Interaction of insulin with colloidal ZnS quantum dots functionalized by various surface capping agents

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Abstract

Interaction of quantum dots (QDs) and proteins strongly influenced by the surface characteristics of the QDs at the protein-QD interface. For a precise control of these surface-related interactions, it is necessary to improve our understanding in this field. In this regard, in the present work, the interaction between the insulin and differently functionalized ZnS quantum dots (QDs) were studied. The ZnS QDs were functionalized with various functional groups of hydroxyl (-OH), carboxyl (-COOH), amine (-NH₂), and amino acid (-COOH and -NH₂). The effect of surface hydrophobicity was also studied by changing the alkyl-chain lengths of mercaptocarboxylic acid capping agents. The interaction between insulin and the ZnS QDs were investigated by fluorescence quenching, synchronous fluorescence, circular dichroism (CD), and thermal aggregation techniques. The results reveal that among the studied QDs, mercaptosuccinic acid functionalized QDs has the strongest interaction ($\Delta G^\circ = -51.50$ kJ/mol at 310 K) with insulin, mercaptoethanol functionalized QDs destabilize insulin by increasing the beta-sheet contents, and only cysteine functionalized QDs improves the insulin stability by increasing the alpha-helix contents of the protein, and. Our results also indicate that by increasing the alkyl-chain length of capping agents, due to an increase in hydrophobicity of the QDs surface, the beta-sheet contents of insulin increase which results in the enhancement of insulin instability.

Keywords: Insulin; Quantum dots; capping agent; ZnS; denaturation.

1. Introduction

Quantum dots (QDs) are defined as luminescent semiconductor nanocrystals whose size are smaller than twice the size of their exciton Bohr radius. Because of the quantum-size confinement effect, QDs show unique optical and electronic properties [1]. Due to these novel properties, the new emerging QDs have been widely used in biomedical applications such as bioimaging [2], drug delivery [3], and therapies [4]. ZnS QDs either solely or as a shell at the surface of Cd-containing QDs, have been utilized in biomedical imaging [5], biosensing [6] and cancer diagnosis [7].

Surface capping agents are commonly used in the synthesis and functionalization of nanomaterials [8]. These materials could also be used for the stabilization and water solubilization of QDs. Surface capping agents increase the stability of QDs through electrostatic repulsion, steric stabilization, or a combination of both mechanisms [9]. In nanomaterials synthesis it is not mandatory the covalent bonding of the capping agent to the nanomaterials surface, however in functionalizing of nanomaterials by a capping agent, the capping agent must be covalently bonded to the nanomaterials surface. In the case of the synthesis and functionalization of chalcogenide QDs, sulfur-containing capping agents are the commonly used ones [6, 10, 11]. In such QDs, sulfur-containing capping agents are attached to the surface of the QDs through S-chalcogen bonds and their functional end group(s) determine(s) the surface characteristics of the QDs. Functional end groups of the capping agents on the surface of QDs can be further used for functionalization of the QDs or attachment of some biological agents to the surface of the QDs such as the attachment of antibodies for the target delivery purposes [12].

In biomedical applications of QDs, such as cancer diagnosis, in order to detection of various diseases the QDs as diagnostic agents are injected directly into the blood vessel and carried by blood to their targets (cancerous cells). Inside of the blood vessel, by exposure of the blood proteins to QDs, these proteins may be adsorbed on the QDs surface, leading to the formation of the protein-QDs conjugates [13-16]. The attached proteins at the surface of QDs (protein corona) undergo some structural changes, and produce the protein aggregates or some toxic proteins [17, 18]. On the other hand, formation of the protein corona at the surface of QDs may alter the normal function of the QDs and also may change the selective binding of the

QD-bioconjugates to their intended target or even in some cases may deliver the QD-bioconjugates to other (unrelated) parts of body, and by this way produces various side effects [19, 20]. Therefore, it is necessary to study the interactions between proteins and nanoparticles in order to gain insight into the underlying mechanisms for designing effective QDs with good bioimaging properties and less toxic side effects [21].

During the interactions between QDs and proteins, the capping agents on the surface of QDs play an important role via their functional groups [22, 23]. Related studies, such as interaction of proteins and viruses with various functionalized colloidal alumina particles [24, 25], protein interactions with differently functionalized Au and silica nanoparticles [26, 27], influence of surface charges during the interaction of gold nanoparticles and electrodes with proteins [28, 29], effects of surface composition on nanoparticle-protein interactions [30], and bovine serum albumin (BSA) adsorption on differently charged polystyrene nanoparticles [31] have been reported.

Although, there are many studies concerning the preparation and functionalization of ZnS QDs with various capping agents [32-37], however, there is no comparative study that evaluates the effects of different capping agents at the surface of ZnS QDs on interaction of these QDs with insulin protein. For this reason in the present study, effect of 2-Mercaptoethanol (ME), Cysteamine hydrochloride (CA), Thioglycolic acid (TGA), L-cysteine (CYS), Mercaptosuccinic acid (MSA), Mercaptopropionic acid (MPA), 6-Mercaptohexanoic acid (MHA) and 11-Mercaptoundecanoic acid (MUA) capping agents at the surface of ZnS QDs on interaction of insulin with these QDs were studied. The capping agents were selected in such a way that the surface functionalization with various functional groups of hydroxyl, carboxyl, amine, and amino acid to become possible. The acidic capping agents were selected in four different alkyl chain lengths in order to study the effect of the alkyl chain length on these interactions. Insulin was selected as a model of blood protein because of its very stable structure, because of the presence of three disulfide bond in its small structure (5808 Da) [38].

