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Surface-Enhanced Raman Spectroscopy Study of the Interaction of the Antitumoral Drug Emodin with Human Serum Albumin

Abstract: Surface-enhanced Raman spectroscopy was employed in this work to study the interaction between the antitumoral drug emodin and human serum albumin (HSA), as well as the influence of fatty acids in this interaction. We demonstrated that the drug/protein interaction can take place through two different binding sites which are probably localized in the IIA and IIIA hydrophobic pockets of HSA and which correspond to Sudlow's I and II binding sites, respectively. The primary interaction site of this drug seems to be site II in the defatted albumin. Fatty acids seem to displace the drug from site II to site I in nondefatted HSA, due to the high affinity of fatty acids for site II. The drug interacts with the protein through its dianionic form in defatted HSA (when placed in the site II) and through its neutral form in the site I of nondefatted albumins. © 2004 Wiley Periodicals, Inc. *Biopolymers* 74: 125–130, 2004

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INTRODUCTION

Emodin (Figure 1) is a naturally occurring anthraquinone extracted from the roots and bark of numerous plants of the genus *Rhamnus*. So far this

drug has been employed as anticancer, anti-agregant, antiulcer, antifeedant, anti-inflammatory, myorelaxant, and antiseptic therapy in different medical applications.^{1,2} In past years many authors reported the antitumoral activity of emodin against

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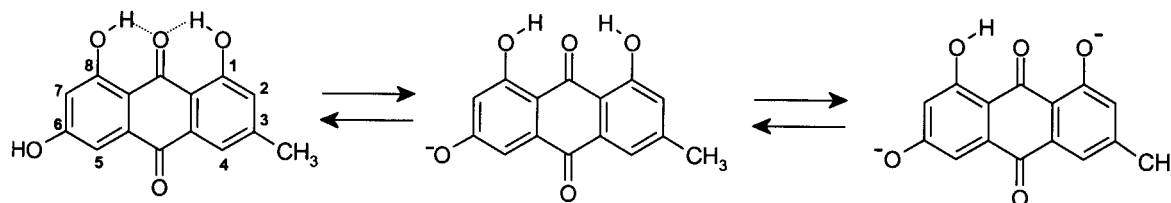


FIGURE 1 Structure, numbering, and acid–base equilibrium of emodin.

certain types of cancers.^{3–6} This action could take place through the inhibition of protein kinase C or protein-tyrosine kinase.^{3–5}

Serum albumin, as the most abundant plasma protein, contributes significantly to many transport and regulatory processes. The protein binds a wide variety of substrates like metals, fatty acids, amino acids, hormones, and an impressive spectrum of therapeutic drugs.⁷ Because of its clinical and pharmaceutical importance, the interaction of serum albumin with a variety of ligands has been studied extensively.^{8–12}

In recent works we studied the interaction between the related molecule hypericin and serum albumins of different origins (human, rat, and bovine) by Raman spectroscopy.^{13,14} In this work we present a similar study carried out for emodin using surface-enhanced Raman spectroscopy (SERS). This technique induces a giant enhancement of the Raman signal together with fluorescence quenching. These features allow for the application of this technique in recognition studies involving highly fluorescent molecules such as emodin and the employment of small concentrations of the biological molecules involved in the interaction. In the present study we examined the interaction of emodin with human serum albumin (HSA), and, on addition, we tested the influence of fatty acids (FA) in the binding of emodin using both defatted (HSA_f) and nondefatted (HSA) human albumins. The influence of FA, or rather their dissociated forms (carboxylates), on the drug binding to albumins was already studied for other albumin ligands.¹⁵

EXPERIMENTAL

Materials

Emodin was purchased from Sigma. HSA and HSA_f used in this study were purchased from Sigma and used without further purification. All reagents were of analytical degree and triply distilled water was always employed for the solutions.

Samples for Surface-Enhanced Raman Spectroscopy Measurements

SERS measurements were carried out on silver colloids prepared using the Lee–Meisel method.¹⁶ Samples for macro-SERS experiments were prepared by solving 0.6 mg of HSA in 100 μL of water. Then, an aliquot of a 10^{-3}M emodin solution in dimethylsulfoxide (DMSO) (DMSO) was added to obtain 1:4 or 1:2 emodin/albumin complexes. At these relative concentrations a complete interaction of the drug with the protein is ensured within approximately 12 h. Samples containing only drug were also aged for the same time under the same conditions. The colloid was activated before being added to the drug–protein complex solution by adding 30 μL of an aqueous 0.5M sodium nitrate solution to 1 mL of the original colloid. Afterward, 270 μL of this activated colloid was added to the solution of the drug/albumin complex, leading to a drug/albumin concentration ratio of $6 \times 10^{-6}\text{M}/2.4 \times 10^{-5}\text{M}$ (in 1:4 complexes) and $1.2 \times 10^{-5}\text{M}/2.4 \times 10^{-5}\text{M}$ (in the 1:2 complex). The pH of the final mixture was about 6.5 in all cases and the DMSO in the sample was at a concentration of 0.8–1.6% (v/v). SERS of emodin at different pH were obtained by adjusting the final pH with HNO_3 or NaOH.

