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Biomolecular interactions: essential instrumentation methods

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1. ABSTRACT

The main goal of this review is to outline the basic principles and applications of the broad range of modern biophysical technical methods used to study the different aspects of protein–ligand interactions by discussing such aspects as newer systems, unusual approaches and highly used techniques.

2. INTRODUCTION

Molecular self-assembly is a powerful approach for creating novel supramolecular arquitectures. It is mediated by weak non-covalent bonds-notably hydrogen bonds. Although these bonds are relatively insignificant in isolation, when combined together as a whole, they govern the structural conformation of all biological macromolecules (1). Perhaps, one of the most important self-assembly processes are protein-protein and protein-ligand interactions (2). These kinds of interactions are ubiquitous in the natural world. The study of interactions between proteins and ligands is essential for understanding biological systems.

In contrast to protein interfaces, ligand-binding pockets are smaller, typically covering several hundred $Å^2$. Although very large ligands may be located on a more planar surface, to increase interaction strength, the majority of pockets are concave in shape so that they can firmly grasp or partially envelop their cognate ligands; hence, the name pocket. A very recent comparison between experimental and artificial quasi-spherical structures of single-domain proteins has proposed that packing by hydrogen-bonded secondary structures is crucial for the formation of protein interfaces and ligand-binding pockets (3). The key elements in protein-ligand interactions are complementarily (i.e. electrical charge) and structural compatibility (hydrophobicity, steric). Like hands and gloves, both the size and the correct orientation, i.e. chirality, are important in order to have a complementary and compatible fitting (4). Essential for high biological activity are a good geometric fit, the Emil Fischer "lock-and-key" principle, and a high degree of complementarity of hydrophobic and polar parts of both entities, namely, the binding site of the protein and the ligand. However, this

short characterization is only part of the story: ligand and binding site flexibility, distortion energies, desolvation effects, entropy, molecular electrostatic field complementarity, and other effects are often equally important. One area where molecular recognition has made a relatively limited impact so far is in toxicology. The observed toxicity is likely to be governed by specific protein ligand interactions, but the ability to predict potential liabilities remains low (5). However, these subjects must address the new challenges evoked by recent discoveries that should be overcome. Recent years have seen a significant change in this situation. Thus, the interpretations of data in the extensive literature on the unfolding of proteins in aqueous solution by amphiphilic molecules (surfactants) have been recently analyzed (6 , 7). During the 2000s, enhanced methods were revised for improving assumptions associated with incomplete equilibrium constants, contradict model-free interpretation of the data and misuse of the van't Hoff relation are really common. Consequently, a new analysis of unfolding via the Gibbs–Duhem equation with the correct Phase Rule constraints it was proposed (8). In addition, the notion that proteins are simply three-dimensional, compact objects has been questioned: while proteins need to keep their specific native fold structure thermally stable, the native fold displays the ability to perform large amplitude conformational changes that allow proper function. Indeed, the possibility that proteins may be better characterized by fractal geometry rather than as a compact three-dimensional object has appeared in a variety of measurements. This appears to be the case for protein surfaces, for which a fractal dimension of 2.1 to 2.4 is widely accepted. Nevertheless, the fractal dimension of the protein itself based on several estimates has been argued to lie near 2.5 (9). In this sense, it appears that when viewed as fractals, proteins exhibit an unexpected universal behavior (10) than can be parameterized by the equation:

$$\frac{2}{d_s}+\frac{1}{d_f}=1+\frac{b}{ln(N)}$$

Where N is the number of amino acids along the protein backbone; d_s the spectral dimension; d_f, the fractal dimension and the parameter b is a molecular fit parameter weakly depends on temperature and interactions (11). Also the hydrophobicity and polarizability distributions in protein interior follow fractal scaling. All β-proteins (thermophilic, mesophilic alike) are found to have maximum amount of unused hydrophobicity, while all α-proteins have been found to have minimum polarizability. It is also proved that origin of α-helices are possibly not hydrophobic but electrostatic; whereas β-sheets are predominantly hydrophobic in nature (12).

Early in this century, we are witnessing an incredible development of the life sciences. This has aroused and attracted the interest of scientists from different fields. This approach must be based on knowledge of approaches, tools and techniques of biophysics. We can now expect an even more dramatic rate of progress, particularly in throughput of protein nano-arrays, lipoparticles, development of novel pharmaceuticals, control of colloidal stability in foods and pharmaceuticals, construction of nanodevices, etc (13). Consequently, the aim of this review is to evaluate and offer a general overview about the current knowledge on protein surfactant interactions supplied by different experimental techniques. In the compilation of this review an attempt has been made to give appropriate recognition to the current interest in normal and emergency applications of protein–surfactant systems, by discussing such aspects as newer systems, unusual approaches and highly used techniques including information about the physical principles and effectiveness of selected techniques. For this purpose we have focused on the experimental point of view. The reviewer is organized by techniques with the same format: beginning with a small introduction to the experimental set and working principles and a discussion of the specific approaches, applications and papers that are mainly based on it. It is almost impossible to cover all current techniques; consequently we have chosen those more popular and accessible in most laboratories all over the world. Further, because of space limitations and the recent upsurge of interest in biotechnology and biomedical areas the cited articles represent only a small fraction of the colossal growth of the total number published in recent years.

3. EXPERIMENTAL TECHNIQUES: FUNDAMENTALS AND APPLICATIONS

3.1. Zeta Potential

When an electric field is applied across an electrolyte, charged particles suspended in the electrolyte are attracted towards the electrode of opposite charge. Viscous forces tend to oppose this movement. When equilibrium is reached between these two opposing forces, the particles move with constant velocity, which is commonly referred to as its electrophoretic mobility, η. From electrophoretic mobility zeta potential can be obtained by using the Henry equation:

$$\xi=\frac{3\mu_E\eta}{2\varepsilon_0\varepsilon_r f(\kappa a)}$$

where a is the particle radius, κ the Debye length, ε_0 the permittivity of vacuum, ε_r the relative permittivity and η the viscosity of the solvent. The function $f(\kappa a)$ depends on the particle shape, size and media. Two values are generally used as approximations for $f(\kappa a)$: 1.5 for particles in polar media (maximum value) and 1 for particles in non-polar media (minimum value) (14, 15).

ζ-potential measurements have been used in studying the interaction of a range of penicillins, nafcillin, cloxacillin, dicloxacillin, and flucloxacillin with human serum albumin (HSA) in aqueous solution at pH 7.4 at 25 °C(16). The interest in the interactions of penicillins with HSA arises from reports of allergic reactions in some subjects to penicillin and other β-lactam antibiotics and to evidence of haptenation of penicillin by conjugation with

Table 1. Number of Adsorption Sites, N1, and Standard Gibbs Energy of Adsorption, , of some surfactants onto proteins

System		ΔG^0_{ads} (kJ mol^{-1})	*Ref.*
Cloxacillin/Human serum albumin	8.34×10^{15}	-7.7	(104)
Dicloxacillin/Human serum albumin	2.37×10^{16}	-10.2	(104)
Sodium perfluorooctanoate/Lysozyme	1.90×10^{16}	-15.1	(105)
Sodium octanoate/Hemoglobin	4.4×10^{14}	-12.6	(105)
Sodium octanoate/Catalase	9.5×10^{12}	-18.2	(105)
Lithium perfluorooctanoate/Myoglobin	1.9×10^{16}	-25.3	(106)
Sodium dodecanoate/Ovalbumin	7.0×10^{15}	-20.9	(106)

serum proteins, resulting in the formation of IgE antibodies. The interaction of penicillins with HSA arises both from the hydrophobic nature of the drugs and the presence of hydrophobic cavities in the protein structure. The ζ-potentials of HSA became more negative as the negatively charged penicillins bind to it. The variations of ζ-potential with drug concentration have been interpreted in terms of a modification of the theory of Ottewill and Watanabe (OW) to take into account the cooperativity of drug binding in terms of the Hill equation. The Hill coefficients for the drugs were all greater than unity and diagnostic of positive cooperativity on binding. The plots and the Gibbs energies per drug molecule bound ($\Delta G_{\bar{\upsilon}}$) as a function of the number of drug molecules bound per protein molecule are closer to the experimental values from equilibrium dialysis experiments than when the Langmuir isotherm is used. The modified OW theory was consistent with the clustering of drug molecules on the polypeptide chain of the protein and a description of the resulting complexes in which the protein is covered in micelle-like aggregates.

