason for the observed increase in antioxidant activity for smaller NPs. However, the size dependent antioxidant behaviour of D-QDs towards reactive oxygen species (ROS) is least explored despite the presence of high density of surface V_O at smaller D-QDs.

In this article we report, for the first time, the rational designing of D-QDs of different size possessing well defined physico-chemical properties exhibiting dual potential as metabolic enzyme inhibitor and antioxidant capability. For obtaining D-QDs of different sizes we have adopted defect rich seed mediated growth¹⁷. We have grown three different sizes of monodispersed D-QDs from a defect rich seed QDs obtained by rapid microwave heating. Our experimental results suggest that both enzyme inhibition and antioxidant property has a good correlation with the size of D-QDs and hence the size dependent surface defect density.

2. Experimental Details

Chemicals: All the precursors for synthesis of D-QDs and the assay chemicals are commercially available. The absolute ethanol (Analytical Reagent, China, Assay: 99.99 %) was the solvent used for D-QDs synthesis. The raw precursors $Zn(OAc)_2$ (99.99 %, metal basis) and LiOH ($\geq 98\%$, reagent grade) from Sigma Aldrich, USA., were used for synthesis as received without further purification. The C-ZnO NPs were purchased from Sigma Aldrich, USA. Potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), sodium potassium tartarate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, sodium hydroxide (NaOH), sodium chloride (NaCl) and porcine pancreatic α -amylase was bought from HiMedia Laboratories, Mumbai, India. α -glucosidase, p-nitrophenyl α -D-glucopyranoside (pNPGP), 3,5-dinitrosalicylic acid (DNSA), riboflavin, ethylenediaminetetraacetic acid (EDTA), nitroblue tetrazolium (NBT) were bought from Sigma Aldrich, USA. The standard enzyme inhibitor, Acarbose was procured from Bayer Pharmaceuticals, India.

Synthesis of D-QDs: The D-QDs were synthesised as per the previous report¹⁷. For a typical synthesis 0.05 M $Zn(OAc)_2$ was mixed with 0.2 M LiOH (3 mL each) in a quartz reaction vial. A polymer cap was used to seal the reaction vial and microwave reaction was initiated by using a microwave reactor (CEM Discover) with 2.45 GHz operating frequency. The reaction temperature ($75 \pm 5^\circ C$) was maintained for one minute under high magnetic stirring followed by cooling to room temperature using compressed air flow. The solution obtained was used as a D-QDs seed and the different size of D-QDs were obtained by adding different concentration of metal precursors $Zn(OAc)_2$ to seed QDs followed by microwave heating till $50^\circ C$ for one minute. All the samples for activity studies were prepared from the as prepared solution by appropriate dilution

to achieve desired concentrations, which was expressed in $\mu g/mL$.

Characterization: Powder X-ray diffraction (XRD) data were collected using a Philips (PANalytical) powder diffractometer, with Cu K_α radiation as X-ray source. The normal and high resolution transmission electron micrographs were taken using a JEOL JEM-2100F high-resolution transmission electron microscope (HR-TEM) operating at 200 kV. PL and PLE spectra of D-QDs and enzymes were recorded at room temperature using HORIBA Jobin Yvon Fluorolog 3 spectrofluorometer. Surface charge on the D-QDs and C-ZnO NPs were determined in terms of zeta potential using zeta potential analyzer (ZetaPALS, Brookhaven Instruments Corporation, USA).

DPPH Radical Scavenging Assay: The scavenging of DPPH radical was initiated by addition of 20 μL of different concentration of each sample with 80 μL of ethanolic solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH, 100 μM) in a 96 well plate which was incubated in dark room at room temperature for 20 min²⁸. The change in absorbance value was measured at 517 nm. The radical scavenging activity of samples was found out using following formula:

$$\% \text{ Radical scavenging} = (A_{517 \text{ Control}} - A_{517 \text{ Test}}) / A_{517 \text{ Control}} \times 100 \quad (1)$$

EPR Measurements: The DPPH^{*} scavenging activity of D-QDs was measured using previously described method²⁹. An ethanol solution of 200 μL of D-QDs (varying concentration) was added to 800 μL of DPPH (100 μM) in ethanol solution (ethanol itself is used for collecting control spectra). The solutions were then transferred into a quartz capillary tube and fitted into the cavity of the electron paramagnetic resonance (EPR) spectrometer, after vigorous mixing for 10 sec. The X-band (9.44 GHz) EPR trace of the DPPH^{*} spin adduct was measured using JES-FA200 EPR spectrometer (JEOL, Tokyo, Japan) exactly 1 min later at $25^\circ C$. Measurement conditions: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 5 mW, gain 6.3×10^5 and sweep time of 2 min.

Superoxide Radical Scavenging Assay: Different concentration of 100 μL of D-QDs sample was added to 100 μL riboflavin solution (20 μg), 200 μL EDTA solution (12 mM), 200 μL ethanol and 100 μL NBT solution (0.1 mg). To this, 3 ml of phosphate buffer (50 mM) was added to dilute the reaction mixture, which was further illuminated for 5 min. The absorbance of solution was measured at 540 nm³⁰.

$$\% \text{ Radical scavenging} = (A_{540 \text{ Control}} - A_{540 \text{ Test}}) / A_{540 \text{ Control}} \times 100 \quad (2)$$

Porcine Pancreatic α -amylase Inhibition Assay: The amylase activity was assayed using chromogenic DNSA as reported earlier³¹. Different concentration of each samples were incubated with porcine pancreatic α -amylase (50 $\mu g \text{ mL}^{-1}$) for 30 minutes at $37^\circ C$. 1% starch solution was used as a substrate. Samples without α -amylase were taken as controls and test readings were subtracted from the absorbance of these controls. Reducing sugar was estimated by interpolation of obtained absorbance value of DNSA for different concentration of D-QDs at 540 nm (A_{540}) in the standard graph. The percentage of enzyme inhibition was calculated by using the formula:

$$\% \text{ Enzyme inhibition} = (A_{540 \text{ Control}} - A_{540 \text{ Test}}) / A_{540 \text{ Control}} \times 100 \quad (3)$$

The K_m and V_m for porcine pancreatic α -amylase inhibition by D-QDs and C-ZnO NPs was found out by using Michaelis–Menten and Lineweaver–Burk equations. The different starch concentration ($1\text{--}5\text{ mg mL}^{-1}$) was incubated in the presence of samples and porcine pancreatic α -amylase for 10 min and DNSA was used for estimation of residual enzyme activity.

