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Narrative Review

A Comprehensive Review of the Aggregated Proteins through Routine Optical Studies Aiming to be Replaced by Aurone-specific Derivatives

Sirvan Abbasbeigi [1*]

[1] Molecular Biology/Biochemistry Field of Study, Islamic Azad University (IAU), Science and Research Branch, Sanandaj, Kurdistan, Iran

*** Corresponding Author:** Sirvan Abbasbeigi, Molecular Biology/Biochemistry Field of Study, Islamic Azad University (IAU), Science and Research Branch, Sanandaj, Kurdistan, Iran. E-mail: nemesis.student@gmail.com

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Abstract

Students often recognize biochemistry as a research-oriented system. It is effortless to see the spread of this view because everyday students pay more attention to what their instructors concentrated on and witness professors' basic research in the laboratory. Meanwhile, biochemistry science depends on research activities that are fundamental and theoretical, but always alongside very practical or applied aspects as well. In amyloid studies nearly 30 years ago, many probes have emerged exhibiting various responses to amyloids, such as intensity changes, shifts in fluorescence maxima, and variations in lifetimes, among many others. These probes have shed light on a variety of topics, including the kinetics of amyloid aggregation, the effectiveness of amyloid aggregation inhibitors, the elucidation of binding sites in amyloid structures, and the staining of amyloid aggregates in vitro, ex vivo, and in vivo. Therefore, this study is considered as one of the most debatable subjects currently being used in different investigations. This review is based on published studies associated with aggregated protein probes and detectors (whether synthetic or intrinsic ones). It has also tried to cover the common concern introducing the most applicable ones In vitro/vivo. Furthermore, this glance tries to look into several well-known chemical and biochemical methods to follow protein aggregation assembly compared with lately used protocols. Interestingly, the provided content does not intend to prioritize either intrinsic staining methods or synthetic ones but also attempts to illustrate a parallel pathway and ultimately express an expanded point of view.

Keywords: Biochemistry, Protein, Aggregation, Probe, Synthetic, Intrinsic Characteristics

Introduction

Proteins play notable roles inside cells. In fact, most proteins have to be spherical to perform their biological function[1] (Figure 1). The stability of the folded proteins in the environment outside the living organism and identifying factors that comprise stability are significant for their use in medicine and industry, respectively.[2] Considering the various factors such as proper molecule concentration, pH, ionic strength, oxidation and reduction potential, and temperature can lead to negative consequences in the native state.[3] The conditions mentioned earlier must be considered carefully to work in extracellular circumstances and also to prevent changes in covalent and non-covalent protein forms.[4] These modifications can be directed to changes in the spatial structure of the protein, aggregation, and finally, loss of biological activity in the proteins.[2,5] At the molecular level, many of the biological mechanisms of cells are performed by proteins; proteins accomplish their functions by interacting with each other in an exact and controlled manner.[6] The molecular basis of most diseases is due to defects in the normal function of related proteins, through which the mechanisms of cell life are possible.[7] If the function of proteins is abnormal, it can cause various diseases. Any change in the spatial structure of proteins not associated with peptide bond destruction is considered abnormal.[8] It means that the final stage of abnormality leads to the formation of a completely free polypeptide structure. The protein misfolding can occur under various physical conditions such as high or low temperatures and pH treatments.[9,10] The most common way to estimate the stability of a spatial protein structure can be to shift the balance toward protein abnormalities. The old methods of measuring stability are thermal

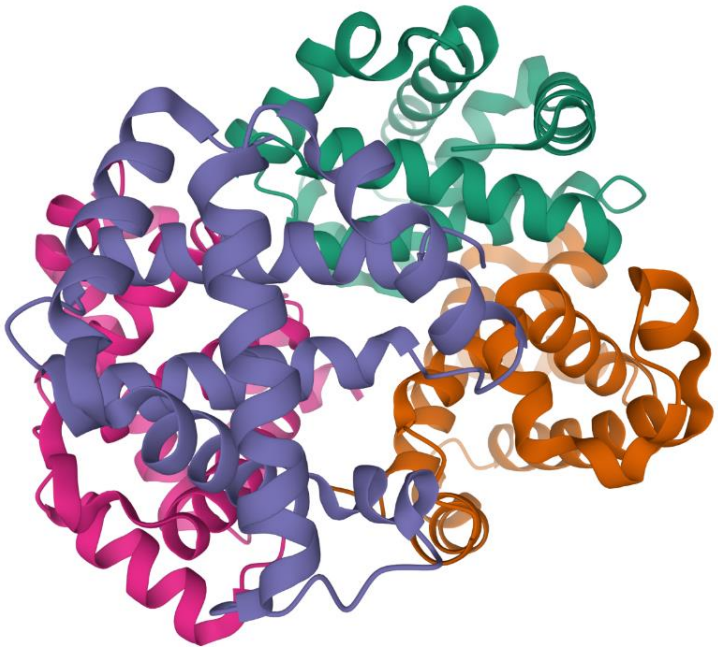


Figure 1. The Crystal Structure of Recombinant Human Adult Hemoglobin. This illustration created by Protein Data Bank | PDB ID: 6KYE.

