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Synthesis, Characterization and Spectroscopic Studies on the Interaction Between *β*-Casein and Co(II) Schiff Base Complex

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In this study, Co(II) complex of a Schiff base was synthesized and characterized. The *in vitro* binding behavior of this complex with β-Casein (β-CN) was investigated using biophysical techniques. For evaluation the thermodynamics parameters of interactions between Schiff base complex and β-CN, the fluorescence data at different temperatures were obtained. Based on the results, the complex shows a significant binding affinity to β-CN and the process is enthalpy driven. The β-CN conformation was also changed through inducing a more unfolded structure. Fluorescence resonance energy transfer was used to estimate the distance between donor (β-CN) and acceptor (complex). Also, ligand and its complex were reacted with different bacteria (*Escherichia coli*, *Bacillus subbtilis, staphylococcus aureus*) to examin the inhibitory action of compounds. The results showed that the antimicrobial activity of metal complex is more than that of ligand. Our results confirm the ability of β-CN as a protein carrier for Co(II) complex to deliver it to the target molecules.

Keywords: β-Casein binding, Schiff base complex, Fluorescence quenching, Thermodynamic parameters, Secondary structure

INTRODUCTION

Due to the growing knowledge of the significance of food in preventing disease, modern technologies for enriching different types of foods and drinks have been recommended using several types of bioactive compounds to produce supplementary foods. Due to the growing utilization of low-fat and non-fat foods, addition of lipid-soluble additives to foods seems to be more necessary compared to that of water-souble additeves. However, formulation of lipid-soluble additives are very challenging. Numerous hydrophobic compounds, such as essential fatty acids and lipid-soluble vitamins are sensitized to oxidation and they need to conserving from destructive agents. In recent years, using nano-carriers to deliver these nutritions has increased their stability and solubility. Proteins of milk have significant functional properties such as ability to bond to hydrophilic molecules, ability to organize gels, interact with biopolymers, and stabilize emulsions. Due to these properties, casein is known as a perfect tool for delivery and enticement of bioactive molecules [1].

β-Casein (β-CN), classified among four major caseins in bovine milk, contains 209 residues in an individual chain with the molecular weight of 23.983 kDa. Bovine β-CN is an extremely amphiphilic protein consisting of a hydrophilic N-terminal segment with a hydrophobic C-terminal segment and a group consists of five phosphoryl residues [2]. Radius of gyration (R_g) of single β-CN molecules are 4.6 nm and its isoelectric pH (pI) is 5.33. The micelles including about 16-60 molecules of β-CN have a R_g value of 7.2 to 13.4 nm [3]. It must be attended that bovine β-CN is recognized as a dynamical protein with a flexible and non-condensed conformation [4].

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Previous studies have shown that the average accumulation number of β-CN micelle is within 45.80 [5]. Therefore, β-CN micelles have been used as the potential carriers for lipid-soluble drugs such as sucrose esters, vitamins A, K, E and D [6]. Also, β-CN nanoparticles can capture and carry hydrophobic chemotherapeutics [7-9]. The simple digestion of β-CN guaranties the easy liberation of encapsulated chemotherapeutics drugs in the stomach [10]. Compounds with a functional group containing -C=N- (azomethine functional group) are well-known in the role of Schiff bases, which are generally prepared by the condensation reaction of carbonyl groups and primary amines [11]. Schiff bases are mainly studied because of their sensitivity to the metallic ions, synthetic flexibility and selectivity [12]. The origin of anticancer activity of Schiff bases has been ascribed to their ability to cleave DNA with intercalating between DNA base pairs [13]. As Schiff bases are potentially anticancer drugs, studies on their properties in binding to the plasma carrier proteins would be of particular importance.

Schiff bases and their metal complexes have a diversity of biological applications in medicinal and pharmaceutical region. They have antimicrobial, antibacterial, antifungal, antioxidant, antimalarial and anticancer properties [14,15]. The purpose of this study was to explore the potential of bovine β-casein micelles as a carrier for bioactive Schiff base complex. At first, the Schiff base complex (Scheme 1) was synthesized and characterized by ^{1}H NMR, ^{13}C NMR and FT-IR spectroscopies. After that, the β-CN interaction of the complex in aqueous solutions was explored using fluorescence, UV-Vis spectroscopy and circular dichroism mthods.

EXPERIMENTAL

Materials

Bovine milk β-CN (98%, Sigma Company) was dissolved in pH 7.0 phosphate buffer comprising 80.0 mM NaCl, 5.60 mM Na_2HPO_4 and 3.10 mM NaH_2PO_4 with an ionic strength of 0.10. 2-Hydroxy-3-methoxybenzaldehyde and arginine were obtained from Merck chemical company and used without further purification. All the salts employed for buffer prepration were of analytic quality and dissolved in doubled distilled water.

