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STUDY OF INTERACTION BETWEEN TRYPTOPHAN, TYROSINE, AND PHENYLALANINE SEPARATELY WITH SILVER NANOPARTICLES BY FLUORESCENCE QUENCHING METHOD

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Using the spectroscopic method, the individual interaction of the three biochemically important amino acids, which are constituents of protein, namely, tryptophan, tyrosine, and phenylalanine with biologically synthesized silver nanoparticles has been investigated. The obtained UV-Vis spectra show the formation of ground-state complexes between tryptophan, tyrosine, and phenylalanine with silver nanoparticles. Silver nanoparticles possess the ability to quench the intrinsic fluorescence of the aforesaid amino acids by a dynamic quenching process. The binding constant, number of binding sites, and corresponding thermodynamic parameters (ΔH, ΔS, and ΔG) based on the interaction system were calculated for 293, 303, and 313 K. In the case of tryptophan and phenylalanine, with increase in temperature, the binding constant K was found to decrease; conversely, it was found to increase with increase in temperature in the case of tyrosine. The thermodynamic results revealed that the binding process was spontaneous; hydrogen bonding and van der Waals interaction were the predominant forces responsible for the complex stabilization in the case of tryptophan and phenylalanine, respectively, whereas in the case of tyrosine, hydrophobic interaction was the sole force conferring stability. Moreover, the Förster non-radiation energy transfer theory has been applied to calculate the average binding distance among the above amino acids and silver nanoparticles. The results show a binding distance of <7 nm, which ensures that energy transfer does occur between the said amino acids and silver nanoparticles.

Keywords: *amino acids, silver nanoparticles, fungi, fluorescence spectroscopy.*

Introduction. Under normal conditions human beings can synthesize L-tyrosine when phenylalanine is supplied from an exogenous source. Patients suffering from the genetic disease phenylketonuria are severely deficient in the enzyme phenylalanine hydroxylase, which affects the synthesis of tyrosine from phenylalanine; in that case, tyrosine can be considered as an essential amino acid [1]. Tyrosine is a precursor for the biosynthesis of thyroxine, melanin, and neurotransmitters like dopamine and norepinephrine [1]. It was reported that if tyrosine is not supplied properly to the human body, there is a chance of development of symptoms such as depression, hypertension, stress, memory loss, and also the possibility of contracting Parkinson's disease. L-tryptophan is also an essential amino acid and plays a crucial role in protein biosynthesis; it is an important precursor for the biosynthesis of various biologically active compounds like serotonin, melatonin, and quinolinic acid and the coenzymes nicotinamide adenine dinucleotide (NAD) and NAD phosphate (NADP). Tryptophan plays a unique role as a natural remedy for diseases like depression, pain, insomnia, hyperactivity, and eating disorders [2], but both excessive intake and deficiency in tryptophan are detrimental to health [3]. The aromatic amino acid L-phenylalanine is also an essential amino acid and is required for protein synthesis and also for synthesis of DOPA from which dopamine, norepinephrine, and epinephrine have been synthesized. Whenever intake of this amino acid is found to be excessive, this interferes with the production of serotonin and other aromatic amino acids [4].

Nanotechnology is gradually influencing all fields of sciences and our daily lives in the 21st century and also creating the upsurge in innovation in almost all areas of industries. The unique physicochemical, optical [5], electrical [6], catalytic [7, 8], sensing [9, 10], and antimicrobial functionalities [11] of silver nanoparticles compared to the bulk metal have attracted considerable attention over the past decades.

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Silver nanoparticles show various applications in the fields of biolabelling [12], sensors, drug delivery [13], polarizing filters [14], electrical batteries, staining pigments, and health sciences in the form of antimicrobial and anticancer agents [15–17]. Although silver nanoparticles may show cytotoxicity [18] and can trigger oxidative stress [17], the market for silver nanoparticles is rapidly expanding based on the demand for consumer products in our daily lives, but consumers are ignorant of the impact of silver nanoparticles on the environment and health to a considerable extent [19].

In medical applications, silver nanoparticles are applied to the human body and are circulated through the blood to the target site for their action. During circulation through the blood there may be a chance of interaction between silver nanoparticles and plasma proteins, which may not be tolerated.

It is known that the protein absorption and emission occur mainly due to the presence of three aromatic amino acids, namely, tryptophan, tyrosine, and phenylalanine, in the protein molecule. The interaction between biosynthesized silver nanoparticles and bovine serum albumin has been described in our previous report [20], and the present study is an extension of our previous work, i.e., the study of the interaction between biosynthesized silver nanoparticles and the three individual biologically important amino acids, i.e., tryptophan, tyrosine, and phenylalanine.

