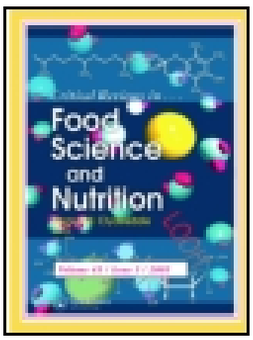

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Analytical techniques for the study of polyphenol--protein interactions

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Abstract

This mini review focuses on advances in biophysical techniques to study polyphenol interactions with proteins. Polyphenols have many beneficial pharmacological properties, as a result of which they have been the subject of intensive studies. The most conventional techniques described here can be divided into three groups: (i) methods used for screening (*in-situ* methods); (ii) methods used to gain insight into the mechanisms of polyphenol--protein interactions; and (iii) methods used to study protein aggregation and precipitation. All of these methods used to study polyphenol--protein interactions are based on modifications to the physicochemical properties of the polyphenols or proteins after binding/ complex formation in solution. To date, numerous review articles have been published in the field of polyphenols. This review will give a brif insight in computational metods and biosensors and cell-based methods, spectroscopic methods including fluorescence emission, UV-vis adsorption, circular dichroism, Fourier transform infrared and mass spectrometry, nuclear magnetic resonance, X-ray diffraction, and light

scattering techniqes including small-angle X-ray scattering and small-angle neutron scattering), and calorimetric techniques (isothermal titration calorimetry and differentiall scanning calorimetry), microscopy, the techniques which have been successfully used for polyphenol-protein interactions. At the end the new methods based on single molecule detection with high potential to study polyphenol-protein interactions will be presented. The advantages and disadvantages of each technique will be discussed as well as the thermodynamic, kinetic or structural parameters, which can be obtained. The other relevant biophysical experimental techniques that have proven to be valuable, such electrochemical methods, hydrodynamic techniques and chromatographic techniques will not be described here.

Key words

polyphenol--protein interactions; *in-situ* techniques; the mechanisms of polyphenol--protein interactions; protein aggregation; single molecule detection

Abbreviations

BSA -- bovine serum albumin

CD -- circular dichroism

DLS -- dynamic light scattering

DSC -- differential scanning calorimetry

EGCG -- (--)-epigallocatechin-3-gallate

FRET -- fluorescence resonance energy transfer

FTIR -- Fourier transform infrared spectrometry

MS -- mass spectrometry

NMR -- nuclear magnetic resonance

ITC -- isothermal titratin calorimetry

SAX -- small-angle X-ray scattering

SANS -- small-angle neutron scattering

STED -- stimulated emission depletion

1. INTRODUCTION

Polyphenols are secondary plant metabolites that are found in fruit and vegetables, and in beverages such as juices, beer and wine. Polyphenols have become an intense focus of research interest in the last few decades due to their beneficial effects on human health, and especially in the treatment and prevention of cancer (Guo et al., 2009; Chen et al., 2011; Weng and Yen, 2012) and cardiovascular diseases (Kuriyama et al., 2006; Mursu et al., 2008). Additionally, it has been suggested that their beneficial effects include activities that are anti-atherogenic, anti-ulcer, antithrombotic, anti-inflammatory, anti-allergenic, anticoagulant, immune modulating, antimicrobial, vasodilatory, analgesic, and antioxidative (Ozdal et al., 2013, and references therein).

As the polyphenols are components of our diet, it is expected that at some stage they will interact with proteins. There has been accumulating literature data that indicate that polyphenols have high binding affinities for salivary proteins, digestive enzymes, plasma proteins, proteins involved in neurodegenerative diseases, and many others. These interactions of polyphenols with proteins can lead to changes in the tertiary and secondary protein structures, and formation of complex aggregates, depending on the size and nature of the proteins (Gonçalves et al., 2007; de Freitas and Mateus, 2013, and references therein).

From the pharmacological point of view, saliva proteins like proline-rich proteins (PRPs), statherin, histatins, muscins and α-amylase have the first contact with polyphenols in the diet. In the digestive tract, the polyphenols inhibit three main classes of digestive enzymes: lipases (McDougall and Stewart, 2005; Yoshikawa et al., 2002), glycosidases (Nakahara et al., 1993; McDougall et al., 2005, Rohn et al., 2002) and proteases (McDougall and Stewart, 2005;

Griffiths, 1984; Gonçalves et al., 2007). The most probable mode of polyphenol action here is denaturation of these digestive enzymes, which will lead to the loss of their catalytic activities and reduced food digestion. A comprehensive review on this subject was published recently (de Freitas and Mateus, 2013).

The plasma proteins are the most studied proteins regarding the binding of polyphenols, because they can provide insight into the metabolism and transport processes of polyphenols (Xiao, 2013; Xiao and Kai, 2012; Hui et al., 2013).

Polyphenols have high binding affinities for serum albumins, which are extracellular proteins that are at high concentrations in blood plasma and that can act as carriers of several drugs with different molecular targets. Serum albumins are a major constituent of the circulatory system, and they commonly serve as a depository and as transport molecules for many exogenous compounds (Peters, 1996).Over the last decade, there has been a growing interest in the development of dietary polyphenols for potential prevention and/or treatment of patients with Alzheimer's disease, as well as for other neurodegenerative conditions. Many polyphenols can inhibit/ prevent the formation of amyloid, and can even disrupt preformed amyloid fibrils. Weak forces between inhibitor and peptide chains, such as hydrogen bonding and hydrophobic and aromatic interactions, have been suggested to be the driving forces in the anti-amyloidogenic roles of polyphenols (Lekmul, 2010; Porot, 2006; Lewy-Sakin, 2009; Feng et al., 2012).

However, these aspects of polyphenols represent only a few of the proteins to which a lot of attention has been given in recent years, as there have also been growing numbers of studies into interactions between polyphenols and proteins involved in different diseases and disorders. Some polyphenols can inhibit the activities of proteolytic enzymes that have crucial therapeutic

effects in disease processes, such as bacterial colonization, inflammation, angiogenesis, and tumor invasion and metastasis (Bras et al., 2010; Signoretto et al., 2012; De et al., 2011). Although various studies have described disease prevention by polyphenols, the exact mechanisms by which this occurs has not been clarified (Bras et al., 2010).

1.1. Bonds involved in polyphenol-protein interactions

There have been many studies on the influence of the structures of polyphenols and proteins, on their molar ratios and concentrations, on the pH and ionic strength of their solutions, and on the temperatures of interactions between polyphenols and proteins. Interest in the study of the molecular mechanisms and structure-activity relationships involved in polyphenol--protein interactions from the pharmacological point of view (e.g., foodstuffs and beverage astringency) has also been increasing, in the search for an understanding of the potential beneficial roles of polyphenols in human health.

The main forces involved in these interactions will depend on the properties of the polyphenols and proteins, and these include hydrogen bonds, hydrophobic and ionic interactions, and van der Waals forces (Oh et al., 1980; Murray et al., 1994; Baxter et al., 1997; Charlton et al., 2002; Haslam, 1996; Prigent et al., 2003; Bourvellec, 2011). For hydrogen bonding, this occurs between H-acceptor sites of proteins and hydroxyl groups of polyphenols. Indeed, it has been suggested that the driving force behind the interactions of low molecular weight flavan-3-ols with proteins like bovine serum albumin (BSA) and gelatin are mainly hydrogen bonds (Simon et al., 2003; Frazier et al., 2006, 2010; de Freitas and Mateus, 2012; Xiao and Kai, 2013). Furthermore, nuclear magnetic resonance (NMR) and molecular modeling studies have shown that the binding of procyanidin dimer B3 to the hydrophilic site of the 14-amino-acid saliva

peptide IB7 appears to be governed by hydrogen bonding between the carbonyl functions of the proline residues of IB7 and both phenol and catechol -OH groups (Simon et al., 2003; de Freitas et al., 2012). Also, thermodynamic studies of the epicatechin--BSA (Frazier et al., 2006; Skrt et al., 2013) and procyanidin--gelatin (Frazier et al., 2010) systems have indicated that hydrogen bonding is a dominant factor in this binding. For interactions between condensed tannins and BSA, hydrogen bonds have been shown to prevail over hydrophobic interactions (Frazier et al., 2010).

Hydrophobic interactions involve the aromatics rings of polyphenols and hydrophobic sites of proteins. Examples here include the pyrrolidine rings of prolyl residues, and the nonpolar side chains of amino acids such as leucine, lysine or proline. Indeed, proteins rich in proline are very effective in binding polyphenols (Hagerman and Buller, 1981; Hagerman et al., 1980; Asquith and Butler, 1986). Prolines provide a flat, rigid, hydrophobic surface that is favorable for face-to-face stacking interactions with other planar hydrophobic surfaces, such as aromatic rings. At the same time, proline interactions with globular proteins are believed to involve only surface-exposed residues (Baxter et al., 1997; Charlton et al., 2002; Murray and Williamson, 1994; de Freitas et al., 2012).