2. Materials and methods

2.1. Materials

All chemicals were of analytical reagent grade and used without further purification. Zinc acetate dihydrate ($\text{Zn}(\text{OAc})_2 \cdot 2\text{H}_2\text{O}$), sodium sulfide ($\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, Purity: $\geq 98.0\%$), sodium hydroxide, 2-Mercaptoethanol (ME), Cysteamine hydrochloride (CA), Thioglycolic acid (TGA), L-cysteine (CYS), Mercaptosuccinic acid (MSA), Mercaptopropionic acid (MPA), 6-Mercaptohexanoic acid (MHA) and 11-Mercaptoundecanoic acid (MUA) were purchased from Merck. Insulin was purchased from Exir pharmaceutical company.

2.2. Methods

2.2.1 ZnS QDs preparation methods

The preparation procedure of the water-soluble ZnS QDs in presence of the various capping agents such as ME [32-34, 39], CA [35], CYS [6], MSA [36], TGA [10], MPA [11, 37] and MUA [40] are previously reported in the literatures. Briefly in the present work, in a three-necked flask, 50 mL aqueous solution of capping agent (0.01 M) was added drop-wise to 50 mL aqueous solution of zinc acetate (0.01 M) under N_2 atmosphere and magnetic stirring. After ~ 30 min aging at this condition, the solution pH was adjusted to 10 by using NaOH solution. Then, to this solution, 10 mL Na_2S solution (0.05 M) was added and aged for about 1h at temperature of 50°C under N_2 atmosphere. For purification, the obtained ZnS QDs were precipitated with ethanol, separated by centrifuging at 20000 rpm, and finally washed with ethanol.

Due to the hydrolysis of S^{2-} at pH 7 ($\text{S}^{2-} + \text{H}_2\text{O} \rightleftharpoons \text{HS}^- + \text{OH}^-$), preparation of ZnS QDs must be done in basic solutions. Based on the literatures, solubility product constants (K_{sp}) value of ZnS (1.6×10^{-24}) is very low than the K_{sp} of $\text{Zn}(\text{OH})_2$ (3×10^{-17}) and for this reason, there is no impurity related to the formation of $\text{Zn}(\text{OH})_2$ in the obtained ZnS QDs [41, 42].

2.2.2. ZnS QDs characterization methods

The optical absorption characteristics of ZnS QDs were determined by a Varian UV-Vis spectrophotometer (Carry 100 Bio). Transmission electron microscopy (TEM) was used to study the particle size and morphology of the ZnS QDs. TEM images of the ZnS QDs samples were taken using a Philips CM30 (300 kV) transmission electron microscope and in order to sample preparation for this experiment, the ZnS QDs were dispersed in ethanol and deposited on an unstained copper grid and dried at room

temperature. The X-ray diffraction patterns of ZnS QDs were obtained using an X-ray diffractometer (XRD, Philips X'pert MPD Pro) with Cu K α irradiation ($\lambda = 1.5418 \text{ \AA}$). Fourier-transform infrared spectroscopy (FTIR) was used to confirm the presence of the desired capping agents on the surface of ZnS QDs samples and also for surveying the bonding mechanism of the capping agents to the surface of ZnS QDs. FTIR spectra were recorded at room temperature by Bruker (V33 spectrophotometer) FT-IR spectrometer using the KBr Pellet technique. Dynamic light scattering (DLS) was used for determination of the hydrodynamic particle size distribution of the ZnS QDs by using a Zeta plus dynamic light scattering (DLS) instrument (Brookhaven Instruments Corporation, USA). To gain insight into the surface charges of the prepared ZnS QDs, the Zeta potentials of the ZnS QDs capped by the different ligands and of insulin in PBS buffer (10 mM, pH 7.4) at 25 °C were determined by using a Zeta plus dynamic light scattering (DLS) instrument (Brookhaven Instruments Corporation, USA). Each of these measurements was repeated three times.

Photoluminescence (PL) emission spectra of the ZnS QDs samples were recorded using Varian, Cary Eclipse fluorescence spectrophotometer. The PL quantum yield (QY) of ZnS-CYS QD was obtained by referencing to 2-Aminopyridine as standard (in 0.05 M H₂SO₄, QY = 60% [43]). The excitation wavelength (λ_{exc}) of this standard sample is 285 nm which is equal with λ_{exc} of ZnS-CYS QDs and for this reason 2-Aminopyridine with concentration of 10 μ M in 0.05 M H₂SO₄ was selected as standard. According to the protocol reported in the literature [44], the room temperature PL QY of ZnS-CYS QDs was calculated using the following equation:

$$\varphi = \varphi' \times \left(\frac{1-10^{-A'}}{1-10^{-A}} \right) \times \left(\frac{I}{I'} \right) \times \left(\frac{n}{n'} \right)^2 \quad (1)$$

where φ and φ' are the PL QY for ZnS-CYS QDs and the standard sample (2-Aminopyridine), respectively; A (for ZnS-CYS QDs) and A' (for standard sample) are the absorbance at the excitation wavelength used for PL; I (for ZnS-CYS QDs) and I' (for standard sample) are the integrated intensities (areas) of emission spectra; n (for ZnS-CYS QDs) and n' (for standard sample) are the refractive indices of the solvents.