abnormalities and solvent abnormalities.[11] Solvent abnormalities include abnormalities with urea and guanidium hydrochloride.[12] This study has concentrated on synthetic compounds (Congo red, Thioflavin T, and polyphenol derivatives) to detect protein aggregation regarding other primary methods such as turbidimetry, fluorescent, and Circular Dichroism (CD) studies. In this review, extensive investigations in PubMed, Scopus, and Google Scholar have been performed using keywords, including misfolded proteins, congo red, thioflavin T/S, polyphenols, flavonoids, and aurone structures. Accordingly, the most important research papers about this subject based on the quality and level of pieces of evidence have been collected, categorized, and discussed.

Protein Aggregation Monitoring

From a scientific point of view, protein accumulation is a marvelous process. Today, it has become clear that protein accumulation is involved in the pathogenesis of many abnormalities.[13,14] In this large group of diseases, known as amyloid diseases, one or more intracellular proteins form amyloid. Those well-known amyloid diseases or neurodegenerative disorders can be listed, including Alzheimer's disease, Parkinson's disease, Huntington's disease, type 2 diabetes, prion disease, etc.[13,15]

Turbidimetry Study

Protein aggregation is when abnormal protein molecules bind together to form unbranched fibers or amorphous aggregates.[16] Conformational and colloidal stability of proteins is among the influential factors controlling the accumulation process.[17] The accumulation and formation of protein masses; especially, in high protein concentrations, is a competitor for the correct folding of the protein. Furthermore, it is the main factor for reducing the efficiency of the correct and functional structure of the protein.[18] Due to the kinetic competition of correct folding and aggregation, partial or complete aggregation inhibition by any factor will increase the folding efficiency and regular activity of the target protein.[16,19] It is noteworthy that abnormal proteins have less solubility than natural proteins in the buffer, thus scattering the reflected light.[20,21] Therefore, the intensity and rate of formation of insoluble protein aggregates can be studied by measuring the apparent increase in light absorption and using the turbidity technique.[22,23] It seems that the rate of absorption or scattering of light at the appropriate wavelength is proportional to the amount of protein present in insoluble protein masses. Regarding the variety in shape, size, density, etc., there is a significant difference in choosing the appropriate optical wavelength for the kinetic study of

insoluble protein aggregates, in which 340 to 600 nm is used more.[24] There is a direct relationship between the apparent absorption and the amount of protein participating in the accumulation process.[25] It means that by observing an increase in the initial protein concentration due to heat or during re-folding of the protein, the apparent absorption at the mentioned wavelengths increases. However, only in a narrow range of visible wavelengths is a perfectly linear relationship with our high correlation coefficient between apparent absorption and protein concentration.[26] Consequently, to study the aggregation processes, the appropriate wavelength should be selected for each protein separately.[19,22,27] A better understanding of the phenomenon of aggregation is essential for applications such as protein refolding, formulation, and stability of pharmaceutical products and finding appropriate solutions to slow down or stop the progression of aggregation-related diseases.