Instrumentation

SERS spectra were recorded in a U-1000 Jobin-Yvon spectrophotometer by excitation with the 514.5-nm line provided by a Spectra Physics Model 165 argon ion laser. Resolution was set to 4 cm^{-1} and a 90° geometry was used to record the data. The laser power at the sample was fixed at 20 mW. The samples for SERS measurements in this Raman spectrophotometer were placed in 1-mm-diameter glass capillaries.

UV-Vis Absorption measurements were done in a UV-2401PC Shimadzu spectrophotometer, using a 1-cm-path-length cell.

RESULTS AND DISCUSSION

UV-Visible Absorption of Emodin and Its Albumin Complexes

In Figure 2a the UV-Vis absorption spectra of free emodin at different pH are plotted. Three emodin

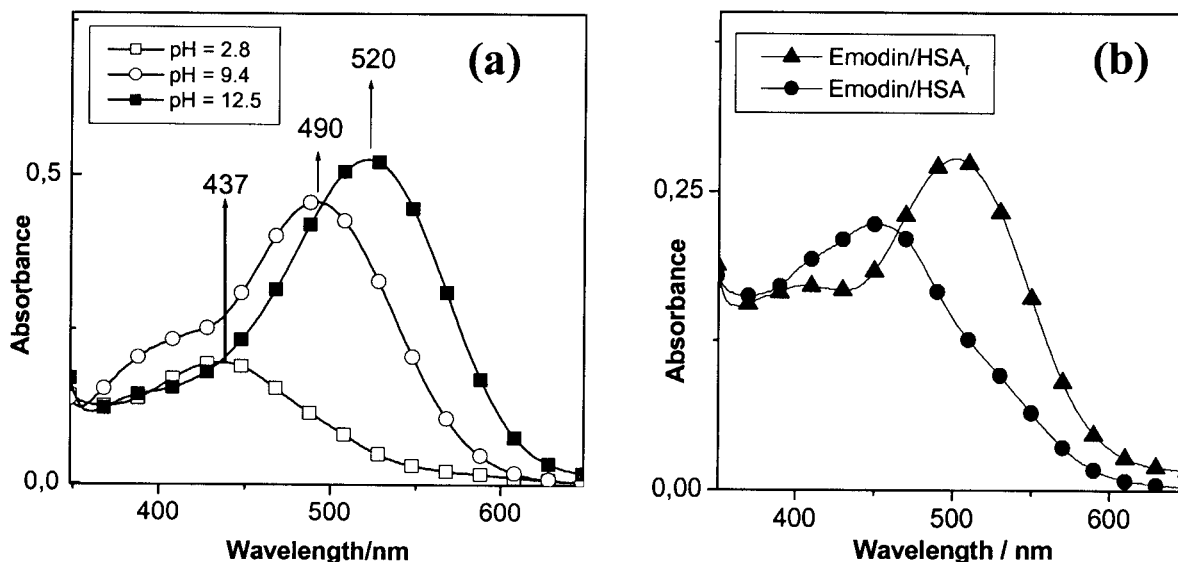


FIGURE 2 (a) UV-visible absorption spectra of emodin ($6 \times 10^{-6}M$) at pH 2.8, 9.4, and 12.5. (b) UV-visible absorption spectra of emodin/albumin complexes ($6 \times 10^{-6}M/2.4 \times 10^{-5}M$).

species (neutral, monoanionic, and dianionic, Figure 1) can be observed with characteristic maxima at 437, 490, and 520 nm, respectively.

In Figure 2b the absorption spectra of emodin/albumin complexes are displayed. For HSA_f (squares) the absorption spectrum is similar to the spectrum at alkaline pH (closed squares in Figure 2a), thus indicating that the drug is under the dianionic state in the complex. However, in the case of HSA, the emodin absorption spectrum is similar to the neutral emodin, with a maximum at 450 nm.