Time-resolved fluorescence spectroscopy was used for determination of the fluorescence lifetime of ZnS-CYS QDs. The PL decay time profile was recorded on a time-correlated single photon counting (TCSPC) FLS920 spectrometer (Edinburgh Instruments, UK), at excitation and emission wavelengths of

285 and 432 nm, respectively. The Fluorescence Analysis Software Technology was used for data analysis and for deconvolution of instrument response function (IRF) from the PL decay time profile of ZnS-CYS QDs.

2.2.3. Interaction of Insulin and ZnS QDs

Fluorescence quenching measurement

The synchronous fluorescence and fluorescence quenching tests were measured using were measured using Cary Eclipse Fluorescence spectrophotometer (Varian, Inc., USA). For these tests, 0.5 mg of insulin is dissolved in 1 mL PBS buffer (10 mM, pH 7.4) and concentration of insulin in this solution is 86 μ M. For tyrosine fluorescence quenching studies, the excitation wavelength was set at 275 nm and the emission scanned from 285 to 400 nm in the presence of the different ZnS QDs. In all of these experiments, the excitation and emission slit widths were fixed at 5 and 10 nm, respectively.

The fluorescence quenching constants of the different ZnS QDs can be obtained using the Stern–Volmer equation [44]:

$$\frac{F_0}{F} = K_{SV}[Q] + 1 \quad (2)$$

where F_0 and F denote the steady-state fluorescence intensities in the absence and presence of the quencher (ZnS QDs), respectively. K_{SV} is the Stern–Volmer quenching constant and $[Q]$ is the concentration of the quencher (ZnS QDs). A larger degree of fluorescence quenching results in the higher K_{SV} value, and with measuring the K_{SV} at different temperatures, it is possible to reveal the fluorescence quenching mechanism.

The number of binding sites (n) and binding constant (K_a) can be extracted from the fluorescence quenching data by using Eq. (3) [44]:

$$\log \left[\frac{F_0 - F}{F} \right] = n \log [Q] + \log K_a \quad (3)$$

From the linear fitting of the plot of $\log [(F_0 - F)/F]$ versus $\log [Q]$, the values of K_a and n can be calculated from the intercept and slope of the linear plot, respectively. Moreover, with measuring the

binding constant (K_a) at different temperatures, the entropy and enthalpy changes can be obtained by using the van't Hoff equation:

$$\log K_a = \frac{-\Delta H^\circ}{2.303RT} + \frac{\Delta S^\circ}{2.303R} \quad (4)$$

where R is the universal gas constant. The Gibbs free energy (ΔG°) is also estimated from the following equation:

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (5)$$

For extracting thermodynamic parameters (ΔH , ΔS and ΔG) from the fluorescence quenching data, fluorescence quenching tests were done at three temperatures of 295, 304 and 310 K.

Synchronous fluorescence measurement

Since the best result for synchronous fluorescence measurements of tyrosine amino acids was obtained at the wavelength interval ($\Delta\lambda = \lambda_{em} - \lambda_{ex}$) of 20 nm, then $\Delta\lambda$ was set to 20 nm and the excitation wavelength was scanned from 220 to 320 nm. The excitation and emission slit widths were kept at 5 and 10 nm, respectively.

Circular Dichroism (CD) spectropolarimetry

In order to determine the insulin secondary structure changes in the presence of the different ZnS QDs, far UV-CD spectra (195–260 nm) of the samples were obtained by using JASCO J-810 spectropolarimeter. The spectra were recorded at 25 °C in a 1 mm quartz cell and concentration of insulin was set to 0.5 mg/mL in PBS buffer (10 mM).

Aggregation measurements

The aggregation of insulin with concentration of 86 μ M in PBS buffer (10 mM) in the presence of the different concentrations of the ZnS QDs were studied by monitoring the relative turbidity of the solution at wavelength of 360 nm and temperature of 343 K by Varian spectrophotometer (Carry 100 Bio). The aggregation kinetic data have been interpreted using a model described by Wang and Kurganov [45]. In the case of the first-order kinetic fitting of this model, the first-order aggregation rate constant (k_1) and the

aggregation lag time (t_0) could be extracted from the slope and x-intercept of a fitted tangent line to the linear part of $\ln[(\tau_{lim} - \tau)/\tau_{lim}]$ versus t plot, respectively, according to the following equation:

$$\ln[(\tau_{lim} - \tau)/\tau_{lim}] = k_1(t_0 - t) \quad (6)$$

where τ is turbidity at time of t and τ_{lim} is the limiting value of turbidity as time tends to infinity.

3. Results

3.1. Characterization of ZnS QDs

Fig. 1 shows the X-ray diffraction patterns of ZnS QDs samples prepared using different capping agents. As this figure shows, all of these QDs have crystalline nature without any impurity. The three major characteristic peaks at 2θ values of 28.7, 47.8 and 57.1 were related to the diffraction from the (111), (220), and (311) planes, respectively, matching the cubic zinc blende crystal structure phase (JCPDS No. 05-0566) [46]. The broadness of the diffraction peaks indicate the nanocrystalline nature of all the samples. The average crystallite sizes of ZnS-ME, ZnS-CA, ZnS-CYS, ZnS-MSA, ZnS-TGA, ZnS-MPA, ZnS-MHA and ZnS-MUA QDs were estimated to be 7.9, 6.7, 6.1, 5.2, 5.3, 4.9, 4.1 and 3.5 nm, respectively from the full-width at half-maximum (FWHM) of the major XRD peak according to the Scherrer's formula [47]:

$$D = \frac{0.9\lambda}{\beta \cos \theta} \quad (7)$$

where D , λ , θ and β are the crystallite size, the wavelength of X-rays, the diffraction angle and the FWHM (in radians), respectively.