Fluorescence Study

Many biochemical systems are photoluminescence, meaning they can be excited by electromagnetic radiation and subsequently emitted by radiation of either the same wavelength or another wavelength.[28] Two types of photoluminescence include fluorescence and phosphorescence.[29] Remarkable information has been obtained about the three-dimensional structure of the protein and the mechanisms involved by using the fluorescence technique.[30] The electrons in the atom or molecule occupy the lowest available energy level, in which case the base is paired with the opposite spin. Electron transitions from the ground state to higher energy levels occur in the range of ultraviolet and visible light.[31] Two groups of fluorophores are used to study the structural properties of proteins by fluorescence. The first group is the intrinsic fluorophores or side chains of aromatic amino acids present in the protein structure. The fluorescence of proteins is derived from the residues of phenylalanine, tyrosine, and tryptophan. In proteins containing all three aromatic amino acids, fluorescence is usually determined by the proportion of tryptophan residues. This is actually because most of these hydrophobic amino acids, including aromatics, relocate to the inner parts of the protein and create a hydrophobic environment as the driving force of folding.[32] In addition, the quantity and quality of intrinsic fluorescence of the proteins are positively affecting by environmental conditions such as solvent type, pH, silencer sequence, polarity, and ionic strength of the solvent.[33] Therefore, intrinsic protein fluorescence is an efficient way to study structural changes in proteins. The second category includes fluorophores that are added to the system (protein solution or any other solution) such as 1-Anilinonaphthalene-8-sulfate (ANS) and 5-(dimethylamino)naphthalene-1-sulfonamide (DNSA) to bind or interact with large molecules to provide somehow valuable information about the spatial structure or even kinetics.[34,35] An external fluorescent agent is remarkable when there is no intrinsic fluorophore in studying the structure of a protein.[36] Secondly, the placement of intrinsic fluorophores in the studied molecule should be such that while changing the spatial structure of the molecule, the position of the intrinsic fluorophore and its spectroscopic properties do not change much.[37] This phenomenon is used as a standard criterion for detecting the mass of partial intermediates of folded proteins.[38] The preferential interaction of these fluorophores with well-balanced equilibrium and kinetic interfaces over the natural state and the fully open state of the protein is well established (Figure 2).[39]

Circular Dichroism (CD) Study

One of the applications of CD is to study and evaluate various concepts related to the structure of proteins.[40,41] The study of the far region in ultraviolet light (mainly 190 to 240 nm) is used to calculate the secondary structure of proteins;[42] in this region, the adsorbent group is a peptide bond. A weak but broad transition ($n \rightarrow \pi^*$) occurs in the 210 nm region, and a strong transition ($\pi \rightarrow \pi^*$) happens at about 190 nm.[39] The type of spectrum recorded in the area varies depending on the pattern of secondary structure. The alpha helix spectrum has two minimum points at 208 and 222 nm. On the other side, beta-sheets are characterized by a minimum signal at a wavelength of 215 nm, subsequently, a non-structured polypeptide chain due to a maximum point at 210 nm.[43,44] In the near-ultraviolet region, aromatic amino acid chains (phenylalanine, tyrosine, and tryptophan) are absorbed in the range of 250 to 290 nm.[40,45] The CD spectrum of the proteins containing all three structures combines the secondary structure's spectral properties.[46] The position of the aromatic amino acids such as phenyl-alanine, tryptophan, and tyrosine reflects the tertiary