Materials and Methods. Tryptophan, tyrosine, and phenylalanine (SRL, India) and silver nitrate (Merck, Germany) were used in the experiment. Stock solutions of tryptophan, tyrosine, and phenylalanine were dissolved in Tris-HCl (50 mM, pH 7.4) buffer to prepare solutions of the desired concentration (2 mM). All other chemicals were of analytical reagent grade, and double distilled water was used throughout.

A Cary spectrophotometer (Agilent) and a Cary Eclipse fluorescence spectrophotometer (Agilent) were used for the spectroscopic binding study of the interaction between the amino acids and silver nanoparticles.

Aspergillus foetidus was used as the potential source for extracellular biosynthesis of silver nanoparticles. For biosynthesis of silver nanoparticles at a 1 mM final concentration, $AgNO_3$ was added to 50 ml of the cell filtrate in a 250 ml Borosil flask and agitated at 303 K in the dark. Various methods for the characterization and standardization of physiochemical parameters for the biosynthesis of silver nanoparticles and also the estimation of biosynthesized nanoparticle concentration have already been described in our previous report [21, 22]. It was found that the dimension of the biosynthesized silver nanoparticles was in the range 20–40 nm.

Green synthesis is an ecofriendly, cost effective, as well as an unmatched alternative to chemical synthesis. The beauty of biosynthesis lies in the fact that it requires no additional reducing and capping agents to confer stability to the biosynthesized nanoparticles, as cellular protein is readily involved in carrying out the dual role. Here, in the case of our biosynthesized nanoparticles, an extracellular live cell filtrate was used as the source of protein. After processing, the biosynthetic method was optimized, an estimate of the concentration of the nanoparticles has also been reported in our earlier publication [22].

The UV-visible absorption spectra were recorded after adding different concentrations of silver nanoparticles (0, 10, 20, 30, 40, 50, and 60 μM) to the tryptophan, tyrosine, and phenylalanine (2 mM) solutions. The intrinsic emission of tryptophan, tyrosine, and phenylalanine was observed at excitation wavelengths of 279, 270, and 260 nm, respectively. The experiments were carried out after adding different concentrations of silver nanoparticles (0, 10, 20, 30, 40, 50, and 60 μM) to the tryptophan, tyrosine, and phenylalanine (2 mM) solutions. The same experiments were repeated at 293, 303, and 313 K.

Results and Discussion. UV-vis absorption spectroscopy is a very simple yet important method in gathering knowledge on the structural change and detecting the possibility of complex formation [23]. The absorption spectra of tryptophan, tyrosine, and phenylalanine in the presence of silver nanoparticles were recorded and are presented in Fig. 1. As per Fig. 1, with the addition of silver nanoparticles the absorbance intensity increased and the absorption maximum was shifted towards the shorter wavelength region, which indicated that tryptophan, tyrosine, and phenylalanine may bind to silver nanoparticles and facilitate complexation.

The values of the apparent association constant, K_{app}, were calculated based on the Benesi and Hildebrand equation from the absorption spectra of the amino acid–nanoparticle (complex) according to the previously described methods [24]. For weak binding affinities, the data were treated using linear reciprocal plots based on the equation [25]

$$\frac{1}{A_{obs} - A_0} = \frac{1}{A_C - A_0} + \frac{1}{K_{app}(A_C - A_0)[SNP]}, \quad (1)$$

where A_0 is the amino acid absorbance in the absence of silver nanoparticles (SNP) and A_C is the recorded absorbance for the amino acids at different concentrations (0, 10, 20, 30, 40, 50, and 60 μM) of silver nanoparticles. Using the double reciprocal plot (R = 0.9958, 0.9968, and 0.9991, respectively, for tryptophan, tyrosine, and phenylalanine, where R is the correlation