Another way of obtaining information between surfactants and proteins is through the variations of zeta potential of formed complexes. The changes of the ζ-potential as a function of surfactant concentration is a consequence of adsorption due to the hydrophobic effect, since the protein and the drug ion have the same sign charge. Consequently, the following equation can be used to calculate the number of adsorption sites N_1:

$$\left(\frac{d\zeta}{d\log c}\right)=\frac{4.606\,k_BT}{ze}\left(\frac{sinh(ze\zeta_1/2k_BT)-sinh(ze\zeta_2/2k_BT)}{\cosh(ze\zeta_2/2k_BT)}\right)\times$$

$$\times\left(\frac{\sqrt{8n_0\varepsilon k_BT}\left[sinh(ze\zeta_1/2k_BT)-sinh(ze\zeta_2/2k_BT)\right]}{ze\,N_1}-1\right)$$

ζ_1 and ζ_2 are selected zeta potentials on the line, c the concentration and n_0 the ionic concentration. The adsorption constant k_2 can be calculated from the equation:

$$\frac{1}{c}=k_2\left(\frac{ze\,N_1}{\sqrt{8n_0\varepsilon k_BT}\left[sinh(ze\zeta_1/2k_BT)-sinh(ze\zeta_2/2k_BT)\right]}-1\right)$$

Here, c is chosen as the concentration at the ζ-potential midpoint between ζ_1 and ζ_2. The standard free energy of adsorption, ΔG^0_{ads}, can be obtained from the equation

$$k_2=\exp(-\Delta G^0_{ads}/k_BT)$$

In all studied systems the standard Gibbs energies of adsorption evaluated from the above equation have shown that Gibbs energies are large and negative at low values of drug concentration where binding to the high energy sites takes place, and become less negative as more drug molecules bind suggesting a saturation process, see Figure 1. As an example, calculated values of the number of adsorption sites and Gibbs energies of different surfactant onto proteins can be consulted in Table 1.

3.2. Equilibrium dialysis

Equilibrium dialysis is based on differences in molecular size. It is mainly recommended for surfactants that strongly absorbs light at a given wavelength. In dialysis, two compartments are separated by a semipermeable membrane being (ideally) perfectly permeable to one interactant, say, the ligand, and perfectly impermeable to the substrate (protein). After equilibrium is achieved, the two compartments are analyzed for their total concentration of ligand. Since the activity of free (unbound) ligand is identical in the two compartments at equilibrium, the analytical data yield the concentration of free ligand and, by difference, the concentration of bound ligand in the compartment containing the substrate. Aliquots of protein in dialysis bags are equilibrated with surfactant solution in a buffer over a wide range of concentrations in screw cap sample tubes held in a thermostated bath for a period of time sufficiently large to reach the equilibrium. The concentrations of the free surfactant are measured with reference to their extinction coefficients (17). The Gibbs energies of binding per mole of surfactant ($\Delta G_{\bar{v}}$) are calculated from the Wyman binding potential (Π) derived from the area under the binding isotherms according to the equation.

$$\Pi=RT\int_0^{\bar{\upsilon}}\bar{\upsilon}\,d\ln[S]$$

where R is the gas constant, T the absolute temperature, $\bar{\upsilon}$ the number of surfactant molecules bound per protein molecule and [S] the free surfactant concentration. The binding potential is related to the apparent binding constant, K_{app}, as follows

$$\Pi=RT\ln\left(1+K_{app}[S]^{\bar{\upsilon}}\right)$$

The Gibbs energy of binding is calculated from

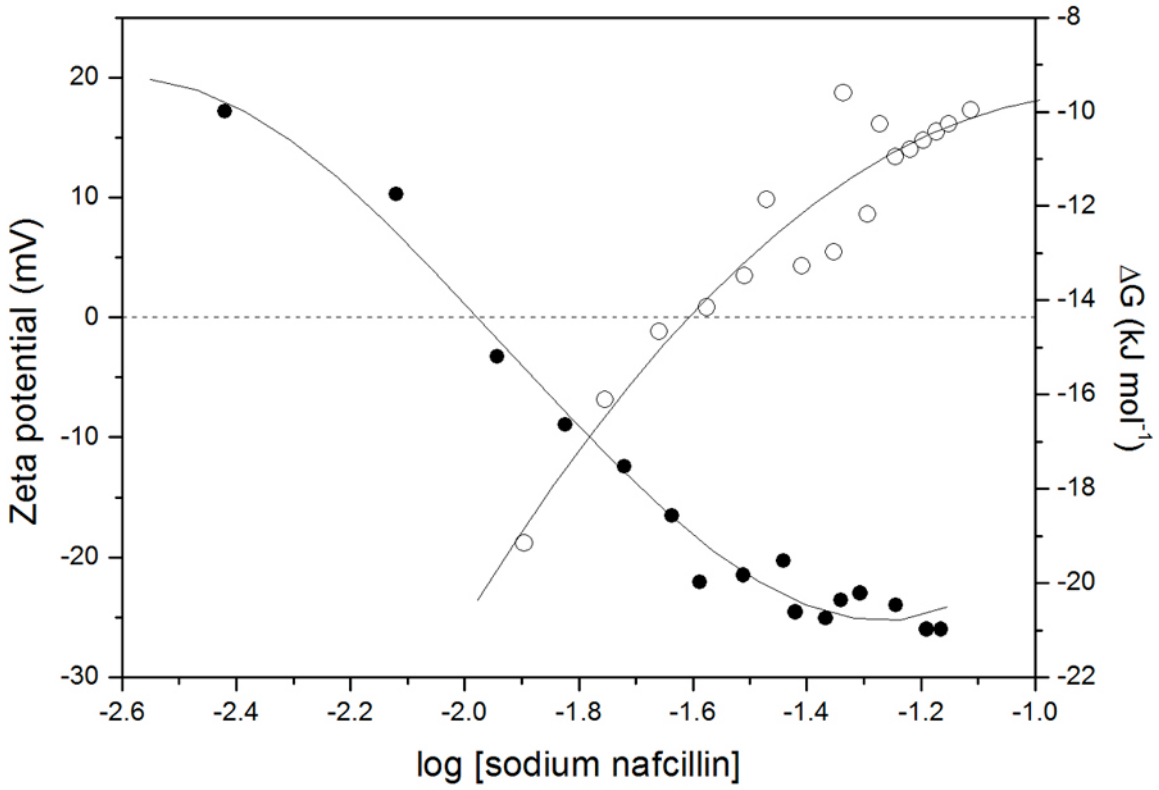

Figure 1. Zeta-Potential (●) and Gibbs energies of the adsorption (○) of bovine catalase (0.125% w/v) in glycine buffer as a function of logarithm of sodium nafcillin concentration.

$$\Delta G_{\bar{\upsilon}} = -\frac{RT}{\bar{\upsilon}} \ln K_{app}$$

As an illustrative example, the adsorption of nafcillin on catalase at different pHs can be useful (17). Obtained isotherms at pH 3.2 reveals an initially binding of the anionic nafcillin to cationic residues, followed by hydrophobic binding at the drug concentration approaching the critical micellar concentration (cmc) of the drug. The isotherm passes through maxima. Such behavior has been attributed to the activity of long-chain ions going through a maximum in the vicinity of the cmc. At pH 7.4 and 10.0, specific binding to cationic sites is lost, and only hydrophobic binding occurs. The number of drug molecules per amino acid residue was 0.37, 5.4, and 4.64 for pH 3.2, 7.4, and 10.0, respectively.

The complexation of nafcillin, cloxacillin, dicloxacillin, and flucloxacillin, with human serum albumin (HSA) in aqueous solution, pH 7.4, has been also analyzed by equilibrium dialysis (18). In this case, the binding process is largely non- specific. The binding isotherms for the drugs range up to $\bar{\upsilon}$ values of 3000 for nafcillin and cloxacillin, 1200 for dicloxacillin, and 2000 for flucloxacillin. Human serum albumin consists of 585 amino acids18 so binding of 3000 drug molecules corresponds to approximately 5 drug molecules per amino acid residue; this suggests clustering of drug molecules along the polypeptide chain. Such a cluster size corresponds closely to the aggregation numbers of the drug micelles, which at an ionic strength of 0.1 M were found to be 5 (cloxacillin), 5 (dicloxacillin), and 4 (flucloxacillin). However, these data do not allow to draw any conclusions about the uniformity of binding along the polypeptide chain, although it is likely that binding is greater (and cluster size larger) around hydrophobic aminoacid residues and in hydrophobic cavities in the protein and less around hydrophilic residues (19).

3.3. Difference UV-vis spectroscopy

Ultraviolet/visible (UV/vis) absorption spectroscopy is a powerful tool and nondestructive technique that requires only small amount of material for analysis. It measures the interaction of the electromagnetic radiation with molecules. Light in the near-ultraviolet (UV) and visible (20) range of the electromagnetic spectrum has an energy of about 150-400 kJ mol^{-1}. Prosthetic groups in proteins frequently have strong electronic absorbance bands that depend on the oxidation, ligation, and conformation states of the chromophores. They are also sensitive to conformational changes of the polypeptide chain into which they are embedded. Steady-state absorption spectroscopy provides information on ligand binding