SERS of Emodin and Its Albumin Complexes

SERS spectra of emodin at different pHs are shown in Figure 3. Usually, the molecular pK_a value is shifted downward when the adsorbate is linked to a metal surface. Thus, the spectra of emodin recorded at pH 4, 7, and 12 (Figure 3a–c) must correspond to the spectra of the neutral, monoanionic, and dianionic emodin species. The main changes observed in these spectra concern the band at 1673 cm^{-1} , which is shifted to 1641 cm^{-1} at alkaline pH, and the bands at $1360\text{--}1250\text{ cm}^{-1}$, which undergo an upward shift. This effect is a consequence of the deprotonation and the electronic resonance increase in the drug. Another interesting effect of the pH is the progressive decrease of the relative SERS enhancement as the pH was raised, deduced by taking the 679 cm^{-1} band of DMSO as an

internal standard. In principle this is in contradiction with the higher absorption of dianionic emodin at 514.5 nm (Figure 2a). Hence, this emodin species could undergo a nonradiative de-excitation by interaction with the metal or by the formation of an ionic pair.

The SERS spectrum of emodin/albumin complexes reveals interesting differences depending on the use of HSA_f or HSA.

The 1:4 emodin/HSA_f complex displays a very weak spectrum (Figure 3g). When this ratio is increased to 1:2 the SERS intensity is increased (Figure 3f). The multiplied SERS of Figure 3e (Figure 3f, x3) shows a higher similarity to the SERS of emodin at alkaline pH (Figure 3c). In contrast, the intensity of the 1:4 emodin/HSA complex is approximately 1 order of magnitude higher (Figure 3d) than the SERS of the 1:2 emodin/HSA_f complex (Figure 3e), as deduced from the comparison of the 679 cm^{-1} band of DMSO. In addition, the SERS spectral pattern of the emodin/HSA complex is very similar to the SERS of emodin recorded at acidic pH (Figure 3a).

The above differences between the SERS of emodin/HSA_f and emodin/HSA complexes indicate the existence of two different binding sites for emodin in human albumin, which depends on the presence of FA in the protein. With respect to this, it is necessary to know some aspects of the binding behavior of albumins.

