RSC Advances

Monitoring the metallofulfenamic – bovine serum albumin interactions: A novel method for metallodrug analysis

Journal:	*RSC Advances*
Manuscript ID:	RA-ART-07-2014-007638.R1
Article Type:	Paper
Date Submitted by the Author:	03-Sep-2014
Complete List of Authors:	Abdelhamid, Hani; NSYSU, chemistry department Wu, Hui Fen; National Sun Yat-Sen University,

Monitoring the metallofulfenamic – bovine serum albumin interactions: A novel method for metallodrug analysis

Hani Nasser Abdelhamid[1,3], Hui-Fen Wu[1,2,4,5,6]*

[1]Department of Chemistry, National Sun Yat-Sen University, Kaohsiung, 804, Taiwan
[2]School of Pharmacy, College of Pharmacy, Kaohsiung Medical University, Kaohsiung, 800, Taiwan
[3]Department of Chemistry, Assuit University, Assuit, 71515, Egypt
[4]Center for Nanoscience and Nanotechnology, National Sun Yat-Sen University, Kaohsiung, 804, Taiwan
[5]Doctoral Degree Program in Marine Biotechnology, National Sun Yat-Sen University and Academia Sinica, Kaohsiung, 80424, Taiwan
[6]Medical Science and Technology, National Sun Yat-Sen University, Kaohsiung, 804, Taiwan
* Corresponding author. Phone: 886-7-5252000-3955. Fax: 886-7-525-3908.
E-mail: hwu@faculty.nsysu.edu.tw
Contract/grant sponsor: Ministry of Science and Technology, Taiwan

Abstract

A new methodology for the drug/metallodrug detection in aqueous solution and their interactions with serum albumin were presented. The interactions of the metals with flufenamic drug (FFA, non-steroidal anti-inflammatory drugs(NSAIDs)) were studied by quantum dot assisted laser desorption/ionization mass spectrometry (QALDI-MS) and Fourier transform infrared (FT-IR). The drug/metallodrug interactions were investigated by many instruments such as fluorescence and 3D fluorescence spectroscopy, Fourier transform infrared and matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS). QALDI-MS offered a soft ionization of the weak interactions of the metallodrugs because there is no disturbance of the non-covalent bonds among the metals with this drug. Fluorescence emission spectra of BSA in the presence of FFA and metalloflufenamic were recorded at the excitation wavelengths 295 nm (tryptophan), clearly showed that the FFA and metalloflufenamic act as quenchers. The quenching of the BSA emission is attributed to changes in the environment of the protein fluorophores after the drug

and their complexes bind to the protein backbones. 3D-fluorescence spectra showed strong interactions with the order of Fe(III)-FFA complex > Cu(II)-FFA complex > FFA according to the quenching of tryptophan emission fluorophore. FTIR studies confirmed the interaction among FFA, metallodrug and protein. Successful detection of the high mass protein of BSA, BSA-FFA and metallodrug-BSA complexes about 66 kDa could be used to probe their non-covalent interactions based on mass shift or the change on these peaks profile.

Keywords: NSAIDs, metallodrug-protein interaction, MALDI, fluorescence.

Introduction

In recent years, considerable efforts have been performed in monitoring biomolecular interactions, such as protein-protein, drug-protein and metallodrug-protein interactions, because it is paramount important for understanding/describing a physiological process, and exert the biological effect e.g. drug-modulating protein-protein interactions [1-6]. Probing metallodrug - biomolecule interactions are important for understanding the roles of metals in biology and the molecular mechanism of metallodrug actions. These studies directed metallo-proteomics and metallomics toward drug-free side effect or to improve the drug efficiency. Measurements of the affinity of drugs or its metallodrug-protein interactions not only provide insight into basic cellular or biological processes but also facilitate the development of chemotherapeutics drugs. Monitoring albumin-metallodrug interactions are very important because once the drug enters the human body, the blood proteins can interact with the drug/metallodrug that would ultimately affect cellular uptake, thus alter biochemical activity of human system. These studies are important because serum albumins (SAs) are the most abundant soluble proteins in human plasma and other mammalians. If the drug enter the body, it could interact with SAs or metals in

the biological fluids. Conventionally, to probe the drug–protein binding processes, bovine serum albumin (BSA) is used as a model compound due to its similarity to human serum albumin (HSA) [7].

Fenamates drugs family (anthranilic acid derivatives) are non-steroidal anti-inflammatory drugs (NSAIDs). Nowadays, they are widely used therapeutic agents for the treatment of many pathophysiological conditions for analgesic, antipyretic, anti-inflammatory, and fever. We selected flufenamic drug (FFA) as a model for this drug family. It is a non-steroidal anti-inflammatory drug (NSAIDs) that can suppress inflammation in a manner similar to steroids but with less side effects. Their mode of action is mainly through inhibition of cyclooxygenases to reduce synthesis of the pro-inflammatory mediator prostaglandin H2 [8] or through fatty acid amide hydrolase and phospholipase A2 [9]. FFA has high affinity toward metals [10-11]. The biological activities of this drug after complexation with metals are increased [12]. Copper-NSAIDs interactions have been studied and are reviewed in Ref. [13]. However, modern studies have revealed that NSAIDs can cause arthritis and pain, cancer and neurodegenerative diseases such as the Alzheimer's disease [14-17]. Recently, NSAIDs were reported for treatment for nonmelanoma skin cancer (NMSC) which includes squamous cell carcinoma (SCC) and basal cell carcinoma (BCC)[18] and the results are no statistically significant effect on NMSC of NSAIDs. However, these studies provide prospective evaluation for the relationship between NSAIDs and NMSC. It was also reported that NSAIDs possess *in vitro* antibacterial against *Escherichia coli* DNA polymerase III β subunit [19].

Probing the metallodrug and metallodrug-protein interactions are a challenge task using matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) due to matrix acidity, interference (<600Da) and cluster formation. A clear knowledge of the coordination

structure of the metallodrug facilitated the development of metal-based drugs. Recently, a novel method for metallodrug ionization of this drug with graphene has been proposed [20]. Modern analytical techniques for metallodrug-protein interactions have been reported in Ref. [21]. Interactions of NSAIDs containing metals i.e metallodrug with SAs proteins are known to exert a great impact on the mode of action of these drugs, including drug metabolism, delivery, cell processing, and targeting. The interactions between some NSAIDs (naproxen, ibuprofen and flurbiprofen) and BSA or HAS using two complementary techniques, isothermal titration calorimetry (ITC) and frontal analysis/capillary electrophoresis (FA/CE). It can be concluded that the main interaction forces are hydrophobic and Van der Waals forces [22].

In this work, probing the non-covalent interactions of flufenamic acid (FFA) with biological metals (Fe(III) and Cu(II)) are demonstrated using quantum dots assisted laser desorption/ionization mass spectrometry (QALDI-MS) and Fourier transform infrared (FTIR). QDs are proposed as a soft matrix to avoid the conventional organic matrix drawbacks [20-23]. Non-covalent interactions among FFA, metalloflufenamic and BSA were investigated using various analytical methods. Data showed the interaction of the metallodrug species with BSA are strongly than the parent drug i.e FFA.

Experimental Section

Chemicals and Methods

The reagents utilized throughout this work were of analytical grade and all solution preparations and dilutions were made using 18 MΩ purified Millipore water (USA). Metal salt $CuSO_4.5H_2O$ and flufenamic acid were purchased from Sigma Company (China). 2,5-dihydroxy benzoic acid, $FeCl_3.6H_2O$ was purchased from Riedel-de-Haën (Seelze, Germany). Sinapinic acid was

purchased from Alfa Aeser (Great Britain). Methanol (HPLC grade) was purchased from Merck Co. All the chemicals were used directly without further purification. De-ionized water was used for preparation of solutions and complexes.

Instruments

Spectroscopy measurements

Fluorescence was performed in F-2700 Fluorescence spectrophotometer (Hitachi Co. Japan). Quantum dots were characterized using transmission electron microscopy (TEM, Philips CM200, Switzerland) at accelerated voltage 300 keV. FTIR measurements were carried out at room temperature on a Perkin Elmer Spectrum 100 FTIR. Raman spectra were recorded using Peak Seeker™ and Peak Seeker Pro™ (Agiltron, USA).

MALDI-TOF Instrument

MALDI-TOF-MS analyses were performed by employing positive ion mode on a time-of-flight mass spectrometer (Microflex, Daltonics Bruker, Bremen) with a 1.25 m flight tube. The metallodrug (<1000 Da) were recorded in positive/reflectron mode, while protein analysis are recorded in positive/linear mode. Desorption/ionization was obtained by using a 337 nm nitrogen laser with a 3 ns pulse width. Available accelerating potential is in the range of +20 kV. The spectra were presented in this article represent averages of 100-200 laser shots. Laser power was adjusted to slightly above the threshold to obtain good resolution and signal-to-noise ratios. Dried droplet is used for sample spotting into MALDI plate. All measurements were averaged over 3 scans to ensure consistency.

