Unfolding of insulin at the surface of ZnO quantum dots

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1 **Abstract**

2 ZnO Quantum dots (QDs) have been used in many biomedical applications such as bioimaging,
3 cancer treatments and etc. Crystallinity, particle size, optical absorption and photoluminescence
4 spectra of ZnO QDs were analyzed by X-ray diffraction (XRD), transmission electron
5 microscopy (TEM), UV-Vis absorption spectroscopy and fluorescence spectroscopy
6 respectively. Interaction of ZnO QDs with insulin was investigated by fluorescence quenching,
7 circular dichroism (CD), isothermal titration calorimetry (ITC) and thermal aggregation tests.
8 The fluorescence quenching results showed a static type quenching along with red shift in
9 synchronize fluorescence (a sign of protein unfolding). CD spectroscopy results also confirmed
10 this unfolding and show a reduction in alpha helices content of insulin in contact with ZnO QDs
11 and their conversion to random coils. According to ITC results, the ΔG , ΔH and binding
12 constant of this interaction are -32.35 kJ/mol, -43.21 kJ/mol and $4.69 \times 10^5 \text{ M}^{-1}$, respectively.
13 Thermal aggregation test showed fast aggregation of insulin in the presence of ZnO QDs.
14 Therefore in biological application of ZnO QDs such as bioimaging, presence of such QDs in
15 vicinity of insulin could unfold this protein.

16 **Keywords:** Insulin; Zinc oxide; Quantum dots

17

1. Introduction

Quantum dots (QDs) are defined as semiconductor nanocrystals with unique optical and electronic properties, whose size are smaller than twice the size of their exciton Bohr radius. Due to their novel properties, QDs have been used in various fields of science and technology [1, 2]. From biological application viewpoints, numerical studies have been reported for the use of QDs in the biological imaging [3], tumor-specific receptors imaging [4, 5], biological sensing [6], drug delivery [7], and therapies [8]. In comparison with the commonly used organic and proteins fluorophores such as Green Fluorescent Protein (GFP), QDs have provide many benefits including high quantum yields, resistance against photobleaching, size-tunable light emission and so on [9].

Due to the high photoluminescence quantum yield, among the other types of QDs, cadmium (Cd) based QDs, such as CdS, CdSe and CdTe have attracted many scientist and technologists attentions in various fields. But from the safety viewpoints, the systematic cytotoxicity assessment of QDs is important for their applications in biology and medicine [10]. There are many scientific reports about toxicological effects of Cd based QDs [11-13]. Main toxicity mechanisms in which QDs may potentially induce their toxic effects are oxidative stress, genotoxicity, neurotoxicity and mitochondrial malfunctions and apoptosis [14]. Therefore it is vital to explore potential utility of other QDs as alternative for Cd based QDs such as zinc based QDs (e.g. ZnO and ZnS) [15].

Over the past few years, ZnO QDs have attracted great interest in many biological applications, ranging from bioimaging to biosensing, tumor destruction and drug delivery [16-

20]. Indeed, several studies have concerned on toxicological effect of ZnO nanomaterials [21-23]. In addition, there have been a number of reports for the interaction of ZnO nanomaterials with proteins such as human serum albumin, bovine serum albumin and transferrin [24-27]. To the best of our knowledge, there is no significant report for the interaction of ZnO QDs with insulin. In order to develop the knowledge on this important subject, it is necessary to investigate the interaction of insulin (as a blood protein model) with ZnO QDs (as biological imaging and sensing material).

Insulin is one of the most important blood proteins, which contains 51 amino acid residues in its structure and mediates glucose uptake. Insulin consists of 21-residues in the A chain and 30 residues in the B chain, which are tethered by one intra-chain (A6-A11) and two inter-chains ((A7-B7) and (A20-B19)) disulfide bonds [28, 29]. Insulin as a control hormone binds to its receptor and regulates metabolism by suppressing gluconeogenesis enzymes. Therefore, induced conformational changes in insulin impair receptor binding and could trigger diabetes mellitus [30].