Murine Pancreatic and Intestinal α -amylase Inhibition Assays:

Swiss male mice were used as the source of murine enzymes with institutional animal ethical committee guidelines. In short, 10 weeks old mice with approximate weight of 20 gm were starved for 12 h, followed by excision of small intestinal and pancreatic tissues which was homogenized in chilled phosphate buffer (10 mM containing 6 mM NaCl) containing protease inhibitors. The tissue homogenates were centrifuged at 10,000 r.p.m for 10 min. Collected supernatant was diluted to get an absorbance of 0.4 (at 280 nm) which was used as a source of enzyme. Percentage inhibition of pancreatic α -amylase and small intestinal α -amylase were also calculated as described earlier.

Porcine α -glucosidase Inhibition Assay: Glucosidase inhibitory activity was evaluated as per our earlier report³². Typically, 100 μL of α -glucosidase (0.1 unit/ml) was mixed with 200 μL of different concentration of D-QDs samples and incubated for 1 hour at 37°C. Enzyme action was initiated by the addition of 10 mM *p*-nitrophenyl- α -D-glucopyranoside (*p*-NPGP) in 100mM phosphate buffer of pH 6.8 and stopped after 10 minutes incubation at 37°C by adding 2 mL of 0.1M Na_2CO_3 . Determination of α -glucosidase activity was carried out by estimation of absorbance by *p*-nitrophenol released from *p*-NPGP at 420nm (A420) using a 96-well multiplate reader (SpectraMax M5, Molecular Devices Corporation, Sunnyvale, CA).

$$\% \text{ Enzyme inhibition} = (A420_{\text{Control}} - A420_{\text{Test}}) / A420_{\text{Control}} \times 100 \quad (4)$$

Murine Intestinal α -glucosidase Inhibition Assay: The Intestinal extract of mice was prepared by aforementioned process for evaluation of murine intestinal α -glucosidase inhibition. Percentage inhibition of murine intestinal α -glucosidase was determined using *p*-NPGP as substrate as per the above protocol.

Statistical Analysis: The statistical analysis was performed using one way analysis of variance (ANOVA) ($P < 0.05$). Results are expressed as means \pm SEM ($n=3$).

Circular Dichroism (CD) Studies: The Jasco J-815 Circular Dichroism spectropolarimeter was used to record the CD spectra. The spectra were recorded at 25 °C using 1nm slit width, scan speed = 40 nm/min and response time = 1 s. All the measurements were performed in a 2 mm path length quartz cell in the range of 190–300 nm. An IC_{50} of D-QDs(1) was incubated with fixed enzyme concentration of 100 $\mu\text{g/ml}$ in phosphate buffer. The CD spectrum of D-QDs in phosphate buffer acquired under the same conditions was used as baseline setting for acquiring the main CD spectra.

Fluorescence Study of Enzymes: HORIBA Jobin Yvon Fluorolog 3 spectrofluorometer was used to acquire the fluorescence spectra of tryptophan at 25°C with a 1.0 cm path length quartz cuvette. A 3.0 nm slit width was set at emission and excitation side. The emission spectra were collected in the range of 280 to 450 nm, with an excitation at 270 nm. The

enzyme concentration used are the same as that in the CD study, while D-QDs(1) concentrations were varied.

3. Results and Discussion

Physico-Chemical Properties of D-QDs

Near-spherical D-QDs with diameter ranging from 3 to 7 nm were prepared by seed mediated growth from defect rich seed QDs¹⁷. A less defective ZnO NPs procured from Sigma Aldrich, USA was used as a control. Fig. 1a, b, c, shows transmission electron micrograph (TEM) of three different diameters of as grown D-QDs labeled as D-QDs(1): size $\sim 3.56 \pm 0.25$ nm, D-QDs(2): size $\sim 5.12 \pm 0.25$ nm, D-QDs(3): size $\sim 7.18 \pm 0.25$ nm and control ZnO NPs (C-ZnO NPs): size $\sim 50 \pm 15$ nm (Fig. 1d). The synthesized D-QDs have good monodispersity as confirmed by particle size distribution profile (Fig. 1a, b, c, bottom right insets). All the samples and control were characterised for the purity and phase using X-ray diffraction (ESI† Fig. S1) studies, which show peaks associated with ZnO wurtzite crystal structure (JCPDS card No. 36-1451). A broadening in the X-ray diffraction peaks were observed for D-QDs, which can be attributed to strained lattice due to high density of surface defects and reduced crystal size. The high resolution transmission electron microscopy (HR-TEM) investigations of D-QDs (Fig. 1e) show a strained lattice with point defects, while the C-ZnO NPs (Fig. 1f) shows a distinct lattice ordering associated with crystalline nanocrystals. This confirms that the D-QDs are rich in defects when compared with C-ZnO NPs.

The normalized photoluminescence (PL) spectra (I/I_{max}) of D-QDs of different size and C-ZnO NPs are shown in Fig. 2a. The observed PL emission spectra can be split into three regions: 1) an UVA emission from the ZnO band edge,^{33, 34} 2) oxygen vacancy (V_{O}) related visible emission³⁵⁻³⁸ and 3) zinc vacancy (V_{Zn}) related near infrared (NIR) emission³⁹. The emission spectra of D-QDs used in the current study possess a weak band edge emission and NIR emission, while a strong visible emission was observed. This confirms that the currently synthesized D-QDs are rich in V_{O} . A bathochromic shift in D-QDs emission was observed with increasing D-QDs size, which can be attributed to quantum confinement effect. The photoluminescence excitation (PLE) spectra (ESI† Fig. S2) of D-QDs also follow a similar trend, which further confirm that the D-QDs were subjected to quantum confinement effect. While in the case of C-ZnO NPs a strong band edge emission was observed, confirming its highly crystalline nature. The intensity ratio of λ_{max} of defect emission (I_{DE}) to λ_{max} of UV emission (I_{BEE}) could be utilized for semi-quantitative determination of defect density in ZnO NPs^{33, 34}. The $I_{\text{DE}}/I_{\text{BEE}}$ value shown in Fig. 2b shows a size dependent change in the case of D-QDs. The $I_{\text{DE}}/I_{\text{BEE}}$ value is very low for C-ZnO NPs which signifies a very low concentration of radiative defect centres in C-ZnO NPs. Fig. 2c shows a photograph of C-ZnO NPs and D-QDs emitting a different colour under a UV lamp (365 nm, 4 watt) excitation. The zeta potential of D-QDs(1), D-QDs(2), and D-QDs(3) of same concentration (10 $\mu\text{g/ml}$) in ethanol were -61.48 ± 9.1 , -54.06 ± 5.06 and -41.39 ± 6.10 mV, which have a size dependent change. The increase in the value of negative zeta potential with decreasing size can be attributed to increase in positive surface charge by increased surface V_{O} density at smaller size (Fig. 2d). In contrast to this, C-ZnO NPs shows a positive zeta potential (19.92 ± 5.02 mV),

this can be due to negative surface charge in the less defective oxygen terminated surface.