Preparation of CdS Quantum dots

CdS quantum dots were prepared as mention in Ref. [24]. Briefly, 400.0 µL of 3-MPA in 15.0 mL deionised water was stirred about 1.0 h. After that, 2.0 mL of 0.01 M cadmium nitrate solution

was added dropwisely under N_2 pressure and stirring for 1.0 h. pH of the solution was adjusted to 9.0 by adding ammonium hydroxide solution. Sodium sulphide solution (2.5 mL, 8.0×10^{-3} M) was quickly added to the above solution at 96 °C and solution was stirred for 2.0 h to get green yellowish CdS QDs.

Metal-drug and Metallodrug-Protein interactions

All solution (Cu(II), Fe(III), FFA, BSA) are prepared in the same concentration 1×10^{-3} M. For metallodrug detection, 1 mL of the drug mixed with Cu(II) and Fe(III) solution in molar ratio 1:2 and 1:3, respectively at pH=7.4 [18]. The mixing solution was incubated for 10 mins prior to analysis. For the interaction between the metallodrug and the protein, equal volume of the drug, metallodrug and protein (1:1, v/v, protein/drug) was incubated for 30 min prior to MALDI analysis. For fluorescence analysis, different volume of drug/metallodrug was added to 1 mL of protein solution and incubated for 10 min before measurements. FTIR of the drug and the metallodrug species were measured in liquid and solid form. For the solid measurement, the mixtures of BSA and FFA or their metallodrug were centrifuged at 10,000 rpm, and then it was washed to remove the unreacted species. The materials were measured after mixed with KBr in disc form.

Results and Discussion

Characterization of CdS quantum dots

CdS quantum dots were prepared and were modified with mercaptoproponic acid (MPA) i.e CdS@MPA. Simply, 3-MPA was stirred about 1.0 h with cadmium nitrate solution to form Cd^{2+}-MPA complex. This reaction takes place under N_2 pressure to avoid oxidation. Then, sodium sulphide solution was quickly added to the above solution at 96 °C and solution was stirred for 2.0 h to get green yellowish CdS@MPA QDs. CdS QDs were characterized using TEM, and

Raman spectroscopy. TEM image (Fig.1A) shows the sizes of CdS QDs. The sizes are in range of 1-5 nm with the average size 3.38 nm based on particles number (inset of Fig.1A). The Raman spectra (Fig.1B) revealed that CdS quantum dots were successfully modified with MPA.. Spectra show a strong peak at 300 cm^{-1} corresponding to Cd-S [25]. Peak at 1450 cm^{-1} (corresponding to C=O) shows shift to 1525 cm^{-1} after MPA capping CdS.

Metallodrug detection in aqueous solution using QALDI-MS

Detecting small molecules (M.Wt < 500Da) using conventional MALDI MS is a challenge as the organic matrices display interferences to produce ambiguous spectra. The spectrum used conventional matrix (2,5-DHB) to detect flufenamic drug (FFA) is shown in Fig.S1A. FFA has nominal mass 281.2 Da. 2,5-DHB produces many interferences with high intensity at *m/z* 137.1,155.2,177.0, 200.0, 264.0 and 273.3 Da corresponding to $[DHB–H_2O+H]^+$, $[DHB+H]^+$, $[DHB+Na]^+$, $[DHB+2Na]^+$, $[2DHB+H–CO_2]^+$ and $[2DHB–2H_2O+H]^+$, respectively [26]. Those peaks influence the small molecules detection in MALDI-MS. The spectrum of FFA (Fig.S1A) show the drug peak at 281.0, and 562.0 Da corresponding to $[FFA+H]^+$ and $[2FFA+H]^+$. These interferences including cluster ions are intense in the metallodrug detection (Fig.S1(B-C). In general, organic matrix are carboxylic acid, it can form cluster ions with the metals. Because of the acidity character, it destroys the weak interactions among the metal and the drug. Thus, the metallodrug detection in aqueous solution using conventional MALDI MS is a big challenge. Thus, we explored the application of CdS QDs for the metallodrug detection in aqueous solution. To detect the drug or their metallodrug is important for biomedicince and environment. The main purpose of this study is to introduce a new methodology to detect the complexation of FFA in aqueous solution at the physiological condition where the drug can interaction with the biological metals such as Cu(II) and Fe(III).

Figure 1

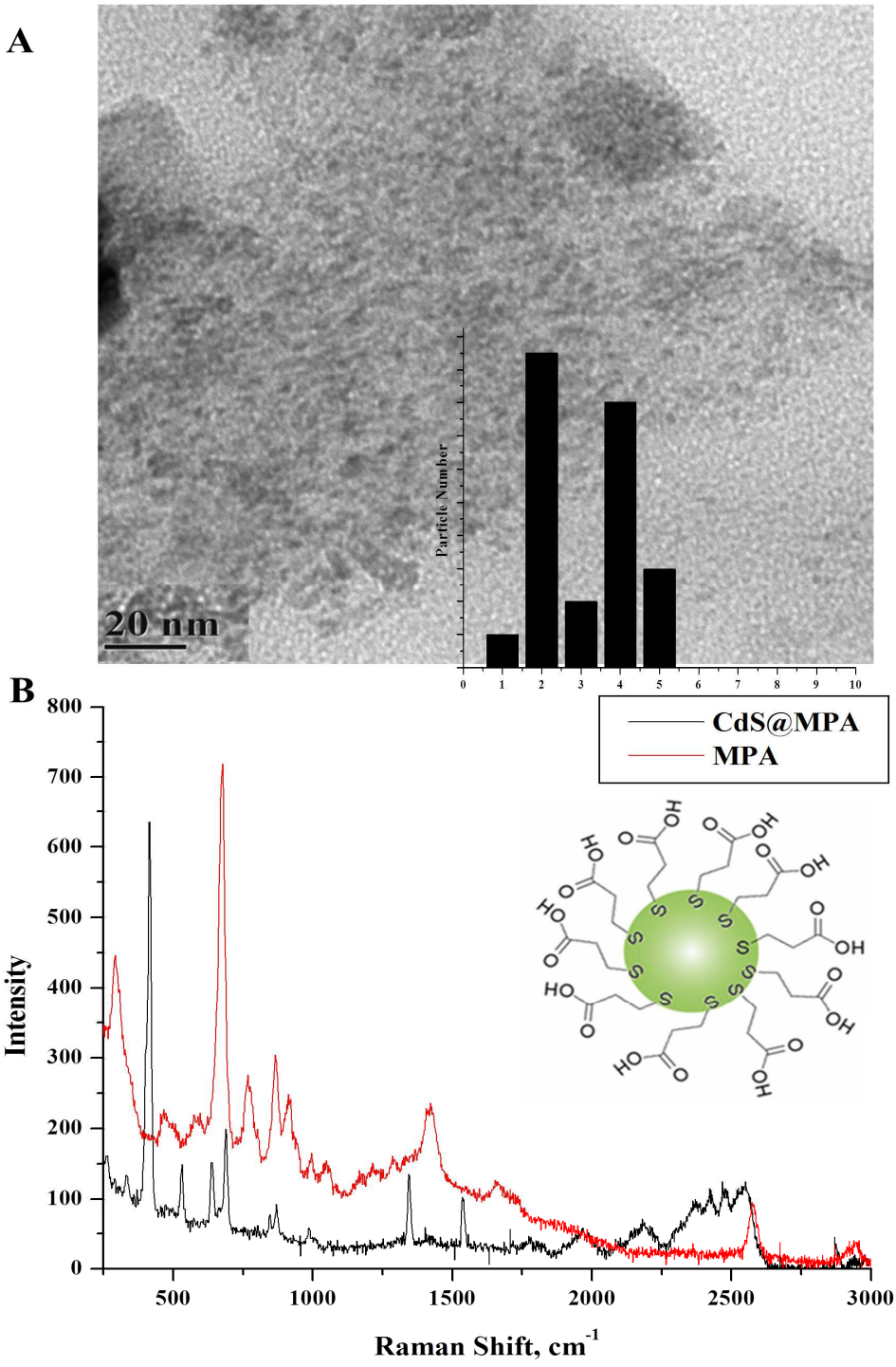


Fig 1: CdS@MPA quantum dots characterization using (A) TEM, the inset represents the size distribution diagram and (B) Raman Spectroscopy.

