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Joint application of micro-Raman and surface-enhanced Raman spectroscopy to the interaction study of the antitumoral anthraquinone drugs danthron and quinizarin with albumins

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We report on the joint application of surface-enhanced Raman scattering (SERS) and micro-Raman techniques to the study of danthron (DT) and quinizarin (QZ) and their complexes with human and bovine albumins. We propose a novel method based on the combination of SERS and micro-Raman spectroscopy by using immobilized Ag nanoparticles, which we have named micro-SERS, to improve the sensitivity of the SERS technique and that could be applied in recognition processes of drugs with large biomolecules. The micro-SERS technique demonstrated clear advantages over direct measurements in air: reproducibility, rapidity and high sensitivity due to a large coverage of the metal surface and good organization of the adsorbate on the surface. Furthermore, the chemical damage of the analyte induced by laser irradiation is substantially reduced. By means of this method, we were able to deduce important information concerning the binding properties of DT and QZ when interacting with human and bovine albumins. For instance, the interaction mechanism can be drastically modified when fatty acids are present in albumins, there are large differences between human and bovine albumins in the binding of the studied drugs related to changes in the amino-acid sequence of the binding pockets of these proteins and the interaction mechanism of the analysed drugs can be different depending on their structure. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: SERS; micro-Raman; danthron; quinizarin; albumin; fatty acids

INTRODUCTION

Dihydroxyquinones are compounds of significant chemical and biochemical interest. These molecules have important applications as a prominent family of pharmaceutically active and biologically relevant chromophores, as an analytical tool for the determination of metals and in many aspects of electrochemistry.[1] Our interest in these molecules arises not only because of their own biological activity, but also because they are the molecular basis of other more active drugs such as hypericin and emodin and hence their study could help in understanding which molecular groups are actually important for the biological activity of drugs based on hydroxyanthraquinone structures.

The vibrational characterization of anthraquinone derivatives must be done by surface-enhanced Raman scattering (SERS) spectroscopy owing to their intense fluorescence emission, as we have already demonstrated in recent studies.[2–4] SERS induces a large enhancement of the Raman signal together with fluorescence quenching, which allows the application of this technique in recognition studies involving highly fluorescent molecules and the employment of low concentrations of the biological molecule involved in the interaction. Here we propose a novel method based on the combination of SERS and micro-Raman spectroscopy, which we have named micro-SERS, to improve the sensitivity of the SERS technique and that could be applied in recognition

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processes of drugs. We applied the micro-SERS technique to the study of quinizarin (QZ) and danthron (DT) and their complexes with human and bovine serum albumins (HSA and BSA).

The interaction of drugs with albumins is an important process in the biological activity of insoluble drugs, as is the case with most anthraquinone and naphthoquinone derivatives, since the transport with serum proteins ensures their delivery in the organism. Hence there is great interest in the application of spectroscopic techniques in the study of drug–albumin interactions. 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.[5,6] Serum albumin as the most abundant plasma protein contributes significantly to many transport and regulatory processes. The protein binds a wide variety of substrates such as metals, fatty acids, amino acids, hormones and large range of therapeutic drugs.[7] Because of its clinical and pharmaceutical importance, the interaction of serum albumin with a variety of ligands has been extensively studied.[8–12]

The application of the micro-SERS technique to the study of other simpler anthraquinone molecules such as DT and QZ (Fig. 1) could help in the understanding of the mechanism of interaction and action of other more active drugs. To accomplish this study, we used both defatted (HSA_f and BSA_f) and non-defatted (HSA) albumins in order to check also the influence of fatty acids (FA) on the binding of drugs by albumins. The influence of FA, or rather their dissociated forms (carboxylates), on drug binding to albumins has been already studied for other albumin ligands, demonstrating that it can dramatically change the protein affinity for these ligands.[13]

EXPERIMENTAL

Materials

DT and QZ were purchased from Sigma. HSA, HSA_f and BSA_f were also purchased from Sigma and used without further purification. All the reagents were of analytical grade and triply distilled water was employed to prepare all solutions.

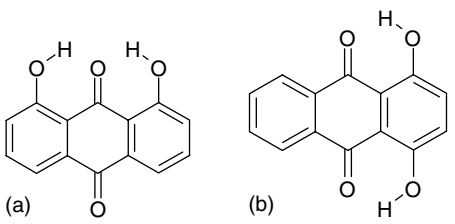

Figure 1. Structures of (a) danthron and (b) quinizarin.

