STUDY OF DRUG TRANSPORTATION BY ESR SPECTROSCOPY

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ABSTRACT

STUDY OF DRUG TRANSPORTATION BY ESR SPECTROSCOPY

The ability to track drug binding and release makes electron spin resonance (ESR) spectroscopy well suited for drug delivery studies. Using the continuous wave cw ESR technique to extract information about the dynamics of the spin labeled drugs we can simultaneously determine the bound and unbound drugs.

In this study, ESR technique was used to detect the binding and release of spin-labeled salicylic acid (SLSA) to and from bovine serum albumin (BSA), and to detect different binding interactions between them. We have labeled salicylic acid with stable nitroxide-based tempo radicals to monitor the BSA bound and unbound conditions of the drug. Studying with the different concentrations of SLSA-BSA binding showed that the drug-protein stoichiometry increases significantly in the physiological range of BSA concentration. Also, during the release of SLSA from BSA, there is an unchanging balance between the bound and unbound SLSA.

In order to study various drug binding interactions, SL-benzoic acid, SL-phenol, SL-benzene, SL-cyclohexane, SL-hexane and SL-methyl were prepared. We showed that the main conjugation in the binding of these drugs to BSA is hydrophobic interaction. In addition, cationic BSA (cBSA) was prepared to investigate the effect of electrostatic interaction on drug binding. The SLSA loading capacity of cBSA is significantly higher than that of BSA, this result indicates the importance of electrostatic interactions for the drug binding.

Finally, we examined the competitive binding behaviors of salicylic acid, ibuprofen and aspirin to BSA. Binding sites of SL-salicylic acid and SL-ibuprofen in BSA show 96% of similarities. In addition, our results showed that binding sites of SL-salicylic acid and SL-aspirin in BSA have 73% of similarities.These results demonstrate that cw ESR spectroscopy with the spin labeling technique is an effective technique for the determination of drug-protein interactions and stoichiometric analysis of drug binding.

ÖZET

KONTROLLÜ İLAÇ TAŞINIMININ ESR SPEKTROSKOPİSİ İLE ÇALIŞILMASI

İlaçların bağlanmasını ve salınımını takip etmek için kullandığımız elektron spin rezonans (ESR) spektroskopisi, ilaç taşınım çalışmaları için uygun bir tekniktir. Spin etiketli ilaçların dinamikleri hakkında bilgi elde etmek için sürekli dalga cw ESR tekniği kullanarak, bağlı ve bağlanmamış ilaçları eş zamanlı olarak belirleyebiliriz.

Bu çalışmada, spin etiketli salisilik asidin (SLSA) sığır serum albüminine (BSA) bağlanması, salınması ve aralarındaki farklı bağlanma etkileşimlerinin belirlenmesi için ESR tekniği kullanılmıştır. BSA’ ya bağlı ve bağlı olmayan durumlarının izlenebilmesi için, salisilik asit kararlı nitroksit bazlı tempo radikalleri ile etiketledik. Farklı konsantrasyonlarda SLSA-BSA bağlanmasını incelediğimizde, ilaç-protein stokiyometrisinin, BSA konsantrasyonunun fizyolojik aralığında önemli ölçüde arttığını gözlemledik. SLSA’ nın BSA’ dan salınmasını incelediğimizde, bağlı ve bağlanmamış SLSA arasında diyaliz boyunca değişmeyen bir denge olduğunu gözlemledik.

Ek olarak çeşitli ilaç bağlanma etkileşimlerinin etkilerini ortaya çıkarmak için, spin etiketli sentezlediğimiz SL benzoik asit, SL-fenol, SL-benzen, SL-siklohekzan, SL-hekzan ve SL-metilin ESR sonuçları, hidrofobik etkileşimin bu ilaçların BSA’ ya bağlanmasında ana etkileşim olduğunu göstermiştir. Elektrostatik etkileşimin ilaç bağlanması üzerindeki etkisini araştırmak için ise, katyonik BSA (cBSA) sentezlendi. cBSA' nın SLSA yükleme kapasitesi, BSA' nınkine kıyasla önemli ölçüde yüksektir bu da ilaç bağlama çalışmaları için elektrostatik etkileşimin önemini gösterir.

Son olarak, salisilik asit, ibuprofen ve aspirinin BSA' ya rekabetçi bağlanma davranışlarını inceledik. BSA içinde SL-salisilik asit ve SL-ibuprofen bağlama bölgeleri % 96 benzerlik göstermektedir. Ek olarak sonuçlarımız, BSA' da SL-salisilik asit ve SL-aspirinin bağlanma bölgelerinin % 73 benzerliğe sahip olduğunu göstermiştir.

Bu sonuçlar, cw ESR spektroskopisinin, spin etiketleme tekniği ile birlikte, ilaç-protein etkileşimlerinin belirlenmesi ve ilaç bağlanma, serbest bırakma dinamiklerinin stokiyometrik analizi için etkili bir teknik olduğunu göstermektedir.

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LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
HAS	Human Serum Albumin
SA	Salicylic acid
Aspirin	Acetylsalicylic acid
ESR	Electron Spin Resonance
TEMPO	2,2,6,6-tetramethylpiperidine-1-oxyl
Tempo 4-Amino	4-amino-2,2,6,6-tetramethylpiperidine-1-
HCl	Hydrogen chloride
DCM	Dichloromethane
EDC	*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride
DMSO	Dimethyl sulfoxide
SL-drugs	Spin Labeled Drugs
SLSA	Spin Labeled Salicylic acid
SL-Benzoic acid	Spin Labeled Benzoic acid
SL-Phenol	Spin Labeled Phenol
SL-Benzene	Spin Labeled Benzene
SL-Cyclohexane	Spin Labeled Cyclohexane
SL-Hexane	Spin Labeled Hexane
SL-Methyl	Spin Labeled Methyl
SL-Ibuprofen	Spin Labeled Ibuprofen
SL-Aspirin	Spin Labeled Aspirin
cBSA	Cationic BSA
Trp	Tryptophan
K_a	Association constant
K_d	Dissociation constant, $1/K_a$
ΔG	Binding free energy

CHAPTER 1

INTRODUCTION

1.1. Bovine Serum Albumin (BSA)

Serum albumin is the most abundant protein in blood plasma. Highly water soluble of Human Serum Albumin (HSA) and Bovine Serum Albumin (BSA) are 65 kDa proteins. Solubility of powder BSA in pure water is 40 mg/mL at room temperature. Serum albumin is produced in the liver and its production depends on nitrogen rich diet. [1]

Albumin has many features and functions in the body:

- Regulation of blood volume by preserving colloid osmotic pressure (COP),
- Transportion of the substrate,
- High buffering capacity,
- Neutralization of the free radicals therefore inactivation of many toxic substances in the body,
- Participation in drug metabolism. Thus, it plays an important role in coagulation and wound healing.[1-2]

BSA involves 583 amino acid residues with three homologous α-helical domains (I-II-III). These three domains were assembled together in a heart-shaped (Figure 1.1). Each of these helical domains divided into two subdomains: A and B. The single polypeptide chain which forms the BSA is composed of nine loops (L1-L9) separated by 17 disulfide bonds. BSA has two tryptophan residues; one at the molecular surface (Trp-134) and another one in the hydrophobic binding pocket (Trp-212) of the serum albumin. Due to the fluorescence emission of these tryptophan residues, structural and functional properties of serum albumin can be determined. Any substance that binds to the albumin alters the fluorescent emission of the albumin. Therefore, binding of ligans to albumin can be detected with this change, also, the structural properties of the albumin can be determined by fluorescence emission if the albumin binds to an extrinsic fluorophore.

One of the most important duties of the BSA is to transport the molecules, ions or atoms in the bloodstream to target molecules or tissues. This is why BSA is often used in

drug delivery systems. The structural similarity of BSA to HSA is also one of the reasons for its frequent use in these systems.[3]

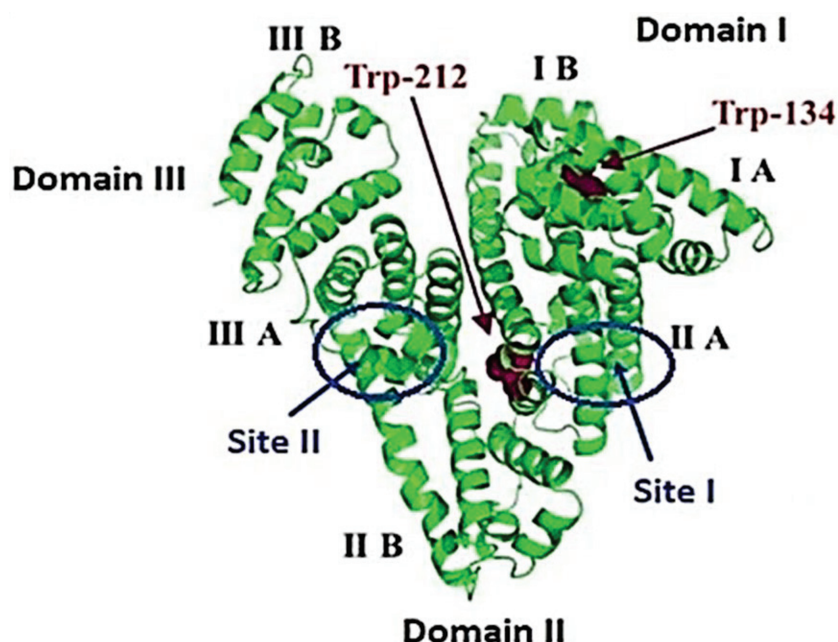

Figure 1.1. Schematic structure of bovine serum albumin showing the binding sites, the domains, the subdomains and the tryptophan residues.[4]

Albumin has two binding sites known as Sudlow sites (Figure 1.1). Drugs generally bind to one of the two Sudlow sites found in subdomains IIA and IIIA. Sudlow site II (subdomain IIIA) is the major binding site of drugs. Site II is hydrophobic and has a high affinity for small aromatic ligands. It is a good model for protein-ligand studies. Sudlow site I (subdomain IIA) consists of several adjacent regions and has a wide variety of ligands. It is a tight binding site for some drugs (warfarin, salicylate). But their ligands do not form a good model system, and competition experiments can give uneven results. This is because binding to Sudlow site I is less specific. Site I has the medium affinity for large and various ligands.[5] The mechanisms of serum albumin binding and release are important in terms of the therapeutic effect of drugs. The ability of the drug to bind to the albumin determines the amount of drug to be administered in the treatment. Protein binding is reversible. Most drugs bind to the protein by various interactions and compete with each other for albumin binding sites.[6] The bound drug may be replaced with a drug having a higher binding capacity than itself, or the structure of the BSA may be altered. Hence, the analysis of the release and binding mechanisms of the drug-BSA complex has high importance in terms of the success of the drug delivery systems.[7]

1.2. Drugs-BSA Interactions

Transportation ability of albumin allows of drugs binding to albumin. Therefore, in the literature, albumin-drugs interactions have been studied intensively. In this thesis, we studied salicylic acid, ibuprofen and aspirin bindings to BSA.

1.2.1. Salicylic Acid-BSA Interactions

Salicylic acid (SA), which is produced from many plants, is part of the salicylate family. It is known that SA has anti-inflammatory, antibacterial, and antifungal properties.[8] SA has shown promising results in the treatment of some diseases like Cardiovascular disease and cancer. SA is barely soluble in water (2 g/L at 20 °C) with a pKa value of 2.97.[9] SA, the active metabolite, can also be derived from another active metabolite, aspirin. Salicylate family drugs have been used since the 5th century AD. Members of salicylate family exhibit a bioavailability of about 50% if the organism is orally administered.[8] The reason for this, salicylic acid is deacetylated. The duration of salicylate deacetylation is 2 to 30 hours. This time varies depending on the concentration of salicylic acid.[10]

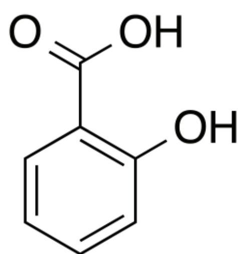

Figure 1.2. Chemical structure of salicylic acid.

The rate of release of salicylic acid is important for the treatment of diseases. In controlled release studies, it has shown that a rapid release of SA in the treatment of various infections has been found appropriate, on the contrary a slower release for cancer and cardiovascular diseases has been found appropriate.[11] SA contains two functional groups, a carboxylic acid (–COOH) group and a hydroxyl (–OH) group (Figure 1.2).

There are many studies in the literature about the binding of SA to BSA. Benividi Ali et al. used differential pulse voltammetry (DPV) and UV-vis spectrophotometry (UV-

Vis) to detect the protein-drug interactions.[12] In addition, chemometric methods that strongly compatible to experimental measurements were also used. These methods are multivariate curve resolution-alternating least squares (MCR-ALS) and parallel factor analysis (PARAFAC).[12] For all these compound methods, predetermined algorithms are used. Thus, stoichiometric and electrochemical behaviors of the components were determined. Molecular modeling was used to confirm the parameters of the determined binding regions. As a result, the load on the BSA surface was an important factor in the attachment of SA to BSA. Electrostatic repulsion between SA and BSA leads to a reduction in hydrophobic interactions. It was emphasized that this may affect SA and BSA interaction and binding.[12] Y. Ni et al. examined the interactions between salicylic acid, warfarin, ibuprofen and bovine serum albumin using spectrofluorimetry. [13] Results showed that the interaction between SA and BSA can be altered by a possible interaction between ibuprofen and SL. Data were analyzed by parallel factor analysis (PARAFAC) algorithm.[13] SA was determined to have two low-affinity binding sites in BSA. One of them is a high affinity binding site for ibuprofen in site II and the other is a low binding site for ibuprofen in the site I.[13]

1.2.2. Acetylsalicylic Acid (Aspirin)-BSA Interactions

Acetylsalicylic acid (Aspirin) is a commonly consumed analgesic and antipyretic drug.[14] Acetylsalicylic acid's structure is given in figure 1.3.

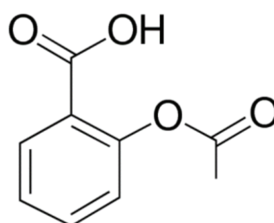

Figure 1.3. Chemical structure of acetylsalicylic acid.

Aspirin contains two functional groups, a carboxylic acid (–COOH) group and ester group. When it is taken orally, approximately 50% of the drug is absorbed and the rest undergoes hydrolysis and the aspirin is converted to the salicylate.[14] Water solubility of aspirin is about 3 mg/ml with a pKa value of 3.5. Depending on the concentration of salicylate, the plasma half-life of aspirin ranges from 2 to 30 hours. At least 80% of the

circulating salicylate binds to the proteins found in the plasma.[14] The short half-life of aspirin doesn't create any disadvantages in terms of binding to protein. Nevertheless, the large-scale binding of salicylate to protein provides a competitive interaction with many drugs.[15]

As regards the studies of aspirin-BSA interactions in the literature, Kalpana et al. used gel filtration and equilibrium dialysis methods to examine the Drugs-BSA interactions.[16] The results were characterized by parameters such as the percentage of a bound drug, relationship constant, binding constant, and free energy exchange. The effect of competitive behavior of hydroxyzine and acetylsalicylic acid on the binding of phenoxazine derivatives to albumin was investigated for the binding sites of BSA. According to results, hydrophobicity was not the only interaction in the binding of the drug to proteins as in salicylic acid but also ionic bonding, hydrogen bonding or steric effects, etc. which are needed to be taken into consideration.[16] Ni et al. used fluorescence spectroscopy to investigate the interaction of aspirin and ibuprofen with bovine serum albumin (BSA).[17] Thermodynamic data were obtained with fluorescence spectroscopy results (ΔH, ΔS, ΔG).[17] These data showed that binding of aspirin to BSA included van der Waals interactions and hydrogen bonds. In addition, the competitive interaction of aspirin and ibuprofen for the binding sites of BSA was investigated by three-way stimulation-emission fluorescence spectra and parallel factor analysis chemometry (PARAFAC).[17] When we look at the competitive studies of ibuprofen with aspirin, the ibuprofen-BSA solution was more stable than the aspirin-BSA solution.[17]

1.2.3. Ibuprofen-BSA Interactions

Ibuprofen is an acid derivative of α-methyl-4-(2-methylpropyl) benzene-acetic acid (non-steroidalanti-inflammatory-drugs (NSAIDs)).[18] Some parts of the structure of ibuprofen are the side group isobutane, propionic acid, and phenyl ring. Abbreviations of these functional groups formed the name of ibuprofen (Figure 1.4).

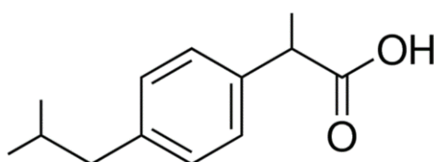

Figure 1.4. Chemical structure of ibuprofen.