The analysis of the FFA and their metallodrug is shown in Fig.2A. The structure of FFA, Cu(II)-FFA, Fe(III)-FFA is presented in the inset of Fig. 2B. The peaks at *m/z* 282.0 and 264.0 are assigned as $[FFA+H]^+$ and $[FFA-H_2O+H]^+$, respectively. Comparing with the previous method of graphene assisted laser desorption/ionization mass spectrometry (GALDI-MS) for detecting the FFA drug [18], the QALDI-MS is a more hard ionization technique as the dimer ion peak (*m/z* 562.0) of FA is absent. While the QALDI-MS can achieve higher resolution because the π-π interactions are absent [18]. The QALDI MS is superior over than the organic matrices because they produce interferences and cluster ions, and disturb non-covalent interaction (Fig.S1).

Generally, the forces govern the interactions for the metallodrug or metallodrug-protein is non-covalent nature and can be classified into four categories: ionic or electrostatic interactions, hydrogen bonds, hydrophobic interactions and Van Der Waals forces. Using MALDI-MS methods, these interactions are labile as the conventional matrix destroys these interactions due to matrix acidity or solvent effect [18, 23]. The copper divalent complex is an octahedral complex showing peaks at *m/z* 660.5 Da corresponding to $[^{63}Cu(FA-H)_2(H_2O)_2+H]^+$ and the structure is proposed in inset of Fig.2B. While the Fe(III) complexes show peak at *m/z* 895.8 corresponding to $[^{56}Fe(FA-H)_3-HF+H]^+$ and the structure is proposed in inset of Fig.2B. Our previous method [18] based on graphene (GALDI-MS) as platform for metallodrug ionization shows coordination of water molecules that indicate the QALDI-MS using CdS quantum dots as matrix is a more hard ionization method compared with the GALDI-MS, but soft than conventional MALDI due to the drawbacks such as acidity, interference, and cluster formation (Fig.S1). The limit of detection and the peaks assignments of these complexes are tabulated in Table 1. The spectra show insignificant detection of the drug i.e FFA after complexation (Fig.2A(b-c)) at molar ratio 1:2-1:3 for Cu(II) and Fe(III), respectively. That reveals complete complexation with the biological

metals at these ratios [20]. Although the structure of the drug and their complexes could be detected from QALDI-MS, the binding sites could not be revealed. Thus, Fourier transform infrared (FTIR) spectra (Fig.3A) were applied. The spectra show peaks at wavenumber 3450 and 1600 cm^{-1} corresponding to O-H and C=O, respectively. While the metallodrugs show changes in the OH peaks (broader band) and carbonyl groups (shifting to 1700 cm^{-1}) as shown in Fig.3A-B. The FTIR spectra can confirm the formation of metal complexation via carboxylic groups. The bending area (1600-450 cm^{-1}) reveals changes of the peak boarding and small shift that confirm the complexation. QALDI-MS (Fig.2) and FTIR (Fig.3A) indicate that FFA can form complexation in physiological condition in a short time. Thus, the probability of the metallodrug and drug interactions with serum albumin is high.

Monitoring Metallo-Flufenamic-Bovine Serum Albumin Interactions by Various Analytical Methods:-

It is well known that a drug present in the blood binds plasmatic proteins, such as albumin. Since FFA is able to interact with the metals (Fe(III) and Cu(II)) in the biological fluid as proved above, the free drug and its metallodrug are able to interact efficiently with SAs. Thus, it is paramount important for therapeutical and biomedicine interest. However, we must mention that controlled conditions are necessary to minimize the experimental artifacts. For instance, the experiment take place at the physiological pH =7.4 and in water. In this study, bovine serum albumins (BSA) are the selected proteins [27].

Fig 2

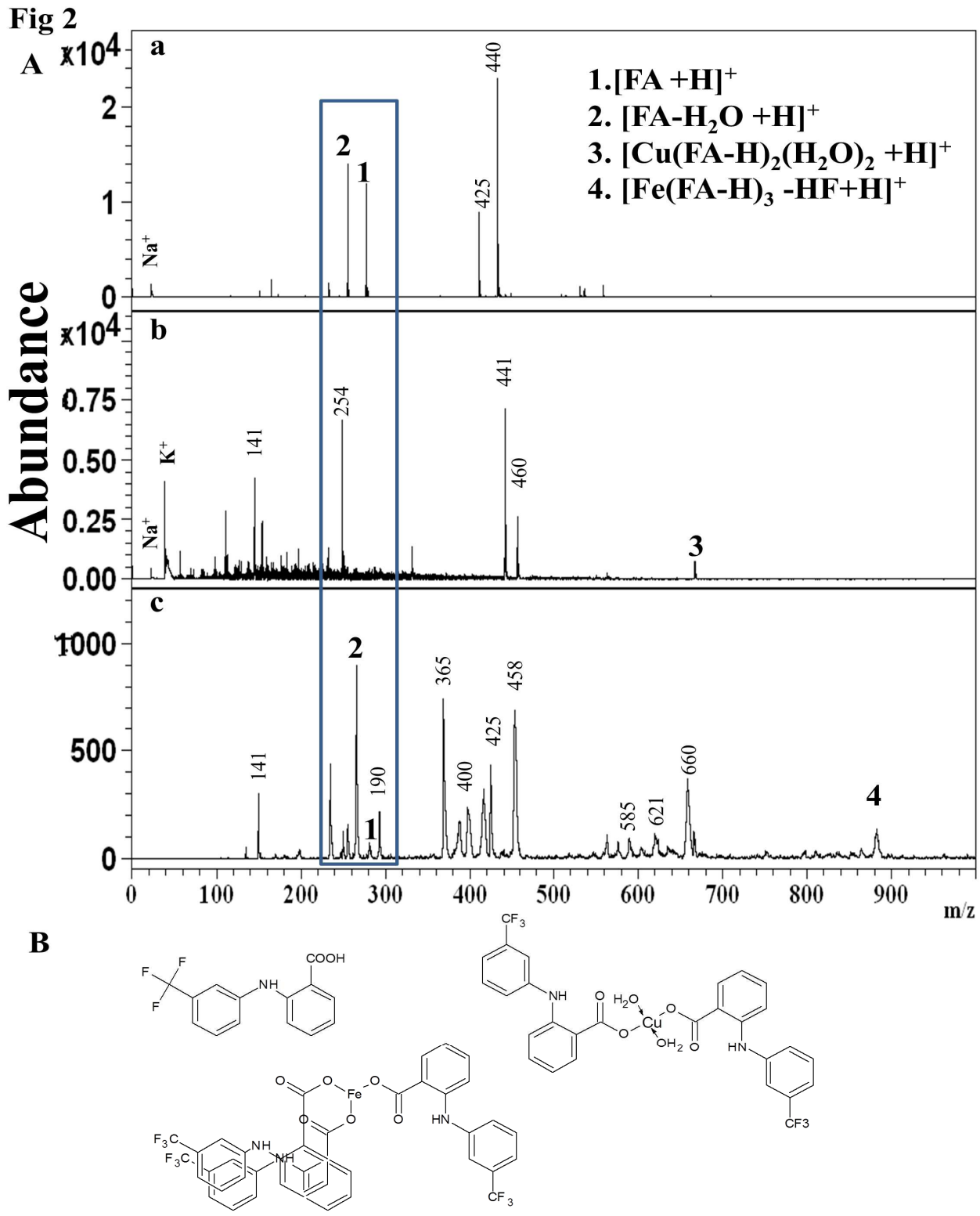


Fig 2: A) QALDI-MS analysis of (a) FFA, (b) Cu(II)-FFA complex, and (c) Fe(III)-FFA complex, and (B) chemical structure of the drug and its metallodrug.