Samples for surface-enhanced Raman spectroscopic measurements

SERS measurements were carried out on silver colloids prepared using the Lee–Meisel method.[14] Samples of the complexes were prepared by dissolving 1 mg of the albumin in 100 µl of water. Then, 3.7 µl of a 10^{-3} M drug solution in dimethyl sulfoxide (DMSO) was added to obtain 1 : 4 drug–albumin complexes. At these relative concentrations, complete interaction of the drug with the protein is assumed. The above mixtures were left for 15 h in order to allow the interaction of drugs with proteins. Samples containing only the 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. This activation consisted in partial aggregation of the colloidal particles by adding 30 µl of an aqueous 0.5 M sodium nitrate solution to 1 ml of the original colloid. Subsequently, 270 µl of this activated colloid were added to the mixture, leading to further dissolution of the above complex to a final ratio of $10^{-5} : 4 \times 10^{-5}$ M for the drugs–albumin complexes. Although the samples prepared in such a way were stable for several days, they were left for 1 h at room temperature before starting the measurements to allow the complete diffusion of the complex to the surface. This step is important in biomolecules of large size, which need more time for complete diffusion through the bulk. The pH of the final mixture was about 6.5 in all cases and the DMSO concentration in the sample was 1%.

The final samples for micro-SERS experiments were prepared in the following way: 20 µl of the activated suspension prepared by the method described above were placed on a glass slide cover and dried at room temperature. In this way, a large amount of analyte migrates to the surface, leading to a large surface coverage. The same suspension was placed on a glass slide provided with a groove (2 cm diameter and 0.38 mm deep), which was then covered with the cover glass containing the dried sample with the side containing the dried colloid facing downwards and the suspension placed in the groove as depicted in Fig. 2.

This method leads to an increase in the sensitivity of micro-SERS since the excess of molecules and ions adsorbed on the metal nanoparticles is desorbed, passing to the solution in contact with the liquid phase. Hence an equilibrium with a suspension of the same characteristics is established in such a way that a large concentration of adsorbed molecules remains at the surface. This method is particularly useful for very expensive analytes or poorly soluble molecules, which cannot diffuse well through the aqueous solution to the metal surface.

One of the main advantages of this method is that the chemical photodegradation of the analysed biological materials is overcome because the metal nanoparticles are in contact with the aqueous solution and the heat originating from the laser irradiation is dissipated by the solvent. In addition, a high homogeneous distribution of adsorbate molecules on the metal is achieved, since the molecules

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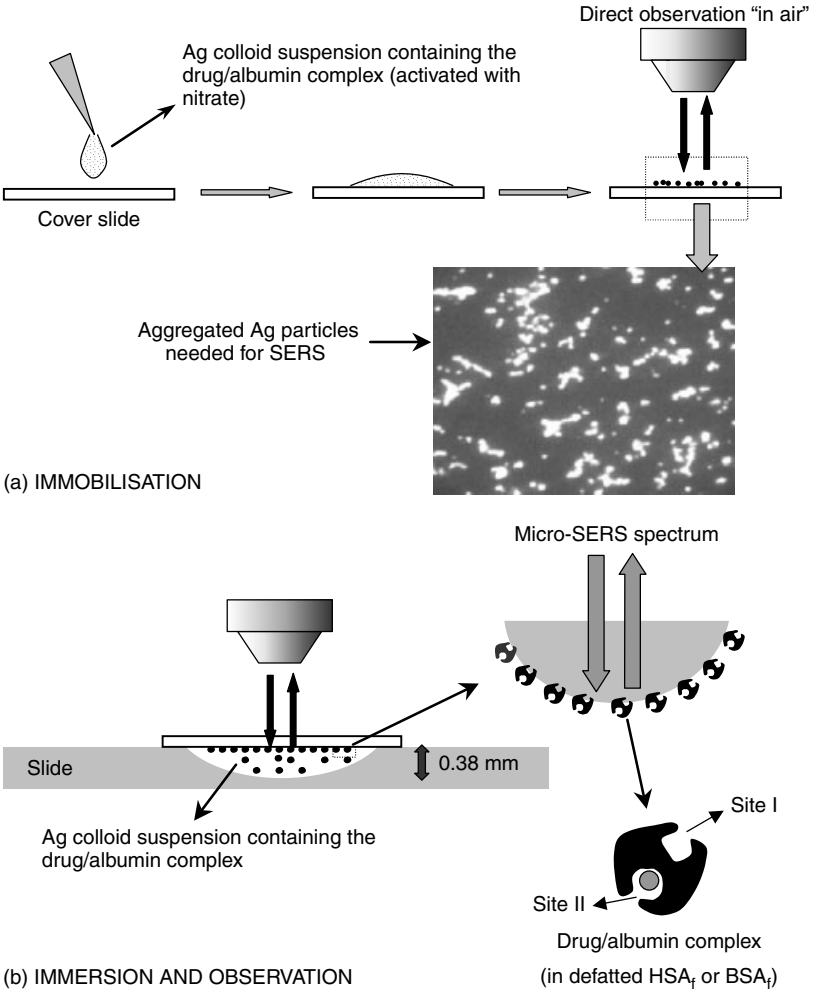

