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Study on the Interaction of Formononetin and Herring Sperm DNA by Electrochemical Methods and Spectroscopy

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The interaction of formononetin (FN) with herring sperm DNA has been studied by electrochemical methods, UV-Vis spectroscopy and fluorescence. On a poly L-cysteine film modified electrode (PLC/GCE), FN showed a couple of well-defined voltammetric peaks in 0.1 M acetate buffer of pH 5.3. As adding the herring sperm DNA into above solution, the peak current of FN decreased and no new peaks appeared. Based on the electrochemical results, the binding constant and binding ratio between FN and herring sperm DNA were determined to be 1.98×10^4 M^{-3} and 1:3, respectively. The UV-Vis spectroscopy indicated that the maximum absorption of FN had obviously hyperchromic effect and red shift after the interaction of DNA and FN. The interaction of DNA and FN can also make the fluorescence intensity of FN enhancement. The electrochemical and spectroscopic results indicated that the interaction of FN to DNA was an intercalative mode.

Keywords: Formononetin; DNA; Interaction; Spectroscopy; Electrochemistry

1. INTRODUCTION

Flavonoids represent a diverse group of over 4000 ubiquitous secondary plant metabolites and they play a multifunctional role in living organisms[1,2]. Among various types of flavonoids, isoflavonoids are a subclass of flavonoid and are abundant in legumes. Isoflavonoids have a basic skeleton of 3-phenylchromen-4-one core structure which was biogenetically derived by an aryl migration mechanism from the 2-phenylchroman skeleton of flavones[2-6]. Over the past decades, isoflavones have sparked tremendous interest worldwide due to their potential health-enhancing benefits in human, such as remedying menopausal discomfort and osteoporosis[7,8], promoting cardiovascular health[9,10], chemopreventing breast and prostate cancer[11-14], protecting the brain from damage through increase in endogenous antioxidant mechanisms[15] and reducing alcohol intake

in heavy drinkers[16]. Formononetin (7-hydroxy-4-methoxyisoflavone), one of the predominant methoxylated herbal isoflavones, is the major isoflavone constituent of Astragalus membranaceus (named Ougi in Japan and Huang Qi in China) and Leguminosae plant Trifolium pretense L. (red clover) [17-20]. Astragalus membranaceus is a well-known traditional Chinese medicine. Scientific evidence indicates that the extract of these herbs possess a wide spectrum of pharmacological properties and has been used clinically to treat different diseases with a long history of thousands of years [21-25]. Numerous clinical and nutritional studies have shown that formononetin possesses diverse biological activities, such as antidiabetic, hypolipidemic, immunomodulatory, neuroprotective roles, cardioprotective, antioxidant, antitumorigenic, antiviral, and estrogenic effects[26-33]. Additionally, it has been reported that formononetin potentiates epirubicin-induced apoptosis via reactive oxygen species production in Hela cells in vitro[34]. Li et al. explored the potential benefits of formononetin on traumatic brain injury rodent model[35]. Furthermore, formononetin is reported to produce a chronic antihypertensive effect by regulating the expressions of contractile receptors and endothelial nitric oxide synthase spontaneously in rat arteries [36]. The chemical structure of formononetin is shown in Fig. 1.

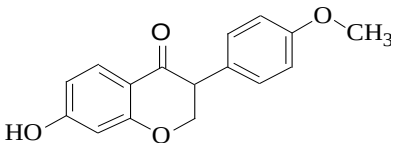

Figure 1. Chemical structure of formononetin

Up to now, many efforts have been focused on the therapeutic efficacy or the separation of formononetin from the crude extracts. Whereas, based on our best knowledge, there is insufficient information on the interaction of FN with DNA. Herein, there is an urgent need for further clarification of the mechanism involved.