Radical Scavenging Assays

The potential of D-QDs for reducing oxidative stress was determined by using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH[•]) scavenging assays. The half maximal inhibitory concentration (IC₅₀) of different size D-QDs, C-ZnO NPs and a standard antioxidant ascorbic acid are shown in Fig. 3a. Scavenging of DPPH[•] by all tested ZnO NPs indicated their antioxidant potential which were significantly superior as compared to standard antioxidant, ascorbic acid (IC₅₀ ~57.24 µg/mL). D-QDs(1) (IC₅₀ ~ 1.59 µg/mL) and D-QDs(2) (IC₅₀ ~ 1.77 µg/mL) showed comparable activity while D-QDs(3) (IC₅₀ ~ 3.96 µg/mL) showed slightly lower activity. C-ZnO NPs on the other hand showed a lower activity (IC₅₀ ~ 6.34 µg/mL).

The results of superoxide radical scavenging activity are shown in Fig. 3b. A similar trend was observed in case of the superoxide radical scavenging activity as well where D-QDs(1) (IC₅₀ ~ 0.03 µg/mL) and D-QDs(2) (IC₅₀ ~ 0.04 µg/mL) showed comparable activity, which was superior to that of D-QDs(3) (IC₅₀ ~ 0.06 µg/mL). However, all the tested D-QDs were found to be extremely potent compared to C-ZnO NPs (IC₅₀ ~ 0.20 µg/mL) as well as ascorbic acid (IC₅₀ ~ 81.54 µg/mL).

Mechanistic Study of Radical Scavenging by D-QDs

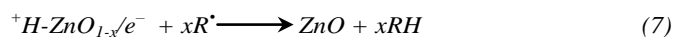
The mechanism of anti-radical property of D-QDs were studied using a stable DPPH[•]. Here onwards for mechanistic study D-QDs(1) was used as a representative for D-QDs, unless otherwise mentioned. The addition of different concentration of D-QDs ethanol solution to a 100 µM DPPH[•] in ethanol solution resulted in the formation of 2,2-diphenyl-1-picrylhydrazine (DPPH-H). The electron paramagnetic resonance (EPR) spectroscopy was employed for monitoring the reduction of DPPH[•] to DPPH-H in the presence of D-QDs. The reduction in the integrated electron paramagnetic signal intensity of DPPH[•] in the presence of D-QDs was considered as direct evidence for the formation of DPPH-H. Fig. 3c shows the first derivative EPR spectrum without D-QDs and with D-QDs, its corresponding colour change from violet (DPPH[•]) to pale orange (DPPH-H) is shown as the bottom left inset. A linear Stern-Volmer (SV) plot of integrated EPR absorption intensity (top right inset of Fig. 3c.) with different concentration of D-QDs shows a static nature for DPPH[•] quenching.

This observation of anti-radical activity of D-QDs is similar to the recent report by Schrauben *et al.*, where they observed that a solution containing reduced metal oxide NPs can transfer a proton (H⁺) and an electron (e⁻) to organic radicals, suggesting a proton coupled electron transfer (PCET) reaction⁴⁰. This observation of PCET in reduced metal oxide NPs supports our observation on radical scavenging by D-QDs. In the case of D-QDs the oxygen vacancies may be present as singly ionized (V_O[•]) or doubly ionized (V_O^{••}) states as shown below⁴¹.



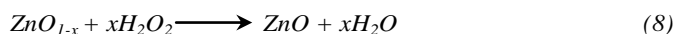
From Equation (5) and (6), it is clear that the existence of V_O[•] or V_O^{••} will result in the generation of an electron. This e⁻ along with the H⁺ from the solution matrix can be used for initiating PCET in the case of D-QDs. Thus the presence oxygen vacancies in D-QDs will endow it with radical scavenging property via PCET reaction.

Therefore, the above observation of anti-radical activity by D-QDs can follow the reaction scheme as shown in Equation (7), where R[•] = organic free radical.



Further we have checked the scavenging potential of D-QDs towards a non-radical reactive oxygen species, H₂O₂. The PL spectroscopy results show that in the presence of H₂O₂ a reduction in the defect emission was observed for D-QDs(2) (Fig. 3d).

At a higher concentration of H₂O₂ almost all the defect emission has vanished (ESI† Movie 1). This implies the oxidation of V_O by H₂O₂, which resulted in the healing of V_O sites responsible for defect emission (Equation (8)).



The SV plot for PL quenching by H₂O₂ shows an upward positive curve, which means the coexistence of both static and dynamic nature for luminescence quenching by H₂O₂.