Other interactions include ionic bonds between positively charged groups of proteins, such as the ε-amino groups of lysine, and negatively charged hydroxyl groups of polyphenols, although these appear to have minor roles in polyphenol--protein interactions (Bourvellec, 2011). Ionic binding has been excluded at acidic and neutral pH (below the pKa values of the phenolic groups) due to the absence of charged groups (Oh et al., 1980). De Freitas and Mateus (2012) observed that the formation of insoluble aggregates between BSA and grape-seed procyanidin

oligomers decreased with increases in ionic strength. Here, an 80% decrease in precipitate was observed when the ionic strength increased from 0.07 M to 0.5 M (de Freitas and Mateus, 2012; Bourvellec, 2011). Sastry and Rao (1990) showed that increasing salt concentrations decrease the binding between 5-*O*-caffeoylquinic acid and the polyphenol-free 11S protein of sunflower seed. In this case, aggregation phenomena are affected by ionic factors, and hydrophilic interactions are dominant over hydrophobic ones. Globular proteins with acidic isoelectric points, such as BSA, are precipitated more readily by tannin at pH 4.9 than at pH 7.8, and basic proteins such as lysozyme have higher affinities for polyphenol binding at higher pH (Hagerman and Butler, 1981). Rawel et al. (2005) also reported higher affinities of BSA for ferulic and caffeoylquinic acids close to the isoelectric point of BSA. However, reduction of the pH from 7 to 4 or 3 had no effects in studies of the binding of 5-*O*-caffeoylquinic acid to BSA (Prigent et al., 2003). Frazier et al. (2006) also failed to find any effects of pH on binding of (-)-epicatechin to BSA. This is also supported by data from Papadopoulou et al. (2005) and Charlton et al. (2002), such that electrostatic interactions are not a major factor in forming the (-)-epicatechin/BSA complex (Bourvellec, 2011).

In conclusion, it is generally accepted that hydrogen bonding and hydrophobic interactions occur during polyphenol--protein recognition, and that these depend on the polyphenol and protein structures and the medium conditions (Oh et al., 1980; Charlton et al., 2002; Frazie et al. 2003; Hagerman et al., 1998, Artz et al., 1987).

In studing polyphenol--protein interactions, we should take into consideration that polyphenols can also self-associate in aqueous solution (i.e., stacking involving aromatic rings), and form colloids, which can influence their protein affinities (Charlton et al., 2002; Pianet et al.,

2008). Proanthocyanidins can aggregate in alcoholic solution, which depends on their structural features and on the ethanol content (Poncet-Legrand et al., 2003). The formation of colloidal particles depends on their critical aggregation concentration, which is a function of the experimental conditions (Zanchi et al., 2007). These authors suggested that at low concentrations, the polyphenols act as single molecules that can interact individually with proteins, while above the critical aggregation concentration, they might interact with proteins as an aggregate, in a more nonspecific way (de Freitas, 2012)

2. METHODS

As indicated above, the interactions of polyphenols with proteins have become a well-studied topic in recent years. These interactions should be seen in terms of small-molecule interactions with proteins. Numerous physicochemical methods have been developed over many years to study the stoichiometry of such binding, and to determine binding affinities, binding energies, conformational changes, kinetics constants, and aggregate formation, for example. Mostly of these studies were designed to investigate noncovalent interactions between small molecules and proteins. However, all of these methods give complementary, but fragmentary, evidence, so there is the need to combine them to address a phenomenon in its entirety. The use of global methods has thus allowed the identification of the driving forces of polyphenol--protein interactions, and the study of their structure/property relationships (Haslam, 1996; Murray et al., 1994; Charlton et al., 2002; Baxter et al., 1997; Xiao and Kai, 2013).

Studies on noncovalent polyphenol--protein interactions have given insight into this mechanism, which involves weak associations that are modulated mostly by hydrogen bonds and hydrophobic interactions (Bourvellec, 2011). Numerous structures of protein targets have

become available through crystallography, NMR and bioinformatics methods, and there is an increasing demand for computational tools that can identify and analyze active interaction sites and suggest potential drug molecules (e.g., polyphenols) that can bind to these sites specifically. The recent addition of advanced physical methods, such as small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS), can now provide information on intramolecular conformations of polyphenol--protein complexes, and have demonstrated the integration of polyphenol moieties inside protein molecules (Zanchi et al., 2008).

Covalent interactions have instead received much less attention, although they have been demonstrated to be important in the structures and reactivity of polyphenol--protein complexes.

2.1. Analytical techniques for small-molecule--protein interactions

2.1.1. In-silico (computational) methods

In-silico-based methods appear to have been the fastest-growing analytical methods over recent decades. These can help in the identification of drug targets via the use of bioinformatics tools if the structure of a protein is available from X-ray crystallography or NMR. *In-silico*-based methods can also be used to analyze target structures for possible binding, and for active sites. This can generate candidate molecules to be checked for structural similarities to known drugs, which can be docked with the target *in silico*, and ranked according to potential binding affinities, with the aim to further optimize these molecules to improve their binding characteristics. By the combination of experimental and informatics techniques, the chance of success through these processes increases, from the identification of novel targets and the elucidation of their functions, to the discovery and development of lead compounds with the desired properties.

Computational tools thus offer the advantage of delivering new drug candidates more quickly and at lower cost. The major roles of such computation in the drug-discovery process are: (1) virtual screening and *de-novo* drug design; (2) *in-silico* ADME/T (i.e., absorption, distribution, metabolism, excretion and toxicity) prediction; and (3) advanced methods for determining ligand--protein binding (Figure 1). *In-silico*-based methods are described in recently published comprehensive review (Fechner, 2014).

2.1.2. Biosensors

Classically, a biosensor has been described as "a self-contained analytical device that incorporates a biologically active material in intimate contact with an appropriate transduction element for the purpose of detecting (reversibly and selectively) the concentration or activity of chemical species in any type of sample" (Arnold, 1988). In these terms, biosensors can be grouped according to their biological element or their transduction element. Despite their different transduction principles (e.g., electrochemical, acoustic, calorimetric, optical; see below), all such biosensors share specific properties (De Corcuera and Cavalieri, 2003). They usually require one of the interacting substances to be immobilized on a surface, which can be either the small molecule or the protein, to create the sensing element. While all such sensor-based techniques only need a small amount of a protein target to be immobilized (a few micrograms), the amounts of small molecules needed can be very high (up to high micromolar concentrations), depending on the affinity of the small-molecule--protein interaction (Figure 2) (Fechner, 2014).

2.1.2.1. Electrochemical transduction sensors

Electrochemistry offers a wide variety of possibilities for the investigation of small-molecule--protein interactions. The most important of these are the use of ampermetric, potentiometric and impedance transducers. One main advantage of electrochemical transduction is the possibility of using relatively simple and cost-effective equipment. The observed signals are usually not strongly dependent on the size of the analyte, which is useful for small-molecule detection. A disadvantage is that the analyte must be redox active when square-wave voltammetry is performed. However, this problem can be overcome by immobilizing a redox-active component on a sensing surface in close proximity to the analyte (Fechner, 2014). More recently the affinities of small molecules towards the amyloid β peptide that has important functions in Alzheimer's disease have been determined using impedance spectroscopy (Grabowska, 2010).

2.1.2.2. Acoustic

Electroacoustic devices used in biosensors are based on detection of a change in the mass-density, elastic, viscoelastic, electric, or dielectric properties of a membrane made of chemically interactive materials in contact with a piezoelectric material. Quartz crystal microbalance sensors measure the mass per unit area by measuring the change in the frequency of a quartz crystal resonator. The combination of such resonance frequency and dissipation measurements is most widely used for analyte detection in the liquid phase. For a more detailed overview, the reader is referred to one of the recent reviews (Cheng, 2012; Speight, 2012).

Quartz crystal microbalance measurements require a small amount of analyte, and they can also be performed in non-transparent solutions. High concentrations of solvent, which are sometimes required in small-molecule screening, do not disturb these measurements. The disadvantages here are the low degree of high-throughput capability, and that the detected sensor

signal is directly dependent on the mass of the detected molecule, which is, by definition, very small. Another notable recent example where a quartz crystal microbalance was used to investigate small-molecule binding to an immobilized protein is the binding of the antioxidant catechin (from green tea) to troponin C (a marker for cardiac failure) (Tadana et al., 2010). Tadana et al. (2010) revealed that several catechin derivatives from green tea bind to the immobilized troponin C subunits, and the affinity constants of these interactions were calculated from the resulting frequency changes (Fechner, 2014).

2.1.2.3. Optical sensors

Optical sensors are based on light of a suitable wavelength for irradiation of a chromophore. Fiber optic probes are used, on the tip of which enzymes and dyes (which are often fluorescent) are co-immobilized. These probes consist of at least two fibers. One is connected to the light source of a given wavelength range to produce the excitation wave; the other is connected to a photodiode, to detect any change in optical density at the appropriate wavelength (De Corcuera and Cavalieri, 2003).

Surface plasmon resonance is an evanescence-based technique, which detects any change in the refractive index of a surface upon binding of an analyte to a sensitive layer. Surface-plasmon-resonance-based techniques can be used to determine kinetic constants directly, and can use a wide variety of surface chemistry (North et al., 2010). One advantage of this technique is that it is very well accepted by the scientific community, especially for determining kinetic rate constants; alternatively, a disadvantage of surface plasmon resonance is that the size of the analyte affects the amount of signal generated by the sensor.

Reflectometric techniques do not only monitor refractive-index changes upon binding, but also changes in the physical thickness. Sensors based on reflectometry have been used for fragment-based screening. Using biolayer interferometry, reproducible matches were observed for targets JNK1, which has been implicated in diabetes, and IF4E, an important modulator of disease progression in oncology (Wartchow et al., 2011). In addition to overlapping matches obtained from surface plasmon resonance and biochemical assays, compounds uniquely identified with biolayer interferometry have been observed (Wartchow et al., 2011).