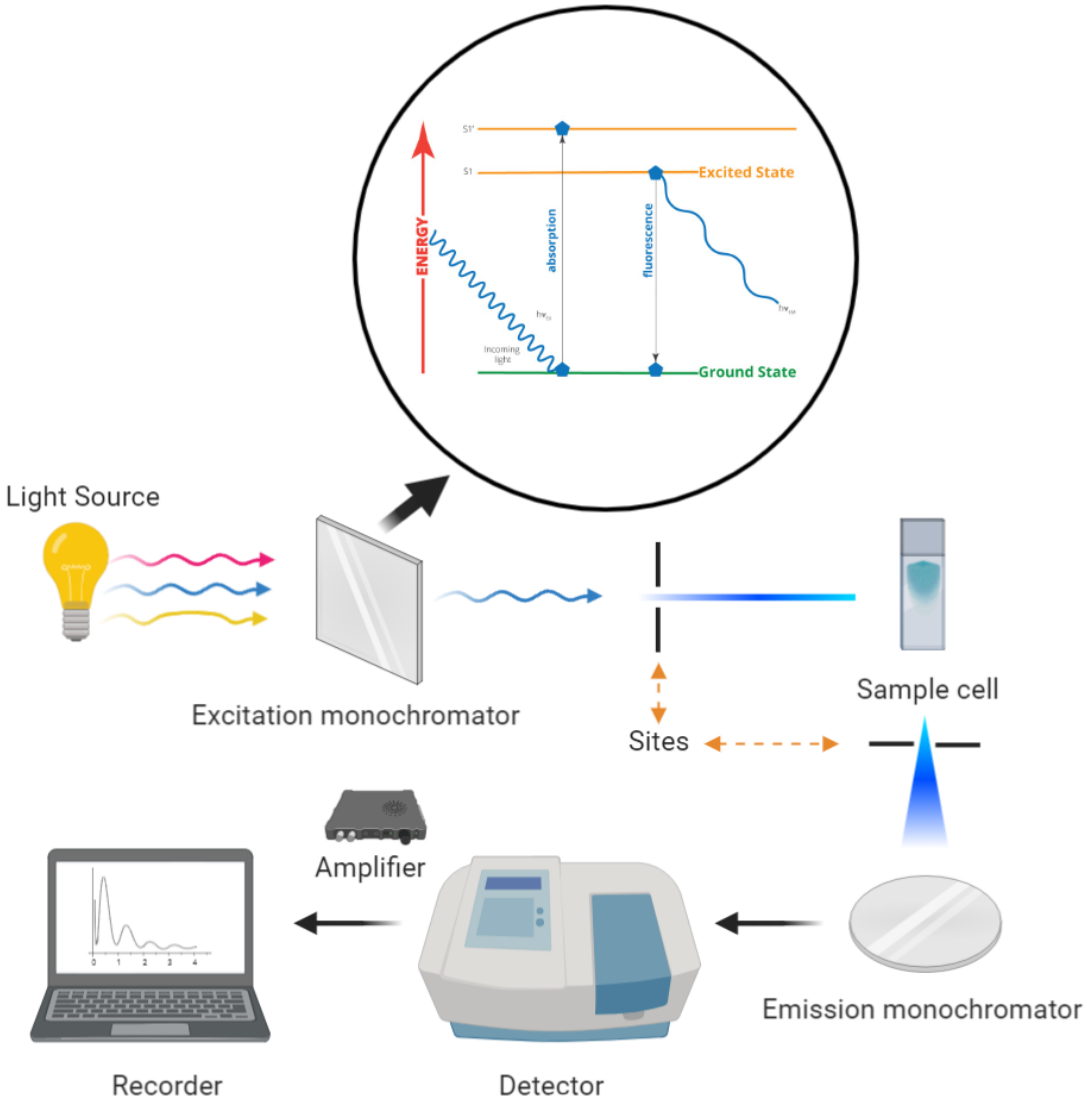


Figure 2. Fluorescence Spectroscopy Uses a Beam of Light that Excites the Electrons in Molecules of Certain Compounds and Causes Them to Emit Light. That light is directed towards a filter and onto a detector for measurement and identification of the molecule or changes in the molecule. This illustration created by BioRender.

structure of the proteins.[47,48] A trace of the tertiary and native structure of the proteins can be obtainable by placing the side chain of the mentioned amino acid units in asymmetric environments with the help of the circular dichroism technique.[49] Amino acids have circular dichroism absorption spectra at specific wavelengths; for example, tryptophan peaks at about 290 nm, tyrosine at 275-285 nm, and phenylalanine at 255-275 nm.[50]

Misfolded Protein Detectors

Congo Red (CR)

Congo red compound with an IUPAC name disodium; 4-amino-3-[[4-[4-[(1-amino-4-sulfonatonaphthalen-2 yl) diazenyl]phenyl]phenyl]diazenyl]naphthalene-1-sulfonate is a salt that dissolves easily in water but dissolves more easily in organic conditions such as ethanol. Congo red tint is characterized by binding to amyloid fibrils;[51] therefore, the presence of amyloid can be proven by this stain in turn. Congo red absorption in the presence of amyloid fibrils finds a shift to higher wavelengths;[52] however, the mechanism of interaction between concord and amyloid fibrils is still not well understood. Congo red is a flat molecule with both polar and non-polar sections (Figure 3). This molecule has several active groups by which it interacts with amyloid fibrils.[53] For example, Congo red molecules are stabilized by electrostatic interactions between negatively charged sulfate groups and positively charged amino acid roots or by the forces of turbulence, and Congo red molecules enter the beta plate surface.[54]

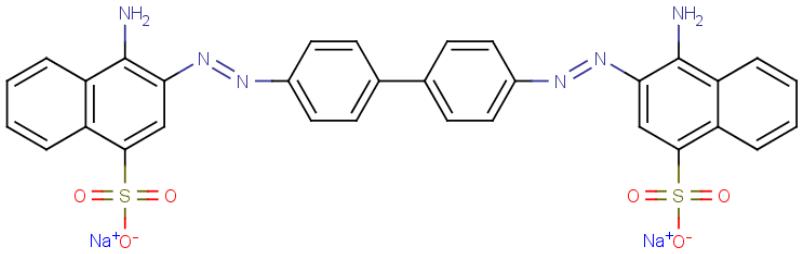


Figure 3. Congo Red Core Structure. This illustration created by MarvinSketch.