Figure. 3

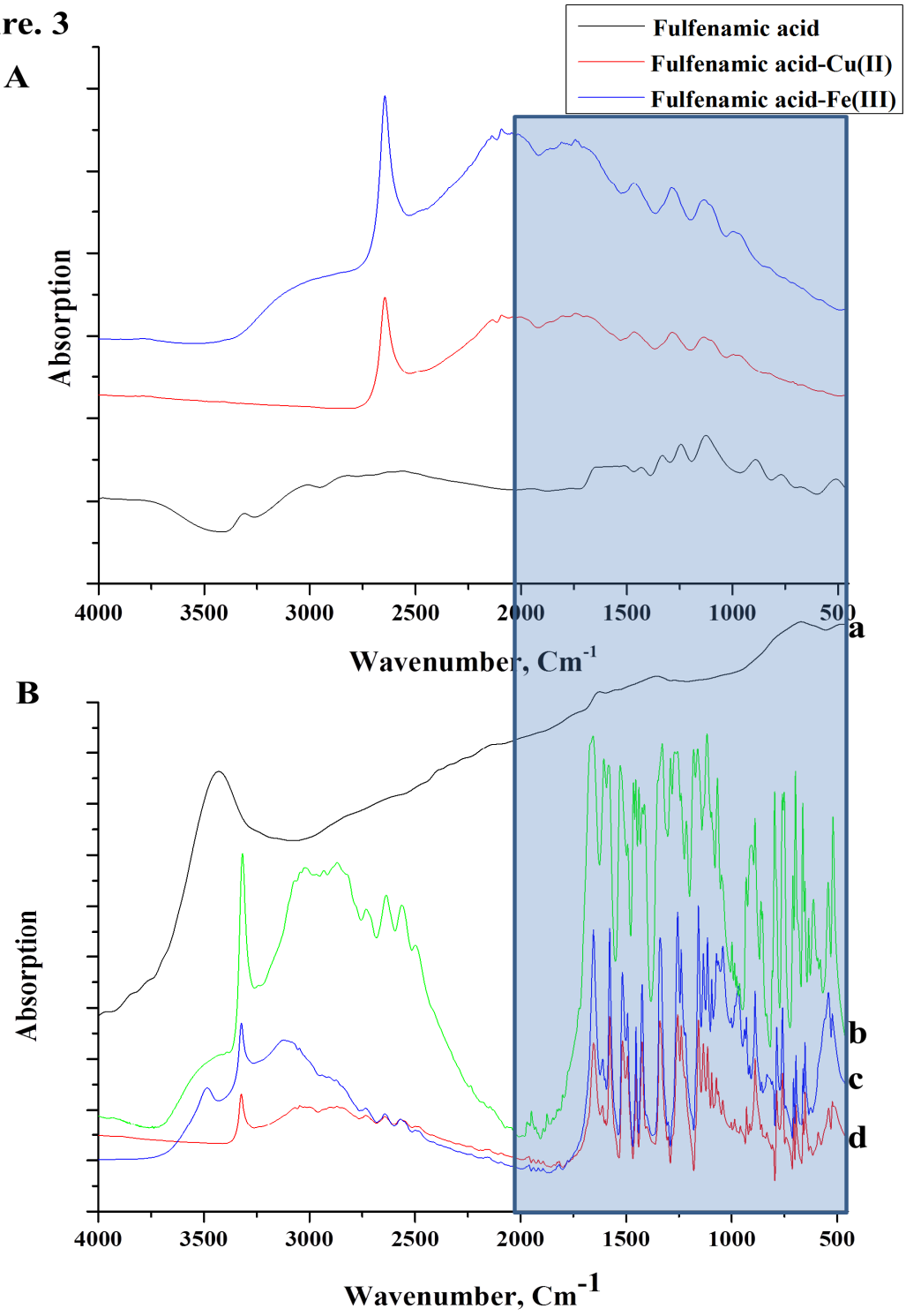


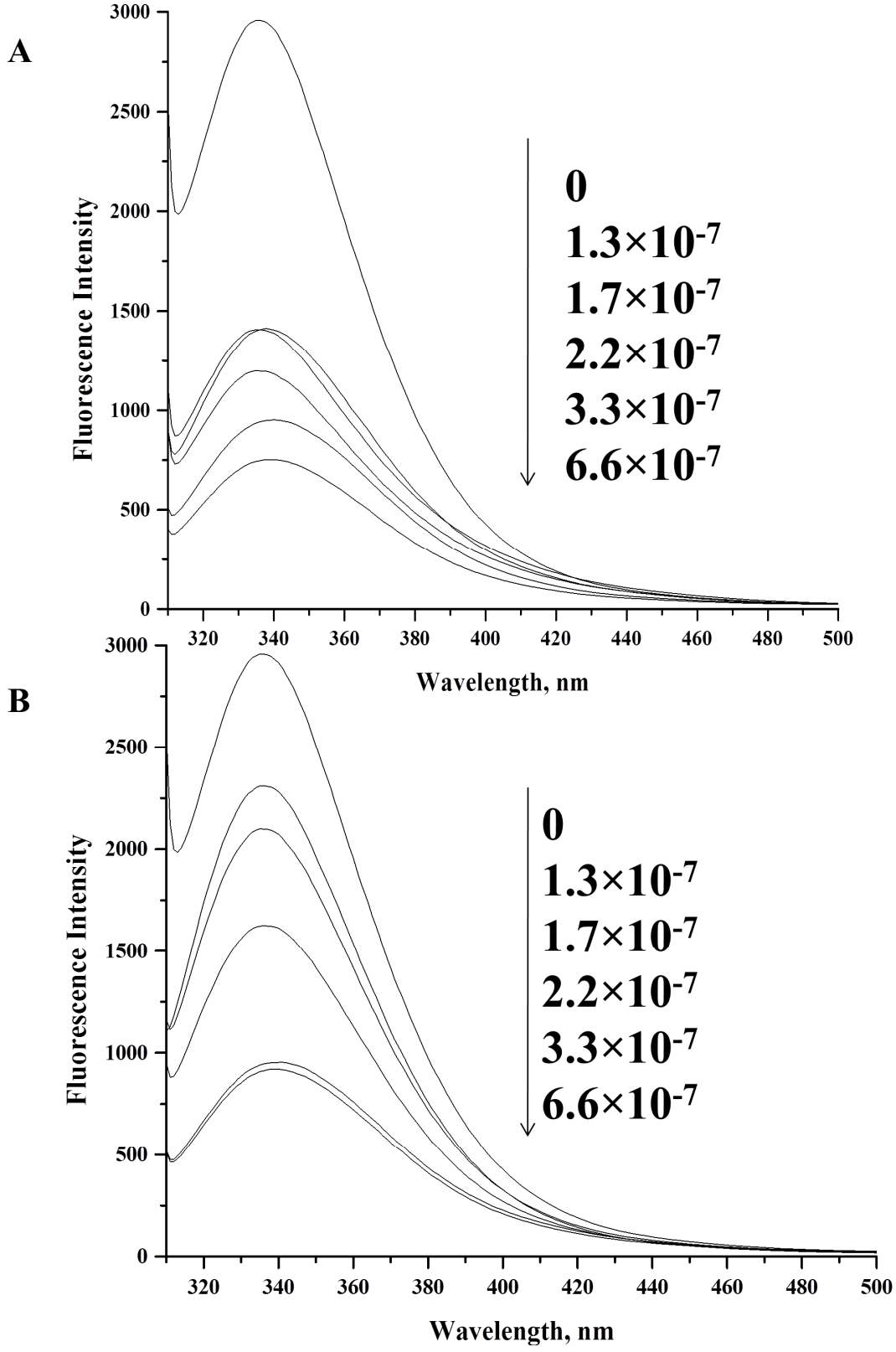
Fig 3: FTIR spectra for (A) the drug interactions with biological metals, and (B) the protein BSA (a) interactions with (b) FFA, (c) Cu(II)-FFA and (d) Fe(III)-FFA.

Fluorescence Spectroscopy

The SAs structure shows eight and half loops due to disulfurbridges and cystine residues and all vertebrates show the same loop structure [27]. The intrinsic fluorescence emissions of any protein are due to excitation of the aromatic amino acids i.e tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe). Typically, tryptophan has a wavelength of maximum UV absorption at 295 nm and an emission peak ranging from ca. 300-350 nm. Conformational state of a folded protein can be measured using tryptophan fluorescence emission [28]. At 295 nm, the tryptophan emission spectrum of bovine serum albumin is dominant over the other weaker tyrosine and phenylalanine fluorescence [29].

The fluorescence emission spectra of the drug and its complexes with bovine serum albumin (BSA) are plotted in Fig. 4(A-C). It can be seen that the addition of the drug or metallodrug cause a gradually decrease in the emission intensity of BSA. The quenching of the fluorescence and the slight shift of the maximum emission wavelength of the drug/metallodrug and BSA (from 335 to 334 nm for flufenamic acid, to 333 nm for Cu(II)-complexes, and to 332 nm for Fe(III)-complexes) indicate that the binding of the three ligands to BSA protein altered the microenvironment of Trp residue, and thus the conformation of BSA.