Deoxyribonucleic acid (DNA) is an important biological macromolecule with specific three-dimensional structure, which is consisted of three parts: a phosphate acid group, a basic group and a sugar unit. It is associated with many kinds of biological phenomena and plays a central role in replication and transcription of the genetic information. DNA can interact with many endogenous and exogenous substances including many drugs [37-41]. Thus, the investigations of the interactions of the drugs with DNA are of great importance in life sciences from both a therapeutic and a scientific point of view [42-45]. There are primarily two modes of DNA-drug binding-covalent and non–covalent [46]. Plenty of methods have been applied to examine the DNA-drug interactions, such as fluorescence spectroscopy [47], ultraviolen/visible spectroscopy and viscosimetric technique[48], resonance light–scattering[49-51] and electroanalytical methods[40,41]. Among these methods, voltammetry and spectrophotometry are very common and reasonable ones.

This paper aims at the investigation of the interaction between FN and DNA by spectroscopic and electrochemical methods in vitro. We fabricated the GCE modified with poly L-cysteine film for the voltammetric determination. The modified electrode exhibited striking electrocatalytic activity

towards the redox of FN, and consequently significantly improves the sensitivity of the detection of FN. The experimental results have proved that FN could interact with DNA mainly by intercalative mode. We believe this will inspire further study on the mechanism of interactions of DNA with this class of isoflavonoids as well as deeper understanding on pharmacological effects of formononetin. The knowledge gained from this paper can provide fruitful information for the development of pharmaceutical design and new therapeutic reagents for tumors and other diseases.

2. EXPERIMENTAL

2.1. Materials and reagents

Formononetin, kindly provided by Aladdin Chemistry Co. Ltd.(Shanghai, China), was used without further purification. Herring sperm DNA was purchased from Beijing Biodee Biochnology, the purity of DNA was verified by the absorbance ratio value in the range of 1.8 ~ 1.9 for A_{260}/A_{280}, indicating that DNA is sufficiently free of protein and RNA [52]. The molar concentration of the DNA was detected spectrophotometrically according to the molar extinction coefficient value of 6600 L $mol^{-1}cm^{-1}$ at 260 nm[53]. The DNA stock solution was prepared by dissolving DNA in triple-distilled water and then stored at 4℃. Stock solution (7.45×10^{-4} M) FN was prepared in ethanol and stored at 4℃. Standard solutions were prepared daily by diluting the stock solution with the selected supporting electrolyte. A 0.1 M acetate buffer ($C_{HAc\text{-}NaAc}$) was prepared by mixing different amounts of 0.1 M HAc and 0.1 M NaAc to the required pH. L-cysteine was provided by Sinopharm Chemical Reagent Co., Ltd.(Shanghai, China). All other reagents purchased commercially were analytical grade and used without further purification. Triply distilled water was used throughout. All solutions under investigation were deaerated by bubbling highly pure nitrogen prior to measurements.

2.2. Methods and Instrumentation

2.2.1. Instrumentation

A computerized voltammetric analyzer CHI660E electrochemical workstation (Shanghai Chenhua Instruments Co., Ltd., China) was used for the voltammetric measurements. The working electrode was a bare or modified GCE; a saturated calomel electrode (SCE) and a platinum wire were utilized as reference and counter electrode. UV spectroscopy was performed by PC for data processing UV TU-1810 spectrophotometer (Beijing, China). The quartz cell with a 1.0 cm path length was used. Fluorescence measurements were performed on a LS-30 (Perkin Elmer, USA) fluorescence spectrophotometer. All pH-metric measurements were made on a model pHS-25 digital acidometer (Shanghai Leici Instrument Factory, China) was used for pH measurement. All experiments were performed at the ambient temperature of the laboratory (25±0.5℃).

2.2.2. Preparation of the modified electrode

The bare GCE was pretreated according to the previous literature [54] before modification. Then the anodic immobilization of L-cysteine (4×10^{-3} M) in a phosphate buffered saline (PBS, pH = 7.0) was performed by CV. The film was grown on a GCE by 32 segments of cyclic voltammetric scans in the range of -0.8 ~ 2.4V at 140 $mV\cdot s^{-1}$. After modification, a uniform adherent blue polymer was formed, which indicated that L-cysteine was electropolymerized on the surface of GCE.