Figure 2. Experimental set-up for micro-SERS measurements.

are constricted to interact with the surface during the drying process. Subsequently, when the dried Ag nanoparticles are soaked in the Ag colloidal suspension placed in the groove, the diffusion process occurs in the opposite direction, from the metal to the bulk, thus leading to better organization of the adsorbate on the metal surface, which renders a more homogeneous coverage.

The immobilized silver nanoparticles were examined with the Raman microscope by focusing the laser beam into the Ag nanoparticles placed under the cover glass, as depicted in Fig. 2. The micro-Raman spectra of the dried nanoparticles were also recorded in air for the same cover glasses which were not put in contact with the aqueous colloid suspension, obtaining very different spectra depending on the analysed aggregate (spectra not shown) due to the photothermal decomposition of the sample induced by the laser irradiation. Despite this, the SERS analysis in air of metallic particles is an experimental device currently employed in single-molecule detection but the results are not reliable in some cases, as also found by other workers.[15]

Instrumentation

Raman spectra were recorded by means of a Renishaw RM1000 Raman microscope system equipped with a diode laser operating at 785 nm and a Leica microscope and an electrically refrigerated CCD camera. The spectra shown here were obtained by using an NPLAN 100× objective. The laser output was 25 mW. The resolution was set to 4 cm^{-1} and one scan of 10 sec was recorded.

RESULTS

Mico-SERS of danthron

Micro-SERS spectra of DT and the different complexes with albumins are displayed in Fig. 3. A previous study of this molecule at different pH values was carried out in order to relate the interaction of this molecule with the proteins with possible changes in the protonation state of the ligand, as was also observed for other similar molecules.[5,16] More details about the acidic behaviour of DT and QZ on Ag colloids will be published elsewhere.[17]

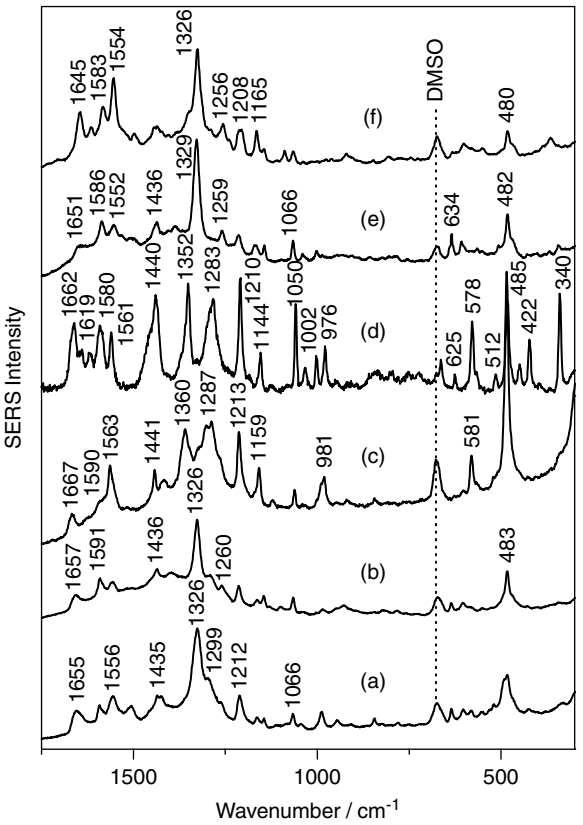

Figure 3. Micro-SERS spectra of DT (10^{-5} M) at pH (a), 11.5 (b) 6.5 and (c) 3.5 and SERS spectra of the following complexes: (d) DT–HSA; (e) DT–BSA_f; and (f) DT–HSA_f. All complexes at a 1 : 4 concentration ratio (10^{-5} : 4×10^{-5} M).