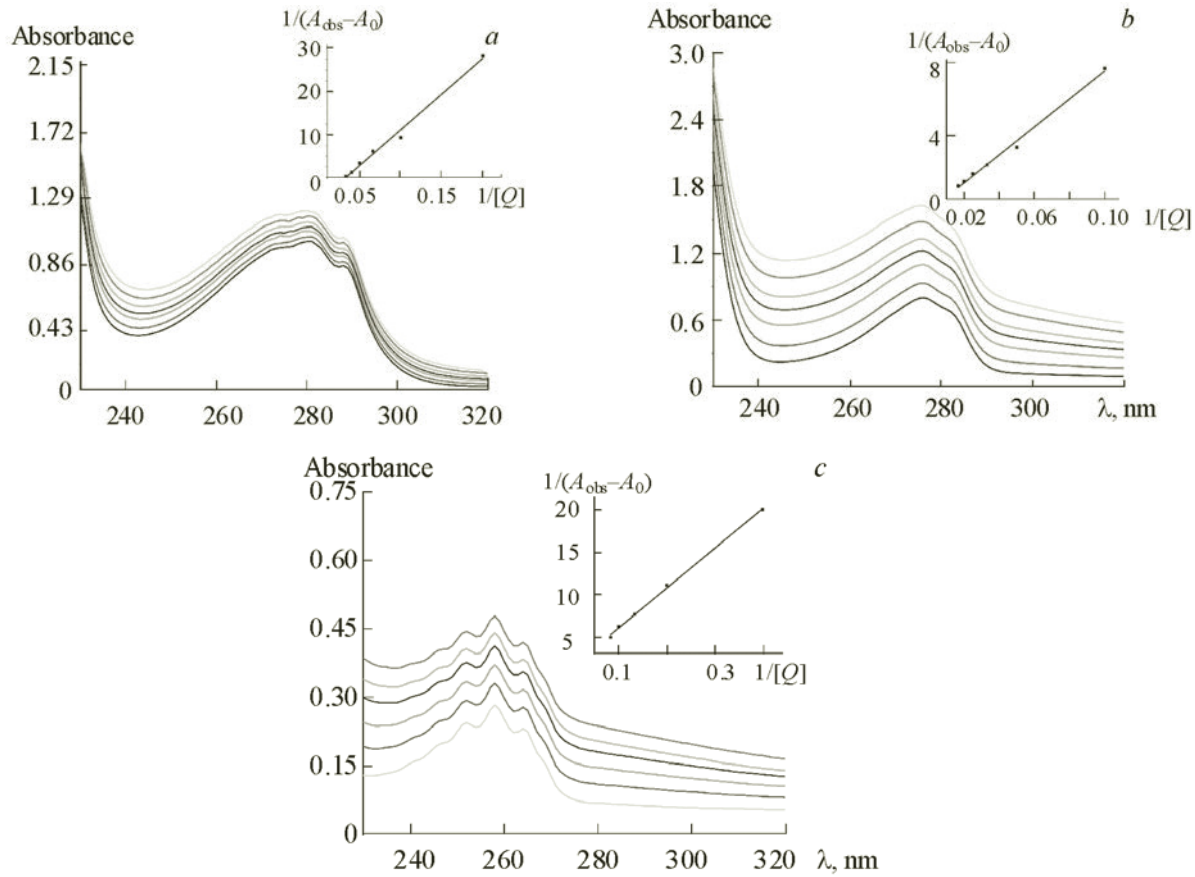

Fig. 1. Absorption spectra of tryptophan (a), tyrosine (b), and phenylalanine (c) in the presence of silver nanoparticles (0, 10, 20, 30, 40, 50, and 60 μM). Insets show determination of apparent association constants (K_{app}) of tryptophan, tyrosine, and phenylalanine with silver nanoparticles.

coefficient) of $1/(A_{obs} - A_0)$ vs 1/[Q], which was found to be linear, we found the values of the apparent association constant (K_{app}) to be 2.54×10^3, 1.80×10^3, and 3.14×10^4 L/mol, respectively, for tryptophan, tyrosine, and phenylalanine (Fig. 1, insets) from the ratio of the intercept to the slope [24]. From the values of the apparent association constant estimated, it is clear that a low K_{app} value is indicative of weak complex formation between silver nanoparticles and the mentioned amino acids. From the above results, we observed that for phenylalanine and tyrosine the apparent association constants (K_{app}) were highest and lowest, respectively.

It was evident that tryptophan, tyrosine, and phenylalanine have strong fluorescence emission peaks at 365, 300, and 280 nm, respectively, when excited at 279, 270, and 260 nm wavelength, respectively. Figure 2 shows the emission spectra of tryptophan, tyrosine, and phenylalanine in the presence of different concentrations of silver nanoparticles. It was observed that the fluorescence intensity of tryptophan, tyrosine, and phenylalanine decreased sharply with increasing concentration of silver nanoparticles. These data indicated that silver nanoparticles could interact with tryptophan, tyrosine, and phenylalanine and thereby quench the fluorescence intensity.