For preparing of β-CN micelle solutions, the β-CN solution was filtrated *via* a 0.45 μm aporous membrane. Next, they were dialyzed versus the phosphate buffer for 24 h at 4 ºC, to prevent formation of Ca^{2+} bridge using 1 mM EDTA solution [16]. After dialysis, the β-CN concentration was specified spectrophotometrically by employing $\varepsilon = 0.44$ ml mg^{-1} cm^{-1} at 280 nm [17].

Synthesis of the Schiff Base Complex

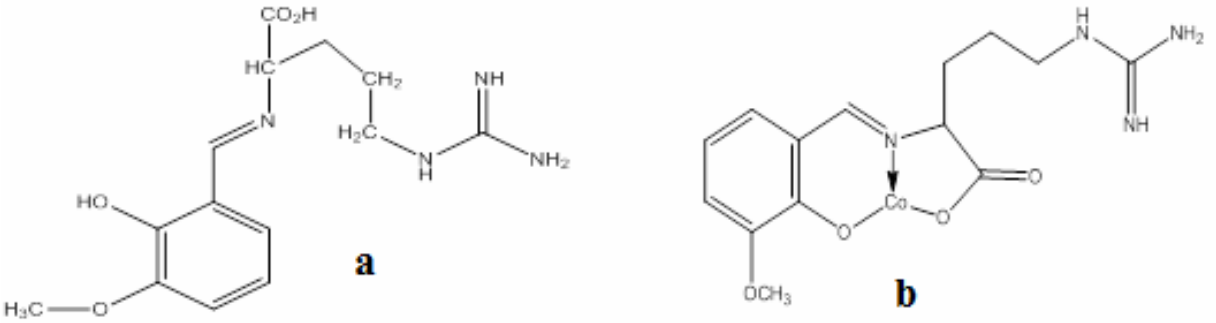
An ethanolic solution of 2-hydroxy-3-methoxybenzaldehyde (1 mmol) was added dropwisely to a firmly stirred solution of arginine (1 mmol) in 20 ml ethanol. The resulting solution was refluxed for 3 h. The light-yellow crystals were obtained on slow evaporation of the solvent.

In order to form the metal complex, the ligand (1 mmol) in distilled acetone was added to a solution of cobalt(II) acetate (2 mmol) in distilled ethanol dropwise with consistent stirring for 2 h. The reaction mixture was evaporated, and the obtained precipitate was washed and dried *in vacuo*.

Reaction was detected using thin layer chromatography (TLC). Melting point was controlled on a apparatus Electrothermal IA9100. A THERMO IR100 FT-IR spectrometer was used to record the FT-IR spectrum to investigate the functional groups of Schiff base ligand. NMR spectra of Schiff base ligand were registered in DMSO-d_6 as standard on a Bruker Vector 33 NMR spectrometer (400 MHz, SF:300) to recognize its molecular structure.

Antimicrobial Activity

In order to examine the possibility of the synthesized products against different strains of bacteria, the antibacterial activity analysis was performed. Disc diffusion procedure was used for the antibacterial activity analysis of the synthesized products (ligand and its complex) against *staphylococcus aureus, Bacillus Subbtilis* and *Escherichia coli*. The medium was sterilized at 121 °C for 30 min. After mixing with distilled water, the nutritious agar was dispersed uniformly. The medium was transferred to the petri plates where the filter paper discs were located. The



Scheme 1. Chemical structures of (a) Schiff base ligand and (b) Cobalt(II) complex

compounds were placed individually in separate discs of the filter paper and were remained for 24 h at 37 °C [18,19]. The ligand and its complex showed significant antibacterial activities which created obvious regions of prohibition in the form of circles. The regions for each compound were evaluated in mm. The results were compared with the Rifamipicin (standard drug).

Protein Binding Studies

Fluorescence measurements were carried out using a spectrophotometer (HITACHI F-2500) with the following excitation wavelengths at 280 and 295 nm, along with the excitation and emission wavelengths registered from 300 to 500 nm. The excitation and emission bandwidths were fixed at 5 nm.

Synchronous fluorescence spectroscopy was performed through simultaneously scanning the emission and excitation monochromators. The obtained spectra have shown the Tyr and Trp residues of the protein when the wavelength interval ($\Delta\lambda$) had been 60 and 15 nm, respectively. The absorption spectra were registered at ambient temperature on a SPEKOL 1500 UV-Vis spectrophotometer eqqiped with 1 cm quartz cells.