It is a widely used drug and the weekly use rate of ibuprofen has been reported as 17%.[19] Ibuprofen is almost insoluble (21 mg/L at 25 °C) in water with a pKa value of 5.2.[18] It was reported in 2006 by the Food and Drug Administration that aspirin may affect antiplatelet potency when ibuprofen is co-administered with low-dose aspirin.[20] This information has shown that in clinical trials, the competitive effects of aspirin and ibuprofen, may be effective in the prevention of certain diseases. The competitive binding of ibuprofen and other drugs to the albumin is one of the subjects to be observed in this study.

As regards the studies of ibuprofen-BSA interactions in the literature, Wybranowski et al. used fluorescence anisotropy to study the interaction between HAS and ibuprofen.[21] Ochratoxin A (OTA) was tightly bound to albumin. The fluorescence anisotropy method revealed that the free fraction of OTA was higher than that of the bound fractions of OTA competing with ibuprofen. In other words, ibuprofen decreases the bound fraction of OTA at HSA.[21]

1.3. Electron Spin Resonance (ESR) Spectroscopy

According to the principle of the Pauli Exclusion, an orbit cannot occupy more than two electrons. The spin of these electrons must be opposite. Since the spin directions are opposite, the magnetic moments of the two spins in an orbital cancel each other out.

Electron spin resonance (ESR) also known as electron paramagnetic resonance (EPR) is a spectroscopic technique that is used to detect and characterize materials with unpaired electrons. If electrons are paired, the net magnetic moment becomes zero. Presence of a net magnetic moment originated from electrons depends on the presence of the unpaired electron.[22] Magnetic moment of an unpaired electron can interact with an external magnetic field and it can be aligned parallel or antiparallel to applied magnetic field (Figure 1.5).

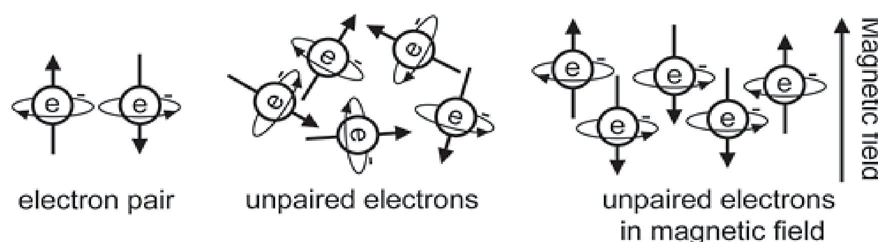

Figure 1.5. Electron spin motions in the absence and presence of the magnetic field.[22]

ESR spectroscopy measures the energy differences between the electron spin states in the presence of magnetic field. Interaction between the magnetic moment (μ_B) of an unpaired electron and external magnetic field (B_0) called electron Zeeman interaction splits the energy state of electron into two (Figure 1.6). Energy values of electron spin states are calculator with this formula:

$$E = m_s g \beta B_0 \tag{1.1}$$

where m_s is spin quantum number, g is a constant factor and β Bohr magneton equal to 9.2700949 x 10^{-24} JT^{-1}. In the case of resonance, the microwave energy is absorbed to excite the unpaired electron from lower to upper state. In other words, when the difference between energy levels of electron spin states (ΔE) is equal to the energy of the microwave source, a resonance condition occurred and the microwave energy is absorbed:

$$\Delta E = h\nu = \mu_B B_0 \tag{1.2}$$

where h is Planck's constant and ν is the frequency of the radiation.

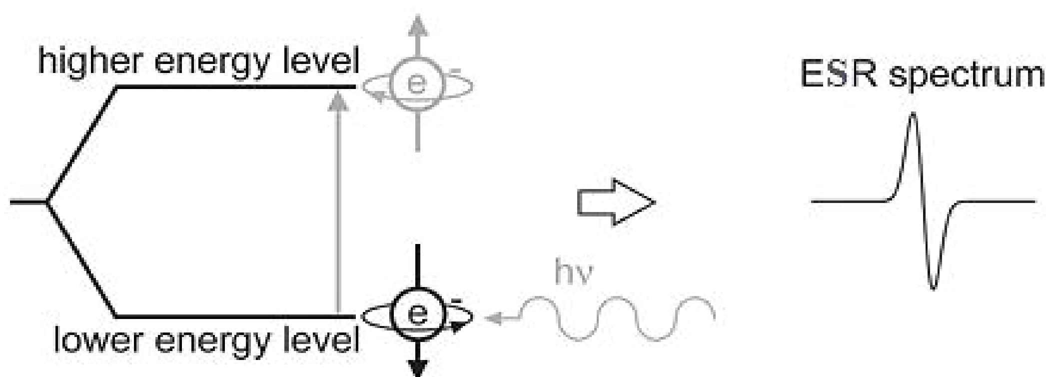

Figure 1.6. The energy-level scheme of an unpaired electron showing ESR absorption and corresponding spectrum with one line.[22]

Generally, absorption spectrum is converted into the ESR spectrum after taking its first derivative. ESR technique depends on also the magnetic properties of the nucleus which interacts with the unpaired electron. The line splitting of the ESR spectrum is determined by the interaction between the nucleus and unpaired electron called as hyperfine coupling.

For example, for the nitroxyl radical the usual splitting in three hyperfine lines comes from the nitrogen nucleus (^{14}N) with a nuclear spin number I = 1. The formula "2nI+1" can be used for calculating number of splitting where the 'n' is the spin-active nuclei around electron (Figure 1.7).

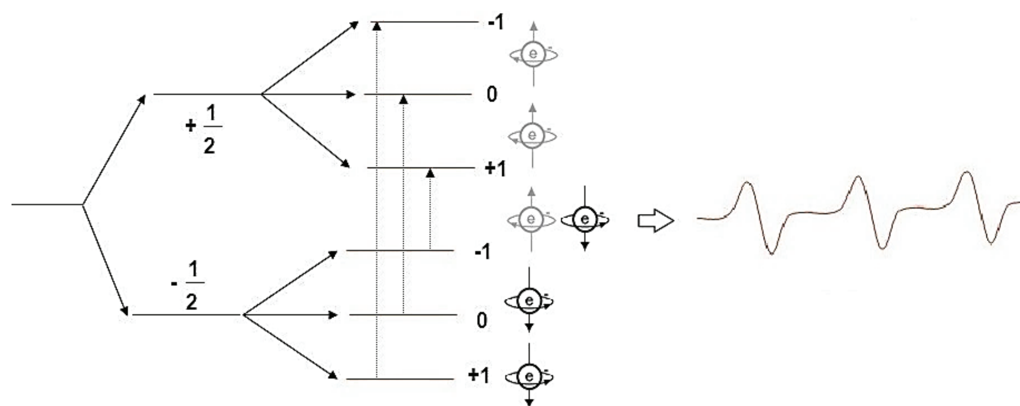

Figure1.7. The energy level scheme of unpaired electron coupled with a nucleus with I = 1/2, showing two resonant transitions and corresponding spectrum with two lines.[22]

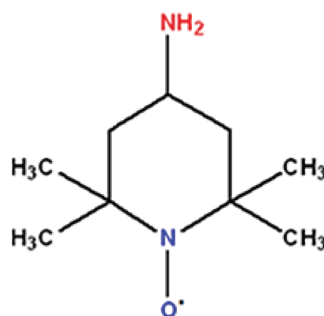

Figure 1.8. Structure of 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (4-Amino Tempo). Red-labeled part is functional group, blue-labeled part is radical group.

Usually, nitroxyl radicals are used as spin labels (SL) or spin probes. Spin labels are covalently attached to the macromolecules. On the other hand, spin probes interact with the macromolecules via weaker forces. 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) based radicals are frequently used spin labels or probes for protein studies. There are different tempo radicals commercially available with different functional groups (–OH, $–NH_2$, –COOH, –O, –NCS, etc.). These functional groups are used to connect TEMPO to other molecules. We have used 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (4-Amino Tempo) in our study (Figure 1.8).

Spin labeling method is used together with ESR spectroscopy to investigate the binding states of small molecules to large biomolecules.[23-26] Using the cw ESR spectroscopy, we can identify bound and unbound drugs at the same time due to their different dynamics behaviours. The rotational dynamics of spin labeled molecules determines the line shape of the ESR spectrum. When spin labeled drugs rotate freely in solution, their spectra have three narrow signals with rotational correlation times (τ_c) between 0.01 - 0.1 ns. When spin-labeled drugs bind to a large biomacromolecule, such as protein, the free electron of nitroxyl radicals move slowly. Because the protein restricts the spin movements of the unpaired electron. Therefore, the spectrum line expands, the signal amplitude decreases and the τc value increases. Stocker et al. showed that ESR spectra vary according to the rotational correlation times of free radicals (Figure 1.9).[27] In other words, immobilized drugs (bound) have broad signals coming from restricted rotational motion. Mobilized drugs (free) have three sharp line signals coming from freely rotational motion.

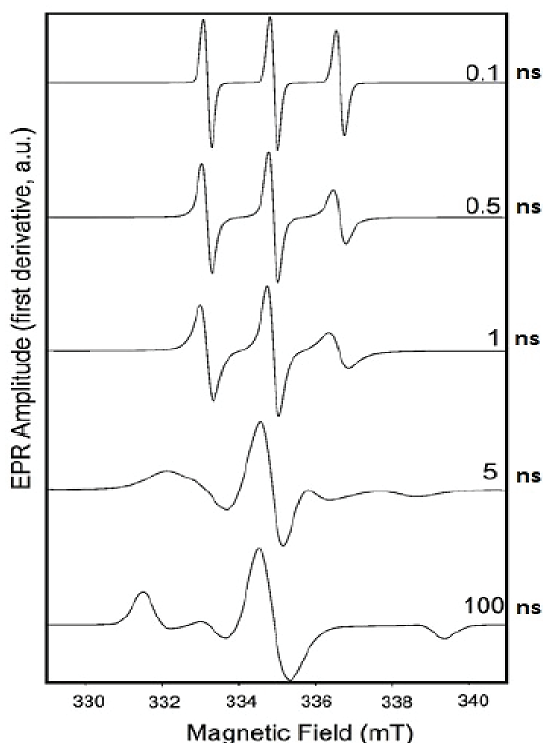

Figure 1.9. Examples of simulated X-band ESR spectra of a nitroxide spin label.[27]

CHAPTER 2

EXPERIMENTAL

2.1. Reagents And Instruments

Bovine serum albumin (fatty acid free), 4-amino salicylic acid (99%), Tempo-4-amino (97%), 4-amino benzoic acid (99%), 3-amino phenol (98%), phenyl isothiocyanate (97%), cyclohexyl isothiocyanate (98%), hexyl isothiocyanate (95%), ibuprofen (98%), acetylsalicylic acid (99%), *N*-(3-dimethylaminopropyl)-*N'* ethylcarbodiimide hydrochloride (EDC, 98%), hydrochloric acid (37%, 12.2 M), dichloromethane (99.8%), trimethylamine (99.5%), ethyl acetate (99.5%), methanol (99.8%) were purchased from Sigma Aldrich. Thiophosgene (99%) was purchased from Merck.

All reagents were used without further purification. All solvents were of analytical reagent grade. Double distilled water was used throughout the experiments. The samples were measured on a Varian Cary Eclipse Fluorescence spectrophotometer equipped with 1.0 cm path length quartz cuvettes and a CMS 8400 (Adani) benchtop X-band ESR spectrometer with a TE_{102} resonator cavity.[26]

2.2. Synthesis of Spin Labeled Molecules

In order to obtain the spin label drugs we followed different methods. In the first method we synthesized isothiocyanate forms of drugs then labeled the drugs. In the second group we used EDC crosslinking technique.

2.2.1. Synthesis of Spin Labeled Salicylic Acid (SLSA)

4-Amino salicylic acid (0.01 mol) was dissolved in 32 mL water and then acidified with 3.4 mL HCl (12.2 M) in a 100 mL reaction tube with a stopcock. Thereafter, thiophosgene (0.044 mol) was introduced. After stirring for 1 hour at 0 °C (in an ice bath),

the solution was stirred for 2.5 hours at 25 °C. The solution was filtered and dried under vacuum.[28] Isothiocyanate salicylic acid was isolated as a white powder. The yield of the experiment was 50%.[26] Tempo-4-amino (0.625 mmol) and 4-isothiocyanate salicylic acid (0.46 mmol) were dissolved in 20 mL DCM in a 50 mL flask. A few drops of triethylamine were added and stirred overnight at 25 °C. Tempo attached salicylic acid (spin labeled salicylic acid, SLSA) was purified with column chromatography (ethyl acetate:methanol, 15:1, 13:1, 12:1 and 10:1). The yield of the experiment was 46%.[26]

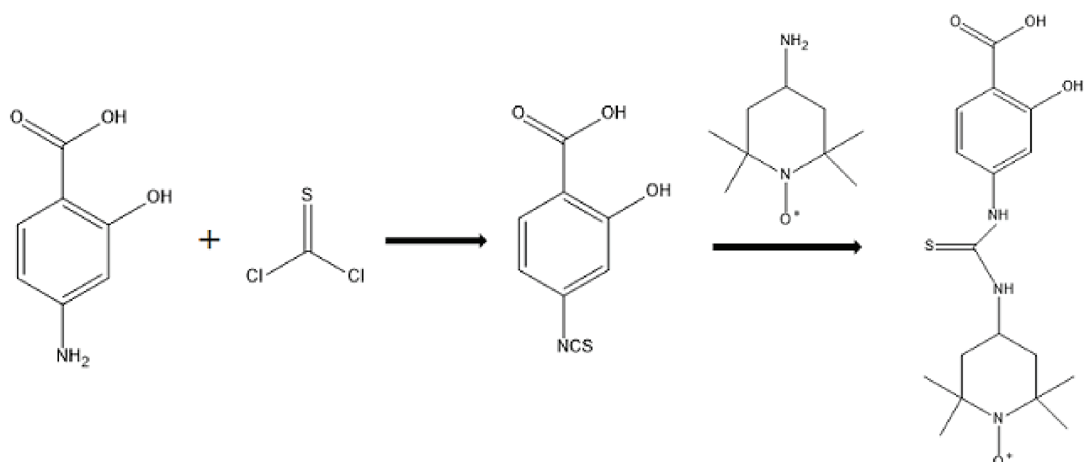

Figure 2.1. Synthesis mechanism of spin labeled Salicylic acid (SLSA).

2.2.2. Synthesis of Spin Labeled Benzoic Acid (SL-Benzoic Acid)

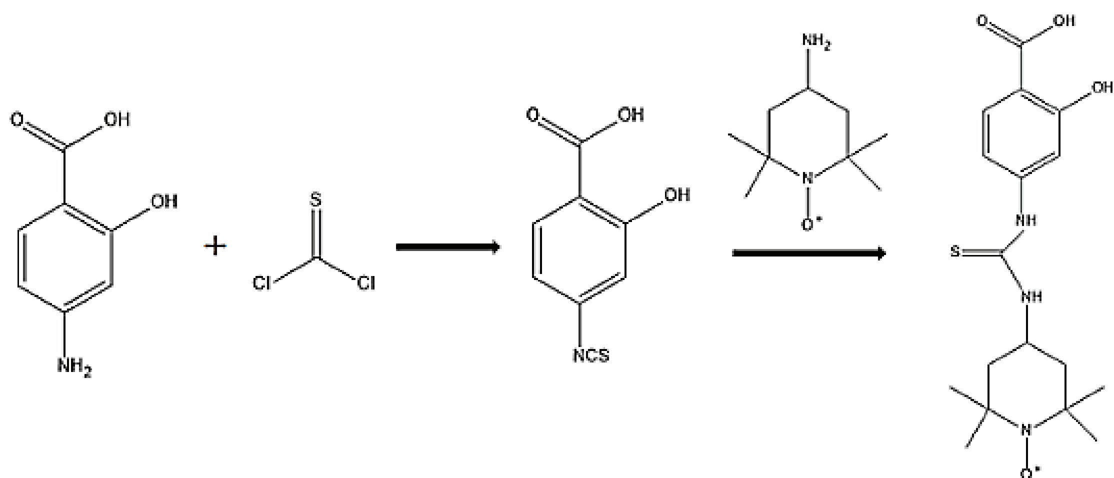

Figure 2.2. Synthesis mechanism of spin labeled Benzoic acid (SL-Benzoic acid).