In the SERS spectrum of DT at pH 3.5 [Fig. 3(c)], a set of bands at 1667, 1590, 1563, 1441, 1360, 1302, 1287, 1213 and 485 cm^{-1} is observed, which corresponds to the neutral form of danthron.[17] On the other hand, the SERS spectra at pH 6.5 and 11.5 [Fig. 3(b) and (a)] are similar with respect to the position and relative intensity of the bands attributed to the C═O and C—OH groups, indicating that they mainly correspond to the monoionized form of DT. However, the dianionic form of DT could correspond to the small, new bands increasing at alkaline pH at 1505, 986, 844 or 580 cm^{-1}. However, the SERS spectrum at acidic pH [Fig. 3(c)] shows many differences characteristic of the transition from neutral to monoionized danthron which are the shift downwards of the band at 1667 cm^{-1} to 1655 cm^{-1}, the disappearance of bands observed at 1360 and 581 cm^{-1} and the appearance of a strong band at 1326 cm^{-1}. All these changes are related to deprotonation of the molecule since they involve bands attributed to C—OH bending or C═O stretching motions,[16] which are very sensitive to the internal H-bonds.

The SERS spectra of DT–BSA_f and DT–HSA_f complexes [Fig. 3(e) and (f)] are similar to those at pH 6.5 and 11.5 [Fig. 3(a) and (b)], except for some small differences, indicating that DT interacts with those proteins through its dissociated form. In the DT–HSA_f complex [Fig. 3(f)], further shifts downwards are observed for the bands at 1655 and 1593 cm^{-1} to 1645 and 1583 cm^{-1}, respectively. In addition, new bands are observed at 1614 and 1348 cm^{-1}. The latter changes could be due to the second ionization of danthron, which could not be observed when the free molecule is adsorbed on Ag, but which can occur when DT is interacting with HSA_f.

In contrast, the SERS spectrum of the DT–HSA complex [Fig. 3(d)] resembles that of DT at low pH [Fig. 3(c)], where DT is in the neutral form. This clearly suggests that the drug is in the neutral form in the complex with non-defatted HSA. In spite of the similarity found between the SERS spectra of the DT–HSA complex and that of DT at acidic pH, small shifts of the bands are observed, together with numerous new bands appearing at 1629, 1619, 1025, 1002, 850–700, 625, 512, 448, 422 and 340 cm^{-1}. Many of these new bands probably correspond to the protein, whereas others may be due to the structural modification undergone by DT in the complex with HSA.

Micro-SERS of quinizarin

Micro-SERS spectra of QZ and its different complexes with albumins are shown in Fig. 4. As in the case of DT, the changes observed on decreasing the pH [Fig. 4(a)–(c)] are attributed to a change in the protonation state of QZ. In this context, the shift of the C═O stretching band downwards, together with the shift upwards of the two intense bands at 1392 and 1232 cm^{-1} observed at high pH [Fig. 4(a)] to 1403 and 1270 cm^{-1} must be a consequence of deprotonation of QZ. The SERS spectra recorded at neutral and alkaline pH reveal differences between them, which may be due to a second ionization of the molecule.

The SERS spectrum of the QZ–HSA_f complex [Fig. 4(f)] in general shows a pattern closer to that of QZ at pH 11.5 [Fig. 4(a)], indicating that QZ interacts with HSA_f through its dianionic form. For instance, the C═O stretching is further shifted towards lower wavenumbers (1616 cm^{-1}) in the QZ–HSA_f complex [Fig. 4(f)], and new bands are observed at 1374, 1338, 1269 and 1253 cm^{-1}. These changes indicate that QZ could lose the remaining proton in order to interact as the dianionic form with HSA_f.

However, the SERS spectral pattern of the QZ–BSA_f complex [Fig. 4(e)] approaches that of QZ at pH 6.5 [Fig. 4(b)], owing to the lower intensity of the 1224 cm^{-1} band and the higher intensity of the bands at 1073, 655 and 466 cm^{-1}, which suggest a different interaction mechanism of the drug in each case. In contrast, the SERS spectrum of the QZ–HSA complex [Fig. 4(d)] is more similar to that of QZ at low pH [Fig. 4(c)], where QZ is assumed to be in the neutral form, as demonstrated by the existence of bands at 1642, 1404, 1368, 1341, 1262 and 1144 cm^{-1}. As in the case of DT, this result indicates that QZ interacts with non-defatted albumins in its neutral form.