Attached proteins to the surface of nanoparticles (protein corona) may change nanoparticle properties. These proteins may alter selective binding of nanoparticle bioconjugates to its intended target or even in some cases could delivery nanoparticles bioconjugates to other sites producing side effects [31, 32]. On the other hand, proteins on the nanoparticle surface are observed partially unfolded which these unfolded proteins have potential to produce protein aggregates or some toxic proteins [33-35]. In addition, unfolded proteins lose their normal functions and may bind to sites that are different from its conventional receptor or target. The present work has been focused on the study of the interaction between insulin and ZnO QDs by

- 1 using fluorescence, isothermal titration calorimetry (ITC) and circular dichroism (CD)
- 2 spectroscopy techniques. With these techniques we extract fluorescence quenching mechanism,
- 3 thermodynamic properties and possible protein unfolding data involved in these interactions.

2. Materials and methods

2.1. Materials

Zinc acetate dihydrate ($\text{Zn}(\text{OAc})_2 \cdot 2\text{H}_2\text{O}$), dimethyl sulfoxide (DMSO), absolute ethanol, ethyl acetate and tetramethylammonium hydroxide (TMAH) ($\text{N}(\text{Me})_4\text{OH} \cdot 5\text{H}_2\text{O}$) were purchased from Merck. Insulin was purchased from Lilly Company.

2.2. ZnO QDs preparation

ZnO QDs were prepared according to the method reported by Gemalin research group [36, 37]. In a typical synthetic procedure, 22 mg $\text{Zn}(\text{OAc})_2 \cdot 2\text{H}_2\text{O}$ was dissolved in 1 mL DMSO. In another container, 27.5 mg TMAH was added to 1 mL absolute ethanol to obtain TMAH ethanolic solution. Ethanolic solution of TMAH was added drop wise to the $\text{Zn}(\text{OAc})_2 \cdot 2\text{H}_2\text{O}$ solution. The ZnO QDs were precipitated with adding adequate amount of ethyl acetate. For aqueous solubilization of QDs, suitable amounts of TMAH aqueous solution (25wt%) was added until all precipitates dissolve [38].

2.3. Methods

2.3.1. ZnO QDs characterization methods

UV-Vis absorption

The optical absorption characteristics of ZnO QDs were obtained by using Varian UV-vis absorption spectroscopy (model: Carry 100 Bio).

Transmission Electron Microscopy (TEM)

The particle size and morphology of the prepared ZnO QDs was studied using Philips CM30 transmission electron microscopy (TEM)

X-Ray Diffraction (XRD)

To determine the crystal structure, the X-ray diffraction patterns of ZnO QDs were recorded using Philips X'Pert MPD Pro X-ray diffractometer with Co K_α irradiation ($\lambda = 1.54018 \text{ \AA}$).

Fluorescence spectroscopy

Fluorescence excitation and emission characteristics of ZnO QDs were recorded on Varian, Cary Eclipse fluorescence spectrophotometer. For recording of fluorescence emission spectra, excitation wavelength was set to 344 nm and for recording of fluorescence excitation spectra, emission wavelength was set to 570 nm.

2.3.2. Interaction of Insulin and ZnO QDs

Fluorescence quenching measurement

The fluorescence quenching and the synchronize fluorescence were measured using a fluorescence spectrophotometer (Cary Eclipse; Varian). For these purposes, 0.5 mg insulin was dissolved in 1 ml PBS buffer (10 mM, pH 7.4). Insulin has no Trp amino acid therefore only tyrosine fluorescence quenching was studied. For tyrosine fluorescence quenching measurements, the excitation wavelength was set at 275 nm and emission was measured in the range of 285–400 nm in the presence of desired amounts of ZnO QDs, with fixed excitation and emission slit widths of 5 and 10 nm respectively.

With analyzing the fluorescence quenching data by using the well-known Stern–Volmer equation one could obtain the fluorescence quenching strength [39]:

$$\frac{F_0}{F} = K_{SV}[Q] + 1 \quad (1)$$

Where F_0 and F denote the steady-state fluorescence intensities in the absence and presence of the quenching (ZnO QDs) respectively. K_{SV} is the Stern–Volmer quenching constant and $[Q]$ is the concentration of the quenching (ZnO QDs). With increasing K_{SV} , the fluorescence quenching strength increases. Therefore with surveying the K_{SV} in different temperatures one could judges about the mechanism of quenching.

The binding constant and number of binding sites can be extract from fluorescence quenching data given by equation (2) [39].

$$\log \left[(F_0 - F)/F \right] = n \log [Q] + \log K \quad (2)$$

By linear fitting the plot of $\log [(F_0 - F)/F]$ versus $\log [Q]$, the values of n and K can be obtained from the slope and intercept, respectively.