4-Amino benzoic acid (0.01 mol) was used as a starting chemical and the procedure of synthesis of SL-salicylic acid was applied. The yield of 4-isothiocyanate

benzoic acid is 50%.[26]Tempo-4-amino (0.625 mmol) and 4-isothiocyanate benzoic acid (0.46 mmol) were dissolved in 10 mL DCM. 10 drops of triethylamine were added and stirred for 2 days at 25 °C. SL-benzoic acid was purified by column chromatography (hexane:ethyl acetate 4:1 and 3:1). The yield is 25%. [26]

2.2.3. Synthesis of Spin Labeled Phenol (SL-Phenol)

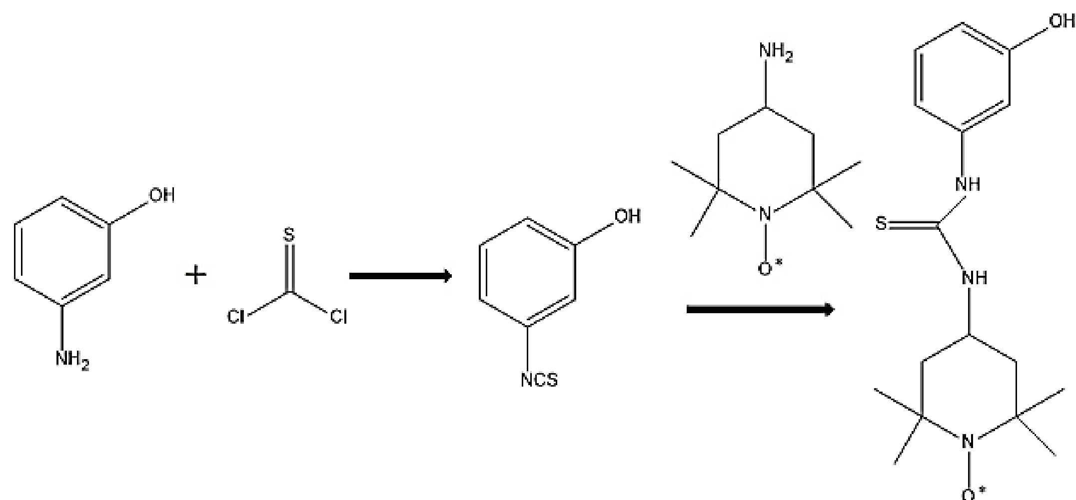

Figure 2.3. Synthesis mechanism of spin labeled Phenol (SL-Phenol).

3-Amino phenol (0.01 mol) was dissolved in 32 mL water and then acidified with 3.4 mL HCl (12.2 M). Thereafter, thiophosgene (0.044 mol) was introduced. After stirring for 1 hour at 0 °C, the solution was stirred for 2.5 hours at 25 °C. This was followed by extraction with saturated 3-isothiocyanate phenol solution and saturated dichloromethane (3x30 mL) to remove starting materials and by-products. The organic solvent was removed by rotary evaporation and dried under vacuum. The yield is 45%.[26]

Tempo-4-amino (0.625 mmol) and 3-isothiocyanate phenol (0.46 mmol) were dissolved in 10 mL DCM. A few drops of triethylamine were added and stirred for 2 days at 25 °C. SL-Phenol was purified by flash column chromatography (hexane:ethyl acetate 4:1, 3:1 and 2:1). The yield is 37%.[26]

2.2.4. Synthesis of Spin Labeled Benzene (SL-Benzene)

Tempo-4-amino (0.625 mmol) and phenyl isothiocyanate (0.46 mmol, 55 mL) were dissolved in 10 mL DCM. 7 drops of triethylamine were added and stirred overnight

at 25 °C. SL benzene was purified by flash column chromatography (hexane:ethyl acetate 4:1, 3:1 and 2:1). The yield is 55%. [26]

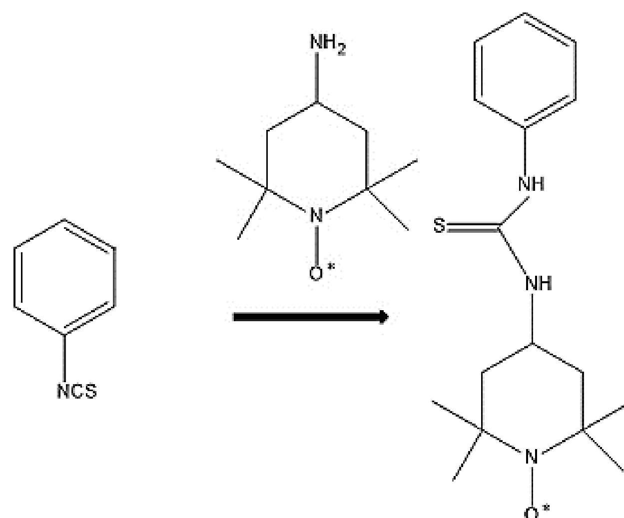

Figure 2.4. Synthesis mechanism of spin labeled Benzene (SL-Benzene).

2.2.5. Synthesis of Spin Labeled Cyclohexane (SL-Cyclohexane)

Tempo-4 amino (0.625 mmol) and cyclohexyl isothiocyanate (0.46 mmol, 15.3 mL) were dissolved in 10 mL DCM. A few drops of triethylamine were added and stirred overnight at 25 °C. SL-cyclohexane was purified by column chromatography (hexane:ethyl acetate 4:1 and 3:1). The yield is 25%.[26]

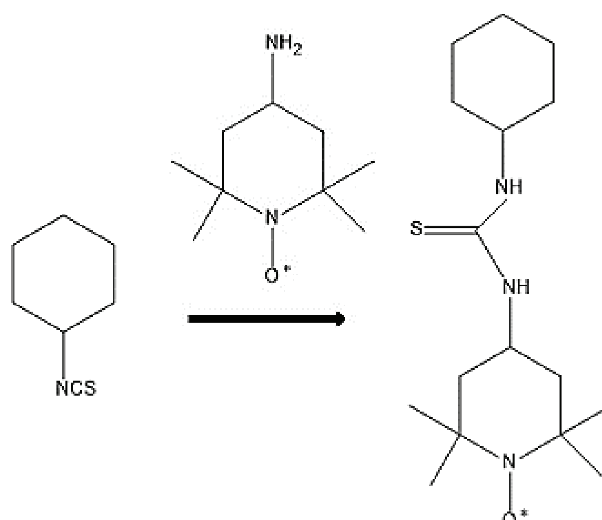

Figure 2.5. Synthesis mechanism of spin labeled Cyclohexane (SL-Cyclohexane).

2.2.6. Synthesis of Spin Labeled Hexane (SL-Hexane)

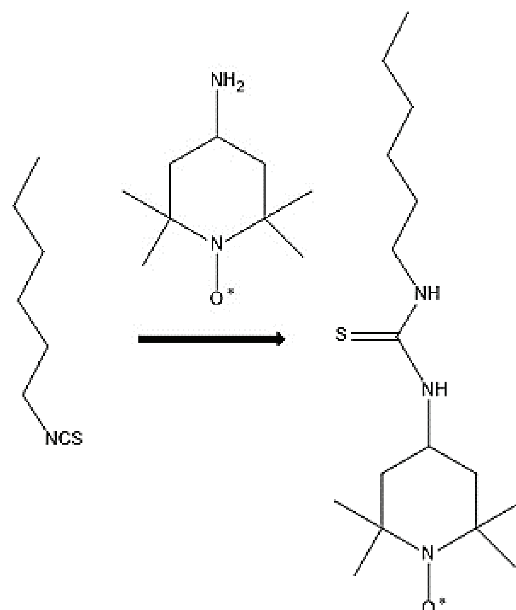

Figure 2.6. Synthesis mechanism of spin labeled Hexane (SL-Hexane).

Tempo-4-amino (0.625 mmol) and hexyl isothiocyanate (0.46 mmol, 13.2 mL) were dissolved in 10 mL DCM. A few drops of triethylamine were added and stirred overnight at 25 °C. SL-hexane was purified by column chromatography (hexane:ethyl acetate 4:1 and 3:1). The yield is 28%.[26]

2.2.7. Synthesis of Spin Labeled Methyl (SL-Methyl)

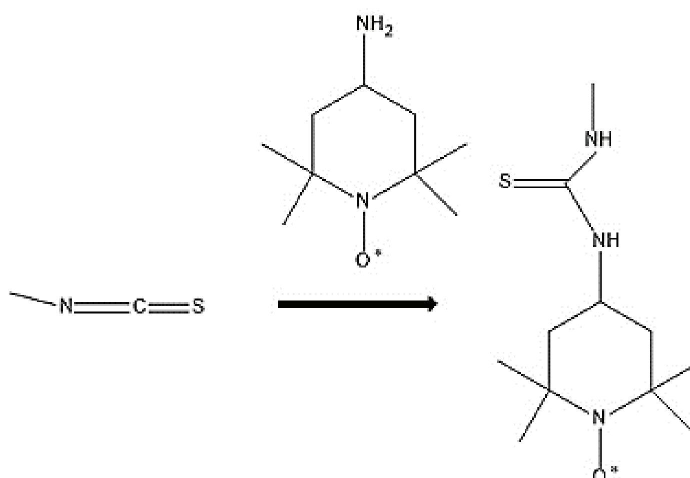

Figure 2.7. Synthesis mechanism of spin labeled Methyl (SL-Methyl).

Tempo-4-amino (0.625 mmol) and methyl isothiocyanate (0.46 mmol, 31.5 mL) were dissolved in 10 mL DCM. A few drops of triethylamine were added and stirred overnight at 25 °C. SL-methyl was purified by column chromatography (hexane:ethyl acetate 3:1 and 2:1). The yield is 32%.[26]

2.2.8. Synthesis of Spin Labeled Ibuprofen (SL-Ibuprofen)

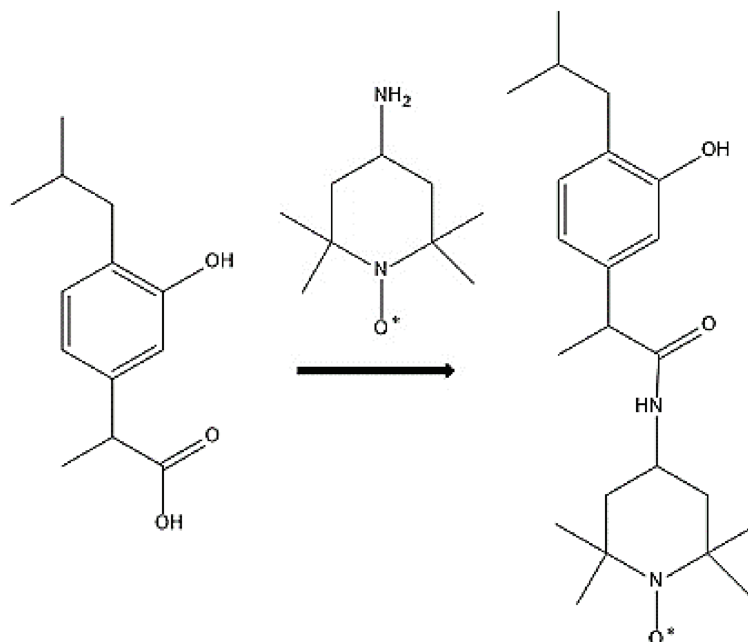

Figure 2.8. Synthesis mechanism of spin labeled ibuprofen (SL-ibuprofen).

Tempo-4-amino (0.2 M), ibuprofen (0.2 M) and EDC (0.2 M) were mixed in DCM at 1:1:1 ratio under argon and stirred for 18 hours at 25 °C. SL-ibuprofen was purified with column chromatography (hexane:ethyl acetate 5:1, 4:1, 3:1 and 2:1). The yield is 69%.[26]

2.2.9. Synthesis of Spin Labeled Aspirin (SL-Aspirin)

Tempo-4-amino (0.2 M), aspirin (0.2 M) and EDC (0.2 M) were mixed in DCM at a 1:1:1 ratio under argon and stirred for 18 hours at 25 °C. SL-aspirin was purified by column chromatography (hexane:ethyl acetate 4:1, 3:1 and 2.3:1). The yield is 58%. [26]

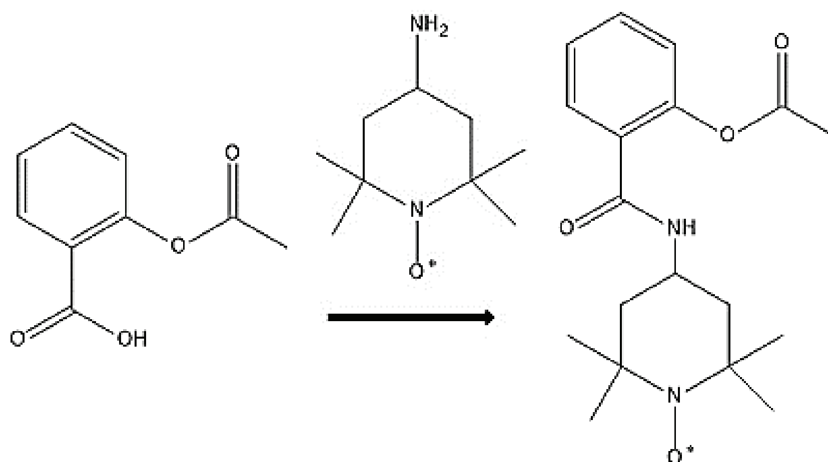

Figure 2.9. Synthesis mechanism of spin labeled Aspirin (SL-Aspirin).

2.3. Preparation of cBSA

The cationic BSA (cBSA) was prepared following a previously reported procedure.[29] BSA (50 mg, 0.15 mmol) was dissolved in 5 mL ethylenediamine aqueous solution (50mM, pH:4.75) at 25 °C. After that EDC (410 mg, 2.1 mmol) was introduced. After stirring for 2 hours at 25 °C, the reaction was stopped by adding acetate-buffer (360 mL, 4 M, pH:4.75). The colorless solution was concentrated by ultrafiltration (Amicon Ultra 50 kDa MWCO), washed three times with acetate-buffer (100 mM, pH:4.75) and five times with Milli-Q water and subsequently lyophilized. We obtained cBSA as white powder. The yield of the experiment was 95%. A Malvern dynamic light scattering (DLS) Nano-ZS instrument was used for zeta potential measurements.[26]

2.4. Binding of Spin Labeled Molecules to BSA and to cBSA

In different concentrations, anionic BSA and cBSA solutions (pH = 7.4) were prepared in phosphate buffer (0.1 M). Low concentrations of phosphoric acid and dihydrogen phosphate ions are present in the blood.[30] Phosphate buffer is therefore often used in biological reactions. The stock solutions of spin labeled drugs were prepared in DMSO solution to be 1 M. To prepare different ratios of spin labeled drugs in BSA, different quantities of spin labeled drugs were added to protein solution with constant stirring (DMSO concentration is below 1% by volume). The solutions were allowed to equilibrate at 37 °C for 10 minutes, before which ESR measurements were taken.[26]

2.5. Fluorescence Measurements

Varian Cary Eclipse Fluorescence Spectrophotometer was used for fluorescence measurements. Specimens were placed in 1.0 cm long quartz cuvettes. Upon excitation at 278 nm, the emission spectra were collected in the range of 300-550 nm. The slit width was 5 nm for both excitation and emission. Reference measurements of all spin labeled drugs and BSA were taken in phosphate buffer.

2.6. ESR Measurements

A CMS 8400 (Adani) benchtop spectrometer provided with a TE_{102} resonator cavity was used for all X-band ESR measurements at a microwave frequency of ~ 9.4 GHz at 25 °C. Measurements were performed in quartz capillary sample tubes. The shown ESR spectra were normalized to the intensity of the high field line of the unbound spin labeled molecules. All spectra were simulated using the Matlab-based Easyspin 4.5.5 software package.[31]

CHAPTER 3

RESULTS AND DISCUSSION

In this study, our primary focus is on the use of ESR spectroscopy to investigate drug binding and release. For this, we applied spin labeling technique to the drugs. Binding and release of spin labeled drugs were monitored in BSA protein solution. The ESR signal of the drug bound with protein is broader than the released drug. Due to this difference between signals, we can observe the bound and released drug with ESR spectroscopy.

Secondly, we examined the potential for drugs to behave competitively while binding to the BSA protein in different combinations. As an alternative method again, we used ESR spectroscopy. SA was used as the main drug for binding and release experiments to BSA. Other spin labeled derivatives of ibuprofen, aspirin and salicylic acid have been used to study competitive effects.

3.1. Analysis of Binding and Release Studies of SLSA to/from BSA

Analysis of drug binding and releasing was studied with fluorescence and ESR spectroscopy.

3.1.1. Fluorescence Spectroscopy Studies on the Binding of SA and SLSA to BSA

In order to compare the binding ability of SA and SLSA to BSA, fluorescence emission spectra of BSA were recorded in the absence and presence of SA and SLSA (Figure 3.1). Intrinsic tryptophan fluorescences (Trp-212 and Trp-134) dominate the BSA fluorescence spectrum with a maximum at 350 nm. Moreover, SA has a characteristic fluorescence emission spectrum with a maximum at around 410 nm. However,

fluorescence emission intensity of SLSA is about 11 times weaker than that of SA because spin labeling of SA quenched the fluorescent signal of the SA due to the interaction between the nitroxide radical and the fluorophore (Figure 3.1, dashed lines).