2.1.2.4. Calorimetric transducers

Calorimetric transducers measure the heat of a biochemical reaction at the sensing element. These devices can be classified according to the way the heat is transferred. The principles involved are described below (see 'Isothermal titration calorimetry'), whereby isothermal calorimeters maintain a reaction cell at constant temperature using Joule heating or Peltier effects (De Corcuera and Cavalieri, 2003)

2.1.3. Cell-based methods

Cell-based methods have emerged from the advances in molecular biology methods and genetic engineering. These methods can be regarded as a link between the relatively artificial methods, which rely on the use of isolated ligands and proteins, and the whole-animal models. This means they are closer to reality than fully artificial systems, and they are easier to handle and require less intensive care than animal models. Most commonly, a transgenic cell line is created by introducing new genetic material. This new genetic material is designed as a 'reporter gene', which makes the cell generate a detectable signal (e.g., fluorescence, antibiotic resistance) when exposed to a specific stimulus, e.g. the chemical stimulus of a small molecule (Himmleret al,

1993). The reporter gene can either be temporarily introduced (i.e., transient transfection) or can be persistent (i.e., stable transfection), to generate a 'reporter cell line'. Either bacterial or eukaryotic cells can be used for this process.

With these reporter-gene assays, as well as detecting a single small-molecule species, it is also possible to detect whole groups of substance classes, including their metabolites that share the same effects (and not necessarily with structural similarities). This behavior has been referred to as 'effect-directed analysis' (Schmitt et al., 2013), which indicates that it is not possible to determine whether a small molecule itself has the observed effect, or whether the small molecule must be metabolized to have that effect. It is even possible to use genetically unmodified cells and monitor their 'behavior' (e.g., morphology, growth, change in refractive index caused by second-messenger activity) when exposed to small molecules (Figure 3). A comprehensive overview of methods that provide deeper insight into such cellular effects for small-molecule characterization can be found in Feng et al. (2009).

2.2. Analytical techniques for understanding the mechanisms of small-molecule--protein interactions

2.2.1. Fluorescence emission spectrometry

Fluorescence emission spectrometry has been widely used to investigate interactions between polyphenols and proteins. Proteins are considered to have intrinsic emission fluorescence, which is mainly due to their tryptophan, tyrosine, and phenylalanine residues. The fluorescence emission spectral characteristics change when these aromatic residues are exposed to different solvent conditions. There have been numerous studies of polyphenol--protein interactions performed by fluorescence quenching (Liang et al., 2013). From the fluorescence emission

spectrometry (fluorescence quenching of intrinsic protein fluorescence by polyphenols) the Stern-Volmer constant, the number of binding sites and the van't Hoff enthalpy of binding can be obtaine (Figure 4). In the combination with other methods such as isothermal titration calorimety the thermodynamic profile of inteactions and the nature of polyphenol--protein interactions can be evaluated.

2.2.2. UV--Vis absorption spectrometry

UV-Vis absoption spectrometry is a relatively easy and widespread technique that is used to explore structural changes of proteins and their formation of complexes. The absorption spectra of proteins show two main absorption peaks: at 200 nm, which reflects the framework conformation of the protein; and at 280 nm, which represent the aromatic amino acids. The polyphenols also absorb in the UV/Vis range. Their UV/Vis spectra are generally attributed to electronic transitions between π-type molecular orbitals (Anouar, 2011).

The study of interactions between procyanidin B3 and lysozyme is an example of this technique, where procyanidin B3 has strong absorption at around 200 nm. After its interaction with lysozyme, the intensity peak of lysozyme at 200 nm decreases markedly, as its maximum absorption wavelength shows an apparent red shift (from 200 nm to 205 nm). These results indicate that the conformation of the peptide backbone unfolds and the hydrophobicity of the microenvironment changes because of the strong binding of B3 to lysozyme (Glazer & Smith, 1961). Meanwhile, a slight decrease in absorbance at around 280 nm confirms the complex formation, through the interaction of procyanidin B3 with lysozyme (Liang et al., 2013).

2.2.3. Circular dichroism

Circular dichroism (CD) arises from the difference in adsorption of left and right circularly polarized light. CD is a valuable tool, especially for showing changes in the conformation of proteins. The far-UV CD spectrum of proteins reflects the characteristics of their secondary structure. As such, CD spectra can be readily used to estimate the fraction of a molecule that is in an α-helix conformation, a β-sheet conformation, a β-turn conformation, or some other conformation (e.g., a random coil conformation) (Greenfield, 2006; Whitmore, 2008). The near-UV CD spectrum (>250 nm) of proteins provides information on their tertiary structure, due to the absorption, dipole orientation, and nature of the surrounding environment of the phenylalanine, tyrosine, cysteine (or S-S disulfide bridges) and tryptophan amino-acid residues. The near-UV CD spectrum cannot be assigned to any particular three-dimensional structure. Additionally, the near-UV CD spectra provide structural information on the nature of the prosthetic groups in proteins; e.g., the heme groups in hemoglobin and cytochrome c. CD gives less specific structural information than X-ray crystallography and protein NMR spectroscopy, but it is a quick method that does not require large amounts of a protein or extensive data processing. Thus CD can be used to survey a large number of solvent conditions, varying temperature, pH and salinity, and the presence of various cofactors. CD spectroscopy has often been used in combination with other techniques to study polyphenol binding to proteins, to obtain the binding constant, stoichiometry of binding and other thermodynamic parameters in addition to insight into the structural changes induced by this binding (Figure 5) (Skrt et al., 2013; Brás et al., 2010).

Of the many examples in the literature of the study of polyphenol--protein binding using CD spectroscopy, only a few can be mentioned here. The CD spectrum of native lysozyme

shows two negative bands, at 208 nm and 222 nm that are characteristics of the α-helical structure of the protein (Brás et al., 2010). Rocyanidin B3 binding to lysozyme causes a red-shift of the band at 208 nm. These changes demonstrate that the binding of rocyanidin B3 to lysozyme disrupts the native secondary structure of the protein upon association. The content of the protein secondary structure elements can then be estimated by applying different deconvolution algorithms (Wallace, 2009).

Synchrotron radiation CD spectroscopy extends the use of laboratory-based CD spectroscopy through the high light flux from a synchrotron, which enables the collection of data at lower wavelengths, detection of spectra with higher signal-to-noise ratios, and measurements to be carried out in strongly absorbing nonchiral components, such as salts, buffers, lipids and detergents (Wallace, 2009).

2.2.4. Fourier transform infrared spectroscopy

Fourier transform infrared (FTIR) spectroscopy is a technique that can provide great insights into the molecular mechanisms of polyphenol--protein interactions. It is noninvasive, and it can provide potent dynamic and structural information of such interactions. The advantages of FTIR spectroscopy are that it can be applied to both soluble proteins and membrane proteins, it is cost effective, and it looks simultaneously at the whole protein as well as at specific sites. Also, there is no need to label the protein, and there is no perturbation of the original protein by an inserted label (Wu and Bird, 2010). FTIR spectroscopy is very well suited for the study of different sizes and types of proteins. In contrast, using NMR spectroscopy it is not possible to study large proteins, and using X-ray crystallography it is difficult to study membrane proteins (Mildvan and Cohn, 1996).

As an alternative to transmission-mode techniques, there is the attenuated total reflection technique (Rudbeck et al., 2009; Wright and Varderkooi, 1997). In this technique, the IR beam is guided in an IR-transparent crystal by total reflection. The protein under study can be in direct contact with a bulk water phase, as this is not sensed by the IR beam. The sample can therefore be manipulated during the experiment; e.g., by the addition of agents which might change the properties of the protein (Kakihana and Okamoto, 1984). In the literature, there are many examples of the use of this attenuated total reflection technique for studying polyphenol--protein interactions (e.g., Kanakis, et al. 2011). Polyphenol--β-lactoglobulin complex formation was characterised using IR spectroscopy and its derivative methods. As there was no major spectral shift upon polyphenol interactions for the protein amide I band at 1660 cm^{-1} (mainly C = O stretch) and the protein amide II band at 1530 cm^{-1} (C--N stretching, coupled with N--H bending modes) (Kanakis, 2011), the difference spectra (i.e., [protein solution + polyphenol solution] -- [protein solution]) were obtained to monitor the intensity variations of these vibrations.

Similarly, IR self-deconvolution with second derivative resolution enhancement and curve-fitting procedures has been used to determine protein secondary structures in the presence of polyphenols (Hasni, 2011).

2.2.5. Nuclear magnetic resonance

NMR is mainly used to determine the structures of molecules (Morris 1986; Becker et al., 2007). With the development of this technique, NMR has become suitable for investigations into interactions between small molecules and proteins. Rademacher et al. (2008, 2011) used NMR to study norovirus infection. Virus infection processes of cells with virus-like particles have also been investigated (Mari et al., 2005; Pereira et al., 2009). Potenza et al. (2011) reported on a

NMR study of living cancer cells. Furthermore, information about aggregation states of investigated proteins (e.g., important for Alzheimer's disease) (Tycko, 2011) and about protein dynamics (Hussain, 2013; Krushelnitsky et al., 2013) and stability can be obtained. More detailed insights into such applications of NMR techniques is provided in more-specialized NMR reviews (Meyer and Peters, 2003; Cala et al., 2013; Bewley and Shahzad-ul-Hussan, 2013; Bhunia et al., 2012).