Evaluating of the binding constant (K) in different temperatures enable us to obtain some thermodynamic functions, namely, change in entropy (ΔS°) and enthalpy (ΔH°) by using the van't Hoff equation:

$$\log K = \frac{-\Delta H^\circ}{2.303RT} + \frac{\Delta S^\circ}{2.303R} \quad (3)$$

Where K is the binding constant at the corresponding temperature and R is the gas constant. By linear fitting the plot of $\log K$ versus $1/T$ the values ΔS° and ΔH° can be obtained from the intercept and slope respectively. The Gibbs free energy (ΔG°) is estimated from the following equation:

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

For extracting thermodynamic parameters (ΔH , ΔS and ΔG) from fluorescence quenching data, this test was done in three different temperatures of 295, 304 and 310 K.

Synchronous fluorescence measurement

For this test, the difference between excitation and emission wavelength ($\Delta\lambda$) was set at 30 nm [40] (for tyrosine amino acids) then the excitation wavelength was scanned from 220 to 320 nm. The excitation and emission slit widths were set to 5 and 10 nm respectively.

Isothermal Titration Calorimetry (ITC)

In order to explore the interaction of ZnO QDs with insulin, isothermal titration calorimetry (ITC) technique was utilized by using a VP-ITC calorimeter (Microcal Inc, USA). All solutions (buffer, insulin and ZnO QDs solution (dissolved in buffer)) were degassed at 25 °C while stirring, before experiment. The sample and reference cell were filled with 1.8 ml of insulin (15 mM) and 1.8 ml of PBS buffer solution (10 mM, pH 7.4) respectively. Each cell was coin shaped and adiabatic. The syringe was filled with 300 ml of ZnO QDs (600 mM) which was injected into the sample cell in 30 steps. The heat exchange was recorded once for interaction of ZnO QDs with insulin and once for dilution of insulin. Then dilution heat was subtracted from

interaction heat, the acquired curves were fitted in one set of site model, and thermodynamic parameters including number of binding sites (N), affinity constant (K), enthalpy (ΔH) and entropy (ΔS) changes were obtained by frequent curve fitting of binding isotherms [41]. The Gibbs free energy (ΔG) change was calculated from following equation ($\Delta G = \Delta H - T\Delta S$).

Circular Dichroism (CD) spectropolarimetry

To determine the protein secondary structure, far UV-CD spectra (195–260 nm) of samples were carried out at 25 °C using a JASCO J-810 spectropolarimeter. The spectra were recorded in a 1 mm quartz cell and step size of 1 nm (accumulative 3). For this analysis the Concentration of insulin was 0.5 mg/mL in pbs buffer (10 mM).

Aggregation measurements

The aggregation of insulin with concentration of 0.5 mg/ml in pbs buffer (10 mM) in the presence and absence of ZnO QDs were studied by monitoring the relative turbidity at wavelength of 360 nm and temperature of 343 K with using Varian spectrophotometer (model : Cary-100). Duration of the lag phase was obtained as the time-intercept of fitted line to the linear portion of turbidity versus time plot, which appeared after the lag phase[42].

The kinetic data were fitted to the model described by Wang and Kurganov [43]. In the case of first order kinetic fitting of this model, effective first order aggregation rate constant, k_1 , could be extract from the slope of line of $\ln[(\tau_{lim} - \tau)/\tau_{lim}]$ versus $(t_0 - t)$ plot according to the equation (5):

$$\ln[(\tau_{lim} - \tau)/\tau_{lim}] = k_1(t_0 - t) \quad (5)$$

- 1 Where τ is turbidity at time of t , τ_{lim} is the limiting value of turbidity as time tends to
- 2 infinity and t_0 is a lag time during which the absorbance is effectively zero.
- 3

3. Results and discussion

In some of the biomedical application of ZnO QDs such as bioimaging, the QDs are injected directly into the blood media as a bioimaging agent and carried by blood to its targets (cancerous cells) for detection of some diseases such as cancer. In blood media ZnO QDs could meet blood proteins such as insulin, albumin and etc and interact with them [17, 44]. In interaction with such high active surface materials, the proteins of blood could undergo structural changes or unfolded. The unfolded proteins usually lose their normal function and may produce some toxic materials. Therefore it is necessary to study this interaction in order to develop the knowledge about this interaction for designing of QDs with good bioimaging properties along with less toxic side effect (i.e. protein unfolding). In this work, we have studied the interaction of ZnO QDs with insulin as a model of blood protein.