Comparing the IR spectra of the pure capping agents (Fig. S1A) with that of the differently capped ZnS-QDs (Fig. S1B), mode of the capping agents' attachment to the surface of the QDs is deduced. For pure acidic capping agents (MSA, TGA, MPA, MHA and MUA) there are six main peaks at about 3400, 3000, 2500, 1700, 900 and 1400 cm^{-1} related to the stretching modes of $-\text{O}-\text{H}$, $-\text{C}-\text{H}$, $-\text{S}-\text{H}$, $-\text{C}=\text{O}$, $-\text{C}-\text{O}$ and bending mode of $-\text{CH}_2$, respectively. Five main absorption bands of the pure ME are at about 3400, 3000, 2500, 900 and 1400 cm^{-1} , corresponding to the stretching vibrations of $-\text{O}-\text{H}$, $-\text{C}-\text{H}$, $-\text{S}-\text{H}$, $-\text{C}-\text{O}$ and bending vibration of $-\text{CH}_2$, respectively. The most pronounced IR absorption bands of CA and CYS capping agents appear at about 3400, 3000, 2500, 1200, 1600 and 1400 cm^{-1} attribute to the stretching modes of $-\text{N}-\text{H}$, $-\text{C}-$

H, -S-H, -C-N and bending modes of -NH_2 and -CH_2 , respectively. Comparison the IR spectra of the pure capping agents with that of the ZnS-QDs functionalized with different capping agents shows the complete removal of -S-H peak at about 2600 cm^{-1} in all cases. From this observation it can be concluded that all capping agents are attached to the surface of ZnS-QDs via disulfide bonds between sulfur atoms of the capping agents and sulfur atoms of the ZnS-QDs.

Fig. 2A shows the optical absorption spectra of the ZnS QDs functionalized with different capping agents. The optical absorption spectra of ZnS QDs depend on their particle size [48]. The method purposed by Lippens [49] was used to estimate the ZnS QDs particle size from their band-gap energy. The band-gap energy (E_g) values for all samples may be estimated by using Tauc's equation [50]:

$$\alpha h\nu = A(h\nu - E_g)^{n/2} \quad (8)$$

where α , h , ν and A are the absorption coefficient, Planck's constant, light frequency and constant value, respectively. The value of exponent n is 1 for a direct-band gap semiconductor and for an indirect-band gap semiconductor, n is 4. The plots of $(\alpha h\nu)^2$ versus $h\nu$ for all samples are shown in Fig. S2 and the E_g values can be calculated from the x-intercept of a tangent line to the linear part of the plots. The estimated band-gap energy (E_g) of ZnS-ME, ZnS-CA, ZnS-CYS, ZnS-MSA, ZnS-TGA, ZnS-MPA, ZnS-MHA and ZnS-MUA samples are 3.84, 3.86, 3.90, 3.99, 4.07, 4.08, 4.10 and 4.13 eV, respectively and therefore their size are estimated to be 6.7, 6.1, 5.5, 4.7, 4.1, 4.1, 3.9 and 3.7 nm, respectively.

Fig. 2B shows the fluorescence emission spectra of the ZnS QDs samples. The emission peak of ZnS-ME, ZnS-CA, ZnS-CYS, ZnS-MSA, ZnS-TGA, ZnS-MPA, ZnS-MHA and ZnS-MUA QDs were located at wavelengths of 445, 442, 432, 423, 418, 407, 404 and 402 nm, respectively. It must be noticed that the excitation wavelengths for these emission spectra were 290, 290, 285, 280, 275, 275, 275 and 270 nm, respectively. With varying the surface capping agents of the prepared QDs, their sizes change and as previously reported [51] by reducing the size of quantum dots, their PL emission spectra shift to shorter wavelengths (blue shift). Furthermore, all of the prepared QDs exhibit surface defect-related PL properties which this effect is highly sensitive to the composition of the surface capping agent and with varying the surface capping agents, the PL properties of the QDs changes [52, 53].

As would be seen in subsequent sections, among the obtained ZnS QDs, in interaction with insulin ZnS-CYS QDs act as the best QD and for this reason, only the fluorescence lifetime and the PL QY of this QD was measured. As Fig. S3 shows after deconvolution of IRF signal from the fluorescence decay profile of ZnS-CYS QDs, the fluorescence lifetime of this QD was calculated to be about 4.5 ns. Based on the results of Fig. 3, the integrated intensities (areas) of the emission spectrum of 2-aminopyridin standard (I') is 2.72 times of that of ZnS-CYS QDs (I), as absorbance (A and A') and the solvent refractive index (n and n') of the standard and ZnS-CYS QDs samples are equal, so from the Eq. 1, the PL QY of ZnS-CYS QDs was calculated to be about 22%.