Enzyme Inhibition Assays

The enzyme inhibition assays suggest a likely interaction of D-QDs with α-amylase and α-glucosidase. In porcine pancreatic α-amylase inhibition, all of the tested ZnO nanoparticle samples were found to be potent inhibitors of porcine pancreatic α-amylase (Fig. 4a). It is important to note that all of them exhibited superior activity as compared to acarbose, a standard inhibitor. Among this D-QDs shows maximum inhibition activity, the activity of D-QDs samples are in the order D-QDs(1) (IC₅₀ ~ 0.11 µg/mL) > D-QDs(2) (IC₅₀ ~ 0.17 µg/mL) > D-QDs(3) (IC₅₀ ~ 0.21 µg/mL) which is following a decreasing trend with respect to reduction in particle size and increase in I_{DE}/I_{BEE}. However, C-ZnO NPs showed a lower enzyme activity (IC₅₀ ~ 0.34 µg/mL). The effect of D-QDs on the inhibition kinetics of α-amylase was studied which was supported by a double reciprocal Lineweaver–Burk plot (Fig. 4b) revealing that the mode of D-QDs inhibition is uncompetitive (a type of reversible inhibition) with decrease in both the apparent K_m (Michaelis constant) and V_m (maximum enzyme reaction rate). Further, the kinetic analysis when compared to the uninhibited enzyme activity (V_m of 2.82 and a K_m of 79.84) was found to decrease on incubation with D-QDs. The D-QDs(1) showed an enhanced decrease of V_m (0.11) and K_m (2.54) while D-QDs(2) showed a relatively lower decrease in the V_m (0.36) and K_m (10.27). C-ZnO NPs exhibited a V_m and K_m equivalent to 1.29 and 58.61 respectively. D-QDs(3) showed relatively higher V_m (1.10) and K_m (57.36) as compared to D-QDs(1) and D-QDs(2). However, V_m and K_m was found to be lower in all D-QDs samples as compared C-ZnO NPs. From the above results, it could be noted that incubation of α-amylase with D-QDs resulted in a size dependent inactivation (Fig. 4). Hence, the study suggests that D-QDs are promising candidate to control release of glucose by inhibiting the breakdown of starch by metabolic

enzymes and could be developed as a lead anti-diabetic nanomaterial.

Murine Pancreatic and Intestinal α -amylase Inhibition: Inhibitory activity against murine pancreatic and intestinal α -amylase (Fig. 4c and 4d) indicated extremely superior activity for D-QDs and C-ZnO NPs when compared to acarbose ($IC_{50} \sim 60.23 \mu\text{g/mL}$ for murine pancreatic α -amylase and $IC_{50} \sim 54.83 \mu\text{g/mL}$ for intestinal α -amylase). All of the samples tested showed comparable activity against both pancreatic ($IC_{50} \sim 0.11 \mu\text{g/mL}$ for D-QDs(1)) and intestinal ($IC_{50} \sim 0.11 \mu\text{g/mL}$ for D-QDs(1)) amylase respectively, most superior being D-QDs(1) followed by D-QDs(2), D-QDs(3) and C-ZnO NPs.

α -glucosidase Inhibition: Inhibition studies against α -glucosidase (Fig. 5a) revealed that the compounds tested were superior inhibitors as compared to acarbose ($IC_{50} \sim 1.33 \mu\text{g/mL}$). D-QDs(1) ($IC_{50} \sim 0.11 \mu\text{g/mL}$) was found to be excellent inhibitor as compared to D-QDs(2) ($IC_{50} \sim 0.18 \mu\text{g/mL}$) and D-QDs(3) ($IC_{50} \sim 0.20 \mu\text{g/mL}$). However, C-ZnO NPs showed a lower activity ($IC_{50} \sim 0.33 \mu\text{g/mL}$).

Murine Intestinal α -glucosidase Inhibition: ZnO nanoparticles showed excellent inhibitory potential against murine intestinal α -glucosidase (Fig. 5b) the highest being for D-QDs(1) ($IC_{50} \sim 0.12 \mu\text{g/mL}$) followed by D-QDs(2) ($IC_{50} \sim 0.20 \mu\text{g/mL}$) and D-QDs(3) ($IC_{50} \sim 0.32 \mu\text{g/mL}$) which were considerably superior as compared to C-ZnO NPs nanoparticles ($IC_{50} \sim 0.52 \mu\text{g/mL}$) as well as the standard inhibitor, acarbose ($IC_{50} \sim 1.34 \mu\text{g/mL}$).

Mechanistic study of enzyme inhibition by D-ZnO QDs

Circular dichroism (CD) and fluorescence spectroscopy were used to investigate on the nature of interaction of α -glucosidase and α -amylase with D-QDs. Initially, CD spectroscopy was used as it gave detailed secondary structure information of enzyme⁴². Generally α -helical content of enzyme shows two characteristic minima at 208 and 222 nm⁴³. Any variation in the conformation of α -helical content of enzyme can be found in CD spectra, either as a diminished minimum or a blue shift. Similarly, in the present study we observed a decrease in the negative humped peaks at 222 nm and 208 nm in the presence of D-QDs when compared with the control enzymes (Fig. 6a and 6b). This results clearly suggests a potential interaction of D-QDs with the α -helix of enzyme, which resulted in a conformational change in the enzyme secondary structure.

In order to look into the nature of interaction of D-QDs with the enzymes, PL spectroscopy was used. The active site of both α -glucosidase and α -amylase contains residues of amino acid tryptophan (Trp)^{44, 45}. A strong intrinsic UVA fluorescence from these enzymes is an indication for the presence of Trp residue at the active site of enzyme. Thus the interaction of D-QDs with the enzyme can be determined from the reduction in the intrinsic UVA fluorescence⁴⁶. The SV plot is included in the inset of Fig. 6a and 6b, which shows decay in the Trp fluorescence intensity for α -glucosidase and α -amylase in the presence of different concentration of D-QDs. The upward positive curve of SV plot can be related to both dynamic and static interaction of D-QDs with α -glucosidase and α -amylase. The time correlated single photon counting (TCSPC) for Trp emission at 345nm is shown in Fig. S3. The biexponential fit shows a fast decay component of 1.45 ns for α -amylase in the

presence of D-QDs(1), while for uninhibited α -amylase it was 1.67 ns. Similarly, in the case of slow decay component, the presence of D-QDs with α -amylase shows an evident decrease in the lifetime, i.e. 3.78 ns when compared with the uninhibited α -amylase lifetime of 4.13 ns. These results were in correlation with the observed PL quenching, which could result in the reduction of the lifetime for Trp emission. Hence, it can be concluded that there was an apparent interaction of D-QDs with the enzyme. Therefore, CD, PL and TCSPC investigations together confirm the interaction of D-QDs with the enzymes which resulted in the observed enzyme inhibition.

The electrostatic interaction between positively charged V_O and negatively charged enzyme site can be the possible reason for the observed enzyme inhibition activity. The D-QDs used in the present study have size dependence in surface V_O formation, where the surface defect density increases with particle size reduction. As a result of this an increased positive surface charge density was observed with reduction in D-QDs particle size, as evidenced by I_{DE}/I_{BEE} versus zeta potential value (Fig. 2d). Hence, an increase in positive charge of D-QDs surface with decreasing size will enhance the electrostatic interaction of smaller D-QDs with negatively charged enzyme sites. This electrostatic interaction may result in the conformational change of enzyme active site thereby inhibiting substrate binding. This could be the possible mechanism for enhanced enzyme inhibition activity with decreasing particle size of D-QDs.