3.1. Characterization of ZnO QDs

Fig. 1a shows the X-ray diffraction pattern of ZnO QDs. The QDs have crystalline nature without any impurity peaks other than ZnO peaks. The peaks at 2θ values of 31.3, 34.1, 36, 46.9, 56.2, 62.4 and 67.6 in the XRD pattern were related to the diffraction from the (100), (002), (101), (102), (110), (103) and (112) lattice planes respectively which well matches with the hexagonal ZnO wurtzite crystal structure ((JCPDS card No. 36-1451) with lattice constants of $a = 3.249$, $c = 5.206$ Å) [45]. Size and morphology of the ZnO QDs were studied with Transmission Electron Microscopy (TEM) as shown in Fig. 1b. According to TEM results the QDs were nearly monodisperse with spherical shape. Size of the prepared ZnO QDs was about 6 nm. According to literatures zinc oxide nanoparticles show quantum size effects in the size range

of ≤ 7 nm [46]. Fig. 1c shows the fluorescence excitation and emission characteristics of the ZnO QDs. As can be seen from this figure the prepared QDs have the emission peak at wavelength of 569 nm and the maximum of excitation peak was located in wavelength of 343 nm. The symmetrical shape of fluorescence excitation and emission peaks of the ZnO QDs indicates that the QDs are monodisperse.

Fig. 1d shows the optical absorption spectra of the ZnO QDs, in which this quantum dots have maximum absorption in wavelength of 337.5 nm. According to Meulenkamp work, ZnO QDs with this absorption spectra have nearly 5 nm size [47]. The deconvolution of this absorption peak shows that the ZnO QDs don't have any absorption in the tyrosine amino acid fluorescence emission region and thus fluorescence quenching doesn't interfere with other quenching phenomena. Absorption spectra of the ZnO QDs are summation of absorption from two phenomena: absorption due to the electronic transitions and absorption due to the Rayleigh scattering (according to Rayleigh scattering theory [48]). So Absorption spectra was deconvoluted based on these two effects and as Fig. 1d shows summation of them has reliable agreement with our experimental results. Presence of particles with different size (as shown in TEM image) and effect of other scattering effect such as Mie scattering create some deviation between predicted and experimental results [49].

3.2. Interaction of insulin with ZnO QDs

Fluorescence quenching studies

Fig. 1a shows that in presence of the ZnO QDs, the intrinsic fluorescence intensity of tyrosine amino acids of insulin decreases. From these data one could extract the fluorescence

1 quenching mechanism, binding constant, number of binding sites and thermodynamic functions
2 (ΔG° , ΔH° and ΔS°) of the interactions between ZnO QDs and insulin.

3 Two general mechanisms are involved in fluorescence quenching: dynamic and static
4 quenching. At dynamic quenching regime, the collision of the quencher with excited fluorophore
5 causes the fluorescence quenching of the fluorophore and in this type of quenching with
6 increasing temperature the collision between quencher and fluorophore increases and thereby the
7 quenching enhances. On the other hand, at static quenching, binding of quencher to fluorophore
8 is the main reason of the quenching. Therefore in this type of quenching with increasing the
9 temperature this binding may be weakened and thereby the quenching strength decreases. Then
10 with studying of the quenching strength in different temperatures one could extract the
11 quenching mechanisms.

12 Fig. 2b shows linear fitting of the plots of F_0/F versus $[Q]$. From the slope of these plots, the K_{SV}
13 values were obtained in different temperatures and summarized in Table 1. According to the
14 results, the fluorescence quenching mechanism for the interaction of ZnO QDs with insulin is
15 static type and therefore insulin has been absorbed on the ZnO QDs surface.

16 The value of K as a function of temperature, which obtained from the Fig. 2c, is summarized
17 in Table 1. This table shows with increasing the temperature, the binding constant (K) decreases
18 and thus the interaction between the QDs and insulin decreases at high temperatures (a sign of
19 static quenching).

20 The thermodynamic functions, which obtained from the Fig. 2d, were summarized in Table
21 1. The negative sign of free energy (ΔG°) indicates that the binding process occurs

spontaneously. The negative sign of entropy (ΔS°) indicates that with absorption of insulin to the QDs surface, entropy decreases. Although unfolding of insulin at the QDs surface increases entropy but the absorption of insulin to the QDs surface decreases entropy with decreasing the number of free molecules in solution.