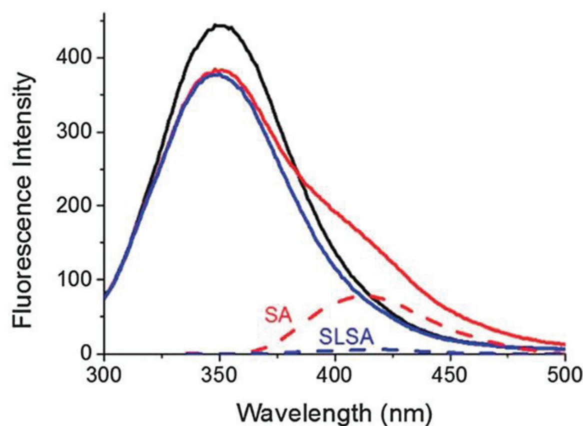

Figure 3.1. Fluorescence emission spectra of BSA (black) in the presence of SA (red) and SLSA (blue). The concentration of BSA is $2x10^{-7}$ M in the 0.1 M phosphate buffer at a drug/BSA ratio of 1. The dashed lines belong to the fluorescence emission of SA (red) and SLSA (blue) in the buffer solutions without BSA.[25]

In general, the fluorescence intensity of BSA decreases when other molecules bind to BSA due to the distortion of the microenvironment around the tryptophan residues of BSA.[17, 32] Ni et al. showed that the addition of SA to BSA decreases the fluorescence intensity of BSA at 350 nm, and a second peak appears at ~ 410 nm.[17] Figure 3.1 shows that the fluorescence intensity of BSA (2×10^{-4} mM) decreases upon addition of SA and SLSA at a drug/BSA ratio of 1. However, the second peak at 410 nm appears only upon addition of SA. Unambiguously, the decreasing ratios in fluorescence intensity of BSA are very similar after SA and SLSA contributions. This observation suggested that BSA binding abilities of SA and SLSA are very comparable.

Furthermore, Figure 3.2 showed the fluorescence emission spectra of BSA (1.5×10^{-7} M) in the presence of SA with different concentrations (0–4.3×10^{-7} M) at 303 K. The fluorescence band intensity of BSA decreases (quenches) upon addition of SA. At the same time, a second peak corresponding to SA appears gradually.

The quenching is generally characterized by the Stern–Volmer equation.[33]

$$F_0/F = 1 + K_{SV}[Q] \tag{3.1}$$

where F_0 and F are the fluorescence intensities of the BSA before and after the addition of the SA (quencher), respectively. [Q] is the concentration of the quencher (SA). K_{SV} is the dynamic quenching constant. The K_{SV} value of the SA–BSA complex was found to be 5.4 x 10^5 M within the range of 0–2 x 10^{-7} M SA concentrations. The quenching behavior of BSA in the presence of SA slowed down above the 2 x 10^{-7} M SA concentration (the ratio of SA/BSA is 1.5) and the quenching ceased after 3.2 x 10^{-7} M SA concentration which is the limit of detection (the ratio of SA/BSA is 2.4) (Figure 3.2).

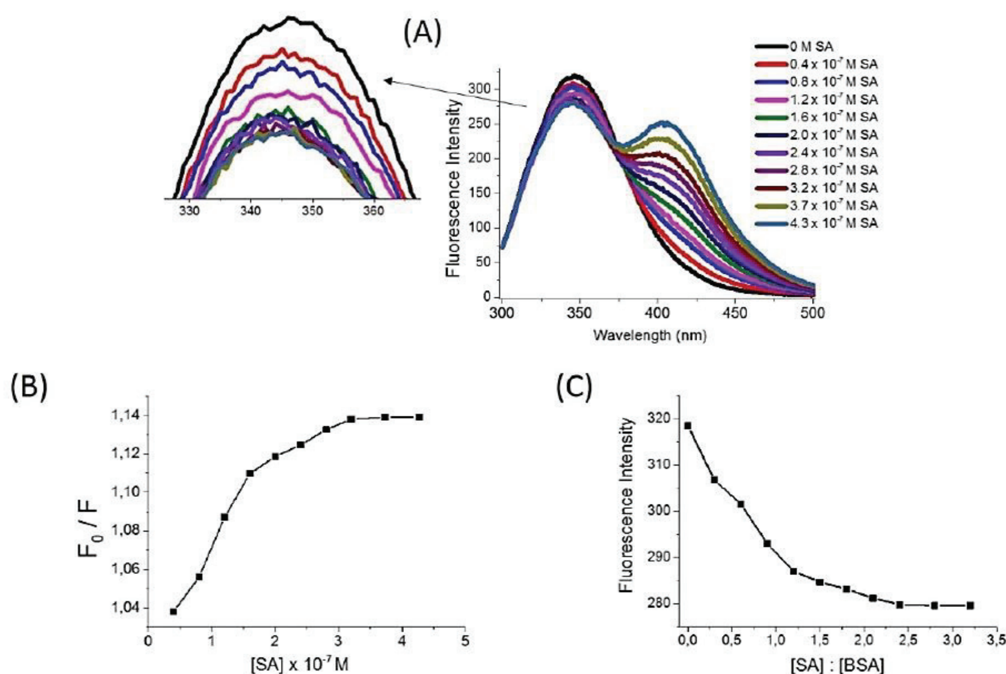

Figure 3.2. (A) Fluorescence emission spectra of BSA (1.5 x 10^{-7} M) in the presence of Salicylic acid (SA) with different concentrations (0-4.3 x 10^{-7} M) at 303 K. (B) Stern-Volmer plot of the SA-BSA complexes. F_0 and F are the fluorescence intensities of the BSA before and after the addition of the SA (quencher), respectively. (C) Fluorescence quenching ceased above the ratio of ~2:1 for SA-BSA.[25]

3.1.2. ESR spectroscopy studies on the binding of SLSA to BSA

In order to study SLSA–BSA binding in detail, first we characterized the ESR spectrum of SLSA. X-band cw ESR spectra of 0.6 mM SLSA and tempo-4-amino in 0.1 M phosphate buffer at 25°C are presented in Figure 3.3. The usual splitting in three hyperfine lines comes from the nitrogen nucleus with nuclear spin I = 1 of the nitroxide

group. The ESR parameters of SLSA and Tempo- 4-amino (isotropic g-values (g_{iso}), isotropic hyperfine coupling constants (A_{iso}) and rotational correlation times (τ_R) were derived from their spectral simulations. Both have similar g-values and hyperfine values (g_{iso}: 2.00554 vs. 2.00552 and A_{iso}: 1.70 mT vs. 1.68 mT, for SLSA vs. Tempo-4-amino, respectively).

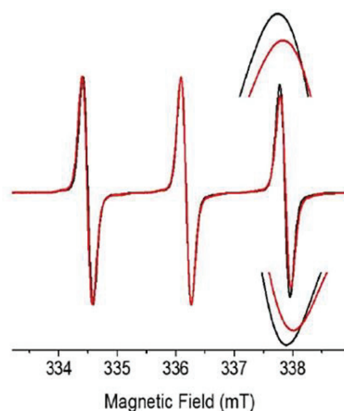

Figure 3.3. ESR spectra of SLSA (red) and tempo-4-amino (black) in 0.1 M phosphate buffer solutions containing 1% (v/v) DMSO.[25]

The line shape of an ESR spectrum contains information about the rotational dynamics of a paramagnetic centre. For both samples, the low-field and central lines of spectra are almost equal, but the high field line is less intense for the SLSA. This indicates the slower rotational motion of SLSA (τ_R = 0.07) compared to the rotational motion of tempo-4-amino (τ_R = 0.02 ns) (Figure 3.3). This suggests that binding of SA to tempo-4-amino restricts the motion of the radical centre.

Next, we studied SLSA binding to BSA using cw ESR spectroscopy (Figure 3.4 and Figure 3.5). In solution, the nitroxide line shape of the cw ESR spectrum of the spin labeled drug is strongly influenced by the protein binding. Rotational freedom of the radical center on the molecule significantly decreases upon macromolecule binding so that the rotational correlation time increases from the ps to ms range as a result of the immobilization strength.[34] In a typical room temperature ESR spectrum, the unbound (mobilized) spin labeled drug possesses sharp three-line signals, signatures of freely tumbling motion. In contrast, the protein bound (immobilized) spin labeled drug gives signals with broad outer hyperfine features stemming from restricted rotational motion (Figure 3.5). Therefore, the ESR spectroscopy technique allows simultaneous detection of bound and unbound spin labeled drug molecules from their corresponding signals.

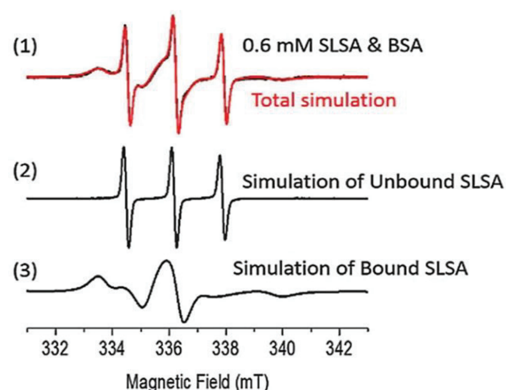

Figure 3.4. (1) Cw ESR spectrum of 0.6 mM SLSA in BSA solution (1 : 1) (black) and its simulation (red). The simulation of the spectrum was obtained by the addition of simulated unbound SLSA (2) and simulated bound SLSA (3).[25]

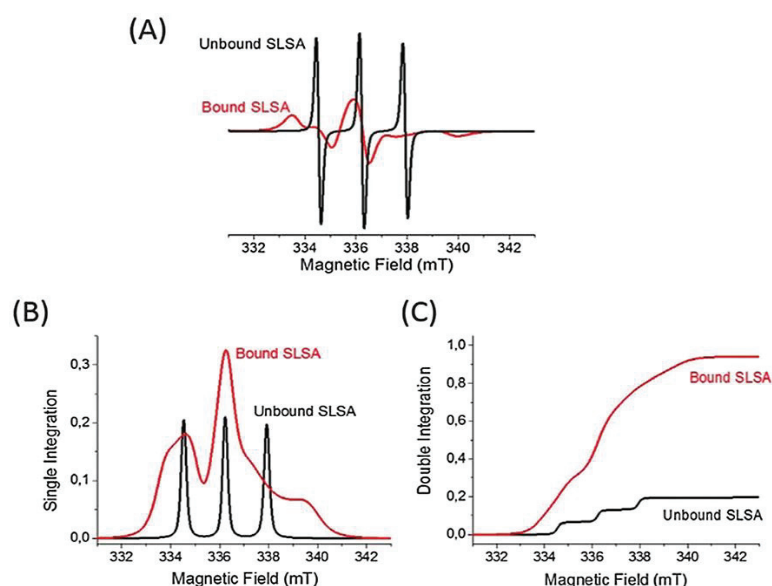

Figure 3.5. (A) Superposition of simulations of unbound (black) and bound (red) fractions of the ESR spectrum belongs to 0.6 mM SLSA in BSA solution (1 : 1). Single integrations (B) and double integrations (C) of ESR spectra in (A). Double integration is performed to determine the proportion of the bound and unbound SLSA to the total.[25]

Moreover, the ratio of bound to unbound spin labeled drugs, and the number of bound SLSA molecules per albumin molecule can be calculated from the areas under the ESR signals (obtained by double integrations of ESR spectra, Figure 3.5):

$$\text{Bound[SLSA]/[BSA]} = \frac{\text{Bound Area}}{\text{Bound Area} + \text{Unbound Area}} \times \frac{[\text{SLSA}]}{[\text{BSA}]} \quad (3.2)$$

Figure 3.4 shows the cw ESR spectrum of 0.6 mM SLSA in the BSA solution at a SLSA/BSA ratio of 1 with the corresponding simulation. The simulation of the spectrum was obtained by the addition of the simulated three line spectrum (unbound SLSA, τ_R = 0.07 ns) and simulated broad signals (bound SLSA, τ_R = 10 ns). Double integrations of the simulated ESR spectra of unbound and bound SLSA revealed that ~ 80% of SLSA is bound to albumin (Figure 3.5). As a control experiment, a mixture of Tempo-4-amino and BSA solution was measured and only three sharp signals coming from free Tempo-4-amino radicals were obtained (Figure 3.6). This indicates that Tempo-4-amino does not bind to BSA by itself.

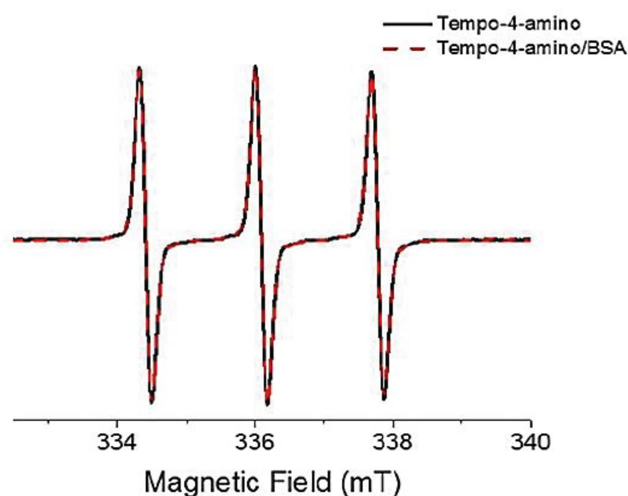

Figure 3.6. ESR spectra of 0.5 mM Tempo-4-amino (black) and 0.5 mM Tempo-4-amino/BSA complex (1:1) (red dashed line) in 0.1 M phosphate buffer solutions.[25]

3.1.3. Concentration Effects On The Protein Binding

In solution, the extent of drug–protein conjugation increases when the concentrations of drug and protein increase according to the collision theory.[35] Figure 3.7 (A) shows the cw ESR spectrum of a concentrated drug–protein solution with 3.0 mM SLSA in 3.0 mM BSA solution (1 : 1) (red line). Most of the SLSA are bound to BSA with a fraction of bound SLSA as 0.95 (Figure 3.7 (B)). When the SLSA–BSA solution is gradually diluted with buffer while keeping the SLSA/BSA ratio at unity, the fraction of bound SLSA decreases from 0.95 to 0.50 at 3.0 mM and 0.1 mM BSA, respectively. This suggests that dilution causes the release of SLSA from BSA.

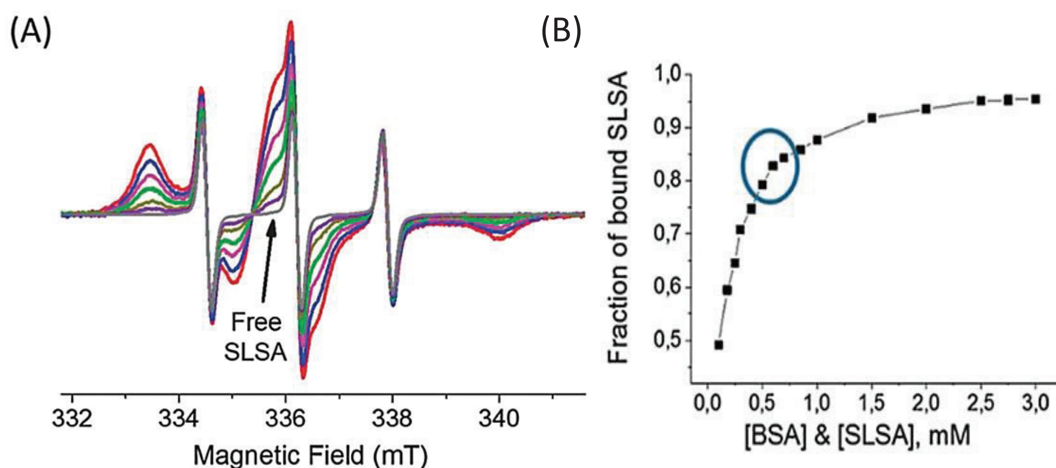

Figure 3.7. (A) Cw ESR spectrum of SLSA in BSA solution at a SLSA/BSA ratio of 1. The concentrations of SLSA and BSA from top to bottom are 3.0, 2.5, 2.0, 1.5, 1.0, 0.5 and 0.25 mM, and the free 0.6 mM SLSA in buffer without BSA (gray line). The ESR spectra are normalized to the intensity of the high field line of the unbound SLSA. (B) Fraction of bound SLSA obtained from simulated spectra of 3.0, 2.75, 2.5, 2.0, 1.5, 1.0, 0.85, 0.7, 0.6, 0.5, 0.4, 0.3, 0.25, 0.18 and 0.10 mM SLSA in BSA solutions at a SLSA/BSA ratio of 1. The circle shows the concentrations of BSA in the physiological range (0.5–0.7 mM BSA).[25]

Usually, drug binding is a reversible process in which drugs bind to the albumin with weak chemical bonds, such as hydrogen bonds or van der Waals forces.[36-37] We have also an equilibrium between bound and unbound SLSA in the BSA solution. During the process of diluting the system to lower concentrations, the collision probability of unbound SLSA and BSA decreases which reduces the formation of SLSA–BSA conjugation. In order to maintain the equilibrium between bound and unbound drugs, BSA releases more SLSA. Thus, the fraction of unbound SLSA increases and the fraction of bound SLSA decreases regularly. When we plotted the fraction of bound SLSA in 1:1 SLSA:BSA solutions of varying BSA concentrations (0.1 mM–3.0 mM) we obtained a saturation curve. The bound fraction is saturated just above the physiological concentrations of albumin (0.5–0.7mM). However, below the physiological concentrations of albumin the fraction of bound SLSA decreases promptly.