Fluorescence quenching denotes any process that decreases the fluorescence intensity of a sample. A variety of molecular interactions results in quenching, including excited-state reactions, molecular rearrangements, energy transfer, ground-state complex formation, and collisional quenching [26]. Fluorescence quenching can be dynamic, due to collisional encounters between the fluorophore and quencher, or static, due to the formation of a ground-state complex between the fluorophore and quencher.

Static and dynamic quenching can be differentiated on the basis of interaction studies with variations in temperature and viscosity or excited-state lifetime. Higher temperatures will result in faster diffusion and hence larger amounts of collisional quenching, which results from the dissociation of weakly bound complexes. The quenching mechanism may be represented by the Stern–Volmer equation [27, 28]

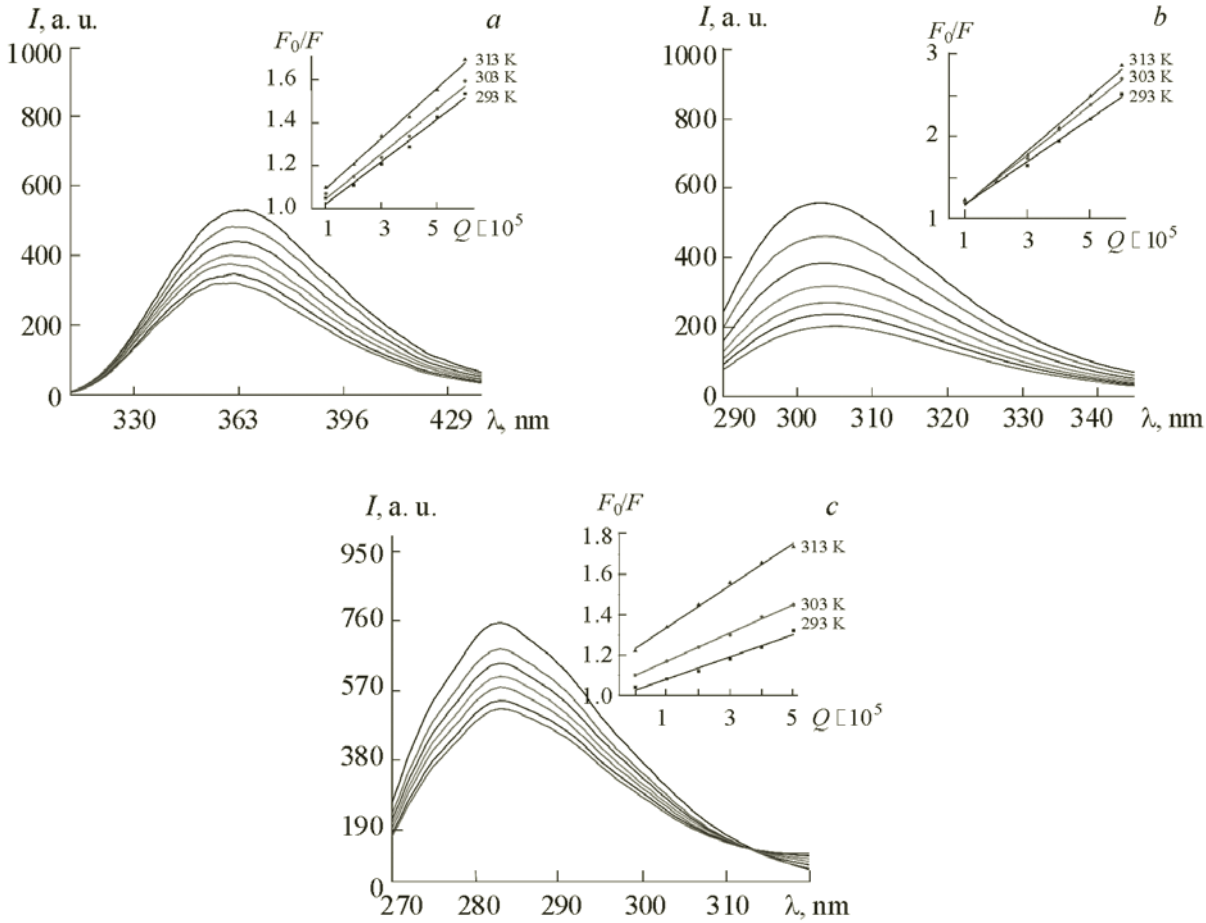

Fig. 2. Emission spectra of tryptophan (a), tyrosine (b), and phenylalanine (c) in the presence of silver nanoparticles (0, 10, 20, 30, 40, 50, and 60 μM). Insets show Stern–Volmer plot of amino acids–silver nanoparticles interaction.