Synchronize fluorescence study

By measuring synchronous fluorescence intensity of the fluorophore amino acid residues of protein before and after the addition of ligand one could obtain some useful data about the conformational or unfolding of the protein from the possible shifts in maximum wavelength of fluorescence. In this method red shift or blue shift in the maximum wavelength of fluorescence indicate decreasing or increasing in hydrophobicity of surrounding environment of the fluorophore amino acid respectively [50]. Fig. 3 shows that in the presences of ZnO QDs the synchronous fluorescence of tyrosine red shifts and therefore hydrophobicity of surrounding environment of this amino acid decreased which indicates penetration of water to protein or unfolding of insulin.

Isothermal Titration Calorimetry (ITC)

Fig 4 shows the ITC results of insulin upon interaction with ZnO QDs which indicates features of exothermic interactions. The obtained thermodynamic parameters from this study were summarized at Table 2. According to these results, the interaction of insulin with ZnO QDs is exothermic (favorable) (i.e., $\Delta H < 0$) whereas the change in the entropy is negative and unfavorable. Therefore in this interaction, enthalpy plays main role. The negative value of enthalpy is directly related to weak van der Waals and/or electrostatic interactions during the

protein–nanoparticles interactions [27]. N (number of binding sites) value of 1 indicates that each ZnO QDs is surrounded only by one insulin protein.

In ITC results, the plot of exchanged heat versus ligand ratio shows a semi-biphasic feature, indicating that the binding events in solution could be best described by two distinct sets of interaction. As reported in literatures [51], reactivity of ZnO QDs depends on their particle size and our TEM result shows that in as prepared ZnO QDs there are particles with different sizes so we think the semi-biphasic feature of ITC results was originated from the presence of ZnO QDs with different sizes and reactivity.

Circular dichroism spectroscopy

For precise determination of the secondary structure and unfolding of insulin in the presence of ZnO QDs the circular dichroism spectroscopy was used in far-UV region. Fig. 5 shows α -helical contents of insulin decrease and random coil contents increases upon interaction with ZnO QDs. Table 3 quantitatively summarized CD data. The CD results show nearly 15% increase in random coil content and about 27% decrease in alpha helix content of insulin after interaction with ZnO QDs with 168 nM concentration, indicating insulin unfolding at the surface of ZnO QDs. Therefore presences of ZnO QDs cause unfolding of insulin.

Thermal aggregation study

Thermal aggregation study of protein at wavelength of 360 nm is a useful method to show the resistance of proteins to thermal denaturation. A thermal aggregation curve is composed of three phases, a lag phase, a logarithmic phase and a plateau phase. A large lag phase and a slow slope logarithmic phase indicate a more stable protein [52]. With increasing of protein unfolding,

1 thermal aggregation of that protein occurs in shorter lag phase along with fast slope. As shown in
2 Fig. 6 and Table 4, with increasing of the concentration of ZnO QDs, the lag phase of thermal
3 aggregation curve of insulin decreases and the rate of aggregation increases. The larger lag time
4 is one of indicators of chaperone applicability [44-45]. All of these indicate chaperone disability
5 and partial unfolding of insulin in presence of the ZnO QDs.

7 4. Conclusion

8 In this study interaction of insulin (as a model of the blood protein) with ZnO QDs was
9 investigated to survey how ZnO QDs alter insulin structure. QDs, because of their small size,
10 have high surface area, vast active sites and high surface curvature, in interaction with proteins
11 may tremendously alter protein folding, these are the general features of all QDs and toxicities or
12 side effects related to these characters of QDs are the same in all types of QDs [53]. On the other
13 hand each QD has its own properties and therefore the interaction between protein and QDs may
14 change from one QD to another QD, such as Cd based QDs which main toxicity of them is
15 related to the release of poisonous cadmium ion by them or metal sulfide QDs may have
16 different interaction with proteins than to metal oxide QDs [54, 55].

17 In this study the results show that the structure of insulin protein near the ZnO QDs surface
18 was denatured. According to the results of fluorescence quenching study, the fluorescence
19 quenching is of static type and unfolded insulin binds to the surface of ZnO QDs. As the results
20 of circular dichroism showed secondary structure of insulin undergoes some changes in presence
21 of ZnO QDs in such way that α -helical contents of insulin decrease and random coil contents

1 increase. In order to study the effects of these changes on insulin stability, thermal aggregation
2 test was used. Results of thermal aggregation shows that aggregation of insulin in presence of
3 ZnO QDs occurs in short lag phase with fast aggregation rate. Therefore in overall our results
4 showed that the presence of ZnO QDs in vicinity of insulin could change secondary and tertiary
5 structure of insulin and thereby unstable this protein, so in applications (such as bioimaging) that
6 these QDs meet insulin it's necessary the suitable surface functionalizing of these QDs for
7 diminishing of this effect.