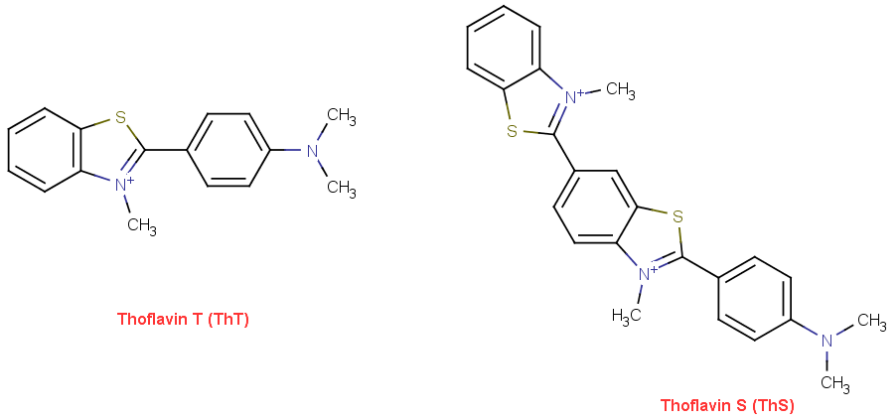


Figure 4. Thioflavin T (ThT) and Thioflavin S (ThS) Core Structures. This illustration created by MarvinSketch.

There is evidence that Congo red interacts with myeloid fibrils of different proteins by different mechanisms.[52]

Thioflavin T (ThT)

Thioflavin T (ThT) is a benzothiazole salt that is gathered by two benzothiazole rings, benzene and also a dimethylamine group.[55] Subsequently, it is formed by the methylation of Dehydrothiotoluidine via methanol in the presence of chloric acid.[56] In addition to Congo red, other pigments that bind specifically to amyloid fibrils can be counted, such as ThT.[57] This dye is commonly used to detect abnormal fibrils both in the laboratory and in the living environment.[58,59] In 1959, two scientists named Vassar and Culling introduced ThT, which can bind to amyloid fibrils.[60] Additionally, it is demonstrated that this compound can form micelles in aqueous media, followed by an increase in fluorescence emission after specific binds to the amyloid aggregations.[61] The structure of thioflavin T has a hydrophobic end along with a dimethylamino structure attached to a phenyl group and also connected to another polar structure, referring to the benzothiazole group composed of the polar N and S directions (Figure 4).[62] This hydrophobic and polar structure for thioflavin T allows the formation of micelles in aqueous media. In an aqueous medium, these rings rotate around the C-C bond quickly and have low fluorescence activities. By entering an environment with high viscosity, fluorescence emission increases due to the limited rotation of the rings relative to each other.[63] the ThT micelles are bound by hydrophobic and ionic interactions along amyloid fibrils, which increase fluorescence emission.[56] Interestingly, ThT does not interact with natively folded and unfolded proteins and interacts explicitly with proteins in the amyloid state.[56,64]

Synthetic Probe

There have been some reports that a drinking compound (*R*) may help protect against Alzheimer's disease.[65] The health benefits of using *R* are due to polyphenolic compounds (called resveratrol) that are part of this drink.[66,67] *R* contains a wide range of polyphenols that may interact with specific peptides and proteins.[68] Likewise, these compounds have a wide range of properties for the prevention and treatment of various diseases and also have correlated