$$F_0/F = 1 + k_q\tau_0[Q] = 1 + K_{SV}[Q] , \quad (2)$$

where F_0 and F denote the fluorescence intensities in the absence and presence of a quencher, k_q is the bimolecular quenching rate constant, τ_0 is the average lifetime of the molecule in the absence of the quencher, which is about 10^{-8} s, K_{SV} is the Stern–Volmer quenching constant, and [Q] is the concentration of the quencher [29, 30]. The values of K_{SV} decrease with increase in temperature for static quenching, and the opposite is seen for dynamic quenching. The Stern–Volmer plots for the quenching of fluorescence of tryptophan, tyrosine, and phenylalanine separately by silver nanoparticles at different temperatures are presented in the inset in Fig. 2. The calculated results are shown in Table 1. In the case of dynamic quenching it is known that the maximum scatter quenching collision constant of various quenchers with the biomolecule is approximately 1×10^{10} L/mol/s. The experimental quenching constant was found to increase gradually with increasing temperature. Hence, we can say that in all three cases the quenching mechanism is dynamic in nature [31].

Binding constant (K) and number of binding sites (n). When small molecules interact independently with a set of equivalent sites on a biomolecule, the equilibrium between the biomolecule and small molecules is expressed by Eq. (3). The number of binding sites (n) and binding constant (K) of interaction between silver nanoparticles and each of the three individual amino acids tryptophan, tyrosine, and phenylalanine separately can be calculated by the same equation [32]:

$$\log [(F_0 - F)/F] = \log K + n \log [Q] . \quad (3)$$

A plot of log $[(F_0 - F)/F]$ vs log [Q] forms a straight line, whose slope represents n (the number of binding sites between silver nanoparticles and each of the three amino acids: tryptophan, tyrosine, and phenylalanine), and the length of the intercept on the Y axis represents log K. Figure 3 denotes the double logarithm plots and Table 2 gives the corresponding results.