RESULTS AND DISCUSSION

Spectral Data of the Synthesized Schiff Base Ligand

The synthesized Schiff base is a colored solid and water soluble material which is persistent and has the melting point of 146 °C. Its structure was proved through spectroscopic data (^{1}H NMR, ^{13}C NMR and FT-IR). Yellow solid, m.p.: 146 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.50 (m, 2H), 1.70-1.90 (m, 2H), 3.19 (m, 2H), 3.91 (m, 1H), 6.57 (t, 1H, Ar-H), 6.90 (m, 2H, Ar-H), 7.23 (t, 1H, Ar-H), 7.79 (br, 3H, NH_2, C=N<u>H</u>), 8.39 (s, 1H, C<u>H</u>=N), 9.0 (s, 1H, NH), 10.50 (s, 1H, carboxylic OH), 14.30 (s, br, 1H, phenolic OH); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 25.9, 31.6, 56.0, 56.5, 69.4, 114.7, 115.1, 117.1, 117.9, 119.2, 120.5, 123.1, 124.3, 149.2, 150.2, 152.1, 157.9, 159.7, 164.4, 174.6, 192.4.

The ^{1}H NMR spectra affirm the identity of the synthesized Schiff base, until identify its structure. The ^{1}H NMR spectrum of the synthesized Schiff base (Scheme 1a) exhibited the azomethine protons at 8.39 ppm as a singlet peak [20,21]. The compound has aromatic rings, the hydrogens of the aromatic rings were confirmed in the range of 6.57-7.23 ppm.

The FT-IR spectrum of the synthesized Schiff base is represented in Fig. 1. The remarkable infra red bands of the compound are specified in Table 1. The FT-IR spectrum displays a band allocated on azomethine stretching vibrations at at 1543 cm^{-1} affirming the Schiff base structure [22-24]. The bands at 1629 and 1221 cm^{-1} are attributed to C=O and C-O stretching vibrations, respectively. The broad bands located at 3168 and 3350 cm^{-1} are corresponded to N-H and O-H groups, respectively [25].

Antibacterial Activity Analysis

The antibacterial activity analysis was performed by using disc diffusion procedure. Three bacterial strains were used: *Staphylococcus aureus, Bacillus subbtilis* and

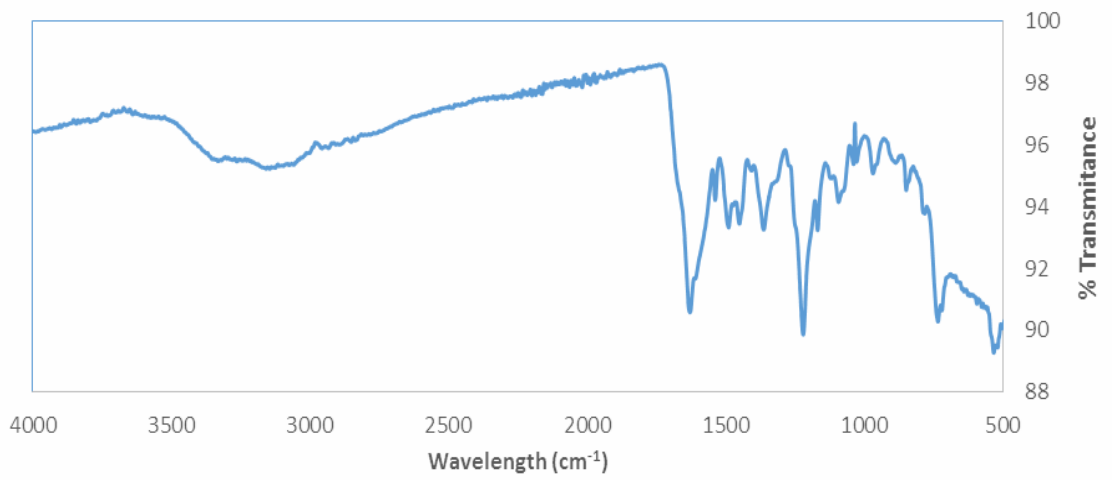


Fig. 1. FT-IR spectrum of Schiff base ligand.