When investigating the interactions between small molecules and proteins using NMR, changes in the chemical shifts of the protein signals are monitored after binding of the small molecules to the proteins. Two-dimensional NMR (e.g., heteronuclear single quantum coherence spectroscopy, heteronuclear multiple-bond correlation spectroscopy) can help to identify protein regions that small molecules interact with, although these techniques can be time consuming and challenging in terms of signal interpretation. Alternatively, signals of small molecules that interact with substantially larger proteins can be monitored, and there are several fast one-dimensional approaches that can provide sufficient information (e.g., saturation transfer difference spectroscopy, water-ligand observed via gradient spectroscopy). For these techniques, nuclear Overhauser effects can be used to transfer magnetization from the protein to the small molecule. Three particular techniques for the investigation of small-molecule--protein interactions are transferred nuclear Overhauser effect spectroscopy, saturation transfer difference spectroscopy, and water-ligand observed via gradient spectroscopy (Pellecchia et al., 2008; Bieri et al, 2011). The major disadvantage of these techniques, however, is the time that is needed to monitor the chemical shifts of the proteins to identify the binding domains (Cavanagh et al., 2007; Billeter et al., 2008; Lescop et al., 2009). In addition, the amounts of protein that are

needed for NMR are still relatively high. Recently, the combination of NMR with molecular modeling has also been used successfully to understand binding processes at the molecular level (Gonçalves et al., 2011).

2.2.6. X-ray diffraction

X-ray diffraction is best known as a standard technique for determination of molecular structures. This technique can be used to resolve chemical structures that range from a few daltons (Fitzpatrick et al., 1993) to a theoretically unrestricted upper molecular weight (Blundell et al, 2002), and it can be used to resolve complexes containing different molecules. This resolution of complexes thus makes X-ray diffraction perfectly suited for investigations into small-molecule--protein interactions.

The basic principles of X-ray diffraction have changed little since its development over 60 years ago (Kendrew et al., 1958), although it has been extended and varied. X-ray diffraction uses an X-ray irradiation source to produce a beam (alternatively, electrons or neutrons can be used; Smyth and Martin, 2000; Wlodawer et al., 2008; Blakeley et al., 2004), which is then diffracted by the sample of interest. The diffraction patterns can then be used to reconstruct the three-dimensional structure of the sample. For this, the sample has to be in a crystallized state.

When using X-ray diffraction to investigate small-molecule--protein interactions, there are several possible ways to prepare the relevant samples: (i) the small molecule and the protein can be co-crystallized; (ii) the protein can be crystallized alone, and then the crystal can be soaked in a solution of the small molecule; and (iii) the protein can be crystallized with a low-affinity ligand, and then be soaked in a higher-affinity ligand. Each of these methods of creating the sample to be diffracted has advantages and disadvantages, however, and as for NMR,

structural information for both the protein and the small molecule is obtained (Turnbull and Emsley, 2013). Crystallizing the protein alone and then soaking it with small molecules is generally regarded as the most resource-effective method, as the crystallization process has to be optimized only once, and it is even possible to compare the structures of the apo-protein with the small-molecule--protein complexes. However, the soaking can destroy the crystal entirely, or it can lead to false-negative results if there is not enough structural flexibility left for the binding of the small molecule within the crystal (Skarzynski and Thorpe, 2006). Co-crystallization with a small molecule has the advantage that the small-molecule--protein complex is often more stable than the apo-protein. Additionally, the crystallization process can be considered closer to the native state, as it is performed in the liquid phase. This method is less suitable, however, for low-affinity interactions, when the high concentrations of the small molecule required can disrupt the crystallization process (Hassell et al., 2007). All of these crystallization methods can help to provide the three-dimensional structure of the protein, the binding pocket of the protein, and the small molecule, and as such, false-positive results are not obtained. It is also possible to investigate very low affinity interactions, whereby affinities down to 5 mmol L^{-1} create no particular problems (Davies and Tickle 2012). On the other hand, large amounts of very pure protein are needed, and the crystallization process can be challenging (or even impossible in the worst-case scenario) and time consuming, and therefore the throughput will always be limited (Chayen and Saridakis, 2008). However, the latest developments in robotics and laboratory automation have increased the throughput, and X-rays can also be used in primary screening (Chilingaryan et al., 2012; Erlanson, 2012).

2.2.7. Mass spectrometry

Mass spectrometry is a method for detection and identification of unknown molecules that can range from small molecules to nanoparticles. These methods are sensitive, fast, have low sample consumption, and can be easily automated, and thus used for high-throughput approaches. The basic principle of mass spectrometry remains the same for investigating small-molecule--protein interactions: instead of a single molecule, the small-molecule--protein complex is ionized and then detected (Lim and Lord, 2002). However, there are some challenges associated with using mass spectrometry for this purpose, because the structure of the protein should remain in its native state, and be minimally affected by the ionization. In the worst case scenario, ionization can cause the interaction between the small molecule and the protein to be completely destroyed. This can be especially problematic for hydrophobic interactions, because no water is present in the gaseous phase and some additives can strengthen the ionic and electrostatic forces, while weakening hydrophobic and van der Waals forces. Another challenge is that the buffer should be compatible with both the protein and the ionization technique required for mass spectrometry, otherwise peak broadening and 'ion suppression' can occur (Annesley, 2003). In many cases, it is not possible to find a compatible buffer system. A related challenge is that sometimes the shift in mass as a result of the small molecule in relation to the larger mass of the protein is not sufficient to clearly discriminate between the protein itself and the small-molecule--protein complex. However, if these challenges can be overcome, it is possible to determine the binding stoichiometry and even its affinity with low sample consumption (less than femtomolar) (Kitova et al., 2012), and very rapidly. Probably the most promising ionization method is electron-spray ionization mass spectrometry (ESI-MS) (Fenn, 2002). NanoESI-MS was used to reveal the

allosteric mechanism of the binding of different inhibitors to fructose 1,6-bisphosphatase, which is a potential target in type 2 diabetes. Fructose 1,6-bisphosphatase is a tetrameric enzyme that consists of four identical subunits. The nanoESI-MS spectra showed the formation of a noncovalent complex with four ligands (Cubrilovic et al., 2013). The Hill coefficient was determined by titration, and positive cooperativity was shown. ESI-MS has also been used in fragment-based screening for drug discovery (Maple et al., 2012). Fully automated nanoESI-MS methods with high throughput can be designed to screen hundreds of potential drug candidates in a short time. An example can be seen for the 157 phenylpyrazole-derived compounds that were screened against the anti-apoptotic protein Bcl-xL, a protein that has a function in tumour progression (Figure 7) (Maple et al., 2012). Another interesting application is the coupling of affinity columns to mass spectrometry; with this approach, it has been possible to find new ligands for the nuclear receptors (Riu et al., 2011). This method has a huge potential to study the polyphenol-protein interactions.

2.2.8. Isothermal titration calorimetry

Calorimetric methods are one of the oldest methods used in analytical (and bioanalytical) chemistry. Calorimetry has been used in numerous applications, and a detailed overview of the current applications is given in Chai et al. (2010). In particular, the advantages of calorimetric methods have attracted much interest regarding drug discovery and drug design (Ladbury et al., 2010). Isothemal titration calorimetry (ITC) directly measures the heat of interactions between two reactants; e.g., for a small molecule and a protein. The main advantage of calorimetric methods for investigating small-molecule--protein interactions is the amount of information generated per sample; it is possible to gain information about enthalpy, entropy, affinity, specific

heat capacity, and stoichiometry in a single analysis (Figure 8). As it was mentioned before, the affinity of polyphenols binding to protein (constant of binding) can be obtained by applying the above mentioned *in-silico* and spectroscopic methods. As ITC information is generated in a homogenous phase and without labelling, the information can be assumed to be particularly accurate when compared with the equivalent information obtained by other methods.

Additionally, the molecular weights of the measured reactants do not affect the strength of the signal. The basic principle of this method is explained here; for a more detailed explanation of the functional principles, see Perozzo et al. (2004).

However, ITC has some disadvantages. These analyses usually take several hours, with limited potential for automation or high-throughput screening, and the amounts of the reactants needed are usually relatively high. Although this might not be a problem in terms of the small molecule, the availability of the protein is limited in some cases. Other limitations of these methods are the result of the intrinsic properties of the measured system; namely, the product of the affinity and the concentration of the protein (i.e., the Wiseman constant), on which the affinity has the greatest effect, and which should be approximately 10 to 100 in the optimum case. These disadvantages are encountered as a result of recent developments that have been focused on the reduction of the volumes of the measurement cells down to a few hundred microliters, which reduces the amount of protein needed to around 10 μg (Falconer and Collins, 2010). Another technique that is known as continuous ITC uses continuous injection of the small molecule, which leads to more data points and thus reduces the statistical error and the time needed per analysis (Markova and Hallen, 2004). For high-affinity systems, the limiting Wiseman constant can be circumvented by varying the experimental conditions, e.g., the pH or

temperature, to reduce the affinity of the system (Doyle, 1995). A more elegant method of circumventing this limitation is displacement titration, where a lower-affinity small molecule is pre-incubated with the protein and then displaced by titration with the higher-affinity small molecule (Velazquez-Campoy and Freire, 2006).

Recent examples where ITC has been used in combination with other techniques to investigate small-molecule--protein interactions include tea catechines as inhibitors of catalase (Pal et al., 2014) and lipase (Wu et al., 2013). Liu et al. (2014) investigated the binding of (-)-epigallocatechin-3-gallate to bovine lactoferrin (Liu et al., 2014).

2.2.9. Differential scanning calorimetry

Differential scanning calorimetry (DSC) is a thermodynamic technique for studing thermally induced transitions of biological macromolecules, such as transitions between folded and unfolded states of a protein, between single-stranded and double-stranded DNA, and between phases for lipids. Reviews on this technique are available (Bruylants et al., 2005 and references therein).