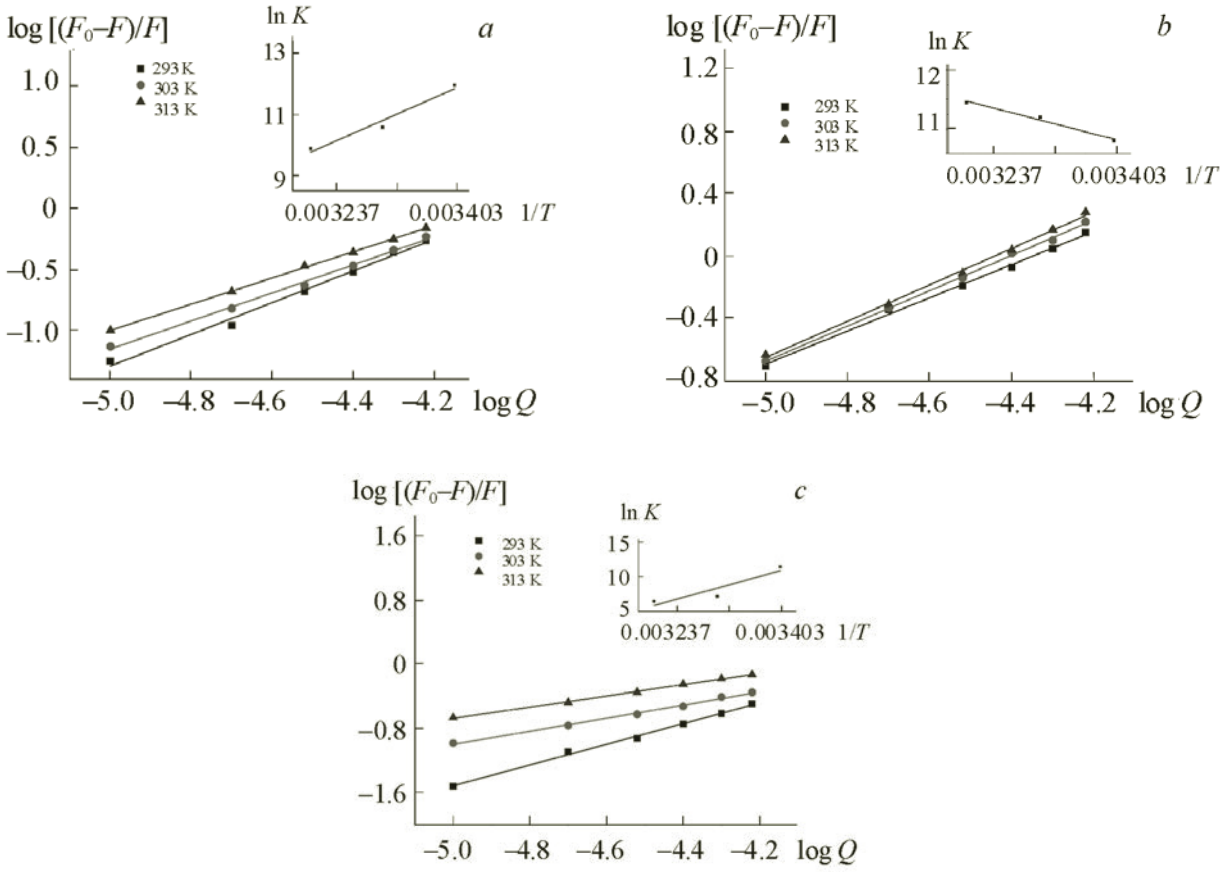

Fig. 3. Calculation of binding constant (K) and number of binding site (n) for amino acids–silver nanoparticles interaction at different temperatures for tryptophan (a), tyrosine (b), and phenylalanine (c). Insets show Van't Hoff plot for the interaction of amino acids–silver nanoparticles.

TABLE 1. Stern–Volmer Constants (K_{SV}, L/mol) and Quenching Constants (k_q, (L/mol/s) × 10^8) of Tryptophan, Tyrosine, and Phenylalanine by Silver Nanoparticles (R is correlation coefficient).

T, K	R	K_{SV},	k_q	R	K_{SV},	k_q	R	K_{SV},	k_q
	Tryptophan			Tyrosine			Phenylalanine		
293	0.9929	0.099	0.099	0.9977	0.2606	0.2606	0.9911	0.055	0.055
303	0.9948	0.106	0.106	0.9987	0.3017	0.3017	0.9988	0.070	0.070
313	0.9984	0.118	0.118	0.9966	0.3297	0.3297	0.9980	0.105	0.105

The values of n at the experimental temperatures were approximately equal to 1, which shows that there is a single binding site in tryptophan, tyrosine, as well as phenylalanine separately for silver nanoparticles, and that the binding is dependent on the temperature ranging from 293 to 313 K. From the experimental results (Table 2) it is observed that in the case of tryptophan and phenylalanine, the binding constant K decreases with increase in temperature, which suggests that the stability of the complexes decreases with increase in temperature but, amazingly, in the case of tyrosine K, increases with increasing temperature, indicating higher stability of the tryptophan complex formed with temperature increase.

Thermodynamic parameters and nature of binding forces. We worked out the thermodynamic forces responsible for the binding of nanomaterials to tryptophan, tyrosine, and phenylalanine. Possible interactive forces between the biomolecule

TABLE 2. Binding Constants (K, (L/mol) × 10^4) and Number of Binding Sites (n) of Phenylalanine Binding by Silver Nanoparticles (R is correlation coefficient)

T, K	R	K	n	R	K	n	R	K	n
	Tryptophan			Tyrosine			Phenylalanine		
293	0.9956	15.84	1.3	0.9982	4.07	1.06	0.9983	9.12×10^4	1.29
303	0.9979	3.98	1.15	0.9992	9.33	1.13	0.9977	1.20×10^3	0.82
313	0.9997	1.99	1.07	0.9987	14.45	1.16	0.9991	3.89×10^2	0.69

TABLE 3. Thermodynamic Parameters [ΔH (kJ/mol), ΔS (J/mol/K), and ΔG (kJ/mol)] for Binding of Silver Nanoparticles to Tryptophan, Tyrosine, and Phenylalanine (R is correlation coefficient).