A recent report on the inhibitory activity of surface-functionalized metal-chalcogenide QDs shows that nanoscale inhibitors are active even at very low concentration, thereby eliminating its toxicity concerns⁴⁷. However, in order to consider D-QDs for biomedical applications, the biocompatibility study is inevitable. Hence, we have studied the biocompatibility of the current samples on human embryonic kidney (HEK 293) cell line by using thiazolyl blue tetrazolium bromide (MTT) assay (see Fig. S4). All the tested samples were found to show good cell viability at a very high concentration ($\sim 100 \mu\text{g/mL}$), when compared with the very low IC_{50} value ($< 2 \mu\text{g/mL}$) for enzyme inhibition and radical scavenging activity reported in this paper.

The summary of physico-chemical properties of sample studied in the current work (Table 1) shows the size dependence in the radical scavenging activity and enzyme inhibition activity. The reduction in I_{DE}/I_{BEE} with increasing size implies a size dependent V_O formation. Similarly with the reduction in I_{DE}/I_{BEE} ratio an increase in the IC_{50} of D-QDs for radical scavenging and enzyme inhibition were observed. This suggests that the size dependence in surface V_O formation can be the underlying reason for the observed increase in activity for smaller size D-QDs. Therefore, these D-QDs can be considered as an alternative strategy for efficient management and treatment of diabetes apart from the available anti-diabetic therapeutics⁴⁸.

4. Conclusions

Therefore, following conclusions were drawn from this study, 1) the antioxidant and enzyme inhibition activity of D-QDs have size dependence (i.e., it increases with decreasing D-QDs size), 2) the size dependent enzyme inhibition and antioxidant properties could be due to the size dependence in V_O formation. In addition to this, our benchmark antioxidant and enzyme inhibition assay results revealed that smaller size

D-QDs (1) is significantly superior, having four times lesser IC_{50} value ($\sim 1.59 \mu\text{g/mL}$) for antioxidant activity and three times lesser IC_{50} value ($\sim 0.11 \mu\text{g/mL}$) for enzyme inhibition activity when compared to C-ZnO NPs (Table 1). Thus the understanding gained from this study will pave the way towards rational design of safe and effective anti-diabetic nanomedicine through NPs size and surface defect engineering.

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Contributions

A.A., A.R.K. conceived the research and designed the experiments. A.R.K supervised the research. A.A. performed the materials synthesis and characterization. S.G., P.A.M. performed the free radical scavenging, enzyme inhibition assays. A.A., S.G. performed the mechanistic study. A.A., A.R.K., S.G., B.A.C., M.N.G. analyzed the data. A.A., A.R.K., S.G. wrote the paper.

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†**Electronic Supplementary Information (ESI) available:** XRD, PLE, supplementary video demonstrating defect luminescence quenching in the presence of H_2O_2 . See DOI: 10.1039/b000000x/

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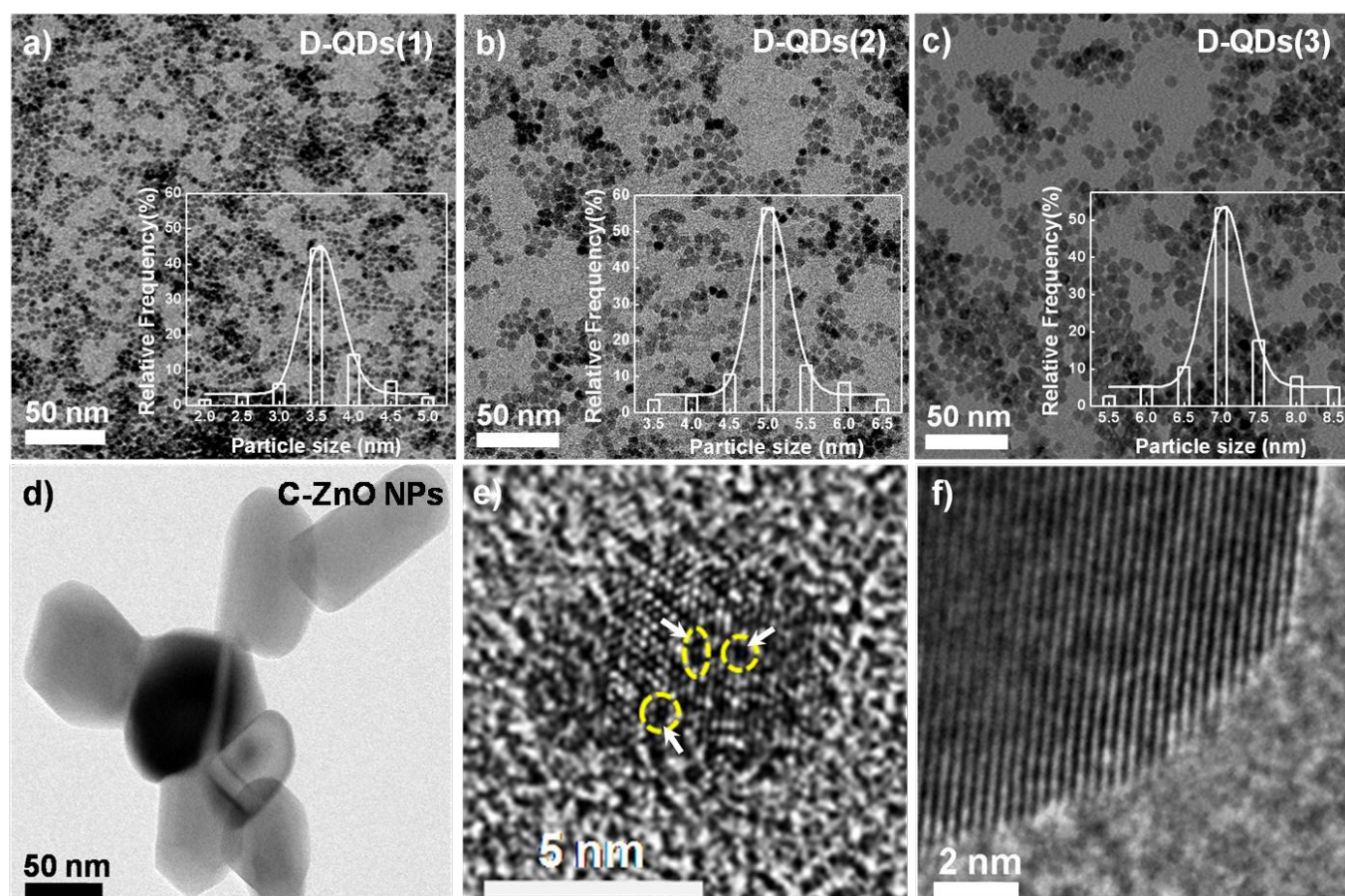