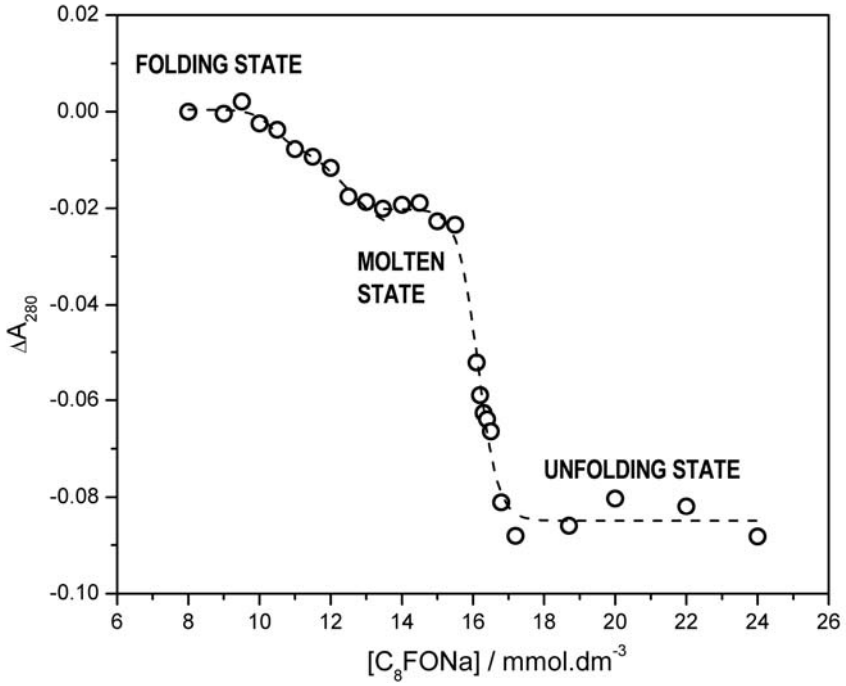

Figure 2. Absorbance variation ($\Delta A_{280} = A_{280} - A^{N}_{280}$, where A^{N}_{280} is the absorbance of protein native state) of Human Serum Albumin 280 nm as a function of sodium perfluorooctanoate concentration. The HSA concentration was 0.0188 mM.

equilibria, from which the Gibbs free energy differences between the ligated and unligated states can be computed. Time-resolved absorption spectroscopy allows one to detect short-lived intermediate states that may not get populated significantly under equilibrium conditions, but may nevertheless be of crucial importance for biological function (21). This technique involves measuring the difference in the extinction between two protein solutions of identical protein concentration, but to one solution is added a known amount of a ligand whose effect on protein conformation is to be investigated. The difference spectrum is measured mainly in the ultraviolet region because the spectra of proteins is due to the chromophores in only the three amino acid residues with aromatic nuclei, phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) (22). The extinction of these residues depends slightly on their environment which is often different in the native state of a protein than in a denatured state. Hence if a substance brings about a conformation change the extinction of the protein solution at some wavelengths will differ from a solution of the same concentration of native protein. The development of a difference spectrum is very positive evidence for a conformation change induced by an added ligand.

In folded native proteins, the aromatic residues that are buried in the hydrophobic core of the molecule also show a small red shift in their absorbance, which is revealed when they become exposed to the aqueous solvent upon unfolding. The thermodynamic stability of a protein is a fundamental parameter that describes the energetic property of the protein structure. The experimental determination of thermodynamic stabilities requires the evaluation of conformational transitions induced by temperature or by chemical denaturants (such as urea or Guanidine Hydrochloride, GdnCl) (23). Spectroscopic probes sensitive to protein conformations are frequently employed for this purpose. Though less popular than circular dichroism (CD) and fluorescence, UV-vis absorption is also known to be sensitive to the conformational changes in proteins (24). Protein unfolding transitions can thus be measured by following the absorbance changes of Trp or Tyr at 287-292 nm as a function of temperature or denaturant concentration, see Figure 2.

UV-vis absorption spectroscopy is a powerful technique by which ligand binding equilibria can be studied. The technique also offers time resolution over the entire range of time scales relevant for biomolecular dynamic. Steady-state absorption spectroscopy provides information on ligand binding equilibria; the energy barriers those have to be surmounted in the reaction, that is Gibbs free energy differences between the ligated and unligated states, can also be computed (21). Time-resolved absorption spectroscopy allows one to detect short-lived intermediate states that may not get populated significantly under equilibrium conditions, but may nevertheless be of crucial importance for biological function. A crucial prerequisite is that a suitable chromophore exits that is sensitive to ligation changes (25).

UV difference spectroscopy has been also utilized to study the interaction of the drugs with proteins. Addition

of propranolol (a widely used betablocker) results in the development of an absorption band in the region of 410 nm that is positive with respect to native haemoglobin, arising from the extended π-electron system of the porphyrin ring. For the HSA/propranolol systems the main peaks were observed at 410, 413, and 417 nm for pHs 2.5, 4.9, and 8.0, respectively. The UV absorption of human serum albumin (HSA) increased smoothly with increase of concentration of added propranolol hydrochloride at all pHs as a consequence of adsorption of the drug ions. In contrast, an abrupt change in these properties was observed for the human haemoglobin (HH)/propranolol system at a concentration of approximately 0.05 mol kg^{-1} at pH 2.5. This change has been attributed to a tetramer to dimer transition arising from the increase of concentration of drug which functions as an electrolyte (26).

3.4. Fluorescence Spectroscopy

Fluorescence is the process by which electronically excited molecules decay to the ground state via the emission of a photon without any change in spin multiplicity. Emission is detected by spectrofluorimeters and occurs at longer wavelengths than the corresponding absorbance band. The quantum yield of fluorescence emission is defined as the ratio of photons emitted through fluorescence to the number of photons absorbed. Its maximum value is 1, although other processes contribute to deactivation of excited molecules and lower values are observed. In proteins the principal fluorophore is the indole side chain of tryptophan with a contribution that exceeds the other aromatic residues, and dominates the fluorescence of proteins between 300 and 400 nm. Folded states of proteins show different fluorescence spectra to unfolded states due to the influence of local environment and solvent. In the folded state tryptophan emission shows a blue shift towards 330 nm from an unfolded value of 350 nm. Changes in intensity and wavelength reflect the influence of local environment on emission. Other aromatics side chains or molecules such as heme may quench fluorescence whilst collisional quenching by small molecules such as oxygen, iodide and acrylamide is an important effect (27). Collisional quenching can determine the accessibility of fluorophores in proteins via the Stern–Volmer analysis. When drug molecules bind independently to a set of equivalent sites on a protein, the equilibrium between free and bound molecules is given by the equation:

$$log\frac{F_0 - F}{F} = log\ K + n\ log[Q]$$

where F_0, F, are the steady-state fluorescence intensities in the absence and presence of quencher; K and n are the binding constant and the number of binding sites, respectively. Thus, a plot of $log\ (F_0 - F/F)$ versus $log[Q]$ can be used to determine K as well as n.

The Förster theory of molecular resonance energy transfer (FRET) points out: in addition to radiation and re-absorption, a transfer of energy could also take place through direct electrodynamic interaction between the primarily excited molecule and its neighbors. According to this theory, the distance r of binding between the drug molecual and the protein can be calculated by the equation:

$$E = \frac{R_0^6}{R_0^6 + r^6}$$

where E is the efficiency of transfer between the donor and the acceptor, R_0 is the critical distance when the efficiency of transfer is 50%.

$$R_0^6 = 8.79 \times 10^{-25} K^2 n^{-4} \phi J$$

where K^2 is the space factor of orientation; n the refracted index of medium; ϕ the fluorescence quantum yield of the donor; J the effect of the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor, which could be calculated by the equation

$$J = \frac{\int_0^\infty F\ (\lambda)\varepsilon(\lambda)\lambda^4 d\lambda}{\int_0^\infty F(\lambda)d\lambda}$$

where F (λ) is the corrected fluorescence intensity of the donor in the wavelength range $\lambda - (\lambda - \Delta\lambda)$; $\varepsilon(\lambda)$ is the extinction coefficient of the acceptor at λ. The efficiency of transfer (E) could be obtained by the equation: $E = 1 - (F/F_0)$.

The above analysis have been widely used for different systems, as an example for the system tetracycline-lysozyme the values obtained were: 5.21×10^4, 1.2 and 1.93 for binding constant (K), binding site (n) and binding distance (r) respectively (28).

3.5. Circular Dichroism Spectroscopy

Dichroism is the phenomenon in which light absorption differs for different directions of polarization. Circular dichroism (CD) is the difference in absorption of left (A_L) and right (A_R) circularly polarized light, usually by a solution containing the molecules of interest. Optically active samples have distinct molar extinction coefficients for left (ε_L) and right (ε_R) circularly polarized light ($\varepsilon_L \neq \varepsilon_R$). The difference between ε_L and ε_R may be expressed as $\Delta\varepsilon$. Accordingly to the Lambert-Beer Law the difference in the absorbance of left and right circularly polarized light ΔA, can be given by,

$$\Delta A = A_L - A_R = \Delta\varepsilon cl$$

ΔA, is a measure of Circular Dichroism (CD), but it is generally reported in terms of the ellipticity (θ) in degrees

$$\theta = tan^{-1}(b/a)$$

where *b* and *a* are the minor and major axes of the resulting ellipse. There is a simple numerical relationship between ΔA and θ (in degrees): $\theta = 33\Delta A$. If $\Delta\varepsilon$, ΔA or θ is plotted against wavelength, λ, a CD spectrum may be obtained. However to compare the results from different samples it is necessary to consider the molarity.

$$[\theta] = \frac{M\theta}{cl}$$

where $[\theta]$ is the molar ellipticity in degree cm^2 mol^{-1}, *M* is the molecular weight; *c* is the concentration in g cm^{-3} and *l* is the length path in cm. Ellipticities are typically in the range 10 mdeg, corresponding to a difference in absorbance, ΔA, of the order of 10^{-4}. This difference can be determined quite accurately with modern instrumentation. However, it can be calculated from CD. The amount of protein or peptide required for CD can be gauged from the need to keep the absorbance less than the unit. Typical cell path lengths for far-UV work are in the range 0.01 to 0.05 cm and the protein concentrations are in the range 0.2 to 1 mg/ml.