T, K	R	ΔH	ΔS	ΔG	R	ΔH	ΔS	ΔG	R	ΔH	ΔS	ΔG
	Tryptophan				Tyrosine				Phenylalanine			
293	0.9819	–86.05	–193.96	–29.21	0.9927	53.62	270.87	–25.74	0.9237	–209.51	–622.38	–27.15
303		–86.05	–193.96	–27.27		53.62	270.87	–28.44		–209.51	–622.38	–20.93
313		–86.05	–193.96	–25.34		53.62	270.87	–31.15		–209.51	–622.38	–14.70

and nanomaterials are hydrogen bonding, van der Waals, hydrophobic, hydrophilic, and electrostatic interactions [33]. Parameters such as changes in enthalpy (ΔH) and entropy (ΔS) of binding interactions help to determine the type of binding interactions, and the Gibbs free energy change (ΔG) helps to compute the spontaneity of the binding process.

The thermodynamic parameters ΔH and ΔS can be determined using the Van't Hoff equation

$$\ln k = -\Delta S/RT + \Delta S/R \,, \tag{4}$$

where k is the binding constant at the relevant temperature (Fig. 3, insets), and ΔH and ΔS can be determined from the slopes and intercepts of the linear Van't Hoff plots. The Gibbs free energy (ΔG) can be calculated from the equation

$$\Delta G = \Delta H - T\Delta S = -RT \ln k \,. \tag{5}$$

According to the enthalpy and entropy changes, the mode of interaction between small molecules and biomolecules can be summarized as $\Delta H > 0$ and $\Delta S > 0$, indicating hydrophobic forces; $\Delta H < 0$ and $\Delta S < 0$, indicating van der Waals interactions and hydrogen bonds; $\Delta H \approx 0$ and $\Delta S > 0$, indicating electrostatic interactions [33]. The thermodynamic parameter values are shown in Table 3. ΔG, ΔS, and ΔH were found to be negative for silver nanoparticles–tryptophan and silver nanoparticles–phenylalanine complexes. Therefore, the formation of biosynthesized silver nanoparticles–tryptophan and silver nanoparticles–phenylalanine complexes is a spontaneous and exothermic reaction followed by a negative ΔS value. According to the previous study [33–36], negative values of ΔS and ΔH suggest that weak forces like van der Waals and hydrogen bonding are primary factors in this binding process. The negative value of ΔG suggests that the interaction between the biomolecule and nanomaterial is spontaneous. In the case of silver nanoparticles–tyrosine complex, ΔG was negative, but surprisingly both ΔS and ΔH were positive, which signifies hydrophobic forces are the main stabilizing force.

Energy transfer. The importance of energy transfer in biochemistry is that the transfer efficiency can be used to evaluate the distance between the ligand and tryptophan residues in the protein [37]. The overlap of the UV-vis absorption spectra of silver nanoparticles with the fluorescence emission spectra of each of the three individual amino acids (tryptophan, tyrosine, and phenylalanine) is shown in Fig. 4. According to the Forster theory of molecular resonance energy transfer [38], the intercenter distance (r) of binding between silver nanoparticles and the individual amino acids, i.e., tryptophan, tyrosine, and phenylalanine, and the efficiency E of energy transfer between the donor (biomolecule) and acceptor (nanoparticles), can be calculated using the following equations:

$$E = 1 - F/F_0 = R_0^6/(R_0^6 + r^6) \,, \tag{6}$$

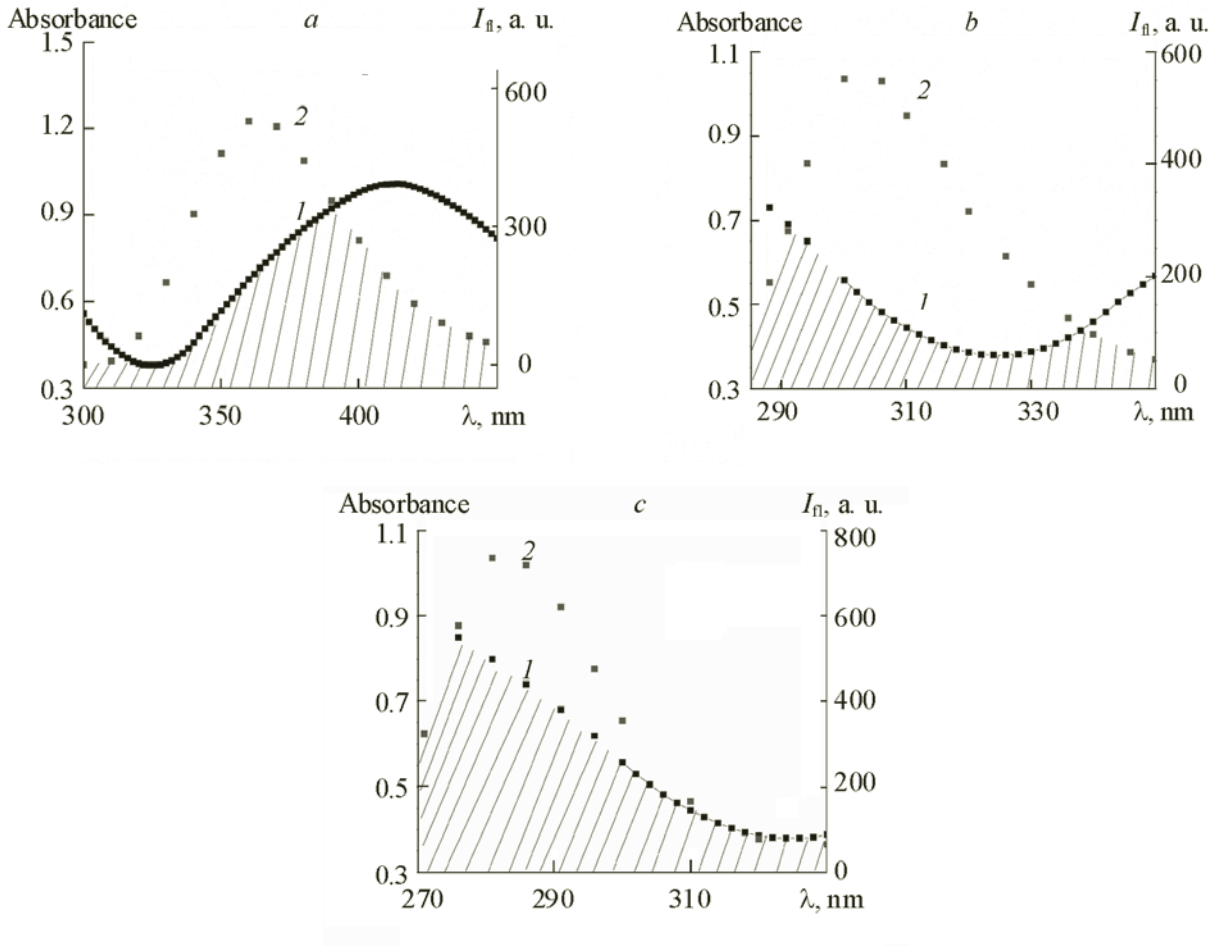

Fig. 4. The overlap plot of the fluorescence emission spectra (1) of amino acids tryptophan (a), tyrosine (b), and phenylalanine (c) and the UV absorption spectra (2) of silver nanoparticles.

$$R_0^6 = 8.8 \times 10^{-25} k^2 N^{-4} \phi J , \tag{7}$$

where E is the efficiency of transfer between the donor and acceptor and R_0 is the critical distance when the efficiency of transfer is 50%; F_0 and F are the fluorescence intensities of the amino acids in the absence and in the presence of the quencher (silver nanoparticles), respectively [39], k^2 is the spatial orientation factor of the dipole, N is the refracted index of the medium, and ϕ is the fluorescence quantum yield of the donor. In this case, $K^2 = 0.476$ [40], $N = 1.336$, and $\phi = 0.118$ [41]. J can be obtained from the following equation:

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

where J is the extent of the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor, and $F(\lambda)$ is the fluorescence intensity of the acceptor at wavelength λ.

According to the above equations and experimental results of our earlier report [35], we have obtained $J = 2.00 \times 10^{-14}$ cm^3 × L/mol, $E = 0.05$, and $R_0 = 2.70$ nm. From Eq. (6), the intercenter distances between the donor and the acceptor molecule are found to be $r = 3.96$, 3.50, and 3.95 nm ($r < 7$) for the three amino acids tryptophan, tyrosine, and phenylalanine, respectively, which indicates that there exist a nonradiative mode of energy transfer from each of the amino acids tryptophan, tyrosine, and phenylalanine, respectively, to silver nanoparticles [42].

Conclusions. In the present work, we focused on the interaction of three amino acids tryptophan, tyrosine, and phenylalanine separately with biosynthesized silver nanoparticles following UV-vis spectroscopy and fluorescence quenching techniques, and the results ascertain that silver nanoparticles can interact individually with each of the three amino acids. This investigation suggests that nanoparticles not only can interact with protein molecules but can also interact with amino acids tryptophan, tyrosine, and phenylalanine responsible for protein absorption and emission.

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