9 **Acknowledgment**

10 The support of the University of Tehran, Center of Excellence in Biothermodynamics
11 (CEBiotherm), Iran National Science Foundation (INSF), Iran National Elites Foundation
12 (INEF), UNESCO Chair on Interdisciplinary Research in Diabetes at University of Tehran and
13 Iran Society of Biophysical Society is gratefully acknowledged. The authors would also like to
14 especially thank Dr. M. Farnia for his helpful comments and discussions.

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Fig. 1. Characterization of the ZnO QDs: (a) XRD pattern, (b) TEM image (c) UV-Visible absorption spectra and (d) fluorescence excitation and emission spectra of the ZnO QDs.

Fig. 2. Tyrosine fluorescence of insulin at interaction with ZnO QDs. (a) fluorescence quenching in presence of ZnO QDs with different concentrations (A=0nM-K=210nM), (b) Stern–Volmer plots, (c) plots of $\log [(F_0 - F)/F]$ versus $\log [ZnO \text{ QDs}]$, and (d) van't Hoff plot for insulin-ZnO QDs system.

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3 **Fig. 3.** Synchronous fluorescence spectra of tyrosine amino acids of insulin at $\Delta\lambda=30$ in absence
4 and presence of ZnO QDs at different concentrations (A=0nM-K=210nM).

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Fig. 4. ITC data from the titration of insulin with ZnO QDs.

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3 **Fig. 5.** Circular dichroism spectra of insulin in the absence and in the presence of the ZnO QDs
4 at different concentrations.

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Fig. 6. Thermal aggregation of insulin in the presence and the absence of the ZnO QDs at different concentrations.

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2 **Table 1**

3 Binding (Stern–Volmer quenching constant, binding constant and number of binding sites) and
4 thermodynamic parameters for interaction of insulin with ZnO QDs.

T(K)	$K_{SV}(M^{-1})$	$K(M^{-1})$	n	$\Delta H^{\circ} (kJ/mol)$	$\Delta S^{\circ} (J/molK)$	$\Delta G^{\circ} (kJ/mol)$
295	2.36×10^6	3.98×10^6	1.04	-55.4	-63.5	-36.7
304	1.41×10^6	1.56×10^6	1.01	-55.4	-63.5	-36.1
310	1.25×10^6	1.07×10^6	0.99	-55.4	-63.5	-35.7

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2 **Table 2**

3 Thermodynamic parameters of insulin-ZnO QDs interactions obtained by ITC.

Complex	K_a (binding constant, M^{-1})	N (number of binding sites)	ΔH (kJ/mol)	$-T\Delta S$ (kJ/mol)	ΔG (kJ/mol)
Insulin-ZnO QDs	$4.69(\pm 2.1) \times 10^5$	1 ± 0.11	-43.21 ± 1.12	10.86	-32.35

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1 **Table 3**

2 Secondary structure content of insulin in the presence of the ZnO QDs with different
3 concentrations.

	insulin	Insulin + ZnO QDs (42 nM)	Insulin + ZnO QDs (84 nM)	Insulin + ZnO QDs (126 nM)	Insulin + ZnO QDs (168 nM)
Alpha Helix	39.65%	33.05%	31.26%	30.21%	28.87%
Antiparallel	6.12%	7.11%	7.02%	7.28%	7.43%
Parallel	7.84%	9.23%	10.31%	10.51%	10.76%
Beta-Turn	14.17%	15.38%	15.88%	15.75%	16.18%
Random Coil	32.21%	35.24%	35.61%	36.25%	37.03%

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1 **Table 4**

2 Thermal aggregation parameters of insulin in the absence and the presence of the ZnO QDs at
3 different concentrations.

Sample	Lag time (min)	$k_1(\text{min}^{-1})$
Insulin	62	46.22
Insulin + ZnO QDs (42 nM)	53	49.56
Insulin + ZnO QDs (84 nM)	40	54.81
Insulin + ZnO QDs (126 nM)	36	56.34

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