CD bands of peptides and proteins appear in two spectral regions, the far and near-UV region. The amide region or the far-UV or (170-250 nm) is dominated by contributions of the peptide bonds, whereas CD bands in the near-UV region (250-300 nm) are originated from aromatic amino acids. In addition, disulphide bonds give rise to several CD bands. Peptides and proteins that lack of amino acids chromosphere (e. g., prosthetic groups) do not exhibit absorption or CD bands at wavelengths above 300 nm.

CD experiments of biomolecules provide not only information about their secondary structure but also it can be used to follow the interaction of ligands to proteins, peptides or nucleic acids. The CD spectrum of each component in solution is directly proportional to its concentration, and the total spectrum arises from the sum of all spectra components. If ligand binding induces extrinsic optical activity in the chromophores of the bound ligand, an induced CD signal is observed, which is directly proportional to the amount of macromolecule-ligand complex formed, and hence it can be used to construct a binding isotherm. Alternatively, ligand binding may result in a conformational change in the macromolecule, and the resultant change in its intrinsic CD signal allows quantifying the binding. For this reason CD spectroscopy has been emerged as an important tool for drug discovery, enabling screening for ligand and drug binding, and detection of potential candidates for new pharmaceuticals. Pharmacological and pharmacodynamic properties of biological active natural and synthetic compounds are crucially determinate via their binding to proteins of the human body. Several spectroscopic techniques are available to study these mainly non-covalent interactions. CD spectroscopy, being sensitive to the chirality of ligand molecules induced by the asymmetry protein environment, has widely and successfully been applied for many decades. Chiral conformation of the ligand due to conformational adaptation to its binding site, or interaction between ligand molecules held in chiral arrangement relative to each other by the protein sites, results in one or more induced CD bands with different shape, sign and intensity. These extrinsic Cotton effects present in light absorbing region of optically active or inactive ligand molecules give qualitative and quantitative information of the binding process. It can provide valuable data on the stereochemistry, location and nature of the binding sites (29).

CD has been used in combination with other biophysical tools, such as Potenciometry and Pendant Drop Tensiometry, in order to study the influence of polifluorinated amphiphiles, in concrete sodium perfluorooctanoate (SPFO) in its interaction with Human Serum Album (HSA) and Immunoglobulin G (IgG) proteins. These types of amphiphiles self associated in discrete objects, such vesicles and tubules and may be used in pharmaceuticals. HSA-SPFO studies performed by CD suggested a compactation of the protein due to the association with the surfactant given by an observed increase of α-helix content (30). Finally it has been shown that a conformational transition was observed as a function of temperature, these data were analyzed to obtain the thermodynamics parameters of unfolding. The results indicate that the presence of surfactant drastically changes the melting unfolding, acting as a structure stabilizer and delaying the unfolding process (31). On contrary IgG-SPFO studies have shown that such interaction lead to the destruction of the native structure of IgG and the formation of unfolded protein-surfactant complexes even at low surfactant concentration.

A practical example in the field of drug development was the development of protein kinase inhibitors for cancer's treatment. Since alpha 1-acid glycoprotein (AAG) is the principal plasma binding component of some kinase inhibitors, it has been evaluated the binding of a series of marketed and experimental kinase inhibitors to AAG by using CD spectroscopy approach (32). It has also been used in high-throughput monitoring of protein folding (33). In this direction, one interesting approach has been presented recently in a review, which focuses on the combination of biochromatography and CD as an effective approach for the characterization of albumin binding sites and their enantioselectivity. Furthermore, its applications to the study of changes in the binding properties of the protein arising by the reversible or covalent binding of drugs are discussed, and examples of physiological relevance reported. Perspectives of these studies reside in supporting the development of new drugs, which require miniaturization to facilitate the screening of classes of compounds for their binding to the target protein, and a deeper characterization of the mechanisms involved in the molecular recognition (34).

Crystal structures have opened the door to understanding the mechanism and ligand specificities of

Monoamine-oxidases (MAO A and MAO B). Their functional properties suggest flexibility in MAO that is likely to influence catalysis under different cellular conditions. The flexibility indicated by altered oxidation kinetics and a changed redox potential in the presence of a substrate was confirmed by CD spectroscopy. CD also demonstrated alterations in the conformation of aromatic residues during reduction of MAO A and after covalent modification of the flavin (35).

CD experiments have been very useful to provide some important information in Wilson disease. This disease is an autosomal recessive disorder of copper metabolism which results in the toxic accumulation of copper in the liver and brain, causing the hepatic and/or neurological symptoms. It has been observed by CD that zinc also binds to Wilson disease copper-binding domain [WCBD] which conformational changes completely different from those induced by copper. Finally, a chimeric protein consisting of the WCBD and truncated ZntA, a zinc-transporting ATPase lacking the N-terminal domain, has been constructed and analyzed for metal ion selectivity. These results suggest that the core determines the metal ion specificity of P-type ATPases, and the N-terminal metal-binding domain may play a regulatory role (36, 37, 38).

3.6. Vibration Spectroscopy: Infrared and Raman

Both Raman and Infrared spectroscopy provide information on the energies of molecular vibrations. However, the two methods are based on different mechanism of molecular interaction with electromagnetic radiation, and they are governed by different selection rules. Infrared spectroscopy probes the characteristic vibrational bands of chemical groups as the atoms move with respect to one another in response to an oscillating electromagnetic field of the appropriate frequency. The normal modes of a particular molecule or group depend on the molecular structure, interatomic forces (bond strengths) and masses of the atoms concerned, but they will only be IR active if the vibrational mode involves a change in dipole moment. The application of infrared and to protein secondary structure has undergone a renaissance with the development of Fourier transform (FT-IR) spectrometers and improved computers (39). Raman spectroscopy is based on the polarizability of some molecules. The electric field induces an electric dipole in some molecules. This oscillating electric dipole will radiate in all directions at the frequency of the electric field. If the imposed field is due to light, then the molecule will scatter a fraction of that light beam, without change of frequency. This is the basis for elastic or Rayleigh scattering of light. In addition, if the electrical polarization of the molecule involves stretching or bending of chemical bonds, then this might excite normal mode bending or stretching vibrations in the molecule itself. In this case, the induced frequency of dipole oscillation may be reduced by an amount corresponding to the normal mode frequency, and the resulting frequency of the scattered light will be reduced accordingly because some of the light energy must be used to excite the molecular vibration. This shift in frequency is the basis for Raman spectroscopy (40).

Nine normal modes are allowed for the amide band of proteins. There are called A, B and I-VII in order of decreasing frequency. The amide bands I (80% C=O stretch, near 1650 cm^{-1}), II (60% N-H bend and 40% C-N stretch, near 1550 cm^{-1}), and III (40% C-N stretch, 30% N-H bend, near 1300 cm^{-1}) are generally employed to study protein structure. The amide I and III bands have appreciable Raman intensities with visible light excitation. In practice, the amide I band in FT-IR and the amide I and III bands in Raman are primarily used to assign secondary structures to proteins. Corrections between of particular frequencies with secondary structures has been made by reference to spectra of homo-polypeptides and proteins with primarily α-helices and β-sheet structures, theoretical calculations, synthetic peptides and proteins with known three-dimensional structures. Raman also provides information on aromatic residues in the region below about 1620 cm^{-1}.

It is known that proteins which adopt an α-helical conformation have strong amide I bands between 1650 and 1655 cm^{-1}. The hydrogen-bonding strengths in β-sheets are more variable due to their flexibility and tendency to twist. A strong band between 1612 and 1640 $cm^{-1}$and a weaker band about 1685 cm^{-1} are commonly observed for β-sheets, although weak bands at somewhat lower frequencies (1665-1670 cm^{-1}) have also been observed. Identification of these bands is aided by $^1H \rightarrow ^2D$ exchange experiments as the amide backbone protons in β-sheets exchange quite slowly at room temperature and the difference in frequency between the amide I and amide I' (in D_2O) bands is large. Only a single strong band at 1670 cm^{-1} is observed in Raman spectra. The amide I band of turns overlap those of helices and sheets making assignment of this component solely from amide I frequencies difficult. Unordered or random structure is generally assigned to the band near 1665 cm^{-1} in the Raman and 1645 cm^{-1} in the IR. The latter is close to the frequencies associated with α-helix.

Due to the large contribution of the N-H bend to the amide II mode, deuteration results in a substantial shift to lower frequency (≈1460 cm^{-1}). In IR, a strong amide II band is observed at 1540-1550 cm^{-1} and a weaker shoulder at 1510-1525 cm^{-1}. Peptides and proteins with an antiparallel β-sheet structure is found at somewhat higher frequencies (1530-1550 cm^{-1}). Inspection of both amide I and II bands provides little help in distinguishing between turn and sheet conformation. In favorable situations, however, the amide II band may assist in assigning random structure and allow to a more accurate estimation of the helix and random components.