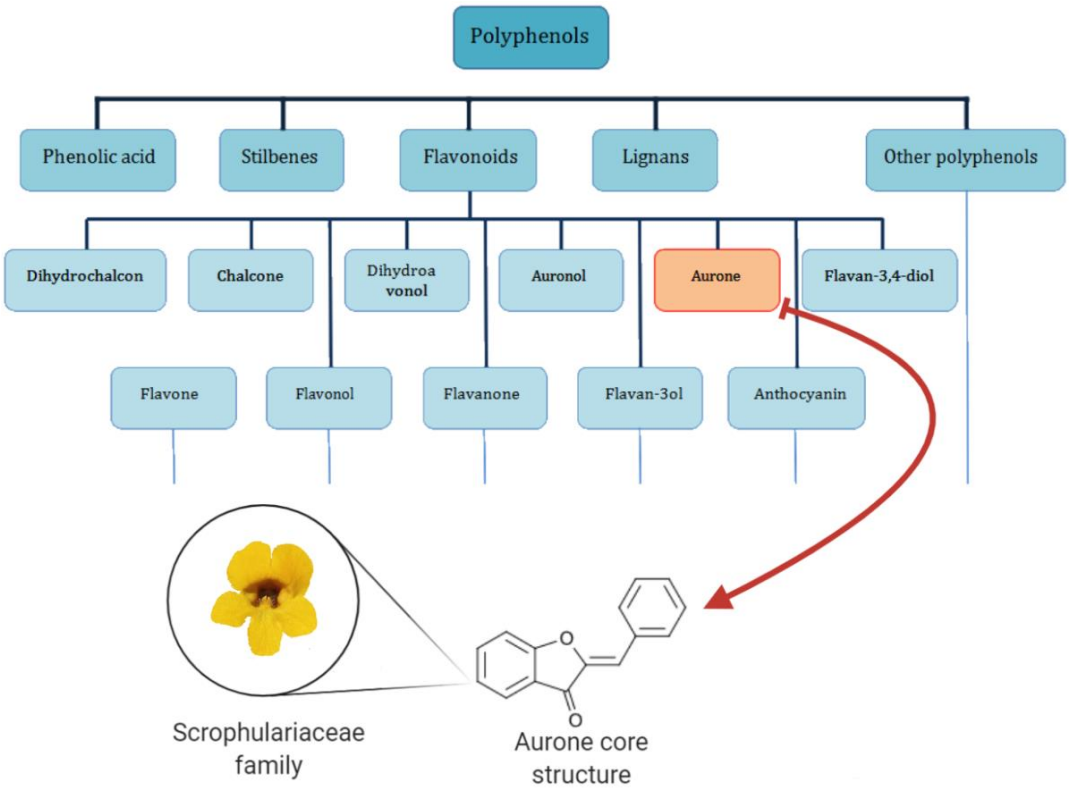
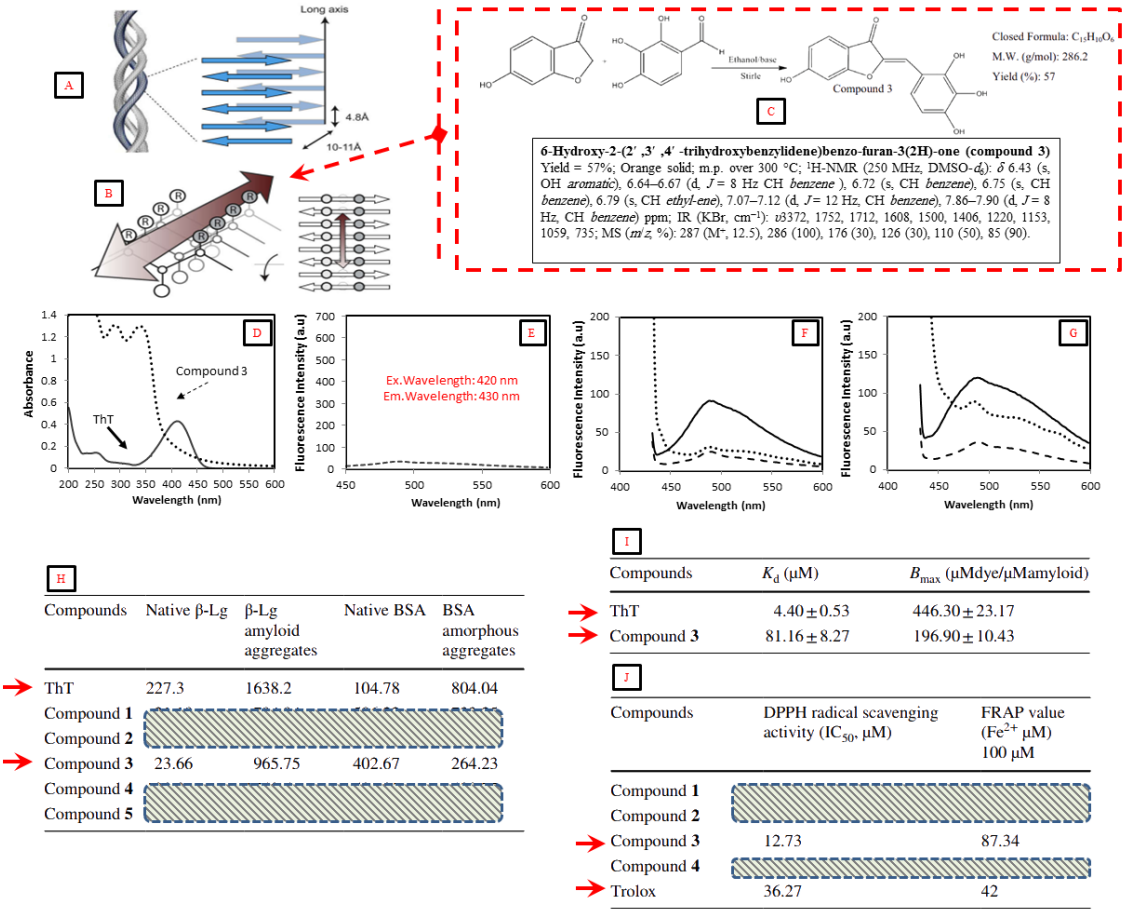