Fig. 1 TEM images of different size D-QDs (a, b, c) and d) C-ZnO NPs, the bottom right insets shows the size distribution histograms of the corresponding TEM images, e) the HR-TEM image of D-QDs (dotted oval and circle show the exact location of some point defects, arrow is for eye guide) and f) HR-TEM image of C-ZnO NPs. Particle size measurement of TEM images shown were analyzed using Image J software from ~ 100 NPs.

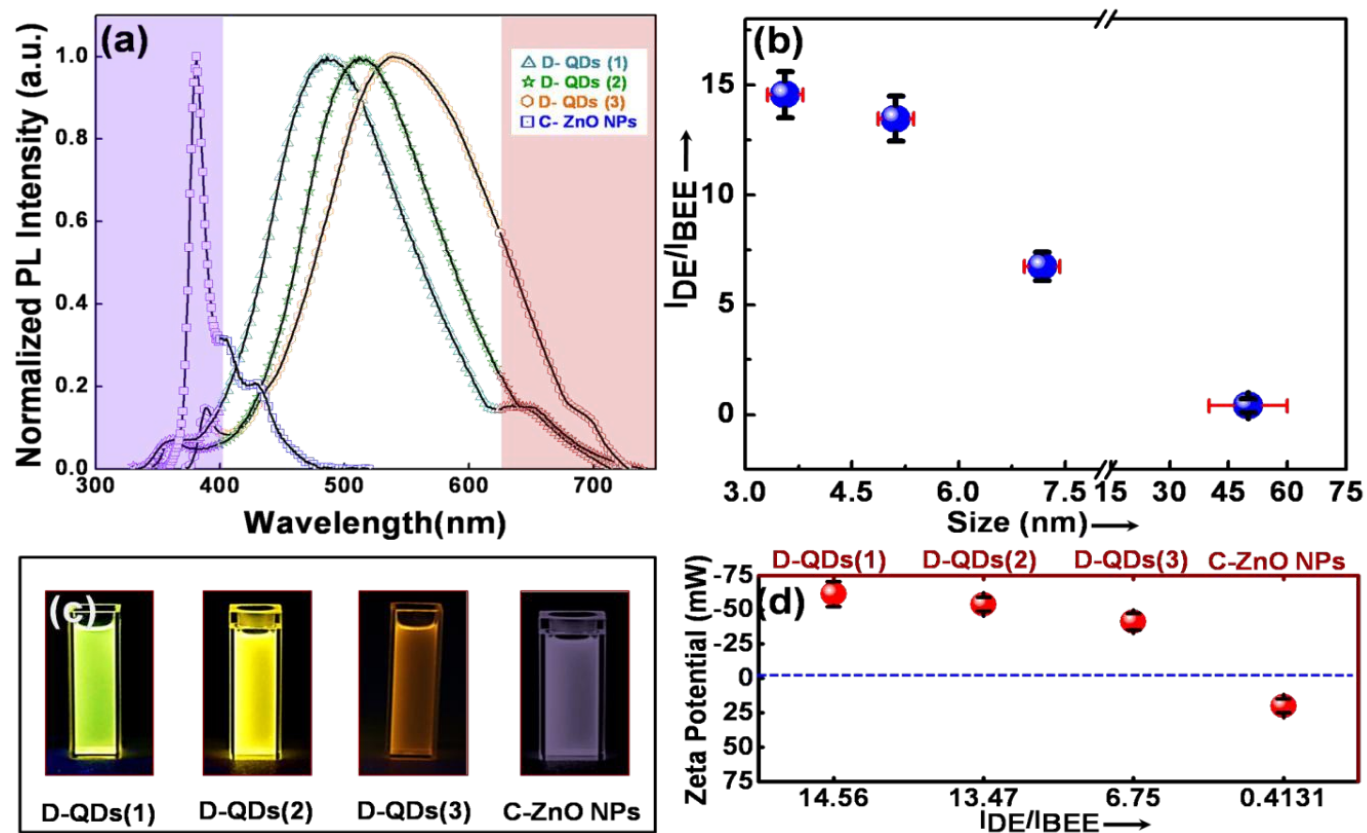


Fig. 2 a) Normalized photoluminescence emission spectra of D-QDs of different size and C-ZnO NPs, b) the I_{DE}/I_{BEE} value for D-QDs of different size and C-ZnO NPs, c) photographs of D-QDs of different size and C-ZnO NPs in ethanolic solution under irradiation by a 365 nm handheld UV lamp, d) the zeta potential for D-QDs of different size and C-ZnO NPs and its corresponding I_{DE}/I_{BEE} value (x-axis is not scaled).