The hydrodynamic particle size distribution of the ZnS QDs was shown in Fig. S4. The mean particle size of ZnS-ME, ZnS-CA, ZnS-CYS, ZnS-MSA, ZnS-TGA, ZnS-MPA, ZnS-MHA and ZnS-MUA samples were estimated to be 14, 11.4, 7.9, 6.2, 5.8, 5.5, 5.1 and 4.6 nm, respectively. Particle size and morphology of the ZnS-CYS QDs were determined by Transmission Electron Microscopy (TEM) as shown in Fig. 4. According to the TEM results the QDs were nearly monodisperse with spherical shape. The size of the ZnS-CYS QD was measured in the range of 4-7 nm.

The zeta potentials of ZnS-ME, ZnS-CA, ZnS-CYS, ZnS-MSA, ZnS-TGA, ZnS-MPA, ZnS-MHA and ZnS-MUA QDs and insulin protein are -18.7, +29.1, -30.6, -54.1, -49.3, -46.2, -32.3, -31.9, and -21.2 mV, respectively. Although insulin contains positively and negatively charged amino acids in its structure, however, overall charge of this protein at pH 7.4 is negative. pK_a value of MSA is about 3.3 [54] and pK_a value of TGA is about 3.68 [42]. Since with the increase of the alkyl chain length from TGA to MUA, the pK_a increases therefore among the mercaptocarboxylic acid capping agents (MSA, TGA, MPA, MHA, and MUA), MSA has the lowest pK_a and because of the dissociation of its carboxyl group, ZnS-MSA acquires the lowest zeta potential. The only difference between MPA and CYS capping agents is the presence of $-NH_2$ group at the position of the alpha carbon, which due to the slight protonation of this group, the zeta potential of ZnS-CYS is higher than that of ZnS-MPA. The $-OH$ group of ME capping agent is slightly dissociated at pH 7.4 and for this reason among the negatively charged QDs, the ZnS-ME QD has the highest zeta potential. Because of the protonation of $-NH_2$ group in CA capping agent, the ZnS-CA QD

acquires positive charge. Schematic illustrations of the surface charges of the ZnS-CYS QDs have been shown in Fig.7.

3.2. Interaction of insulin with ZnS QDs

Static and dynamic quenching are two general types of fluorescence quenching mechanisms. The dynamic quenching occurs from the collisions between quencher molecules and fluorophores. For dynamic quenching, the quenching strength increases with the increase of temperature because higher temperature leads to the increasing of the collision between quencher and fluorophore. However, in the static quenching, the adsorption or binding of quencher molecules to fluorophore causes fluorescence quenching. For the static quenching, increasing of the temperature leads to the dissociation of the bond between quencher and fluorophore and thereby the quenching constant decreases. Therefore, dynamic and static quenching can be distinguished by measuring the values of the Stern–Volmer quenching constant at different temperatures. For determination of the quenching mechanism and thermodynamic parameters of the interaction between insulin and the ZnS QDs, the QDs as ligand were added in low concentrations (0-242 nM) to the insulin solution (86 μ M) and the PL decay of the tyrosine amino acids of insulin was studied.

Fig. S5 clearly indicates that the fluorescence of the tyrosine amino acids of insulin is quenched in the presence of the ZnS QDs. The inset of Fig. S5 (a) also shows the emission region of the ZnS QDs. This pattern was observed for all of the ZnS QDs, so the excitation wavelength of 275 nm for tyrosine amino acids could also excite the ZnS QDs. The observed evolution in the PL emission of the QDs is not related to the adsorption of insulin to the QDs periphery, indeed this is due to the increasing of the QDs concentrations from 0 to 242 nM. From the fluorescence quenching data for tyrosine amino acids of insulin, the Stern–Volmer quenching constant (K_{SV}), binding constant (K_a), number of binding sites (n) and values of the thermodynamic functions (ΔG° , ΔH° and ΔS°) for the interactions of the different ZnS QDs with insulin have been extracted, which the results are summarized in Table 1.

At different temperatures, the K_{SV} values were obtained from the slope of the linearly fitted line to the F_0/F versus $[Q]$ plots according to the Eq. (2). As the results of Table 1 indicate, except for ZnS-ME QDs, with increasing temperature the K_{SV} values decrease in other cases. As a result, the fluorescence quenching mechanism for the interaction of insulin with the ZnS-CA, ZnS-CYS, ZnS-MSA, ZnS-TGA, ZnS-MPA,

ZnS-MHA and ZnS-MUA QDs is static. It can be concluded that insulin is adsorbed on the surface of these QDs. However, for ZnS-ME QDs, the K_{SV} values increases with increasing temperature. Therefore, the fluorescence quenching mechanism for this QDs is dynamic and with the collision of this QDs to insulin, the intrinsic fluorescence of insulin quenches. Because of the dynamic quenching nature of interaction between insulin and ZnS-ME QDs that is not possible determination of K_a and thermodynamic parameters using Eq. 3, 4 and 5.

From the values of K_a and ΔG° for the interactions of the ZnS QDs with insulin, it is concluded that the ZnS-MSA QDs has the strongest and ZnS-CA QDs has the weakest interaction with insulin. The interaction strengths of insulin with the functionalized ZnS QDs show the following trend: ZnS-MSA > ZnS-TGA > ZnS-CYS > ZnS-CA QDs. In the case of the interactions of ZnS-TGA, ZnS-MPA, ZnS-MHA and ZnS-MUA QDs with insulin, with increasing the alkyl-chain length of the capping agents (i.e. increasing the hydrophobicity) the interaction strength decreases.