Figure. 4


A
Fluorescence Intensity
Wavelength, nm
0
1.3×10-7
1.7×10-7
2.2×10-7
3.3×10-7
6.6×10-7
B
Fluorescence Intensity
Wavelength, nm
0
1.3×10-7
1.7×10-7
2.2×10-7
3.3×10-7
6.6×10-7

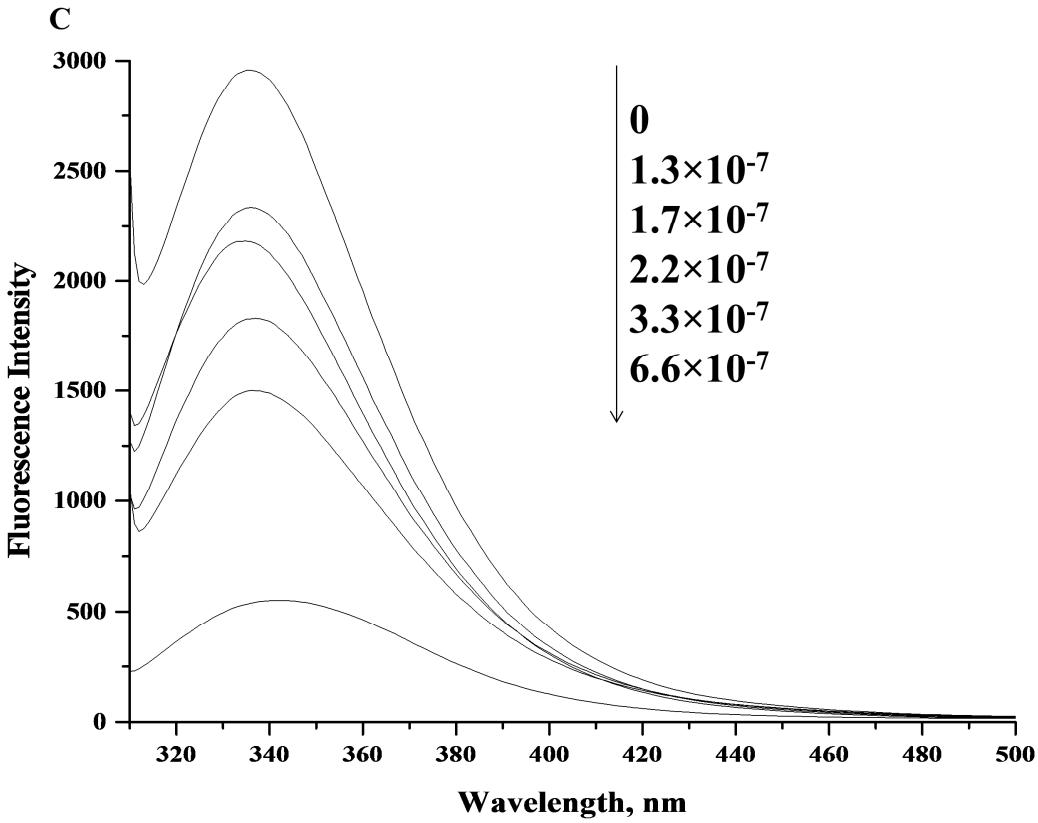


Fig 4: Tryptophan emission fluorescence of BSA upon addition of (A) FFA, (B) Cu(II)-FFA complex and (C) Fe(III)-FFA complex.

Another study using time-resolved/steady-state fluorescence measurements had shown the preferential binding of naproxen to a synthetic pseudopeptidic receptor built using Phe, i.e., bearing an aromatic ring, compared to another model synthesized using Lys, i.e., lacking such aromatic ring but with a basic binding site [31]. Fluorescence spectroscopy is a superior technique to probe the interaction of drug and their metallodrug with protein.

Figure 5

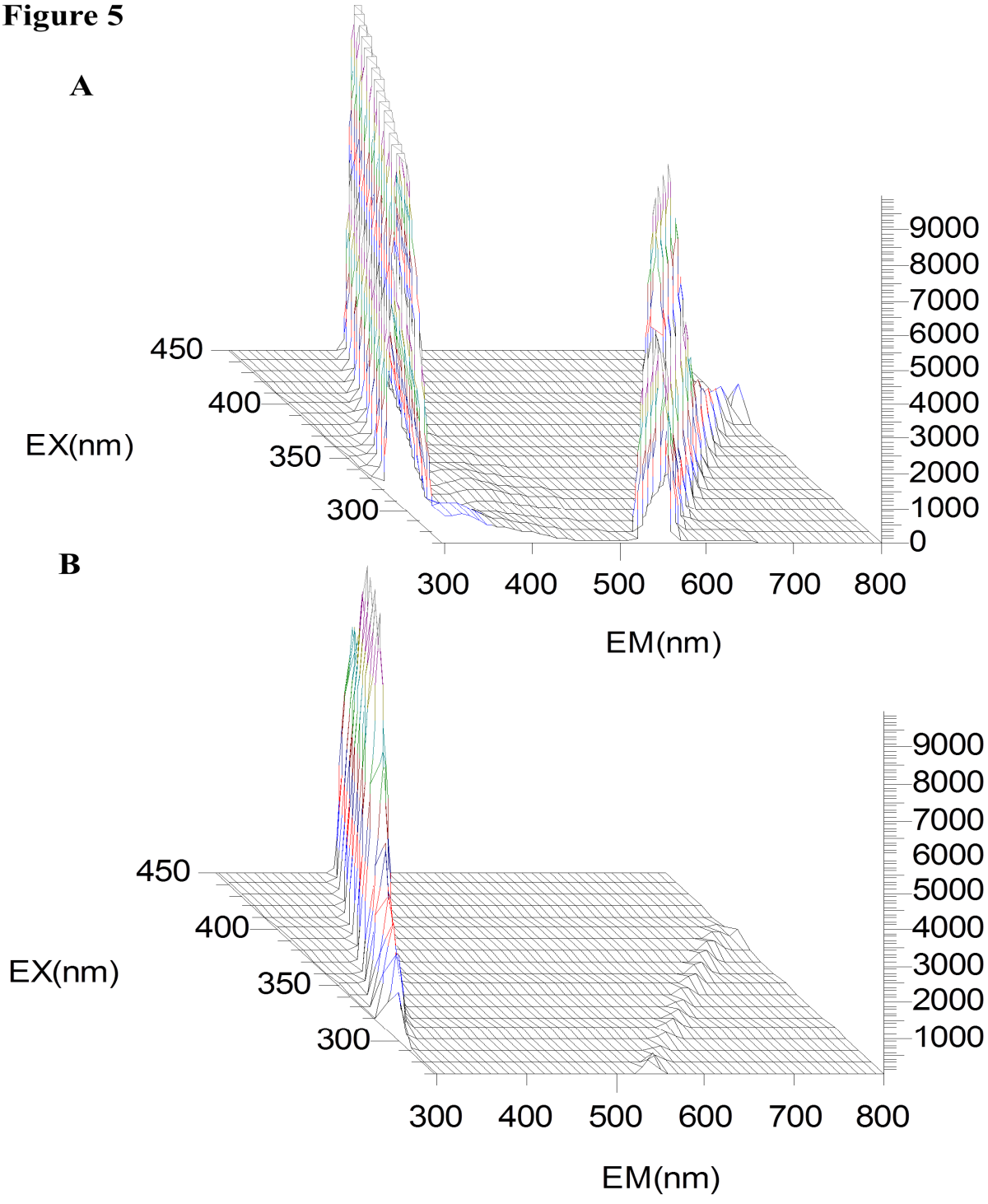

A
EX(nm)
450
400
350
300
300 400 500 600 700 800
EM(nm)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
B
EX(nm)
450
400
350
300
300 400 500 600 700 800
EM(nm)
9000
8000
7000
6000
5000
4000
3000
2000
1000