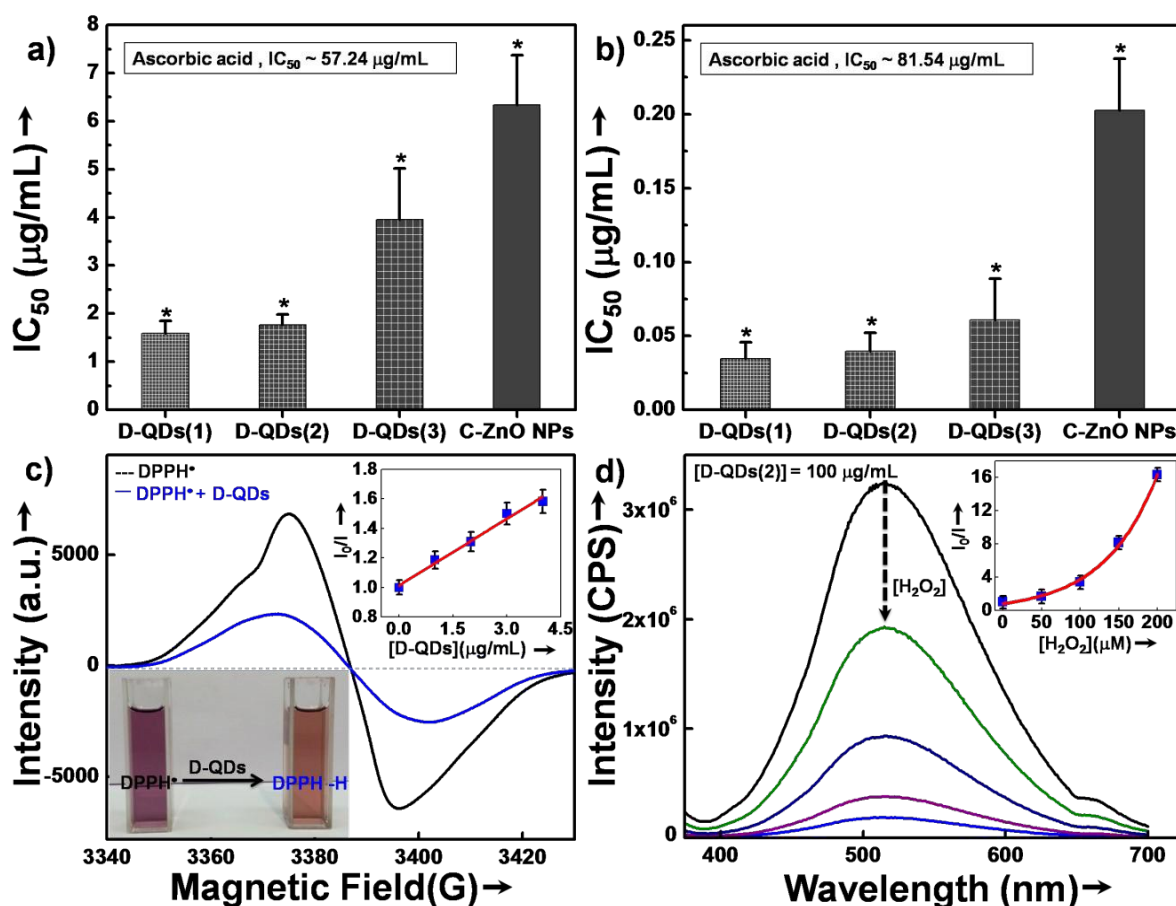


Fig. 3 a) DPPH $^{\bullet}$ scavenging assay for different size D-QDs and C-ZnO NPs b) superoxide radical scavenging assay for different size D-QDs and C-ZnO NPs. c) A typical EPR spectrum for DPPH $^{\bullet}$ with and without D-QDs (the bottom left inset shows the photograph for DPPH $^{\bullet}$ in ethanol, with and without D-QDs and the upper right inset shows the S.V. plot for integrated EPR intensity with different concentration of D-QDs), d) PL spectra showing defect luminescence quenching of D-QDs in the presence of different concentration of H $_2$ O $_2$ (the upper right inset, the S.V. plot for variation in integrated PL intensity with different H $_2$ O $_2$ concentration). (* $p < 0.05$, $n = 3$)

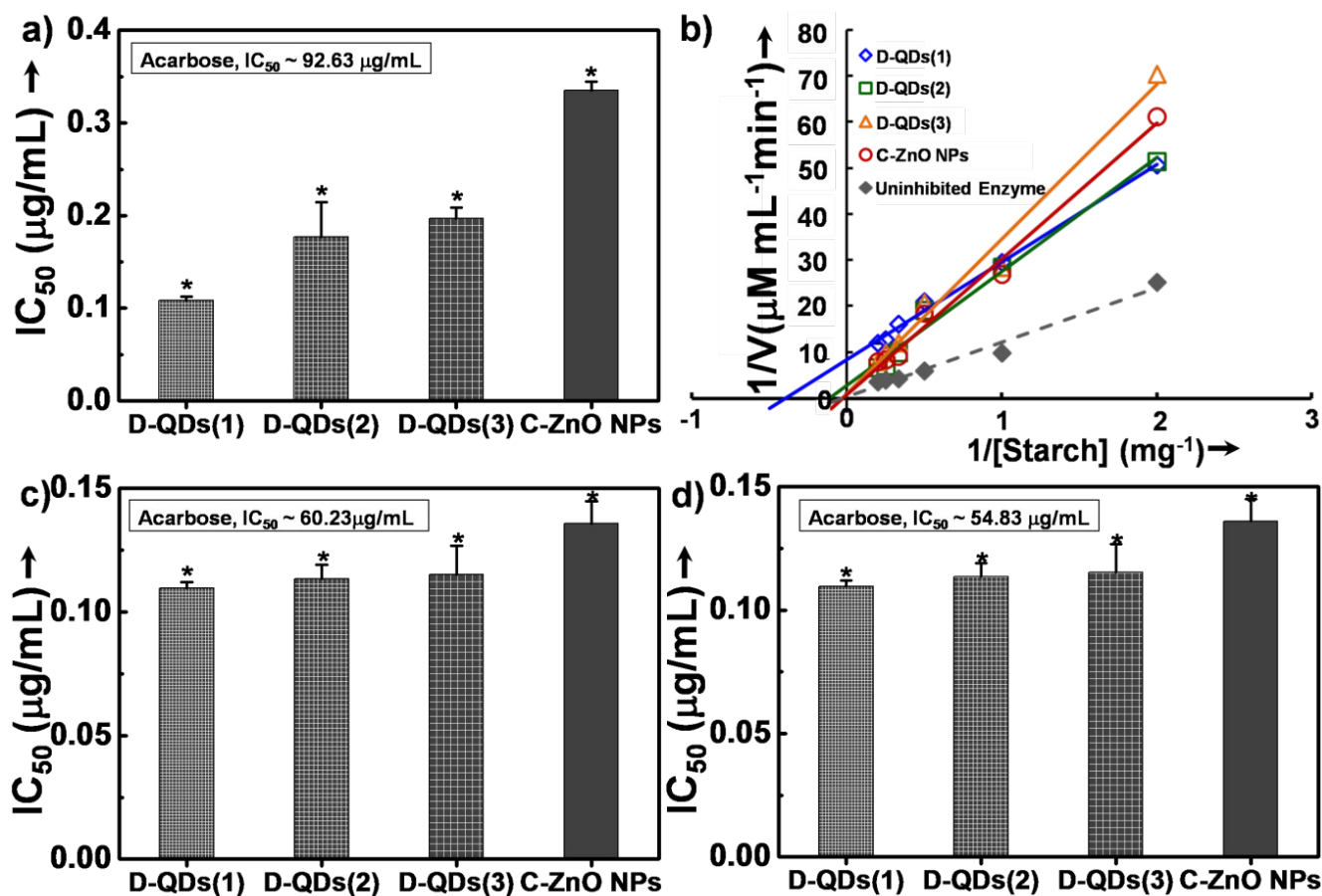


Fig. 4 a) Enzyme inhibition assay of porcine pancreatic α -amylase, b) kinetic analysis of porcine pancreatic α -amylase inhibition by different size D-QDs and C-ZnO NPs, c) enzyme inhibition assay of murine pancreatic α -amylase and d) murine intestinal α -amylase. (* $p < 0.05$, $n = 3$)