DSC measures the excess heat capacity of a solution (C_p) of the molecule of interest as a function of temperature. The transition is recognized as a sharp endothermic peak that is centered at the T_m, and the maximum in C_p occurs directly at the T_m. Integration of the C_p *versus* T curve yields the transition enthalpy (ΔH°_m), and the shift in the baseline yields the ΔC_p. DSC is the only method for direct determination of ΔH°_m (Figure 9). The ΔH°_m is a net value from a combination of endothermic contributions, such as the disruption of hydrogen bonds, and exothermic contributions, such as the disruption of hydrophobic interactions. The sharpness of the transition peak can be measured as the width at half-peak height, and this provides an index of the

cooperative nature of the transition from native to denatured state (i.e., a two-state or multistate process). If denaturation occurs within a narrow temperature range, the transition is considered to be highly cooperative. Fitting of the progress curve will yield the van't Hoff enthalpy (ΔH_{vH}), which can be different from the calorimetric enthalpy, ΔH°_{m}. As indicated above, the calorimetric enthalpy is the total enthalpy change, which includes the contributions from all of the processes and is determined independently of any model, while the corresponding van't Hoff enthalpy assumes a simple two-state transition. By comparing these two enthalpies, this allows determination of whether the transition occurs as a two-state or a multistate process. The interactions of ligands or proteins with proteins induce changes in the intramolecular and intermolecular interactions, and in the dynamics of all of the components present. An increase in T_m will be observed if a ligand binds preferentially to the native state of a protein, as this will be stabilized. A decrease in T_m relative to T_m^{o} will be observed if the ligand binds to the unfolded state of the protein. The DSC method for measuring binding constants is indirect, compared, for example, to ITC (www.uic.edu; Bruylants et al., 2005).

2.3. Analytical techniques for small-molecule--protein aggregates

2.3.1. Light scattering

Protein aggregation can be directly measured by light scattering techniques, which include dynamic light scatterering (DLS) and nephelometry (Figure 10). From DLS measurements, the translational diffusion coefficient of particles related to the hydrodynamic radius of aggregates can be determined. An example of polyphenol-induced protein aggregation is the formation of lysozyme--procyanidin B3 aggregates (McRae and Kennedy, 2011). An increase in the size of these aggregates was observed after addition of procyanidin B3, and then the size increased

slowly with further increasing procyanidin B3, which indicated that the complex is formed by the strong binding affinity of procyanidin B3 to proteins.

Nephelometry is used to detect the amounts of insoluble aggregates, based on the amount of light scattered at 90° (Liang et al., 2013). Nephelometry can be conducted in the same manner as fluorescence quenching assays, except for the detection method. During nephelometry, a fluorometer can be used as a 90° light-scattering photometer, with both excitation and emission wavelengths that are the same (400 nm), which enables the determination of light scattered by the particles in solution. At this wavelength, protein and procyanidins do not absorb the incident light. The relative aggregation is calculated for each analysis, as the ratio between the scatter intensity of the measured sample and that of the most turbid sample (Bras et al., 2010). The concentration and size of the dispersed insoluble aggregates increases with increasing molar concentrations, as in the case of procyanidin B3, which indicates the aggregation interaction between lysozyme and procyanidin B3 (McRae and Kennedy, 2011).

2.3.2. Small-angle X-ray scattering and small-angle neutron scattering

Small-angle scattering is a fundamental method for structure analysis of biological macromolecules, nanocomposites, alloys, and synthetic polymers (Svergun and Koch et al., 2003). SAXS and SANS are complementary techniques to small-angle scattering (Neylon, 2008; Petoukhov and Svergun, 2007). In the case of X-rays, the scattering contrast originates from spatial fluctuations of the electron density, while neutron contrast arises from the atomic nuclei without any systematic dependence on atomic number. As a result, these techniques provide complementary structural information.

These methods have been used to study the physical state of tannins in water--ethanol model mixtures, and to study mixtures of human salivary PRPII-1 and tannins in model water--ethanol solutions (Zanchi et al., 2008). PRPs are major components of stimulated saliva production in the mouth, and they interact with the tannins in food. SAXS in combination with ESI-MS and DLS has been used to study the interactions of EGCG with a basic salivary PRP, B35(Canon, 2013; Pascal et al., 2008). Astringency is one of the most important organoleptic qualities of numerous beverages, including red wines. It is generally believed to originate from interactions between tannins and salivary PRPs. Furthermore, the interactions between a glycosylated PRP, known as PRPII-1, and flavan-3-ols have been studied in aqueous solutions and at the colloidal level by DLS and SAXS (Canon, 2013; Pascal et al., 2008).

The adventage of SAXS/SANS measurements is that it can be easily performed in different conditions by adding ligands or binding partners, and by changing physical and/or chemical characteristics of the solvent to provide information on the structural responses. The technique provides kinetic information about processes like folding and assembly and also allows one to analyze macromolecular interactions (Svergung and Koch et al., 2013).

2.3.3. Microscopy

2.3.3.1. Atomic force microscopy

Atomic force microscopy is a useful techniques to follow protein aggregation, which can be induced or prevent by different small molecules, including the polyphenols. Aggregation and fibrillation of proteins, and the subsequent pathological deposition of fibrillized protein, are related to many age-related diseases, like Alzheimer's disease and type 2 diabetes mellitus. Due to the awarenes that a healthy diet rich in polyphenols slows down neurodegenerative diseases,

several classes of amyloid inhibitors have recently been tested to understand the mechanisms of amyloid fibril formation. However, it has become apparent that amyloid fibrils and oligomers assemble and have their cytotoxic effects at the cell membranes, rather than in bulk solution. In the literature, atomic force microscopy has been used to follow protein aggregation. This has shown that EGCG is a much less efficient amyloid inhibitor at a phospholipid interface than in bulk solution (Engel et al., 2012). Moreover, EGCG cannot promote the disaggregation of existing amyloid fibrils at a phospholipid interface, in contrast to its behavior in bulk solution. These results indicate that interfaces significantly affect the efficiency of inhibition by EGCG inhibitors, and they should therefore be considered during the design and testing of amyloid inhibitors (Engel et al., 2012).

2.4. New methods to study small-molecule--protein interactions

2.4.1. Ultrafast dynamics of single molecules

The detection of individual molecules has found widespread application in molecular biology, photochemistry, polymer chemistry, and quantum optics and super-resolution microscopy. Tracking of an individual molecule in time has allowed the identification of discrete molecular photodynamic steps, the action of molecular motors, and protein folding and diffusion, which can be taken down to the picosecond level. However, methods to study ultrafast electronic and vibrational molecular dynamics at the level of individual molecules have emerged only recently (Brinks et al., 2014). In the review published by Brinks et al. (2014), several examples of femtosecond single-molecule spectroscopy were presented, starting with basic pump-probe spectroscopy in a confocal detection scheme, followed by deterministic coherent control approaches using pulse shapers and ultra-broad-band laser systems. Brinks et al. (2014)

presented the detection of both electronic and vibrational femtosecond dynamics of individual fluorophores at room temperature, to show electronic (de)coherence, vibrational wavepacket interference, and quantum control (Figure 11). Finally, they also present two-color phase shaping, as applied to photosynthetic light-harvesting complexes. This has allowed the investigation of persistent coherence in photosynthetic complexes under physiological conditions, at the level of individual complexes (Brinks et al., 2010, 2014).

2.4.2. Quantum dots in bioanalysis

Semiconductor quantum dots are brightly luminescent nanoparticles that have found numerous an application in bioanalysis and bioimaging, and a comprehensive review was published recently (Petryayeva et al., 2013). These authors highlighted recent developments in these areas, in the context of specific methods for fluorescence spectroscopy and imaging. Quantum dots have been used in combination with steady-state and time-resolved spectroscopic techniques, to develop a variety of assays, bioprobes, and biosensors that function through changes in quantum-dot photoluminescence intensity, polarization, and lifetime.

Fluorescence resonance energy transfer (FRET) describes the excitation energy exchange between two adjacent molecules over distances that typically range from 2 nm to 10 nm. This process depends on the dipole-dipole coupling of these molecules, and its probability of occurrence cannot be proven directly. Fluorescence is mainly used for quantification, as it represents a concurring process of relaxation of the excited singlet state, so that the probability of fluorescence decreases as the probability of FRET increases. This reflects the closer proximity of the molecules, or an orientation of the donor and acceptor transition dipoles that facilitates FRET (Mueller et al., 2013). With the discovery of fluorescent proteins, and with their improvement

toward spectral variants and their usability in plant cells, a tool box for *in-vivo* FRET analyses in plant cells was provided. FRET then became applicable for *in-vivo* detection of protein-protein interactions and for the monitoring of conformational dynamics.

Recently, FRET couples of two fluorescent proteins have been supplemented by additional fluorescent proteins for FRET cascades, to monitor more complex arrangements. Novel FRET couples that involve switchable fluorescent proteins promise to increase the use of FRET, through combinations with photoactivation-based super-resolution microscopy (Mueller et al., 2013). Quantum dots can be combined with flow cytometry, including traditional cell analyses and spectrally encoded barcode-based assay technologies. The advantages of quantum dots is that they can be used across different platforms for biological fluorescence imaging, which now include: epifluorescence, confocal, and two-photon excitation microscopy; single particle tracking and fluorescence correlation spectroscopy; super-resolution imaging; near-field scanning optical microscopy; and fluorescence lifetime imaging microscopy (Petryayeva et al., 2013).