Fig.5

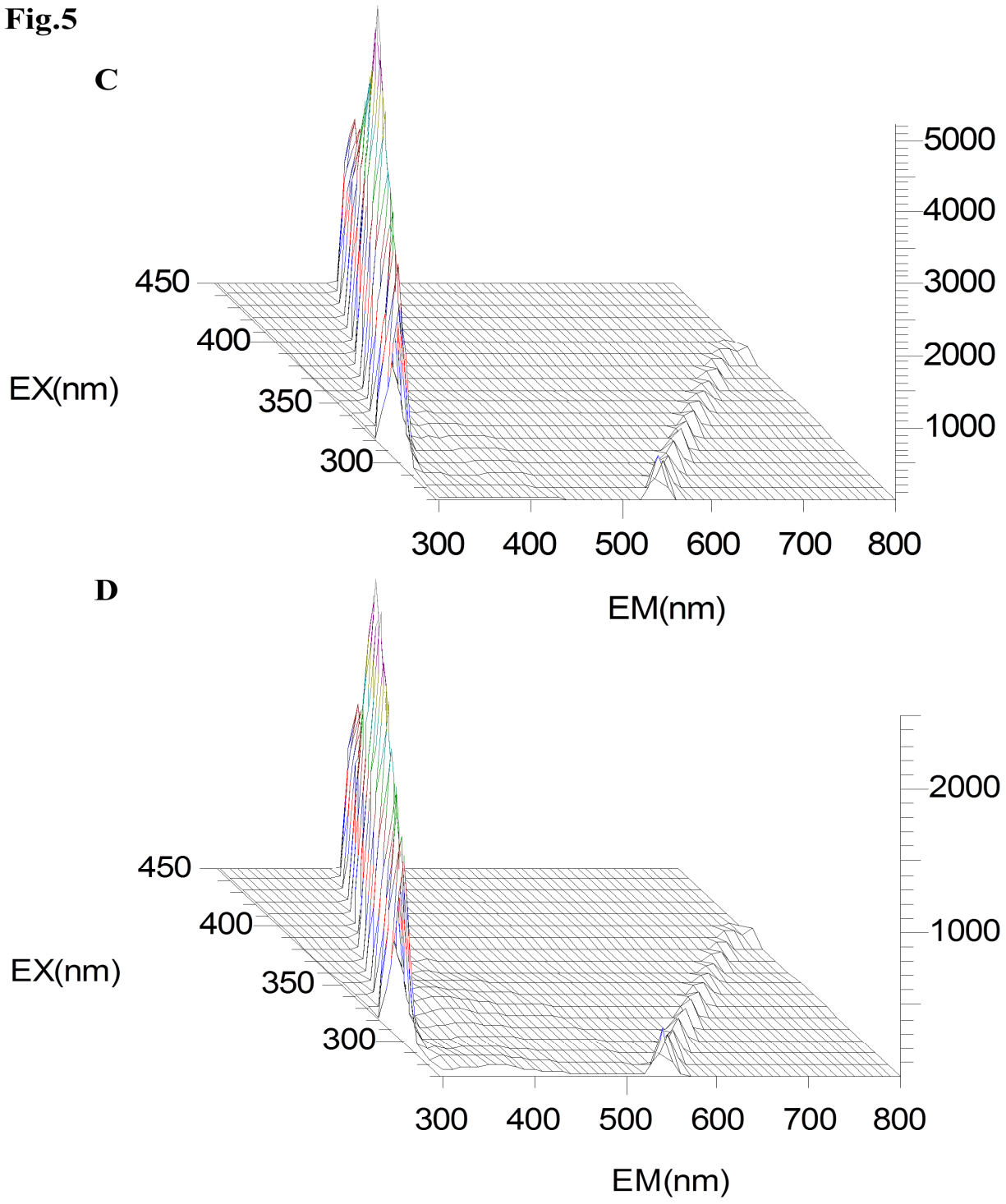


Fig 5: 3D-Flourescence spectra of (A) BSA with volume ratio 1:1 , (B) Flufenamic drug, (C) Cu(II)-Flufenamic, and (D) Fe(III)-Flufenamic complex.

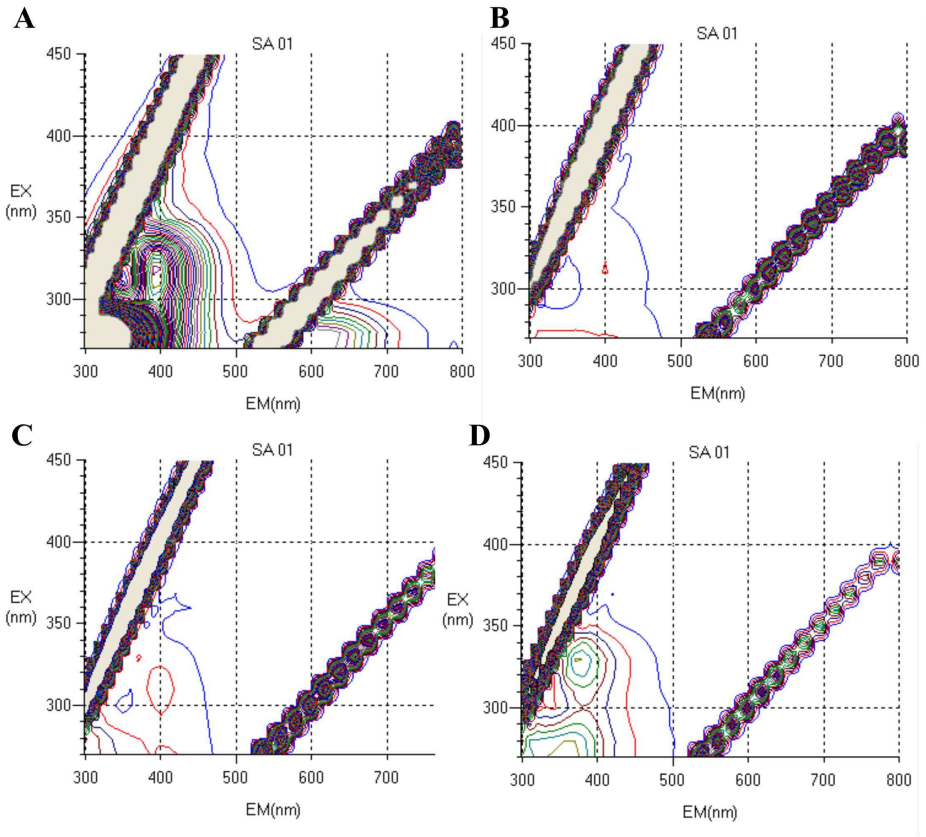


Fig 6: 2D-Flourescence spectra of (A) BSA with volume ratio 1:1 , (B) Flufenamic drug, (C) Cu(II)-Flufenamic, and (D) Fe(III)-Flufenamic complex.

2D and 3D-Flourescence

BSA has two tryptophan moieties (Trp 135 and Trp 214), located in subdomains IA and IIA, respectively [32]. Fig. 5(A-D) and Fig.6(A-D) display the subdomains of the protein and protein-drug/metallodrug interactions. Fig. 5A shows the two subdomains IA and IIA of the protein. Flufenamic-protein at 1:1 (v/v, protein/drug) shows selectivity toward domain IIA and no effect of domain IA (Fig.5B). While, metallodrug (Cu(II) & Fe(III)) show affinity to both domains as

shown in Fig. 5(C-D) and Fig.6(C-D). 2D and 3D fluorescence spectra of the two metallodrug (Fig. 5(C-D)) reveal the high binding affinity of Fe(III)-flufenamic complex compared to the Cu(II)-flufenamic complex. FFA (pK_a= 3.88) being α-aryl carboxylic acids and ay neutral pH are in their anionic form and they bind, preferably, the subdomain II of the albumin, as widely reported.

Fourier Transform Infrared Spectra

FTIR is a powerful tool to probe the drug-protein interactions [33]. It can show the binding sites between the drug and the protein. The FTIR spectra of BSA in liquid (Fig.S2) and solid ((Fig.3B)) show peaks about 3375, 1700, and 1050 cm^{-1} corresponding to O-H, C=O, and C-O, respectively. Flufenamic drug (Fig.3B) shows shift of O-H and C=O to 3600 and 1715 cm^{-1}, respectively that indicate there are interactions between the drug and the BSA protein. While, the metallodrug (Cu(II) and Fe(III)) show no or slightly shift with a change in the peaks profile (Fig.3B(b-c)). These observations indicate that the interactions between the flufenamic-BSA interactions are completely different than metallodrug-BSA interactions. The bending areas in solid form (Fig.3B) reveal the interaction via the enhancement of the peak intensities. Thermodynamic measurement using isothermal titration calorimetry (ITC) for the interaction between BSA and naproxenor flurbiprofen were reported [34]. The data revealed that the interaction can be a favorable number of hydrogen bond or Vander Waals interactions between BSA and naproxenor flurbiprofen [34]. However, the data indicated also that the ibuprofen undergoes hydrogen bonding reinforced by a small hydrophobic contribution. The importance of thermodynamic investigation, it have to use only when all the side reactions is well known i.e low or intermediate complexity in order to obtain the true thermodynamic binding constant.

Thus, it is really difficult for interactions between a common drug and a complex protein such as the albumin [35].