Like the amide II bands, deuterium substitution of the ^1H atom shifts the amide III band to lower wavenumbers (960-1000 cm^{-1}). In the infrared, the amide III band is normally quite weak and occurs in a region of mixed vibrations (C-H bending, Tyr, Phe ring vibrations) that are not readily correlated to protein secondary structure. In the Raman spectrum amide III band is stronger. Although it appears in a region of the spectrum that contains a number of unrelated vibrational bands, the amide III components can be identified by their shift in

D_2O. When used in combination with the amide I band, their assignment may permit a distinction between β and disordered structure that is generally not possible based on the amide I band alone.

The primary problem in IR spectral analysis of proteins is that the bands are a complex composite of overlapping component bands that represent different structural elements. The width of the component bands is usually greater than the separation between the maxima of adjacent peaks and cannot be resolved by simple inspection of the spectra. While resolution enhancement of IR and Raman spectra does not increase the instrumental resolution, it does lead to separation of the underlying band structure and permits individual components to be visualized. The successful application of resolution enhancement techniques requires careful attention to the elimination of water vapor and random noise from the spectrum. The later can produce artifacts indistinguishable from amide bands after application of enhancement techniques. The absence of artifacts must be verified by a careful inspection of the resolution enhanced protein spectrum adjacent to the amide I band (1700-1800 cm^{-1}) where peaks are not generally expected.

The bibliography offers us a huge number of applications of vibration spectroscopy in the field of protein surfactant interactions. Thus, Raman spectroscopy has indicated that lysozyme subjected to SDS is no longer in its native state. The amide I peak is shifted toward higher wavenumbers from 1660 cm^{-1}. Thus the secondary structure of lysozyme in the complex salt has a larger contribution of β- structure than the neat additional changes in the environment of the polypeptide's side groups. It was concluded that in the complex salt the lysozyme molecule is still in a compact conformation but with a changed secondary structure compared to its soluble and its crystal, native conformation (41). The dipalmitoylphosphatidylcholine (DPPC)/phosphatidylglycerol (PG) multilayers, reconstituted with various synthetic peptides for modeling human lung surfactant was studied by means of vobrational Raman spectroscopy with the aim of evaluate its thermotropic behavior. The temperature dependence of several spectral peak height intensity ratios and integrated intensity parameters determined from the lipid acyl chain methylene and methyl C-H stretching modes have reflected the inter- and intrachain order characteristic of the reconstituted bilayer assemblies (42). It was confirmed from FT-Raman studies that sodium dodecyl sulfate has a strong effect on the secondary structure of BSA. Some ordered structures of BSA, such as α-helix, changed into unordered structures with increasing concentration of SDS. These results indicate that BSA underwent conformational unfolding during binding and formation of the BSA–SDS complex. The excess negative charge on the protein–SDS complex was thought to completely transform the macromolecule into a hydrophilic polyelectrolyte, which made the protein–SDS complex very non-surface active (43). On the other hand, it was demonstrated that the interaction between Mb and SDS should not be viewed as a simple protein-ligand-binding process, but as a multipathway and multistep process The analysis has lead to a detailed scheme for the formation of Mb/SDS complexes which includes the stable species observed at equilibrium and additional reaction intermediates (44). Also, the role of the hydrophobicity of hydrogenated and fluorinated surfactants has on fibrinogen structure was checked (45).

On the whole, Raman and IR spectroscopy have the following notable advantages (46): are non destructive techniques, are applicable to samples of virtually any morphological form, small volumes are required, the time scale is very short in comparison to the times of fluorescence and there exist a large database of IR and Raman spectra of proteins for which reliable band assignments have been made. Instead, the disadvantages are: the spectra resolution is still inferior to that of high-field magnetic resonance spectroscopy, the Raman process on inelastic light scattering is inherently weak compared to other light absorption and emission processes, thus, considerable effort in sample purification and care in sample handling are necessary.

3.6. Photon correlation spectroscopy: Static and Dynamic Light Scattering

Among the scattering techniques, laser light scattering has been well developed and widely applied in studying polymers in solution or colloids in suspension, including proteins and the corresponding aggregation behavior in aqueous solution. In laser light scattering, we study the interaction of electromagnetic waves with matter by measuring the changes in the number (intensity), the direction (momemtum), and the frequency (energy) of each type of photon in the incident and the emerging light beams (47). Light scattering occurs on molecules bathed in the electromagnetic field of the light beam because of the difference in the dielectric properties of the material and the surrounding media, and the varying field induces oscillating dipoles in the particles radiating light in all directions. Depending on the size of the photon-interacted particles (d) and the wavelength of incident light beams (λ_0), the light scattering can be classified into Mie ($d >> \lambda_0$), Tyndall ($d \approx \lambda_0$), and Rayleigh scattering ($d \leq 0.05\lambda_0$). It has been utilized in many areas of science to determine particle size, molecular weight, particle or macromolecule shapes, their diffusion coefficients, particle aggregation, mobility, etc. The intensity of scattered electromagnetic field depends on the ratio between the particle size and the incident light wavelength (λ), and the shorter the λ value, the smaller the particles. The short-term intensity fluctuations (dynamics) of the scattered light arise from the fact that the scattering particles are in the motion. These motions cause short-term fluctuations in the measured intensity of the scattered light. Various terms have been used for this phenomenon: photon correlation spectroscopy (PCS), dynamic light scattering (DLS) or static light scattering (SELS). The pace of the movement is inversely proportional to the particle size (the smaller the particles are, the faster their motion or diffusion), and the pace can be detected by analyzing the time dependency of the light intensity fluctuations scattered from the particles when they are illuminated with a laser beam (48). PCS has been used

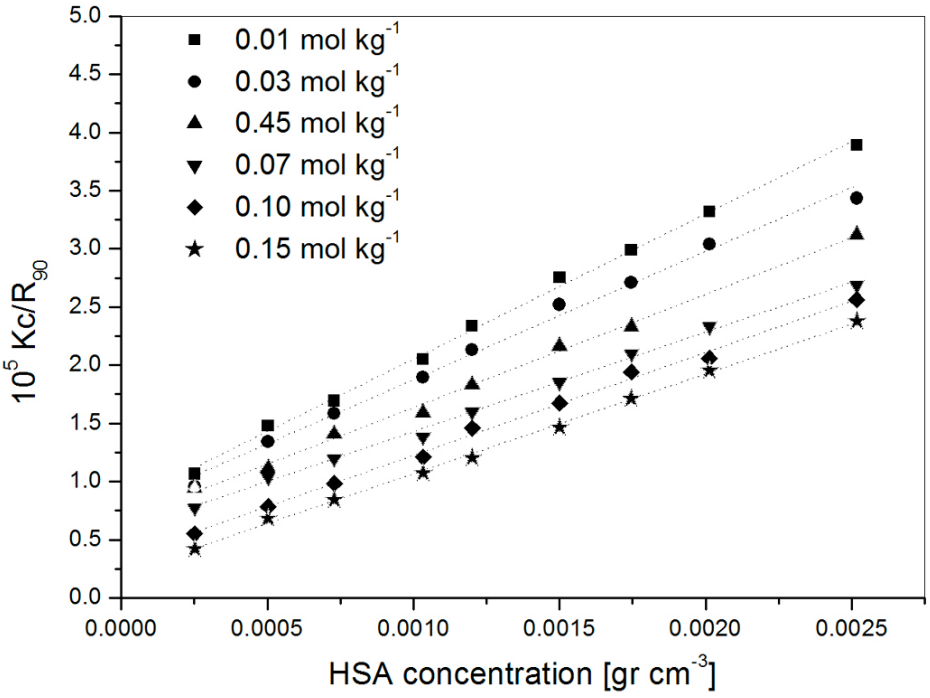

Figure 3. Kc/R_{90}, as a function of the human serum albumin (HSA) concentration in aqueous solutions of sodium nafcillin.

to investigate many phenomena in protein systems such as hydrodynamic radii, shape, diffusion, hydration, oligomerisation, aggregation, agglutination, complexing and as interior dynamics (49). Light scattering intensity is monitored either in the microsecond or in the second time range domain. This is the basic difference between dynamic light scattering (DLS) and static light scattering (SLS), respectively. Fluctuations in the intensity of light scattered by a small volume of a solution, at some angle relative to the incident laser beam, in the microsecond time range are directly related to the Brownian motion of the solute. Averaging the intensity over the second time range interval will cause a loss of the solute dynamic properties information; that is why light scattering is named either static or dynamic (50). SLS provides a direct measure of molecular mass. For globular proteins smaller than ~500 kDa, the intensity of the scattered light is uniform in all directions, so it is only necessary to measure scattering at a single angle, generally 90º. For higher masses or extended proteins (rod-like or unfolded) the scattering varies significantly with angle. By measuring the scattering at additional angles, direct absolute measurements of masses up into the MDa range can be made, and the root mean square radius, related to the geometric size, can also be determined. SLS is very useful for determining whether the native state of a protein is a monomer or a higher oligomer, and for measuring the masses of aggregates or other non-native species. It also can be used for measuring the stoichiometry of complexes between different proteins.