2.2.3. Interaction of FN with DNA in Solution

FN and different concentrations of DNA were mixed in 0.1 M acetate buffer solution (pH = 5.3), then the voltammograms of the mixture were record after equilibration period of 15 minutes. For binding studies, the experiments were carried out by keeping the concentration of FN constant, while varying the DNA concentration.

3. RESULTS AND DISCUSSION

3.1. Electrochemical oxidation of FN

The electrochemical behavior of FN at PLC/GCE was investigated employing CV and DPV. The cyclic voltammograms of 0.1 M acetate buffer of pH 5.3 containing $1.49x10^{-4}$ M FN at a bare (Fig. 2, a) and modified electrode (Fig. 2, b) at scan rate of 180$mV\cdot s^{-1}$ are shown in Fig. 2. At bare GCE, FN exhibited poor current response, while a well-defined redox wave of FN was observed on the PLC/GCE with an oxidation peak (E_{pa}) at 0.323V and a reduction peak (E_{pc}) at 0.238V when the potential scan window was from -0.2 to 0.6V and the peak currents increased significantly. The potential difference between cathode and anode ($\Delta E_p = E_{pa} - E_{pc} = 85mV$) and the current ratio (i_{pa}/i_{pc}) ≈ 1.2 indicated that the electrochemical process of FN at the modified electrode was quasi-reversible. Based on the formula $|E_p - E_{p/2}| = 56.5/n$ [55], the electron transfer number (n) of redox reaction can be calculated to be approximately 1. The voltammograms in succession cycles of FN showed that with increasing number of scans, the redox currents decreased, indicating the significant adsorption of FN on the modified electrode. In order to find the best conditions to enhance the determination sensitivity, different supporting electrolyte, for instance, PBS, Tris-HCl buffer, Britton-Robinson buffer and acetate buffer were investigated. Considering the sensitivity, we choose the anodic peak as focus to examine. Results suggested that only the measurements made in acetate buffer produce stable response and the best defined peak was obtained at pH 5.3. Moreover, the oxidation peak potential shifted negatively with a concomitant increase in solution pH over the interval of pH 3.8 ~ 6.2 in acetate buffer with the regression equation was expressed as $E_{pa}(V) = -0.0653pH + 0.6581$ with $r = 0.999$. From the slope value, it can be concluded that the number of the electrons transferred in the oxidation of FN at PLC/GCE is equal to that of protons.

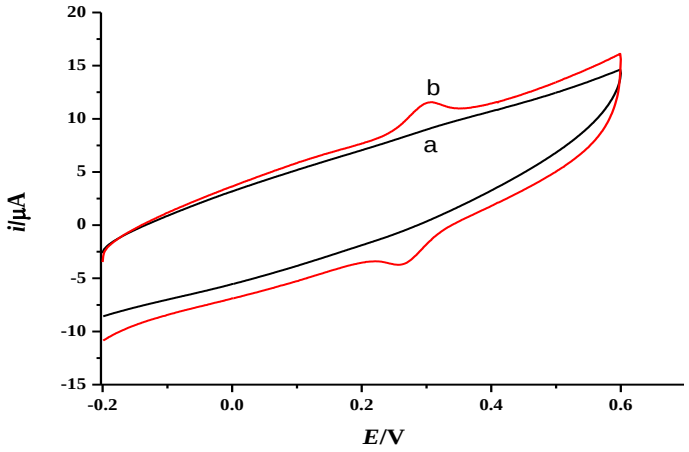

Figure 2. Cyclic voltammograms of FN at bare GCE (a) and PLC/GCE (b) of 1.49×10^{-4} M FN in 0.1 M acetate buffer (pH = 5.3), scan rate: 180mV·s^{-1}

The effect of the scanning rate on the peak of FN has also investigated by CV method. It is evident from Fig. 3 that there was a concomitant rise in the oxidation peak current with increments in scan rate from 60 to 300mV·s^{-1} beyond which its increase was irregular. In addition, the higher the scan rate, then the greater the potential separation observed between the reduction and oxidation peaks. The plots of i_{pa} versus v yielded a straightly line both without and with DNA (r =0.997 and r = 0.995, respectively), which demonstrating an adsorption-controlled electrode process[56]. The slope of the linear for the tests without DNA (8.4023 μA s·V^{-1}) was larger than that with DNA (5.444 μA s·V^{-1}), suggesting that the diffusion coefficient of the free form of FN was larger than that of the form of FN complex with DNA.