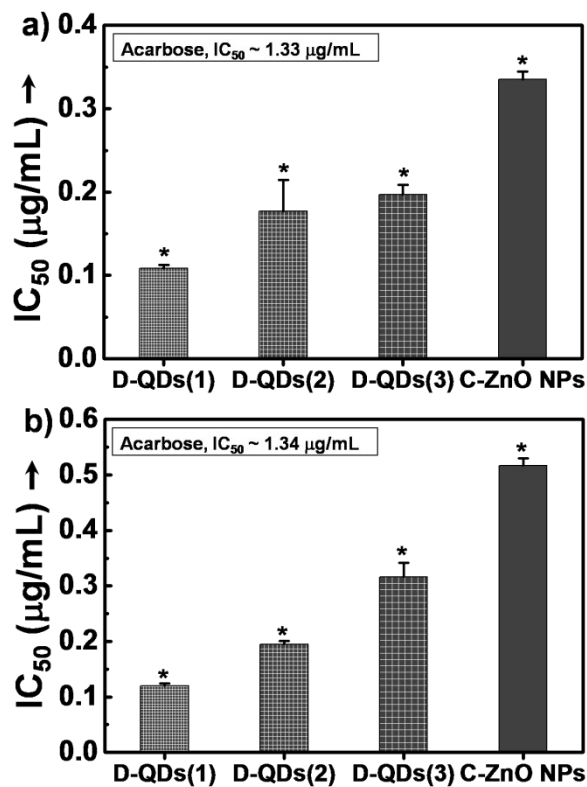


Fig. 5 a) Inhibition assay of porcine α -glucosidase and b) murine intestinal α -glucosidase with different size D-QDs and C-ZnO NPs. (* $p < 0.05$, $n = 3$).

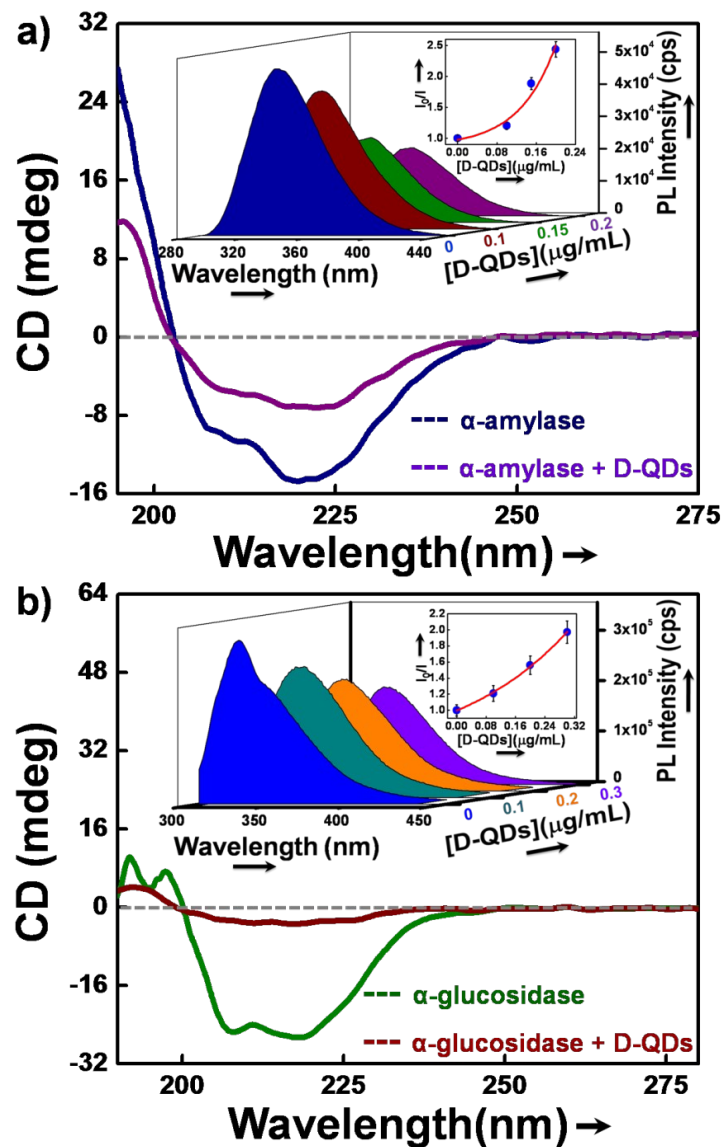


Fig. 6 a) CD spectra of α -amylase and b) α -glucosidase with D-QDs and without D-QDs, the inset shows the quenching of tryptophan PL with different concentration of D-QDs and its corresponding S.V plot is given as top right inset.

Table 1: Summary of the physico-chemical properties of nanoparticles used in this work.

Labels	Size ^a (nm)	I _{DE} /I _{BEE} ratio	Zeta-potential* (mV)	IC ₅₀ * for DPPH* (μg/mL)	IC ₅₀ * for α- amylase ^b (μg/mL)
D-QDs(1)	3.56 ± 0.25	14.56	-61.48 ± 9.10	1.59	0.11
D-QDs(2)	5.12 ± 0.25	13.47	-54.06 ± 5.06	1.77	0.17
D-QDs(3)	7.18 ± 0.25	10.75	-41.39 ± 6.10	3.96	0.21
C-ZnO NPs	~50 ± 15	0.41	19.92 ± 5.02	6.34	0.34

a) Particle size measured from TEM images shown in Figure 1(main text); b) Enzyme source is porcine pancreas. (*p < 0.05, n = 3).