Figure 5. Polyphenols Family, Aurone Forms the Core for a Family of Derivatives which are Known Collectively as Aurones. Aurones are plant flavonoids that provide yellow color to the flowers of some popular ornamental plants, such as snapdragon and cosmos. Aurones including 4'-chloro-2 hydroxyaurone (C15H11O3Cl) and 4'-chloroaurone (C15H9O2Cl) can also be found in the brown alga Spatoglossum variable. Most aurones are in a (Z)-configuration, which is the more stable configuration according to Austin Model 1 computation, but there are also some in the (E)-configurations such as (E)-3'-O-β-d-glucopyranosyl-4,5,6,4'-tetrahydroxy-7,2'-dimethoxyaurone, found in Gomphrena agrestis. This illustration created by BioRender.

to neuroprotective effects both *In vitro* and *In vivo*.[69] In other supportive directions, epidemiological studies demonstrated that vegetables and fruits have a protective effect against cancer[70] and cardiovascular disease,[71] which demands further and renewed investigation, particularly in this field of study.

According to the information available so far, multiple hypotheses about the beneficial effects of vegetables and fruits have been expressed.[72] For example, one of those theories stated that vegetables and fruits are involved in nutrient and micronutrient compounds, which induce protective effects for herb categories.[73] Plant polyphenols are a large group of antioxidants in the plant diet that have been identified as acceptable candidates to be further investigated.[74] All phenols contain derivatives of intermediate phenylalanines or precursors similar to shikimic acid (pathway in plants) that can be divided into at least ten different categories based on chemical structure, aromatic ring structure, and one or more hydroxy groups[75,76] (Figure 5).

Flavonoids are among the largest groups of low-mass natural polyphenolic compounds distributed throughout the world.[77] Flavonoids are widely propagated in plants as secondary metabolites, but the presence and distribution of these compounds depend on a role in plant organs are varying widely.[78] These compounds are found in almost all parts of the plant, including fruits,[79] seeds,[80] stems,[81] leaves,[82] flowers,[83] roots,[84] bark,[85,86] and wood.[86] Flavonoid compounds are commonly found in nature in conjunction with organic acids and sugars, which has led to a wide range of bioflavonoids (about 4,000 species). According to some studies, lignin[87] and tannins[88] have beneficial physiological properties in maintaining human health; they are considered as polymer forms of these substances one after the other.[89-91] The flavonoid family has several members whose two-aromatic ring structure (A and B) is associated with a three-carbon chain, and the carbon skeleton is usually represented as (Ar-C_3-Ar).[92] The three-carbon chain may open to form 1, 3-diphenyl propane derivatives or become a part of a 5- or 6-membered ring structure (C-ring). Those compounds belonging to 1, 3 diphenyl propane are considered as



Compounds	Native β-Lg	β-Lg amyloid aggregates	Native BSA	BSA amorphous aggregates
ThT	227.3	1638.2	104.78	804.04
Compound **1**				
Compound **2**				
Compound **3**	23.66	965.75	402.67	264.23
Compound **4**				
Compound **5**				

Compounds	K_d (μM)	B_{max} (μMdye/μMamyloid)
ThT	4.40±0.53	446.30±23.17
Compound **3**	81.16±8.27	196.90±10.43

Compounds	DPPH radical scavenging activity (IC_{50}, μM)	FRAP value (Fe^{2+} μM) 100 μM
Compound **1**		
Compound **2**		
Compound **3**	12.73	87.34
Compound **4**		
Trolox	36.27	42

Figure 6. Proposed Aurone Derivatives, A & B) The β-sheet (also β-pleated sheet) is a common motif of regular secondary structure in proteins. Beta sheets consist of beta strands (also β-strand) connected laterally by at least two or three backbone hydrogen bonds, forming a generally twisted, pleated sheet. A β-strand is a stretch of polypeptide chain typically 3 to 10 amino acids long with a backbone in an extended conformation. The supramolecular association of β-sheets has been implicated in the formation of the protein aggregates and fibrils observed in many human diseases. C) Chemical structure of a synthesized compound, used in the following studies. D) Study of UV absorption spectrum related to a compound compared with Thioflavin T. E) Fluorescence emission spectra of a synthesized compound compared with Thioflavin T (Regardless fibrils). F) Investigation of fluorescence emission modifications. [Synthetic compound alone (dashed line), a synthetic compound with native beta-lactoglobulin protein (dotted line), and synthetic compound with beta-lactoglobulin amyloid (continuous line)] (This test repeated in five different concentrations as following: 0, 0.05, 0.10, 0.15, and 0.20 mg/ml). G) Investigation of fluorescence emission modifications. [Synthetic compound alone (dashed line), a synthetic compound with native bovine serum albumin protein (dotted line), and synthetic compound with BSA aggregates (continuous line)] (This test repeated in five different concentrations as following: 0, 0.05, 0.10, 0.15, and 0.20 mg/ml). H) Curve slope of binding fluorescence of compounds 1–5 in the presence of β-Lg and BSA (native, amyloid, and amorphous aggregates); however, Thioflavin T and compound 3 reported only. I) The dissociation constant of dye-amyloid complexed (K_d), the maximum existence of compounds in saturated concentration (B_{max}). J) Antioxidant activities of the synthetic compounds; however, Thioflavin T and compound 3 were reported only.