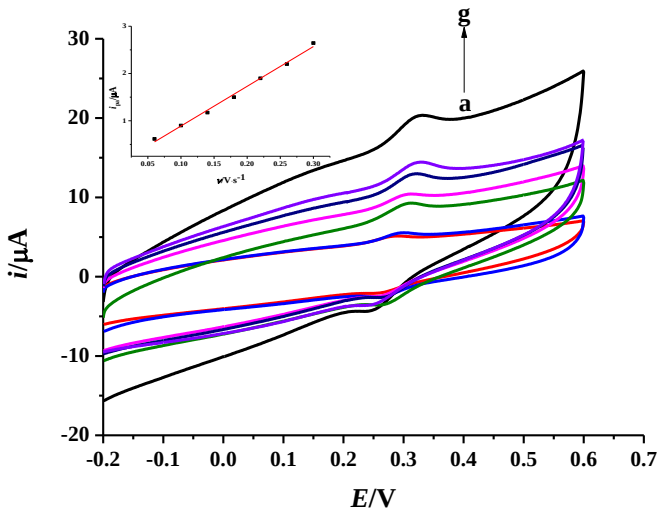

Figure 3. Effect of the scan rates on the CVs in 0.1 M acetate buffer containing 1.49×10^{-4} M FN (curves a to g are 60, 100, 140, 180, 220, 260, 300 mV·s^{-1}, respectively) Inset: Plot of i_{pa} versus v.

3.2. DNA–FN interaction

DPV method has been employed to the interaction between DNA and FN because it provides higher sensitivity and better peak resolution for investigating the electrochemical behavior of biological systems[57]. A DPV of 7.45×10^{-5} M FN in the absence and presence of DNA is shown in Fig. 4. As can be seen, the peak current exhibits a distinct weakness while the peak potential shifted more positively with that in the absence of DNA. The duration over which FN is in binding to DNA has a remarkable influence on the value of peak current. A time of 15 min is the optimal waiting time.

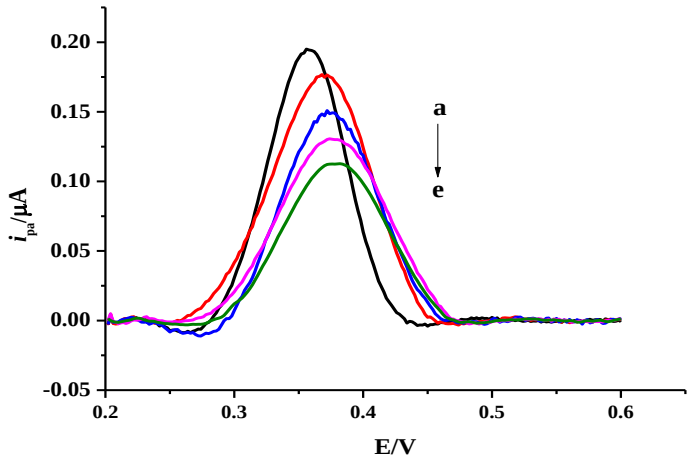

Figure 4. DPV of 7.45×10^{-5} M FN in absence (a) and presence of (b) 0.08, (c) 0.12, (d) 0.16, (e) 0.2 mg·mL^{-1} DNA in acetate buffer of pH 5.3.

There are several possible reasons responsible for the drop in peak current. One possible explanation is based on an increase in viscosity of the solution or a blockage of the electrode surface by DNA adsorption[58]. In order to eliminate this possibility, a special CV experiment was carried out in $K_4[Fe(CN)_6]$ solution in absence and presence of DNA at the PLC/GCE. Due to coulombic repulsion between the negative charges, the $[Fe(CN)_6]^{4-}$ ions did not interact with DNA. It was found that the peak currents were almost equal. Thus, it could be concluded that the change in viscosity of solution or DNA adsorption cannot fully account for the peak attenuation.