2.4.3. Super-resolution imaging

Single-molecule fluorescence spectroscopy and super-resolution microscopy are important techniques due to their resolution at the level of biochemical and cellular processes. The Nobel Prize in Chemistry 2014 was jointly awarded to Eric Betzig, Stefan Hell, and William E. Moerner “for the development of super-resolved fluorescence microscopy” (Möckl et al. 2014). Conventional fluorescence microscopy techniques (e.g., confocal microscopy) do not allowed observation of details at the near-molecular scale, which instead become visualized with super-resolution optical techniques. These techniques retain the advantages of fluorescent marker

technology, and have a spatial resolution that approaches 10 nm, meaning that objects that are just over 10 nm apart can be distinguished, which was previously only attainable with electron microscopy (Soeller et al., 2013). Demands placed on the photophysical properties of the fluorophores are stringent, and these drive the choice of the appropriate probes in terms of the spectroscopic parameters, redox properties, size, water solubility, photostability, and several other factors, as indicated in a recent review (Ha and Tinnefeld, 2012). Fluorescent probes, as both organic fluorophores and fluorescent proteins, are used in super-resolution imaging, particle tracking, and single-molecule FRET (Ha and Tinnefeld, 2012). The stimulated emission depletion (STED) fluorescence microscopy techniques that utilize narrowing of fluorescent spot by modulation of transitions between two molecular states provides super resolution imaging by selectively deactivating fluorophores (Harke et al., 2008).

3. CONCLUSIONS

Polyphenol--protein interactions can be studied using many different physicochemical and biochemical methods that are used more in general for the study of biopolymer--ligand interactions. Methods with high-throughput capabilities, e.g., *in-silico* screening, mass spectrometry, biosensors, and cell-based systems, are probably best suited for primary screening. Afterwards, positive screening targets need to be further investigated using other methods. For a general understanding of how single small (polyphenol) molecules interact with their target proteins, methods such as fluorescence emission spectrometry, UV-Vis adsorption spectroscopy, CD and FTIR, in additional to calorimetric methods such as ITC and DSC, are generally used, while methods such as mass spectrometry, X-ray diffraction, and NMR are probably the most promising. Methods like DLS, SAXS, SANS and microscopy are recommended for protein

aggregation studies. Newly developed techniques based on observation of single molecules, such as super-resolution confocal microscopy, will be the next step in the study of small-molecule--protein interactions. Other factors that will affect the decision of what method to choose include the availability of the method and the related costs. The specialization and expertise of the operators of such equipment will also contribute to the decision as to which method to use.

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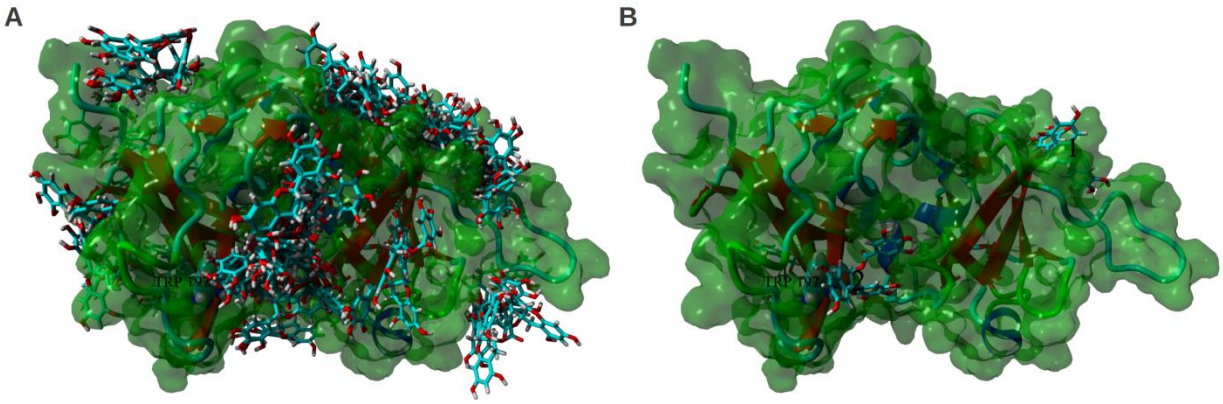

Figure 1. Autodock blind-docking of the (--)-epigallocatechin-3-gallate (EGCG) to the Staphylococcus enterotoxin B (SEB) protein target. (A) 100 EGCG poses. (B) The four selected EGCG poses and the assignment of the binding sites. Adapted from Benedik et al. (2014).

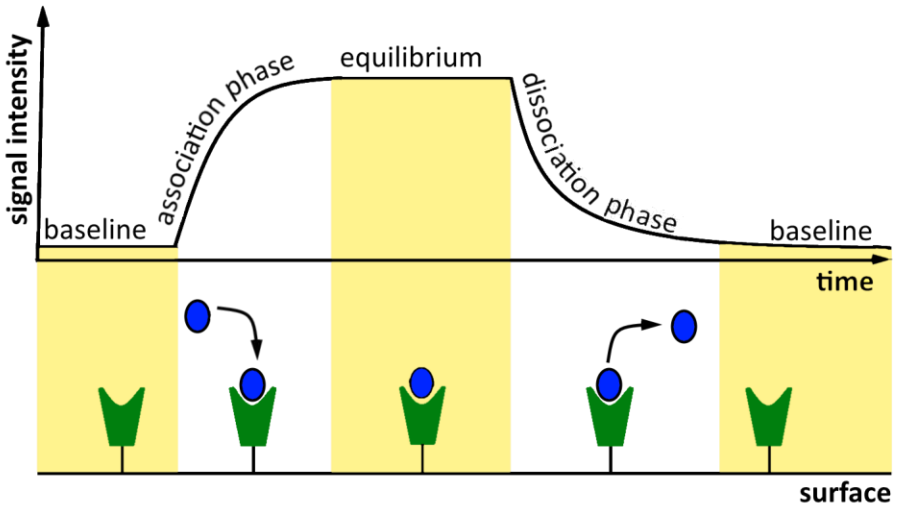

Figure 2: Shematic sensogram consists of base line, binding of small molecule to the surface -- association phase, equilibrium phase, dissociation phase and baseline. Adapted from Fechner (2014).

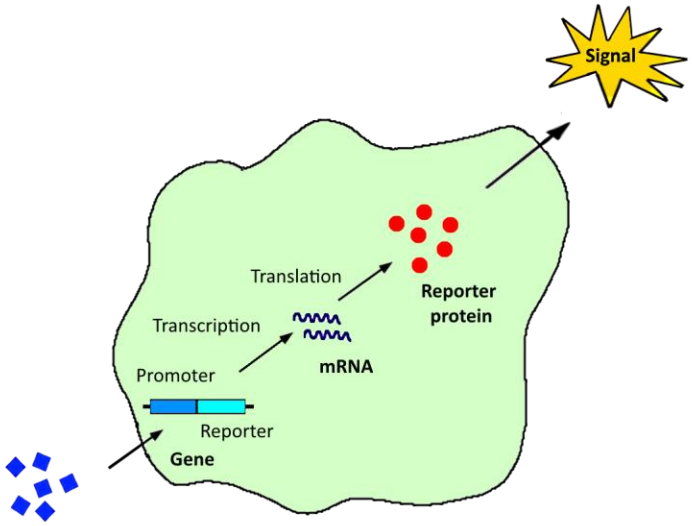

Figure 3: Reporter gen assay. Adapted from Feng et al. (2009).

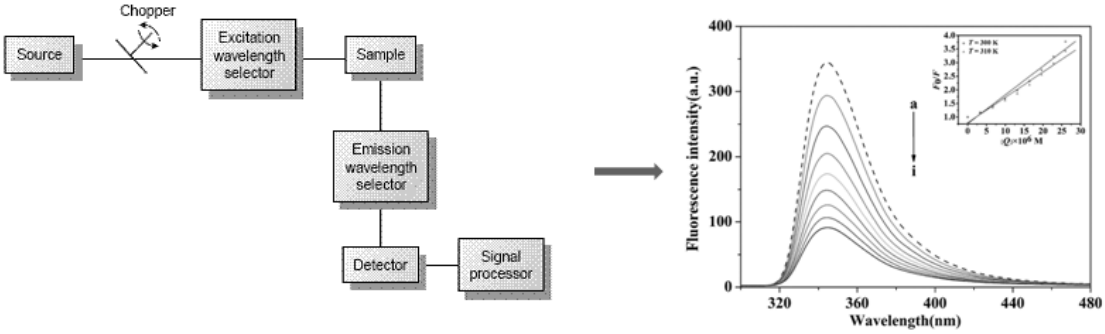

Figure 4: Principle of fluoriescence measurements. Adapted from manufacturer's informational materials.