The negative sign of free energy (ΔG°) indicates that the interaction of ZnS QDs with insulin occurs spontaneously. For ZnS-CA, ZnS-CYS and specially ZnS-MSA QDs, the entropy change (ΔS°) increases. The increase of the entropy is attributed to the unfolding of the protein. Since the interaction of the ZnS-MSA QD with insulin is endothermic, increasing of the entropy play the main role in this interaction. Due to the decrease in the entropy of the interaction of the ZnS-TGA, ZnS-MPA, ZnS-MHA and ZnS-MUA QDs with insulin, the exothermic nature of the interactions play the significant role. The entropy decrease may be resulted from the accumulation of the protein molecules at the surface of the QDs.

The red shift in the synchronous fluorescence spectra of a fluorophore shows a decrease in the hydrophobicity of the environment surrounding that fluorophore, and on the other hand, the blue shift in the synchronous fluorescence spectra indicates an increase in the hydrophobicity of microenvironment around the fluorophore [55]. Therefore, the synchronous fluorescence study of the fluorophore amino acid residues of a protein in the presence of a ligand, can reveal any conformational changes or unfolding in the protein structure as a result of the interaction with that ligand. The synchronous fluorescence spectra of the tyrosine amino acids of insulin for the interactions of the different ZnS QDs with this protein are shown in Fig. S6.

The synchronous fluorescence spectra of insulin show a blue shift in the presence of the ZnS-CYS QDs, while in the presence of the other QDs, these spectra show a red shift. It can be concluded that the structure of insulin may be more compacted in the presence of the ZnS-CYS QDs. However, in presence of the other QDs, the structure of insulin may be denatured. The largest red shift in the synchronous fluorescence spectra of insulin is observed for the interaction of this protein with ZnS-TGA QDs.

Fig. 5a shows the CD spectra changes of insulin in the presence of ZnS QDs with various surface functional groups. Among the ZnS QDs, only the ZnS-CYS QDs increases the alpha-helix contents of insulin and decreases the random coil contents of this protein, and thereby leading to the insulin stabilization. This behavior is in good agreement with the synchronous fluorescence results. The presence of the ZnS-ME, ZnS-CA and ZnS-TGA QDs remarkably increase the random-coil and beta-sheet contents of insulin and decrease the alpha-helix contents of the protein, resulting in the insulin destabilization. The largest increment in the beta-sheet contents of insulin was observed in the interaction of ZnS-ME QDs with insulin. In the case of the ZnS-MSA QD, the presence of this QD decreases the alpha-helix contents of insulin and increases only the random-coil contents of this protein.

The effect of the length of alkyl-chain of capping agents (or the effect surface hydrophobicity changes) on the protein secondary structure were studied using the CD spectra of insulin in the presence of the ZnS-TGA, ZnS-MPA, ZnS-MHA and ZnS-MUA QDs (Fig. 5b). As Fig. 5b indicates with increasing the surface hydrophobicity of the interacting QDs, the beta-sheet contents of insulin increases. As reported previously the capping agents with long alkyl-chain are suitable for the synthesis of QDs with good stability (due to the increasing of the steric repulsion with increasing the length of alkyl-chain), however these capping agents have more surface hydrophobicity, which cause the aggregation of proteins in biological applications and interaction with proteins.

Thermal aggregation study of a protein is a useful method to show the resistance of that protein against thermal denaturation. With increasing of the protein thermal stability, its thermal aggregation lag time increases and the logarithmic phase slope of the protein thermal aggregation decreases, and with the unfolding of the protein, the protein thermal aggregation occurs in shorter lag phase along with the sharp

logarithmic phase slope [56]. Fig. 6a and 6b show the effect of surface functional group of the ZnS QDs and the effect of the alkyl-chain length of capping agents on the thermal aggregation profile of insulin, respectively. Results of Fig. 6 (the lag time (t_0) and the aggregation rate constant (k_1)) are summarized in Table 2. In the presence of the ZnS-CYS QDs no aggregation was observed for insulin after 100 min, therefore the presence of ZnS-CYS QDs significantly increases the insulin thermal stability. Among the QDs with different surface functional group, the ZnS-ME QD has the worst effect on the thermal stability of insulin.

Fig. 6b shows that in interaction between insulin and the ZnS QDs, with increasing the surface hydrophobicity of the QDs (by increasing the alkyl chain length of capping agents) the thermal aggregation of insulin occurs sooner (small lag time). These results have a good agreement with the CD results, and indicate that in biomedical applications of the ZnS QDs and in interaction of these materials with biomolecules such as proteins, the surface functional groups and hydrophobicity of the QDs play significant roles.

4. Discussion

Fig.7 shows the molecular structure of the different capping agents, and schematic illustration of the surface characteristics of the ZnS QDs and insulin. Based on the zeta potential measurements and according to the molecular structure of capping agents, it can be concluded that when surface of the ZnS QDs is capped with ME, CYS, MHA, MUA, MPA, TGA, and MSA capping agents, the surface charge of the related ZnS QDs is negative due to the dissociation of carboxyl (-COOH) and hydroxyl (-OH) functional groups in aqueous solution. When the ZnS QDs surface is capped with the basic CA capping agent, the surface charge is positive due to the protonation of its amine (-NH₂) functional groups. In electrostatic interactions of insulin with the charged QDs surface, the residues of the protein with an opposite charge with respect to the QDs surface are attracted, and residues with same charge are repulsed from the QDs surface. Opposite electrostatic interactions of different parts of protein may force the protein to denature. CYS capping agent is an amino acid that contains both -NH₂ and -COOH functional groups in its structure. Although ZnS-CYS QDs have the negative net charge, however, due to the dissociation of -COOH and