Another possibility is that the change of the electrochemical kinetics of FN in the presence of DNA. The major electrochemical kinetic parameters including α (the electron transfer coefficient) and k_s (the standard rate constant) of FN either in absence or in presence of DNA could be obtained from the following equation[59]:

$$E = E_0 + \frac{RT}{\alpha nF}\ln\frac{RTk_s}{\alpha nF} - \frac{RT}{\alpha nF}\ln\upsilon \qquad (1)$$

Where E_0 is the formal potential which can be obtained from the intercept of the E_{pa} versus v plot by extrapolation to the vertical axis at $v = 0$. Other symbols have their usual meanings. The values of α and k_s were evaluated from the plot of E_{pa} vs. ln v and found to be 0.51 and 836.21 s^{-1} in absence

of DNA and 0.50 and 793.89 s^{-1} in presence of DNA. Obviously, the addition of DNA does not significantly influence the kinetics of the FN redox reactions at PLC/GCE.

Therefore, the decrease in oxidation signals of FN with increasing of DNA is ascribed to the formation of an electrochemically inactive DNA-FN adducts with large molecular weight, resulting in a distinct weakness in equilibrium concentration of free FN and the apparent diffusion coefficients[60].

In the case of the peak potential, as reported by Bard and coworkers[61], the interaction will be an intercalative mode if the peak potential shifts towards positive direction. Hence, the conclusion that FN could intercalate into the DNA base pairs was drawn.

Furthermore, chronocoulometry was performed to estimate the diffusion coefficient for free FN or DNA–FN complex. The chronocoulometric response of diffusion reactant can be described as below[62]:

$$Q = 2nFAc(Dt)^{1/2}/\pi^{1/2} + Q_{dl} + Q_{ads} \qquad (2)$$

Where D ($cm^2 \cdot s^{-1}$) is diffusion coefficient, n is the number of electrons transferred in reaction, c ($mol \cdot cm^{-3}$) is the concentration of FN, A (cm^2) is the surface area of the working electrode, F is the Faraday constant (96,487 $C \cdot mol^{-1}$), t is the pulse width, Q_{dl} is the double-layer charge and Q_{ads} is the faradaic component due to the adsorbed species. The plots of Q (μC) against $t^{1/2}$ are shown in Fig. 5. Based on our experimental data, the linear regression equations related to FN and DNA–FN complex were concluded as Q (μC) = 0.1247 + 28.17 $t^{1/2}$ ($s^{1/2}$), r = 0.996; Q (μC) = 0.04602 + 17.33 $t^{1/2}$ ($s^{1/2}$), r = 0.998, respectively. Obviously, after the addition of DNA, the slope decreases, indicating a decrease in diffusion rate. According to Eq. (2), if we know n, c and A, the diffusion coefficient can be determined from the slope of the plot of Q *vs.* $t^{1/2}$. Therefore, the diffusion coefficient of $2.92 \times 10^{-11} cm^2 \cdot s^{-1}$ was calculated for free FN and of 1.11×10^{-11} $cm^2 \cdot s^{-1}$ for the DNA-FN complex.