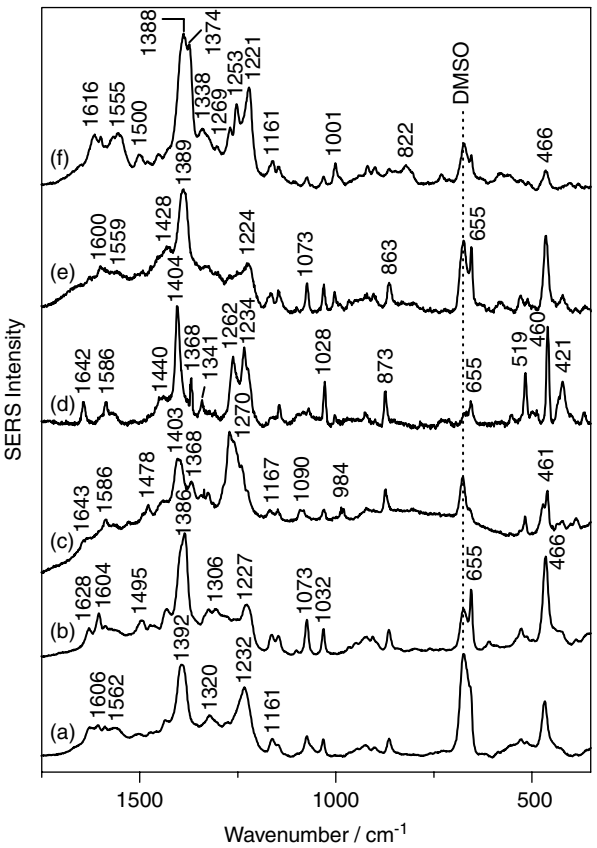

Figure 4. Micro-SERS spectra of QZ (10^{-5} M) at pH (a), 11.5 (b) 6.5 and (c) 3.5 and SERS spectra of the following complexes: (d) QZ–HSA; (e) QZ–BSA_f; and (f) QZ–HSA_f. All complexes at a 1 : 4 concentration ratio (10^{-5} : 4×10^{-5} M).

The effect of the protein in the complex with HSA leads to evident spectral changes for neutral QZ, e.g. the enhancement of the ν(C=O) band at 1642 cm^{-1}, the disappearance of the band at 1478 cm^{-1} and the downward shift of the broad band at 1270 cm^{-1} to 1262 and 1234 cm^{-1}. The latter changes can be attributed to the establishment of H-bonds between the molecules and amino acid residues existing in the site I cavity through the keto and hydroxyl groups of QZ.

DISCUSSION

The differences found between the SERS of drug–HSA_f, drug–BSA_f and drug–HSA complexes indicate the existence of different binding sites for anthraquinones in albumin. On the other hand, the affinity of these sites depends on the presence of other ligands in the protein such as fatty acids. With respect to this, it is necessary to know some aspects of the binding behaviour of albumins.

It is generally accepted that the primary binding sites of small aromatic ligands in HSA are localized in subdomains IIA and IIIA,[18] which correspond to the I and II Sudlow's sites.[19] Thus, anthraquinones may interact with albumin with one or two of these sites. On the other hand, primary binding sites of albumins corresponding to fatty acids are placed in subdomains IB, IIIA and IIIB,[17,19] the IIIA binding site being the most important.[20]. Since the presence of FA in HSA dramatically changes the structure of anthraquinones in complexes with HSA, we suggest that the primary binding site of these molecules in albumin corresponds to site II, placed in the IIIA subdomain, which coincides with the primary binding site of long-chain FA. Hence, in the absence of FA, anthraquinones seem to interact with defatted albumins through site II, most probably through electrostatic interaction involving the basic amino acid residues mainly localized in the pocket entrance.[18]

In the presence of FA, site II could be occupied by one or two fatty acid molecule(s), depending on the FA length,[20] thus displacing the ligand to site I of subdomain IIA. Since the SERS spectral profile of anthraquinones in this site is similar to that observed for the drug at low pH, i.e. to neutral ligands, we suppose that the drug is located 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 through another primary binding site, IB, may favour the interaction of the drug with site I, since this union induces a relative rotation of domains I and II of HSA which leaves this site more accessible for the union with drugs.[20] Similar results were found for the related molecule emodin,[22] indicating that the dihydroxyanthraquinone drugs exhibit similar binding properties with respect to albumins irrespective of the distribution of the OH groups.