MALDI-Mass spectrometry

The interaction among FFA and their metallodrug with BSA were also reported using concentional MALDI (Fig.7). Bovine serum albumin (BSA) possesses 583 residues and a molecular weight of 66 kDa. The interactions between metallodrugs with BSA are confirmed by the changes in the mass spectra before and after addition of the drug/metallodrugs (Fig.7). Differences in the *m/z* values, change in the profile or appearing/disappearing of the protein peaks can be used to identify the new complexes formed [36]. MALDI-MS is a powerful tool that can be used to study the intact non-covalent interactions [37–41]. However, the applicability of conventional MALDI-MS is limited to study the non-covalent bonding interactions because the detection of MALDI-MS requires the crystallization of the sample with the matrix. Furthermore, the energy required for desorption/ionization of these complexes are not yet clearly known. Thus, conventional MALDI-MS failed to detect the non-covalent interactions due to the dissociation of these types of interactions. The acidity of the organic matrix plays a significant role on the disturbing of the non-covalent interactions. MALDI-MS spectra in Fig.7 (A-B) reveal strong interactions between the flufenamic drug and BSA (Fig.7B). Due to the intrinsic acidity of the flufenamic drug, the matrix acidity plays no effect. Furthermore, the flufenamic acid is an anionic drug that show mainly electrostatic interactions which can be easy detected in the gas phase. MALDI-MS spectra (Fig.7C-D) indicate that there are real interactions between the metallodrug with the BSA protein as the protein profile also shows a change. The native structure of any proteins tends to produce multiply charged ions covering a fewer charges. When

the protein undergoes interactions, unfolded protein would have accessibility of many of the protonation sites thus increase charged ions peak. MALDI-MS spectra (Fig.7) in the cases of Cu(II)-drug (Fig.7C) and Fe(III)-drug (Fig.7D) complexes show several new peaks that indicate change of protein conformation. Metallodrug of Cu(II) and Fe(III) may provide sufficient energy for the protein to unfold and create a wider charge state distribution centering on more highly charged ions. For instance peaks at 44760.8 Da corresponding to [2BSA+3H] $^{3+}$.The fluorescence spectra (Fig. 4), 3D-flourescence spectra (Fig. 5) and FTIR spectra (Fig. 6) reveal that the detected complexes by MALDI-MS is a result of specific binding, and not due to non-specific aggregation in the MALDI-MS plume. It is worthy to mention that the Cu(II) interact with human serum albumin (HSA) at pH 7.4 and it coordination via imidazole side chains that can cause the net charge of the protein to be more positive [42]. In general, opsonization (reaction with plasma proteins) of the hydrophobic compounds (metalloflufenamic) occurs more rapidly compared to that of the hydrophilic one. The peak at 44760.8 Da was assigned corresponding to $[2BSA+3H]^{3+}$. Recently, X-Ray crystallography showed that flufenamic acid and its Cu(II) complexes exhibit good binding affinity to human or bovine serum albumin protein with high binding constant values [43]. The interaction of 3,4_,7,8-Tetrahydroxyflavone (3,4_,7,8-tetraHF) and, human serum albumin (HSA) was probed by mass spectrometric approaches [44]. Overt than other technique such as UV or fluorescence measurement, mass spectrometry is simple technique to study the drug-protein interactions [44]. However, fluorescence spectroscopy is important where quantification analysis of the binding is required [45]. For more details about the merits of the current method for characterization of drug–target interactions by mass spectrometry and chemoproteomic target binding studies were reviewed by Bantscheff et.al. [46]. Recently, Monrad et.al [47] investigated the covalent reaction of 1-β-O-acyl glucuronides of ibuprofen and several

NSAID analogues with human serum albumin (HSA) protein *in vitro* under concentrations encountered in therapy. They observed a stable transacylation and glycosylation adducts. The data revealed that AG-induced modification of plasma proteins during treatment with carboxylic acid containing drugs such as ibuprofen can take place in a number of different ways that lead to covalent adducts.

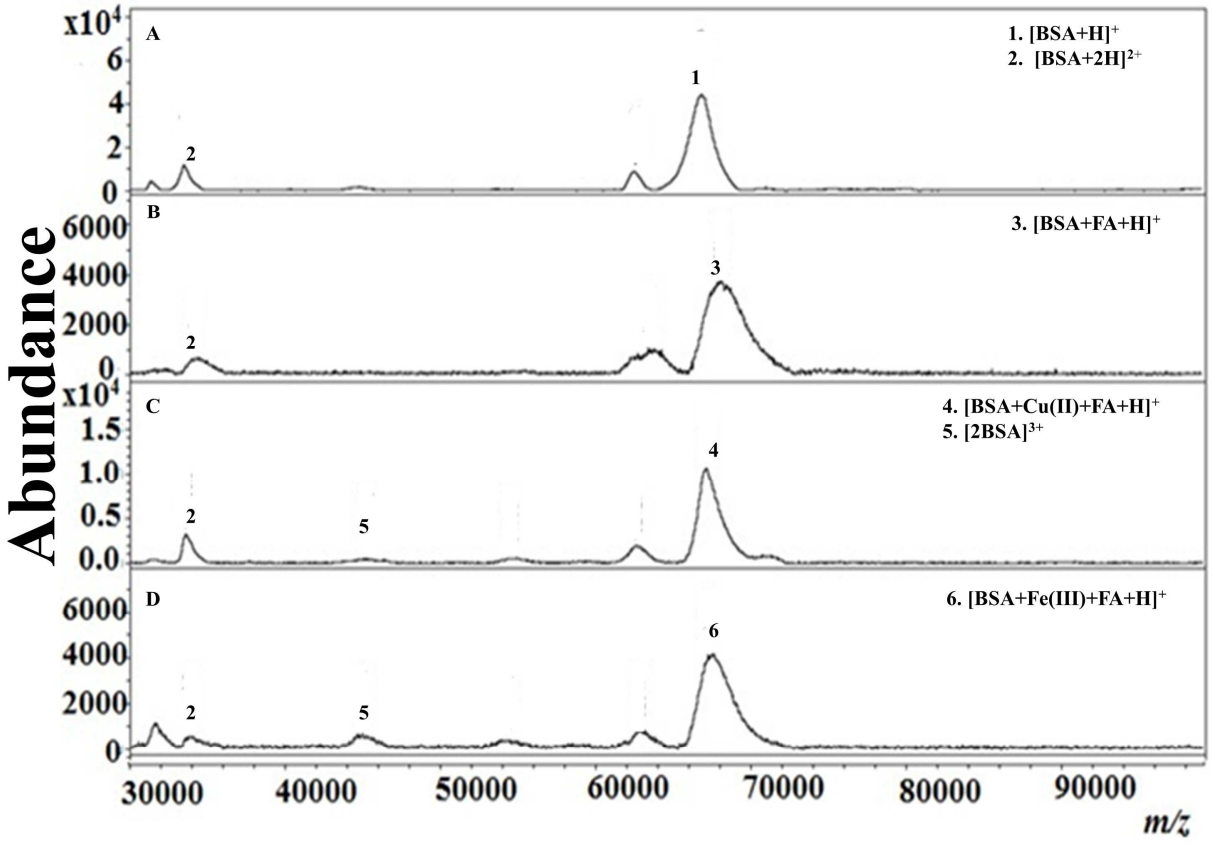


Fig 7: MALDI spectra of (A) BSA, (B) Flufenamic-BSA interaction, (C) Cu(II)-Flufenamic-BSA complex, (D) Fe(III)-Flufenamic-BSA complex.

Conclusion

QALDI-MS is a powerful ionization method to investigate the non-covalent interactions of fulfenamic acid and biological metals such as Cu(II) and Fe(III). The complete interaction

profiles between anionic NSAIDs i.e FFA and BSA were evaluated using MALDI, FTIR and fluorescence spectroscopy. These technique show ability of flufenamic drug to form metallodrug with biological metals (Cu(II) and Fe(III)) at physiological environment. According to the quenching rate of the fluorescence of BSA, the interaction of metallodrug is stronger than the parent drug. MALDI-MS reveal direct and sensitive view of the parent/metallodrugs interactions with BSA. These interactions are important for the therapeutical and chemoproteomic studies.

Acknowledgements

The authors are grateful to the Ministry of Science and Technology of Taiwan for the financial support.

References

1. M.R. Arkin, J.A. Wells.. *Nature Reviews Drug Discovery*. 2004, **3**,301-317.
2. S. Fukui, T. Feizi, C. Galustian, A.M. Lawson, W. Chai W. Nature Biotechnology.2002, **20**, 1011 – 1017.
3. C. Bovet, D. Moras, M. Ruff, S. Eiler, R. Zenobi, F. Granger, R. Wenzel, A. Nazabal. *Anal. Chem*. 2008, **80**, 7833–7839.
4. W.T. Chen, C.K. Chiang, C.H. Lee, H.T. Chang H T. *Anal. Chem*. 2012, **84**, 1924–1930.
5. E. Boeri, K. Barylyuk, Y. Yang, R. Zenobi. *Anal. Chem*. 2012, **83**, 9251-9259.
6. C. Bich, S. Maedler, K. Chiesa, F. DeGiacomo, N. Bogliotti, R. Zenobi. *Anal. Chem.* 2010, **82**, 172–179
7. X.M. He, D.C. Carter, Nature 1992, **358**, 209-215.
8. C.A. Dinarello, Cell, 2010, **140**, 935–950.