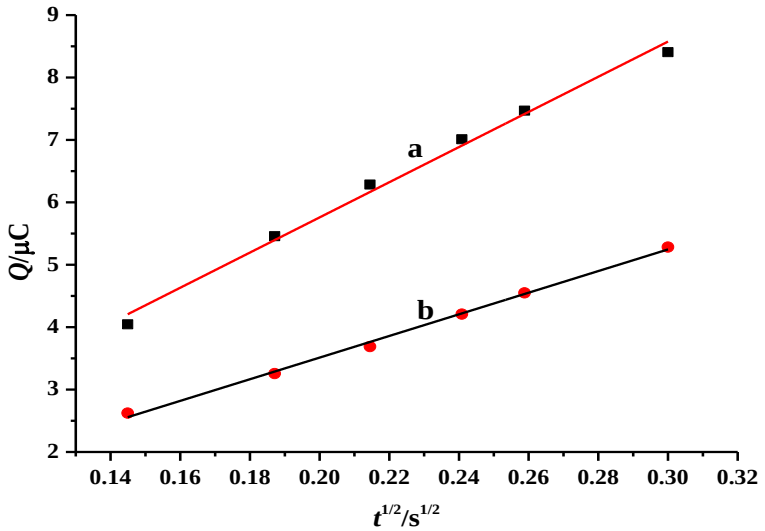

Figure 5. Dependence of charges on the square roots of time: (a) 7.45×10^{-5} M FN and (b) 7.45×10^{-5} M FN mixing with 0.2 $mg \cdot mL^{-1}$ DNA.

3.3. Determination of binding constant and binding ratio

Assuming that FN and DNA produces only a single complex, DNA-mFN, with the reaction scheme [63]

DNA + mFN = DNA–mFN

where m is the binding ratio. The equilibrium constant (β) can be deduced according to the following equations:

$$\beta = \left[\mathrm{DNA-mFN}\right] / \left\{\left[\mathrm{DNA}\right]\left[\mathrm{mFN}\right]^{\mathrm{m}}\right\} \quad (3)$$

Because of

$$C_{\mathrm{DNA}} = \left[\mathrm{DNA-mFN}\right] + \left[\mathrm{DNA}\right] \quad (4)$$

$$\Delta i_{\max} = K C_{\mathrm{DNA}} \quad (5)$$

$$\Delta i = K\left[\mathrm{DNA-mFN}\right] \quad (6)$$

Therefore:

$$\Delta i_{\max} - \Delta i = K\left(C_{\mathrm{DNA}} - \left[\mathrm{DNA-mFN}\right]\right) \quad (7)$$

$$\Delta i_{\max} - \Delta i = K\left[\mathrm{DNA}\right] \quad (8)$$

From Eqs. (3), (5), and (7) we get

$$1/\Delta i = \left(1/\Delta i_{\max}\right) + \left\{\left[1/\left(\beta \Delta i_{\max}\right)\right] \times \left(1/\left[\mathrm{FN}\right]^{\mathrm{m}}\right)\right\} \quad (9)$$

or

$$\log\left[\Delta i/\left(\Delta i_{\max} - \Delta i\right)\right] = \log\beta + \mathrm{m}\log\left[\mathrm{FN}\right] \quad (10)$$

Where Δi is the difference in peak current of FN in presence and absence of DNA, and $\Delta i_{\max}$ corresponds to the value when the concentration of FN is extremely higher than that of DNA. C_{DNA}, [DNA] and [DNA-mFN] are the total, free and bound concentrations of DNA in the solution, respectively.

If DNA interacts with FN to form a single complex, then the plot of log[Δi/ ($\Delta i_{\max}$-Δi)] versus log[FN] for DNA-FN interaction shows linearity. The values of binding constant and binding ratio are deduced from the intercept and slope of the straight line, and these values are found to be 1.98×10^{4} M^{-3} and 3. Thus, the formation of a stable 1:3 complex (DNA: 3FN) is proposed.

3.4. Spectroscopic investigations

3.4.1. UV-Vis absorption studies

UV-Vis absorption is a simple but effective technique generally used to testify the interaction of molecules with DNA. Fig. 6 showed the UV-Vis absorption spectra of FN in absence and presence of varying amounts of DNA in acetate buffer (pH 5.3). It can be seen that the maximum absorbance of FN was located around at 305 nm. With gradually increasing concentration of DNA, FN showed a progressively lower absorbance followed by a slight red shift of 4 nm. The reason for the hypochromic effect could be the interaction between the electronic states of the intercalating chromophore and those

of the DNA bases [61,64]. The changes of absorption spectra of FN endow evidence for the intercalative binding between FN and DNA.