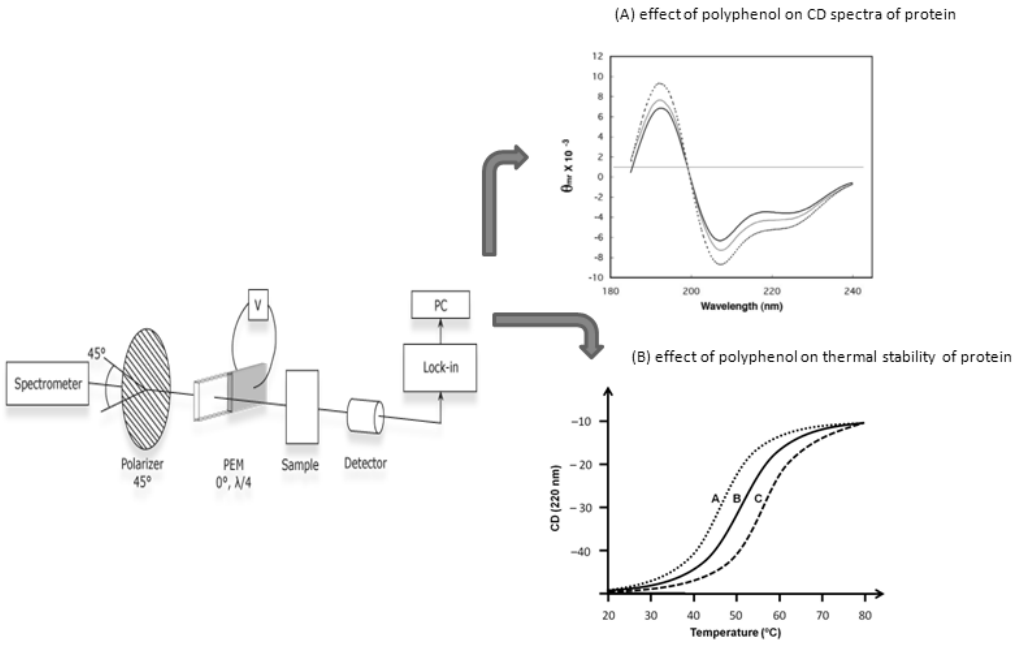

Figure 5: Principle of CD measurements. Effect of polyphenols on CD spectra of protein at constant temperature (A) and effect of ligand on the thermal profile of protein at constant wavelength (B). Adapted from manufacturer's informational materials.

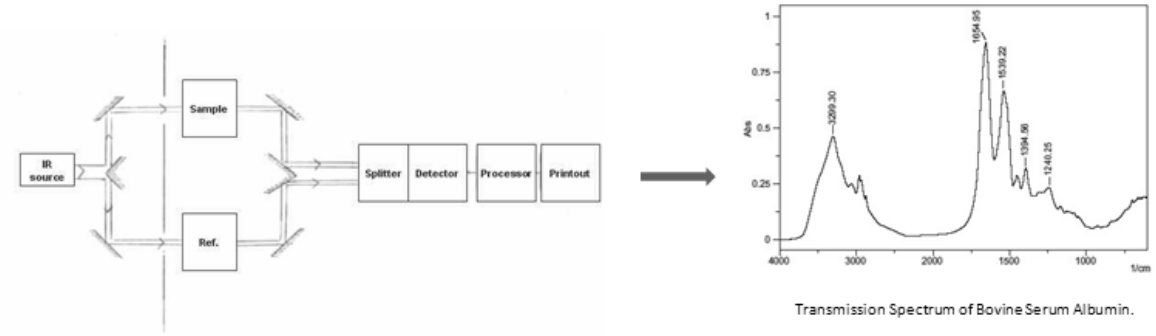

Figure 6: Principle of IR measurements and transmission spectrum of bovine serum albumin.

Adapted from manufacturer's informational materials.

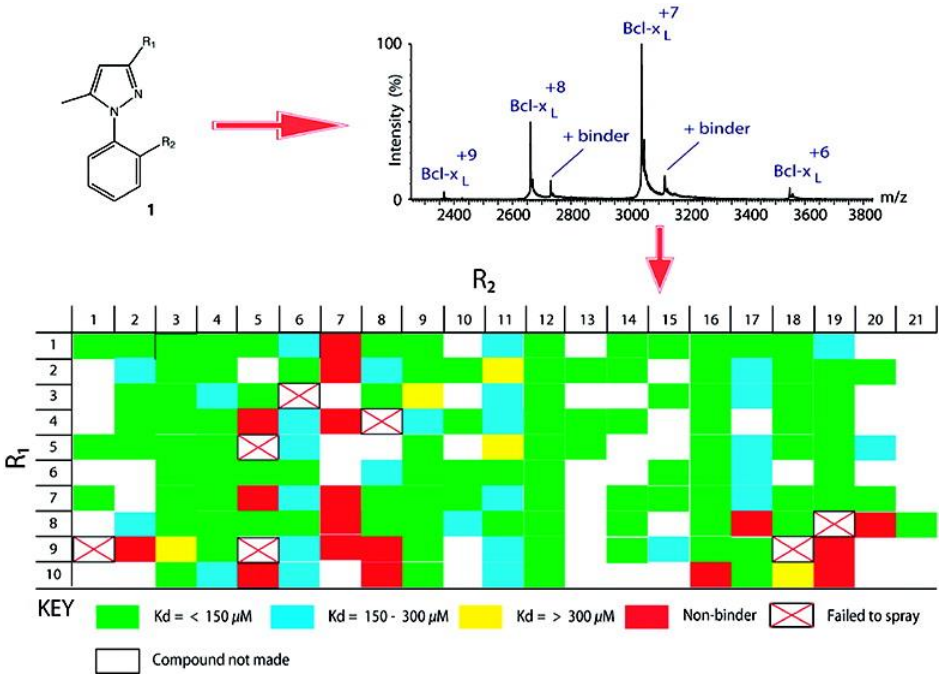

Figure 7: Results from the MS screen of libery of phenylpyrasole-derived compounds against Bcl-x_L at 2:1 ligand/protein ratio. Each row corresponds to a diferent chemical functionality at R_1 and each column to a different chemical functionality at R_2. Compounds have been color-coded according to their measured K_D values and are grouped as shown in the key. *"Reprinted with permission from Maple et al., 2012. Copyright 2012 American Chemical Society."*

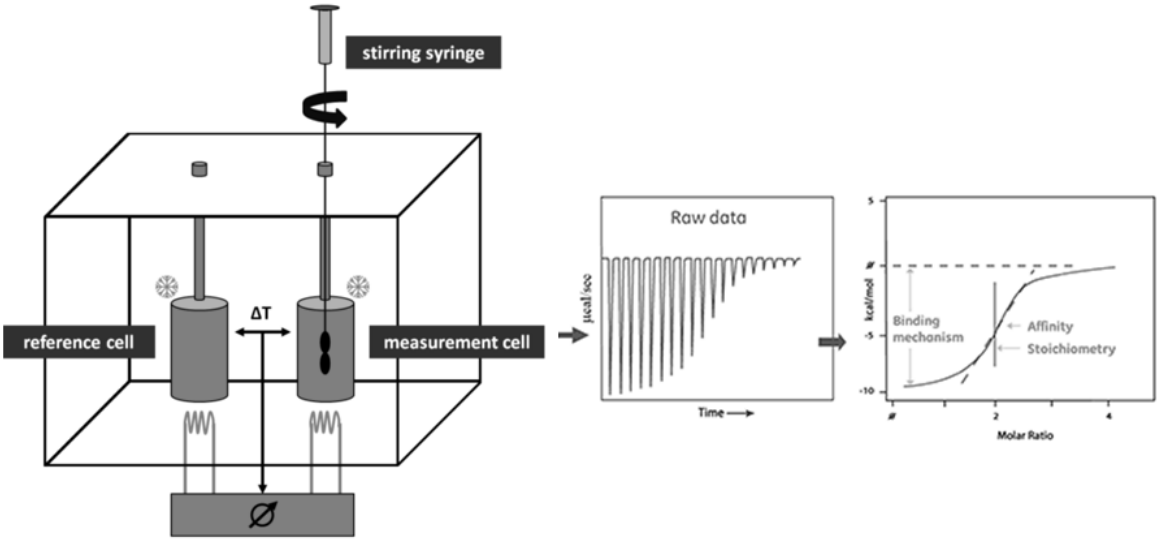

Figure 8: Principle of ITC meaurements and thermodynamic parameters obtained. Adapted from manufacturer's informational materials.

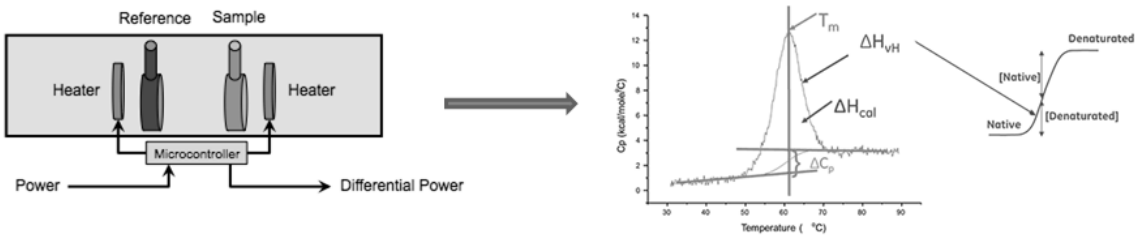

Figure 9: Principle of DSC measurements and thermodynamic parameters obtained from thermogram. Adapted from manufacturer's informational materials.