Chalcones or Chalconoids,[93] a 5-membered ring is classified as Aurone or Auronoids,[94] and the 6-membered ring is categorized as phenyl benzopyran derivatives. In other words, depending on the phenyl bond's position from the B-ring to the benzopyrene fraction, they can be divided into three distinct categories, including flavonoids, iso-flavonoids, and neo-flavonoids.[95] In the view of heterocyclic ring oxidation, this group may be divided into subgroups.[96] Equivalently, different countable processes such as hydroxylation, methoxylation, glycosylation, acylation, and rarely sulfation (sulfurylation) can be a reason for the wide range of structural diversity with flavonoids in nature.[89,91] The flavonoid family is a subgroup of polyphenolic compounds, according to different substitutions and their position in the family; it is also divided into 13 other subgroups[97] (Figure 5).

The yellow color of flowers is generally related to carotenoid pigments. However, the bright color of Scrophulariaceae and composite plants is due to a small group of flavonoids called aurone.[98] The structure of

aurones consists of (2Z)-2-benzylidene-1-benzofuran-3(2H)-one and several hydroxyl groups in their aromatic rings.[98] The word aurone is derived from a Latin word (aurum) meaning gold, and the choice of this name is on account of the presence of yellow and gold pigments in this combination.[99] Since aurone is a member of the flavonoid family and has been mentioned in various studies as an analgesic, antioxidant, anti-mutant, anti-diabetic, antiviral, anticancer agent;[100] therefore, a synthetic compound of the aurone family generated from sulfuretin derivatives that describing the antiviral, anticancer, and tyrosinase inhibitory effects respectively.[101] The sulfuretin compound itself has been tested as a protective agent on nerve cells and a compound against the formation of free radicals. The introduced candidate, which was selected as the proper one in our previous study, in this review, has been chosen to determine the positional of synthetic compounds for imaging the Proteinopathy disease.[13,14] Mainly, selected from Polyphenol family, Flavonoid subfamily, and ultimately Aurone core structure.[14] Considering so many reliable papers that have been published, it is clear that we still need further requirements (both fundamental and clinical) as well as a comprehensive global conclusion to claim that we can treat AD brain or neurodegenerative disorders *In vivo*[102] (Figure 6).

Conclusion

To shortly paraphrase, in the past, some experimental measurements on biomolecules have included studying their interaction with electromagnetic beams of all lengths; including X-rays, ultraviolet-visible rays, and infrared rays. It has been experimentally observed that when light shines on solutions or crystals of a molecule, at least two processes occur: light scattering and light absorption. Both processes have led to the development of fundamental methods for the characterization and analysis of biomolecules. We now use the term spectroscopy to refer to the laws that study electromagnetic radiation interaction with matter. Absorption of ultraviolet-visible light by molecules is an exciting process for measuring concentration and for illuminating molecular structure. The absorption process depends on two factors: A) the properties of the beam (wavelength, energy, etc.) and B) the structural properties of the absorbing molecules (atoms, functional groups, etc.). The interaction of electromagnetic radiation with molecules is a quantum process and has been mathematically described by quantum mechanics; thus, the beam is divided into single and separate energy packets called photons. Besides, molecules have a certain excitation level and only accept packets with a certain energy level, so only an individual electron transfer will be possible. In some molecules, light emission with longer wavelengths occurs after the adsorption process. This process is called fluorescent properties, which depend on the molecular structure and environmental factors. It can help to describe the biological analysis of essential molecules in addition to the dynamic processes that are taking place between molecules. Last but not least, it is briefly attempted to evaluate some of the most common methods for studying aggregated proteins, and a glace into a comparison of possible solutions toward monitoring the abnormal protein structures by synthetic probes from the past until now.

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