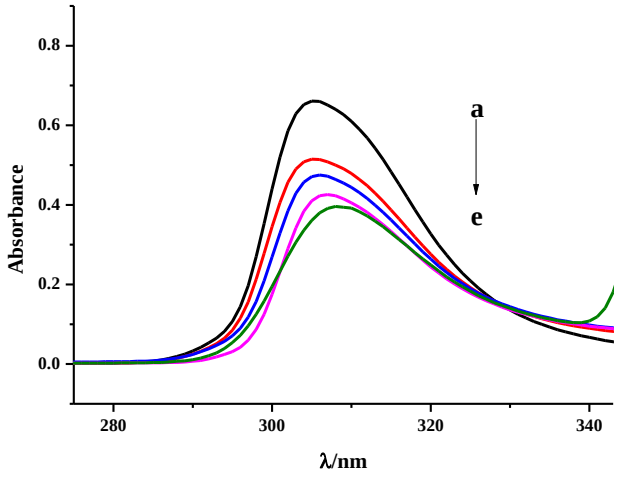

Figure 6. UV-Vis absorption spectra of 7.45×10^{-5} M FN in presence of different concentrations of DNA. The DNA concentrations were (a) 0, (b) 0.08, (c) 0.12, (d) 0.16, (e) 0.2 mg·mL^{-1}

3.4.2. Fluorescence studies

Additionally, the fluorescence features of FN ($\lambda_{max,ex}$ = 318nm, $\lambda_{max,ex}$ = 479nm) were used for the investigation of DNA-FN interaction. As evident from Fig. 7, there was a concomitant rise in intensity of emission with increments in DNA concentrations. These spectral characteristics are largely attributed to the intercalation of FN into the bases of DNA and the hydrophobic circumstance of DNA could help to enhance the fluorescence quantum yield of FN, which leaded to the fluorescence enhancement of FN[65]. This was in accordance with the results obtained by the electrochemical methods.

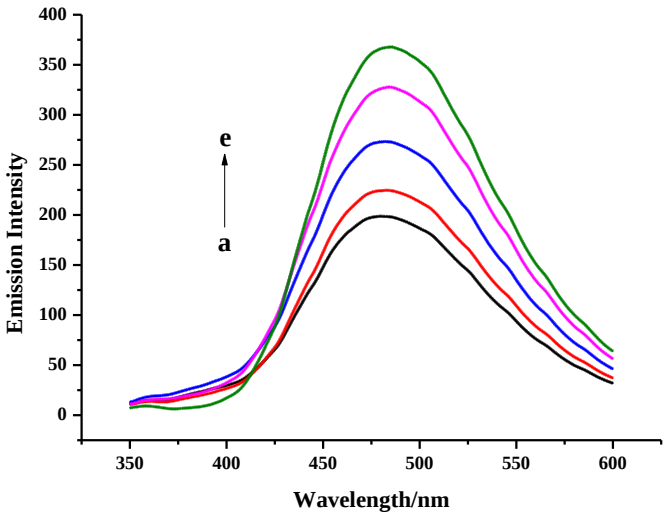

Figure 7. Fluorescence emission spectra of 7.45×10^{-5} M FN in the presence of DNA in acetate buffer solution (0.1M, pH 5.3). C_{DNA} = 0, 0.08, 0.12, 0.16, 0.2 mg·mL^{-1} for curves a–e.

4. CONCLUSION

This paper investigated the electrochemical behaviors of formononetin and its interaction with DNA by electrochemical and spectroscopic methods. The interaction between FN and DNA results in a series of variations in the cyclic behavior and spectra characteristics. All the experimental results suggested that the principal interaction mode of FN with DNA is dominated by intercalative mode. FN binding DNA formed a complex of DNA-3FN with $\beta = 1.98\times10^4\ M^{-3}$. These investigations indicated that the electrochemical method coupled with spectroscopic techniques provide significant promise to characterize the mechanism of binding of DNA to compounds which in turn be a beneficial guide to design DNA targeted compounds. The results may serve as a reference for the study of isoflavones with DNA in the natural environment of living cells.

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