Static light scattering provides a direct measure of molecular mass. For globular proteins smaller than ~500 kDa, the intensity of the scattered light is uniform in all directions, so it is only necessary to measure scattering at a single angle, generally 90º. For higher masses or extended proteins (rod-like or unfolded) the scattering varies significantly with angle. By measuring the scattering at additional angles, direct absolute measurements of masses up into the MDa range can be made, and the root mean square radius, related to the geometric size, can also be determined. SLS is very useful for determining whether the native state of a protein is a monomer or a higher oligomer, and for measuring the masses of aggregates or other non-native species. It also can be used for measuring the stoichiometry of complexes between different proteins (51). The static light scattering data in the limit of small concentrations, or volume fractions, and for small values of the scattering angle, can be analyzed using the Debye equation

$$\frac{Kc}{R_{90}} = \frac{1}{M_W} + 2A_2c$$

where c is the concentration of the protein, M_W is the molar mass, A_2 is the second virial coefficient, K is an optical constant, and R_{90} is the Rayleigh ratio at 90º. By using this procedure, stoichiometry of the drug-protein complex can be inferred. Some examples can illustrate this use. Figure 3 shows static light scattering data for HSA in aqueous solutions containing between 0.015 and 0.15 $molkg^{-1}$ nafcillin(52). In this treatment of the data nafcillin has been considered as an electrolyte, so the system is acting as a binary system formed by the protein complex as solute, and

the nafcillin water as solvent. Molar masses, derived from the intercepts of Figure 3 increased linearly with concentration of added drug. The molar mass obtained using the Debye equation for the HSA in pure water was 7.9×10^4 g mol^{-1}, which corresponds to an association number $N = M_W/M_0$ (where M_0 is the molecular weight of the HSA, 6.641×10^4 g mol^{-1}) of 1.19 indicating only limited association. The average number of nafcillin molecules adsorbed onto the protein can be obtained from the difference between the molar mass of HSA (6.6×10^4 g mol^{-1}) and that of the complex, divided by the molar mass of nafcillin (454.5 g mol^{-1}). The average number of nafcillin molecules adsorbed onto HSA derived by this method changes with nafcillin concentration from 171 (at 0.015 mol kg^{-1}) to 1075 (at 0.15 mol kg^{-1}). Based on these results it can be deduced that at small drug concentrations, individual drug molecules bind to the protein which results in small changes in protein structure. On the other hand, drug molecules at higher concentrations aggregate to form micelle-like clusters along the unfolded polypeptide chains of the protein. HSA has exhibit the same pattern in the presence of other drugs such as cloxacillin, dicloxacillin (53) and verapamil (54). This behavior has been studied not only by experimental techniques but also by a theoretical formalism based on the combination of the Brunauer–Emmet–Teller multilayer adsorption model with an electrolytic adsorbate. The experimental binding processes are correctly predicted by this noncooperative binding model and it is demonstrated to be due to the ionic character of the drug. The effect of the hydrophobic interactions between drug monomers is also a matter of study, and it is proven that this number increases with increasing hydrophobic character of the drug molecule (55).

Common problems of protein solutions explorations by Dynamic Light Scattering (DLS) were analyzed in several reviews (56-58). The quality of the DLS investigations are improved with the accumulation of information and experience which illustrate the possibility of this technique towards investigations devoted to specific biological systems (59-63).

Measurement of the protein translation diffusion coefficients (D_T) in the solution is relatively simple, but it is important in the task of the DLS investigations. These data permit to estimate the size of protein particles and, in some cases, to determine their molecular shape. The values of the translation diffusion coefficients (D_T, from 2.5×10^{-6} cm^{2}s^{-1} to 2.2×10^{-8} cm^{2}s^{-1}) and the corresponding hydrodynamic radii (R_h, from 0.9 to 98 nm) of a variety of proteins are determined(48). In addition to the studies of globular and fibrillar proteins, separated protein fragments, protein complexes and oligomers were also explored. The R_h of globular proteins is close to the true geometrical size (R) of macromolecules, as $R_h = R + \kappa^{-1}$, where κ is the Debye-Huckel parameter and the thickness (κ^{-1}) of the electrical double layer (shear layer) is relative small because of high polarity and charging of polyelectrolyte molecules (proteins, DNA, etc.) especially far from the point of zero charge (or the isoelectric point on electrophoresis). However, for proteins which shape is strongly different from the spherical one, the hydrodynamic size can be considered as only a conventional or effective magnitude dependent on the solution characteristics (pH, T and ionic strength). The changes in protein D_T values are linked to both variations in the microstructure of individual particles and the overall properties of object, for example the oligomerisation or the aggregation degree. To study the proteins behavior in different states and in different media additional experiments are necessary, i.e.: decay rate angle dependence measurements (Γ); image correlation spectroscopy methods; image cross-correlation spectroscopy; fluctuation correlation spectroscopy; two-photon fluorescence microscopy; electron microscopy (TEM, SEM) and atomic force microscopy (AFM).

Analysis of the proteins polydispersity (PSD) in aqueous media is one of the overall problems of biological applications of DLS, as its solution gives an answer to many questions about the protein systems. For example, a clear notion about the mean size of particles and the oligomerisation degree can be obtained even from a rough size distribution. Examination of the PSD dynamics allows analyzing the processes in the biological systems at the molecular level. The geometry of oligomer aggregates could differ significantly from the geometry of the units forming these secondary particles (due to spatial changes in the shapes of the molecules and their fractality that may lead to unexpected changes in the scattering ability of secondary particles). Therefore, the relationship between the oligomers diffusion coefficients (or aggregates) and monomers is not a linear function of the number of monomers in the oligomers (notice that $D_T \approx R_h^{-1}$). To solve this problem, one should determine or estimate the shape of secondary particles using other techniques such as: CD, multi-photon fluorescence spectroscopy, X-ray diffraction (XRD), small angle X-ray scattering (SAXS), X-ray photon correlation spectroscopy (XPCS), multi-angle light scattering (MALS), electron microscopy and atomic force microscopy (AFM), among others. Provercher *et al.*(64, 65) developed a special version of CONTIN algorithm to solve the inverse problem for CD to estimate the secondary structure of proteins.
Considering polydisperse systems, a dimensionless parameter of the polydispersity is usually employed:

$$Q = \frac{\sigma^2\tau^2}{2}$$

where σ^2 is the distribution dispersion, τ^2 is the mean correlation time for this distribution. Sometimes the percentage of particles different from the size of a current monodisperse portion is used as a polydispersity criterion. The homogeneity of a solution or a suspension is considered as a good one (narrow PSD) if the polydispersity is lower than 15%, which corresponds to $Q < 0.1$. For a monodisperse system (narrow PSD), the average statistical value of the protein molecular mass (M)

can be determined if the sedimentation coefficient (s) and the specific partial volume (v) are known:

$$M = \frac{sR_gT}{D_T(1-v\rho)}$$

where R_g is the gas constant, ρ is the specific density of the solvent. The v value can be determined by using a densimeter.

$$v = \left[\frac{1}{m_0} - \frac{100}{P_m}\left(\frac{1}{m_1} - \frac{1}{m_0}\right)\right]$$

where V is the densimeter volume, m_0 is the solution mass, m_1 is the solvent mass, $P_m = 100(cV/m_0)$, c is the protein concentration in the solution.

The protein shape determination is a complex problem; however, the application of even single-scattering-angle PCS set-up can be fruitful if the translation friction coefficient f is used. This coefficient is linked with the translation diffusion coefficient by $fD_T = kT$, where k is the Boltzmann constant. The asymmetry parameter of molecules corresponds to f/f_0, where f_0 is the translation friction coefficient of a sphere with the equivalent mass. For globular proteins modeled by an ellipsoid of revolution, f_0 is:

$$f_0 = \frac{3\pi\eta L(p^2-1)^{1/3}}{pln\left[p + (p^2-1)^{1/2}\right]}$$

where L is the contour length of the molecular chain (for proteins, it is the length of the polypeptide chain), p is the ratio of the main axes of the ellipsoid (p > 1) and η is the dynamical viscosity. It should be noted that this equation is valid independently on the shape and the size of particles. The particle (or macromolecule) shape factor can be determined by means of the MALS technique.

Qualitative assessments of the proteins shape were typically performed and published in the literature, e.g.: for biliprotein R-phycoerythrin IV eliminated from marine algae Phyllophora antarctica, R_h determined by using PCS is equal to 5.54 nm for a non-spherical shape of the molecule (66). Information about the shape of biopolymers allows one to elucidate the localization of them in the native structures For instance, simultaneus application of analytical centrifugation and PCS allowed Zgurskaya and Nikaido (67) to determine a large asymmetry of monomers of protein AcrA as a component of a multifunction complex AcrABTolC (Escherichia coli). The p value of this lipoprotein was equal to 8, which allowed the authors to conclude that this lipoprotein locates in periplasm but not in the lipid bilayer. Understanding the mechanisms of protein folding requires knowledge of both the energy landscape and the protein structural dynamics. Bu *et al.* (68) reported a study of nanosecond and picosecond dynamics of native and denatured alpha-lactoalbumin. The picosecond time-scale dynamics showed that the potential barrier to side-chain proton jump motion is reduced in a molten globule and in the denatured state when compared to that of the native protein. The obtained results provided a dynamic view of the native-like topology established in the early stages of protein folding.

More complicated picture can be observed in the case of complex solutions containing, not only proteins but also surfactants, drug, polymers, salts and other compounds affecting the structure of protein molecules and protein–protein interaction (48).