slight protonation of -NH_2 functional groups of CYS, the ZnS-CYS QDs can contain simultaneously the positively and negatively charged parts on its surface (Fig.7). As a result of the interaction of the ZnS-CYS QDs with insulin, the protein is adsorbed to the ZnS-CYS QD surface, and adsorption of the protein to the ZnS-CYS QD surface reduces the protein thermal fluctuations and enhances insulin stabilization by hindering the thermal denaturation. As Fig.7 shows, the only difference between CYS and MPA, is the presence of -NH_2 in carbon alpha position of CYS, however presence of this group and slight protonation of it gives doubly charged nature to ZnS-CYS QDs. There are some reports for increasing protein stability by carbon nanotubes [57], however to the best of our knowledge, in the case of QDs there is no such report, and the increasing insulin stability by ZnS-CYS QDs is the first one.

Based on the ME molecular structure and according to the zeta potential test results, ME capping agent has electrostatic, dipole-dipole and hydrogen bond interactions with insulin. From the CD results it can be concluded that ME, because of its small size and through its hydrophobic alkyl-chain, can penetrate into the hydrophobic pocket of insulin and increases the beta-sheet contents of the protein and thereby destabilizes insulin. In mercaptocarboxylic capping agents with long alkyl-chain such as MHA and MUA, because of their long hydrophobic alkyl chain and the low dissociation of their carboxyl group, nonpolar-nonpolar interactions between their hydrophobic alkyl chain and the hydrophobic parts of insulin play an important role in their interaction with insulin and insulin destabilization [58]. However, in MSA, TGA, and MPA capping agents, because of the high dissociation constant of these capping agents, the QDs capped with them have many negatively charged groups on their surface and in interaction with insulin, the electrostatic interactions are dominant ones.

At this work the interaction of insulin with the differently functionalized ZnS QDs was studied by the fluorescence quenching, synchronous fluorescence, CD, and thermal aggregation techniques and based on the obtained results CYS amino acid was suggested as best capping agent for the functionalization of the ZnS QDs. There are many studies in this field which some of the selected works would be discussed in the following. Khani et al in their work studied the interaction of bovine serum albumin (BSA) with the ME capped ZnS and $\text{Zn}_{(1-x)}\text{Fe}_x\text{S}$ QDs, they proved that the fluorescence quenching of BSA by these QDs is

related to the formation of the QDs–BSA complex and they extracted the thermodynamic parameters of the interaction from the fluorescence quenching data [34]. Ghali studied the interaction of the TGA capped CdS QDs with BSA by fluorescence quenching technique, and demonstrated that because of the static fluorescence quenching, the CdS–BSA complex is formed during the interaction of CdS QDs with BSA [59]. Liang et al worked on the interaction of the TGA capped CdTe QDs with BSA by fluorescence quenching technique, and proved that the hydrophobic force and sulfhydryl group played a key role in this interaction and structural changes of BSA [60]. Shen et al worked on the interaction of the thioacetamine capped CdS QDs with human hemoglobin by CD, fluorescence quenching and Raman techniques, and demonstrated that because of this interaction human hemoglobin was denatured [61]. Xiao et al studied the influence of the CdTe QDs sizes on the interaction of these QDs with HSA by fluorescence quenching and CD techniques, and indicated that the biological activity of HSA was weakened in the present of the CdTe QDs with bigger size [62]. In comparison with these works, the major novelty of the present study is the investigation of the effects of the various functional groups of hydroxyl, amine, carboxyl and amino acid at the surface of the ZnS QDs on the interactions of the ZnS QDs with insulin. The effects of alkyl chain lengths on these interactions have also been studied in the present work. Such comprehensive and comparative study has not yet reported to date in the field of the protein-QDs interactions.

5. Conclusion

In this work, the interaction of insulin with the differently functionalized ZnS QDs was studied. The ZnS QDs were functionalized with various functional groups of hydroxyl (-OH), carboxyl (-COOH), amine (-NH₂), and amino acid (-COOH and -NH₂) and due to the dissociation of -OH and -COOH groups and protonation of -NH₂ group in aqueous solution, these QDs acquires a net charge on their surfaces. In interaction of insulin with positively charged QDs (i.e. Cysteamine capped QDs (CA-ZnS QDs)), positively charged residues of insulin (such as Arginine and Lysine) are repulsed from the QDs surface, and negatively charged residues (such as Aspartic acid and Glutamic acid) are attracted to the QDs surface, because of the formation of such opposite forces the protein undergoes denaturation. This condition is reversed for interaction of insulin with negatively charged QDs (such as Thioglycolic acid capped QDs (TGA-ZnS

QDs)). However, in the interaction of insulin with the L-cysteine capped ZnS QDs (ZnS-CYS), due to the presence of both -NH₂ and -COOH functional groups on the surface of this QDs, ZnS-CYS QDs can contain simultaneously the positively and negatively charged parts on its surface, and for this reason all of the charged residues of insulin can be attracted toward the ZnS-CYS QD surface. Interaction of insulin with ZnS-CYS QDs increases the alpha-helix contents of the protein, which in turn improves the thermal stability of insulin. In interaction between ZnS-ME QDs and insulin, the beta-sheet and random-coil contents of the protein increases and the alpha-helix contents decreases, resulting in the insulin destabilization. It also revealed that in interaction of insulin with the ZnS QDs functionalized by longer alkyl-chain length capping agents, due to the interaction of the hydrophobic parts of the capping agents with hydrophobic core of the protein, the protein undergoes more destabilization, leading to the protein unfolding. In summary, the results of the present work show that the surface capping agents with amino acid functional groups and suitable alkyl-chain lengths are the better choice for nanomaterial functionalization in biomedical applications.