Table 1. Spectroscopic Data (FT-IR) (cm^{-1}) of the Synthesized Schiff Base

Compound	ν (-CH=N-)	ν (C=O)	ν (C-O)	ν (N-H)	ν (O-H)
Synthesized Schiff base	1543	1629	1221	3168	3350

Table 2. Antibacterial Activity Analysis of Ligand and Metal Complex

Compound	Staphylococcus aureus	Bacillus subbtilis	Escherichia coli
Schiff base ligand	6.32 ± 1.31	4.96 ± 2.00	5.85 ± 0.56
Co (II)-complex	14.71 ± 0.45	11.45 ± 1.16	12.58 ± 0.92
Rifampicin	20.00 ± 0.2	18.00 ± 1.2	19.00 ± 0.5 [26]

Escherichia coli. The results determined that the cobalt complex has a greater antibacterial activity compared to the synthesized ligand. The results were compared with the standard drug (Rifamipicin) which displayed greatest activity (Table 2).

Fluorescence Quenching

To decrease the inner filter effect, absorption spectra of β-CN at 280 nm (excitation wavelength) were registered and the fluorescence intensities used in this research were all corrected using Eq. (1) [27]:

$$F_c = F * anti\log\left\{\frac{A_{ex} + A_{em}}{2}\right\} \quad (1)$$

where F and F_c are the initial and corrected intensity of fluorescence, respectively, A_{ex} is absorption of ligand in the excitation wavelength and A_{em} is absorption of complex in the emission wavelength.

The intrinsic fluorescence of β-CN is related to Trp-143

(tryptophan). This tryptophan residue is mainly placed in the hydrophobic region of this protein. When other molecules interact with β-CN, depending the effect of interaction on the protein configuration, the fluorescence of tryptophan may change [28]. Figure 2 displays fluorescence spectra of β-CN in various concentrations of ligand at $\lambda_{ex} = 280$.

It can be noted that Schiff base complex quenched fluorescence of β-CN and maximum emission wavelength has only a weak red shift (about 3 nm) (Fig. 2a). The slight red shift in the fluorescence spectra is correlated with an enhanced polarity of the microenvironment after binding [29].

For analysis of β-CN fluorescence quenching, the fluorescence intensity data of β-CN at 298, 305 and 310 K were analyzed in accordance with the Stern-Volmer equation [30]:

$$\frac{F_0}{F} = 1 + K_q \tau_0 [Q] = 1 + K_{SV}[Q] \quad (2)$$

where F and F_0 are the intensities of fluorescence in the presence and absence of quencher, respectively; K_q is the quenching rate constant for the biomolecule, K_{SV} is the Stern-Volmer dynamic quenching constant, τ_0 is the mean lifetime of the fluorophore without quencher (τ_0 = 3.3 ns for β-CN), and [Q] is the quencher concentration. The plots of F_0/F against Schiff base complex at three different temperatures are presented in Fig. 2b. The quantities of K_{SV} and K_q at different temperatures are given in Table 3.

The data indicated that with increasing temperature, the values of K_{SV} decreased and the order of value of K_q was estimated to be 10^{12} for β-CN-Schiff base complex, which were bigger than the maximal scatter collision quenching constant of different quenchers with biopolymers (2.0×10^{10} l mol^{-1} s^{-1}) [31].

Identification of Binding Parameters

The Scatchard equation is applied to evaluate the binding constant of ligand to protein (K_a) and the binding sites number (n) concerned with the binding interaction; it is known as [32]:

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

Figure 3 shows the plots of log(F0 - F)/F against log[Q] for the interaction between β-CN and Schiff base complex at different temperatures. n and Ka obtained from the plots are listed in Table 4.

It may be concluded from Table 4 that the reducing tendency of K_a with growing temperature is in agreement with K_{SV}'s dependence on temperature as discussed about, a feature that is in accordance with the type of static quenching [30].

Thermodynamic Parameters

The interaction forces between proteins and ligands include H-bonds, hydrophobic and electrostatic interactions, and Van der Waals forces. In accordance with the Van't Hoff equation, when the temperature does not change extremely, the enthalpy (ΔH°) and entropy (ΔS°) changes of the binding reaction can give proof for clarification of the type of interaction. If ΔH° does not alter remarkably through the temperature range specified, the values of ΔH°, ΔS° and ΔG° are calculated by the following equation:

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

where R is the universal gas constant, T is the experiential temperature (K) and K_a is the binding constant. The entropy (ΔS°) and the enthalpy (ΔH°) changes can be achieved from the intercept and slope of the fitted curve of $\ln K_a$ against 1/T, respectively. Later, the Gibbs free energy, ΔG°, was obtained using Eq. (5) [33]:

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

Ross and Subramanian described the sign and value of the thermodynamic variables involved in the protein association processes. The calculated thermodynamic variables are summarized in Table 4.