The experimental results show that SLSA can bind to BSA with very high efficiency at the physiological concentrations of BSA. But, below the physiological concentrations, BSA loses its affinity for SLSA dramatically.

We measured the ESR spectra of 2 mM, 0.8 mM and 0.4 mM of SLSA in BSA solution at a SLSA/BSA ratio of 1 within a period of time after preparation of the samples from 1 minute to 30 minutes (Figure 3.8). Since tuning the ESR cavity after sample insertion and ESR measuring collectively take some time, we cannot collect the data immediately (0 min).

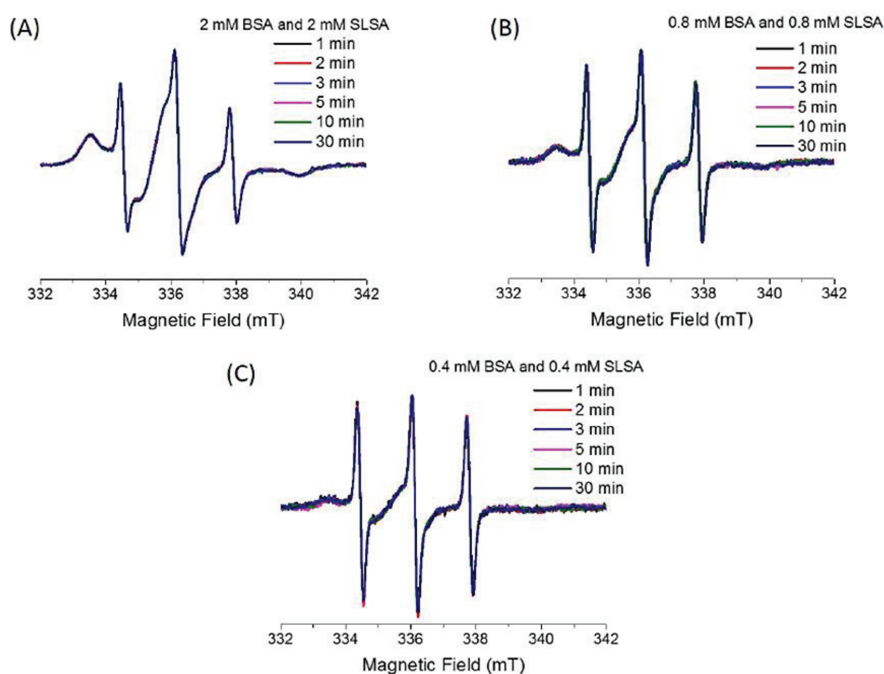

Figure 3.8. ESR spectra of (A) 2 mM SLSA in 2 mM BSA solution, (B) 0.8 mM SLSA in 0.8 mM BSA solution, and (C) 0.4 mM SLSA in 0.4 mM BSA solution within a period of time after preparation the samples from 1 minute to 30 minutes.[25]

Figure 3.8 shows that ESR spectra of different concentrations of SLSA:BSA (1:1) assemblies do not change with time. The bound to free drug ratio is constant within 30 min. Usually, drug–protein binding interaction is a kinetically rapid reversible interaction. Therefore, the quick drug association and dissociation rates in ranges of milliseconds and seconds could not be calculated by the ESR technique.

3.1.4. Binding Stoichiometry of SLSA to BSA

Simulations of ESR spectra of both bound and unbound SLSA were used to obtain a maximum number of bound SLSA to BSA, at physiological albumin concentration. For

this purpose, SLSA was added to 0.5 mM BSA solution to generate a series of samples with different SLSA concentrations from 0.5 to 9.5 mM, and recorded using ESR spectroscopy.

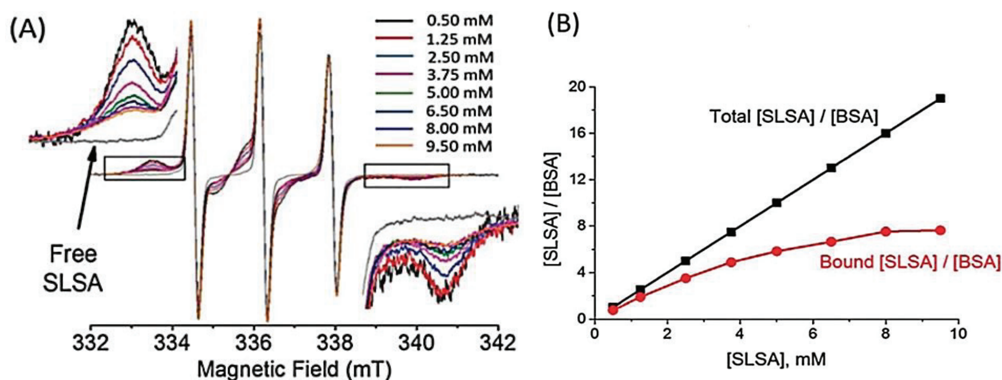

Figure 3.9. (A) Cw ESR spectra of SLSA in a constant 0.5 mM BSA solution at different SLSA/BSA ratios. The concentrations of SLSA are 0.5, 1.25, 2.5,3.75, 5.0, 6.5, 8.0 and 9.5 mM (from top to bottom). 0.5 mM free SLSA in buffer solution is shown by grey color. (B) Ratio of total SLSA concentration to BSA concentration is shown by black line, and the ratio of bound SLSA to BSA concentration is shown by red line. Bound SLSA concentrations are obtained from simulations of ESR spectra from (A).[25]

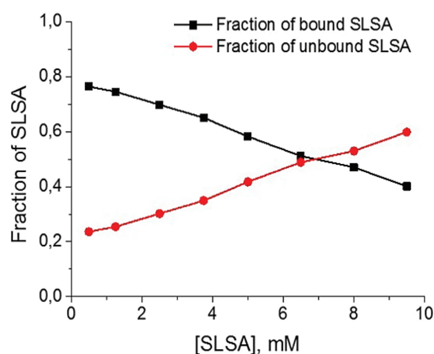

Figure 3.10. Fractions of bound (black) and unbound (red) SLSA in 0.5 mM BSA/buffer solution obtained from simulations of their ESR spectra. The concentrations of SLSA are 0.5, 1.2, 2.5, 3.7, 5.0, 6.5, 8.0 and 9.5 mM.[25]

In the normalized ESR spectra, the intensity of bound SLSA signals decreases with increasing SLSA concentration (Figure 3.9 (A)). After simulations of each spectrum, the fractions of bound SLSA were found to be 0.8 and 0.4 in the presence of 0.5 mM and 9.5 mM SLSA, respectively (Figure 3.10).

Although, increasing the concentration of SLSA decreases the bound fraction of SLSA (Figure 3.10), the number of bound SLSA per BSA increases (Figure 3.9 (B)). Nonetheless, a deviation from the straight line coming from the ratio of total SLSA to BSA concentrations is observed by increasing the concentration of SLSA. Eventually, SLSA forms a saturated complex with BSA at a molar ratio of ~ 7 : 1 (SLSA : BSA) (Figure 3.9 (B)). This indicates that at physiological concentration (0.5–0.7 mM) BSA possesses about 7 binding sites for SLSA (Equation 3.2).

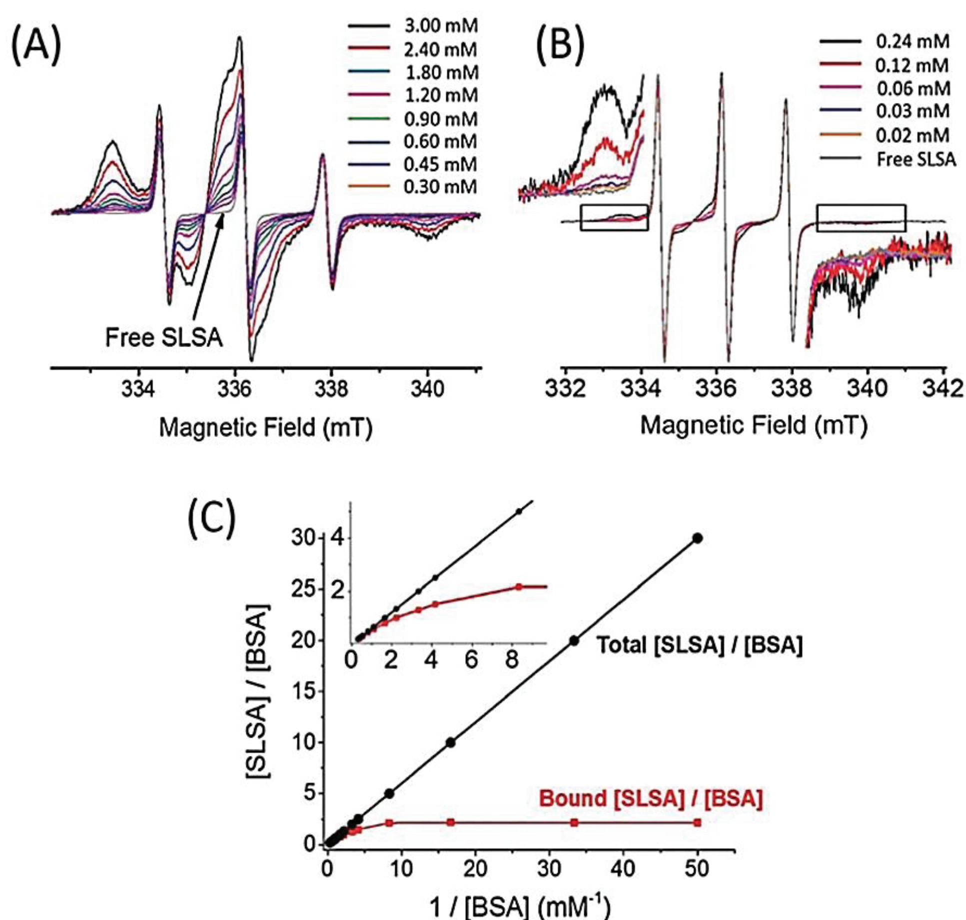

Figure 3.11. Cw ESR spectra of constant 0.6 mM SLSA in different concentrations of BSA. The concentrations of BSA are 3.00, 2.40, 1.80, 1.20, 0.90, 0.60, 0.45 and 0.30 mM in (A) and 0.24, 0.12, 0.06, 0.03 and 0.02 mM in (B). 0.6 mM free SLSA in buffer solution was shown as a reference. The ESR spectra are normalized to the intensity of the high field line of the unbound SLSA. (C) Ratio of total SLSA concentration to BSA concentration is shown by black line and the ratio of protein bound SLSA concentration to BSA concentration is shown by red line. Protein bound SLSA ratio is obtained from simulation of ESR spectra from (A) and (B).[25]

In another experiment, the concentration of BSA was changed while the concentration of SLSA was kept constant at 0.6 mM. ESR spectra of 0.6 mM SLSA in different concentrations of BSA solutions between 3.0–0.3 mM (Figure 3.11 (A)) and 0.24–0.02 mM (Figure 3.11 (B)) show that the bound fraction of SLSA decreases from 0.96 (at 3.0 mM BSA) to 0.08 (at 0.02 mM BSA) (Figure 3.12).

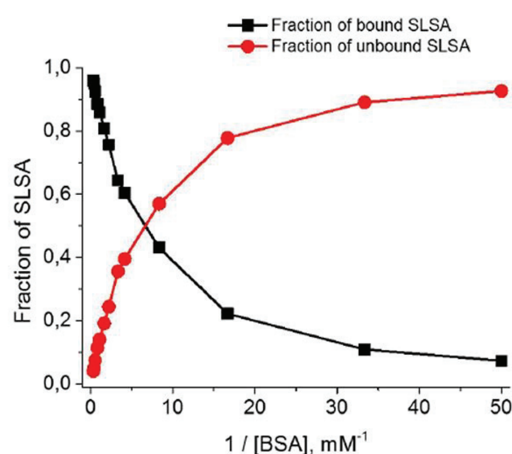

Figure 3.12. Fractions of bound (black) and unbound (red) SL-Salicylic acid in different concentrations of BSA (0.02 – 3.00 mM) obtained from simulations of their ESR spectra. The concentration of SLSA was kept constant at 0.6 mM.[25]

The ratio of bound SLSA/BSA reaches a plateau when the BSA concentration is as low as 0.125 mM. Therefore, at lower BSA concentrations (< 0.125 mM) compared to the physiological range, the maximum binding ratio was found to be ~ 2:1 for the SLSA:BSA interaction (Figure 3.11 (C)).

If we compare the bound fractions of SLSA in two BSA systems (in a physiological range and in a diluted system) at the same molar ratio of 19 : 1 (SLSA : BSA), the bound fractions are 0.40 and 0.12 for the SLSA in 0.5 mM BSA and in 0.03 mM BSA, respectively. Therefore, in the physiological range maximum ~ 7 SLSA molecules bind per BSA, and in a diluted BSA system maximum ~ 2 SLSA molecules bind per BSA even at a higher [SLSA]/[BSA] molar ratio of 30 : 1. Binding stoichiometries of small drugs to albumin obtained by fluorescence spectroscopy are also in line with our ESR results. Ni et al. reported the binding ratios of aspirin–BSA and ibuprofen–BSA as 3 : 1 and 2 : 1, respectively.[17] Also, they studied the binding stoichiometry of SA to BSA and showed that SA primarily binds to site I and may have two additional low affinity binding sites in site I and II.[13]

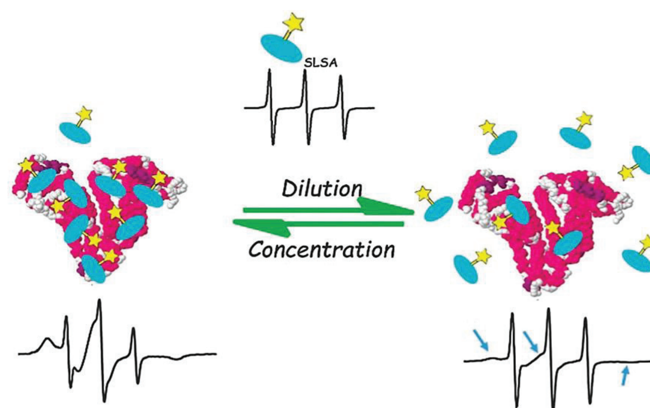

Figure 3.13. Sketch of SLSA binding to BSA at physiological concentrations (left) and at lower concentrations (< 0.125 mM) (right) of BSA as derived from the cw ESR results. spin labeled salicylic acids are shown with turquoise salicylic acid group and yellow tempo group. The ESR spectrum of free SLSA is shown at top.[25]

One should be careful when comparing the results of ESR and fluorescence experiments. Since the fluorescence measurements were done in the presence of around 0.0002 mM BSA, we can compare those results with our ESR results obtained from the diluted BSA system (0.02 mM) not the physiological range (0.5–0.7 mM).

We have schematically summarized our ESR results in Figure 3.13. A combination of SLSA–BSA dilution experiments (Figure 3.7) and stoichiometry analyses (Figure 3.9 and Figure 3.11) shows that albumin can bind to maximum 7 SLSA at the physiological range. However, albumin loses its affinity for SLSA when the concentration of albumin decreases.

3.1.5. SLSA Release Study From BSA

The SLSA release profile of BSA was also investigated at two temperatures 25 °C and 37 °C. 0.5 mM SLSA/BSA (1:1) solution placed in a D-tube dialyzer was measured by ESR spectroscopy as a function of time (Figure 3.14). At time zero the fraction of bound SLSA is about 0.8. Therefore, two types of SLSA (bound and unbound) are present in the dialyzer tube. During the dialysis, ESR line intensities coming from both bound and unbound SLSA decrease together with time (Figure 3.14 (A)). This suggests that the amount of SLSA diffused through the membrane consists of the SLSA released from BSA and a portion of the unbound SLSA.