Proteins in solution have a tendency to aggregate depending on the physicochemical conditions. In the food industry, for example, aggregation (especially oligomerisation) is often irreversible and leads to an undesirable loss of the protein products. Similar phenomena can cause negative effects in the human organism or may be a sign of some serious diseases (such as Alzheimer's and Jacob diseases). Nevertheless, many proteins possess specific activity in the form of dimers, trimmers or other oligomers. Accordingly, investigations of protein aggregation and oligomerisation are of interest from many points of view. The processes of aggregation or oligomerisation can be effectively explored by means of the PCS method since the scattering ability is proportional to the square of the scatterer mass. In the case of strong interaction of particles filling a marked portion of the scattering volume the correlation between the decay rate angle dependence (Γ) and the scattering vector q, $\Gamma = D_T q^2 + 6D_R$, could be written with consideration for dependence of diffusion coefficients on the particle volume fraction Φ:

$$\Gamma_\Phi = D_{T,\Phi}q^2 + 6D_{R,\Phi}$$

Where

$$D_{T,\Phi} = D_T(1 - k_T\Phi)$$
$$D_{R,\Phi} = D_R(1 - k_T\Phi)$$

being k_T and k_R constants.

Aggregation, oligomerisation and other processes occurring with protein molecules depend on several factors such as the nature of proteins, their concentration, solvent type, the availability of interaction with other organic (low and high molecular weight, surfactants, etc.) and inorganic (salts, metal ions, acids, bases, i.e. salinity and pH) compounds (i.e. composition of surroundings as a whole). In addition temperature, pressure, exposition (ageing), external actions (applied electrostatic or electromagnetic fields, UV radiation, sonication, etc.) play an important role in protein-protein interactions. We will mention only certain interesting results related to some human, animal

and food proteins with other biomolecules (DNA, RNA, lipids, etc.), some and some drugs used for delivery and model systems.

All the proteins obtained in the crystalline form are typically studied by means of the XRD method; however, protein function occurs in liquid or semi-liquid media. Clearly, the structures of proteins in the crystalline and native quasi-liquid states can be strongly different. Unfortunately, the XRD data do not allow one to make even presupposed estimation about a number of protein molecules which oligomerized in a biologically active unit. Therefore, many XRD investigations are performed simultaneously with PCS (but applied to the liquid media) (69).The application of two powerful physical methods providing practically unambiguous interpretation of the obtained results is fruitful or even essential for preliminary estimation of the homogeneity of the solution before the crystal growth for the XRD exploration (70). Cysteine proteinase CPP32 obtained from *E. coli* in the soluble form was studied by the XRD (resolution 0.23 nm) and PCS methods. It was shown that an asymmetrical unit of the enzyme contains a tetramer in accordance with the structure of the tetramer protein substrate. Mittl *et al.* (71) postulated that the obtained structural information could be useful to synthesize small inhibitors of CPP32 or to create cysteine proteinase mutants. Human uroporphyrinogen decarboxylase (recombinant of an intracellular enzyme) which catalyzes the fifth stage in the heme biosynthesis was obtained from *E. coli* and purified to a homogeneous state was studied by PCS. It was shown that this protein is a dimer in the monodisperse solution; then this result was confirmed by using sedimentation analysis (72). 4-Oxolate tautomerase studied by PCS and other methods was characterized by the translation diffusion coefficient value and the time of rotational motions (14.5 ns) showing formation of the trimer / dimer structure of $M \approx 41$ kDa(73).

Many of PCS studies have demonstrated the presence of negatively charged, globular or micelle-like structures in human saliva containing proteins. Similar structures were found in parotid saliva to be initially 100–150 nm in diameter, increasing up to 450 nm 50 min after sampling. Schuler *et al.* (74) investigated the colloidal properties of a transferring receptor (isolated from human placenta, hTfR) in detergent free solution by using PCS techniques and analytical ultracentrifugation. In such a solution at 293.2 K, hTfR formed stable aggregates with an apparent hydrodynamic radius of 17 nm. The molecular mass was determined by ultracentrifugation to lie between 1722 ± 87 kDa (sedimentation equilibrium) and 1675 ± 46 kDa (sedimentation velocity). This implied that the aggregates were build up from nine hTfR dimers. Based on model calculations, which were in good agreement with the experimental data, the authors proposed a torus-like structure for the aggregates. Upon pH shift from pH 7.5 to 5.0 or removal of the N-linked carbohydrate chains, formation of larger aggregates was induced. These aggregates could be described in terms of porous fractal structures. The authors (75) proposed a simple model, which accounted for that behavior assuming that the aggregation was mainly because of the reduction of negative surface charge providing repulsive forces between the hTfR units.

Protein aggregation has been recognized to be a pathological indicator for several fatal diseases, such as Alzheimer's disease, transmissible spongiform encephalopathies, Jacob disease, etc. Self assembly (or oligomerisation) usually involves conformational changes of proteins that have acquired an intermediate conformation and can occur even at low protein concentration. Bulone *et al.*(75) has shown that BSA, even at low concentration, exhibits self-association properties related to conformational changes, so providing a very convenient model system to study this class of problems. Obtained results showed that the interaction between the two species of BSA in native and intermediate form was responsible for a decrease in the thermodynamic stability of the solution. This occurred without requiring noticeable conformational changes of the native protein. These results could provide new insights on the 'protein only' hypothesis proposed for the formation of plaques involved in several neurodegenerative diseases. Bonincontro *et al.* (76) studied the effect of solvent viscosity on both translational and rotational dynamics of a simple model protein: the egg white lysozyme. For this, they investigated the dynamical properties of lysozyme in mixtures of water and glycerol by means of parallel measurements by PCS and dielectric relaxation spectroscopy (DRS) at different radiofrequencies.

The PCS technique was applied to study such proteins as α-lactoglobulin (77) and α crystallins (78). The hydrodynamic structure of the α-crystallins (78) and their mutual interaction are the essential parameters characterizing the solution structure at low and high concentration of the protein. Changes in these characteristics, as a function of temperature, can explain the activity of the α-crystallin. Absolute light scattering, PCS and equilibrium sedimentation of diluted solutions as a function of temperature yield the molar mass M, the hydrodynamic radius $R_{h,w}$ of the equivalent hard sphere and the second virial coefficient of the molecules in a temperature range from 2 ºC to 37 ºC.

Photon correlation spectroscopy techniques provide the most obvious methods for obtained quantitative information on size, shape and structure of protein in solution. They are based on interaction between incident radiation (light, X-ray or neutrons) and particles. DLS is a useful tool for studying particle aggregation and, in particular, for monitoring protein self-assembly with high sensitivity and resolution.

SLS and DLS have the following disadvantages: requires a solvent with different refractive index than the dispersed macromolecules, careful removal of extraneous scattering particles (dust), much less accurate for distinguishing small oligomers and applications to many systems of scientific and industrial relevance has been limited due to multiple scattering (highly concentrated solutions), wherein photons are scattered multiple times by the sample before being detected.

3.8. Microcalorimetry

A prerequisite for a deeper understanding of the molecular basis of protein–drug interactions is a thorough characterization and quantification of the energetic governing complex formation (79). All living things have some thermal energy. The amount of heat is related to the sum of processes taking place in the system. Thermal Analysis techniques are based upon the detection of changes in the heat content or the specific heat of a sample with temperature. Presently, two modernized high-accuracy automated types of equipment are available with accompanying convenient software. One is known as "differential scanning calorimetry" (DSC), and the other is known as "isothermal titration calorimetry" (ITC). DSC measures the heat capacity (which at constant pressure is the temperature derivative of enthalpy) of the protein-drug interaction under investigation by incrementally varying the temperature of the system over a specified range. Isothermal titration microcalorimetry measures the heat change that is associated with reactions in solution at a constant temperature and, by the sequential addition of drug to the solution. Both are well-characterized and widely accepted techniques.

Contemporary DSC instruments are characterized not only by high sensitivity but by the high stability of their baseline and the ability to scan aqueous solutions up to and above 100 °C under excess pressure and by supercooling down below 0 °C. The wide operational range is important because changes of many proteins take place over a very broad temperature range. As for the stability of the baseline, this is important since it permits determination of the partial heat capacity of the protein in dilute solution (80). Also, DSC might provide a useful clinical diagnostic tool for screening and disease monitoring by simple analysis of the thermogram of weighted sum of the thermal denaturation of the most abundant plasma proteins (81, 82).

Different thermodynamic and statistical mechanical approaches to describing unfolding, stability and energy of proteins linked to a ligand have been discussed in literature (20, 83-87) . Early studies have demonstrated the usefulness of DSC in understanding the drug-resistance mechanism caused by mutations. Efficiently, DSC analysis allows partitioning of the effects of mutations on native protein structure and on protein-drug interactions because binding free energy change is dissected into stabilities of the free and complexed forms of the protein. Another motivation for DSC studies emerges from the utility of thermal shift measurements in primary of secondary drug screening. DSC measures the cooperative melting of a folded protein structure, which occurs at a temperature, T_m, that reflects the protein`s stabilization energy. When drugs bind to a protein, the energy of ligand binding adds incrementally to the protein`s stabilization energy and the protein`s T_m is increased by an amount that is proportional to the ligand binding energy. Thermodynamic analysis of the ΔT_m, or thermal shift, associated with ligand binding can be semiquantitatively related to ligand binding affinity, subject to reasonable assumptions regarding the ligand binding enthalpies of drug-like compounds (88).