9. L. Bertolacci, E. Romeo, M. Veronesi, P. Magotti, C. Albani, M. Dionisi, C. Lambruschini, R. Scarpelli, A. Cavalli, M. De Vivo. D. Piomelli, G. Garau J. Am. Chem. Soc. 2013, **135**, 22–25.
10. A. Aboul Khier, M. El-Sadek, M. Baraka.Analyst.1987, **112**, 1399-1403.
11. M.I. Albero, C. Sanchez-pedreno, M.S. Garcia, *J.Pharm.Biomed.Anal.* 1995, **19**, 1113-1117.
12. P.C. Andrews, R.L. Ferrero, P.C. Junk, I. Kumar, Q. Luu, K. Nguyen K, W. James, J.W. Taylor. *Dalton Trans.* 2010, **39**, 2861–2868.
13. J.E. Weder, C.T. Dillon, T.W. Hambley, B.J. Kennedy, P.A. Lay, J.R. Biffin, H.L. Regtop, N.M. Davies. *Coord. Chem. Rev*.2002, **232**, 95-126.
14. T. Kawamori, C.V. Rao, K. Seibert, B.S. Reddy. *Cancer Res*. 1998, **58**, 409-412.
15. H.M. Tucker, R.E. Rydel, S. Wright, S. Estus. *J. Neurochem*. 1998, **71**, 506-516.
16. Y.Joo . H.S. Kim, R.S. Woo, C.H. Park, K.Y. Shin, J.P. Lee, K.A. Chang, S. Kim, Y.H. Suh. *Mol Pharmacol*. 2006, **69**, 76–84.
17. M. Asanuma, S. Nishibayashi-Asanuma, I. Miyazaki, M. Kohno, N. Ogawa. *J. Neurochem.* 2001, **76**, 1895–1904.
18. B. Zhang., X. Liang, L. Ye, Y. Wang, PLoS ONE 2014, **9**, e96887. doi:10.1371/journal.pone.0096887
19. Z. Yin, Y. Wang, L.R. Whittell, S. Jergic, M. Liu, E. Harry, N. E. Dixon, M. J. Kelso, J. L. Beck, A. J. Oakley, Chemistry & Biology 2014, **21**, 481–487.
20. H.N. Abdelhamid, H.F.Wu. Anal.Chimica.Acta. 2012,**751**, 94– 104.
21. A.R. Timerbaev, C.G. Hartinger, S.S. Aleksenko, B.K. Keppler *Chem Rev*. 2006, **106**, 2224-2248.

22. C. Ràfols, S.Zarza, E. Bosch, Talanta. 2014, **130**, 241–250

23. H.F. Wu, J. Gopal, H.N. Abdelhamid, H. Nazim. *Proteomics.* 2012, **12**, 2949–2961

24. H.N. Abdelhamid, H.F. Wu *J. Mater. Chem. B*, 2013, **1**, 6094-6106.

25. J.S. Mak, A.A. Farah, F. Chen, A.S. Helmy. *ACS Nano*, 2011, **5**, 3823–3830.

26. (a) K. Tanaka, H.Waki, Y. Ido, S. Akita, Y. Yoshida, T. Yoshida, Rapid Commun. Mass Spectrom. 1988, 2, 151-153; (b) H. N. Abdelhamid, H.F. Wu, *Talanta*, 2013, **115**, 442–450.

27. T.J. Peters, All About Albumin: Biochemistry Genetics, and Medical Applica- tions, Academic Press, Inc., San Diego, 1996.

28. Y.J. Hu, Y. Liu, J.B. Wang. *J. Pharm.Biomed. Anal*. 2004, *36*, 915**–**919.

29. J.T. Vivian, P.R. Callis. *Biophys. J.* 2001, **80**, 2093–109.

30. I. Vayá, R. Pérez-Ruiz, V. Lhiaubet-Vallet, M. C. Jiménez , M. A. Miranda, Chemical Physics Letters 2010, **486**, 147–153

31. M. Isabel Burguete , Ghinwa Fawaz , Francisco Galindo , M. A´ ngeles Izquierdo , Santiago V. Luis, Jean Martı´nez , Xavier J. Salom-Roig, Tetrahedron 2009, **65**, 7801–7808

32. D.C. Carter, J.X. Ho. *Adv. Protein Chem*.1994, **45**, 152-153.

33. A. R. Timerbaev, C. G. Hartinger, S. S. Aleksenko, B. K. Keppler, Chem. Rev. 2006, **106**, 2224-2248

34. C.Ràfols, S. Zarza, E.Bosch, Talanta 2014, **130**, 241–250

35. G. Garrido,C.Ràfols,E.Bosch,Talanta 2011, **84**, 347–354.

36. (a) M. Petkovic´, T .Kamcˇeva. Metallomics 2011; 3, 550–565, (b) C. G. Hartinger, M.Groessl, S. M. Meier, A. Casinif, P. J. Dysond, Chem. Soc. Rev., 2013, **42**, 6186-6199

37. X. Fan, R.C. Beavis. *Org. Mass Spectrom*. 1993, **28**, 1424-1429.

38. M.O. Glocker, S.H.J. Bauer, J. Kast. *J. Mass Spectrom*.1996, **31**, 1221–7.

39. L.R.H. Cohen, K. Strupat, F. Hillenkamp. *J. Am. Soc. Mass Spectrom*.1997, **8**, 1046–52.

40. G. Bolbach. *Curr. Pharm. Des*. 2005, **11**, 2535–57.

41. M. Zehl, G. Allmaier. *Anal. Chem*. 2005, **77**, 103–10.

42. J.H. Viles. *Coord. Chem. Rev*.2012, **256**, 2271-2284.

43. C. Tolia, A.N. Papadopoulos, C.P. Raptopoulou, V. Psycharis, C. Garino, L. Salassa, G. Psomas, *J. Inorgan. Biochem*. 2013, **123**, 53–65

44. J. Ma, L.Y. Wang, M.X. Xie, Journal of Photochemistry and Photobiology A: Chemistry 2012, **243**, 65– 72

45. C. M. Cortez, D. Silva , C. M.C. Silva , S.Missailidis, Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 2012, **95**, 270–275

46. N. Zinn, C. Hopf, G. Drewes, M. Bantscheff, Methods 2012, **57**, 430–440

47. R. N. Monrad, J. C. Errey, C. S. Barry, M. Iqbal, X. Meng, L. Iddon, J. A. Perrie, J. R. Harding, I. D. Wilson, A. V. Stachulskib, B. G. Davis, Chem. Sci., 2014, **5**, 3789–3794.

Figure Captions:

Fig 1: CdS@MPA quantum dots characterization using (A) TEM, inset represent the size distribution diagram and (B) Raman Spectroscopy.

Fig 2: A) QALDI-MS analysis of (a) FFA, (b) Cu(II)-FFA complex, and (c) Fe(III)-FFA complex, and (B) chemical structure of the drug and its metallodrug.

Fig 3: FTIR spectra for (A) the drug interactions with biological metals and (B) the protein BSA (a) interactions with (b) FFA, (c) Cu(II)-FFA and (d) Fe(III)-FFA.

Fig 4: Tryptophan emission fluorescence of BSA upon addition of (A) FFA, (B) Cu(II)-FFA and (C) Fe(III)-FFA complexes.

Fig 5: 3D-Flourescence spectra of (A) BSA with volume ratio 1:1 , (B) Flufenamic drug, (C) Cu(II)-Flufenamic, and (D) Fe(III)-Flufenamic complex.

Fig 6: 2D-Flourescence spectra of (A) BSA with volume ratio 1:1 , (B) Flufenamic drug, (C) Cu(II)-Flufenamic, and (D) Fe(III)-Flufenamic complex.

Fig 7: MALDI spectra of (A) BSA, (B) Flufenamic-BSA interaction, (C) Cu(II)-Flufenamic-BSA complex, (D) Fe(III)-Flufenamic-BSA complex.

Table 1: Peaks assignment and limit of detection for FFA and their metallodrug.

m/z	**Assignments**	**LOD (fmole)**
896.0	$[^{56}Fe(FA\text{-}H)_3+H]^+$	2
660.5	$[^{63}Cu(FA\text{-}H)_2(H_2O)_2+H]^+$	1
425	$[2(FA\text{-}CF_3)+H]^+$	0.5
300.0	$[FA+H_2O+H]^+$	
282.0	$[FA+H]^+$	
264.7	$[FA\text{-}H_2O+H]^+$	

122	$[C_7H_6O_2+H]^+$	
39	K^+	
23	Na^+	