It can be concluded from results that the binding reaction has spontaneously occurred (ΔG° < 0). Additionally, the negative enthalpy (ΔH° < 0) and entropy (ΔS° < 0) demonstrates the important roles of hydrogen bonding and van der Waals interactions in the Schiff base complex binding to β-CN.

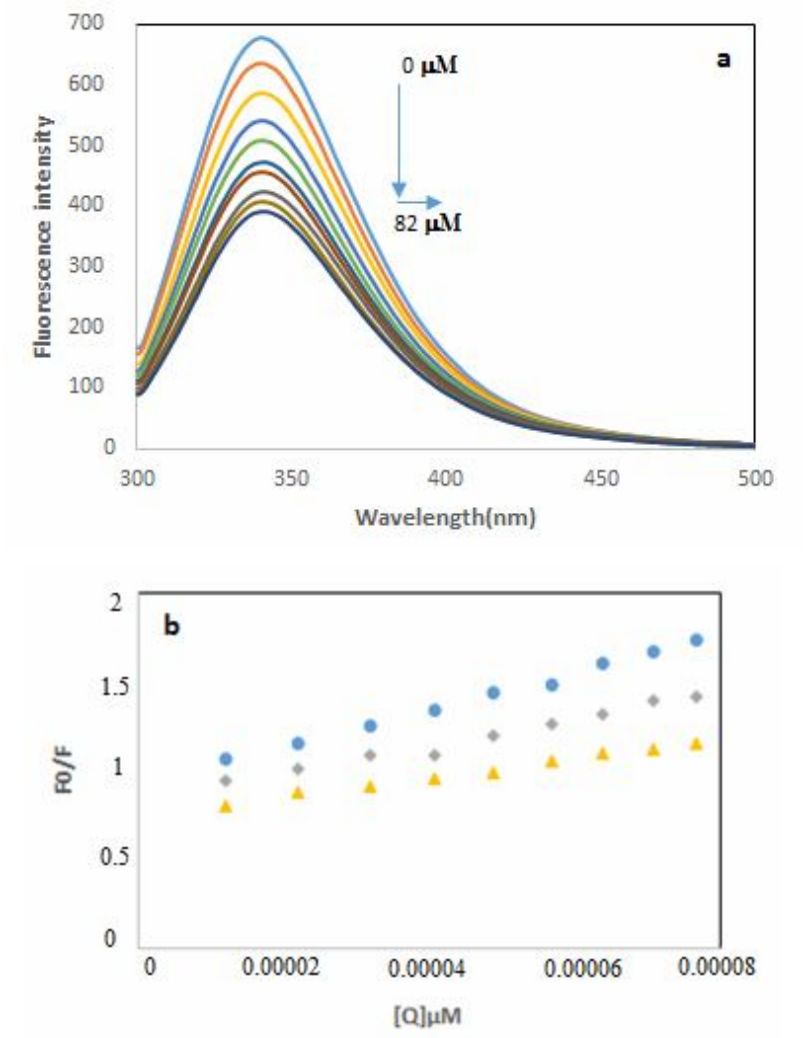


Fig. 2. (a) Emission fluorescence spectra of β-CN at various concentrations (0-82 µM) of Schiff base complex at λ_{ex} = 280 nm, T = 298 K, pH 7.4. (b) Stern-Volmer curves for quenching of β-CN with schiff base complex at 298, 305 and 310 K.

Table 3. Stern-Volmer Constants for the Interaction of β-CN with Complex at Different Temperatures

T (K)	K_{sv} ($l\ mol^{-1}$)	K_q ($l\ mol^{-1}\ s^{-1}$)	R^a
298	9.94×10^4	3.01×10^{13}	0.992
305	7.13×10^4	2.16×10^{13}	0.982
310	4.96×10^4	1.50×10^{13}	0.985