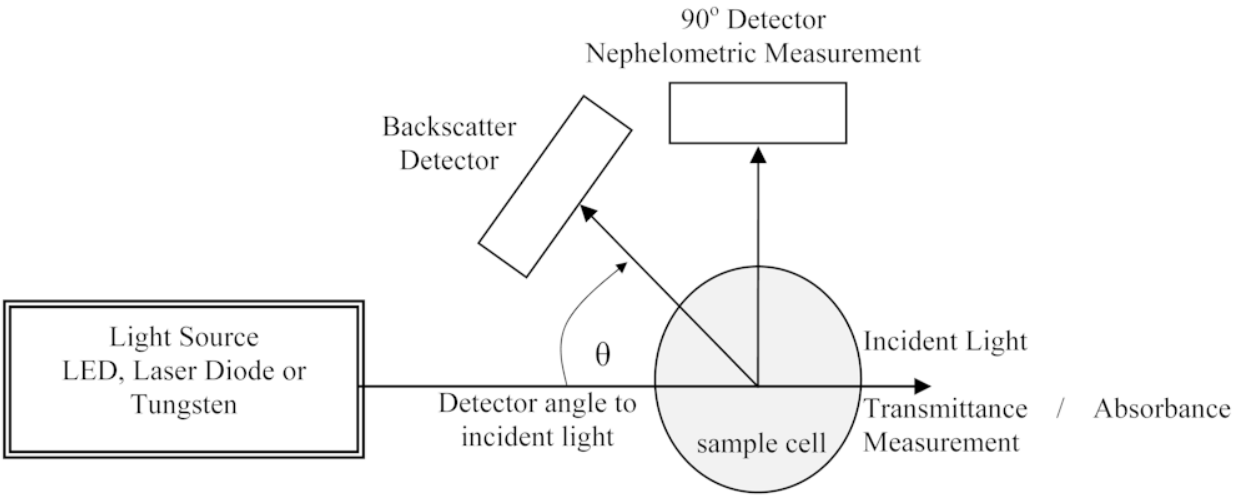

Figure 10: Turbidity measureing techniques. Adapted from Liang et al. (2013).

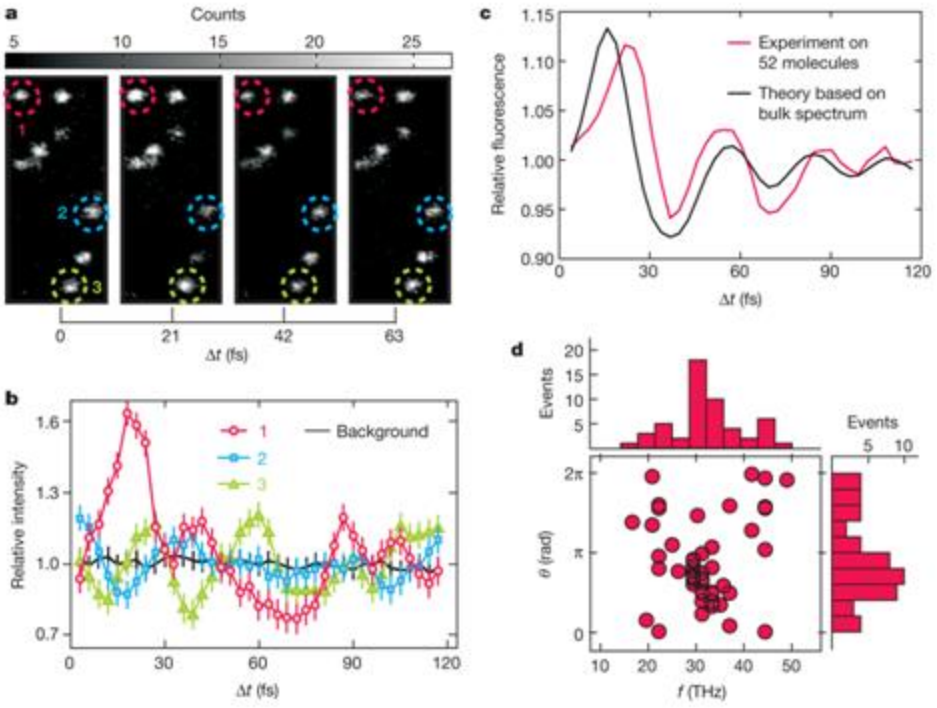

Figure 11: Single-molecule wave-packet interference. **a**, Fluorescence images of single molecules excited with two mutually delayed (Δt), phase-locked laser pulses. **b**, Integrated intensity as a function of Δt for the fluorescence emission of the three molecules marked in **a**. A typical background trace is shown for reference. The traces are normalized to their respective average in order to visualize fluctuations in the intensity. Error bars, ±1 s.d. **c**, Averaged response of 52 molecules compared to the theoretical prediction based on the bulk absorption spectrum.**d**, Result of the Fourier analysis of 52 single-molecule traces. Distributions and scatter plot of the main frequency component (f) and its corresponding phase (θ). Marker size and bin width include the experimental errors. *Reproduced from Ref. Brinks et al. (2010) with permission from Nature Publishing Group.*

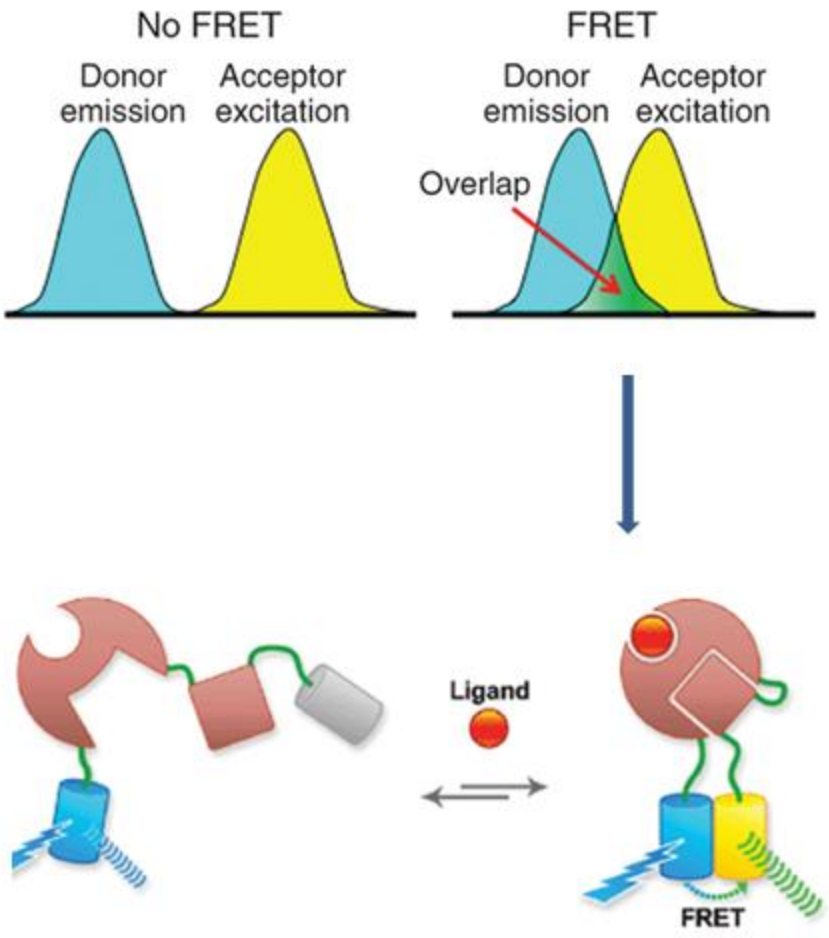

Figure 12: Shematic illustrating the FRET technique. FRET-based biosensors based on a ligand-dependent protein--protein interaction. Adapted from Frommer et al. (2009).

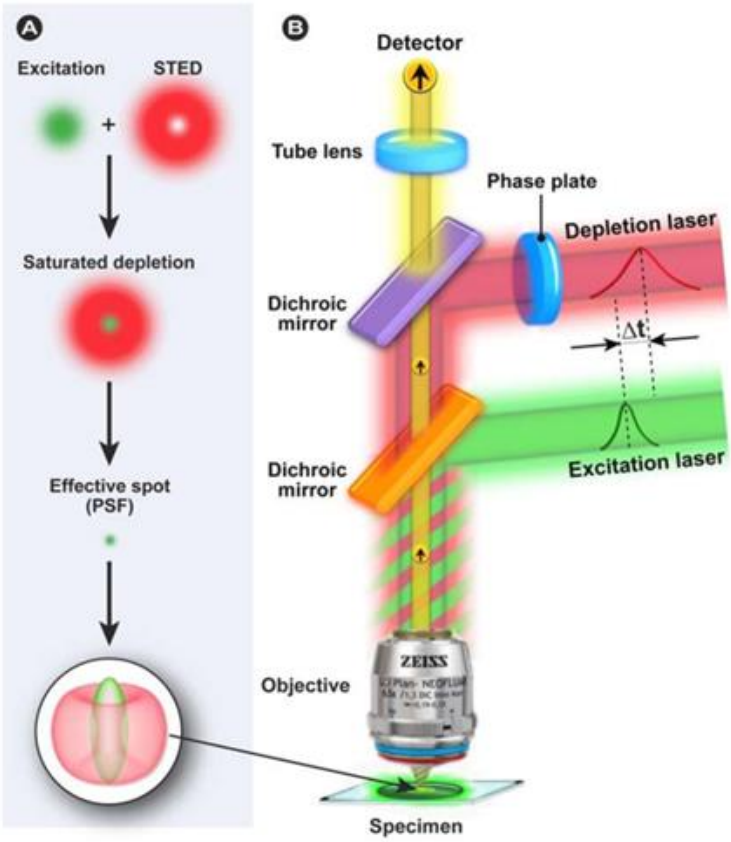

Figure 13: Principle of stimulated emission depletion (STED) microscopy. (A) STED is based on shrinking the excitation focal spot by depleting the outer excited state fluorochromes through stimulated emission with a doughnut-shaped STED beam of red-shifted and Δt time-shifted light (B). In essence the excitation point spread function (PSF) is combined with the PSF of the STED depletion laser (B) to produce a resultant PSF that is smaller than the diffraction limit of light. *Reproduced from Ref Ishikawa-Ankerhold et al. (2012) with permission from Molecular Diversity Preservation International (MDPI).*