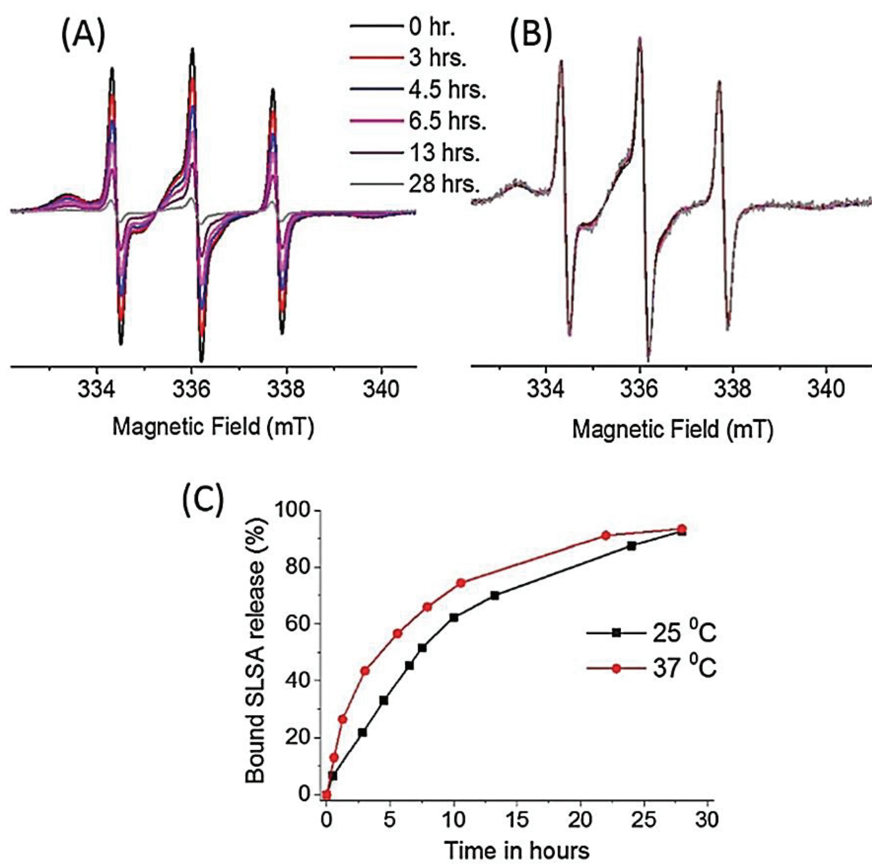

Figure 3.14. (A) Cw ESR spectra of 0.5 mM SLSA/BSA (1 : 1) solution in a dialyzer tube as function of time. (B) Normalized ESR spectra of (A) to the intensity of the high field line of the unbound SLSA. (C) The bound SLSA release profile of BSA with time at 25 °C and 37 °C.[25]

Expectedly, the relative diffusion rate of SLSA increases with temperature. At 37 °C an initial rapid release followed by a slower release was observed. Instead, a relatively steady release rate was observed at 25 °C. Nevertheless, the same amount of bound SLSA (92%) is released from BSA at the end of 28 hours both at 25 °C and 37 °C. In fact, while the total ESR intensity was gradually decreasing, the fraction of bound SLSA (0.8) did not change in the dialyzer tube (Figure 3.14 (B)).

We observe the bound and unbound SLSA signals together during the dialysis. This indicates the existence of an equilibrium between bound and unbound states.

$$\text{SLSA} + \text{BSA} \leftrightarrow \text{SLSA–BSA complex} \qquad (3.3)$$

Since the dialysis removes only the unbound drug from the dialyzer tube, the equilibrium shifts to the left and promote the SLSA release from BSA.

3.1.6. Kinetics of the Binding Process

Drug affinity for protein binding sites can be judged by K_a (association constant), K_d (dissociation constant, $1/K_a$) and ΔG (binding free energy). When the drug–albumin association constant is high, the drug releasing from albumin becomes very difficult. On the other hand, when the drug–albumin association constant is low, the drug loading becomes very weak and the drug is metabolized very quickly. Therefore, a critical range between strong and weak association is necessary for the drug transportation.

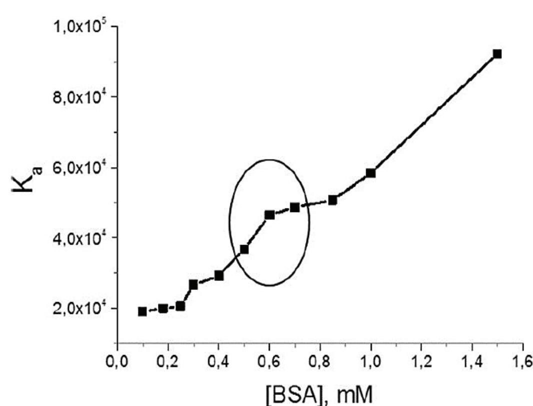

Figure 3.15. The curve of calculated K_a versus [BSA] concentration for the SLSA-BSA assembly. The circle shows the concentrations of BSA in the physiological range (0.5-0.7 mM BSA).[25]

The drug–albumin association constants at different concentrations of albumin were calculated using ESR results from Figure 3.7 (B) (Figure 3.15 and Table 3.1). The binding interaction between a SLSA and a BSA at equilibrium is described by the following:

$$K_a = \frac{[\text{SLSA-BSA complex}]}{[\text{SLSA}] \text{ x } [\text{BSA}]} \tag{3.4}$$

where K_a is the association constant, [SLSA] is the concentration of free drug, [BSA] is the concentration of free protein, and [SLSA–BSA complex] is the concentration of the drug bound to the protein at equilibrium. We calculated the concentrations of [SLSA–BSA complex] and [SLSA] from their corresponding ESR signals. [SLSA–BSA complex] corresponds to bound ESR signals and free [SLSA] corresponds to free ESR

signals. [BSA] concentration is the same as the [SLSA] concentration because of the 1 : 1 ratio of initial [SLSA] : [BSA].

Drug immobilization on the protein is an entropically unfavorable process in an aqueous environment. Differences in the solvation free energies of drug and protein from the drug–protein complex provide the binding free energy of the drug with protein. However, the formation of energetically favorable weak bindings (electrostatic interactions, hydrophobic forces, van der Waals interactions, and hydrogen bonds) between the drug and protein compensates it and leads to increased K_a values. Binding free energy (ΔG) of SLSA with BSA includes noncovalent interactions, and so it can be related to the binding affinity by the following:

$$\Delta G = -RT\ln K_a \tag{3.5}$$

where T is the experimental temperature and R is the gas constant. Table 3.1. summarized the K_a and ΔG values of SLSA–BSA complexes at different BSA concentrations (SLSA : BSA, 1 : 1).

Table 3.1. BSA concentrations, association constants (K_a) and binding free energies (ΔG) of SLSA-BSA complexes.[25]

[BSA], mM	K_a, 10^4 M^{-1}	ΔG, kJ mol^{-1}
0.10	1.91	-24.42
0.18	1.99	-24.54
0.25	2.05	-24.60
0.30	2.66	-25.35
0.40	2.93	-25.48
0.50	3.68	-26.05
0.60	4.65	-26.63
0.70	4.87	-26.74
0.85	5.06	-26.84
1.00	5.84	-27.19
1.50	9.22	-28.32

Figure 3.15 shows that at physiological concentrations the K_a values are about 3.7–4.8 x 10^4 M^{-1}. Below the physiological concentrations of albumin the K_a values decrease to about 2.0 x 10^4 M^{-1}. Above the physiological concentrations of albumin, first

the K_a values remain constant but at a high concentration for example 1.5 mM of BSA the K_a value reaches 9.0 x 10^4 M^{-1}. These different K_a values show that at physiological concentrations of albumin, SLSA is loaded by albumin fairly but not so strongly which is crucial for the drug transportation.

3.2. Analysis of Intermolecular Interactions of SL-Drugs and BSA

Drugs bind to albumin via different forces. In order to understand binding interactions SL-drugs.

3.2.1. Intermolecular Interactions of SL-Salicylic Acid And BSA

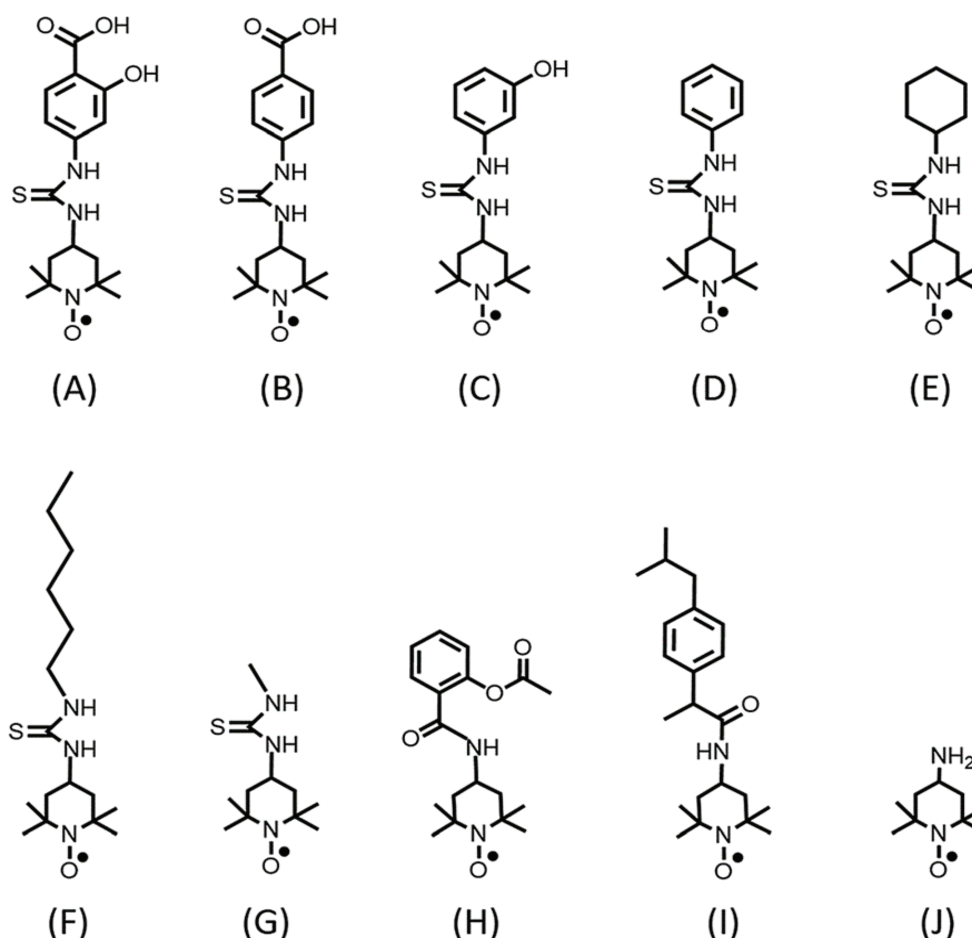

Figure 3.16. Chemical structures of spin labeled (SL) molecules: (A) SL-salicylic acid, (B) SL-benzoic acid, (C) SL-phenol, (D) SL-benzene, (E) SL-cyclohexane, (F) SL-hexane, (G) SL-methyl, (H) SL-aspirin and (I) SL-ibuprofen. Tempo based nitroxide radical: (J) 4-amino Tempo.[26]

Hydrophobic interactions, electrostatic interactions, hydrogen bonding and van der Waals interactions determine the extent of binding of drugs to serum albumin.[38] Here, we study the effect of these intermolecular interactions on the binding of salicylic acid to albumin individually by ESR spectroscopy. In previous section, we showed that SL-salicylic acid binds to albumin by 80% at physiological concentrations. Salicylic acid has a phenyl group and two functional groups, a hydroxyl group and a carboxylic acid group. At pH 7.4 salicylic acid carries a negative charge on the carboxylate group ($pK_a = 2.97$) and contains a hydrogen bond forming group (hydroxyl group). The aromatic group of salicylic acid may interact with other aromatic rings in albumin forming π-π stacking in addition to hydrophobic interactions.

Figure 3.16 shows chemical structures of spin labeled molecules used in this study. SL-salicylic acid has two functional groups (–COOH and –OH) but SL-benzoic acid and SL-phenol have only the –COOH group and the –OH group, respectively. Although SL-benzene and SL-cyclohexane do not have these functional groups, SL-benzene conserves aromaticity but SL-cyclohexane does not.

Therefore, we can control the π-π stacking effect on the formation of a drug-albumin complex by comparing the results of SL-benzene and SL-cyclohexane. The polarity of these molecules is also different. Salicylic acid with two functional groups is expected to be more polar than others. Benzene without two functional groups must be less polar than salicylic acid, benzoic acid and phenol. The polarity of cyclohexane is similar to benzene but it does not have aromaticity. In addition to them, we also synthesized SL-hexane which is more hydrophobic than the others.[39]

The uptake of spin labeled molecules by BSA is shown in Figure 3.17 (A). The ESR spectra are normalized to the intensity of the high field line of the unbound molecule which is the least affected signal by the presence of bound signals of SL-molecules. Simulations of each spectrum of 0.6 mM SL-salicylic acid, SL-benzoic acid, SL-phenol, SL-benzene and SL-cyclohexane in 0.6 mM BSA solutions showed that these molecules extensively bind to albumin with bound fractions of 0.84–0.76 (Figure 3.17 (B)). Since each molecule has a hydrophobic fragment, such as a benzene ring and a cyclohexane ring, these hydrophobic fragments are thought to cause the main effect on the formation of a strong similar molecule–albumin complex (0.84–0.76). In order to study the hydrophobic effect on protein binding, we also measured the SL-hexane/BSA complex. Most of the SL-hexane molecules are bound to BSA with a fraction of bound SL-hexane

as 0.90. Since SL-hexane is more hydrophobic than the others with its aliphatic hexyl chain, the fraction of bound SL-hexane is higher than those of other SL-molecules.

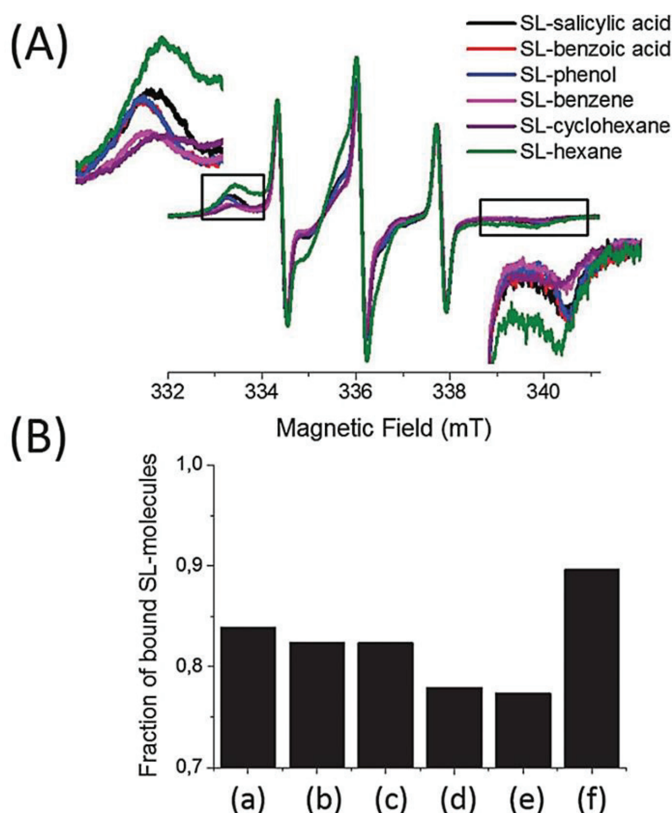

Figure 3.17. (A) Cw ESR spectra of 0.6 mM SL-molecules in BSA solutions (1 : 1), (B) fractions of bound SL-drugs obtained from simulations of spectra from (A): (a) SL-salicylic acid, (b) SL-benzoic acid, (c) SL-phenol, (d) SL-benzene, (e) SL-cyclohexane and (f) SL-hexane.[26]

Nevertheless, these molecules have different bound to unbound molecule ratios with BSA because of their structural differences. Although SL-salicylic acid, SL-benzoic acid, SL-phenol and SL-benzene all have an aromatic benzene ring, the number of functional groups on the molecules is different. SL-salicylic acid binds to BSA slightly more than both SL-benzoic acid and SL-phenol, and evidently more than SL-benzene. The fractions of bound SL-salicylic acid, SL-benzoic acid, SL-phenol, and SL-benzene are 0.84, 0.82, 0.82 and 0.77, respectively. The presence of –COOH and –OH groups on the benzene ring increases the bound ratio slightly in the order of SL-benzene $<$ SL-phenol $\approx$ SL-benzoic acid $<$ SL-salicylic acid. This indicates that the presence of a negative charge carrying group (–COOH) and hydrogen bond forming groups (–COOH and –OH) promotes albumin binding.

Another structural factor which is known to promote protein binding is π-π stacking. Comparison of SL-benzene/BSA and SL-cyclohexane/BSA spectra shows that SL-benzene and SL-cyclohexane bind to BSA very similarly. The fractions of bound SL-benzene and SL-cyclohexane are 0.77 and 0.76, respectively. Since their polarities are similar[39] and π-π stacking is only possible for SL-benzene and not for SL-cyclohexane, these results show that the presence of a π-π stacking interaction between SL-benzene and albumin does not have considerable effect on protein binding.