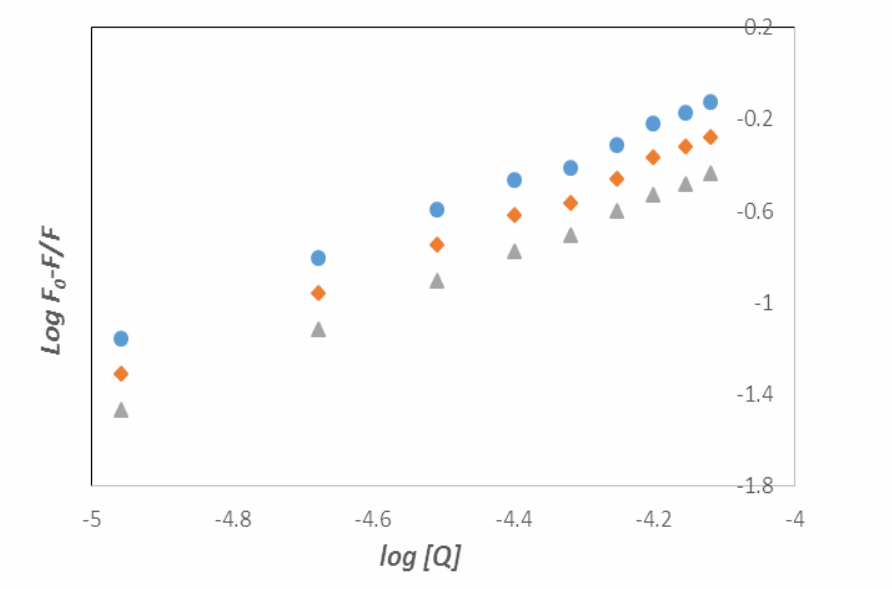


Fig. 3. Plot of log[(F_o - F)/F] *vs.* log[Q] for the fluorescence quenching of β-CN at different concentrations of schiff base complex at different temperatures.

Table 4. Binding Constant (K_a) and Relative Thermodynamic Parameters of the β-CN-Complex System

T (K)	K_a (l mol^{-1})	n	R^a	ΔH° (kJ mol^{-1})	ΔG° (kJ mol^{-1})	ΔS° (J mol^{-1} K^{-1})
298	7.404×10^4	1.21	0.997	-40.320	-27.799	-42.014
305	5.242×10^4	1.21	0.998		-27.505	
310	3.929×10^4	1.22	0.999		-27.295	

[a]Correlation coefficient.

Fluorescence Resonance Energy Transfer (FRET) Between β-CN and Schiff Base Complex

The energy transfer phenomena are classified into radiative and nonradiative energy transfer modes in fluorescence. According to Forster's theory [34,35], the energy transfer depends on the distance between the donor and acceptor (r) and the critical energy transfer distance (R_0).

The relationship between FRET efficiency and distance is as follows:

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \quad (6)$$

where E is energy transfer efficiency; F and F_0 are fluorescence intensities of β-CN in the presence and absence of complex. R_0 is calculated using the following equation:

$$R_0^6 = 8.79 \times 10^{-25}\ K^2\ N^{-4}\ \Phi\ J \quad (7)$$

where K^2 denotes the spatial orientation factor, medium refractive index is given as "*N*" where N = 1.53.Φ is the donor's fluorescence quantum yield in the absence of acceptor which is equal to Φ = 1.49, and J is the effect of spectra overlap between the fluorescence emission spectrum of the donor and absorption spectrum of the acceptor and is given by:

$$J = \Sigma F(\lambda)\, \varepsilon(\lambda)\, \lambda^4\, \Delta\lambda / \Sigma F(\lambda)\, \Delta\lambda \quad (8)$$

where F (λ) is the emission spectrum Fluorescent donor, and ε (λ) denotes molar absorption coefficient of the acceptor. From the above equations the *r* value was estimated to be 3.86 nm. Figure 4 displays an overlap among the absorption spectra of Schiff base complex and the emission spectra of β-CN.

Since the distance between acceptor and donor is found to be lower than 7 nm, there is a high opportunity for nonradiative energy transfer between β-CN and Schiff base complex [36]. Moreover, the value of R_0 is comparatively less than r, accordingly, the static quenching mechanism manages the intrinsic fluorescence quenching.

FT-IR Studies

FTIR has been considerably utilized to collect information about protein conformation and structural changes by measuring the frequencies or energies of molecular vibrations. IR spectra of the pure protein solution and every solution of the Schiff base complex-protein mixture were collected using hydrated films and the difference spectrum was produced by subtracting the spectra of the β-CN solution from that of the Schiff base-β-CN complex by following the method described by Dousseau et al., 1989 [37]. The water subtraction was carried out using 0.1 M NaCl solution as a reference at pH 6.5-7.5. This method is a rough estimate but removes the water content in a satisfactory way [38]. Ligand-protein interactions induce protein conformational change and alter spectral change for protein amide I band at 1600-1700 cm^{-1} (mostly C=O stretch) and amide II band at 1500-1600 cm^{-1} (C-N stretching together with N-H bending modes) [39]. Figure 5 shows the variations of intensity of β-CN amide I and amide II bands, due to binding the Schiff base complex to the C-N, C=O and N-H groups of protein [40].

Such phenomenon also suggested that the arrangement of the polypeptide carbonyl hydrogen bonding model and the secondary structure of β-CN have been changed.