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Fig. 1. XRD patterns of ZnS-QDs stabilized with different capping agents.

Fig. 2. (A) Optical absorption spectra and (B) fluorescence emission spectra of (a) ZnS-ME, (b) ZnS-CA, (c) ZnS-CYS, (d) ZnS-MSA, (e) ZnS-TGA, (f) ZnS-MPA, (g) ZnS-MHA and (h) ZnS-MUA QDs.

Fig. 3. Absorption and fluorescence spectra of 2-Aminopyridine and ZnS-CYS QDs.

Fig. 4. TEM image of ZnS-CYS QDs.

Fig. 5. Circular dichroism spectra of insulin in the absence and in the presence of the ZnS QDs with different surface functional group (a) and different alkyl chain length of capping agents (b).

Fig. 6. Thermal aggregation of insulin in the absence and in the presence of the ZnS QDs with different surface functional group (a) and different alkyl chain length of capping agents (b).

Fig. 7. Molecular structure of the different capping agents, and schematic illustration of the surface characteristics of the ZnS QDs and insulin (Insulin was shown in surface representation and polar, nonpolar negatively charged, and positively charged parts of it were depicted with green, yellow, red, and blue colors, respectively).

Table 1. Binding (Stern–Volmer quenching constant, binding constant and number of binding sites) and thermodynamic parameters for the interaction of insulin with the ZnS QDs.

	$K_{SV}(M^{-1})$	n	$K_a(M^{-1})$	$\Delta H^\circ (kJ/mol)$	$\Delta S^\circ (J/molK)$	$\Delta G^\circ (kJ/mol)$
ZnS-ME						
T=295 K	8.33×10^5	*****	*****	*****	*****	*****
T=303 K	9.56×10^5	*****	*****	*****	*****	*****
T=310 K	1.07×10^6	*****	*****	*****	*****	*****
ZnS-CA						
T=295 K	2.52×10^6	0.9852	1.42×10^6	-32.38	7.89	-34.71
T=303 K	2.23×10^6	0.9491	9.47×10^5	-32.38	7.89	-34.77
T=310 K	1.81×10^6	0.9161	7.53×10^5	-32.38	7.89	-34.83
ZnS-CYS						
T=295 K	2.04×10^6	1.0601	4.24×10^6	-31.58	19.65	-37.39
T=303 K	1.58×10^6	1.0415	2.82×10^6	-31.58	19.65	-37.54
T=310 K	1.58×10^6	1.0195	2.28×10^6	-31.58	19.65	-37.67
ZnS-MSA						
T=295 K	4.92×10^6	1.1245	2.91×10^7	154.85	665.64	-41.51
T=303 K	4.87×10^6	1.2805	2.44×10^8	154.85	665.64	-46.84
T=310 K	4.00×10^6	1.3228	5.87×10^8	154.85	665.64	-51.50
ZnS-TGA						
T=295 K	2.65×10^6	1.1457	2.52×10^7	-86.40	-150.67	-41.96
T=303 K	2.52×10^6	1.1084	1.21×10^7	-86.40	-150.67	-40.75
T=310 K	2.37×10^6	1.0358	4.53×10^6	-86.40	-150.67	-39.69
ZnS-MPA						
T=295 K	1.44×10^6	1.1583	1.49×10^8	-110.18	-236.26	-40.48
T=303 K	1.22×10^6	1.0863	4.37×10^7	-110.18	-236.26	-38.59
T=310 K	1.22×10^6	1.0145	1.70×10^6	-110.18	-236.26	-36.94
ZnS-MHA						
T=295 K	7.71×10^5	1.1584	7.23×10^6	-108.06	-235.33	-38.64
T=303 K	7.52×10^5	1.0683	1.98×10^6	-108.06	-235.33	-36.76
T=310 K	7.50×10^5	1.0093	8.65×10^5	-108.06	-235.33	-35.11
ZnS-MUA						
T=295 K	6.72×10^5	1.0322	1.04×10^6	-106.05	-243.90	-34.11
T=303 K	6.36×10^5	0.9769	3.89×10^5	-106.05	-243.90	-32.16
T=310 K	5.15×10^5	0.8852	1.28×10^5	-106.05	-243.90	-30.45

Table 2. Thermal aggregation parameters of insulin in the absence and the presence of the different ZnS QDs.

	insulin	Insulin + ZnS-ME	Insulin + ZnS-CA	Insulin + ZnS-CYS	Insulin + ZnS-MSA	Insulin + ZnS-TGA	Insulin + ZnS-MPA	Insulin + ZnS-MHA	Insulin + ZnS-MUA
t_0 (min)	42.3	10.11	17.04	*	27.85	20.23	17.16	10.64	7.52
k_1 (min ⁻¹)	0.0150	0.1451	0.0536	*	0.0381	0.0510	0.0821	0.1179	0.1341

*. No aggregation after 100 min