Furthermore, the release profiles of SL-salicylic acid and its spin labeled derivatives (SL-benzoic acid, SL-phenol and SL-benzene) from BSA were studied by ESR spectroscopy (Figure 3.18). 0.5 mM SL-molecules/BSA (1 : 1) solutions were placed in 3mL D-tube dialyzers (MWCO 6–8 kDa) and at pre-fixed times 7 μL samples were removed and measured by ESR spectroscopy. Each time both bound and unbound SL-molecules in albumin solution were detected simultaneously. The ESR line intensities of the SL-molecules coming from both the bound and free fractions decrease gradually. Therefore, the bound fractions of SL-molecules do not change with time in BSA solutions. At 37 °C the relative diffusion rates of SL-salicylic acid and its derivatives are very similar in the SL-molecules/BSA (1 : 1) solution (Figure 3.18). This indicates that the release profiles of SL-salicylic acid and its spin labeled derivatives (SL-benzoic acid, SL-phenol and SL-benzene) from BSA are comparable because of having similar binding interactions with BSA.

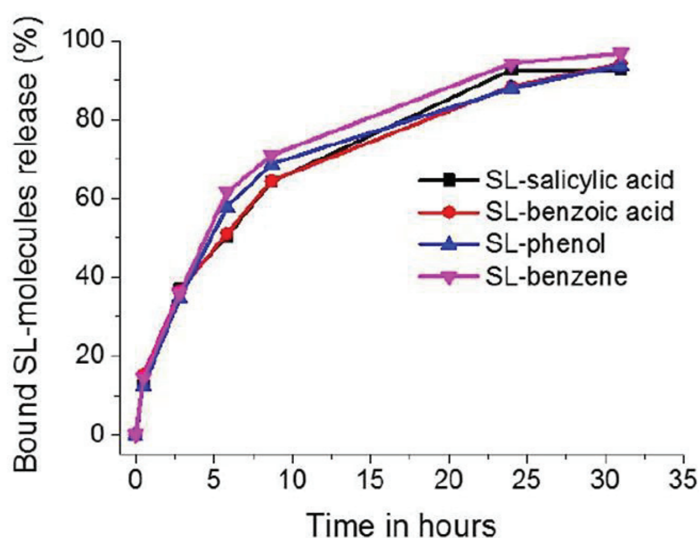

Figure 3.18. The bound SL-salicylic acid, SL-benzoic acid, SL-phenol and SL-benzene release profiles from BSA with time at 37 °C.[26]

Taking the ESR results together, we conclude that hydrophobic interaction is the main force between salicylic acid and BSA. The presence of two functional groups (–COOH and –OH) on the benzene ring has a minute but detectable effect on the formation of drug–protein complexes.

As a control experiment, SL-methyl was synthesized and a mixture of SL-methyl and BSA solution was measured by ESR spectroscopy (Figure 3.19). Since methyl has a very low hydrophobicity compared to hexane, SL-methyl did not bind to albumin and only three sharp signals coming from free SL-methyl were obtained. Moreover, Tempo-4-amino was also mixed with BSA and measured by ESR spectroscopy (Figure 3.19). Again, only three sharp signals were observed, signatures of free radicals.

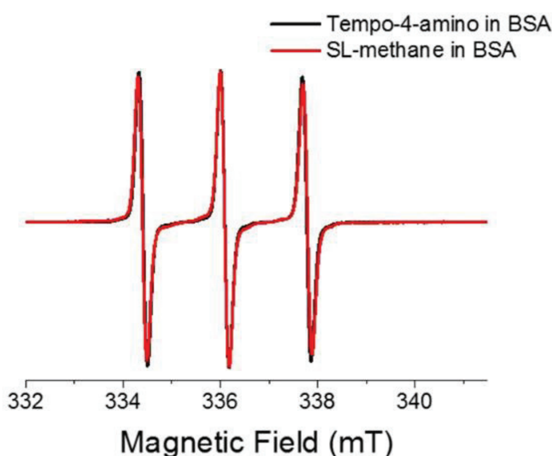

Figure 3.19. ESR spectra of 0.5 mM SL-methyl and Tempo-4-amino in 0.5 mM BSA in 0.1 M phosphate buffer solutions.[26]

3.2.2. Comparison of SL-Salicylic Acid Binding to BSA and to cBSA

Cationic albumin was prepared in order to observe the effect of the net charge of albumin on drug binding. Albumin cationization was achieved by following the procedure that has been previously described.[29] The negative net charge (−19 ± 2) on the BSA was converted into a positive net charge (+23 ± 3) after the reaction of BSA with ethylenediamine at pH 4.75. The uptake of 0.6 mM SL-salicylic acid by 0.6 mM cBSA was monitored by ESR spectroscopy (Figure 3.20 (A)). The bound fraction of SL-salicylic acid increases from 0.84 to 0.89 in BSA and in cBSA, respectively. An obvious increase in the bound fraction of SL-salicylic acid can be explained by the presence of electrostatic attraction between SL-salicylic acid and cBSA.[29, 40]

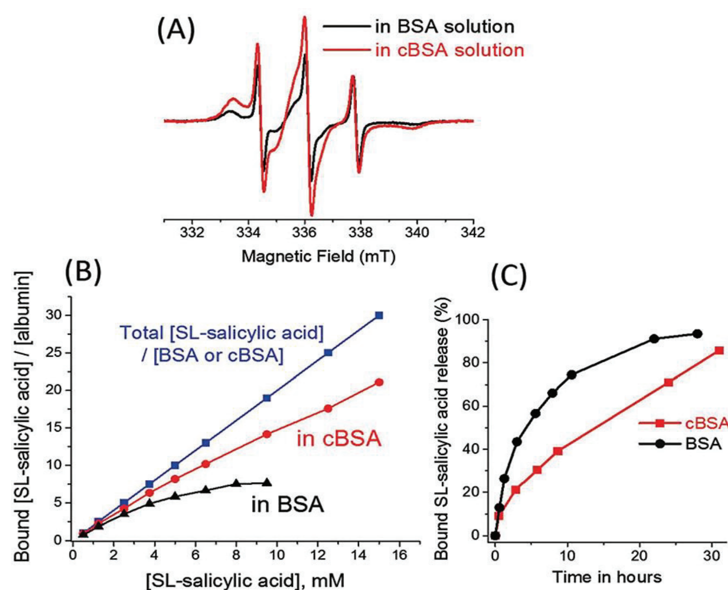

Figure 3.20. (A) Cw ESR spectra of 0.6 mM SL-salicylic acid in BSA solution (black) and in cBSA solution (red) at a drug/albumin ratio of 1. (B) The concentration ratio of bound SL-salicylic acid to BSA saturates (black) with increasing SL-salicylic acid concentration in a constant 0.5 mM BSA solution. The concentration ratio of bound SL-salicylic acid to cBSA increases (red) with increasing SL-salicylic acid concentration in a constant 0.5 mM cBSA solution. (C) The bound SL-salicylic acid release profiles from BSA and from cBSA with time at 37 °C. The data of SL-salicylic acid/BSA in (B) and (C) were taken from figure 3.9.[26]

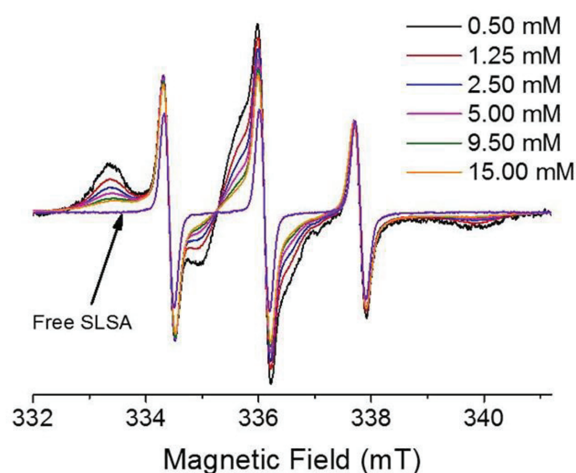

Figure 3.21. Cw ESR spectra of SL-salicylic acid in a constant 0.5 mM cBSA solution at different SL-salicylic acid/BSA ratios. The concentrations of SL-salicylic acids are 0.5, 1.25, 2.5, 5.0, 9.5 and 15 mM .The ESR spectra are normalized to the intensity of the high field line of the unbound SLSA.[26]

In a previous section, it has been shown that a maximum of 7 SL-salicylic acid molecules can bind to each BSA molecule in the physiological concentration range. In order to find out the the loading capacity of cBSA, a series of SL-salicylic acid samples with different concentrations from 0.5 mM to 15 mM were added to 0.5 mM cBSA solution (Figure 3.21). In the normalized ESR spectra, the bound fraction of SL-salicylic acid decreases with increasing SL-salicylic acid concentration (Figure 3.21). Yet, the number of bound SL-salicylic acid per cBSA increases regularly and does not reach a saturation point under the experimental conditions (up to 15 mM SL-salicylic acid) (Figure 3.20(B)). This indicates that cBSA at physiological concentrations has numerous binding sites for SL-salicylic acid. If we compare the bound fractions of SL-salicylic acid in BSA and in cBSA solutions, SL-salicylic acid forms a saturated complex with BSA at a molar ratio of 7 : 1 but each cBSA can load ~ 20 SL-salicylic acid at a [SL-salicylic acid]/[cBSA] molar ratio of 30 : 1. The –COOH and –OH functional groups collectively provide a partial negative charge on SL-salicylic acid. Therefore, SL-salicylic acid can bind to cBSA with a higher efficiency compared to its binding to anionic BSA. Repelling of the negatively charged drug by anionic BSA can be explained in terms of a heterogeneous charge distribution on the BSA molecule. The larger negative domain of the protein hinders the anionic drug binding and also prevents it electrostatically from the attractive cationic domain.[41] Furthermore, theoretical studies of polyelectrolyte (PE) binding onto net-neutral Janus nanospheres (JNSs), with two equally but oppositely charged hemispheres, support the drug binding behaviour of BSA.[42] PE–JNS adsorption at low salt concentration is carried out with PE attraction by the oppositely charged hemisphere and PE repulsion by the similarly charged hemisphere.

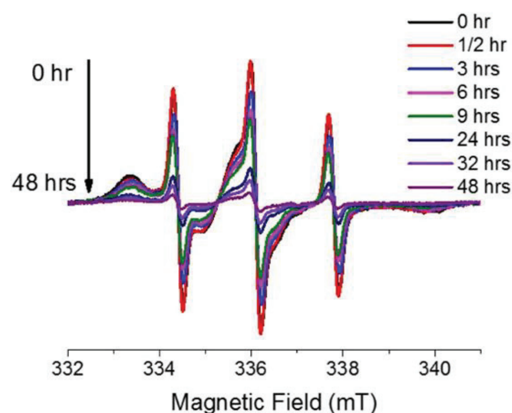

Figure 3.22. Cw ESR spectra of 0.5 mM SLSA/cBSA (1:1) solution in a dialyzer tube as a function of time.[26]

The net charge of albumin also affects the drug release. For the releasing study, 0.5 mM SL-salicylic acid/cBSA (1 : 1) and 0.5 mM SL-salicylic acid/BSA (1:1) solutions were placed in 3 mL D-tube dialyzers (MWCO 6–8 kDa) and at prefixed times 7 μL samples were removed and measured by ESR spectroscopy (Figure 3.22). At 37 °C the relative diffusion rate of SL-salicylic acid in the SL-salicylic acid/cBSA (1 : 1) solution is slower than the diffusion rate of SL-salicylic acid in the SL-salicylic acid/BSA (1:1) solution (Figure 3.20 (C)). This can be explained by the relatively strong attractive forces between cBSA and SL-salicylic acid. Attractive electrostatic forces between cationic albumin and anionic SL-salicylic acid slow down the SL-salicylic acid release. For example, only 30% of bound SL-salicylic acid is released from cBSA after 6 hours, but in the same time interval 57% of bound SL-salicylic acid is released from BSA.

3.2.3 Binding of SL-Ibuprofen, SL-Aspirin and SL-Salicylic Acid to BSA

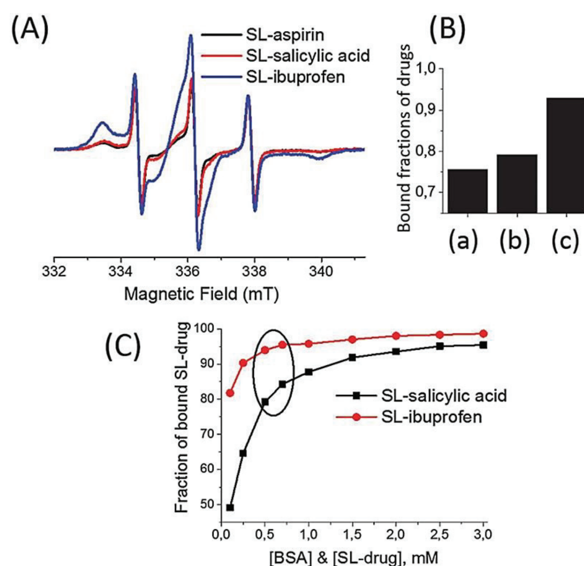

Figure 3.23. (A) Cw ESR spectra of 0.5 mM SL-drugs in 0.5 mM BSA solution: SL-aspirin (black), SL-salicylic acid (red) and SL-ibuprofen (blue). (B) Fractions of bound SL-drugs obtained from simulations of spectra from (A): (a) SL-aspirin, (b) SL-salicylic acid, and (c) SL-ibuprofen. (C) Fractions of bound SL-salicylic acid and SL-ibuprofen obtained from simulations of ESR spectra of SL-drug/BSA solutions (1:1). The concentrations of SL-drug and BSA decrease from 3.0 mM to 0.1 mM for both drugs. The circle shows the concentrations of BSA in the physiological range (0.5–0.7 mM BSA). The data for SL salicylic acid/BSA in (C) were taken from figure 3.7.[26]

In addition to salicylic acid, we also studied ibuprofen and aspirin by ESR spectroscopy. Spin labels were attached to the –COOH groups of ibuprofen and aspirin (Figure 3.16). Figure 3.23 (A) shows the comparison of the ESR spectra of 0.5 mM SL-ibuprofen, SL-aspirin and SL-salicylic acid in 0.5 mM BSA solutions. Most of the SL-ibuprofen is bound to BSA with a fraction of bound SL-ibuprofen as 0.93. But, the bound fractions of SL-salicylic acid and SL-aspirin are 0.79 and 0.76, respectively which are lower than the bound fraction of ibuprofen (Figure 3.23 (B)). The bound fractions of SL-drugs depend on the serum albumin concentration (at a drug : BSA ratio of 1). Therefore, the bound fractions of SL-salicylic acid are 0.84 and 0.79 in 0.6 mM and 0.5 mM BSA solutions, respectively.

In a previous section, we showed that the extent of SL-salicylic acid/BSA conjugation decreases sharply especially below the physiological concentrations (Figure 3.23 (C)). Therefore, we emphasized the importance of the physiological concentration of albumin for drug delivery.

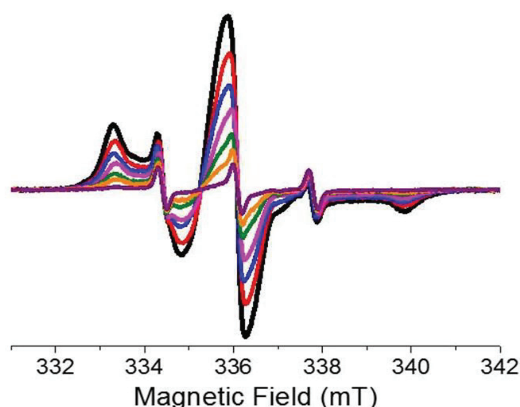

Figure 3.24. Cw ESR spectrum of SL-ibuprofen in BSA solution at a SL-ibuprofen/BSA ratio of 1. The concentrations of SL-ibuprofen and BSA from top to bottom are 3.0, 2.5, 2.0, 1.5, 1.0, 0.5 and 0.25 mM.[26]

Figure 3.24 shows the ESR spectra of SL-ibuprofen in BSA solution at a SL-ibuprofen/BSA ratio of 1. For the concentrated solution (3.0 mM of SL-ibuprofen and BSA), the bound fraction of SL-ibuprofen is 0.99. When the mixture of SL-ibuprofen/BSA solution (1 : 1) is diluted with buffer, the fraction of bound SL-ibuprofen decreases only from 0.99 to 0.82 at 3.0 mM and 0.1 mM concentrations, respectively (Figure 3.23 (C)). As a result, a significant amount of SL-ibuprofens is still bound to BSA in the diluted system. On the other hand, dilution causes a strong release of SL-salicylic

acid from BSA, the fraction of bound SL-salicylic acid decreases from 0.95 to 0.50 at 3.0 mM and 0.1 mM concentrations, respectively.