Enteric-coated bovine serum albumin (BSA) nanospheres carrying cyclodextrin complex were performed for indomethacin delivery. The inclusion complex and the nanospheres were characterized by DSC analysis. The thermal behavior of albumin nanospheres showed no phenomena in the temperature interval. Albumin nanospheres carrying inclusion complex showed disappearance of the endothermal peak of indomethacin indicating the the strong drug interaction with albumin or albumin/betacyclodextrin carrier. The results showed that indomethacin solubility can be increased by complexation with protein/drug interaction with albumin nanospheres (89).

DSC studies on amorphous trehalose-water systems with embedded proteins (myoglobin, lysozyme, BSA, hemoglobin) were performed. From DSC upscans the thermodynamic properties of the matrix (glass transition) and the functional properties of the encapsulated protein (thermal denaturation) were calculated. At high hydration, the presence of the proteins increases the glass transition temperature of the encapsulating matrix. An increase of the protein thermal stability is observed in the whole hydration range, a finding potentially of high biotechnological relevance (90).

DSC thermograms have allowed characterizing the processes involved in synthesis of the antibody-drug conjugate Trastuzumab-DM1 (a semysinthetic ansamaclolide). Lysine residues in CH2 domain of the antibody, due to their greater flexibility and solvent accessibility, are more susceptible to conjugation with the drug species DM1 than are those in other parts of the molecule. The increased susceptibility of the CH2 Lys residues to conjugation leads to disruption in the structural stability of this domain. In addition, T-MCC, an intermediate in the processing of T-DM1, is highly susceptible to covalent aggregation due to formation of intermolecular cross-links involving the maleimidyl moiety and the side chains of nucleophilic amino acid such as Cys, Ser, or Tyr. The conjugation of the drug DM1 to T-MCC greatly increases the stability of the molecule to aggregation behavior (91).

The thermal denaturation of ovalbumin, lysozyme, myoglobin and fibrinogen at different 3-(2-Benzothiazolylthio)-propanesulfonic acid (BTS) concentrations have been investigated using DSC. The thermal denaturation of the four proteins was completely irreversible. All the proteins have different denaturation temperatures, indicating differences in thermal stability. The BTS binds though electrostatic interactions to myoglobin and lysozyme, and through hydrophobic interactions to ovalbumin and fibrinogen. Greater changes in α-helical contents correspond with the BTS higher concentrations. The lysozyme denaturation temperature increases at low concentrations BTS indicating that BTS acts as a structure stabilizer; meanwhile it acts as a destabilizer at higher concentrations in all the proteins studied. A major effect is observed in the case of myoglobin, the protein with the highest α-helical secondary structure (75%). This could be related with the fact that

backbone hydrogen bonds of α-helix are generally slightly weaker than those found in β-sheets. Thus, they are readily attacked by the surrounding BTS molecules (92).

The complex formed due to the interaction of the amphiphilic betablocker acebutolol with fibrinogen in a buffer solution (50mN glycine, pH of 8.5) was investigated. Differential scanning calorimetry measurements of the complexes have shown no reversibility of thermal denaturation as indicated by the three observed peaks and the opposite role that acebutolol plays in the folding different domains of the fibrinogen molecule and the stability of such domains: stability at low concentrations and unfolding at the higher ones in the end D fragment; acts as a potent denaturant for C-terminal of the α chains and denaturant at higher concentrations for the central E domain. On the other hand, transitions from the native to the unfolded state of fibrinogen induced by acebutolol have been found to be a multiple step process with intermediate molten globule states (93). Many more examples of DSC applications can be consulted in the review of S. Vyazovkin (94).

An advantage of ITC over other methods is that it measures the enthalpy change directly. Other techniques, also described below, determine the enthalpy change indirectly. In the standard ITC apparatus, the protein-ligand interaction proceeds in a sample cell of small volume. One component (e.g., protein) of the reaction is placed in the reaction cell, and the other component (e.g., ligand) is added in stepwise fashion by an automated injection system in preset measured amounts for preset measured times. The change in temperature is measured as the amount of differential current that is required to maintain the reaction cell at the same preset temperature as that of a reference cell filled with distilled water or the same buffer solution as the reaction cell. If the protein-ligand interaction is endothermic, more current is required relative to the reference cell. The power that is required, over baseline, comprises the raw data output of the ITC equipment. If the reaction is exothermic, less power is required, which is recorded as a downward deflection in output. The overall interaction between a protein and a ligand is carried out in a sequence of automated titrations. At the end of each run, all of the binding sites are occupied and no further heat of reaction is detected. The raw data obtained for each injection, peak, are integrated, and the integrated heats that are derived from the raw data are plotted against the molar ratio of the interacting species. A best fit of the data is obtained using a non-linear algorithm. This fit allows for the accurate determination of binding constants (K), reaction stoichiometry (n), and a thermodynamic profile of the protein interaction that includes the observed molar calorimetric enthalpy (ΔH), entropy (ΔS), heat capacity (ΔC) of binding and change in free energy (ΔG) (5, 21). The dissection of ΔG into contributions from ΔH and ΔS has improved the understanding of the relationships between thermodynamics, structure and function. Thermodynamic signatures have begun to be assigned to energetic contributions from hydrogen bonding, hydrophobic interactions, conformational changes, electrostatic interactions, molecular flexibility and solvent effects. However, it is often not straightforward to interpret binding thermodynamics and it is essential to realize that the measured parameters actually reflect differences between free macromolecule and free ligand compared with the complex (95, 96).

Firstly, we think that we should highlight the excellent work done by Olsson *et al.* (97). They have collated the results of a large number of published isothermal titration calorimetry (ITC) studies of protein–ligand complexes for which structural information is also available, in order to enhance the possibilities for investigation of the potential relationships between structure and thermodynamics of protein–ligand interactions. Further, they have created an online resource (SCORPIO—Structure/Calorimetry of Reported Protein Interactions Online (http://scorpio.biophysics.ismb.lon.ac.uk/scorpio.html, #233)) making these data available to other researchers.

ITC is a highly effective tool in historical attempts to target protein-protein interactions. Recently it has been described the discovery of the usefulness of benzodiazepine (BZD) as an inhibitor that disrupt the function of the BET family of bromodomains. Isothermal titration calorimetry (ITC) experiments confirmed that BZD bound to both the individual BD1 and BD2 domains with a 1:1 stoichiometry with very similar affinities. However, the affinity purification studies identified only the BET family of proteins as BZD binders despite the likely presence of many other bromodomain containing proteins in cell lysates (98).

ITC experiments show that the interaction of cytochrome *c* with ferulic acid is driven by a moderately favorable entropy increase in combination with a less favorable enthalpy decrease for the first binding site of the protein (99).

The interaction energies of the NimA protein and the antibiotics: metronidazole, tinidazole, ornidazole and dimetrazoleantibiotic were are studied by ITC. Measurements found that one NimA dimer has two antibiotic binding sites which were not affected by mutation. The enthalpy release upon binding to NimA for the four drugs studied was relatively low, because of the drug binding is mainly entropy driven and along with the hydrophobic drug binding site (100).

Evaluation of binding of vismodebig (a selective Hedgehog pathway inhibitor) with human and rat AAGs and albumins have confirmed that binding is rapidly and completely reversible. The data suggest that as compound enters the bloodstream, it will preferentially bind to available binding sites on AAG rather than albumin. These data highlight the utility of ITC techniques in creating a comprehensive picture of protein binding across species by measuring binding affinities for multiple plasma proteins and multiple species. An understanding of these differences could be useful in enabling the translation of efficacy and safety in preclinical models to humans (101).

A recent analysis of the experimental binding thermodynamics provides important insights into the factors governing drug affinity and efficiency. In fact, standard definition of drug efficiency is problematic for comparing drug of disparate size because drug efficiency is itself a function of molecular size. Consequently, just in a few cases the enthalpy is correlated with free energy. It is tempting to speculate that this could be an important consideration as to why some targets are readily amenable to modeling and others are not (102).

ITC studies further demonstrate that quinacrine can serve as a targeting ligand for specific delivery of additional therapeutic molecules or imaging agents to the receptor-overexpressing cancer cells implicated in breast and prostate cancers. Pertinent to this targeting utility, it would be possible to apply the concept of multivalent ligand design, in which even suboptimal targeting capability can be enhanced through multivalent tight binding (103).

4. SUMMARY AND PERSPECTIVE

This review is the result of a collective research work developed by a huge number of scientists in the field of proteins - ligand interactions. A compendium of different experimental techniques that in addition to an introduction to this complex and wonderful world, outlines the impact of these technical developments in disciples such as genetic engineering, personalized medicine, drug delivery, biosensor or biotechnology. The techniques address the great diversity of approaches and current applications. All sections start at a fundamental level and finally deal with the concluding remarks and future perspectives. If the incorporation of new technologies and more efficient methods is a common practice in all fields of science, has been precisely in the study of protein interactions where it has achieved impressive proportions. These new insights complement and extend our knowledge of proteins and their potential applications to unimaginable levels.

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