Circular Dichroism Study

The circular dichroism (CD) spectroscopy is a delicate method to observe the secondary structural alterations in the protein [41]. CD spectra of β-CN without and with diverse concentrations of Schiff base complex are displayed in Fig. 6.

The protein conformation analysis based on CD information (Table 5) suggests that free β-CN has α-helical 36%, β-sheet 0%, turn 34% and random coil 30%. Upon Schiff base complex interaction, significant reduction of α-helix was occurred from 36% to 31% and 7% (in 0.26 and 0.48 μM of Schiff base complex, respectively). The reduction in α-helix was occurred with an increase in the random coil and turn structures. The great decrement of the ***α***-helix with an increment in the random structures is due to a major protein unfolding.

Absorption Study

Since this Schiff base complex has a UV absorption spectrum from 300 to 500 nm, the absorbance titrations at these wavelengths were carried out at constant Schiff base complex concentration (30 mM), whereas the β-CN concentration was changed (Fig. 7).

With increasing the β-CN concentration, Schiff base complex showed an increase with no shift in absorption band. Changes in maximum absorbance evidently support the formation of complex between protein and ligand.

CONCLUSIONS

In this study, the Schiff base complex was synthesized and specified by diverse spectroscopic techniques. Binding of Schiff base complex with β-CN was studied by multi-spectroscopic techniques. The outcomes of the fluorescence studies demonstrated that Schiff base complex is able to bind to β-CN and quench the fluorescence of protein with static mechanism. Binding parameters between β-CN and Schiff base complex such as the number of binding sites and binding constant were determined. The thermodynamic results displayed that hydrogen bonds and Van der Waals

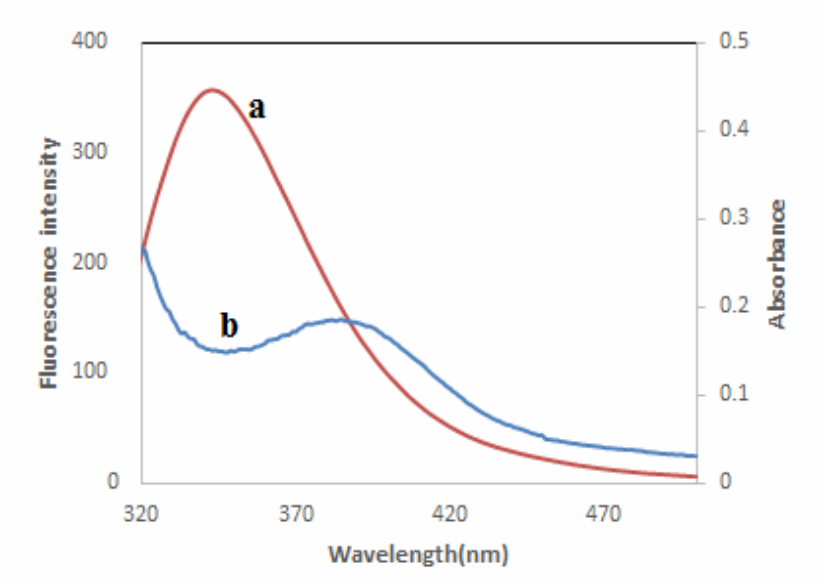


Fig. 4. The overlap of the fluorescence emission spectrum of β-CN (curve a) with the absorption spectrum of schiff base complex (curve b) at 298 K; [β-CN]:[complex] = 1:1.