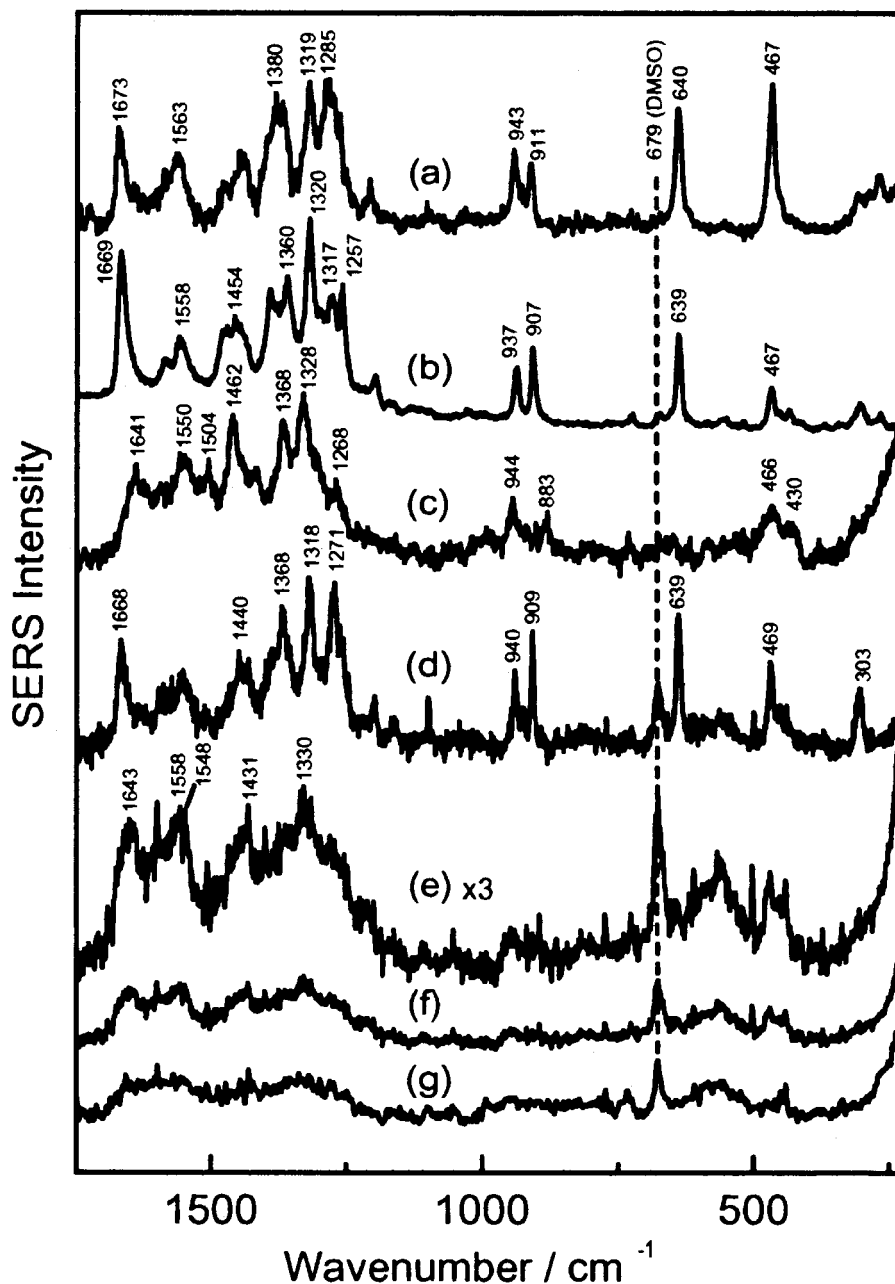


FIGURE 3 SERS spectra of emodin ($6 \times 10^{-6}M$) at pH 4 (a), 7 (b), and 12 (c) together with the SERS spectra of emodin/albumin complexes under the following conditions: (d) 1:4 emodin/HSA ($6 \times 10^{-6}M/2.4 \times 10^{-5}M$); (e) 1:2 emodin/HSA_f ($1.2 \times 10^{-5}M/2.4 \times 10^{-5}M$), threefold intensified *f* spectrum; (f) 1:2 emodin/HSA_f ($1.2 \times 10^{-5}M/2.4 \times 10^{-5}M$); and (g) 1:4 emodin/HSA_f ($6 \times 10^{-6}M/2.4 \times 10^{-5}M$). Excitation at 514.5 nm

It is generally accepted that the primary binding sites of small aromatic ligands in HSA are localized in subdomains IIA and IIIA,¹⁷ which correspond to the I and II Sudlow sites.¹⁸ Thus, emodin may interact with albumin with one or two of these sites.

On the other hand, albumin's primary binding sites corresponding to FA are placed in subdomains IB, IIIA, and IIIB,¹⁷ with IIIA (site II) being the most important.¹⁹ Thus, FA and emodin have a common binding site in site II. Since the presence of FA in

HSA dramatically changes the structure of emodin in the complex with HSA, we suggest that the primary binding site of emodin in defatted albumin corresponds to site II, where emodin may interact through electrostatic interaction with the basic amino acid residues localized in the pocket entrance,¹⁷ since the drug is under the dianionic form in the complex with HSA_f (Figure 3e). Although this site seems very shallow, in our SERS spectra a very weak signal was observed for the 1:4 emodin/HSA_f complex (Figure 3g). This is attributed to the lower photoactivity of emodin under the mono- and dianionic forms.

In the emodin/HSA complex (Figure 3d), site II could be occupied by one or two fatty acid molecules, depending on the fatty acid length,¹⁹ thus displacing the emodin to site I as also occurs with other ligands displaying affinity for both binding sites.^{20,21} Since the SERS of emodin/HSA complex is similar to that of the neutral drug at low pH (Figure 3a), we suggest that the drug is placed in the hydrophobic cavity of site I, interacting with the amino acid residues therein by means of H-bonds and hydrophobic interactions. In addition, the interaction of FA with the IB site, which is another primary binding site of FA, may favor the interaction of the drug with site I, since in the IB subdomain site FA induce a relative rotation of domains I and II of HSA, rendering this site more accessible for the union with drugs.²² Although emodin could be placed in a deeper position in site I than in site II, the higher photoactivity demonstrated by the neutral drug could induce a further SERS intensity increase in the 1:4 emodin/HSA complex (Figure 3d).

CONCLUSIONS

Emodin can interact with human albumin through two different binding sites corresponding to the I and II Sudlow binding sites. The primary interaction site of emodin seems to be site II in defatted albumins, where the drug interacts with the basic amino acid residues existing at the entrance of the cavity through its dianionic form.

In contrast, in the presence of fatty acids the drug seems to be removed from site II and is displaced to site I, where emodin interacts with the protein through its neutral form in the hydrophobic cavity of this site. The drug may interact through the H-bond, affecting both the keto and the hydroxyl groups with amino

acid residues existing in this cavity. Since under physiological conditions the albumin molecules are actually transporting FA, we suggest that emodin could then be located and transported by serum albumins in site I.

In conclusion, SERS spectroscopy has revealed important data concerning the interaction of anthraquinone drugs with albumins of different origins and the influence of other ligands, such as fatty acids, on the interaction of drugs with these important blood carrier proteins.

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