Fixing the 679 cm^{-1} of DMSO as reference, we compared the relative SERS intensities of the drug–albumin complexes. For all the analysed drugs the SERS intensity is further enhanced in the case of the drug–non-defatted HSA complexes [Figs 3(d) and 4(d)]. This is obviously related to the fact that in the latter complexes the drug is supposed to be located in the hydrophobic pocket of site I in subdomain IIA in the neutral form. Under these conditions (neutral molecule placed in a hydrophobic environment), the drug should display a higher Raman cross-section.[23] On the other hand, the comparison with the SERS spectra of the same molecule at acidic pH [Figs 3(c), 4(c) Figs 3(d) and 4(d)] reveals clear differences of the ligand in the complex with respect to the free one, which mainly involve the keto and the adjacent OH groups, and which are related to the formation of H-bonds with H donors existing in the latter cavity as also found for hypericin.[5,6]

The results obtained in this work demonstrate the existence of several differences between HSA_f and BSA_f concerning the binding of the studied drugs. It is usually accepted that the binding properties of site II (in subdomain IIIA) are essentially the same irrespective of the origin of the albumin because the amino acid sequence of the subdomain, mainly the amino acids in direct contact with the ligand, is greatly conserved in all mammalian albumins.[18] Nevertheless, for the drug–HSA_f complex, the

SERS profile changes considerably with respect to the BSA_f complex for both DT and QZ in the sense of a larger electronic delocalization, which is probably due to the second ionization of the drug in the presence of human albumin. This is related to the existence of a higher amount of basic amino acid residues in subdomain IIIA of HSA_f than in the same subdomain of BSA_f.[18] Furthermore, the appearance of bands corresponding to certain aromatic amino acid residues in the SERS spectra of drug–defatted HSA and BSA suggests the interaction of these drugs through an additional mechanism of interaction, such as stacking and H-bond interactions, in addition to the ionic interaction of negatively charged anionic drugs with basic amino acids. For instance, the enhancement of the tyrosine (Tyr) bands[23] at 1616, 1338, 1161 and 822 cm^{-1} in the QZ–HSA_f complex [Fig. 4(f)] indicates that some Tyr residue existing in the site I, most probably Tyr 411 could be involved in the interaction with QZ through an H-bond, as in the case of fatty acids.[21] This interaction could be more effective in the case of the human albumin.

CONCLUSIONS

Micro-SERS on immobilized colloidal particles is a promising technique for studying recognition phenomena in biomolecules as demonstrated by the large sensitivity enhancement of the SERS signal. The immobilization, followed by immersion, method reported in this paper demonstrated clear advantages with respect to direct measurements in air: reproducibility, rapidity and high sensitivity due to a large coverage of the metal surface and the adoption of good organization of the adsorbate on the surface. Furthermore, this method avoids chemical damage of the analyte induced by laser irradiation.

Using this method, we have deduced that DT and QZ can interact with human and bovine albumins through two main different binding sites which are probably localized in the IIA and IIIA hydrophobic pockets of HSA, corresponding to Sudlow's binding sites I and II, respectively. The primary interaction site of these drugs seems to be localized at site II in defatted albumins, where the drug interacts via ionic bonds with the basic amino acid residues existing at the entrance of the cavity through its monoanionic form and, probably, with the Tyr 411 existing in this cavity. In the case of the drug–HSA_f complex, the drug seems to interact with the protein in its dianionic form, demonstrating the existence of clear differences from site II of BSA_f, where the drug seems to interact in its monoanionic form.

In the presence of FA, the ligands seem to be removed from site II, because it is a primary binding site for medium- and long-chain FA, and are displaced to site I, where the molecule interacts with the protein in its neutral form in the hydrophobic cavity of this site through hydrophobic stacking and H-bonds involving its keto and OH groups. Further experiments concerning the association constants of QZ and DT with the studied albumins are in progress in order to establish the importance of the relative position of OH groups in the binding of anthraquinones with albumins.

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 FA, on the interaction of drugs to these important carrier proteins of blood.

Acknowledgements

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