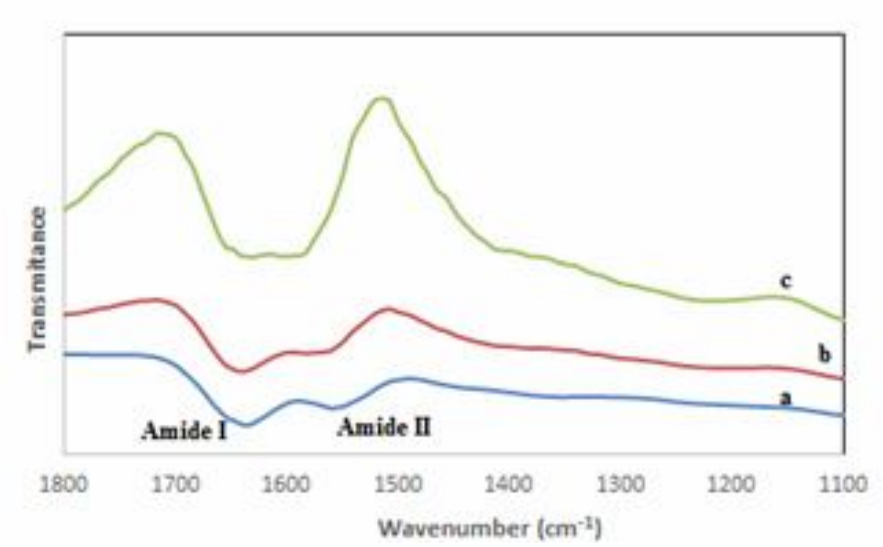


Fig. 5. FT-IRspectra of (a) free β-CN, (b) β-CN + 0.26 μM complex, (c) β-CN + 0.48 μM complex in a pH = 7.4 buffer solution in the region of 1800-1100 cm^{-1}.

forces play a principal role in binding of schiff base complex to β-CN. Binding of Schiff base complex to β-CN persuaded structural alternations in the configuration of the protein and a decrease in α-helix percentage. The antimicrobial activity analysis data showed that antibacterial activity of metal complex is greater than that of its ligand. All results revealed that Schiff base complex bind to β-CN efficiently, which might be a beneficial guide for Schiff base drug design and β-CN and seems to be the promising Schiff base complex carrier.

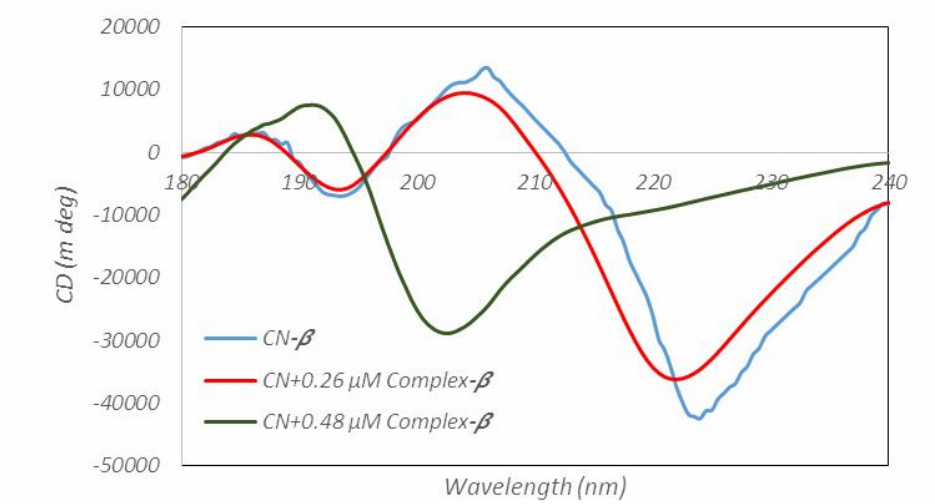


Fig. 6. Far UV-CD spectra of β-CN in the presence and absence of different concentrations of schiff base complex, pH = 7.4.

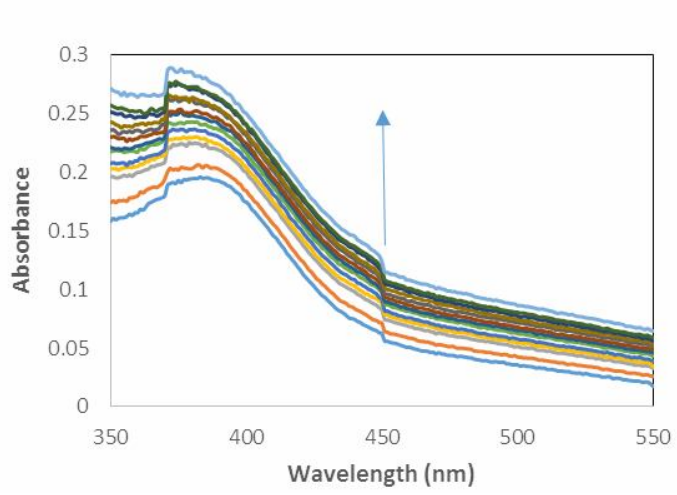


Fig. 7. Absorption spectra of Schiff base complex in the presence of various concentrations of β-CN, pH = 7.4.

Table 5. Secondary Structures of β-CN in the Presence of Different Concentrations of Schiff Base Complex, pH = 7.4

System	α-helix%	β-sheet%	Turn%	Random coil%
β-CN	36.47	0.00	33.80	29.73
β-CN-0.26 μM complex	31.70	0.00	24.70	43.60
β-CN-0.48 μM complex	07.50	17.60	0.00	74.80

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