The drug–albumin association constants can also be calculated from the ESR results. Salicylic acid, aspirin and ibuprofen bind to BSA via noncovalent bonding therefore an equilibrium exists between the bound and free states of the drugs:

$$\text{SL-drug} + \text{BSA} \leftrightarrow \text{SL-drug/BSA complex} \quad (3.5)$$

The areas under the ESR signals of the bound and free drugs provide the concentrations of bound and free drugs in the system. Then, the association constant (K_a) at equilibrium can be calculated from the following equation:

$$K_a = \frac{[\text{SL-bound drug/BSA complex}]}{[\text{SL-free drug}] \text{ x } [\text{BSA}]} \quad (3.6)$$

where [SL-drug] is the concentration of the free drug, [BSA] is the concentration of free protein, and [SL-drug/BSA complex] is the concentration of the drug bound to the protein at equilibrium. The free BSA and free drug concentrations are assumed to be equal by accepting that one BSA takes up one SL-drug in the mixture of drug : albumin with a 1 : 1 molar concentration ratio.

At physiological concentrations of albumin (0.5–0.7 mM), the calculated K_a values of SL-ibuprofen are about 3.8–5.6 x 10^5 M^{-1}, while for SL-salicylic acid and SL-aspirin, the K_a values are about 3.6–4.8 x 10^4 M^{-1}, and 2.6–3.3 x 10^4 M^{-1}, respectively. ESR results show that the binding affinity of SL-ibuprofen to BSA is ~ 10 times higher than the binding affinities of SL-salicylic acid and SL-aspirin to BSA.

Ibuprofen is a more hydrophobic molecule compared to aspirin and salicylic acid. therefore it is not surprising that the more hydrophobic ibuprofen molecule could bind to BSA higher than the binding of aspirin and salicylic acid to BSA.

3.2.4. ESR Studies of Competitive Binding of Drugs with BSA

In the literature, interactions between small molecules and biomacromolecules have been extensively studied by X-ray crystallography.[43-45] Structures of several

albumin-drug complexes have also been resolved by X-ray crystallography.[46] A 3D structure of the albumin crystal revealed that the main drug binding sites in BSA site I and site II are located in the hydrophobic cavities of sub-domains IIA and IIIA, respectively.[46-47] Yang et al. showed 3D structures of both aspirin and salicylic acid bind to HSA at site I.[36] The phenyl group of salicylic acid has mostly hydrophobic interactions with the surrounding aminoacids in site I. Ghuman et al.[46] crystallized an ibuprofen–HSA complex and showed the binding sites of ibuprofen as mainly site II and also to a small extent site I.

Furthermore, fluorescence studies showed that salicylic acid primarily binds to site I as well as to a smaller extent to site II of BSA.[13] Site II is the main binding site for ibuprofen and aspirin.[17]Also, aspirin binds to BSA to a small extent in site I.[17] In addition to these drug high binding sites of BSA, several low affinity binding sites are also available in BSA.[48] Drugs may also compete for these low affinity binding sites in BSA.

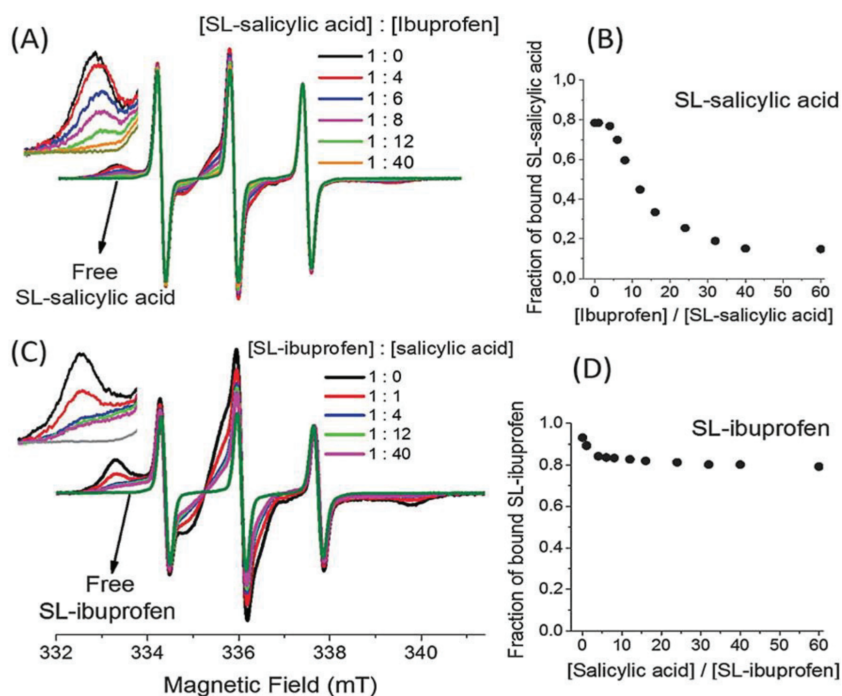

Figure 3.25. (A) ESR spectra of 0.5 mM SL-salicylic acid in 0.5 mM BSA solution in the presence of ibuprofen with different concentrations. (B) The bound fractions of SL-salicylic acids after the addition of ibuprofen obtained from the simulated spectra in (A). (C) ESR spectra of 0.5 mM SL-ibuprofen in 0.5 mM BSA solution in the presence of salicylic acid with different concentrations. (D) The bound fractions of SL-ibuprofen after the addition of salicylic acid obtained from the simulated spectra in (C).[26]

Figure 3.25 (A) shows the ESR spectra of the SL-salicylic acid/BSA complex before and after the addition of ibuprofen with different concentrations. At 0.5 mM SL - salicylic acid/BSA (1:1), the bound fraction of SL-salicylic acid is 0.79. After the addition of ibuprofen, first the bound fraction of SL-salicylic acid does not change (up to 4:1, ibuprofen : SL-salicylic acid) but then it decreases sharply to 0.14 (Figure 3.25 (B)). This shows that ibuprofen first binds to empty sites and then additional ibuprofens displace the bound SL-salicylic acid from BSA. Since the association constant of ibuprofen is higher than that of salicylic acid, ibuprofen can easily replace the salicylic acid. Combining the knowledge from the literature and our results, one can speculate that SL-salicylic acid occupies mainly site I and to a small extent site II. Ibuprofen primarily binds to the empty side of site II and then replaces SL-salicylic acid from site II and also most of the SL-salicylic acid from site I.[13, 17, 46] In contrast, we monitored the state of SL-ibuprofen in a solution of BSA in the presence of different amounts of salicylic acid (Figure 3.25 (C)). At 0.5 mM SL-ibuprofen/BSA (1:1), the bound fraction of SL-ibuprofen is 0.93. After the addition of 0.5 mM and 2.0 mM salicylic acid, an obvious decrease in the bound fraction of SL-ibuprofen from 0.93 to 0.89 and to 0.84, respectively, is observed.

However, the bound fraction of SL-ibuprofen does not change excessively after the addition of salicylic acid; e.g., the bound fraction of SL-ibuprofen reduces only to 0.80 in the presence of 30 mM salicylic acid (Figure 3.25 (D)). This result correlates with the preceding result; the bound fraction of SL-ibuprofen only reduces from 0.93 to 0.80 because salicylic acid with a lower association constant cannot replace ibuprofen easily. Salicylic acid displaces only a part of bound ibuprofens from the very limited sites which have low K_a values for ibuprofen and high K_a values for salicylic acid, probably site I.[13]

We can also find out the percentage of binding sites shared by salicylic acid and ibuprofen. Since additional ibuprofen (30 mM) replaces 83% of bound salicylic acid (0.5 mM), the remaining 17% of bound salicylic acid must either have a higher association constant with BSA or have different binding sites with respect to ibuprofen. To solve the remaining 17% of the bound salicylic acid situation, we used the data obtained from SL-ibuprofen/salicylic acid measurements. The addition of 30 mM salicylic acid to a 0.5 mM SL-ibuprofen/albumin (1:1) solution replaces only 14% of bound SL-ibuprofen. It means 14% of SL-ibuprofen initially occupies the places in which salicylic acid has a higher association constant compared to that of ibuprofen. Therefore, 97% of all sites of salicylic acid and ibuprofen are shared (83% + 14%).

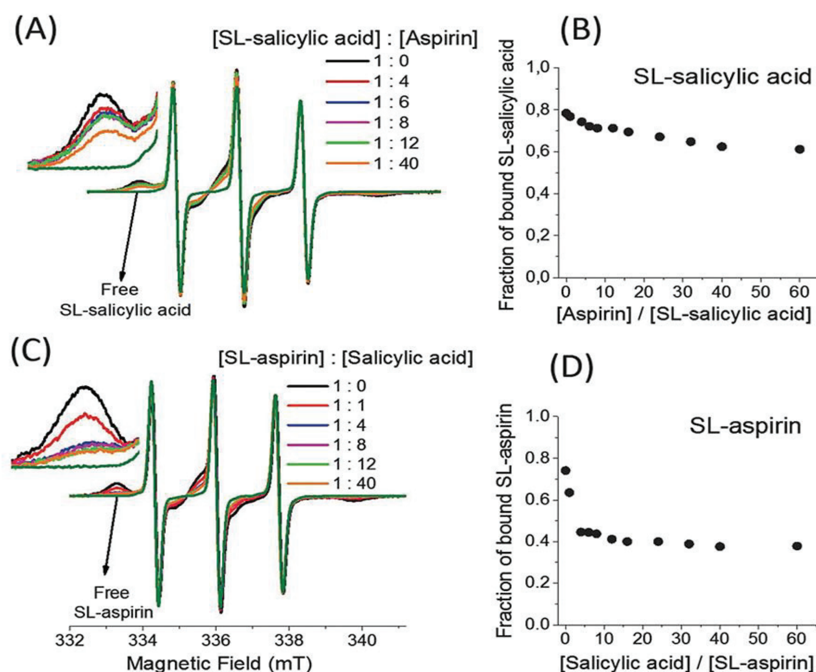

Figure 3.26. (A) ESR spectra of 0.5 mM SL-salicylic acid in 0.5 mM BSA solution in the presence of aspirin with different concentrations. (B) The bound fractions of SL-salicylic acids after the addition of aspirin obtained from the simulated spectra in (A). (C) ESR spectra of 0.5 mM SL-aspirin in 0.5 mM BSA solution in the presence of salicylic acid with different concentrations. (D) The bound fractions of SL-aspirin after the addition of salicylic acid obtained from the simulated spectra in (C).[26]

We also investigate the competitive interaction of salicylic acid and aspirin. Figure 3.26 shows the ESR spectra of 0.5 mM SL-salicylic acid/aspirin (Figure 3.26 (A)) and also SL-aspirin/salicylic acid (Figure 3.26 (C)) mixtures in 0.5 mM BSA solutions. First, in the SL-salicylic acid/aspirin mixtures, aspirin concentration increases from 0.5 mM to 30 mM. The fraction of bound SL-salicylic acid decreases gradually from 0.79 to 0.61 by increasing the ratio of aspirin/SL-salicylic acid from 0 to 60 (Figure 3.26 (B)).

Since the calculated association constant of SL-salicylic acid is slightly higher than that of SL-aspirin, it is expected that aspirin would not replace the SL-salicylic acid completely in BSA. The small amount of replacement of SL-salicylic acid by aspirin can be explained by the weak binding ability of SL-salicylic acid at site II and the higher binding ability of aspirin at site II.[13, 17] The remaining high proportion of SL-salicylic acid locates probably at site I.[17] Second, in the SL-aspirin/salicylic acid mixtures, the addition of salicylic acid rapidly reduces the bound fraction of 0.5 mM SL-aspirin from 0.76 to

0.44 after the addition of 2 mM salicylic acid. It means that the added salicylic acid directly replaces some SL-aspirin but additional salicylic acid (up to 30 mM) only reduces the bound fraction of SL-aspirin to 0.38 (Figure 3.26 (D)). This correlates with the above results; salicylic acid replaces SL-aspirin easily at site I but some of the SL-aspirin located at site II is still bound.[13, 17]

The percentage of binding sites shared by salicylic acid and aspirin can be calculated from the above results. Since additional aspirin (30 mM) replaces only 23% of the bound salicylic acid (0.5 mM), the remaining 77% of the bound salicylic acid must either have a higher association constant with BSA or have different binding sites with respect to aspirin. The addition of 30 mM salicylic acid to 0.5 mM SL-aspirin/albumin (1:1) solution replaces 50% of the bound SL-aspirin. It means 50% of the bound SL-aspirin initially occupies the places where salicylic acid has a higher association constant compared to that of aspirin. Therefore, 73% of all sites of salicylic acid and aspirin are shared (23% + 50%).

The extent of drug transportation is important to determine the dosage of drugs. In the body, drugs can be transported either as free floating molecules or as protein bound molecules. The concentration of free drugs is used to determine the effect of a drug rather than the protein bound concentration. Instead, a protein bound drug can escape from fast metabolism, which is crucial for sustained drug delivery applications. Our ESR results suggested that when two typical non-steroidal anti-inflammatory drugs, ibuprofen and salicylic acid, are co-administered, mostly ibuprofen binds to albumin and salicylic acid floats in the blood. Therefore, salicylic acid is expected to affect faster or/and be metabolized faster than ibuprofen.

CHAPTER 4

CONCLUSIONS

In this study, we presented the application of spin labeling technique and ESR spectroscopy in albumin binding and release studies. When we synthesis the drugs labeled with the stable nitroxide based tempo radicals, we can monitor the diamagnetic drug with ESR spectroscopy. Each spin labeled drug has different ESR signals. The areas under these signals give the concentration of bound and unbound drugs separately.

In our work, we first examined the intermolecular interactions between spin labeled salicylic acid and bovine serum albumin. The results of ESR spectroscopy, which we supported with fluorescence spectroscopy results, showed that as the albumin concentration increases, the bound fraction of SLSA increases. In addition, SLSA affinity of albumin reaches the saturation point just above the physiological concentration range (0.5 -0.7 mM) of albumin, while the bound SLSA fraction in the BSA decreases rapidly just below the physiological concentration range. Depending on this results, we can say that any reduction in albumin concentration may lead to a significant increase in the amount of unbound drug, leading to toxicity.

Another finding is that the amount of binding of SLSA to BSA varies depending on the BSA concentration. For example, in a 0.5 mM BSA solution (within the physiological concentration range), approximately 7 SLSA molecules can be bound to a BSA molecule, but when we reduce the concentration of the BSA solution (<0.125mM), the number of SLSAs bound to a BSA molecule drops to about 2 molecules. In other words, the loading capacity of BSA decreases with decreasing BSA concentration.

The ESR data in the release experiments showed that there was a kind of equilibrium between the bound and unbound SLSA signals. While the intensity of the ESR signals of the bound and unbound SLSA is reduced during the experiment, the concentration ratio of the bound and unbound SLSAs remains constant. That is, the amount of SLSA bound to BSA remains constant as long as the concentration of the BSA does not change. Therefore, SLSA is reversibly bound to the BSA.

In later stages of our experiments we synthesized SLSA derivatives. Our aim was to observe the effects of functional groups and aromaticity on the binding and release of SL-drugs to BSA with differences in ESR signals.The experimental results showed that the most effective interaction between SL-molecules and BSA was hydrophobic interactions. The bound fractions of the spin-labeled molecules are very close to each other (0.76-0.84). However, the effects of structural change on protein variation can be distinguished in ESR spectroscopy.

BSA is known to have anionic charge density. Therefore, the importance of electrostatic interactions by synthesizing cationic BSA (cBSA) was determined. The negatively charged SL-salicylic acid binds more strongly to the cBSA than to the BSA. The SL-salicylic acid loading capacity of cBSA is greater than that of the BSA.

Finally, in order to examine the competitive behavior of drugs, we have simultaneously added SL-drugs to BSA. We analyzed competitive binding behaviors with ESR spectroscopy as an alternative technique. The relation constants of spin labeled drugs with BSA (0.5-0.7 mM) were found to be like SL-Aspirin < SL-salicylic acid < SL-ibuprofen. SL-ibuprofen has ten times higher binding affinity to BSA than SL-salicylic acid. Therefore, when SL-ibuprofen added to the solution, it displaced 82% of the bound SL-salicylic acid. As to the added SL-salicylic acid can occupy about 14% of the bound SL-ibuprofen. All SL-salicylic acid and SL-ibuprofen share 96% of the binding sites of BSA.On the other hand, the relationship constants of SL-aspirin and SL-salicylic acids with BSA are very close to each other. Therefore, the added salicylic acids displaced about 50% of the bound SL-aspirin.The added SL-aspirin occupies only about 23% of the bound SL-salicylic acid. This indicates that SL-salicylic acid and SL-aspirin share 73% of all binding sites of BSA. Therefore, combined use of cw ESR spectroscopy and spin labelled technique have a potential to reveal intermolecular interactions of protein complexes and furthermore it can enlighten us about competative drug binding.

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